LEISHMANIASIS: FROM INNATE AND ADAPTIVE IMMUNITY TO VACCINE DEVELOPMENT

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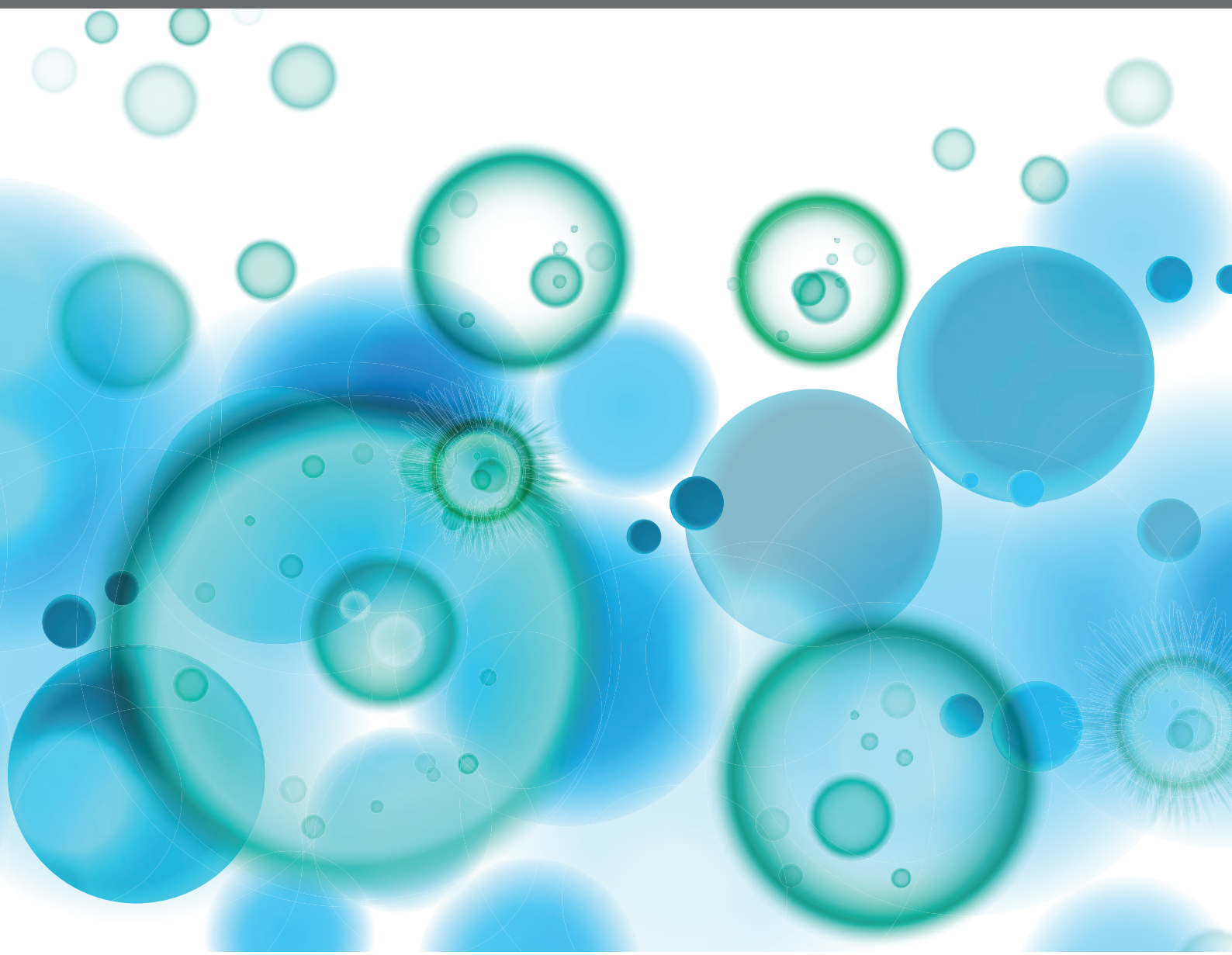

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LEISHMANIASIS: FROM INNATE AND ADAPTIVE IMMUNITY TO VACCINE DEVELOPMENT

Topic Editors:
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The parasitic disease leishmaniasis in its various clinical manifestations from self-resolving skin lesion to deadly systemic infection is a serious health problem in many developing countries and is considered to be a neglected tropical disease by the World Health Organization. To date, a vaccine is lacking and strategies to treat severe forms of leishmaniasis efficiently are missing.

Basic research using animal models of experimental visceral or cutaneous leishmaniasis has allowed to dissect the immune response to parasitic pathogens and has contributed substantially to many important, paradigm-changing insights such as the role of cytokines in helper T-cell differentiation and the impact of myeloid cell subsets on innate and adaptive immunity. One strength of experimental leishmaniasis is that tissue-associated parasites constitute a self-renewing antigen reservoir that needs to be controlled by adaptive and innate branches of the immune response. Therefore, mechanisms involved in wound healing, chronic inflammation, host pathogen interactions and the development of long lasting memory responses can be interrogated.

This research topic aims to cover a broad range of important concepts in adaptive and innate immunity to leishmaniasis and will include recent work, including vaccine development, to understand and fight this tropical disease. We welcome both reviews and original research articles that cover the latest breakthroughs in leishmaniasis research. We recognize that reproducibility is a fundamental aspect of research and thus welcome also confirmatory studies.

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Leishmania infantum Parasites Subvert the Host Inflammatory Response through the Adenosine $A2_A$ Receptor to Promote the Establishment of Infection

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Adenosine is an endogenously released purine nucleoside that signals through four widely expressed G protein-coupled receptors: A1, $A2_A$, $A2_B$, and A3. Of these, $A_{2A}R$ is recognized as mediating major adenosine anti-inflammatory activity. During cutaneous leishmaniasis, adenosine induces immunosuppression, which promotes the establishment of infection. Herein, we demonstrated that $A_{2A}R$ signaling is exploited by *Leishmania infantum* parasites, the etiologic agent that causes Visceral Leishmaniasis, to successfully colonize the vertebrate host. $A_{2A}R$ gene-deleted mice exhibited a well-developed cellular reaction with a strong Th1 immune response in the parasitized organs. An intense infiltration of activated neutrophils into the disease-target organs was observed in $A_{2A}R^{-/-}$ mice. These cells were characterized by high expression of CXCR2 and CD69 on their cell surfaces and increased *cxcl1* expression. Interestingly, this phenotype was mediated by IFN-γ on the basis that a neutralizing antibody specific to this cytokine prevented neutrophilic influx into parasitized organs. In evaluating the immunosuppressive effects, we identified a decreased number of $CD4^+$ $FOXP3^+$ T cells and reduced *il10* expression in $A_{2A}R^{-/-}$ infected mice. During *ex vivo* cell culture, $A_{2A}R^{-/-}$ splenocytes produced smaller amounts of IL-10. In conclusion, we demonstrated that the $A_{2A}R$ signaling pathway is detrimental to development of Th1-type adaptive immunity and that this pathway could be associated with the regulatory process. In particular, it promotes parasite surveillance.

Keywords: visceral leishmaniasis, adenosine, immunoregulation, $A_{2A}R$ signaling, inflammation

INTRODUCTION

Leishmania parasites are the etiological agent of a wide spectrum of diseases in mammals and other vertebrates (1). Among this complex of diseases, Visceral Leishmaniasis (VL), which is caused by *L. donovani* or *L. infantum*, is one of the most severe clinical manifestations of infection with *Leishmania* parasites and is a major cause of human mortality and morbidity worldwide (2–4).

The most effective mechanisms for protection against *Leishmania* involve the generation of $CD4^+$ Th1 cells. These cells secrete IFN-γ, which activates phagocytic cells, such as neutrophils, macrophages, and dendritic cells (DCs), to release reactive oxygen species (ROS) and nitric oxide (NO). These mediators lead to the death of the parasites (5, 6). IL-17, which is produced by the Th17 subset, can act synergistically with IFN-γ to increase the NO production and the anti-Leishmanial ability of the infected macrophages (7). Despite having several microbicidal activities to control parasite growth, the host defense can be subverted by the *Leishmania* parasite to provide a typical microenvironment for initiation and maintenance of successful infection. The mechanisms that are altered could involve those mediated by cellular response [i.e., Th2 subset, regulatory T cells (Tregs)], anti-inflammatory cytokines (IL-10, TGF-β), and some metabolites that have a high capacity to inhibit leukocyte migration and activation (8), including arachidonic acid metabolites (Prostaglandins E and J series) and adenosine (9, 10).

Adenosine is a potent immunomodulatory biomolecule that is produced by the ecto-enzymes CD39 (nucleoside triphosphate dephosphorylase) and CD73 (ecto-5′-nucleotidase), which are highly expressed by several cell types including leukocytes during stress, injury, and infection (11). Under these circumstances, extracellular ATP is hydrolyzed by CD39, which converts ATP or ADP into AMP, and subsequently CD73 rapidly dephosphorylates AMP to adenosine (ADO) (12, 13). After being generated, adenosine modulates the immunological responses through the activation of four G-protein-coupled transmembrane receptors (GPCRs) that can either stimulate (Gs) or inhibit (Gi) adenylyl cyclase, which catalyzes the formation of cyclic AMP (cAMP), which inhibits immune cell function. The adenosine A1 and A3 receptors are high- and low-affinity receptors for adenosine, respectively, and both are coupled to Gi, which decreases the generation of cAMP. By contrast, the high-affinity $A2_A$ and low-affinity $A2_B$ receptors activate adenylyl cyclase, thereby increasing the intracellular levels of cAMP (14, 15). Thus, $A_{2A}R$ and $A_{2B}R$ regulate multiple physiologic responses, including the anti-inflammatory and immunosuppressive effects of ADO. Genetic ablation or pharmacologic inhibition of $A_{2A}R$ or $A_{2B}R$ leads to excessive immune responses (16, 17).

The $A_{2A}R$ is widely distributed on the surfaces of several types of leukocytes, including neutrophils, monocytes, macrophages, DCs, T cells, and natural killer (NK) cells (18). Among its activities, $A_{2A}R$ activation blocks the classical macrophage activation by inhibiting its microbicidal machinery (19), attenuating phagocytosis (20), and blocking the production of ROS by phagocytes (21, 22). Moreover, $A_{2A}R$ signaling reduces the leukocyte recruitment to inflammatory foci (23, 24), induces T cell anergy (25, 26), and promotes both regulatory T cell generation and suppressive functions (27, 28). Furthermore, adenosine, acting through $A_{2A}R$ signaling, inhibits the DC ability to present antigen, thus leading to suppression of the Th17 subset dependent on IL-10 production (29).

In addition to these effects on the host cells, there is increasing evidence that microorganisms escape from the control of the immune system due to the synthesis of adenosine at the site of infection, which favors invasion and dissemination of infectious agents. Several microorganisms, including protozoa (belonging to the genus *Trypanosoma*, *Toxoplasma*, *Trichomonas*, *Giardia*) (30–36), fungi (*Candida parapsilosis*) (37), bacteria (38–40), and worms (*Schistosoma mansoni* and *S. japonicum*) (41, 42) express CD39–CD73-like machinery that may aid pathogen colonization and dissemination. *Leishmania* parasites can also take advantage of ectonucleotidases expressed on their membrane surfaces to escape from immunological surveillance (32, 43–45). In cutaneous leishmaniasis, we previously demonstrated that ADO and AMP present in saliva of *Phlebotomus papatasi*, *a Leishmania vector*, mediate the immunosuppressive effects. ADO and AMP act through $A_{2A}R$ to induce a tolerogenic profile on dendritic cells by sequential production of PGE_2 and IL-10. Both mediators could also act in a paracrine manner to induce Tregs from Teff populations, thus leading to suppression of the immune response and parasite spreading (46).

An interesting issue is that, in general, the visceral *Leishmania* species are less inflammatory than the cutaneous *Leishmania* species (47). Intriguingly, the viscerotropic *Leishmania* species (*L. infantum* and *L. donovani*) demonstrate higher 3′-nucleotidase activity than the cutaneous species (48). Furthermore, patients with VL have high levels of adenosine in their serum, which is related to the ectonucleotidase activities and disease progression (49). In addition, under inflammatory conditions, $A_{2B}R$ is highly expressed in the monocytes from VL patients (50), which suggests that during this disease, the *Leishmania* parasites may use the adenosinergic signaling pathway to evade host immune response, which contributes to their silent growth and survival inside cells. However, the role of high-affinity $A_{2A}R$ receptor on VL remains to be elucidated. In this context, in this study, we demonstrate that the *L. infantum* parasite benefits from purinergic signaling mediated by the $A_{2A}R$ pathway in the host cells to subvert the immune response. Mechanistically, $A_{2A}R$ signaling negatively regulates the migration and activation of neutrophils that are induced by Th1 cells, thus allowing the establishment of the infection caused by parasite in the susceptible BALB/c mice.

MATERIALS AND METHODS

Mice

Female BALB/c (wild type; WT) and BALB/c-$A_{2A}R^{-/-}$ ($A_{2A}R^{-/-}$) mice that weighed between 18 and 22 g were housed in the animal facility of the Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, University of São Paulo (Brazil) in temperature-controlled rooms (22–25°C) and received water and food *ad libitum*. All experiments were conducted in accordance with the National Institutes of Health (NIH) guidelines on the welfare of experimental animals and with the approval of the Ethics Committee of the School of Medicine of Ribeirão Preto (No 196/2011).

Parasites, Infection, and Parasites Load Estimation

Isolate HU-UFS14 of *L. infantum* was cultured in Schneider medium supplemented with 20% heat-inactivated fetal bovine

serum (Gibco®, Life Technologies, Carlsbad, CA, USA), 5% penicillin and streptomycin (all from Sigma-Aldrich, St. Louis, MO, USA), and 2% human male urine. The mice were intravenously infected with 10^7 of the promastigote form of *L. infantum* parasites in the stationary growth phase. The hepatic and splenic parasite burdens were determined using a quantitative limiting dilution assay (51, 52).

Histopathological and Immunohistochemical Analyses

The mice were euthanized 0, 4, and 6 weeks after infection, and their livers were removed. The tissues were fixed in formalin, dehydrated in graded ethanol, and embedded in paraffin. Serial sections (5 μm) were cut and mounted on glass slides that had been precoated with 0.1% poly-L-lysine (Sigma-Aldrich). Histological assessment was performed after routine hematoxylin-eosin staining. The extent of granuloma formation was analyzed in 50 fields per animal, being classified as: none granuloma, which is characterized by some parasitized cells but in the absence of inflammatory cells surrounding them; developing granuloma, which is characterized as parasitized cells surrounded by some inflammatory leukocytes; mature granuloma, in which the fused parasitized cells are surrounded by a mantle of mononuclear and polymorphonuclear cells; and empty granuloma, when no parasites could be seen inside the areas of the granulomatous reaction (53). For immunohistochemical reactions, the paraffin was removed from the tissues, and antigenic recovery was performed by heating in citrate buffer (pH 6.0), for 30 min at 37°C. Endogenous peroxidase was blocked using 3% H_2O_2, the cells were permeabilized with 0.5% Triton, and non-specific reactions were blocked with 1% bovine serum albumin. The sections were incubated overnight with rat anti-mouse Ly6G (clone 1A8) (Biolegend, San Diego, CA, USA), or isotype control antibodies (Abcam, Cambridge, MA, USA), followed by incubation with a biotinylated secondary antibody and avidin-biotin complex (Vector Laboratories, ON, Canada). The reaction was detected with diaminobenzidine, and the sections were counterstained with Mayer's hematoxylin. For the intracellular staining of iNOS, the liver sections were permeabilized with 0.01% saponin and incubated with rabbit anti-mouse iNOS (clone sc-649) (Santa Cruz Biotechnology, Dallas, TX, USA). Afterward, the sections were incubated with an avidin-biotin-peroxidase complex (Vector Laboratories, ON, Canada), and the color was developed using 3,3′-diaminobenzidine (Vector Laboratories). The slides were counterstained with Mayer's hematoxylin. The areas positive for iNOS staining in the hepatic tissue were quantified using IHC Toolbox Software ImageJ (NIH, MacBiophotonics, Boston, MA, USA). The isotype control from iNOS and LY6G staining by immunohistochemistry is showed as Figure S1 in Supplementary Material.

Evaluation of Inflammatory Infiltration in the Liver

The liver leukocytes were recovered using Ficoll-Paque PLUS gradient centrifugation. After processing, the viability was assessed using Trypan blue exclusion and the cell concentration was determined. For cytokine staining, the cells were preincubated with 20 ng/ml of PMA, 500 ng/ml of ionomycin, and Golgi Plug for 6 h; permeabilized using a Cytofix/Cytoperm kit according to the manufacturer's instructions; and stained with α-IL-17A conjugated with Alexa700 and α-IFN-γ conjugated with APC-CY7. For FoxP3 labeling, the Foxp3 Staining Kit was used according to the manufacturer's recommendations. For each sample, data from a minimum of 200,000 cells were acquired using a FACSCanto II flow cytometer and analyzed using FlowJo software (Tree Star, OR, USA).

Splenic Cell Culture and Cytokine Measurement

Single-cell suspensions from the spleens of the $A_{2A}R^{-/-}$ or WT mice at various time points of infection were prepared aseptically, diluted to a concentration of 2×10^6 cells/ml, and dispensed into 48-well plates in a total volume of 500 μl of complete RPMI-1640 medium (1×10^6 cells/well; Gibco) with or without soluble *Leishmania* antigen (SLA) (5 μg/ml). The cell culture supernatants were harvested after 72 h of culture at 37°C in 5% CO_2, and levels of IFN-γ and IL-10 were determined using ELISA with commercial kits (BD Biosciences and R&D Systems, Minneapolis, MN, USA).

For leukocyte identification, the inflammatory cells were gated based on their characteristic size (FSC) and granularity (SSC), and the T lymphocytes ($CD4^+$ $CD3^+$) and neutrophils ($Ly6G^{high}MHCII^-$) were individually identified. For intracellular staining, the cells were previously cultured with PMA (50 ng/ml) and ionomycin for 4 h, permeabilized with a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's guide and stained with APC-Cy7-labeled α-IFN-γ or Alexa700-labeled α-IL-17 and surface-stained with FITC-labeled α-CD3 and PerCP-labeled α-CD4. For the neutrophil activation analysis, the cells were stained with antibodies to α-Ly6G, α-CD11b, α-CXCR2, and α-CD69 with APC, FITC, PERCP, and PEcy7, respectively. Foxp3 labeling were carried out using the Mouse Foxp3 Buffer Set (BD Pharmigen™) and Foxp3 (Alexa 647) antibodies. The isotype controls used were rat IgG2b and rat IgG2a. All antibodies were from BD Biosciences or eBiosciences (San Diego, CA, USA). The cell acquisition was performed using a FACSort flow cytometer. The data were plotted and analyzed using the FlowJo software (Tree Star, Ashland, OR, USA). Gating strategies were determined represented the leukocyte counts were determined by measuring the relative proportions of the leukocyte subpopulations that stained with the specific antibodies in a total of 200,000 acquired events relative to the total leukocyte numbers obtained in a Neubauer chamber. The Strategy gate for identification of inflammatory leukocytes during *L. infantum* infection was showed as Figure S2 in Supplementary Material.

IFN-γ Depletion *In Vivo*

WT and $A_{2A}R^{-/-}$ mice were treated (i.p.) with the monoclonal antibody anti-mouse IFN-γ clone XMG1.2 (BioXCell, West Lebanon, New Hampshire, EUA). Briefly, the mice were given 20 μg of antibody 1 day before and a second dose 24 h after

the infection. From the fourth week post infection, the animals were treated with 10 μg every 3 days for 2 weeks thereafter. The control group was treated with an irrelevant mab IgG following the same schedule.

T Cell Proliferation, T Cell Isolation, and Th1 Differentiation

Spleens from naïve WT or $A_{2A}R^{-/-}$ mice and the cells were filtered through a cell strainer, centrifuged at 500 × *g* at 4°C for 10 min, and resuspended in RPMI-1640 medium at 2.5×10^6 cells/ml. The cells were stained with CSFE and stimulated with plate-bound α-CD3 mAb (2 μg/ml) and α-CD28 (1 μg/ml), or medium for 4 days in a total volume of 500 μl per condition. The lymph proliferation was determined by CFSE staining by flow cytometry assay. For Th1 differentiation, $CD4^+$ T cells were isolated from spleen cell suspensions of naïve WT or $A_{2A}R^{-/-}$ and stimulated with plate-bound α-CD3 (2 μg/ml), α-CD28 (1 μg/ml) (both from BioXCell) for 4 days in RPMI-1640 medium supplemented with 5% FBS (Gibco), 100 U/ml penicillin/100 μg/ml streptomycin, 1 mM sodium pyruvate, non-essential amino acids, L-glutamine and 50 μM 2-mercaptoethanol in the presence of recombinant cytokines. For Th1, differentiation was included IL-12 (5 ng/ml) plus anti-IL-4 (10 μg/ml) in addition to IL-2 (25 U/ml). All recombinant cytokines were obtained from RD and neutralizing antibodies were obtained from BioXCell. After 4 days of culture, differentiated cells were reestimulated with PMA (50 ng/ml), ionomycin (500 ng/ml) (Sigma-Aldrich), and brefeldin A for 4 h.

Quantitative Polymerase Chain Reaction (qPCR)

The total RNA was extracted from the tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the SV Total RNA Isolation System Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Complementary DNA was synthesized using Transcriptase Reverse SuperScript III (Invitrogen). SYBR Green Mix-based quantitative PCR assays were performed using the StepOnePlus Real-time PCR System (Applied Biosystems, Singapore, Malaysia). The mean threshold cycle (C_t) values of duplicate measurements were used to calculate the expression of the target gene, which was normalized to the housekeeping genes *Actb*, *B2m* and *Gapdh* using the $2^{-\Delta\Delta C_t}$ formula. The standard PCR conditions were as follows: 50°C for 2 min, 95°C for 2 min and 40 cycles of 15 s at 95°C, 30 s at 58°C and 30 s at 72°C, followed by a standard denaturation curve.

Statistical Analysis

The data are expressed as the means ± SD and are representative of two to four independent experiments. The results from individual experiments were not combined because they were analyzed individually. The means of the different groups were compared by ANOVA followed by Tukey's honest significant difference test. Comparisons between two groups were performed using Student's *t*-test. The analyses were performed using Prism 5.0 software (GraphPad). Statistical significance was set at $P < 0.05$.

RESULTS

$A_{2A}R$ Contributes to Susceptibility during *In Vivo L. infantum* Infection

To investigate the relevance of $A_{2A}R$ in the course of *L. infantum* infection, $A_{2A}R^{-/-}$ mice and control littermates were intravenously infected with 10^7 promastigote forms of *L. infantum*, and the parasite loads in the spleens and livers were quantified at various times after the infection. Strikingly, and in contrast to the WT counterparts, the spleens of the mice lacking $A_{2A}R$ harbored reduced parasite loads in both periods (**Figure 1A**); whereas, in the livers, fewer parasites were observed only at the sixth wpi (**Figure 1B**). This result suggested that $A_{2A}R$ participates in the establishment of visceral *Leishmania* infection. Surprisingly, the $A_{2A}R^{-/-}$ mice exhibited increased weights of both spleen and liver compared to the WT mice (**Figures 1C,D**), which may be consequence of an inflammatory reaction.

One feature of VL is the formation of granulomas in an attempt to control the spreading of parasite (2). The granulomas can be pathologically classified as: no granuloma reaction, developing

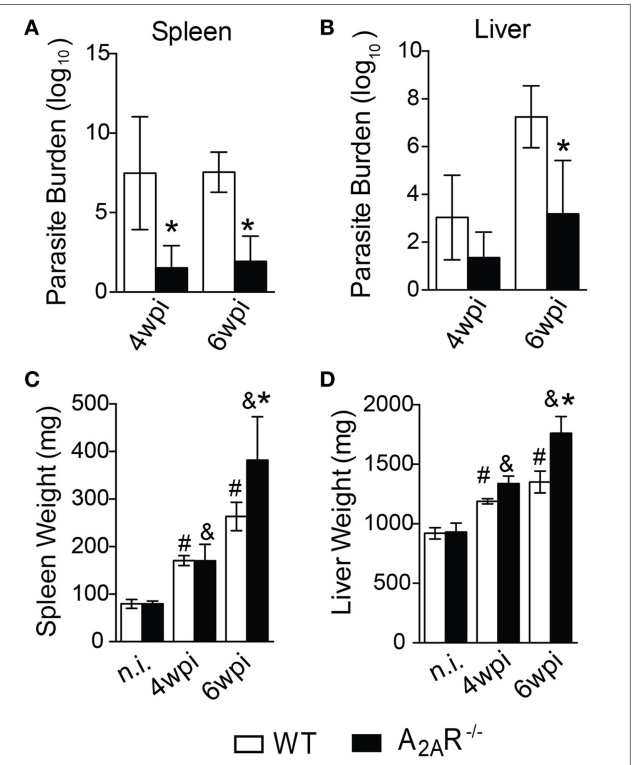

FIGURE 1 | $A_{2A}R$ signaling confers the susceptibility to *Leishmania infantum* infection. BALB/c ($n = 5$) and $A_{2A}R^{-/-}$ mice ($n = 5$) (white bars and black bars, respectively) were infected with 1×10^7 *L. infantum* promastigotes (HU-UFS 14) in stationary growth phase by the intravenous route (i.v.). The parasite burdens (Log10 scale) in the spleens **(A)** and livers **(B)** during the fourth and sixth weeks post infection (wpi) are shown. The spleen **(C)** and liver **(D)** weights (mg of tissue) from the non-infected (n.i.) and infected WT and $A_{2A}R^{-/-}$ groups at the fourth and sixth weeks post infection are shown. The results are expressed as the means ± SD, $^*P < 0.05$ compared to the infected WT group, $^\#P < 0.05$ compared to the non-infected WT group, and $^\&P < 0.05$ compared to the uninfected $A_{2A}R^{-/-}$ group.

granulomas, mature granulomas, and empty granulomas (53). Histopathological analysis demonstrated that the $A_{2A}R^{-/-}$ mice exhibited larger areas with well-formed granulomas at sixth wpi than those observed in the WT mice. These areas included mature granulomas (**Figure 2C**) and empty granulomatous reactions (**Figure 2D**). These results were consistent with the lower parasite numbers detected in the livers (**Figure 1B**) and dearth areas with none granuloma reaction (**Figure 2A**). Moreover, we could not detect any difference with respect to the developing granuloma reactions during the fourth and sixth wpi between the WT and $A_{2A}R^{-/-}$ mice (**Figure 2B**). These data demonstrated that activation of $A_{2A}R$ results in the failure of BALB/c mice to control the *L. infantum* infection possibly due to the generation of a weaker cellular immune response.

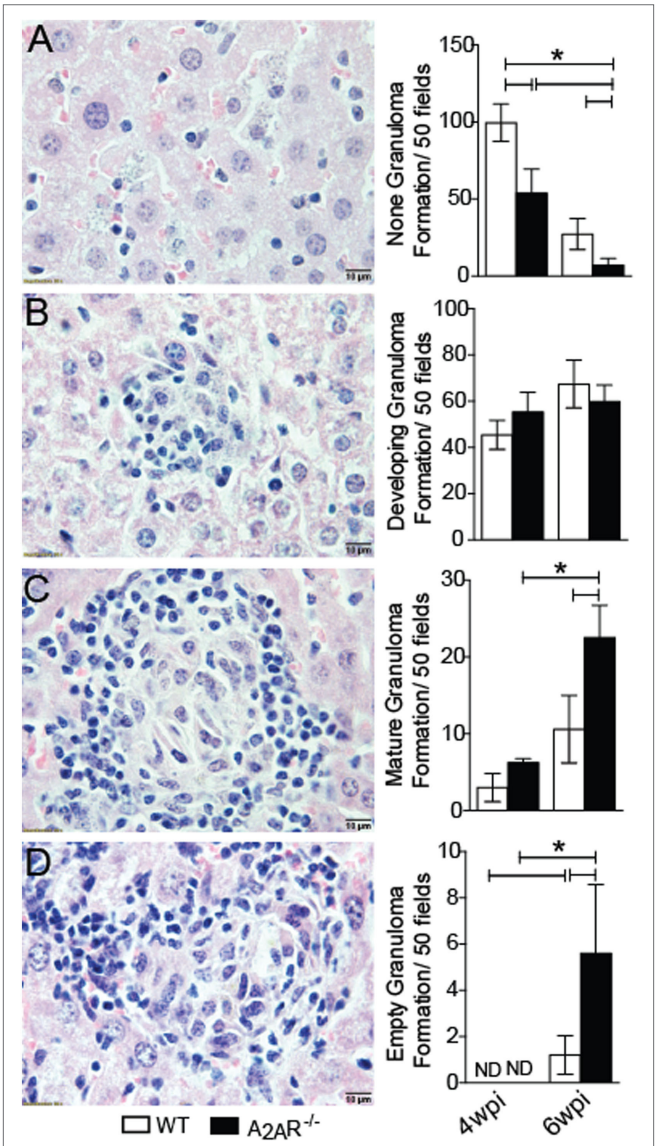

FIGURE 2 | $A_{2A}R$ disturbs the granuloma reaction during *Leishmania infantum* infection. WT and $A_{2A}R^{-/-}$ mice at fourth and sixth weeks post infection were scored for the extent of granuloma formation and classified as: no granuloma **(A)**, developing granuloma **(B)**, mature granuloma **(C)** and empty granuloma **(D)**. The data are expressed as the number of granulomas per 50 high-power fields (magnification 100×). The results are expressed as the means ± SD $^*P < 0.05$. All data are representative of at least three independent experiments.

$A_{2A}R$ Inhibits the Th1 Response during *L. infantum* Infection

Because the development of IFN-γ-producing $CD4^+$ T helper cells is crucial for the control of parasite replication in the target organs of VL, we investigated whether this response could be affected in $A_{2A}R$-dependent manner. The *Ifng* gene expression was upregulated in the livers (**Figure 3B**), but it not altered into spleen (**Figure 3A**), of the $A_{2A}R^{-/-}$ mice at the sixth wpi compared to the control littermates. In terms of protein, IFN-γ production was measured in response to restimulation of the spleen cells from $A_{2A}R^{-/-}$ or WT mice with the SLA. Stimulation of the cells from the WT mice with SLA induced the release of significant amounts of IFN-γ into culture supernatant compared with WT control (medium-without SLA) **Figure 3C**. However, the supernatants of the spleen cells from the $A_{2A}R^{-/-}$ mice contained higher levels of this cytokine by in comparison with supernatants stimulated WT cells. This profile was weakly observed at the fourth wpi, but it was pronounced at the sixth wpi. Moreover, there is a significant difference in the amounts of IFN-γ into culture supernatants from antigen-stimulated $A_{2A}R^{-/-}$ compared with respective $A_{2A}R^{-/-}$ control (medium- without SLA). The *Leishmania* antigen had minimal effects on the basal IFN-γ release in either strain of non-infected mice.

By flow cytometry, we observed that infection with *L. infantum* promoted a significant induction of IFN-γ-producing $CD4^+$ T cells in both BALB/c and $A_{2A}R^{-/-}$ mice compared to their counterparts in terms of both percentages (**Figures 3D,E**) and total number of cells (**Figure 3F**). However, it must be noted that the previously mentioned Th1 profile enhancement was heightened in $A_{2A}R^{-/-}$ mice. Moreover, the infection promoted the expansion of the $CD4^+$ IL-17^+ gated on $CD3^+$ cells, but such population was not affected in the absence of the receptor. There were no differences between the WT and $A_{2A}R^{-/-}$ mice in the frequencies and absolute numbers of the $CD4^+$ IL-17^+ gated on $CD3^+$ (Figures S3A,B in Supplementary Material). We also could not see any difference in the frequency and number of IL-17 production by other $CD3^+$ population, herein characterized as $CD4^-$ IL-17^+ gated on $CD3^+$, that could be $CD8^+$ T, NK^+ T, or $\gamma\delta^+$ T cells between WT and $A_{2A}R^{-/-}$ (Figures S3A,C in Supplementary Material).

A considerable body of evidence has shown that the Th1 subset produces IFN-γ, which in turn induces expression of iNOS (NOS2) in infected phagocytes, which generates NO (54). The resulting production of NO by iNOS represents an important tool to kill *Leishmania* sp parasites (5, 6). By qPCR, we detected greater *Nos2* mRNA expression in the spleens of the $A_{2A}R^{-/-}$ mice than in the WT group at both the fourth and sixth weeks post infection (**Figure 3H**). Likewise, greater areas positive for iNOS staining were observed in the livers of the knockout mice at sixth week post infection than in the WT group (**Figure 3G**). These results suggested that $A_{2A}R$ activation

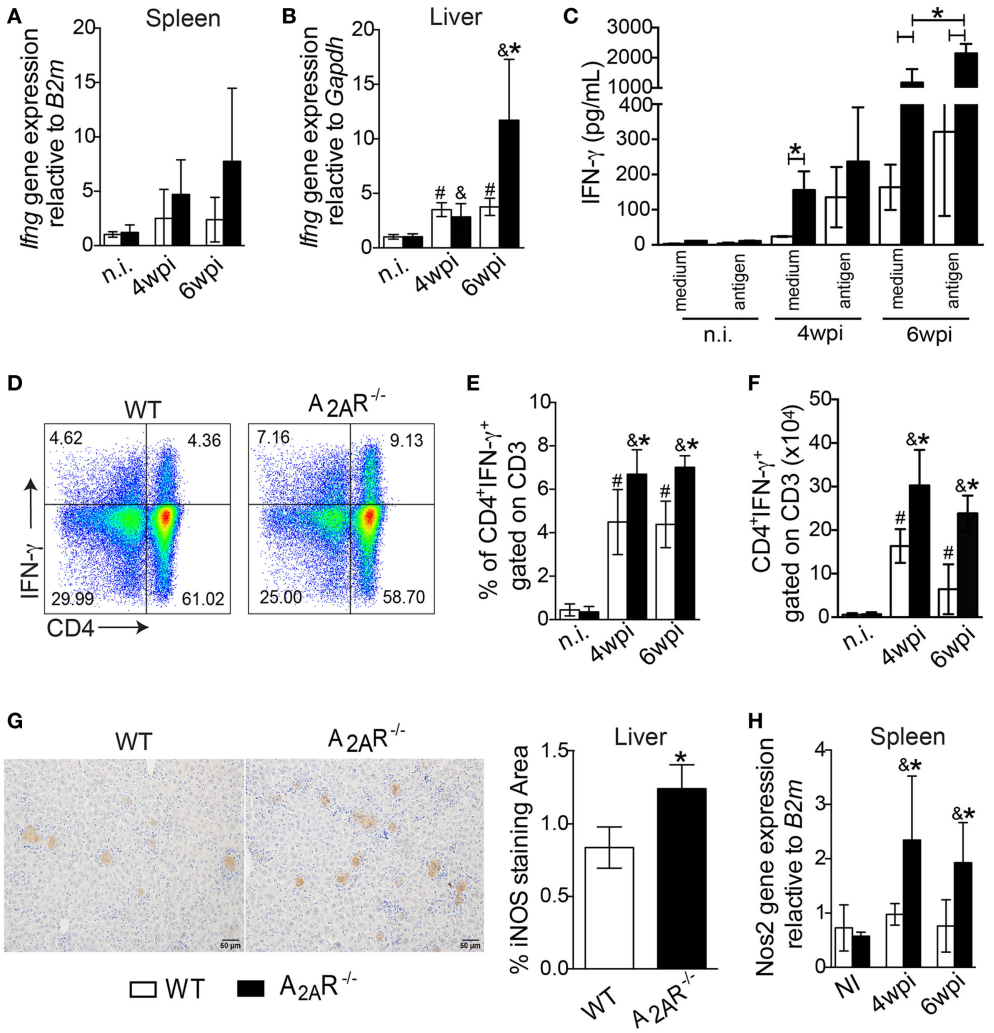

FIGURE 3 | $A_{2A}R$ regulates the establishment of the Th1 immune response in the course of *Leishmania infantum* infection. Fragments from the spleens **(A)** and livers **(B)** from the WT (white bars, $n = 5$) and $A_{2A}R^{-/-}$ (black bars, $n = 5$) mice were subjected to quantitative polymerase chain reaction (qPCR) analysis to evaluate the expression of *ifng* mRNA. The IFN-γ produced by the splenic cells of the non-infected and infected WT and $A_{2A}R^{-/-}$ mice in the presence or absence of *L. infantum* antigens is shown **(C)**. The results are expressed as the means ± SD, $^{*}P < 0.05$ for the comparison with *Leishmania* antigen or medium stimuli. The dot plots represent the frequency of $CD4^+$ IFN-γ^+ gated on $CD3^+$ cells **(D)**. The frequencies **(E)** and absolute numbers **(F)** of $CD4^+$ IFN-γ^+ gated on $CD3^+$ cells that were present in the spleens of the non-infected (n.i.) and infected mice at fourth and sixth weeks of infection are shown. Representative photos of the NOS2 staining by immunohistochemistry in the hepatic tissues from the WT ($n = 5$) and $A_{2A}R^{-/-}$ ($n = 5$) mice at the sixth wpi **(G)**. The photomicrographs are shown at 20X magnification. The positive percentage of NOS2-stained area at the sixth wpi was quantified using ImageJ® software analysis. The mRNA for *nos2* expression in spleen was quantified using qPCR **(H)**. The results are expressed as the means ± SD, $^{\#}P < 0.05$ compared to the non-infected WT, $^{\&}P < 0.05$ compared to the non-infected $A_{2A}R$ group, and $^{*}P < 0.05$ for the comparison of the infected WT group.

downregulated the Th1 responses which could favor the parasite spreading.

$A_{2A}R$ Regulates both Neutrophil Recruitment and Activation through an IFN-γ-Dependent Mechanism

Neutrophils are recruited to *Leishmania* inoculation foci (55) and participate in the restriction of the parasites during VL (56, 57). Because neutrophils both produce and respond to adenosine (58), we addressed whether $A_{2A}R$ signaling affected the neutrophilic inflammation in target organs of the disease.

On the basis of the size (FSC) and granularity (SSC) characteristics, we found a higher frequency of leukocytes with a high side-scatter height, a classical gate for granulocytes, in the infected $A_{2A}R^{-/-}$ mice compared to the infected WT mice (**Figure 4A**). Using specific antibodies to identify the neutrophils, we observed differences in absolute numbers of $Ly6G^+$ $CD11b^+$ cells (neutrophils markers) at fourth wpi and the most notable difference was that the neutrophilic influx observed at sixth wpi that was greater both in percentage (**Figures 4B,C**) and total number (**Figure 4D**) of cells in the infected $A_{2A}R^{-/-}$ mice than in the littermate controls. Interestingly, the strong neutrophilic influx in the absence of $A_{2A}R$ was associated with the increased expression

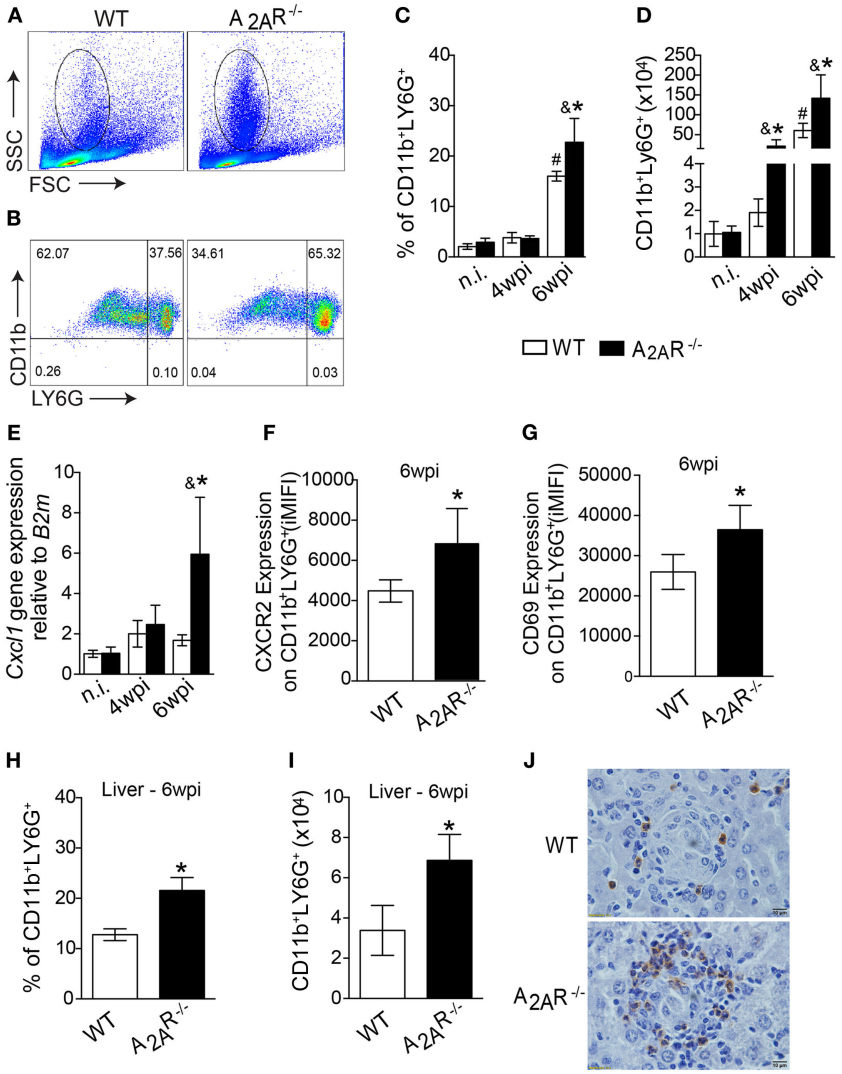

FIGURE 4 | $A_{2A}R$ decreases the neutrophil recruitment to the foci of infections during *Leishmania infantum* infection. Granulocytes were identified according to their size (FSC) and granularity (SSC) **(A)** and further neutrophils were characterized as $CD11b^+$ $Ly6G^+$ cells **(B)** by flow cytometric analysis. The dot-plots represent the frequency **(C)** and the absolute number **(D)** of $CD11b^+$ $LY6G^+$ cells in the spleens of the non-infected (n.i.) and infected (4 wpi and 6 wpi) WT (white bars, $n = 5$) and $A_{2A}R^{-/-}$ (black bars, $n = 5$) mice. *Cxcl1* gene expression in the spleens from non-infected (n.i.) and infected WT and $A_{2A}R^{-/-}$ mice (4 wpi and 6 wpi) **(E)**. Expression of CXCR2 **(F)** and CD69 **(G)** (integrated median fluorescence intensity) on surface of $CD11b^+$ $LY6G^+$ cells at 6 wpi in the spleens of the WT (white bars, $n = 5$) and $A_{2A}R^{-/-}$ (black bars, $n = 5$) animals. The percentages **(H)** and absolute numbers **(I)** of $CD11b^+$ $LY6G^+$ cells in the livers of WT and $A_{2A}R^{-/-}$ mice at sixth wpi determined by flow cytometry are shown. Representative photos of Ly6G staining by immunohistochemistry in the hepatic tissue at the sixth wpi **(J)** are shown. The photomicrographs are shown at 100X magnification. The results are expressed as the means ± SD, $^{\#}P < 0.05$ compared to the non-infected WT group, $^{\&}P < 0.05$ compared to the non-infected $A_{2A}R^{-/-}$ group, and $^{*}P < 0.05$ compared to the infected WT group.

of *cxcl1*, which codes for an important neutrophil chemotactic mediator, by the spleen cells (**Figure 4E**). Subsequently, we used flow cytometry to examine the surface molecules expressed on surface of $CD11b^+$ $Ly6G^+$ cells to determine whether differences in expression of CXCR2 or CD69, relative to neutrophil migration (59–61) and activation (62, 63), respectively, could account for the strong inflammatory process encountered in the tissues of the infected $A_{2A}R^{-/-}$ mice. Consistent with these observations, the neutrophils from the $A_{2A}R^{-/-}$ mice exhibited an enhanced expression of CXCR2, the CXCL1 receptor (**Figure 4F**; Figure S4 in Supplementary Material). Among the neutrophil activation marker, we observed that the integrated median fluorescence intensity (iMFI) for CD69 from the $A_{2A}R^{-/-}$ neutrophils was significantly higher than the iMFI for these markers on the WT neutrophils (**Figure 4G**; Figure S4 in Supplementary Material). Likewise, consistent with the spleen results, the liver sections from the $A_{2A}R^{-/-}$ mice showed a marked increase in the stained neutrophils that infiltrated into the hepatic granulomas (**Figure 4J**). By flow cytometry, higher neutrophils were detected into liver of $A_{2A}R^{-/-}$ mice (**Figures 4H,I**), which suggests that both neutrophil migration and activation may be affected by the adenosine receptor during VL.

It has been determined that IFN-γ directly modulates neutrophil behavior to favor the expression of molecules involved with cell adhesion, migration, activation, and killing activity (64). To assess whether IFN-γ could be responsible for the neutrophilic influx into the target organs of the $A_{2A}R^{-/-}$ mice, we blocked IFN-γ using a specific antibody in the infected mice. The treatment with the α-IFN-γ antibody abrogated the neutrophilic inflammation, as observed by the reduction of percentage (**Figures 5A,B**) and total number (**Figure 5C**) of $CD11b^+$ $LY6G^+$ cells in the spleens of the $A_{2A}R^{-/-}$ mice. In addition, a smaller surface expression of CXCR2 (**Figure 5D**) and CD69 (**Figure 5E**) was observed in the $A_{2A}R^{-/-}$ mice treated with α-IFN-γ than with respective group treated with α-IgG. Interestingly, none of these parameters differed between the WT mice that were treated with α-IFN-γ or anti-IgG control. Together, these data suggested that $A_{2A}R$ may modulate the Th1 subset that is capable of inducing neutrophilic inflammation and controlling the parasite spreading into tissue.

The Immunoregulatory Effect of $A_{2A}R$ Pathway Is Related to the Tregs and IL-10 Production

To determine the mechanism by which $A_{2A}R$ modulates Th1 responses during *L. infantum* infection, we first examined whether T cell-intrinsic $A_{2A}R$ is involved in either T cells proliferation or Th1 generation. CFSE-labeled $CD4^+$ T cells isolated from the spleens of naïve WT or $A_{2A}R^{-/-}$ mice cultured under Th0 and Th1 condition were stimulated with α-CD3 + α-CD28 for 4 days. The proliferation assay was analyzed by CFSE-positivity and Th1 differentiation by either intracellular stained-IFN-γ production or T-bet mRNA expression. The frequency of CFSE-labeled $CD4^+$ T cells between $A_{2A}R^{-/-}$ in the presence of polyclonal stimuli was similar to that in WT cells (Figures S5A,B in Supplementary Material). Moreover, the IFN-γ-production by $A_{2A}R^{-/-}$ $CD4^+$ T cells maintained under Th1 condition were induced in similar levels that those in WT cells (Figure S5C in Supplementary Material). There was no significant difference in T-bet mRNA expression by WT and $A_{2A}R^{-/-}$ $CD4^+$ T cells under Th1 polarizing condition (Figure S5D in Supplementary Material), indicating that T cell-intrinsic $A_{2A}R$ does not affect the either proliferation or differentiation of Th1 cells.

Taking into account that $A_{2A}R$ signaling can restore homeostasis by promoting Tregs generation and immunosupression (27) and inflammatory mediators such as IFN-γ can limit the Treg function and differentiation (65) and that IFN-γ was upregulated in the infectious foci of *L. infantum* (**Figures 3A–F**), we next evaluated whether the Th1-induced inflammation observed in the $A_{2A}R^{-/-}$ mice could be related to compromised Treg functions during infection. The results showed that following the

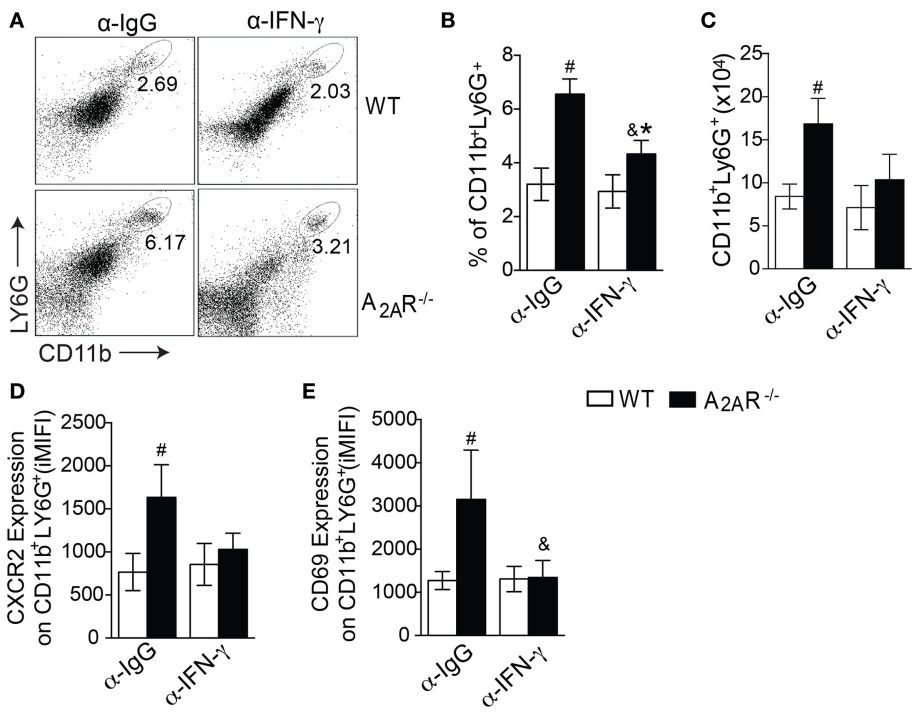

FIGURE 5 | $A_{2A}R$ regulates the neutrophil activation and recruitment into the spleen in an IFN-γ-dependent fashion. The dot plots represent the frequency of $CD11b^+$ $Ly6G^+$ cells gated on granulocytes **(A)**. The frequencies **(B)** and absolute numbers **(C)** of splenic $CD11b^+$ $Ly6G^+$ cells at the sixth wpi in the WT (white bars) and $A_{2A}R^{-/-}$ (black bars) mice that were treated with a control antibody (α-IgG) or interferon-γ neutralizing antibodies (α-IFNγ) ($n = 6$ mice per group). The medians of the integrated median fluorescence intensity of CXCR2 **(D)** and CD69 **(E)** gated on $CD11b^+$ $Ly6G^+$ cells from the non-treated infected mice or those treated with α-IFNγ at the sixth wpi are shown. The results are expressed as the means ± SD, $^{\#}P < 0.05$ compared to the α-IgG-treated WT mice, $^{\&}P < 0.05$ compared to the α-IgG-treated $A_{2A}R^{-/-}$ mice, and $^{*}P < 0.05$ compared to the α-IFN-γ-treated WT group.

infection, the frequency of CD4+ T Foxp3+ cells were reduced in both infected groups at fourth wpi compared with respective naïve littermate control group. However, the reduction of CD4+ T Foxp3+ cells was more pronounced on $A_{2A}R^{-/-}$ mice when compared to infected WT mice (**Figures 6A,B**). In terms of total cells, despite the infection promoting CD4+ T Foxp3+ cells expansion on both infected groups at fourth wpi, it was reduced on infected $A_{2A}R^{-/-}$ mice when compared to infected WT compared with respective non-infected littermate controls (**Figures 6A,B**). Moreover, *foxp3* mRNA expression was reduced in the livers of $A_{2A}R^{-/-}$ mice (**Figure 6C**). We previously demonstrated that adenosine provided an anti-inflammatory activity through a mechanism that was dependent on PGE_2-induced IL-10 release (29). Consistent with the observed Treg reduction, the transcripts of IL-10, an important anti-inflammatory cytokine that is released through $A_{2A}R$ signaling, was reduced in the spleens at fourth wpi (**Figure 6E**) and livers at sixth wpi (**Figure 6F**) of the knockout mice. We observed a similar inhibition in the IL-10 release into supernatants of the *L. infantum* antigen-stimulated spleen leukocytes from the $A_{2A}R^{-/-}$ mice compared with the stimulated cells from the infected WT mice (**Figure 6D**).

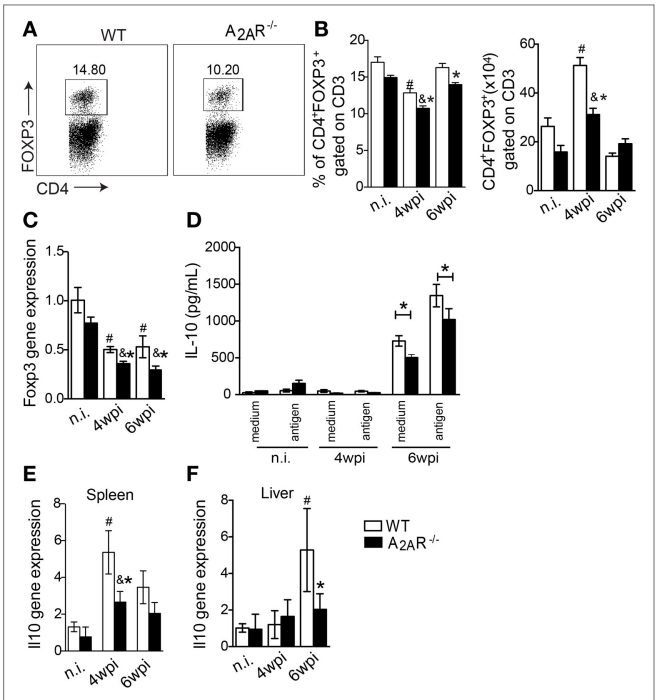

FIGURE 6 | $A_{2A}R$ induces the regulatory T cell population and IL-10 production during *Leishmania infantum* infection. In **(A)** representative dot plots of CD4+ FOXP3+ gated on CD3+ cells in spleen of infected WT or $A_{2A}R^{-/-}$ are shown in each box. The frequencies and absolute numbers of splenic CD4+ FOXP3+ gated on CD3+ cells in non-infected (n.i) and infected WT and $A_{2A}R^{-/-}$ mice (white bars and black bars, respectively) at the fourth and sixth weeks post infection **(B)**. The levels of the mRNA for *foxp3* expression in the livers were determined by quantitative polymerase chain reaction **(C)**. The levels of IL-10 produced by the splenic cells cultured at different phases of infection in the presence or absence of *L. infantum* antigens are shown **(D)**. The *il10* expression in spleens **(E)** and livers **(F)** of the infected and non-infected groups is shown. The results are expressed as the means ± SD. $^{\#}P < 0.05$ compared to the uninfected WT group, $^{\&}P < 0.05$ compared to the non-infected $A_{2A}R^{-/-}$ group, and $^{*}P < 0.05$ compared to the infected WT group.

DISCUSSION

This study revealed the role of $A_{2A}R$ in increasing the susceptibility to *L. infantum* infection during experimental VL. Herein, we demonstrated that the absence of $A_{2A}R$ in the susceptible lineage BALB/c mice increased the cellular immune reaction as shown histopathologically by raised increased areas of mature and empty granulomas into liver. To understand the role of $A_{2A}R$ signaling, we quantified lower parasite burden in the $A_{2A}R^{-/-}$ mice that was accompanied by stronger development of the Th1 pattern of immune response, as indicated by a higher frequency of IFN-γ-producing T cells, increased iNOS expression in the spleens and livers, and decreased Treg numbers and IL-10 release.

Several lines of evidence have shown that the development of mature hepatic granulomas is strictly related to the parasite killing, in that it limits spreading of the parasites to the organs (66). Accordingly, the $A_{2A}R^{-/-}$ mice at the sixth wpi harbored fewer parasites in the livers, and this was related to the elevated numbers of mature granulomas and empty granulomas in the hepatic tissue. Conversely, the susceptibility of WT mice during *L. infantum* infection was accompanied by a higher frequency of no granuloma reactions in both analyzed periods. It is possible that this was due to a decreased capacity to generate the mature granulomas that limit the parasite spreading.

A major requirement for maturation of a granuloma is the IFN-γ production (2, 3). In this sense, the elevated ratio of mature granulomas reflected the increased cellular immune reaction in the $A_{2A}R^{-/-}$ mice, which presented a strong Th1 adaptive immune reaction during *L. infantum* infection. It is no surprise that during a *Leishmania* infection, IFN-γ causes activation of the phagocytes and production of NO, a molecule that is importantly involved in parasite killing (5, 6). However, it is important to note that the increased frequency of mature granulomas was directly accompanied by increased areas of NOS2 staining in the liver, which reflects the importance of IFN-γ in limiting the hepatic infection.

It has been demonstrated that $A_{2A}R$ is upregulated after T lymphocyte activation (25) and attenuates T cell response against cognate stimuli (27). Accordingly, the induction of experimental VL in $A_{2A}R^{-/-}$ mice resulted in an increased Th1 cell frequency during the infection as well as an attenuated response of the splenocytes to *ex vivo* stimulation with *L. infantum* antigen as demonstrated by lower levels of IFN-γ production. Thus, these data could explain the reduced areas of the no granuloma reactions and the increased areas of both mature and empty granulomas on $A_{2A}R^{-/-}$ mice, which reflected the high expression of iNOS into target organs.

The neutrophil recruitment to the VL-infected organs is crucial to controlling the parasite replication (67). Our data demonstrated that the infected $A_{2A}R^{-/-}$ mice displayed an enhanced migration of activated neutrophils into the infected foci, a phenomenon that was accompanied by an increased capacity to control the spreading of the parasites into target organs. It has been shown that Th1 pattern-derived cytokines upregulate $A_{2A}R$

on phagocytes (68). This receptor is mainly involved with inhibition of cell migration, ROS production, and phagocytic activity (20, 22, 69). Interestingly, the absence of $A_{2A}R$ upregulated the expression of the neutrophil chemoattractant *Cxcl1* and its receptor CXCR2 on neutrophils, all of which are events that are involved in neutrophil recruitment. Regarding the activation, CD69 was highly expressed on neutrophils from infected $A_{2A}R^{-/-}$ mice, a phenomenon that could be related to the IFN-γ release (62, 63). Curiously, not only was neutrophil activation abrogated after IFN-γ depletion but also their recruitment was affected in the infected $A_{2A}R^{-/-}$ mice, and this was accompanied by decreased CXCR2 expression. This result demonstrated that the $A_{2A}R$-mediated regulation of neutrophil recruitment during the *L. infantum* infection resulted from the ability of this receptor to attenuate the Th1 immune response.

In addition to repressing the development of the Th1 immune response, $A_{2A}R$ signaling closely associated with the generation of Tregs and the improvement of their suppressor activity by stabilizing FOXP3 expression and inducing the CTLA-4-mediated suppressive effects (28). It is well established in the literature that suppression by the Tregs culminates in susceptibility to infection in several experimental models, including *Leishmania* (70). Accordingly, we demonstrated that the $A_{2A}R^{-/-}$ mice had a reduced frequency of Tregs during infection. It is also known that the Tregs represent a relevant source of adenosine through the action of the ectonucleotidases CD39 and CD73 (71). In addition to expanding the regulatory T cell repertoire, several lines of evidence highlight the importance of $A_{2A}R$ in the context of immune regulation in which it induces release of IL-10 by several types of leukocytes, including DCs, T cells, and Tregs (12, 18, 19, 72, 73). According to the literature, the lower levels of Tregs in $A_{2A}R^{-/-}$ mice are accompanied by lower levels of *il10* mRNA in the organs targeted by the disease. Moreover, the splenocytes of these mice exhibited reduced IL-10 production *ex vivo* following stimulation with the parasite, which suggested that these mice presented an attenuated immunosuppressive potential during experimental VL (72, 74, 75). IL-10 is a potent anti-inflammatory cytokine that is strictly involved with VL progression by impairing the Th1 cell responses, which abrogates the microbicidal mechanisms of the parasitized macrophages (76). Taking these data together, we hypothesize that the $A_{2A}R$-mediated susceptibility in BALB/c mice is based in expanding the numbers of Tregs that, in turn, generate elevated levels of adenosine. This nucleoside may act in a positive feedback loop in which IL-10 is generated by Tregs in an autocrine fashion as well as in paracrine fashion by effector T cells. Moreover, we cannot exclude a role for DCs affecting the Th1 cell polarization based on the recent observation by our group that adenosine, acting through $A_{2A}R$, modulates DC activation as well as the T cell polarization toward to an anti-inflammatory phenotype (46). However, the implications of this process were not aimed in this work. We discard an intrinsic defect of $A_{2A}R$ signaling on Th1 driving, since $CD4^+$ T cells isolated from naïve $A_{2A}R^{-/-}$ under Th1 condition presented similar capacity to express T-bet transcriptional factor and IFN-γ production than those of WT cells.

An important clinical outcome during VL is the development of hepatosplenomegaly (3). The evaluation of the weights of these target organs during the experimental infection revealed that the spleens and livers of the $A_{2A}R^{-/-}$ mice were enlarged compared to the WT counterparts. Thus, we suggest that this phenomenon could be a result of the strong inflammatory response that favored the elimination of the parasites. In agreement with this result, our group observed that *Il17ra*$^{-/-}$ mice, which were more susceptible to infection by *L. infantum*, exhibited less hepatosplenomegaly than the resistant WT group (7). Interestingly, $CD4^+$ T cells-producing IL-17 is also clinically associated with chronic inflammation seen in VL. Symptomatic patients showed a positive correlation between IL-17 and aspartate transaminase levels, indicating development of liver injury in those individuals (77). Therefore, the excessive inflammation triggered during VL could promote tissue damage (herein exemplified by organ enlargement) even though controlling parasite replication. Thus, we suggested adenosine signaling through $A_{2A}R$ limited the inflammation, controlling hepatosplenomegaly but it promotes parasite spread.

We conclude that $A_{2A}R$ is a negative regulator of the Th1 immune response, which may be due to an anti-inflammatory activity mediated by IL-10. In the absence of $A_{2A}R$, BALB/c became resistant to *L. infantum* infection through an exacerbated Th1 immune response, which was responsible for recruiting neutrophils into the foci of the infection. Furthermore, we detected reduced regulatory T cell numbers and IL-10 production in the absence of $A_{2A}R$ signaling. Thus, we suggest that inhibition or blockade of $A_{2A}R$ could enhance the immune system effector functions to address persistent infections.

ETHICS STATEMENT

All experiments were conducted in accordance with the National Institutes of Health (NIH) guidelines on the welfare of experimental animals and with the approval of the Ethics Committee of the School of Medicine of Ribeirão Preto (No 196/2011).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: ML and VC. Performed the experiments: ML, LS, GQ, MF, AS, LB, and VC. Analyzed the data: ML and VC. Contributed reagents/materials/analysis tools: LB, FC, RA, JS, and VC. Wrote the paper: ML, GQ, and VC.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00815/full#supplementary-material.

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IL-4Rα Signaling in Keratinocytes and Early IL-4 Production Are Dispensable for Generating a Curative T Helper 1 Response in *Leishmania major*-Infected C57BL/6 Mice

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Experimental infection with the protozoan parasite *Leishmania major* has been extensively used to understand the mechanisms involved in T helper cell differentiation. Following infection, C57BL/6 mice develop a small self-healing cutaneous lesion and they are able to control parasite burden, a process linked to the development of T helper (Th) 1 cells. The local presence of IL-12 has been reported to be critical in driving Th1 cell differentiation. In addition, the early secretion of IL-4 was reported to potentially contribute to Th1 cell differentiation. Following infection with *L. major*, early keratinocyte-derived IL-4 was suggested to contribute to Th1 cell differentiation. To investigate a putative autocrine role of IL-4 signaling on keratinocytes at the site of infection, we generated C57BL/6 mice deficient for IL-4Rα expression selectively in keratinocytes. Upon infection with *L. major*, these mice could control their inflammatory lesion and parasite load correlating with the development of Th1 effector cells. These data demonstrate that IL-4 signaling on keratinocytes does not contribute to Th1 cell differentiation. To further investigate the source of IL-4 in the skin during the first days after *L. major* infection, we used C57BL/6 IL-4 reporter mice allowing the visualization of IL-4 mRNA expression and protein production. These mice were infected with *L. major*. During the first 3 days after infection, skin IL-4 mRNA expression was observed selectively in mast cells. However, no IL-4 protein production was detectable locally. In addition, early IL-4 blockade locally had no impact on subsequent Th1 cell differentiation and control of the disease. Taken together, the present data rule out a major role for skin IL-4 and keratinocyte IL-4Rα signaling in the development of a Th1 protective immune response following experimental infection with *L. major*.

Keywords: ***Leishmania*, IL-4, IL-4Rα, T helper cell differentiation, T helper 1, T helper 2, keratinocytes, mast cells**

INTRODUCTION

Upon infection, *Leishmania* protozoan parasites can cause a spectrum of diseases ranging from cutaneous, muco-cutaneous to visceral forms. Following *Leishmania major* experimental infection, C57BL/6 mice develop a small cutaneous lesion that is self-healing. Healing of lesion and control of parasite load were shown to result from the differentiation of $CD4^+$ T helper (Th) 1 cells characterized by their secretion of high levels of IFNγ, a cytokine promoting the differentiation of M1 macrophages that kill intracellular parasites. In contrast, following *L. major* infection, BALB/c mice develop non-healing lesions and are not able to control their parasite load. This phenotype was shown to correlate with the development of $CD4^+$ Th2 cells secreting IL-4 and IL-13 cytokines (1, 2). These cytokines induce the differentiation of M2 macrophages that favor parasite survival within macrophages (3). The *L. major* experimental model was the first murine model demonstrating that the discovery of Th1 and Th2 cells subsets by Mosmann et al. *in vitro* (4) had some relevance *in vivo*.

It is well established that the IL-12 produced by antigen presenting cells (APC) is necessary to launch the differentiation of $CD4^+$ Th1 cells that protect against intracellular pathogens, including *Leishmania* (5). In contrast the role of IL-4 in *L. major* susceptibility and Th2 cell differentiation is more controversial. Following infection with *L. major* (LV39), IL-$4^{-/-}$ or IL-$4R\alpha^{-/-}$ mice on a BALB/c genetic background were able to control lesion size and the levels of IFNγ present in draining lymph node (dLN) cells was either very low or remained unchanged compared to that observed in BALB/c wild-type mice (6, 7). These data suggested that IL-4 was critical for *L. major* susceptibility and Th2 cell differentiation. The C57Bl/6x129 IL-$4^{-/-}$ mice used in these studies were backcrossed for six generations onto the BALB/c genetic background. In contrast, following infection with *L. major* LV39 IL-$4^{-/-}$ mice generated with embryonic stem cells of BALB/c origin still developed progressive non-healing lesions that were comparable to those of similarly infected wild-type BALB/c mice (8). Infection of these mice with another strain of *L. major* (IR173) resulted in partial control of lesion size in IL-$4^{-/-}$ mice, while IL-$4R\alpha^{-/-}$ controlled lesion size efficiently (9). Additional studies using IL-4 or IL-4Rα-deficient mice showed that following infection with *L. major* Th2 differentiation could develop in absence of IL-4 (10–12). Specific deletion of IL-4Rα signaling on T cells resulted in a healing phenotype in BALB/c mice associated with increased IFNγ response, suggesting a role for IL-4 and IL-13 in susceptibility following *L. major* infection (13). Collectively, these results indicated that along with IL-4, IL-13, and other factors are involved in the control of Th2 cell differentiation and *L. major* susceptibility (14).

In addition, several lines of evidence suggest that IL-4 may be needed for Th1 cell differentiation. Unlike what was observed following *L. major* infection, IL-4-deficient mice failed to develop Th1 cells in response to infection with *Candida albicans* (15) suggesting a potential role for endogenous IL-4 in Th1 cell differentiation and protective antifungal response. Furthermore, local injection of exogenous recombinant IL-4 within the first 8 h of *L. major* infection in BALB/c mice was sufficient to modify the development of the immune response from an otherwise Th2 immune response into a protective type-1 Th1 response (16). It was hypothesized that IL-4, by acting on dendritic cells, induced their IL-12 secretion (16), a process that had previously been reported on macrophages and DCs *in vitro* (17–19). In addition, dendritic cell-specific IL-4Rα-deficient mice on the BALB/c genetic background developed larger lesions and increased Th2 response, suggesting some protective role for endogenous IL-4 acting on DCs during *L. major* LV39 and IL-81 infection (20). Collectively, these studies suggested that within the first hours of *L. major* infection the transient presence of IL-4 could contribute to the differentiation of $CD4^+$ Th1 cells. In this line, skin keratinocytes present in the footpad of mice infected with *L. major* subcutaneously were identified as an early IL-4 source contributing to the launching of $CD4^+$ Th1 cell differentiation (21). Interestingly, in that study, IL-4 transcription appeared restricted to keratinocytes from C57BL/6 mice and only low IL-4 mRNA levels were observed in BALB/c keratinocytes. Moreover, in the same study, the upregulation of IL-4 mRNA observed in C57BL/6 keratinocytes was shown to be restricted to a very small time window at the onset of infection. Finally, impaired Th1 cell development was observed in C57BL/6 mice following blocking of IL-4 protein with an anti-IL-4 mAb at the cutaneous infection site (21). Targeting IL-4 at the infection site could be of potential interest in the design of vaccines.

Here, we investigated the role of skin IL-4Rα signaling, more specifically the contribution of keratinocyte-derived IL-4Rα signaling during the first days of *L. major* infection and its subsequent impact on the development of a protective type-1 immune response in C57BL/6 mice. To this end, we generated C57BL/6 mice specifically deficient in IL-4Rα in their keratinocytes ($KRT14^{Cre}$IL-$4R\alpha^{-/lox}$). As IL-4 and IL-13 share a common signaling pathway through the IL-4Rα the combined role of both cytokines could be studied in these mice. Following infection with *L. major* in the ear dermis or subcutaneously in the footpad, these mice were fully able to control their lesion size and develop Th1 cells. Furthermore, using IL-4 reporter mice, we identified mast cells as the only skin cells transcribing but not producing IL-4 in C57BL/6 skin early in infection. Our data demonstrate that the presence of IL-4 and IL-4 signaling in the skin during the first days after *L. major* infection are not required for the development of a protective Th1 immune response in C57BL/6 mice.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Charles Rivers. *4get*, and KN2 mice (22, 23) were a gift from Markus Mohrs, Trudeau Institute, USA. Keratinocyte cell-specific 4get/KN2 mice were obtained by the breeding of 4get and KN2 mice. IL-4Rα-deficient ($KRT14^{cre}$IL-$4R\alpha^{-/lox}$) mice on the C57BL/6 genetic background were generated using the *Cre/lox*P system. Briefly, $KRT14^{cre}$ mice on the BALB/c genetic background (Jackson Laboratory) were first backcrossed on the C57BL/6 genetic background for nine generations. The progeny were crossed with IL-$4R\alpha^{-/-}$ C57BL/6 mice and transgenic IL-$4R\alpha^{lox/lox}$ mice (24) to generate

hemizygous KRT14^{cre}IL-4Rα$^{-/lox}$ C57BL/6 mice. Genotyping of KRT14^{cre}IL-4Rα$^{-/lox}$ C57BL/6 mice was performed using specific primers: KRT14 P1, 5'- TTC CTC AGG AGT GTC TTC GC; KRT14 P2, 5'- GTC CAT GTC CTT CCT GAA GC; KRT14 P3, 5'- CAA ATG TTG CTT GTC TGG TG; KRT14 P4, 5'- GTC AGT CGA GTG CAC AGT TT. Characterization was also performed by flow cytometry using the anti-IL-4Rα mAb [anti-CD124, anti-IL-4Rα-PE from (BD, Pharmingen)]. Mice were bred under pathogen-free conditions at the Epalinges Center. 5- to 8-week-old females were used.

Ethics Approval

All animal experimental protocols were approved by the veterinary office regulations of the State of Vaud, Switzerland, authorization 1266.6-7 to FTC and performed in compliance with Swiss ethics laws for animal protection. All mouse experiments performed at the University of Cape Town were performed in strict accordance with the South African national guidelines, as well as the Animal Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (license no. 015/034). All efforts were made to minimize and reduce suffering of animals.

Parasites and Infections

Leishmania major (LV39, MRHO/Sv/59/P strain) or in selected experiments GFP-expressing *L. major* IL-81 (MHOM/IL/81/FEBNI) parasites were cultured at 26°C in complete M199 medium (10% fetal bovine serum, 4% HEPES, 1% penicillin, streptomycin, and neomycin). 10^4 or 2×10^5 *L. major* metacyclic promastigotes were prepared from confluent stationary phase promastigotes by Ficoll gradient density centrifugation as previously described (25) and injected in the ear dermis or footpad. Lesion development was monitored using Vernier caliper, ear lesion diameter was measured for intradermal infection. For IL-4 depletion, 2×10^5 metacyclic parasites (±1 µg 11B11 antibody) were needle injected into the ear dermis in iDMEM in a final volume of 10 µL, or in the hind footpad in a volume of 50 µL. Four hours later, 1 µg of 11B11 mAb was injected intradermally (i.d.) in a final volume of 10 µL. For the induction of a Th2 response in C57BL/6 4get/KN2 mice, 1 mg of αIFNγ (XMG1.2) and αIL-12 were injected intraperitoneally 24, 2 h prior and 48 h post intradermal infection of 2×10^5 *L. major* metacyclic promastigotes.

Determination of Antibody Response

Leishmania major-specific IgG1 and IgG2c levels were determined by ELISA in the sera of mice harvested at the termination of the experiment, as previously described (26), Biotinylated goat anti-mouse IgG2c (Southern Biotech) and biotinylated rat anti-mouse IgG1 (BD Pharmingen) were used. Plates were read at the optical density of 490 nm. Titration curve were performed for all samples.

Isolation of dLN, Spleen, and Ear Mouse Cells

Infected ears, spleen, and dLNs were isolated and processed to single cell suspensions. Briefly, ears were recovered, homogenized in iDMEM containing 0.2 mg/mL Liberase TL (Roche) for 2 h at 37°C and then homogenized and filtered using 40-µm filters (Falcon). dLN and spleen cells were isolated, homogenized, and stained for flow cytometry analysis. For intracellular cytokine staining, 1×10^6 dLN cells were stimulated with 50 µg/mL PMA, 500 µg/mL Ionomycin, and 1 µg/mL Brefeldin A for 4 h prior staining. Live parasites were determined by limiting dilution assay (LDA) as described previously (27).

Cytokine Production

10^6 dLN cells were cultured in presence or absence of UV-irradiated stationary phase parasites (parasites were exposed to UV.C for 3 min, at a distance of 10 cm from the UV lamp) *L. major* promastigotes (MOI of 5:1) for 72 h at 37°C. Supernatants were recovered and IL-4 production measured by ELISA following manufacturer's instructions (BD Biosciences; R&D). IFNγ secretion was detected using a homemade ELISA kit as previously described (28).

Derivation of Mouse Primary Keratinocytes

Primary keratinocytes were isolated from the skin of adult mice tail. Briefly, the skin from the tail was collected and incubated at 4°C for 16 h in a solution of 5 U/mL Dispase (STEMCELL) supplemented with 1% PNS (Gibco) and 0.5% Gentamicin (Gibco). The epidermis was separated from the dermis and treated with 0.2% trypsin (Gibco) for 5 min at 37°C. The reaction was stopped with FCS, and cells were collected by crushing the epidermis with a 100-µm cell strainer (Falcon). 7.5×10^5 cells/mL were plated in a 6-well plate coated with Type I collagen (STEMCELL). Cells were grown for 8 days at 37°C in CnT-57.S medium (CELLnTEC) supplemented with 1% PNS and 0.5% Gentamicin. Medium were removed and cells detached with 200 µL 0.2% trypsin for 5 min at 37°C. Cells were counted and plated at 0.3×10^6 cells/mL in a 96-well plate coated with Type I collagen.

Flow Cytometry

Stained cells were analyzed using a BD LSR II-SORP or Fortessa system (Becton Dickinson) and analyzed with FlowJo software (Tree Star). The following anti-mouse mAbs were used: CD45- PerCPCy5, CD45-APC, CD8-APC, anti-IFNγ-PECy7, and anti-IL-4-FITC (BD Biosciences), CD4-AF700, CD11b APC-eFluor780, and c-kit PECy7 all from (eBiosciences). Anti human CD2 (BioLegend) was used to detect IL-4 production in KN2 mice. To detect IL-4Rα on primary keratinocytes, the following anti-mouse mAbs were used: CD124, anti IL-4Rα-PE (BD, Pharmingen), and CD49f-Brillant Violet 421 (BioLegend). Imaging flow cytometry was performed using an ImageStream cytometer (Amnis, Millipore) at low speed. Data were analyzed with the IDEAS software.

Statistics

All p values were determined with Prism software (GraphPad Software, Inc.) using the Student's t-test for unpaired data or paired data, following prior testing for normal distribution. The degree of significance was indicated as $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Absence of IL-4Rα Signaling in Keratinocytes Does Not Prevent the Development of a Protective Type-1 Immune Response

To investigate the impact of IL-4Rα signaling on keratinocytes in a protective Th1 cell differentiation against cutaneous leishmaniasis, we generated mice on the C57BL/6 genetic background that are genetically deficient in IL-4Rα expression on keratinocytes (KRT14^{Cre}IL-4Rα$^{-/lox}$) using the Cre/LoxP recombination system under control of the *KRT14* locus. In these mice, keratinocytes cannot respond to IL-4 and IL-13, while other cells can. The absence of skin IL-4Rα expression was verified by PCR of naïve ears. KRT14^{Cre}IL-4Rα$^{-/lox}$ expressed both the deleted and WT IL-4Rα allele in addition to the LoxP and Cre bands. The genotype was also validated for IL-4Rα$^{-/lox}$, IL-4Rα$^{+/+}$ (C57BL/6 wild type) control mice as well as for IL-4$^{-/-}$ on the C57BL/6 genetic background (**Figure 1A**). The selective absence of IL-4Rα on KRT14^{Cre}IL-4Rα$^{-/lox}$ keratinocytes was further analyzed by flow cytometry on primary keratinocytes derived from the skin of these mice. IL-4Rα expression was not detected on CD45^{-}CD49f^{+} keratinocytes of KRT14^{Cre}IL-4Rα$^{-/lox}$ mice. As controls, expression of IL-4Rα was detected on keratinocytes of wild-type C57BL/6 mice and was absent on keratinocytes of IL-4Rα$^{-/-}$ mice (**Figure 1B**). As expected, expression of IL-4Rα was, however, detected in splenic CD4^{+} T cells, CD19^{+} B cells, and F480^{+} macrophages of KRT14^{Cre}IL-4Rα$^{-/lox}$ mice (**Figure 1C**). The slightly lower IL-4Rα surface expression levels observed in KRT14^{Cre}IL-4Rα$^{-/lox}$ CD4^{+} T cells, CD19^{+} B cells and F480^{+} macrophages compared to those observed in C57BL/6 wild-type cells are in line with the IL-4Rα$^{-/lox}$ genotype of the control mice. No IL-4Rα expression was observed in IL-4Rα$^{-/-}$ CD4^{+} T cells and CD19^{+} B cells. These data show that KRT14^{Cre}IL-4Rα$^{-/lox}$ mice do not express IL-4Rα selectively on keratinocytes.

To investigate if IL-4Rα signaling in keratinocytes could have an impact on the local microenvironment driving Th1 differentiation

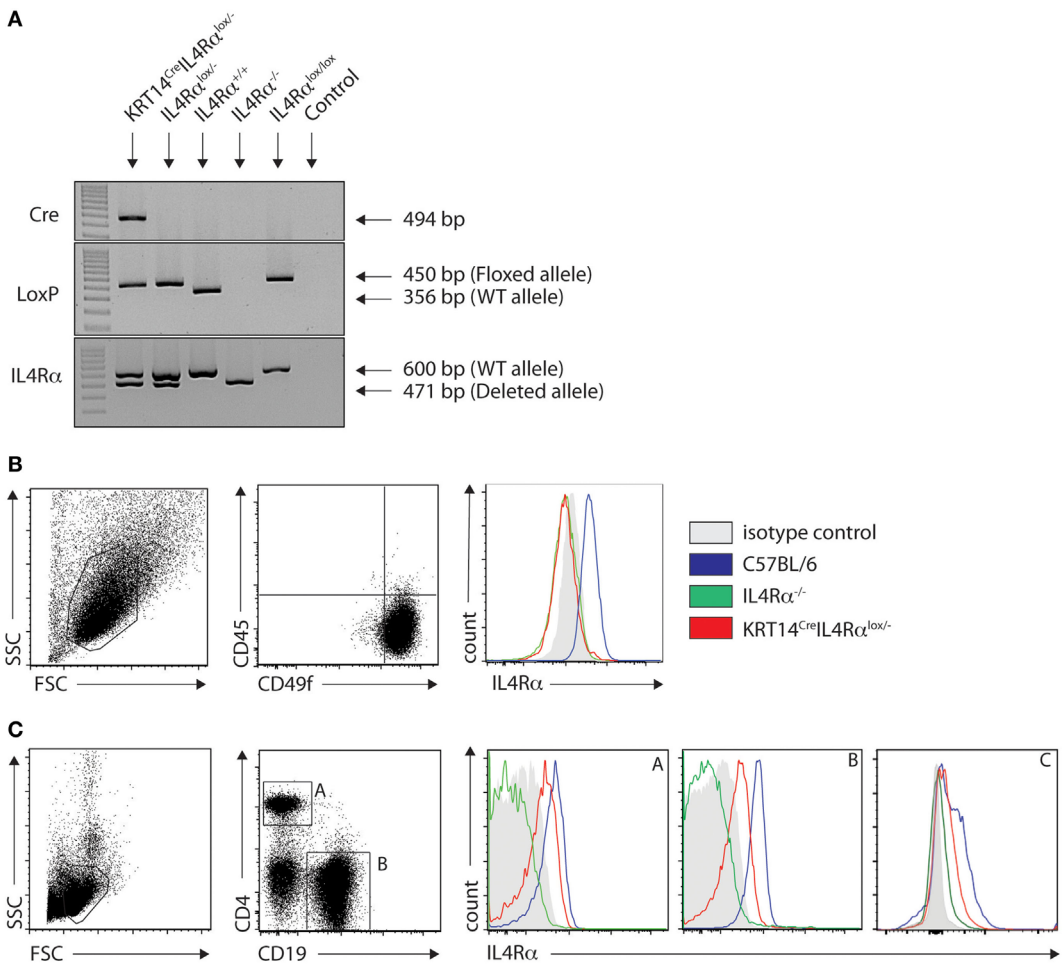

FIGURE 1 | Characterization of KRT14^{cre}IL-4Rα$^{-/lox}$ C57BL/6 mice. **(A)** Genotyping of KRT14^{cre}IL-4Rα$^{-/lox}$ mice, IL-4Rα$^{-/lox}$, IL-4Rα$^{+/+}$, IL-4Rα$^{lox/lox}$, and negative control (H_2O) is shown. The yielded PCR products in base pairs are indicated in the figure. **(B)** IL-4Rα cell surface expression was analyzed by flow cytometry on primary keratinocytes of the indicated mice. **(C)** IL-4Rα expression was analyzed in splenic CD4^{+} T cells (A), CD19^{+} B cells (B) and F480^{+} (C) macrophages of the indicated mice. Data shown are representative of three independent experiments.

during *L. major* infection, KRT14^{Cre}IL-4Rα$^{-/lox}$ on the C57BL/6 genetic background were infected subcutaneously in the footpad with 2×10^5 *L. major*. Lesion development and parasite load in the dLNs and spleen were analyzed and compared to lesion developing in control IL-4Rα$^{-/lox}$, IL-4Rα$^{+/+}$, and IL-4Rα$^{-/-}$ mice all on the C57BL/6 genetic background. *L. major* susceptible BALB/c mice were similarly infected and used for comparison. No difference in lesion development was observed between all groups of mice on the C57BL/6 genetic background, and BALB/c mice developed a significantly larger lesion (**Figure 2A**). Parasite load was also similar in the dLNs of these mice and no parasite dissemination to the spleen was observed, while significantly higher dLN parasite load and the presence of parasite dissemination to the spleen were observed in BALB/c mice (**Figures 2B,C**). Phlebotomine sand flies deposit significantly lower dose of parasites in the mammalian dermis, and it was previously reported that the cellular recruitment differ between subcutaneous infection in the footpad or intradermal infection in mouse ear dermis (29). To assess a potential role for IL-4Rα signaling in keratinocytes on disease outcome in more physiological conditions, we needle inoculated mice with 1×10^4 *L. major* LV39 metacyclic parasites, in the ear dermis of KRT14^{Cre}IL-4Rα$^{-/lox}$ mice. Lesion development and parasite load were compared to those observed in similarly infected IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ mice, IL-4Rα$^{+/+}$ mice all on the C57BL/6 genetic background. No difference in lesion size was observed in all groups of mice. In contrast, similarly infected BALB/c mice developed non-healing lesions (**Figure 2D**). All groups of mice on the C57BL/6 genetic background also controlled parasite load as assessed in the dLN and no parasite dissemination to the spleen was observed while similarly infected BALB/c mice showed higher parasite load in dLN and parasite disseminated to the spleen (**Figures 2E,F**). These data demonstrate that IL-4Rα signaling in keratinocytes is not required for control of lesion development and parasite burden. In addition, total lack of IL-4Rα signaling did not have an impact on the resolution of the infection. The control of parasite load in KRT14^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice suggested that an efficient protective Th1 immune response could develop in these mice in absence of IL-4Rα signaling.

Mice Deficient in IL-4Rα Signaling in Keratinocytes Are Capable to Mount a Th1 Cell Response following *L. major* Infection

To further investigate in details if IL-4Rα signaling in keratinocytes impacted Th1 cell differentiation during cutaneous leishmaniasis, KRT14^{Cre}IL-4Rα$^{-/lox}$ mice and control IL-4Rα$^{-/lox}$ were infected with 10^4 *L. major* (LV39). Four weeks after infection, the frequency of IFNγ and IL-4 producing cells was analyzed by flow cytometry at the site of infection. A high frequency of CD4$^+$IFNγ$^+$ was detected in the ear dermis of KRT14^{Cre}IL-4Rα$^{-/lox}$ mice, with no difference compared to that measured in similarly infected IL-4Rα$^{-/lox}$ control mice (**Figure 3A**). Only very low levels of CD45$^+$CD4$^+$IL-4$^+$ cells were detected in both groups (**Figure 3B**). The frequency of CD4$^+$IFNγ$^+$ cells observed in dLN cells of both groups of mice was also similar (**Figure 3C**) and more elevated than the frequency of CD45$^+$CD4$^+$IL-4$^+$ cells observed in dLN cells (**Figure 3D**) revealing that the CD4$^+$ Th1 cells are able to differentiate in absence of IL-4Rα signaling in keratinocytes.

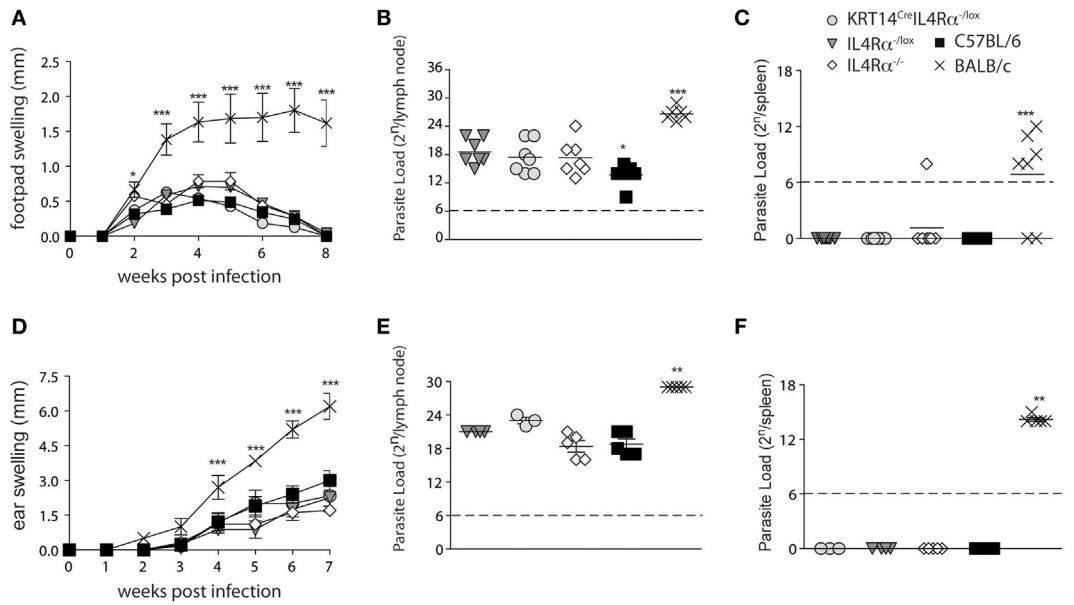

FIGURE 2 | Mice on a C57BL/6 genetic background that are deficient for IL-4Rα in their keratinocytes or on all cells are fully able to control their lesion size and parasite load following s.c. or i.d. *Leishmania major* infection. **(A)** KRT14^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$, C57BL/6, and IL-4Rα$^{-/-}$ mice were infected s.c. with 2×10^5 metacyclic *L. major* LV39 and lesion size was monitored. **(B)** Parasite load in draining lymph nodes (dLNs) and **(C)** spleen was analyzed by limiting dilution assays (LDAs) 8 weeks postinfection. **(D)** The indicated mice were infected in the ear dermis with 10^4 *L. major* and lesion development monitored. **(E)** Parasite load in the dLN or **(F)** spleen was analyzed by LDAs 7 weeks postinfection $^{**}p < 0.01$ $^{***}p < 0.001$ compared to BALB/c. $^{*}p < 0{,}05$ C57BL/6 compared to IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$, and KRT14^{cre}IL-4Rα$^{-/lox}$. These data are representative of two independent experiments.

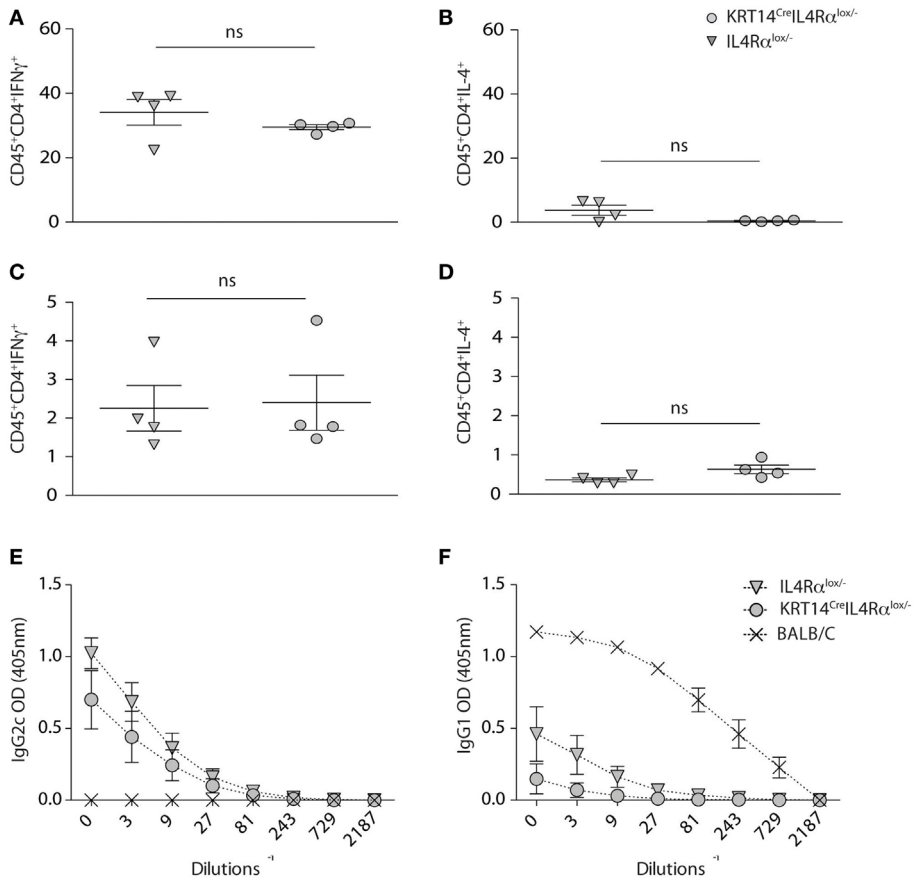

FIGURE 3 | Absence of IL-4Rα signaling selectively on keratinocytes does not impair T helper (Th) 1 cell differentiation. **(A,B)** KRT14^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$ were infected i.d. in the ear pinna with 10^4 *Leishmania major* parasites in the ear dermis. Seven weeks postinfection, the frequency of CD4$^+$IFNγ$^+$ and CD4$^+$IL-4$^+$ T cells at the site of infection (ears) was analyzed by flow cytometry. **(C,D)** Similar analysis was performed on draining lymph node cells at the same time point p.i. **(E)** Serum from these mice was analyzed and compared to that of BALB/c mice similarly infected for the presence of *L. major*-specific IgG2c and **(F)** IgG1. Data shown are representative of two independent experiments.

In line with the elevated levels of IFNγ detected at the site of infection and in dLN cells, high levels of serum IgG2c were observed in KRT14^{Cre}IL-4Rα$^{-/lox}$ and control IL-4Rα$^{-/lox}$ mice (**Figure 3E**). BALB/c mice showed elevated levels of IgG1, correlating with the high production of IL-4 observed in their CD4$^+$ T cells, while sera from KRT14^{Cre}IL-4Rα$^{-/lox}$ and control mice did not show significant IgG1 levels (**Figure 3F**), in line with the absence of Th2 cells detected in dLN and at the site of infection. Similar data were obtained following s.c. infection in the footpads with a higher dose of parasites (2×10^5 *L. major*, data not shown). Taken together, these results show that the absence of IL-4Rα signaling in keratinocytes does not impair the development of a protective Th1 type of immune response during early *L. major* infection.

Detection of IL-4 Expressing Cells in the Ear Dermis of C57BL/6 Mice 1 Day after *L. major* Infection

The lack of impact of IL-4Rα signaling on lesion development, parasite control, and the differentiation of CD4$^+$ Th1 cells at the site of infection and in the dLN firmly demonstrated that IL-4 signaling on keratinocytes was not involved in Th1 cell differentiation following *L. major* infection. However, it did not rule out an early role for keratinocyte-derived IL-4 in Th1 cell differentiation. To visualize the presence of *il-4* mRNA expressing cells in the mouse ear dermis cells of *L. major*-infected mice, we used the bicistronic IL-4 reporter (4get) mice on the C57BL/6 genetic background (22). We first determined if *il-4* mRNA expression was detectable in naive ear skin of C57BL/6 mice. A clear e-GFP-positive population was observed selectively in the CD45$^+$ hematopoietic population. These cells did not express CD11b at their cell surface and stained positive for c-kit, a marker specific for mast cells (**Figure 4A**). Further analysis by imaging flow cytometry confirmed that the e-GFP *il-4*-expressing cells corresponded to mast cells characterized by the expression of CD45, the lack of expression of CD11b, their large size (average of 14.5 μm of diameter) and the presence of numerous granules (**Figure 4B**). We then analyzed the modulation of *il-4* mRNA expressing cells 4 and 16 h following i.d. infection with *L. major,* at a time when expression of *il-4* mRNA by keratinocytes was reported to be the highest following subcutaneous infection in the footpad (21). No e-GFP$^+$ cells were detectable in the CD45-negative

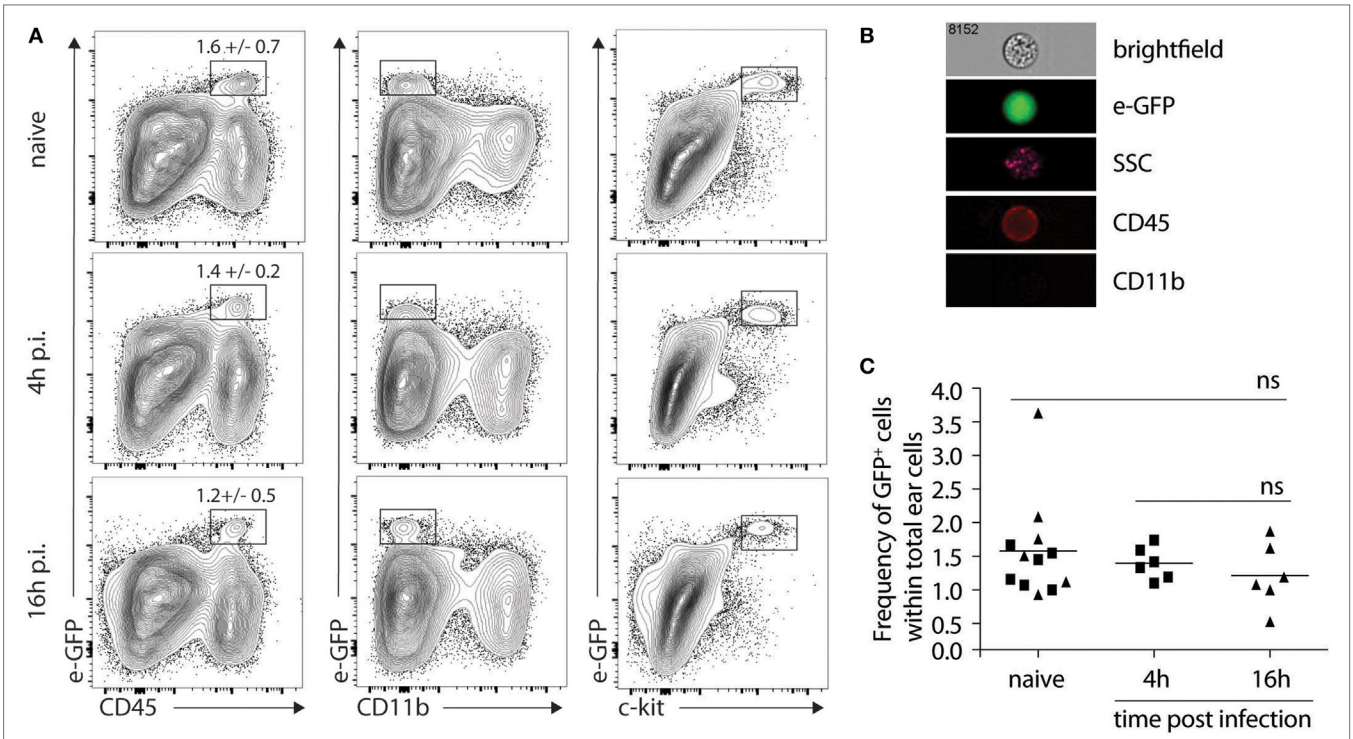

FIGURE 4 | Mast cells express *il-4* in naive and *Leishmania major*-infected ears of C57BL/6 mice. **(A)** C57BL/6 4get mice were inoculated i.d. into the right ear with 2×10^5 metacyclic *L. major*. Ears were recovered 4 or 16 h p.i. processed and analyzed by flow cytometry for e-GFP, CD45, CD11b and c-kit surface expression. As negative controls, naïve ears were also isolated and similarly processed **(B)**. Four hours postinfection, ears were similarly processed, stained with CD45 and CD11b, and analyzed by imaging flow cytometry. The e-GFP positive cells were all $CD45^+CD11b^-$, and showed a high granulosity (SSC) and a bright field phenotype typical of mast cells. A representative picture is shown. n.s: non-significant. **(C)** Frequency of e-GFP positive cells among total ear cells for 4get C57BL/6 at 0 (naïve), 4, or 16 h (▲) p.i. The data are pooled from three independent experiments.

cell population, suggesting that keratinocytes did not express detectable levels of *il-4* mRNA at the time analyzed. Mast cells were the only cell population where *il-4* mRNA, as assessed by e-GFP expression, was detectable 4 and 16 h p.i. (**Figure 4A** lower panels). The frequency of $e\text{-}GFP^+$ cell (approximately 1.5% of total ear cells) between infected and the contralateral uninfected ear was analyzed for each mouse. It did not vary between infected and uninfected ears at the analyzed time points (**Figure 4C**). These data show that most of the *il-4* mRNA expressing cells in the ear during the first 16 h post infection are mast cells and that *L. major* does not modulate *il-4* mRNA expression in these cells early during infection.

Mast Cells Do Not Produce IL-4 upon *L. major* Infection

Mast cells appeared to be the major dermal cells expressing IL-4 early after infection, suggesting that this source of IL-4 could play a role locally. To visualize IL-4 cytokine production, C57BL/6 KN2 (knockin huCD2) IL-4 reporter mice that specifically report the production of IL-4 protein were used. Cells producing IL-4 express a fragment of the human CD2 molecule at the cell surface making it easily detectable by flow cytometry (23). We crossed these KN2 mice with 4get mice to simultaneously detect IL-4 expression and production. First, to ensure that digestion of the ear would not impact IL-4 detection, KN2/4get mice, all on the C57BL/6 genetic background, were treated with anti-IFNγ and anti-IL-12 mAbs at the onset of infection to induce Th2 cell differentiation. Four weeks post infection, ears from mAb-depleted or control mice were isolated, processed, and analyzed by flow cytometry. Treatment with anti-IFNγ and anti-IL-12 promoted the differentiation of Th2 cells. *il-4* mRNA expression ($e\text{-}GFP^+$ cells) and IL-4 protein production (detected by hCD2 expression) were easily detectable (7.9%) in the ear dermis of $CD4^+$ T cells (**Figures 5A,C**). Minimal IL-4 expression or production (0.4%) was detectable in ear cells of similarly infected KN2/4get mice treated with a control mAb (**Figure 5B**) in line with the differentiation of Th1 cells in these mice. 4get/KN2 naïve ears (0 h p.i.) showed detectable *il-4* mRNA expression in $CD45^+$ but not in $CD45^-$ cells and no IL-4 proteins as no $huCD2^+$ cells were detected in either population, suggesting a lack of IL-4 production (**Figure 5D**). Infection did not change the frequency of cells transcribing *il-4* and no IL-4 production was observed 12 and 24 h after *L. major* infection (**Figures 5E–G**). These data suggest that ear skin mast cells transcribe the *il-4* gene but do not produce detectable IL-4 proteins at steady-state conditions, and *L. major* does not induce IL-4 production in the ear dermis early during infection.

Taken together, these results demonstrate that during the first hours of infection, *il-4* gene expression can be detected at the

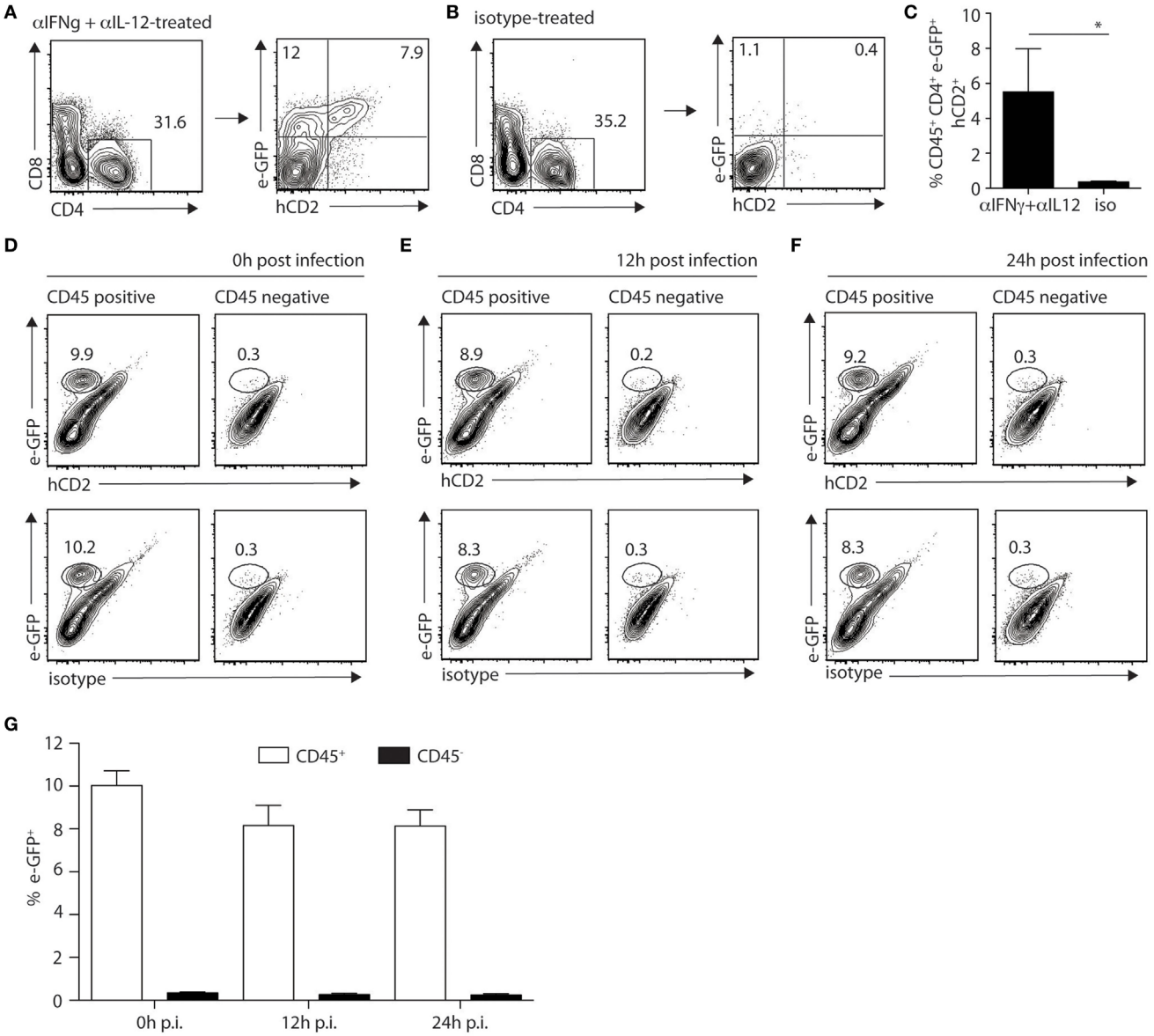

FIGURE 5 | IL-4 production is not detectable locally during the first hours of *Leishmania* infection in mice on the C57BL/6 genetic background. **(A)** C57BL/6 KN2/4get mice were injected intraperitoneally with anti-IFNγ and anti-IL-12 24 and 2 h before and 48 h after infection with 2×10^5 *Leishmania major* i.d. in the ear. Four weeks p.i. ears were isolated and digested prior to labeling with anti-CD4 and anti-CD8 mAbs. $CD4^+$ T cells were analyzed for the expression of *il-4* detected by e-GFP and protein production of IL-4 detected by surface staining with a huCD2 mAb. **(B)** C57BL/6 KN2/4get mice were similarly infected but injected with an isotype control mAb at the onset of infection. Four weeks postinfection, IL-4 expression and production were analyzed by detection of e-GFP and huCD2 staining, respectively. **(C)** Bar graph gives the frequency of $CD45^+CD4^+eGFP^+hCD2^+$ cells (means ± SD of 3–5 animals per group) $^*p < 0.05$. These are representative of three experiments. **(D–F)** C57BL/6 KN2/4get mice were inoculated into the right ear with 2×10^5 metacyclic *L. major.* Ears were recovered 0, 12, and 24 h p.i., processed, and stained with CD45, human CD2, or its isotype control mAbs and analyzed by flow cytometry to detect the expression (e-GFP) and production ($huCD2^+$) of IL-4. Representative flow cytometry profiles are shown **(D–F)** and **(G)** the bar graph shows the frequency of $e\text{-}GFP^+CD45^+$ and $e\text{-}GFP^+CD45^-$ cells (mean ± SD of 4 animals per group). Data are representative of three experiments.

infection site selectively in mast cell, but *il-4* mRNA expression was not modulated during *L. major* infection (analyzed at 0, 4,12,16, and 24 h p.i.). Furthermore, no IL-4 protein production was detectable in the ear dermis during the first 24 h after *L. major* infection.

Local Neutralization of IL-4 Does Not Impair Th1 Development in Response to *L. major* Infection

To further investigate if skin-derived IL-4 played a role in Th1 cell differentiation, C57BL/6 wild-type mice were infected

with 2×10^5 *L. major* in the ear dermis following the protocol described in **Figure 6A**, which is similar to that described previously (21). Two weeks post infection, the frequency of $CD4^+IFN\gamma^+$ and $CD4^+IL\text{-}4^+$ dLN T cells represented in average 0.6 and 0.13% of total $CD4^+$ T cells, respectively. These values were similar to those obtained in mice similarly infected but treated with a control mAb (**Figure 6B**). The levels of IFNγ and IL-4 secretion in dLN cells stimulated *ex vivo* with UV-treated *L. major* 2 weeks after infection also showed no significant differences between the anti-IL-4 and control groups (**Figure 6C**). In line with these data, no difference in parasite load was observed between groups depleted or not of IL-4 at the onset of infection (**Figure 6D**). These results show that neutralization of IL-4 locally in the ear dermis during the first hours of infection at the site of infection does not have an impact on the development of the Type-1 immune response during cutaneous leishmaniasis.

DISCUSSION

The study of the early events leading to the differentiation of Th1 or Th2 cells is of importance and has relevance in the design of vaccines generated against pathogens inducing a specific Th type of immune response. IL-4 and its signaling through interaction with the IL-4/IL-13 receptor has been described as an important cytokine favoring the differentiation of $CD4^+$ Th2 cells and type 2 immunity (30). However, several lines of evidence suggest that IL-4 may also be involved in Th1 cell differentiation (15–18, 31, 32). In this line, it was reported that following s.c. injection in the hind footpad of a very high dose (2×10^7) of *L. major* parasites, keratinocytes transiently upregulated *il4* mRNA mostly within the first 16 h of infection. Blocking of IL-4 at the infection site impaired IFNγ secretion by dLN T cells 7 days later and increased IL-4 secretion by dLN cells 1 week post infection (21).

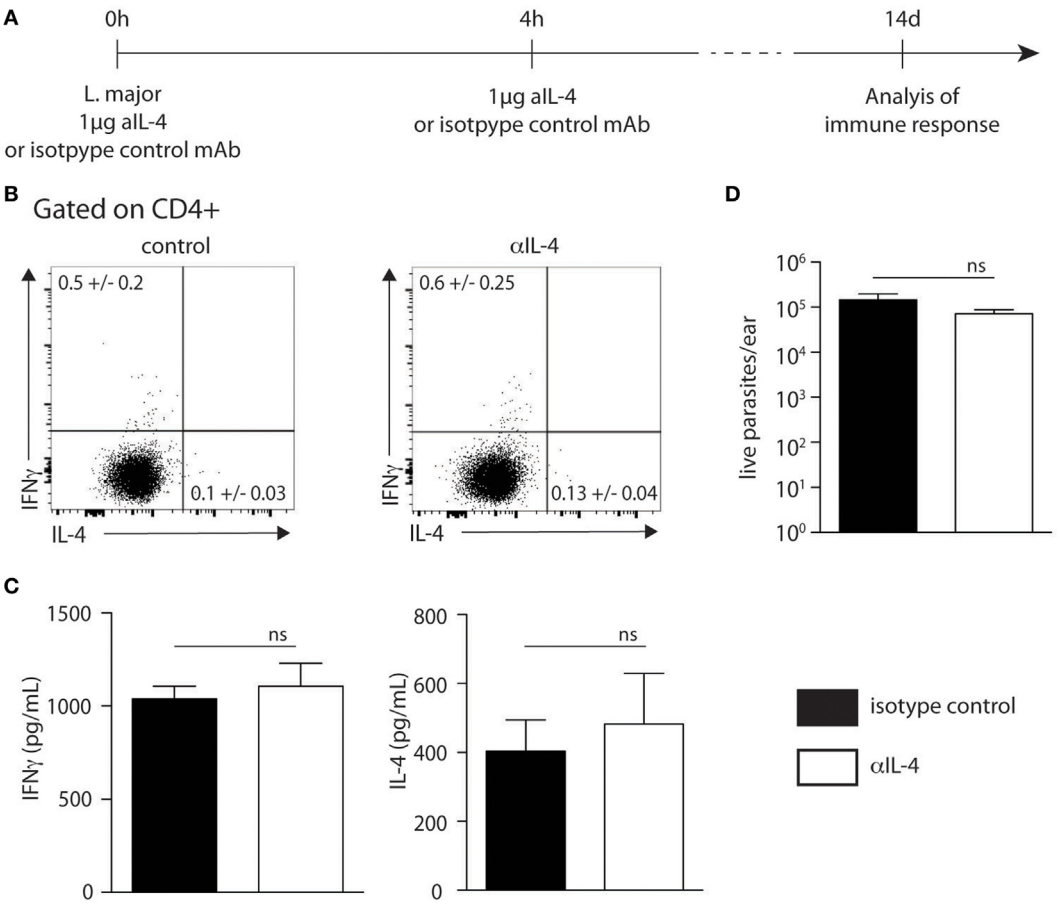

FIGURE 6 | Neutralization of IL-4 prior to infection with *Leishmania major* i.d. does not impact T helper (Th) 1 immune response. **(A)** Neutralization of IL-4 strategy. The anti-IL-4 mAb (11B11) or an isotype control mAb was injected intradermally simultaneously with the infection of 2×10^5 metacyclic *L. major* in the ear dermis. Four hours later, another injection of anti-IL-4 was given i.d. T helper immune response was analyzed 14 days later. **(B)** Two weeks postinfection, cervical draining lymph node (dLN) cells were recovered. The levels of IFNγ and IL-4 were assessed by intracellular staining and analyzed by flow cytometry. A representative plot is shown. **(C)** The levels of IFNγ and IL-4 secretion was quantified by ELISA in supernatants of dLN cells restimulated with UV-irradiated *L. major*. Mean cytokine expression ± SEM is given ($n \geq 3$ mice per group). The data are pooled from two independent experiments. **(D)** Parasite load in the ear analyzed by limiting dilution assay 2 weeks p.i. Data presented as mean ± SEM ($n \geq 3$ mice per group). Data shown are representative of two independent experiments. n.s: non-significant.

In an attempt to analyze if early after *L. major* inoculation, IL-4 secretion by keratinocytes could act in an autocrine way on these cells locally during the first days of infection, we generated mice genetically deficient in the IL-4Rα on keratinocytes. IL-4Rα hemizygosity (-/lox) was used to increase the probability of the floxed allele deletion by the Cre recombinase (24). IL-4 was previously shown to act on structural components of keratinocytes *in vitro*, a process that could be linked to epidermal structural problems (33). We show here that naive KRT14^{Cre}IL-4Rα$^{-/lox}$ mice are viable and have a normal phenotype. Upon infection with *L. major* i.d. in the ear pinna with a low or with a higher dose of parasites, mice with impaired IL-4Rα signaling on keratinocytes were able to resolve their cutaneous lesion, develop protective Th1 cells, and control parasite load. Similar data were obtained following infection of a higher dose of *L. major* subcutaneously in the footpad. These data demonstrate that following *L. major* infection IL-4Rα signaling in keratinocyte does not impair wound healing and is not required for the development of a protective type-1 immune response.

To directly visualize if IL-4 expression was induced in keratinocytes following infection with *L. major*, we used the well characterized IL-4-GFP reporter 4get mice (22). We detected *il-4*-transcribing cells in the ear dermis of naïve C57BL/6 mice and the majority of the positive cells were mast cells and not keratinocytes. Mast cells are tissue-resident cells and it was previously reported that mast cells from BALB/c mice constitutively express *il4* mRNA at steady state (34). The detection of e-GFP^{+} (*il-4* transcribing) mast cells in naïve ears detected here is thus in line with this study. Mast cell stimulation is required for these cells to secrete IL-4 protein. To verify if stimulated mast cells could be the source of the early IL-4 putatively present in C57BL/6 skin of *L. major*-infected mice, we used the KN2 reporter mice on the C57BL/6 genetic background, which allow the visualization of cells that produce IL-4 protein (23). Mast cells did not produce detectable IL-4 levels upon *L. major* infection within the first 24 h p.i. in KN2 mice. It was previously reported that C57BL/6 mice deficient in mast cells (C57BL/6 Cpa^{Cre}) were perfectly able to mount a Th1 type of cell immune response following *L. major* infection (35). Together with our study, these results rule out a role for early mast cell-derived IL-4 in driving Th1 cell differentiation and resolution of lesion size and parasite load following *L. major* infection. In addition, *il4* transcription was not detectable in keratinocytes during the first day of *L. major* infection.

It cannot be excluded that a few cells may locally express *il-4* at low levels at steady state or upon *L. major* infection and that these cells were not detectable using *L. major*-infected 4get mice. Nevertheless, as no detectable IL-4-producing cells were observed in the skin of infected KN2 mice the possibility of IL-4 driving Th1 differentiation following infection with *L. major* is rather unlikely.

Several factors may explain the differences observed between the present report and previous studies reporting a role for IL-4 in Th1 differentiation following *L. major* infection. First, the parasite dose injected differed in the two studies. Ehrchen et al. reported *il-4* mRNA expression by keratinocytes during the first day after infection with a vey high dose (2×10^7) of *L. major* inoculated s.c. in the hind footpad (21). During their blood meal, sand flies deposit a much lower number of parasites ranging from 10–10^4 and it was shown that the size of the parasite inoculum can have an impact on the immune response (36). Here, following infection of reporter mice with 2×10^5 *L. major*, no detectable levels of *il4* mRNA or IL-4 proteins were locally measured during the first days of infection. The parasite dose injected here is 100 times lower than that used in the study by Ehrchen et al., potentially explaining the differences observed. In addition, here, we used infective metacyclic *L. major* rather than stationary phase parasites to infect mice. Furthermore, the sensitivity of the microarray analysis used in the study by Ehrchen et al. is higher than that of the reporter system used here, as qPCR analysis can detect minute amounts of *il-4* mRNA. The absence of IL-4 proteins detected here suggests that if some IL-4 is produced locally following i.d. inoculation of parasites, it is in very low quantities.

A second major difference between the two studies is the site of parasite inoculation. Here, *L. major* was inoculated in the ear dermis, while in the Ehrchen's study it was injected subcutaneously in the hind footpad. Recruitment of innate cells to the site of infection was recently shown to differ whether *L. major* was inoculated in the hind footpad or in the ear dermis of mice, with a distinct impact on the local microenvironment at the site of infection (29). However, here, we show that irrespective of the site of infection, the absence of IL-4Rα on keratinocytes in *L. major*-infected mice did not have any impact on lesion development and immune response.

Distinct *Leishmania* species elicit different immune response. In addition, distinct *L. major* strains also have diverse virulence [reviewed in Ref. (28)]. This could have explained the differences observed in the two studies. However, mice on the C57BL/6 genetic background with total abrogation of IL-4Rα on all cells were fully able to control lesion size and parasite load following either intradermal or subcutaneous infection with 2×10^5 *L. major* of two different strains (LV39 or IR81), a process correlating with the development of functional Th1 cells in response to infection (here and *unpublished data*). Collectively, these data suggest that the difference observed in the two studies may not result from differences in the site of parasite inoculation or *L. major* strain virulence but rather come from differences in the parasite dose inoculated.

Distinct skin microbiota present in different animal facilities may also explain the differences observed between the previous and the present study as microbiota was shown to affect the immune response (37). This is, however, unlikely to be the explanation as the data presented here were obtained in two distinct laboratories with similar results. Furthermore, despite repeated attempts of blocking IL-4 locally using different parasite inoculation dose and injection scheme of anti-IL-4 mAb as well as distinct *L. major* parasite strains (here and *unpublished data*) we could not detect an impact of IL-4 blockade on the immune response and parasite control between 14 and 21 days post infection.

Another study reported the importance of early IL-4 in CD4^{+} Th1 differentiation (16). In this study, BALB/c mice infected with 2×10^5 *L. major* LV39 stationary phase promastigotes were treated with recombinant IL-4 at the time and 8 h post *L. major* infection. Administration of a relatively high dose (1 μg) but not of a lower dose (0.1 μg) of IL-4 instructed resistance in

these otherwise *L. major* susceptible BALB/c mice. One major difference with the present study is that BALB/c mice were used in that study. Furthermore, the dose of IL-4 injected locally may not correspond to cytokine levels present at the site of infection in *L. major*-infected C57BL/6 mice. If such levels of IL-4 protein had been present locally, it would have been detected here using the IL-4 reporter mice. Collectively, these and our data suggest that the early presence of IL-4 may have a different impact on the development of Th1 response depending on the dose of parasite inoculated, the genetic background of the mice, and the local amount of IL-4 protein present at the site of infection.

Altogether, our data provide strong evidence that IL-4 signaling in keratinocytes at the site of infection or in general, is not required for the differentiation of Th1 cells following *L. major* infection and that IL-4 protein production at the onset of infection is not required for Th1 cell differentiation in *L. major*-infected C57BL/6 mice.

ETHICS STATEMENT

All animal experimental protocols were approved by the veterinary office regulations of the State of Vaud, Switzerland, authorization 1266.6-7 to FT-C and performed in compliance with Swiss ethics laws for animal protection. All mouse experiments performed at the University of Cape Town were performed in strict accordance with the South African national guidelines, as well as the Animal Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (license no. 015/034). All efforts were made to minimize and reduce suffering of animals.

AUTHOR CONTRIBUTIONS

MD, BH, MG, KP, BM-S, and RH performed the experiments and contributed to their analysis. BH, MG, and KP made the figures. FT-C, RG, and FB contributed to the conceptual design and provided funding for this project. FT-C wrote the manuscript with comments from BH, MD, KP, RH, RG, and FB.

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Galectin-1 Impairs the Generation of Anti-Parasitic Th1 Cell Responses in the Liver during Experimental Visceral Leishmaniasis

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Many infectious diseases are characterized by the development of immunoregulatory pathways that contribute to pathogen persistence and associated disease symptoms. In diseases caused by intracellular parasites, such as visceral leishmaniasis (VL), various immune modulators have the capacity to negatively impact protective CD4$^+$ T cell functions. Galectin-1 is widely expressed on immune cells and has previously been shown to suppress inflammatory responses and promote the development of CD4$^+$ T cells with immunoregulatory characteristics. Here, we investigated the role of galectin-1 in experimental VL caused by infection of C57BL/6 mice with *Leishmania donovani*. Mice lacking galectin-1 expression exhibited enhanced tissue-specific control of parasite growth in the liver, associated with an augmented Th1 cell response. However, unlike reports in other experimental models, we found little role for galectin-1 in the generation of IL-10-producing Th1 (Tr1) cells, and instead report that galectin-1 suppressed hepatic Th1 cell development. Furthermore, we found relatively early effects of galectin-1 deficiency on parasite growth, suggesting involvement of innate immune cells. However, experiments investigating the impact of galectin-1 deficiency on dendritic cells indicated that they were not responsible for the phenotypes observed in galectin-1-deficient mice. Instead, studies examining galectin-1 expression by CD4$^+$ T cells supported a T cell intrinsic role for galectin-1 in the suppression of hepatic Th1 cell development during experimental VL. Together, our findings provide new information on the roles of galectin-1 during parasitic infection and indicate an important role for this molecule in tissue-specific Th1 cell development, but not CD4$^+$ T cell IL-10 production.

Keywords: ***Leishmania*, visceral leishmaniasis, galectin-1, T cells, inflammation**

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INTRODUCTION

Dysregulated cellular immune responses are a feature of many chronic infectious diseases (1). Visceral leishmaniasis (VL) caused by the protozoan parasites *Leishmania donovani* and *L.infantum* (*chagasi*) represents one such disease. VL is present in the Indian sub-continent, the Americas, Mediterranean Basin, and East Africa. There are between 200,000 and 400,000 VL cases and

around 30,000 deaths each year (2). In VL patients, *Leishmania* parasites rapidly infect macrophages throughout the viscera and become established in the liver, spleen, lymph nodes, and bone marrow (BM) (3). To date, much of our knowledge regarding host immune responses to *L. donovani* comes from studies in genetically susceptible C57BL/6 and BALB/c mice, which display tissue-specific immune responses to infection. The liver is a site of acute, resolving infection, while chronic infections are established in the spleen and BM (3, 4). These disparate responses appear to reflect aspects of asymptomatic infection and fulminant disease in humans, respectively (3, 4). While the immune mechanisms involved in hepatic parasite control have been extensively characterized, the underlying causes of parasite persistence in the spleen and BM are less well understood. The establishment of protective immunity is critically dependent on the generation of pro-inflammatory $CD4^+$ T cells producing IFNγ and TNF (5, 6). These Th1 cells subsequently promote antimicrobial activity in parasitized macrophages (7). However, chronic disease is characterized by the establishment of potent immunoregulatory networks causing profound impairment in these protective immune responses (4).

A better understanding of immunoregulatory networks will be crucial for future efforts to treat chronic infection. One of the most potent immunoregulatory molecules identified to date in both mouse models of VL and VL patients is IL-10. While IL-10 signaling appears to be necessary for restricting tissue damage that occurs as a result of excessive inflammation (8), both experimental (9, 10) and clinical (11–15) data suggest that this immunoregulatory cytokine contributes to the establishment and/or maintenance of chronic infection during VL. Similar roles for IL-10 have also been described in other infectious diseases, including tuberculosis (16), toxoplasmosis (17), and malaria (18). In C57BL/6 mice infected with *Plasmodium chabaudi* AS, IL-10 deficiency had a minimal impact on parasite growth but caused significant pathology, as indicated by increased anemia and liver damage (19).

Galectin-1 is the prototypical member of a large family of β-galactoside-binding proteins, collectively known as galectins, involved in a wide range of immunomodulatory functions (20). Indeed, all immune cells express galectins to varying extents, though they are notably upregulated on activated B cells, NK cells, macrophages, and both conventional T cells and $FoxP3^+$ regulatory T (Treg) cells (21). The pleiotropic nature of galectin-1 arises, in part, on the distribution of the functionally disparate intracellular and extracellular forms of the molecule on different cell populations (20). Intracellular galectin-1 exists primarily in monomeric form and regulates cell growth *via* interactions with Ras family proteins (22). Conversely, the dimeric form of galectin-1 is responsible for lectin activity, which acts as a negative regulator of immune responses (23). Upon secretion, galectin-1 spontaneously dimerizes, whereupon the stability and functionality of the protein is critically dependent on rapid binding to extracellular glycan ligands (23, 24). Previously described functions for galectin-1 in the context of effector T cell regulation include the induction of apoptosis in effector lymphocytes (25–27) and the promotion of immunoregulatory T cell phenotypes (28–30). In addition, $Foxp3^+$ Treg cell suppressive dysfunction has been reported in galectin-1-deficient ($Lgals1^{-/-}$) mice (31), suggesting that galectin-1 is required for optimal Treg cell function. $Lgals1^{-/-}$ mice also exhibit increased pro-inflammatory cytokine production (32), and are more susceptible to autoimmune disease than their wild-type (WT) counterparts (31). Recombinant galectin-1 has been tested as a therapeutic agent in various models of inflammatory disease including arthritis (33), hepatitis (34), type-1 diabetes (35), and graft-versus-host disease (36). Conversely, galectin-1 has been implicated in the promotion of cancer cell immune evasion (37, 38), and blockade of tumor-derived galectin-1 promotes tumor rejection *via* the augmentation of pro-inflammatory T cell responses (39). Similarly, galectin-1 exacerbates disease in models of Hodgkin's lymphoma by inducing Th2 polarization and expansion of Treg cell populations that impair antitumor responses (40). Neutralizing antibodies (41) and effective inhibitors of galectin-1 binding (42) are currently being evaluated as therapeutic agents in clinical trials aimed at treating various cancers.

One of the important consequences of galectin-1 interactions with T cells is the polarization of naïve and effector T cells to a regulatory phenotype. Naïve T cells stimulated with recombinant galectin-1 *in vitro* rapidly differentiate into an IL-10-producing Th1 (Tr1) cell phenotype (28). This process occurs in either the presence or absence of APC (29), suggesting that galectin-1 can act directly or indirectly on T cells to alter their function. Galectin-1 can additionally enhance the production of IL-10 by Tr1 cells *via* the generation of tolerogenic dendritic cells (DCs) by ligation to CD43 on the DC surface and the subsequent promotion of IL-27 secretion (30), which stimulates IL-10 production by Tr1 cells. This mechanism of galectin-1 immunoregulatory function was shown to contribute to enhanced parasite control, survival, and Th1 effector function in $Lgals1^{-/-}$ mice infected with *Trypanosoma cruzi* (43). However, in this latter study, galectin-1 promoted DC-mediated induction of Treg cells rather than Tr1 cells.

To determine whether similar mechanisms of galectin-1-mediated immune regulation influenced disease outcome in another important parasitic disease, we infected $Lgals1^{-/-}$ mice and WT controls with *L. donovani* and assessed control of parasite growth and associated immune responses. Here, we show that galectin-1 suppressed control of hepatic parasite growth without modulating the induction of Tr1 cells. Instead, galectin-1 restricted optimal Th1 cell effector function in the liver. These findings extend our understanding of the diverse roles for galectin-1 in infectious diseases, as well as providing insight into how modulation of galectin-1 may be harnessed for therapeutic advantage.

MATERIALS AND METHODS

Infections

Leishmania donovani (LV9; MHOM/ET/67/HU3) was originally isolated from a patient in Ethiopia in 1967 and subsequently maintained by animal passage (44). The parasite line was transferred from the London School of Hygiene and Tropical Medicine (London, UK) to QIMR Berghofer in 2002 and maintained by passage in $B6.Rag1^{-/-}$ mice. Amastigotes were isolated from

chronically infected passage animals. Experimental mice were infected by injection of 2×10^7 amastigotes i.v. *via* the lateral tail vein. Cohorts were culled at respective time-points post-infection (p.i.) by CO_2 asphyxiation and bled *via* cardiac puncture. Spleens were removed and livers perfused then removed, with parasite burden determined *via* histological assessment of Giemsa-stained (Diff-Quick; Lab Aids) liver and spleen tissue impressions and expressed in Leishman–Donovan units; calculated as the number of parasites per 1,000 nuclei multiplied by the organ weight. Hepatic, splenic, and BM mononuclear populations were isolated as previously described (10, 45).

Mice

Inbred female C57BL/6 and congenic B6.CD45.1 mice, 6 weeks of age, were purchased from the Animal Resource Centre (Canning Vale, WA, Australia). C57BL/6 mice with a specific deletion of the gene-encoding galectin-1 (43) (*Lgals1*$^{-/-}$) were obtained from the Jackson Laboratory and, along with B6.CD11c.DOG mice (46), were bred in-house at QIMR Berghofer (Brisbane, QLD, Australia). All mice were age- and sex-matched and maintained in-house under pathogen-free conditions. All animal procedures were conducted with the approval of the QIMR Animal Ethics Committee under the animal ethics number A02-634M and in accordance with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (Australian NHMRC, Canberra).

Generation of Mixed BM Chimeric Mice

Chimeric mice were generated by lethally irradiating mice with two doses of 5.5 cGy and subsequently engrafting with 10^6 freshly isolated BM cells i.v. *via* the lateral tail vein, as previously described (47). To examine the consequences of antigen presentation by DC in the absence of galectin-1 signaling, a 50:50 mix of B6.CD11c.DOG and either *Lgals1*$^{-/-}$ or congenic WT B6.CD45.1 BM cells were engrafted into a WT recipient. Mice were maintained on pyrimethamine/neomycin sulfate for 2 weeks post-engraftment and infected approximately 8–12 weeks thereafter, as previously described (48). Following cellular reconstitution and subsequent infection, mice harboring a B6.CD11c.DOG hematopoietic compartment received 8 ng/g body weight diphtheria toxin (DT) intraperitoneally (i.p.) every 3 days over a 2-week period, whereupon animals were euthanized. This strategy resulted in all B6.CD11c.DOG DC being depleted by DT, leaving behind only WT or *Lgals1*$^{-/-}$ DC for antigen presentation to T cells.

A second set of mixed BM chimeras were generated as above, except that irradiated recipients were engrafted with a 90:10 mix of congenic (CD45.1) WT (90%) and either *Lgals1*$^{-/-}$ or WT (both CD45.2) (10%) BM cells. This strategy allowed *Lgals1*$^{-/-}$ CD4$^+$ T cells activity to be measured in a predominantly WT immunological background and compared with appropriate WT control CD4$^+$ T cells following infection.

Flow Cytometry

All organ-derived mononuclear cells were prepared as described previously (10, 47, 49). Fluorescently conjugated mAbs against CD4 (GK1.5), CD8α (53-6.7), TCRβ (H57-597), B220 (RA3-6B2), CD19 (6D5), Foxp3 (MF-14), IFNγ (XMG1.2), IL-10 (JES5-16E3), TNF (MP6-XT22), CD11c (N418), CD11b (M1/70), MHC-II (M5/114.15.2), F4/80 (BM8), Ly6C (HK1.4), Ly6G (IA8), NK1.1 (PK136), CD45.1 (A20), CD45.2 (104) (Biolegend, San Diego, CA, USA), and Galectin-1 (R&D Systems, Minneapolis, MN, USA) were used. Dead cells were excluded from analysis using LIVE/DEAD Fixable Aqua Stain (Invitrogen), as per the manufacturer's instruction. Both cell surface and intracellular staining was undertaken according to methods described previously (10), with all samples acquired on a BD LSRFortessa (BD Biosciences). Gating strategies used for analysis are outlined in the manuscript. For analysis of intracellular IL-10, cells were stimulated for 3 h at 37°C and 5% CO_2 in the presence of PMA and ionomycin in addition to Brefeldin A, as described previously (10).

Statistical Analysis

Statistical analysis was performed exclusively in GraphPad Prism 5 and 6 (GraphPad Software, La Jolla, CA, USA). A non-parametric, unpaired Mann–Whitney test was used for comparisons between two groups. A p value of <0.05 was considered significant. Graphs depict mean ± SEM.

RESULTS

Galectin-1 Is Upregulated on T Cell Subsets following Establishment of *L. donovani* Infection

Antigen-presenting cells expressing galectin-1 have previously been shown to promote an IL-10-mediated immunoregulatory pathway (30). To determine whether T cells expressing galectin-1 might also play immunoregulatory roles, T cell subsets from livers and spleens of *L. donovani*-infected C57BL/6 mice at day 56 p.i. were assessed for galectin-1 expression (**Figure 1A**). We chose this time point because infection had largely resolved in the liver and effective CD4$^+$ T cell-mediated, concomitant immunity was established. In contrast, the spleen was a site of chronic infection associated with dysregulated CD4$^+$ T cell responses (50, 51). Thus, day 56 p.i. represents two extremes of infection outcome. Elevated numbers of galectin-1-expressing CD8$^+$ and CD4$^+$ T cells, including Foxp3$^+$ Treg cells, were observed in both liver (**Figure 1B**) and spleen (**Figure 1C**), although increased frequencies of galectin-1-expressing T cell subsets was only found in the latter tissue. However, the level of expression on a per-cell basis [i.e., the galectin-1 mean fluorescent intensity (MFI) value] was significantly ($p < 0.05$ and $p < 0.01$) higher than naïve equivalents for all populations, except hepatic Foxp3$^+$ Treg cells (**Figures 1B,C**).

Given previous association between galectin-1 and IL-10-producing T cells (28–30), and the importance of Tr1 cells for disease outcome in experimental VL (10), the level of galectin-1 expression on IL-10 and IFNγ-producing CD4$^+$ effector cells was next examined (**Figure 2A**). We found that a greater frequency of IL-10$^+$IFNγ$^+$ Tr1 cells expressed galectin-1, compared with IL-10$^-$ IFNγ$^+$ Th1 cells, in the liver (**Figure 2B**) and spleen (**Figure 2C**), but expression was only significantly ($p < 0.01$) higher on a per-cell basis in the liver. However, given the lower number of

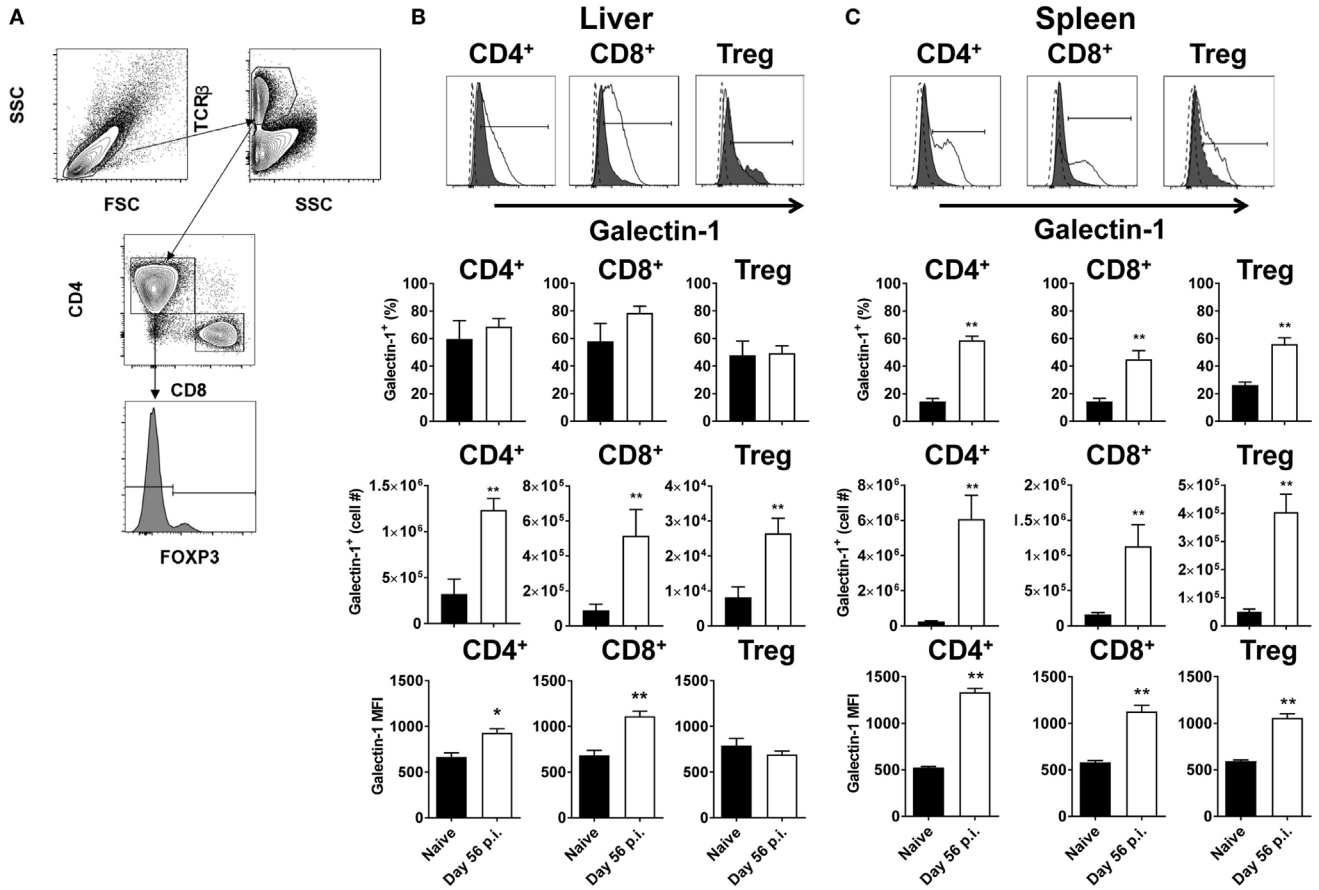

FIGURE 1 | Galectin-1 expression on T cell subsets during established infection. Galectin-1 expression on T cell subsets from C57BL/6 mice ($n = 5$) infected with *Leishmania donovani* (day 56 p.i.) was assessed by flow cytometry and compared with naïve control animals. The gating strategy used to identify conventional ($Foxp3^-$) $CD4^+$, $CD8^+$, and regulatory ($Foxp3^+$) T cells is shown **(A)**. The frequency and number of galectin-1-positive cells, as well as the relative expression of galectin-1 determined by mean fluorescent intensity (MFI) from histograms shown for the liver **(B)** and spleen **(C)** were measured, as indicated. The dotted histograms represent staining with the isotype control antibody, dark gray histograms reflect cells from naïve animals, and white histograms reflect cells from infected animals. These data represent one of two separate experiments ($^*p < 0.05$, $^{**}p < 0.01$; Mann–Whitney test, relative to naïve).

Tr1 cells, relative to Th1 cells (**Figure 2A**), the number of Tr1 cells expressing galectin-1 was significantly ($p < 0.01$) lower than Th1 cells in both organs studied (**Figures 2B,C**). Thus, galectin-1 was highly expressed by Tr1 cells, but the number of Th1 cells expressing this molecule in both tissues was significantly greater at the day 56 p.i. time point examined.

Tissue-Specific Upregulation of Galectin-1 on Non-T Cell Populations

We next investigated galectin-1 expression patterns on antigen-presenting cell populations at the same time point (i.e., day 56 p.i.). Galectin-1 expression (as determined by MFI) was substantially upregulated on hepatic (**Figure 3**) and splenic (**Figure 4**) macrophages, compared to naïve counterparts. Galectin-1 expression on monocytes did not change in the liver (**Figure 3**) and decreased in the spleen (**Figure 4**), but due to increased leukocyte numbers in these tissues as a result of infection (47), the number of monocytes expressing galectin-1 was increased in both tissues (**Figures 3B** and **4**). Hence, in the spleen, there were more monocytes expressing lower levels of galectin-1 in infected mice, compared to naïve counterparts. Other tissue-specific discrepancies in galectin-1 expression were observed on DC subsets, with increased expression noted on splenic $CD8^+$ and $CD8^-$ DC, but not on the same DC subsets in the liver (**Figures 3B** and **4**). A small subpopulation of B cells was found to express galetin-1 during established infection in both the liver and spleen, although this population and galectin-1 expression was more prominent in the spleen (**Figures 3B** and **4**). Therefore, galectin-1 was expressed by multiple antigen-presenting cell populations during established *L. donovani* infection, but expression varied, depending on cell type and tissue location.

The Impact of Galectin-1-Deficiency on *L. donovani* Infection

Given the increased expression of galectin-1 on key immune cell populations in infected tissues, the influence of galectin-1 on *L. donovani* infection was assessed using mice lacking galectin-1 expression (*Lgals1*$^{-/-}$). *Lgals1*$^{-/-}$ mice exhibited

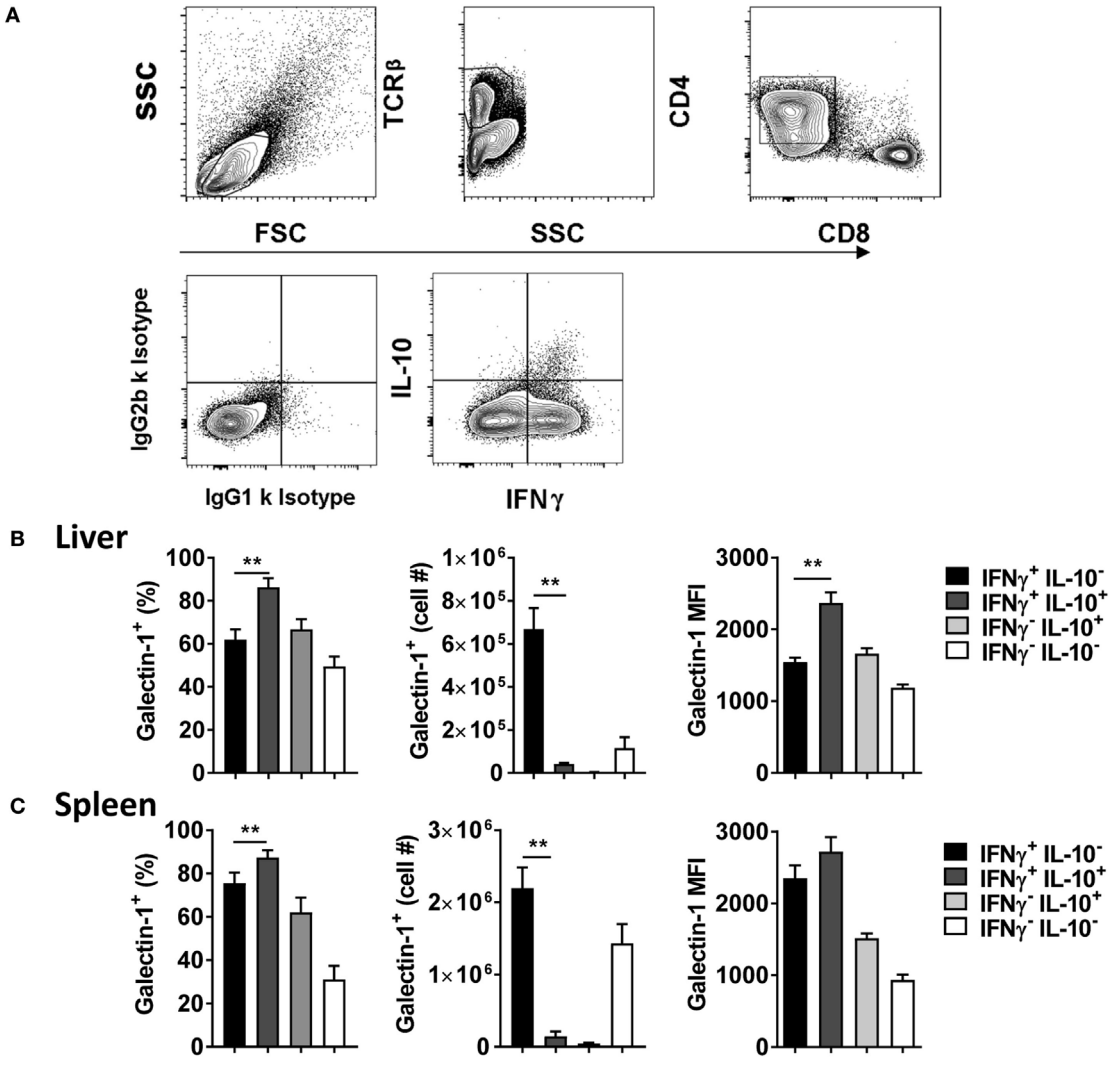

FIGURE 2 | Galectin-1 expression on IFNγ- and IL-10-producing CD4+ T cells during established infection. IFNγ and IL-10 production by CD4+ T cells from C57BL/6 mice ($n = 5$) infected with *Leishmania donovani* (day 56 p.i.) was assessed by flow cytometry. The gating strategy used to identify cytokine-producing CD4+ T cells is shown **(A)**. The frequency and number of galectin-1-positive cytokine-producing cells, as well as the relative expression of galectin-1 determined by mean fluorescent intensity (MFI), on these cells in the liver **(B)** and spleen **(C)** were measured, as indicated. These data represent one of two separate experiments (**$p < 0.01$; Mann–Whitney test between IL-10− IFNγ+ and IL-10+ IFNγ+ CD4+ T cells only is shown).

significantly ($p < 0.05$ and $p < 0.01$) reduced parasite burdens in the liver during the acute phase of infection, as compared to WT control animals (**Figure 5A**). However, splenic parasite burdens remained unchanged between *Lgals1*$^{-/-}$ mice and controls across the course of infection (**Figure 5B**). Galectin-1 has previously been associated with increased IL-10 production in conventional and regulatory CD4+ T cell subsets (30). In addition, we recently reported that IL-10 production by Tr1 cells suppressed anti-parasitic immunity in the liver of *L. donovani*-infected mice (10). Therefore, subsequent cellular analysis focused on alterations in pro- and anti-inflammatory responses by key CD4+ T cell subsets in the liver and spleen. An increased frequency of IFNγ-producing CD4+ T cells was found in the livers of *Lgals1*$^{-/-}$ mice at day 14 p.i., which translated to significant ($p < 0.05$) increase in the total number of IFNγ-producing cells in this organ, compared with control animals (**Figure 5C**). Despite a similar trend in the spleen, differences in frequency and number of IFNγ-producing cells were not significantly different (**Figure 5C**). Both regulatory (Foxp3+) and conventional (Foxp3−) CD4+ T cells have the potential to produce IL-10 during *L. donovani* infection (10). However, no difference in the frequency or number of either of these IL-10-producing CD4+ T cells was observed in the liver (**Figure 5C**) or spleen (**Figure 5D**) at days 14 p.i. Together, these data indicate that galectin-1 modulates protective CD4+ T cell responses during acute, hepatic *L. donovani* infection by limiting numbers of IFNγ-producing CD4+ T cell numbers, and not by promoting CD4+ T cell IL-10 production.

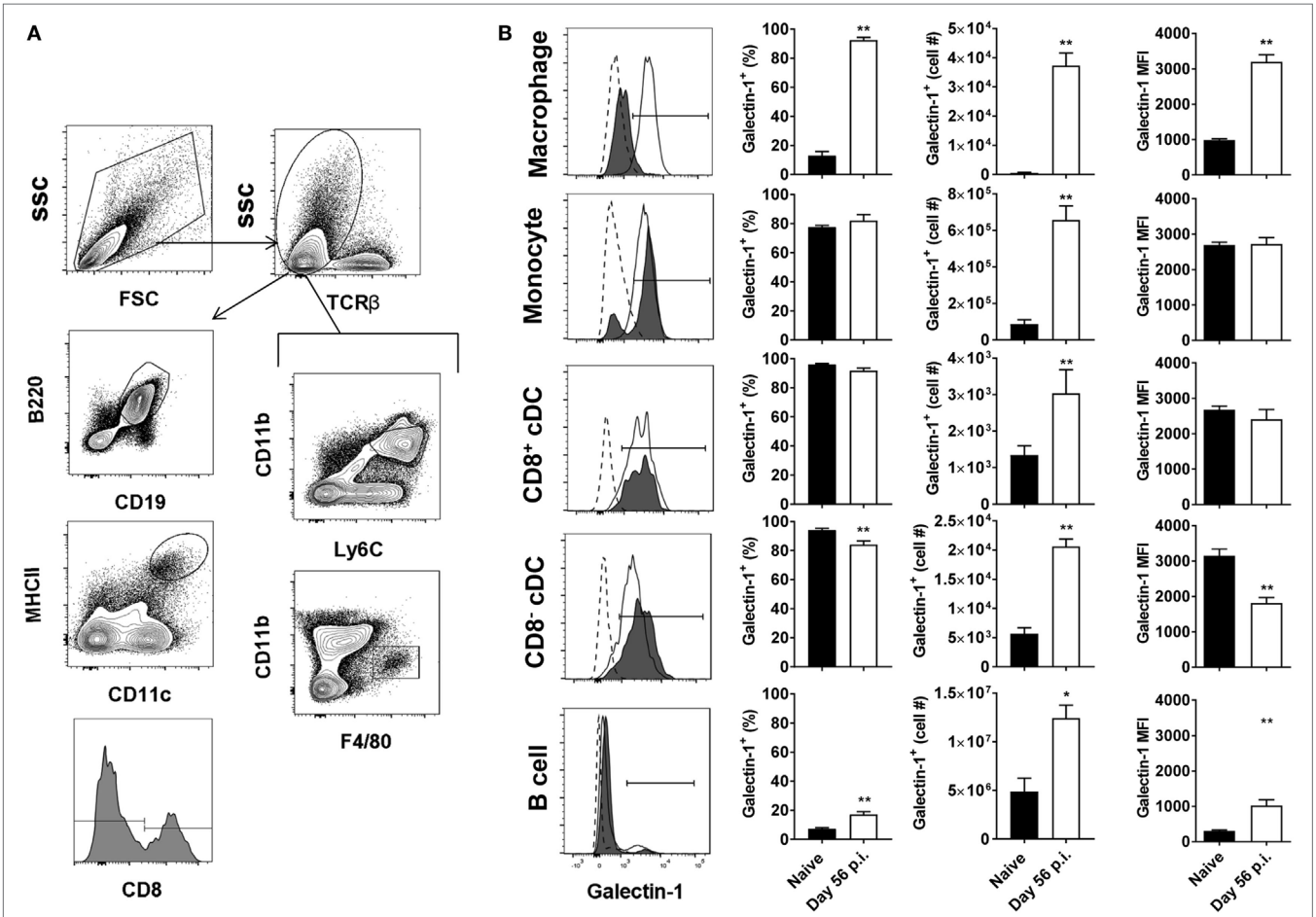

FIGURE 3 | Galectin-1 expression on hepatic antigen-presenting cells during established infection. Galectin-1 expression on antigen-presenting cells from C57BL/6 mice ($n = 5$) infected with *Leishmania donovani* (day 56 p.i.) was assessed by flow cytometry and compared with naïve control animals. The gating strategy used to identify antigen-presenting cells in the liver is shown **(A)**. The frequency and number of galectin-1-positive cells, as well as the relative expression of galectin-1 determined by mean fluorescent intensity (MFI) from histograms, were measured, as indicated **(B)**. Dotted histograms reflect isotype control antibody staining, dark gray histograms reflect cells from naïve animals, and white histograms reflect cells from infected animals. These data represent one of two separate experiments (**$p < 0.01$; Mann–Whitney test, relative to naïve).

Galectin-1 Expression Early during *L. donovani* Infection

Given that enhanced control of parasite growth in the liver was observed early after infection, we next examined galectin-1 expression on immune cell populations at day 14 p.i. (**Figure 6**). Strikingly, the number of galectin-1-positive cells in the liver was elevated for all immune cell subsets examined (**Figure 6A**), while only numbers of $CD8^+$ T cells and $CD8^-$ DCs expressing galectin-1 in the spleen were increased (**Figure 6B**), relative to cells from naïve mice. Of note, the ratio of galectin-1-positive $CD4^+$ T cells to galectin-1-positive Treg cells was higher in the liver, compared to the spleen, in naïve animals (143 ± 23 versus 15 ± 3 for liver and spleen, respectively), and these ratios did not change significantly following *L. donovani* infection. Similar to day 56 p.i., the number of Tr1 cells expressing galectin-1 was significantly lower than Th1 cells in both liver (**Figure 6C**) and spleen (**Figure 6D**). It should be noted that the number of galectin-1-positive $CD4^+$ T cells lacking both IFNγ and IL-10 expression were the most numerous $CD4^+$ T cell subset in both tissues studied at this time point (**Figures 6C,D**). Therefore, galectin-1 expression increased on a broader range of immune cells in the liver than the spleen, and the biggest change in cell numbers occurred in liver $CD4^+$ T cells. Given these findings, and the importance of DCs for priming $CD4^+$ T cells in experimental VL (47), we next focused on investigating the role of galectin-1 on hepatic DCs and $CD4^+$ T cells.

Galectin-1 Directly Influences Hepatic Th1 Cell Function during Early *L. donovani* Infection

To determine whether galectin-1 mediates its immunoregulatory function through DCs, we generated BM chimeras that comprised a 50:50 mix of CD11c.DOG and WT (CD45.1) or *Lgals1*$^{-/-}$ cells. This allowed us to deplete transgenic DCs *via*

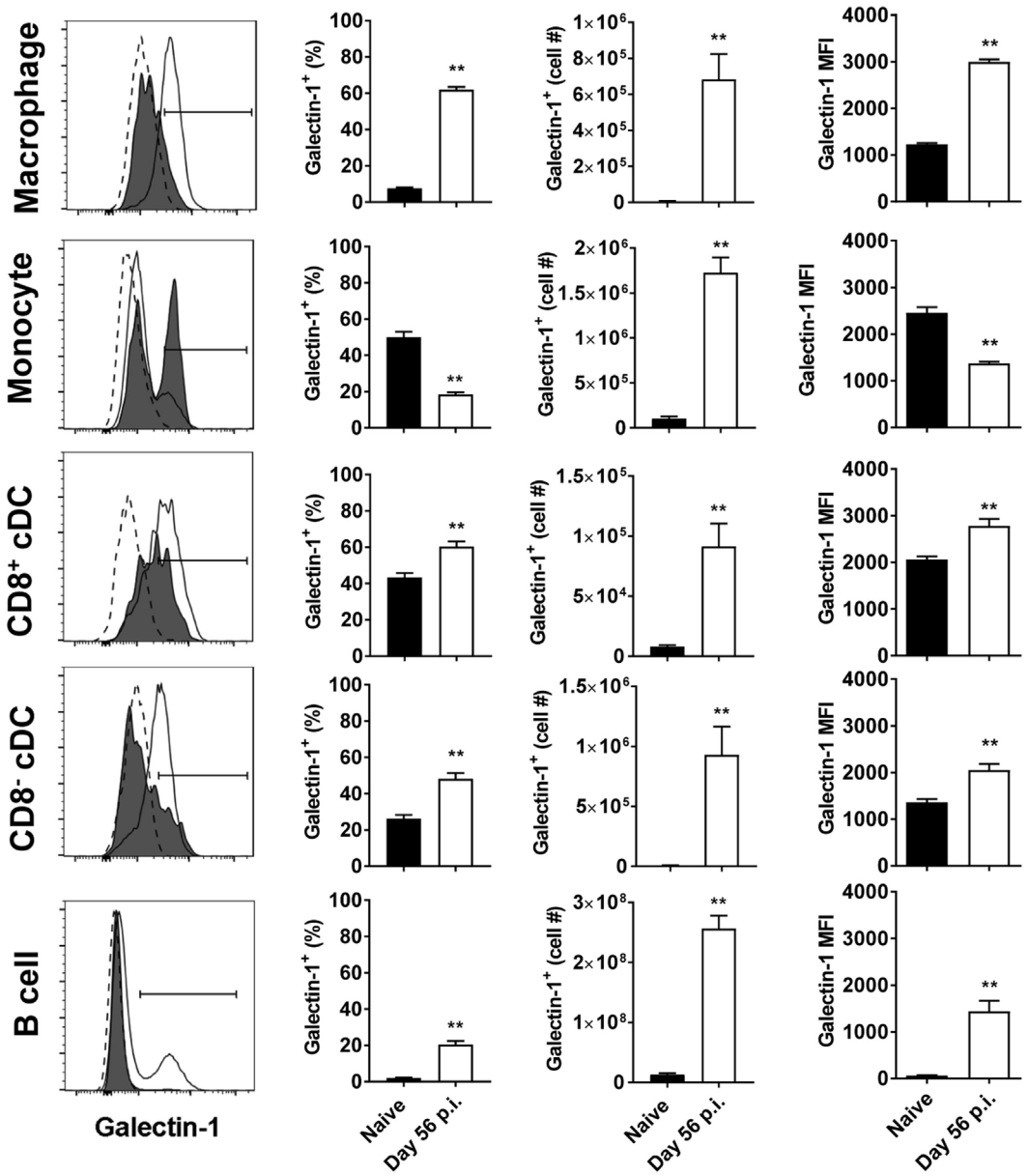

FIGURE 4 | Galectin-1 expression on splenic antigen-presenting cells during established infection. Galectin-1 expression on antigen-presenting cells from C57BL/6 mice ($n = 5$) infected with *Leishmania donovani* (day 56 p.i.) was assessed by flow cytometry and compared with naïve control animals. The gating strategy used to identify antigen-presenting cells in the spleen was the same as shown in **Figure 3A**. The frequency and number of galectin-1-positive cells, as well as the relative expression of galectin-1 determined by mean fluorescent intensity (MFI) from histograms, were measured, as indicated. Dotted histograms reflect isotype control antibody staining, dark gray histograms reflect cells from naïve animals, and white histograms reflect cells from infected animals. These data represent one of two separate experiments (**$p < 0.01$; Mann–Whitney test, relative to naïve).

DT administration, leaving behind only WT or *Lgals1*$^{-/-}$ DC for T cell priming, and thus enabling us to determine the impact of DC-expressed galectin-1 on CD4$^+$ T cell activation in these animals (**Figure 7A**). We found no impact on parasite burden in the liver at day 14 p.i., regardless of whether DCs expressed galectin-1 or not (**Figure 7B**). Furthermore, we found no differences in the frequency of IFNγ-producing CD4$^+$ T cells or Tr1 cells in these mice (**Figure 7C**), indicating that galectin-1 expression by DCs was not responsible for the improved control of parasite growth observed in *Lgals1*$^{-/-}$ mice (**Figure 5A**). Interestingly, we found an increase in the frequency of Treg cells in mice with galectin-1-deficient DCs (**Figure 7C**).

A potential caveat with the above experiment was that the *Lgals1*$^{-/-}$ CD4$^+$ T cells were present in the irradiated recipients receiving CD11c.DOG and *Lgals1*$^{-/-}$ cells and may have influenced results. To address this, and also test whether there was a

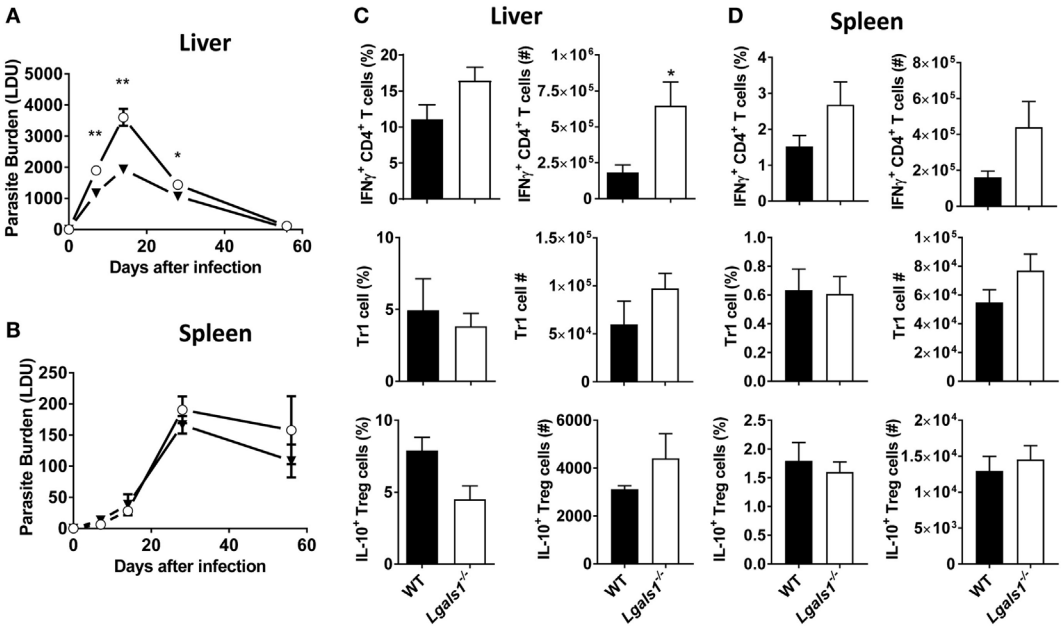

FIGURE 5 | Impact of galectin-1-deficiency on control of *Leishmania donovani*. Parasite burdens in C57BL/6 [wild-type (WT); open circles] and *Lgals1*$^{-/-}$ (closed triangles) mice (n = 4–5 per time point) infected with *L. donovani* were measured in the liver **(A)** and spleen **(B)**, as indicated, on days 7, 14, 28, and 56 p.i. The frequencies and total numbers of IFNγ-producing CD4$^+$ T cells, Tr1 cells, and IL-10-producing Treg cells in the liver **(C)** and spleen **(D)** at day 14 p.i. were measured, as indicated. These data represent one of three separate experiments (*$p < 0.05$, **$p < 0.01$; Mann–Whitney test, *Lgals1*$^{-/-}$ relative to WT at time-point assessed).

T cell intrinsic role for galectin-1 during *L. donovani* infection, we generated another set of BM chimeras that comprised a 90:10 mix of WT (CD45.1) and *Lgals1*$^{-/-}$ cells. This allowed *Lgals1*$^{-/-}$ CD4$^+$ T cell activity to be measured in a predominantly WT immunological background and compared with appropriate WT control CD4$^+$ T cells following infection (**Figure 8A**). We found a small, but significant ($p < 0.05$) difference in the frequency of hepatic IFNγ-producing *Lgals1*$^{-/-}$ CD4$^+$ T cells, compared to WT CD4$^+$ T cells, but no difference in Tr1 cell frequency in the 90:10 chimeras after 14 days of *L. donovani* infection (**Figure 8B**). However, we found an increased frequency of galectin-1-deficient hepatic Treg cells (**Figure 8B**), suggesting that similar galectin-1-mediated regulatory circuits established during acute *T. cruzi* infection (43) were unlikely to explain improved control of parasite growth in *L. donovani*-infected *Lgals1*$^{-/-}$ mice. Of note, despite engrafting mice with a 90:10 mix of WT and *Lgals1*$^{-/-}$ BM, respectively, we consistently found that *Lgals1*$^{-/-}$ leukocytes comprised 20–25% of the immune cell compartment (**Figure 8A**), although the ratio of CD4$^+$ T cells in WT and *Lgals1*$^{-/-}$ compartments remained the same (approximately 30%). Together, these results indicate the enhanced CD4$^+$ T cell IFNγ response observed in the liver of *Lgals1*$^{-/-}$ animals involved direct galectin-1 signaling to CD4$^+$ T cells. Furthermore, the lack of effect on hepatic CD4$^+$ T cell IFNγ production in mice with galectin-1-deficient DC (i.e., DT-treated CD11c.DOG:*Lgals1*$^{-/-}$ chimeras), compared to controls (**Figure 7C**), indicated that direct galectin-1 signaling by DCs was not responsible for the increased hepatic CD4$^+$ T cell IFNγ production observed in *Lgals1*$^{-/-}$ mice (**Figure 5C**).

DISCUSSION

A substantial amount of work on the immunoregulatory activity of galectin-1, and indeed the other members of the galectin family, has been conducted in the context of cancer and associated illnesses (20). As a result, the anti-inflammatory properties of galectin-1 have been well described (28, 29, 52). Thus, the primary focus of this study was to outline the contribution of galectin-1 signaling during experimental VL, with specific focus on the involvement of galectin-1 in CD4$^+$ T cell IFNγ and IL-10 production. Our studies centered on CD4$^+$ T cells because we and others have previously identified CD4$^+$ T cells, but not CD8$^+$ T cells, to be critical for control of *L. donovani* growth during primary infection (47, 53). CD8$^+$ T cells are important for protection against re-infection the liver (53), and protection in some vaccination strategies (54, 55). Here, we report that galectin-1 plays a limited role in promoting IL-10 production by CD4$^+$ T cells during *L. donovani* infection. However, we found galectin-1-mediated suppression of effector cytokine production by hepatic Th1 cells following infection. This finding was at odds with the proposed immunoregulatory network involving galectin-1 outlined by others both *in vitro* (29), and more recently *in vivo* (43). The latter study reported that *Lgals1*$^{-/-}$ mice had reduced mortality and parasite burden following *T. cruzi* infection, compared to WT controls. However, protection was associated with disruption of an immunoregulatory mechanism involving DC-mediated expansion of Treg cells (43). We found the impact of galectin-1 signaling on Foxp3$^+$ Treg cell IL-10

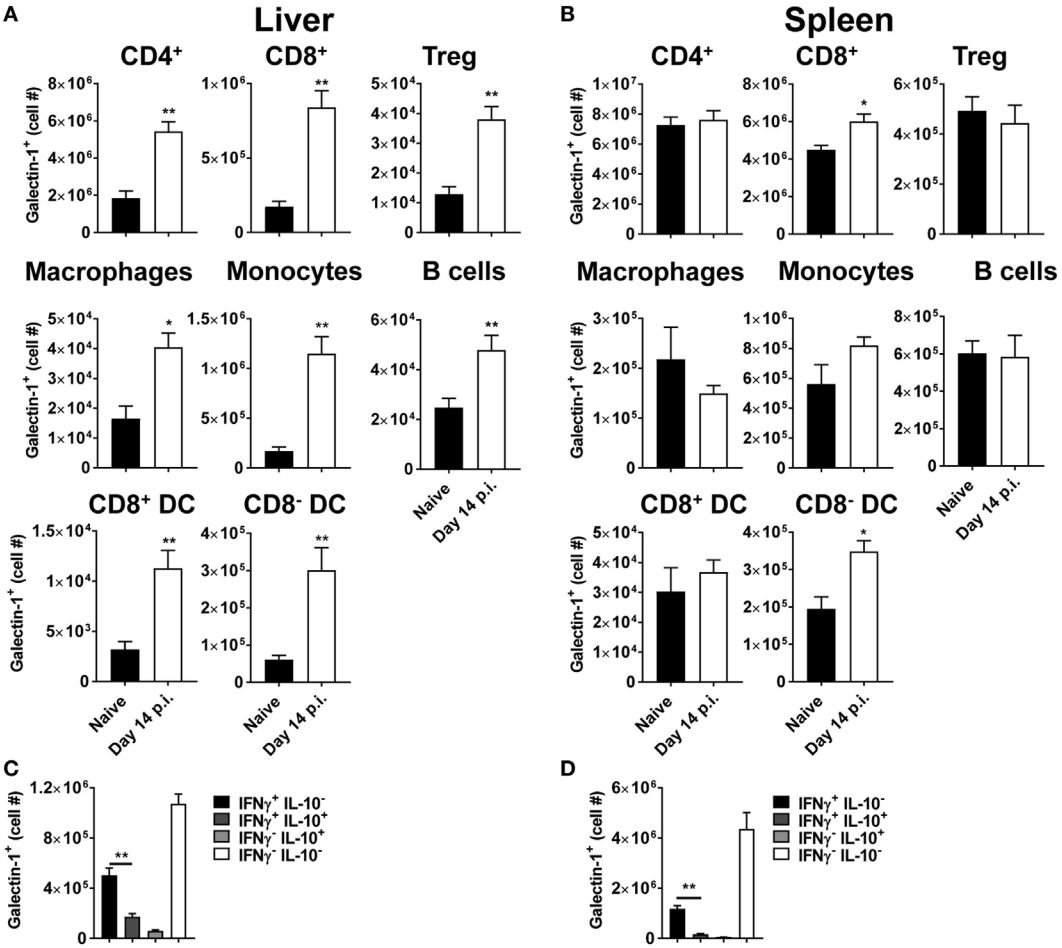

FIGURE 6 | Galectin-1 expression on immune cells early during *Leishmania donovani* infection. Galectin-1 expression on immune from C57BL/6 mice ($n = 5$) infected with *L. donovani* on day 14 p.i. was assessed by flow cytometry and compared with naïve control animals. The gating strategy used to identify cells was the same as shown in **Figures 1A** and **3A**. The number of galectin-1-positive cells in the liver **(A)** and spleen **(B)**, as well as the number of galectin-1-positive cytokine-producing cells in the liver **(C)** and spleen **(D)**, were measured, as indicated. These data represent one of two separate experiments [$^{**}p < 0.01$; Mann–Whitney test, relative to naïve **(A,B)**; Mann–Whitney test between IL-10$^-$ IFNγ$^+$ and IL-10$^+$ IFNγ$^+$ CD4$^+$ T cells only is shown **(C,D)**].

production was minimal in experimental VL. Given galectin-1's role in promoting Treg cell suppressive functions (43), our results indicate either a different role for galectin-1 in hepatic parasite clearance during *L. donovani* infection or an IL-10-independent mechanism of galectin-1-mediated Treg cell modulation. Our data from BM chimeric mice showed increased frequencies of Treg cells when either DCs or Treg cells lacked galectin-1 expression. This finding is at odds with what has been reported during acute *T. cruzi* infection (43), where DC galectin-1 expression promoted Treg cell function, and suggests the distinct roles for galectin-1 in different inflammatory settings. In addition, Treg cells have previously been shown to use multiple mechanisms of immune suppression, including TGFβ, IL-35, and CTLA4 (56, 57), and the impact of galectin-1 deficiency on these features of Treg cell functions during experimental VL still needs to be addressed. Another unexplored galectin-1-mediated mechanism of immune regulation in our study was the induction of apoptosis. Again, this needs examination, given the initial reports on the role of galectin-1 in T cell apoptosis (25). Similarly, we have previously reported that γδ T cell-derived IL-17A suppressed early hepatic anti-parasitic immunity following *L. donovani* infection (10, 47, 49), and although we have never observed IL-10 production by these cells, we cannot exclude a role for galectin-1 in this response.

Despite the limited impact of galectin-1 deficiency on Tr1 cell development during *L. donovani* infection, Th1 cell numbers in the liver were increased in *Lgals1*$^{-/-}$ mice 14 days after *L. donovani* infection, and this correlated with decreased parasite load during acute hepatic infection. Therefore, the action of galectin-1 may depend on the extent of antigen availability and consequent T cell activation status, rather than the induction of IL-10 during experimental VL. Furthermore, changes in galectin-1 expression by various immune cells were most prominent in the liver during the first 14 days of infection, which may have contributed to the tissue-specific impact on parasite control we observed. Our findings with mixed BM chimeras indicate that galectin-1 influences IFNγ production by CD4$^+$ T cells during the acute

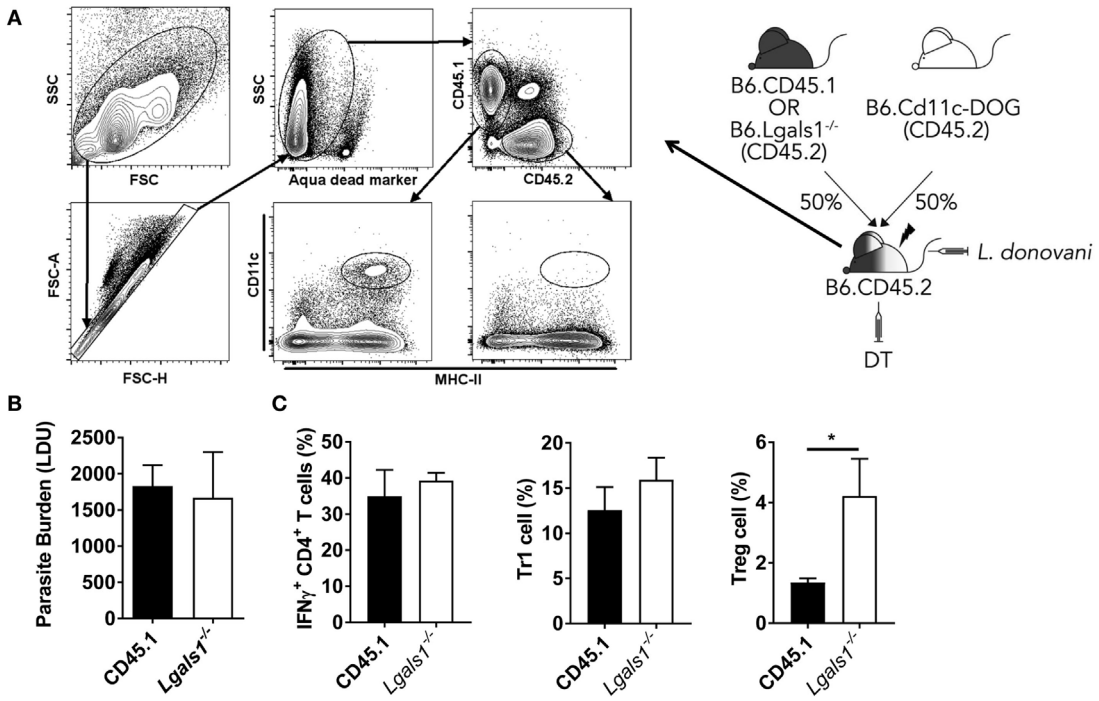

FIGURE 7 | Galectin-1 expression by dendritic cell (DC) has limited impact on infection outcome in the liver. Mixed bone marrow (BM) chimeras including a CD11c.DOG compartment were generated as shown. These mice were infected 3 months after BM engraftment, and received diphtheria toxin (DT) every 2 days from day −1 to day 13 p.i., and DC depletion was confirmed by flow cytometry **(A)**. Liver parasite burdens in DT-treated CD11c.DOG/B6.CD45.1 (CD45.1) and CD11c.DOG/*Lgals1*$^{-/-}$ (*Lgals1*$^{-/-}$) chimeras were measured at day 14 p.i., as indicated **(B)**. The frequencies of IFNγ-producing CD4$^+$ T cells, Tr1 cells, and Treg cells in the liver were measured at the same time **(C)**. These data represent one of two separate experiments (n = 5 mice per group in each; no differences between groups were found, as determined by Mann–Whitney test).

phase of hepatic *L. donovani* infection in a cell intrinsic manner. Previous studies (30, 43) suggest that DCs mediate immunoregulatory effects of galectin-1. This notion was supported by improved parasite clearance early during infection in the liver in *Lgals1*$^{-/-}$ animals, at a time when effector CD4$^+$ T cell responses were being initiated. However, depletion of DC in the presence of either WT or galectin-1-deficient cells did not influence effector T cell function following *L. donovani* infection. Therefore, our data indicate that DC-expressed galectin-1 is unlikely to contribute to the augmented Th1 responses observed in the liver of *Lgals1*$^{-/-}$ animals. However, we cannot exclude the possibility that galectin-1 expressed by other potential antigen-presenting cells, such as macrophages and/or monocytes, might influence T cells responses during experimental VL, an idea supported by the high levels of galectin-1 expression by these cells in the liver following infection. In addition, distinct requirements for DC activity in specific tissue sites by discrete DC subsets in cancer models and *T. cruzi* infection may also help explain lack of requirement for DC-dependent galectin-1-mediated immune regulation in experimental VL.

It is not clear why galectin-1 is expressed on multiple immune cell populations in the liver during *L. donovani* infection, and only influences parasite control in this tissue at the acute stage of infection. One potential explanation is that galectin-1 is preferentially expressed by migrating CD4$^+$ T cell populations that would be expected to be more prominent in the liver during experimental VL. Alternatively, the extent of immune dysregulation in the chronically infected spleen means that abrogation of galectin-1 activity is not, by itself, enough to overcome the *status quo* of impaired cellular responses in this organ. Conversely, the liver, with its delicate balance of pro- and anti-inflammatory responses (4, 50, 51), could be susceptible to more subtle alterations in the immunoregulatory environment. One caveat that must be highlighted, however, is that the flow cytometric analysis of galectin-1 expression in our study does not discern between dimeric and monomeric galectin-1, with obvious implications for interpretation of function. Thus, the mechanism by which galectin-1 impairs parasite clearance remains unclear. Our data indicate a CD4$^+$ T cell intrinsic role for galectin-1 in suppressing IFNγ production. As mentioned previously, monomeric galectin-1 can modulate cell functions by interacting with Ras family proteins (22), and given that suppression of Ras protein activity by farnesyltransferase inhibitors caused reduced IFNγ production by mouse and human CD4$^+$ T cells (58), the manipulation of Ras protein by galectin-1 in CD4$^+$ T cells is one possible mechanism for inhibition of IFNγ production in *L. donovani*-infected mice. An important finding in our studies was that *Lgals1*$^{-/-}$ mice exhibit improved parasite clearance prior to the establishment of strong effector T cell activity (day 7 p.i.), thus indicating a broader immunoregulatory potential of galectin-1,

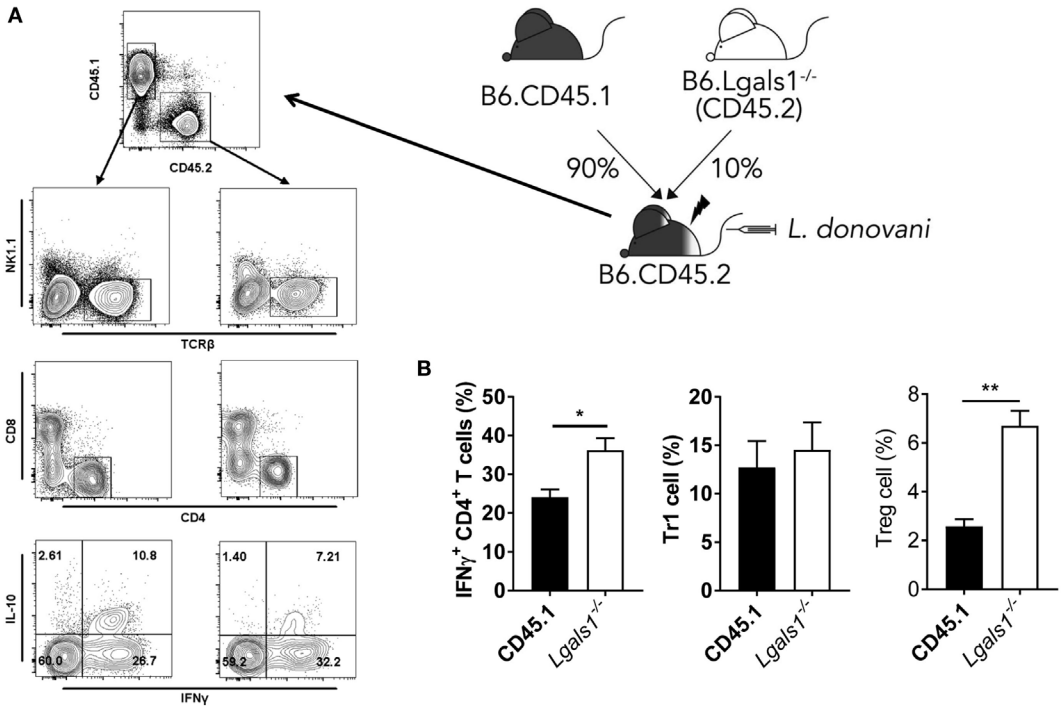

FIGURE 8 | CD4+ T cell intrinsic galectin-1-deficiency promotes CD4+ T cell IFNγ production in the liver. Mixed bone marrow (BM) CD45.1/*Lgals1*$^{-/-}$ (90:10) chimeras were generated as shown. Mice were infected with *Leishmania donovani* 3 months after BM engraftment **(A)**. The frequencies of IFNγ-producing CD4+ T cells, Tr1 cells, and Treg cells in the liver were measured at day 14 p.i. **(B)**. Treg cells were gated as described in **Figure 1A**. These data represent one of two separate experiments [$n = 5$ mice per group in each; $^{**}p < 0.01$; Mann–Whitney test, wild-type (CD45.1) relative to *Lgals1*$^{-/-}$ IFNγ+ CD4+ T cell frequency at day 14 p.i.].

and the extent of which appears to depend on the infected tissue. Both the precise mechanism of galectin-1-mediated suppression of IFNγ production by CD4+ T cells and the broader roles of galectin-1 on other immune cells require further study. One approach could be to block CD45R, as this has previously been shown to be required for galectin-1-mediated functions (25), and might provide novel insights to the mechanism of action of galectin-1 in experimental VL.

In summary, we demonstrate that galectin-1 is an important immunoregulatory molecule in experimental VL. Unexpectedly, galectin-1 did not appear to play a major role in Tr1 cell development or function. The main effect of galectin-1 on anti-parasitic immunity appeared to the suppression of Th1 cells. However, the relatively early effects of galectin-1-deficiency during infection also suggest effects of galectin-1 on innate cell activity, although this is unlikely to be on DCs. Together, these findings provide new insights into the role of galectin-1 in modulating immunity during infectious diseases and highlight both tissue- and disease-specific roles for this molecule.

ETHICS STATEMENT

All animal procedures were conducted with the approval of the QIMR Animal Ethics Committee under the animal ethics number A02-634M and in accordance with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (Australian NHMRC, Canberra).

AUTHOR CONTRIBUTIONS

PB, MO, and CRE designed, performed, and analyzed the work, and wrote the paper. FR, RK, CLE, RF, SN, MS, YW, FA, and AH performed the work and analyzed data.

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REVIEW
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The Equivocal Role of Th17 Cells and Neutrophils on Immunopathogenesis of Leishmaniasis

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Advances in the understanding of leishmaniasis progression indicate that cellular interactions more complex than the Th1/Th2 paradigm define the course of infection. Th17 cells are a crucial modulator of adaptive immunity against *Leishmania* parasites acting mainly on neutrophil recruitment and playing a dual role at the site of infection. This review describes the roles of both these cell types in linking innate defense responses to the establishment of specific immunity. We focus on the Th17–neutrophil interaction as a crucial component of anti-*Leishmania* immunity, and the clinical evolution of cutaneous or visceral leishmaniasis. To date, information obtained through experimental models and patient evaluations suggests that the influence of the presence of interleukin (IL)-17 (the main cytokine produced by Th17 cells) and neutrophils during *Leishmania* infections is strictly dependent on the tissue (skin or liver/spleen) and parasite species. Also, the time at which neutrophils are recruited, and the persistence of IL-17 in the infection microenvironment, may also be significant. A clearer understanding of these interactions will enable better measurement of the influence of IL-17 and its regulators, and contribute to the identification of disease/resistance biomarkers.

Keywords: cutaneous leishmaniasis, visceral leishmaniasis, immunity, T helper 17, interleukin-17, neutrophil, immunopathogenesis

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INTRODUCTION

Leishmania parasites are transmitted to mammals by female phlebotomine sandflies and cause a group of diseases with symptoms defined mainly by the parasite species and the host's ability to develop and control immune responses (1). Sickness results from uncontrolled infection, and the wide spectrum of clinical manifestations (from healing cutaneous lesions to fatal visceral infections) is associated with the parasite species involved, among other factors (2). The main clinical forms are cutaneous (e.g., *Leishmania major*, *Leishmania tropica*, *Leishmania mexicana*, and *Leishmania braziliensis*), mucocutaneous (e.g., *L. braziliensis*), and visceral (*Leishmania donovani* and *Leishmania infantum*) leishmaniasis (3). Visceral leishmaniasis (VL) is the most severe form and may compromise important organs and tissues, such as the liver, spleen, bone marrow, and lymph nodes, leading to hepatosplenomegaly, lymphadenopathy, anemia, constant fever, and immunosuppression (4). Cutaneous leishmaniasis (CL) is characterized in most cases by well-delimited ulcerated skin lesions with raised borders (5). Mucocutaneous leishmaniasis (ML) usually compromises mucosal regions of the nose, mouth, and pharynx, and is associated with disfiguring and psychosocial sequelae (6, 7). There are estimated to be 12 million people currently

living with leishmaniasis, and the World Health Organization estimates that there are approximately 0.7–1.2 million new cases of CL and 0.2–0.4 million cases of VL per year (6). While CL and ML are associated with social stigma, VL may result in fatality rates of 10–20% if not treated (6).

The parasites have a digenetic lifecycle and two distinct morphologies: the promastigote in the sandfly vector midgut, and the amastigote in mammalian host phagocytes. The motile, flagellated promastigotes exist, multiply, and develop extracellularly in the alimentary tract of blood-sucking female sandflies and are transmitted into the mammalian host during blood meals. Inside the host, they infect macrophages in the reticuloendothelial tissue and differentiate into non-motile amastigotes, and multiply as such in phagolysosomal vacuoles (8).

The interaction of inflammatory and regulatory responses delimited by cell-mediated immune responses drives disease expression and may result in asymptomatic infection, self-healing, or chronic leishmaniasis (1). Pathogenesis follows a complex set of interactions between factors triggered by the host's innate and acquired immune responses, which are strongly influenced by some aspects such as the host's genetic background (9, 10), infecting species, dose and route of inoculation (1), and sandfly saliva components (8). Only 2–3% of individuals infected with parasites of the *Viannia* subgenus (*L. braziliensis*, *Leishmania guyanensis*, *Leishmania panamensis*, and *Leishmania peruviana*) develop skin lesions (11) and less than 10% of those infected with *L. donovani* or *L. infantum* develop active VL (12). The relationship established between *Leishmania* parasites and the vertebrate host can lead to a self-healing infection or to the clinical manifestations of leishmaniasis with different severity grades (4). The inflammatory responses mediate disease presentation and, in both forms of the disease, the different clinical manifestations are a function of parasite replication and the efficacy of the immune response generated (5). Clinical cure is associated with the activation of macrophages into a leishmanicidal state mediated by some specific proinflammatory cytokines (1). However, there is as yet no consensus on the mechanisms that lead to susceptibility in humans.

Cellular immunity generated by the T helper type 1 profile is considered to be the key mediator of resistance to *Leishmania*. A protective immune response against CL caused by *L. major*, *L. mexicana*, or *Leishmania amazonensis*, as well as VL caused by *L. donovani* or *L. infantum*, depends on the development of the proinflammatory T cell profile (13). However, exacerbated Th1 responses may cause severe tissue damage and are also responsible for the clinical presentation of the disease. High levels of Th1 cytokines in people with localized CL lesions are associated with disease outcome, because individuals infected with *L. braziliensis*, who do not develop the disease, produce less interferon (IFN) γ and tumor necrosis factor (TNF) (14). *L. braziliensis* infections are characterized by excessive production of proinflammatory cytokines such as IFNγ, TNF and interleukin (IL)-6 (12) and have lower levels of $Foxp3^+$ cells (regulatory lineages) and IL-10 than those infected by other *Leishmania* species (15). In fact, the absence of IL-10-mediated regulation of Th1 is more significant in disease development due to *L. braziliensis* infection than the Th2 polarization itself (16). Indeed, high levels of IL-4 are not observed in patients with severe diffuse CL, suggesting Th2 responses may have less influence on disease progression in humans than in animal models (17).

Interleukin-10 is an important regulatory cytokine that inhibits phagocytosis and affects the ability of macrophages to kill intracellular parasites, contributing to the growth and spread of *Leishmania* (18). IL-10 sources have been identified as $CD4^+$/$CD25^+$ T cells (Th2) (19, 20), $CD4^+/CD25^-/FoxP3^+$ regulatory T cells (Tregs) (21), and $CD4^+/CD25^-/FoxP3^-$ T cells (Th1) (16), stimulated by the recognition of amastigote antibodies (22). Together with other cytokines from these regulatory populations, IL-10 plays a central role in promoting an alternative kind of macrophage activation, which increases arginase expression and facilitates parasite expansion (13).

A successful cellular immune response, which allows parasite elimination without tissue damage, requires a balance between the set of cytokines secreted in the cellular response, beginning with the recognition of the parasite by antigen-presenting cells (APCs) and antigen presentation, the production of Th1 differentiation cytokines, the activation of IFNγ/TNF-producing Th1 cells, and classical macrophage activation (23), ending with the activation of Th2 cells, which mainly secrete IL-10 and block nitric oxide (NO) production by macrophages (13). However, even in the presence of effector molecules such as IFNγ, TNF, and NO during active disease in humans, parasite multiplication persists in many cases. Ansari et al. (24) suggested that despite high IFNγ levels during *L. donovani* infection, the host fails to control the disease due to an incomplete response to IFNγ. Similarly, high titers of TNF have been observed in sera from patients with active VL and diffuse CL, characterized by high parasite loads (25). The biological network acting for equilibrium between parasite clearance and tissue preservation in humans involves substantial participation from effector T cells other than Th1 and Th2 (26).

Helper T cell responses are now known to include four T cell subsets: Th1, Th2, Th17, and Tregs. Th17 cells have recently emerged as an independent T cell subset that may play an essential role in protecting against certain extracellular pathogens (27). However, this relatively newly discovered (28) T cell population has been demonstrated to influence the balance between inflammatory and anti-inflammatory cytokines, which must be orchestrated in the course of infection to guide a successful effector response to the intracellular *Leishmania* protozoa (13). Particular interest in the Th17 type remains focused on its main cytokine, IL-17. Although it is also produced by other cells including $CD8^+$ T cells and neutrophils, IL-17 is mostly produced by Th17 cells. IL-17 is part of a complex mechanistic web that involves up- and downregulation of anti- and proinflammatory cytokines as well as interferences in the genetic background of the host. Furthermore, IL-17 performs an important function in neutrophil recruitment (29). Since neutrophils are regarded as an important element during *Leishmania* infection (30–33), it would be reasonable to consider that Th17 cells might have a significant role in the complex diseases caused by these parasites. The crosstalk between the neutrophils and APCs present at the site of infection contributes to the type and magnitude of the specific immune response that will develop (30, 34). Nevertheless,

at the site of infection, the protecting or damaging role of the neutrophil depends on the parasite species, host, and phase of infection (35). Thus, the effects of Th17/IL-17 are still unclear, as disease-promoting and protective responses have both been attributed to their influence. In this scenario, the Th17 cell activities in the context of the different clinical forms of leishmaniasis, interaction with regulatory cytokines, as well as host particularities, remain to be explored (13, 36).

Although many excellent reviews have discussed the cellular immune responses elicited against the different forms of leishmaniasis (35, 37, 38) or the roles of Th17/IL-17 in infectious and non-infectious diseases (39–42), reviews of the participation of IL-17 and Th17 participation in immunopathogenesis and control of the main clinical forms of leishmaniasis remain scarce (36). In the present review, we identify the Th17–neutrophil interaction as a crucial component of anti-*Leishmania* immunity. A better understanding of immune complexity will contribute to the identification of disease/resistance biomarkers and influence the development of vaccines and immunotherapies for leishmaniasis.

Th17 CELLS AND IL-17

The recent recognition of Th17 cells has provided new insights into the mechanisms that are important in autoimmune diseases and antimicrobial host defenses (28, 43). Th17 cells represent a subset of CD4 effector T cells distinct from Th1 and Th2 lineages, and mediate powerful effects on stromal cells; this results in the production of inflammatory cytokines and recruitment of leukocytes, especially neutrophils, creating a link between innate and adaptive immunity (29). *In vivo* studies indicate that IL-17 is an especially potent activator of neutrophils, both through expansion of the lineage and through their recruitment *via* chemokine expression regulation (44). IL-17 cooperates with other cytokines secreted by Th17 (such as IL-17F,[1] IL-21, and IL-22) to induce tissue inflammation, leading to different effector functions depending on the pathogen (47).

Th17 cell differentiation in both humans and mice is mediated by the activation of naïve T cells in the presence of a combination of TGF-β, IL-6, IL-1β, and IL-23 (48, 49). IL-6 acts in concert with TGF-β to induce the development of Th17 effector cells. Together, TGF-β and IL-21 upregulate the IL-23 and IL-23 receptors, a decisive step in the full differentiation and maintenance of Th17 cells (50). Although the requirement of TGF-β for the classical generation of Th17 cells *in vitro* and *in vivo* has been demonstrated, as well as the endogenous production of TGF-β for autocrine stimulation (51), it has also been shown that Th17 cells can be generated independently of TGF-β (52), in an alternative manner. Thus, the availability of IL-23 and/or TGF-β has emerged as a determinant of Th17 effector phenotypes; in other words, an abundance of TGF-β with a relative lack of IL-23 favors the generation of "classical" Th17 cells, while the presence of IL-23 alone promotes the generation of "alternative" Th17 cells. Transcription factors that are differentially expressed in "classical" vs. "alternative" Th17 cells will cause differential cytokine expression and effector functions. When "classical" Th17 cells were compared with alternatively induced Th17 cells, the "alternative" Th17 cells were more pathogenic (51, 52) (**Figure 1**).

Th17 differentiation is dependent on the steroid receptor-type nuclear receptor (RORγt) (47), as the transduction of naïve T cells with a retroviral vector containing RORγt induces the development of IL-17-producing T cells (53). The IL-23 receptor activates signal transducer and activator of transcription (STAT) 3, which induces RORγt (54). Conversely, in murine models, the generation of Th17 cells is inhibited by IL-4, IL-10, and IFNγ, possibly *via* downregulation of the IL-23 receptor (54, 55). The reciprocity of this antagonism from Th1/Th2 cells remains poorly understood, but as well as Th1 being counterbalanced by Th2 cells (56), Th17 cells are reciprocally related to Foxp3$^+$ Tregs. The presence of TGF-β activates the transcription factor Foxp3 in naïve T cells, whereas the presence of IL-6 suppresses Foxp3, and combined with TGF-β induces RORγt, leading to Th17 differentiation. Thus, the balance between Th17 cells and Foxp3$^+$ Tregs is mediated by the antagonistic interaction of the transcription factors Foxp3 and RORγt [reviewed by Korn et al. (47)].

Th17 is primarily known for its enhancement of host protection against extracellular bacteria and fungi, which requires robust inflammatory infiltrates that are not efficiently cleared by Th1 and Th2 responses (47, 57). IL-17 acts by activating iNOS and inducing the expression of granulocyte macrophage colony-stimulating factor, IL-1β, IL-6, IL-8, TNF, and several chemokines, which collaborate to potentiate the inflammatory reaction (58). The induction of these inflammatory mediators suggests that IL-17 plays a role in intracellular parasite infections. Indeed, the differentiation of Th17 subsets has been noticed during infections of *Mycobacterium tuberculosis* (59), *Francisella tularensis*, and *Listeria monocytogenes* (60). The role of IL-17 is also essential for protection against *Trypanosoma cruzi* in the acute phase of Chagas disease (61, 62), suggesting that Th17 cells are important in the successful clearance of kinestoplastid protozoans such as *Leishmania*. However, although Th17 cells and IL-17 have been shown to protect against some intracellular pathogens including *Leishmania* (63, 64), many recent findings point to them as being responsible for excessive inflammation and pathology (65, 66). Currently available data from VL and CL studies are discussed below.

IL-17 in VL

Active VL is associated with the upregulation of Th2 cytokines and disease progression. Neutrophil depletion during experimental VL results in enhanced parasite loads (67). The effector role of IL-17A is mediated by the accumulation of neutrophils, the enhancement of IFNγ secretion, and the increased production of antimicrobial peptides, acute phase proteins, mucins,

[1]IL-17 is the founding member of the IL-17 family of cytokines, which comprises IL-17A (commonly known as IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F (45). The most widely investigated and understood cytokines of this family are IL-17A and IL-17F, which play an essential role in host defenses against microbial infections and are implicated in various inflammatory conditions such as autoimmune diseases, metabolic disorders, and cancer (46).

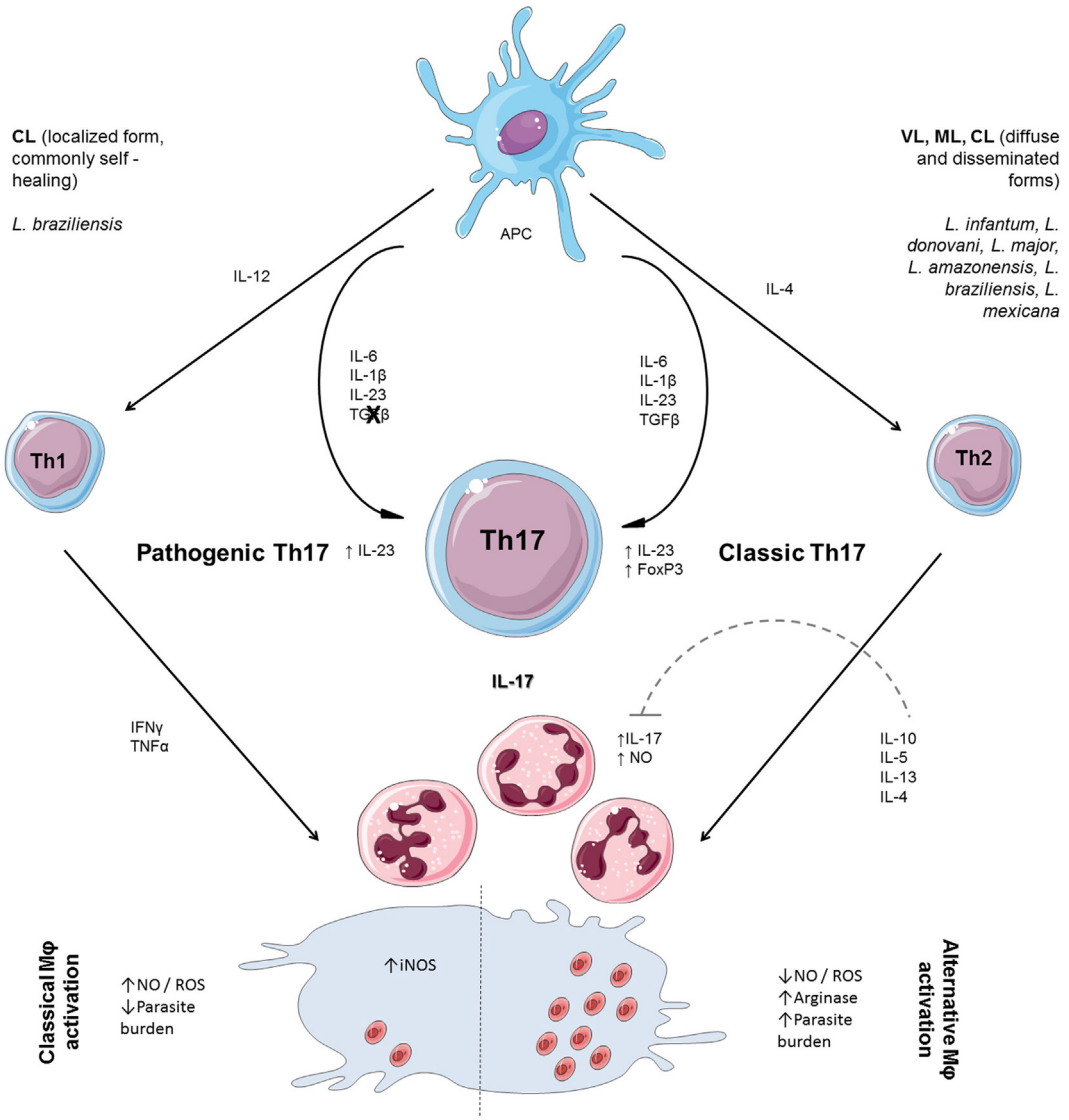

FIGURE 1 | T helper cell polarization and participation of neutrophils on effector responses to *Leishmania*. Th17 cell differentiation is mediated by activation of naïve T cells in a combination of TGF-β, interleukin (IL)-6, IL-1β, and IL-23. TGF-β and IL-21 upregulate the IL-23 and IL-23 receptor, decisive for the maintenance of Th17 cells. Th17 cells can also be generated independently of TGF-β, in an alternative manner. When "classical" Th17 cells were compared with alternatively induced Th17 cells, the "alternative" Th17 cells were more pathogenic. Naïve $CD4^+$ T cells differentiate to the Th1-cell lineage in the presence of IL-12. Interferon (IFN) γ secreted by Th1 activates inducible NO synthase (iNOS) and enhances production of nitric oxide (NO) and reactive oxygen species (ROS), required for parasite clearance. The production of ROS in the infection microenvironment is enhanced by the presence of neutrophils recruited by IL-17 and, in the absence of IL-10 regulation, may worsen tissue injury. The secretion of IL-4 leads to transcription of Th2 genes (IL-10 and IL-13) broadly associated with susceptibility to *Leishmania* infection through alternative macrophage activation, favoring parasite persistence. IL-10 is an important regulatory cytokine that controls Th17 differentiation and regulates NO production. The influx of neutrophils plays a positive role by participating in parasite killing and is limited by the presence of IL-10.

and matrix metalloproteases (45). As IL-17 is associated with neutrophilic inflammation and enhancement of the proinflammatory response, the positive role of this cytokine in VL has been investigated. It is already known that *L. donovani* and *L. infantum* stimulate the differentiation of Th17 cells in peripheral blood mononuclear cells from healthy subjects, as well as the production of IL-6 and IL-23 required for Th17 maintenance (68). A cohort study of individuals with VL showed that IL-17 is strongly associated with protection. This protective role is associated with the increase of CXCL chemokines, which serve as potent chemoattractants for neutrophils and Th1 cells. Thus, naturally resistant subjects, showing greater IL-17 responses, would react more rapidly to *L. donovani*, not only by attracting innate immunity responses, but also by recruiting Th1 cells to tissues (68). In BALB/c mouse models of *L. donovani* infection, parasite clearance was associated with the secretion of Th1 cytokines along with IL-17 and IL-23. The blocking of stimuli by anti-IL-17 and anti-IL-23 led to increased parasite loads in the liver and spleen. Subsequent administration of recombinant IL-17 generated higher levels of IFNγ and NO than recombinant

IL-23 did, leading to a stronger association of IL-17 with parasite clearance (69). These data corroborated previous findings (68) that Th17 potentiates the Th1 response. Depending on the infectant parasite load, the speed of Th1 recruitment and the quantity of NO generation may determine disease progression or natural control.

mRNA analysis revealed greater IL-17A production in VL patients than in healthy controls, in a study performed by Nascimento et al. (55). In the same study, the highest production of IL-17A coincided with the parasitic peak in infected C57BL/6 mice. These data demonstrate the stimulation of IL-17 production by *L. infantum*, promoting the control of parasite replication by strengthening T helper type 1 responses and NO production. The authors also demonstrate that the absence of IL-17 signaling leads to the generation of a Treg/IL-10-dominated response and an impaired Th1 profile, resulting in parasite growth. However, although IL-17 receptor *knockout* mice were more susceptible to infection and also exhibited reduced inflammatory infiltration, the high levels of this cytokine observed in humans was not sufficient to generate IFNγ/NO levels that lead to natural recovery from VL (55). Thus, although the presence of this cytokine has been demonstrated, the significance of its participation in the effector response seems to be relative to the host.

Neutrophil influx mediated by IL-17A was revealed by Quirino et al. to be an important mediator of parasite clearance during VL (4). In this work, the authors indicate that IL-27 is an important regulator of Th1 and Th17 profiles in a VL mouse model. Composed of two subunits (IL-27p28 and Ebi3) that bind to IL-27 receptor, leading to STAT-1 (Th1) and -3 (Th17) activation, IL-27 plays a role in the regulation of the immune response (4). The authors observed that Ebi3$^{-/-}$ mice present a peak in neutrophil migration at 4 weeks post-infection, both in the spleen and in the liver. These data reinforce the previous findings of Lopez Kostka et al. (70) that suggest that the IL-17–neutrophil axis has more of an influence in the recovery from VL infection after its establishment (when the antigens are already recognized and presented by DCs and macrophages) than in the prevention of infection. Neutrophils recruited by DCs in a toll-like receptor 2 (TLR2)-dependent manner are efficient producers of NO and provide a prototypal Th1 and Th17 environment, leading to a decrease in parasite number in C57BL/6 mice infected with *L. infantum*. Furthermore, TLR2$^{-/-}$ mice are unable to recruit neutrophils in this way and had higher levels of IL-10, indicating that a non-responsive neutrophil state is associated with parasite persistence (71). The presence of neutrophil infiltrate, however, may cause damage to the tissue, and seems to be most significant in skin, since the Th17 markers IL-23, IL-17, and RORγt were highly expressed during post kala-azar dermal leishmaniasis (PKDL) compared with controls (72).

The cell source of IL-17A expression is another variant to be studied. IL-17A-knockout C57BL/6 mice are highly resistant to VL infection, showing fewer parasites in the liver and spleen. This phenotype was associated with enhanced IFNγ production by T cells and decreased accumulation of neutrophils and monocytes, resulting in fewer granulomas. The source of IL-17 was mainly γλ T cells, which commonly express markers for CD3, but not CD4 or CD8 (67), and are differentiated by IL-1β and IL-23, suggesting a different mode of activation to the conventional TGF-β and IL-6 that promote CD4^{+}IL-17^{+} cells. IL-17 produced by γλ T cells suppresses NO-mediated parasite control in macrophages in the liver within 7 days of infection, contributing to susceptibility in C57BL/6 mice (73). The mechanisms that lead to counter-regulation between IFNγ and IL-17 secreted by γλ T cells remain to be elucidated.

IL-17 in CL

Leishmania infection strongly stimulates IL-17 production. Elevated levels of IL-17 have been measured in patients with CL and ML, showing that Th17 activation is generated by *L. major* (74, 75), *L. braziliensis* (64), *L. tropica* (26), *L. panamensis* (64), *L. guyanensis*, *L. amazonensis*, and *Leishmania naiffi* (66) infections, acting on macrophage activation and neutrophil recruitment. However, clinical studies indicate differences in immune responses during CL between the subgenera *Viannia* and *Leishmania* (76). For example, higher levels of Th17 were found in the sera of patients infected with *L. (Viannia) guyanensis* than in those infected *L. (Leishmania) amazonensis* (66). Nevertheless, the mechanisms of immune evasion used by *L. amazonensis* are known to block cellular immune responses (37) and may also occur by decreasing Th17 activation. Thus, the influence of Th17/IL-17 on disease depends on the species. As studies on IL-17 are relatively recent, most performed to date have evaluated *L. major* models or *L. braziliensis* infections in patients. Comparing IL-17 participation in other *Leishmania* species seems, therefore, to be an interesting avenue of investigation.

Proinflammatory responses, especially Th1, are a required component in controlling leishmaniasis. However, in the case of CL and ML, the immune response itself contributes to lesion progress. In both these forms, patients develop intense inflammatory responses that damage tissues, despite the low number of parasites in lesions (25), so the disease is mostly associated with a failure of anti-inflammatory cytokine secretion, especially IL-10 (77). IFNγ and IL-17 levels are substantially elevated in mice lacking the capacity to respond to IL-10. According to Gonzalez-Lombana et al. (2), in these mice, IFNγ does not seem to contribute to pathology as much as IL-17, since the former promotes mostly monocyte infiltration, while the latter recruits neutrophils, constituting a nonspecific response. Interestingly, blocking IFNγ increases IL-17 level and pathology, supporting the hypothesis that, in C57BL/6 mice, IFNγ may be critical for downregulating the responses of IL-17 as well as IL-10 (2). In another study, self-healing of the infection in mice correlated with the expansion of IFNγ and IL-17-producing CD4 cells, suggesting the existence of other active mechanisms to regulate local inflammation (7).

In experimental models, lesion progression is related to IL-17-mediated neutrophil recruitment. During the course of infection with *L. major*, BALB/c (a susceptible strain) CD4^{+} cells and neutrophils produced more IL-17 than did cells from (resistant) C57BL/6 mice. IL-17-deficient mice had decreased neutrophil infiltration and smaller lesions than controls (70). Anderson et al. (74) found an association between severe pathology and the presence of IL-17^{+}CD4^{+} cells, which was also linked

to an increase in cellular infiltrate expressing macrophage and neutrophil markers. However, despite contributing to lesion exacerbation, no beneficial effect of controlling parasite replication was observed. Conversely, neutrophils have been thought to contribute to parasite clearance in the early stages of experimental *Leishmania* infection (30), and their expression is closely, but not uniquely, related to IL-17 secretion (78). Thus, neutrophil participation must be better studied and linked to IL-17 associations with leishmaniasis. In resistant strains of mice, the number of polymorphonuclear leukocytes decreases to 2% within 72 h of infection, when the specific immune response is defined. In contrast, insusceptible BALB/c mice, neutrophils continue being recruited and have been detected in large numbers at the site of infection more than 10 days after parasite inoculation (30). Pedraza-Zamora et al. (79) showed that in BALB/c mice, Th17 cells and neutrophils both increased up to 90 days post-infection with *L. mexicana*. This suggests that chronic inflammation throughout *L. mexicana* infection is a consequence of neutrophil recruitment together with Th17 cell differentiation throughout the late phase of the infection. C57BL/6 mice show a more controlled response of these cells and a more resistant infection profile. Together, these data suggest that not only the level of IL-17 but also its persistence during infection resolution, is a decisive factor in disease progression. The excessive activation and maintenance of Th17 by the presence of neutrophils and Th1 may cause a damaging block of regulatory populations and IL-10 secretion, which is necessary to stop inflammation. For example, the study performed by Lopes Kostka et al. (70) suggested that DC-derived IL-23 maintains IL-17 production, thus being responsible for disease progression.

mRNA expression of RORγt and IL-17 is observed in patients with ML, and the synergy of IL-17 and IFNγ is suggested by a positive correlation between IL-17 and IFNγ mRNA expression in ML lesions caused by *L. major* (75), contrasting with the antagonism observed in C57BL/6 mice (2) mentioned earlier. Enzyme-linked immunosorbent assays showed that IL-17 secretion by peripheral blood mononuclear cells was elevated in patients with CL and ML compared with uninfected individuals, and downmodulated by IL-10 and TGF-β (65, 80) similarly to that seen with Th1/IFNγ, providing further evidence for the existence of IL-17/IFNγ synergy in humans. de Assis Souza et al. (63) showed that levels of IL-17 were significantly higher in patients infected by *L. braziliensis* during active disease than in those who recovered without intervention. Interestingly, the authors associated this decreased expression during healing to a suppressive role exerted by high NO levels. During treatment, NO levels decrease and allow more IL-17 secretion (63). In addition, the number of IL-17$^+$CD4$^+$ cells at lesion sites in patients with CL and ML directly correlates with the number of inflammatory cells, supporting their participation in neutrophil infiltration at lesion sites (65).

Although these important studies have linked the presence of IL-17 to more severe clinical presentations of disease, neutrophils are the first line of defense and are also capable of producing NO and killing parasites (32). Therefore, the presence of neutrophils in this inflammatory context can indicate either protection or injury. The reversal of immunodeficiency in *L. panamensis*-infected mice leads to an increase in IFNγ and IL-17, allowing parasite control and elimination (64). This suggests that in the absence of anti-inflammatory cytokines (such as IL-10, IL-13, and IL-4), Th1/Th17 cells proliferate simultaneously and are responsible for parasite elimination, although neutrophil migration, and its role in this situation (3.5 weeks post-infection), was not assessed. This observation reinforces the differences in immunomodulation between parasite species. Interestingly, Novoa et al. (81) also found a trend toward increased levels of IL-17 in human subclinical *L. braziliensis* infections compared with symptomatic CL, suggesting no pathological role of IL-17 in CL or ML. Although those authors associated spontaneous cure to a weak type 1 immune response (due to quick parasite control by innate immune responses), IL-17 levels were measured in the study only 96 h after soluble antigen stimuli, when the cellular immune response was probably being established. The expression of IL-27 (which can act as regulator of IL-17) was also higher in patients with CL caused by *L. braziliensis* than in subclinical individuals (81), reinforcing the idea that the Th17 profile contributed to cure in this study, and supporting a role of IL-17 in parasite depletion. Further investigations encompassing the absence of other IL-17 regulatory mechanisms (such as IL-13) in this situation are necessary.

NEUTROPHILS IN INNATE AND ADAPTIVE IMMUNITY TO *Leishmania*

Neutrophils are cells that operate in innate immunity as the first line of defense, and play an important role in the elimination of pathogens (82). Toxic granule release from the site of infection, secretion of cytokines, phagocytosis, and formation of fibrous structures, known as NETs (neutrophil extracellular traps) are some of the mechanisms used by these cells to eliminate microorganisms (82, 83). In addition to their role in the elimination of pathogens, neutrophils can also participate in immune regulation as a source of cytokines, including IL-2, IL-10, IFNγ, and TNF, thereby establishing a link between innate and adaptive immunity during infection, and even participating in the presentation of antigens to regulate T cell proliferation and antibody production by B cells (84). In fact, murine *L. (L.) amazonensis*-infected macrophages cocultured with neutrophils were better able to destroy the intracellular amastigotes (34). Since no significant difference in *L. (L.) amazonensis* parasite destruction was observed between co-cultures from susceptible BALB/c and resistant C3H/HePas mice, this leishmanicidal activity seemed not to depend on adaptive immunity, which determines an individual's resistance profile (34). However, due to the effector functions in the inflammatory process, neutrophils also need to be regulated because their excessive activity can lead to tissue destruction and even chronic inflammation (82).

Recent studies have addressed the role of neutrophils in *Leishmania* spp. infection. These cells may behave protectively or as enhancers of infection, depending on the parasite species, host, and stage of infection (32, 34). *In vitro* studies demonstrated that several *Leishmania* species are able to induce the formation of NETs, which are basically DNA structures coated with

antimicrobial molecules, released by neutrophils (85). These NETs are involved in the sequestration, immobilization, and killing of parasites [reviewed by Bardoel et al. (86)]. However, the efficacy of this immune strategy varies according to the *Leishmania* species involved. For instance, promastigotes of *L. amazonensis* showed susceptibility to NETs, while *L. donovani*, *L. infantum*, and *L. mexicana* were able to escape from this "trap" using different mechanisms (82). Furthermore, the same promastigote surface lipophosphoglycan that is involved in the activation of NETs (as for *L. amazonensis*) may also be associated with parasite resistance to killing through NETs, as occurs with *L. donovani* (87).

There is no consensus on the activity of neutrophils during infection by *Leishmania* species, as besides the complexity of species involved, there is heterogeneity between studies, which explore different host species, host strains, and tissue types, all factors that may interfere directly with the type of response to the infection (32). In an example of how the host strain (genetic background) may change its response, Tacchini-Cottier et al. (33) demonstrated that neutrophil-depleted C57BL/6 mice exhibited a normal course of infection caused by *L. major*, whereas neutrophil depletion in BALB/c resulted in blockage of the early characteristic IL-4 response, thus leading to an inhibition of Th2-type response. Currently, a specific neutropenic mouse strain (Genista) is being used; Hurrell et al. (82) demonstrated that Genista mice, as well as mice with transient neutrophil depletion, are able to control the parasite load and lesion size when infected with *L. mexicana*, indicating that parasite sequestration by neutrophils is associated with disease progression in mice.

Neutrophils that are recruited and that participate in the early stages of infection behave very differently to those associated with the late immune response against *Leishmania* parasites. In the initial phase of infection, neutrophils are recruited as a response to chemotactic substances released from the tissue lesion or from the sandfly's saliva, and also by stimuli from the parasites (32). DCs and neutrophils have been shown by microscopy studies to infiltrate the site of parasite inoculation within a few hours after sandfly bite, where they engulf the parasites. By 48 h after infection, most parasites at the inoculation site are found within macrophages (88). DCs that were recovered from the skin 1 day after infection appeared to have been infected by these parasitized neutrophils (32). *In vitro* and *in vivo* kinetics studies showed that interactions with early infected or not infected neutrophils (from the acute infiltrate), or even with their apoptotic corpses, may impair the innate response of DCs, thus delaying the activation of a specific immune response *via* $CD4^+$ T cells (32). This response may be crucial for parasite depletion before symptom presentation, resulting in subclinical progression. The parasites may enter directly into the macrophages through receptor recognition, or may be transferred silently *via* neutrophils (89). Parasites may be transferred from neutrophils to macrophages in the following two ways: the first involves the release of the parasite from an infected apoptotic neutrophil, in which the free parasite is phagocytosed by the macrophage; the second suggests that the macrophage engulfs live infected neutrophils, referred to as "Trojan horses" (90, 91). Mollinedo et al. (92) have demonstrated viable *L. major* and *L. donovani* promastigotes inside human neutrophils, and they suggest that this is linked to inhibition of the oxidative burst, as well as prevention of early fusion of lysosomes with parasite-containing phagosomes. The interaction and previous uptake of these early neutrophils by macrophages can drive the development of IL-10-high and IL-12-low macrophages, which are especially permissive to the survival and proliferation of *Leishmania* parasites (32).

Antigen presentation by neutrophils is also inefficient in eliciting adequate T cell differentiation. Recent work revealed that human leukocyte antigen–antigen D related (HLA-DR) positive neutrophils from subjects with VL did not stimulate T-cell proliferation, but they did show higher expression of programmed cell death ligand-1 than other neutrophils, and lymphocytes of the same subjects showed high expression of programmed cell death protein-1, reinforcing the mechanisms of apoptosis that favor *Leishmania* (93). This population of neutrophils also showed elevated expression of IL-10 mRNA and protein, and transcripts encoding arginase-1, which is involved in the suppression of T cell responses (94). Conversely, after *in vitro* infection with *L. braziliensis*, neutrophils from patients with CL produce more reactive oxygen species (ROS) and higher levels of chemokines associated with recruitment of neutrophils and Th1-type cells than neutrophils from healthy control subjects (95), highlighting that the mechanisms used by parasites to explore neutrophils in their favor vary by species.

After the first acute neutrophilic influx, a second wave of neutrophil recruitment to the site of infection occurs approximately 1 week after the first contact with parasites; this is especially mediated by IL-17. Studies using BALB/c IL-17-deficient mice suggest that this cytokine is not the main recruitment factor involved in early stages of infection, but acts beside IL-8 and granulocyte-colony-stimulating factor. However, after 4 weeks of infection, low levels of IL-17 lead to a low number of neutrophils (70). Thus, the role of IL-17 in both leishmaniasis forms (cutaneous and visceral) may be strictly associated with neutrophil function, infection stage, and antioxidant capacity, as well as IL-10 regulation. When the neutrophil is engulfed by an infected macrophage, parasite death is increased *via* an elevation in ROS production. However, this second response is also associated with lesion progression, especially in the mucosal form of leishmaniasis (2, 30). According to Gonzalez-Lombana et al. (2), the key point in the healing of a lesion in ML is the downregulation of IL-17 by IL-10 and IFNγ. The huge concentration of granules or products from neutrophils recruited by IL-17 in the site of infection promotes tissue lesioning. In this context, the authors suggest that the IL-17 pathway is an important therapeutic target for the treatment of severe leishmaniasis in patients in whom IL-10 regulatory function is compromised.

In experiments performed in BALB/c mice, Rousseau et al. (96) demonstrated that neutrophils are involved in the early phase of VL by controlling the parasite growth in spleen but not in liver. The authors also found that these cells appear to have no significant effect in late infection in either of these organs. Finally, Almeida et al. (97) found an association between the severity of canine VL, superoxide production and neutrophil apoptosis, observing that neutrophil function alters according

to disease stage. Initially, there is a high level of ROS production, ensuring resistance to infection. However, in the late phase of the disease, ROS production overcomes the antioxidant capacity of the cell, leading to oxidative stress followed by neutrophil apoptosis. The increase in apoptosis and the consequent decrease in oxidative activity results in a predisposition to coinfections, commonly seen in severe cases of canine VL (98). In support of this hypothesis, treatments based on antioxidants have generated good results in patients with leishmaniasis (98). In short, like in CL, neutrophils are part of the complex web that governs the establishment and development of VL in different hosts.

Further studies are needed to clarify the role of neutrophils in the control or proliferation of different species of *Leishmania*. Because the infection phase is crucial, time-dependent investigations of ROS production should be carried out, and the regulatory/inflammatory profiles of Th17 cell participation should be explored in different hosts, considering tissue peculiarities.

Th17 AND NEUTROPHILS CAN CONTRIBUTE TO PARASITE CLEARANCE OR DISEASE

Recent studies have advanced our knowledge about the influences of each parasite/host factor in the pathogenesis of human leishmaniasis. The characterization of the $CD4^+$ T helper cell population Th17 has further added to the complexity of host–pathogen interactions, previously thought to be determined by Th1 or Th2 polarization. Currently, participation of innate immune responses and complicated interactions between cytokines secreted by different T cell profiles, as well as the diversity of *Leishmania* species and hosts, are known to be relevant to disease outcome.

Neutrophils can potently protect against or enhance infections and can likewise behave as Trojan horses, supporting the invasion of macrophages by *Leishmania*. In addition to DCs and other components of the innate defense system, neutrophils also regulate T cell polarization, through cytokine secretion, thus establishing a link between innate and adaptive immunity during parasitic infection. Although neutrophil presence has been mostly associated with parasite depletion and cure in *L. donovani* and *L. infantum* infections, the infiltration of these cells is suggested to cause more severe disease presentations in tegumentary forms of leishmaniasis. The reviewed data show that regulatory cytokines such as IL-10 and IL-4 are needed to promote the equilibrium in ROS production, which is enhanced by the presence of neutrophils in chronic infections. In the liver and spleen, *L. infantum/donovani* elicits anti-inflammatory cytokines and stimulates arginase production, leading to control of inflammation while neutrophils and activate macrophages kill parasites. The data also suggest that neutrophil participation depends on the timing of their recruitment, the tissue infected, and their duration at the infection site.

Current understanding of the role of Th17 cells in the progression of pathogenesis or contribution to host-protective immunity is evolving rapidly. We now know that *Leishmania* infections trigger $CD4^+$/IL-17^+ T cell differentiation in mice and humans. The IL-17 function in neutrophil recruitment has been widely demonstrated in experimental models of leishmaniasis, in which strong inflammatory infiltrates are observed. Although mice are more dependent than humans on IL-17 to combat *L. infantum*/*L. donovani* infections, IL-17 has also been associated with improved recovery from VL in humans. Conversely, higher levels of IL-17 during commitment in skin in patients with PKDL, CL, and ML than in healthy controls suggest that in this tissue, IL-17 contributes less to healing than in other tissues. Most of the current data link the IL-17–neutrophil axis to disease development or lesion exacerbation in CL. Nevertheless, differences in immunomodulation between parasite species, and contradictory results in humans, point to IL-17 stimulatory (IL-23) or regulatory (e.g., IL-27, IL-10, and IL-13) cytokines, which may be important in determining how long IL-17 remains present. Similarly to the role of IL-12 in the context of Th1 generation, identifying the role of these cytokines in the development or maintenance of Th17 is essential for understanding the influence of its persistence at lesion sites.

AUTHOR CONTRIBUTIONS

SG-d-A and MP-C concept and drafted the paper. SG-d-A, RS, and LT-S acquired, interpreted data, and wrote the paper. SG-d-A, RS, VL, RM, and MP-C revised critically for intellectual content. All the authors approved the final version to be published. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Immune Checkpoint Targets for Host-Directed Therapy to Prevent and Treat Leishmaniasis

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Leishmaniasis encompasses a group of diseases caused by protozoan parasites belonging to the genus *Leishmania*. These diseases range from life threatening visceral forms to self-healing cutaneous lesions, and each disease manifestations can progress to complications involving dissemination of parasites to skin or mucosal tissue. A feature of leishmaniasis is the key role host immune responses play in disease outcome. T cells are critical for controlling parasite growth. However, they can also contribute to disease onset and progression. For example, potent regulatory T cell responses can develop that suppress antiparasitic immunity. Alternatively, hyperactivated $CD4^+$ or $CD8^+$ T cells can be generated that cause damage to host tissues. There is no licensed human vaccine and drug treatment options are often limited and problematic. Hence, there is an urgent need for new strategies to improve the efficacy of current vaccine candidates and/or enhance both antiparasitic drug effectiveness and subsequent immunity in treated individuals. Here, we describe our current understanding about host immune responses contributing to disease protection and progression in the various forms of leishmaniasis. We also discuss how this knowledge may be used to develop new strategies for host-directed immune therapy to prevent or treat leishmaniasis. Given the major advances made in immune therapy in the cancer and autoimmune fields in recent years, there are significant opportunities to ride on the back of these successes in the infectious disease domain. Conversely, the rapid progress in our understanding about host immune responses during leishmaniasis is also providing opportunities to develop novel immunotherapy strategies that could have broad applications in diseases characterized by inflammation or immune dysfunction.

Keywords: leishmaniasis, immunoregulation, immunotherapy, pathology, cell-mediated immunity

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LEISHMANIASIS

Leishmaniasis describes a collection of neglected tropical diseases caused by protozoan parasites of the genus *Leishmania* that are transmitted by female *Phlebotomine* sand flies (1). It largely affects the poorest populations living in developing countries and is prevalent throughout the tropical and subtropical regions of Africa, Asia, the Mediterranean, Southern Europe, and South

and Central America. Globally, 350 million people are at risk of developing leishmaniasis and 1.5–2 million new cases occur annually (2). The clinical spectrum of leishmaniasis ranges from the life-threatening visceral form to self-healing cutaneous lesions or a more serious mucosal manifestation.

Visceral leishmaniasis (VL), also known as kala-azar, typically involves long-term, low-grade fever, enlarged spleen and liver, weight loss, pancytopenia, and hypergammaglobulinemia [reviewed in Ref. (3)]. Untreated VL cases are almost always fatal, and more than 90% of cases occur in India, Bangladesh, Nepal, Sudan, Ethiopia, and Brazil with an estimated incidence of at least 500,000 new cases and 50,000 deaths each year (2–4). Of note, the state of Bihar in north east India has been the focus of most VL cases for many years (5), but recent efforts toward elimination, and civil unrest in Southern Sudan, have now made the latter region the source of most cases (6). Post kala-azar dermal leishmaniasis (PKDL), which can be a late cutaneous manifestation of VL, either following drug treatment or sometimes independent of VL development, is confined to the Indian subcontinent [India, Nepal and Bangladesh, and East Africa (Sudan)]. It presents as an accumulation of heavily infected macrophages in the skin (7–9), which appear as nodules, papules, or hypopigmented macules. PKDL can appear from 6 months to years after apparent VL cure in the Indian subcontinent, but can also occur earlier (within 6 months) or along with VL in the Sudan, where the incidence of this disease is higher. PKDL heals spontaneously in a proportion of cases in Africa, but rarely in Indian VL patients and requires prolonged treatment. Since PKDL patients harbor increased parasite numbers in their skin, they are thought to act as parasite reservoir and play an important role in disease transmission in endemic regions. As such, they may be an important population to target with effective host-directed immunotherapies.

Cutaneous leishmaniasis (CL) is characterized by the development of ulcerative skin lesions containing parasites and is the most common form of disease occurring mainly in Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia, and Syria (2, 10). Cutaneous lesions are generally localized and may persist for months to years or heal spontaneously within weeks. The development of disfiguring scars at the affected skin areas following healing is a major concern.

Mucocutaneous leishmaniasis (MCL) is prevalent in Bolivia, Brazil, Peru, and Ethiopia (WHO, 2014) and is caused by *L. baziliensis*, *L. panamensis*, and *L. aethiopica*. These species metastasize to mucosal tissue in the mouth and upper respiratory tract, leading to localized tissue destruction. MCL can present from several months to years after the development of a cutaneous lesion. Diffused cutaneous leishmaniasis (DCL), which is more common in central and South America, is thought to occur in immunosuppressed individuals, where parasites can readily disseminate to subcutaneous tissue. As both MCL and DCL are associated with strong and weak host inflammatory responses, respectively, which appear to contribute to disease pathology, they also have potential for improved treatment involving host-directed immune therapy.

IMMUNOLOGICAL CHARACTERISTICS OF DISEASE

Visceral Leishmaniasis

Studies in experimental VL in mice, caused by infection with the human parasites *L. donovani* or *L. infantum*, show the development of antiparasitic IFNγ-producing, Tbet$^+$ CD4$^+$ T (Th1) cells is critical for resistance against infection (11). Many VL patients fail to generate potent cell-mediated immune responses against parasite antigens and this is thought to be an underlying cause of disease. However, enhanced IFNγ mRNA expression in the spleen and bone marrow, as well as increased circulatory plasma IFNγ, TNF and IL-12 in VL patients, suggests that they do not lack a protective Th1 response, but instead, immunosuppressive mechanism are established to prevent parasite killing (12–15). Importantly, antigen-specific responses in whole blood assays indicate that VL patient CD4$^+$ T cells have the capacity to produce IFNγ in response to parasite antigen (16–18). Therefore, attention has now focused on regulatory mechanisms that prevent Th1 cell-mediated control of parasite growth.

CD4$^+$ T cell IL-10 production has emerged as an important mechanism to dampen T cell activation in parasitic infections, including in humans with VL (15, 19, 20). Importantly, most T cell-derived IL-10 is not produced by thymus-derived Foxp3-expressing regulatory T (Treg) cells. Instead, the IL-10 producing CD4$^+$ T cells often co-produce IFNγ and have been designated type 1 regulatory (Tr1) cells (15). They are increasingly recognized as a critical regulatory CD4$^+$ T cell subset that protects tissue from inflammation (21–23). However, Tr1 cells also appear to promote infection by suppressing Th1 cell-mediated immunity. The role of IL-10 in immune suppression and disease progression is well documented in both experimental and human VL (15, 24–28). Human VL is associated with enhanced IL-10 plasma levels, increased IL-10 mRNA expression in lymph nodes, bone marrow, and spleen, and readily detected IL-10 produced by whole blood cells from VL patients following parasite antigen stimulation (15, 28). IL-10 dampens major histocompatibility complex (MHC) class II expression on APC and downregulates TNF and nitric oxide (NO) production, leading to reduced parasite clearance and suppressed activation of Th1 cells (29). Neutralization of IL-10 in VL patient sera can suppress *L. donovani* replication in macrophages (15, 30), and IL-10 blockade in splenic aspirate cultures from VL patients can limit parasite replication and enhance Th1 cell cytokine production (24). IL-10 can also modulate immune responses by promoting T cell exhaustion (31, 32).

A number of immune checkpoint molecules have also been identified on CD4$^+$ T cells from experimental VL models and VL patients (**Table 1**). These include CTLA-4 (CD152) and PD-1, which are negative regulators of T cells and are expressed on exhausted or anergic T cells during chronic infection. CTLA-4 binds to the costimulatory ligands B7-1 (CD80) and B7-2 (CD86), with much higher affinity than CD28, while PD-1 interacts with PD-1 ligand 1 (PD-L1; B7.H1) and PD-L2. Activation of CTLA-4 leads to increased levels of TGFβ, as well as apoptosis of CD4$^+$ T cells in murine VL (33). In mice infected with *L. donovani*,

TABLE 1 | Immune checkpoint molecules tested for therapeutic effects in leishmaniasis.

	Disease				Biological system		Reference
	Visceral leishmaniasis	Cutaneous leishmaniasis	Diffused cutaneous leishmaniasis	Mucocutaneous leishmaniasis	Human	Preclinical	
IL-10	Y	Y			Y	Y	(15, 24, 26, 27, 41–45)
PD-1	Y	Y	Y		Y	Y	(38, 40, 46–50)
PDL-1/2		Y					(49)
CTLA-4	Y				Y	Y	(34–37, 51, 52)
OX40	Y	Y				Y	(37, 53, 54)
CD40	Y	Y				Y	(36, 55–57)
CD28	Y					Y	(36)
CD80/86		Y				Y	(58–61)
ICOS		Y				Y	(62)

Y, indicates immune checkpoint molecule tested in the disease indicated.

CTLA-4 blockade decreased parasite burden in both liver and spleen, associated with increased frequencies of IFNγ and IL-4 producing cells, and an accelerated hepatic granulomatous response (34, 35). CTLA-4 blockade has also been shown to increase the efficiency of chemotherapy in *L. donovani* infected mice (36, 37). Similarly, blockade of PD-1 or PD-L1 resulted in enhanced parasite clearance and increased pro-inflammatory cytokine production in experimental VL (38–40). Thus, these studies clearly show the therapeutic potential of targeting immune checkpoint molecules for host-directed immune therapy in VL.

Another important regulatory cytokine involved in VL is IL-27, which is composed of the EBI-3 and p28 sub-units. IL-27 belongs to IL-6/12 cytokine family and was originally described as a co-factor for Th1 cell differentiation, along with IL-12 (63, 64). IL-27 promotes T cell IL-10 production in mice, which is further amplified by autocrine IL-21 production (65, 66). IL-27 receptor-deficient mice infected with *L. donovani* developed enhanced Th1 responses, but this was associated with severe liver pathology (67). Patients with active VL also presented with enhanced IL-27 plasma levels, as well as increased mRNA transcripts encoding EBI-3 and p28 in splenic aspirates (28). IL-27 produced by $CD14^+$ cells, along with IL-21 from T cell sources, promoted the differentiation and expansion of Ag-specific, IL-10–producing T cells in VL patients. Importantly, pro-inflammatory cytokines, such as IFNγ, act on macrophages and stimulate IL-27 production, suggesting a feedback mechanism to stimulate IL-10 production to control IFNγ levels and protect host tissue. IL-27 has also been associated with suppression of $CD4^+$ T cell IL-17A and IL-22 secretion (68, 69). Since *L. donovani* antigen-stimulated production of both IL-17A and IL-22 by PBMC in an apparent disease-resistant Sudanese population, these cytokines were proposed to be protective following *L. donovani* infection and, therefore, elevated IL-27 in VL patients might not only promote disease by increasing IL-10 production, but also by regulating IL-17 production (70). Studies with Indian VL patients showed low levels of IL-17A mRNA transcripts, as well as the IL-17-related transcription factor RORγT during active disease (28). However, there was no direct evidence that this Th17 response was suppressed by IL-27. Furthermore, there is evidence from experimental VL that the impact of IL-17A may depend of the stage of infection, whereby this cytokine impedes antiparasitic immunity early (71), but is protective following establishment of infection (72). Hence, immune dysfunction in VL patients appears to involve multiple immune regulatory pathways that differ both spatially and temporally, and identifying which can be safely and effectively targeted for clinical advantage should be a major research priority.

Other immunosuppressive mechanisms established during VL may be mediated through regulatory T cells. Regulatory T cells can be classified as thymus-derived $CD4^+CD25^+FoxP3^+$ T (Treg) cells and inducible regulatory T cells that include conventional T cells that convert to $FoxP3^+$ regulatory T cells in peripheral tissues, as well as Tr1 cells (73, 74). To maintain immune homeostatic conditions, Treg cells limit the activity of potentially self-reactive T cell responses and prevent immune-mediated pathology and autoimmunity (75, 76). However, these same mechanisms may also contribute to impaired pathogen clearance during parasitic infection. Treg cells function by secreting regulatory cytokines such IL-10 and TGFβ, as well as expressing inhibitory molecules such as CTLA-4 and IL-35 (77). Treg cells express high levels of CD25 (IL-2R), thereby allowing them to form the high affinity receptor for IL-2, which allows them to deprive conventional T cells of this important growth factor, thus causing apoptosis (78). However, there is little evidence for the involvement of Treg cells in human or experimental VL. Studies from VL patients in Bihar, India showed no accumulation of Treg cells in the spleen or blood, and the frequency of these cells did not change during the course of infection (15, 79). Moreover, $FoxP3^-$ T cells were the major source of IL-10 mRNA in VL patient spleens, and this finding was in accordance with murine VL studies where IL-10 secretion by $FoxP3^-CD4^+$ T cells correlated with disease severity (19). However, other studies have reported the accumulation of Treg cells at sites of infection and suggested their possible role in disease pathogenesis in both human and experimental VL. One study from India suggested that Treg cells were a major source of IL-10 in the bone marrow of VL patients and that IL-10 secretion from Treg cells suppressed conventional T cells (80). In a different study with Indian VL patients, production of IL-10 and TGFβ by Treg cells was positively correlated with parasite load (81). Similarly, TGFβ-producing Treg cells were shown to accumulate in infected tissues in a murine model of VL (82). Thus, further investigation is needed to establish whether Treg cells

are involved in the pathogenesis of VL and whether they can be modulated for therapeutic advantage. Interestingly, TGFβ is also secreted by macrophages and dendritic cells (DCs) during experimental VL. A cathepsin B-like cysteine protease present in *L. donovani* can activate TGFβ (83), which, in turn, activates arginase-1, leading to enhanced L-ornithine production and reduced NO secretion, thereby promoting parasite survival in infected cells (84, 85). Human VL patients have enhanced TGFβ plasma levels (13) during active disease, suggesting a possible role in pathogenesis. However, more research is needed to better understanding the precise mechanisms of TGFβ-mediated suppression of antiparasitic immunity before it can be considered as an immune therapy target.

The development of regulatory DC subsets following *L. donovani* infection can also have a major impact on T cell responses during VL. These regulatory DCs are capable of producing anti-inflammatory cytokines, such as IL-10, TGFβ, and IL-27 (86). In experimental VL, it was shown that IL-10^{+}IL-27^{+} DCs were able to promote IL-10 production by Th1 cells *in vivo* and identified this cell population as a potential target for immunotherapy (87). Furthermore, CD11c^{lo}CD45RB^{+} DCs in the spleen of *L. donovani*-infected mice had high levels of IL-10 production, compared to CD11c^{hi} populations, and displayed features of immature DCs, including low expression of co-stimulatory molecules and intracellular MHC class II (88). These DCs also produced IL-10 when stimulated with lipopolysaccharide and promoted Treg cell IL-10 production capable of inhibiting mixed lymphocyte reactions driven by conventional DCs (89). The inhibitory effects of these regulatory DCs could be reversed by IL-10 signaling blockade, indicating that IL-10 production was a critical regulatory mechanisms employed by this DC subset (88). Therefore, both cognate and soluble cytokine signals between effector CD4^{+} T cells, DCs, and infected macrophages have key roles in determining whether parasite growth is controlled and/or disease develops, making these interactions promising targets for immune therapy (**Figure 1**).

CD8^{+} T cells can kill *Leishmania*-infected macrophages by secreting cytolytic enzymes (90, 91). However, studies with human VL blood samples suggest that CD8^{+} T cells have an anergic or exhausted phenotype, as indicated by high expression of IL-10, CTLA-4, and PD-1, which may hamper the protective efficiency of these cells during active disease (51). A better understanding of role of CD8^{+} T cells during VL is needed if the antiparasitic potential of these cells through vaccination or immune therapy can be exploited.

POST KALA-AZAR DERMAL LEISHMANIASIS

Post kala-azar dermal leishmaniasis often develops as a cutaneous complication of VL in apparently cured patients, but can also develop independent of VL. The pathogenesis of PKDL remains poorly understood. It has been postulated that immune suppression may allow multiplication of latent parasites from the viscera or residing in the skin (8). Similar to clinical VL, elevated IFNγ and TNF levels are found in lesions of PKDL patients, with the concurrent presence of the immunosuppressive cytokines IL-10 and TGFβ (92). Despite the presence of high IFN-γ and TNF in these tissues, there is reduced expression of IFNγ and TNF receptors in Indian PKDL patients (92, 93), while genetic polymorphisms in the IFNγ receptor 1 gene promoter region have been reported and found to be associated with susceptibility to PKDL in Sudanese patients (94, 95). Treg cells have also been associated with PKDL in the Indian subcontinent and elevated FoxP3, CD25, and CTLA-4 mRNA expression has been reported in the skin of patients. Furthermore, Foxp3, CD25, and IL-10 mRNA levels directly correlated with parasite load in these PKDL patients (96). Since PKDL either develops soon after VL or independent of VL in the Sudanese population, but takes longer to develop after VL in the Indian subcontinent, the immunopathology of PKDL is likely to differ in these populations. PKDL patients from the Sudan display immune responses similar to cured VL patients and their PBMC proliferate in response to parasite antigens and CD4^{+} T cells secrete IFNγ and IL-10 (97, 98). However, PKDL patients from the Indian subcontinent have high numbers of CD8^{+} T cells in their lesions and circulation, along with increased antigen-induced IL-10 production by circulating CD8^{+} T cells and impaired antigen-induced proliferation (99, 100). Studies with Indian PKDL patients have also demonstrated enhanced Th17 cell responses by analyzing mRNA and protein expression of Th17-related IL-23, IL-17A, and RORγt (101). Stimulation of PKDL patient PBMCs with

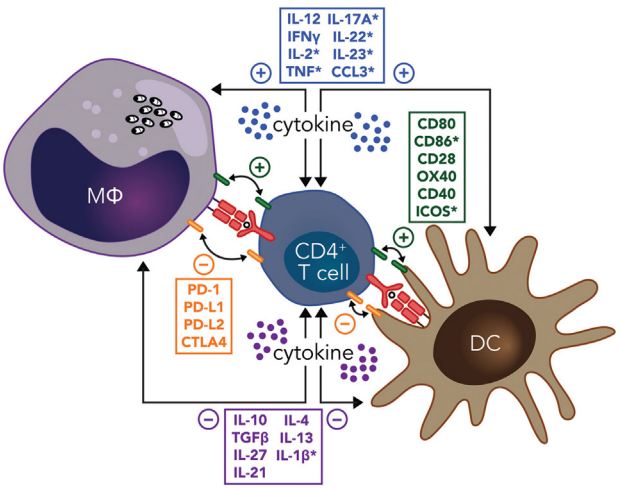

FIGURE 1 | Potential immunotherapy targets to manipulate CD4^{+} T cell-dendritic cell (DC) and CD4^{+} T cell-infected macrophage (MΦ) interactions during leishmaniasis include both cognate and soluble cytokine signals. Primary signals between CD4^{+} T cells and macrophages or DCs through major histocompatibility complex class II antigen presentation of parasite peptide to the T cell receptor are indicated (red), as are both positive (green), and negative (orange) costimulatory signals. Positive (blue) and negative (purple) soluble cytokine signals are also show. Note that many of these signals are bidirectional, as indicated by the double-ended arrows. In addition, molecules highlighted by an asterisk have been reported to have the opposite effects in different type of *Leishmania* species infections. Amastigotes residing in macrophages are shown in black.

parasite antigens resulted in IL-17A and IL-23 production, while stimulation with recombinant IL-17A enhanced TNF and NO production. Hence, these data suggest that enhanced Th17 responses may have a role in parasite clearance during PKDL. However, it is still not clear whether regulatory cytokines and/or other mechanisms suppress IL-17-mediated protective responses during active disease. This knowledge is important if we wish to manipulate this immunoregulatory pathway to improve antiparasitic immunity.

CUTANEOUS LEISHMANIASIS

Cutaneous leishmaniasis is caused by several *Leishmania* species, including *Leishmania major*, *L. braziliensis*, *L. mexicana*, *and L. amazonensis.* Cell-mediated immune responses at the site of cutaneous lesions are of primary importance in determining the outcome of disease. Furthermore, in murine models of CL caused by *L. major*, the genetic background of mice also determines disease outcome. In C57BL/6 mice, Th1 cell responses promote a self-healing process, while Th2 responses are associated with parasite persistence in the lesions of BALB/c mice [reviewed in Ref. (102, 103)]. In humans infected with *L. major*, cutaneous lesions have been associated with high IFN-γ, IL-10, and IL-12 mRNA accumulation, indicative of a mixed $CD4^+$ T cell response. Several immune checkpoint molecules have also been identified in experimental CL studies that can modify $CD4^+$ T cell responses to favor parasite clearance (**Table 1**), again demonstrating immune checkpoint blockade as a potential approach to improve disease treatments.

Following transmission of *L. major* to mice *via* sand fly bites or needle, neutrophils rapidly infiltrate the bite site and capture injected parasites (104). Neutrophils rapidly express apoptotic markers following *L. major* uptake, which attracts monocytes and DCs to the site of infection for removal of apoptotic cells (105, 106). This allows the uptake of parasites into phagocytic cells without triggering inflammation, and thereby enabling establishment of infection. Infected neutrophils also express chemokines, such as CCL-3, to attract DCs to the site of infection (107). This may help to stimulate Th1 cell responses following activation of DCs through interaction of DC-SIGN on DC and specific glycans on neutrophils (108). In addition, CCL3 can induce IL-12 secretion by macrophages in C57BL/6 mice, but not in BALB/c mice (107). Given these latter pro-inflammatory properties of neutrophils, any modifications of neutrophil functions may have to be directed specifically toward their activity as a "Trojan horse" for establishment of infection. However, it is important to remember that the role of neutrophils is critically dependent on the *Leishmania* species in question, the parasite lifecycle stage and stage of infection, when trying to manipulate neutrophil functions.

Following *L. major* infection, complement-dependent platelet activation, including the release of platelet-derived growth factors, can stimulate the release of CCL2/MCP-1 by leukocytes and mesenchymal cells, leading to recruitment of $Ly6C^+$ inflammatory monocytes, which can capture and kill parasites *via* oxidative burst (109). Importantly, these monocytes can migrate to lymph nodes and differentiate into specialized DC subsets during *L. major* infection. These monocytes-derived DCs secret high levels of IL-12 and stimulate *L. major*-specific Th1 cell responses, suggesting a contribution to protection against disease (110, 111). Monocytes expressing high levels of CCR2 can also capture *L. major* at the site of infection in C57BL/6 mice, then migrate to draining lymph nodes and differentiate into inducible nitric oxide (iNOS)-producing DC that also promote Th1 cell-mediated protection (112). Hence, the promotion of these activities in the context of vaccination or drug treatment may be desirable.

NK cells are also recruited to the site of infection in mice infected with *L. major* and produce IFN-γ, which can amplify DC IL-12 production required for the development of strong Th1 cell responses (113). However, NK cells can also produce IL-10 during *L. donovani* infection (114), suggesting they can play either antiparasitic or immunoregulatory roles during infection. Depending upon the dose of infection, $CD8^+$ T cells also produce IFN-γ in murine models of CL, which can also help shape early adaptive immune responses associated with protection (115–118). However, resolution of infection following *L. major* infection is primarily associated with $CD4^+$ T cell-mediated immunity (119–121). Despite healing of cutaneous lesions, parasites continue to persist at the original site of infection, in part due to IL-10-mediated mechanisms, and these persisting parasites are thought to help maintain effector memory $CD4^+$ T cells (T_{EM}) that protect against re-infection (122, 123). This T_{EM} response is lost if parasites are eliminated, as shown by studies in which mice were manipulated to achieve sterile cure (122). Thus, concomitant immunity is compromised and protection against a secondary challenge can be lost in the absence of persisting parasites (124). However, there is also evidence that a pool of long-lasting central memory $CD4^+$ T cells (T_{CM}) can develop in absence of persisting parasites, and that these can acquire effector functions after re-infection leading to protection (125). These T_{CM} cells require additional IL-12 signals to develop into fully functional Th1 cells, and in absence of this signal, they can convert into IL-4-producing Th2 cells (126). T_{CM} cells appear to be generated early during infection, and not only help in controlling secondary infections, but also contribute to clearance of primary infection (127). Hence, these findings suggest both T_{EM} and T_{CM} cells participate in maintaining immunity to *L. major* infection, but only the T_{EM} require persistent parasite antigen. Therefore, vaccines designed to protect against leishmaniasis should target the expansion of long-lasting T_{CM} cells, rather than short lived T_{EM} cells.

More recently, skin resident memory (T_{RM}) $CD8^+$ T cells have been shown to provide protection against *L. major* infection, independent of circulatory $CD4^+$ T cells, by recruiting inflammatory monocytes, which rapidly control parasite growth *via* reactive oxygen species (ROS) and NO generation (128). Thus, these T_{RM} cells also represent a potential target cell population for vaccination. Treg cells appear to play a role in *L. major* persistence in C57BL/6 mice by suppressing $CD4^+$ T cell effector functions through IL-10-mediated immunosuppressive mechanisms (122). The IL-10 produced by these Treg cells can also promote parasite persistence by modulating APC function and/or inhibiting parasite killing mechanisms in infected

macrophages. Thus, the activity of Treg cells at the site of infection can promote concomitant immunity, but also allow parasites to persist. Therefore, although Treg cells could be targeted for immunomodulation, care would have to be taken to ensure that long-term protection was not compromised.

In non-healing CL caused by *L. major* Seidman strain in C57BL/6 mice, Nlrp3 inflammasome-dependent IL-1β activation plays an important role in determining disease outcome (129). The activation of the Nlrp3 inflammasome enhanced IL-1β activation through caspase-1 cleavage, which caused recruitment of neutrophils to the site of infection, and ultimately resulted in the suppression of immunity, which was confirmed by using neutropenic Genista mice (129). Nlrp3 can promote Th2 cell development in non-healing cutaneous lesions caused by *L. major* infection in BALB/c mice (130). Thus, this inflammasome and related cell signaling pathways are potential targets for immune therapy to treat and promote healing of cutaneous lesions in human CL (see also below). However, inflammasome- and caspase-1-dependent IL-1-β production has been shown to provide resistance against *L. amazonensis* infection in mice by triggering NO production (131), thus emphasizing the need for careful consideration in choosing appropriate targets for immune modulation in specific disease settings.

MUCOCUTANEOUS LEISHMANIASIS

Although self-cure is often the outcome of CL, some patients infected with *L. braziliensis, L. panamensis*, and *L. aethiopica* can develop MCL after resolution of their primary lesion, characterized by chronic inflammation of the nasal mucosa and by a hyperactive T-cell response (132, 133), associated with high levels of pro-inflammatory cytokines, such as IFN-γ and TNF-α, and decreased levels of IL-10 and TGF-β (132, 134, 135). Thus, a poorly regulated T cell response is an underlying cause of disease pathogenesis in MCL patients. In patients infected with *L. braziliensis,* the number of $CD8^+$ T cells recruited to lesions increased as disease progressed, and these cells expressed high levels of granzymes and perforin, indicating they had elevated cytolytic activity (136). In fact, these $CD8^+$ T cells have now been shown to contribute to inflammation and disease pathology *via* perforin-mediated cytotoxicity (137). In mice co-infected with lymphocytic choriomeningitis virus and *L. braziliensis*, it was found that perforin-mediated $CD8^+$ T cell cytotoxicity in the lesion resulted in enhanced recruitment of neutrophils and monocytes, which produced IL-1-β that contributed to immunopathology and disease severity (138). Importantly, pharmacological blockade of Nlrp3 reduced inflammation caused by cytotoxic $CD8^+$ T cells in this mouse model of MCL, thus identifying this inflammasome, as well as $CD8^+$ T cell-mediated cytotoxicity, as potential targets for immunotherapy. This was supported by additional data from the same study, using skin biopsies and PBMCs from CL patients infected with *L. braziliensis*, which IL-1β was highly expressed in skin lesions and blockade of the Nlrp3 inflammasome prevented the IL-1-β secretion from skin biopsies, suggesting a similar pathogenic mechanism might be operating during clinical MCL.

In addition to $CD8^+$ T cell-mediated pathology, Th17 cells have also been associated with pathogenesis in MCL patients (139). MCL lesions were found to have elevated IL-17A mRNA, as well as TGF-β, ROR-γT, and IL-23 mRNA levels, which are associated with Th17 cell differentiation. Interestingly, IL-17 was not only produced by $CD4^+$ T cells but also by $CD8^+$ T cells, $CD14^+$, and $CCR6^+$ Cells. The enhanced production of IL-17 was associated with infiltration and recruitment of neutrophils into the lesion, suggesting that IL-17 may promote inflammatory responses in MCL patients. Thus, IL-17 production could be a therapeutic target in MCL patients to reduce tissue pathology.

DIFFUSE CUTANEOUS LEISHMANIASIS

Diffused cutaneous leishmaniasis is a severe manifestation of CL characterized by a defective cellular immune response to *Leishmania* antigens (140). However, this unresponsiveness is restricted to antiparasitic immune responses, as responses to unrelated antigens remain intact (141, 142). DCL patients have high parasite numbers within skin lesions, which has been associated with low levels of IFN-γ and IL-2 mRNA, and concurrent high levels of IL-10, IL-4, and IL-5 mRNA in lesions (135). Therapeutic cure was associated with enhanced IFNγ production, but low IL-10 expression (143), indicating the requirement for a classical Th1 cells response for favorable clinical outcomes. This disease is also associated with high antibody titers and plasma TGF-β [reviewed in Ref. (102)]. IL-10 and TGF-β, along with Treg cells, can antagonize IFN-γ and TNF activities, resulting in impaired microbicidal activities in infected macrophages [reviewed in Ref. (144)]. However, it is not clear in DCL whether high antigen exposure causes T cell unresponsiveness or if impaired T cell responses promote localized parasite growth in the skin [reviewed in Ref. (144)].

Diffused cutaneous leishmaniasis patients respond poorly to conventional drug treatment (145), but some degree of treatment success has been achieved with immune modulation using IFN-γ combined with viable BCG and antimonial drug (146, 147). Since unresponsive T cells often express inhibitory molecules, such as PD-1, CTLA-4, and LAG-3, these may make attractive targets for immune therapy in DCL patients. IL-1-β has also been associated with disease severity in *L. mexicana*-infected DCL patients (148), making it another potential therapeutic target. Again, care will need to be taken to ensure the promotion of antiparasitic immunity in this context is not at the expense of protection against tissue damage.

LEISHMANIZATION

The fact that *L. major*-induced cutaneous lesions often heal spontaneously and protect against future infection is the basis for leishmanization, which involves inoculation with live, virulent parasites in an unexposed part of body to produce a controlled lesion. This strategy has been practiced successfully in the former Soviet Union, Middle East, and Israel, and likely

provides protection in humans because it mimics a natural infection, including allowing parasite persistence and development of concomitant immunity. The protection provided by leishmanization is essentially T cell-mediated, whereby IFN-γ-producing $CD4^+$ T cells are recruited to dermal sites of infection where they perform effector functions, including the promotion of microbicidal mechanisms in infected macrophages (123). Importantly, the success of leishmanization depends on the viability and infectivity of injected parasite. Parasites that lost virulence stimulated delayed-type hypersensitive reactions, but did not provide protection from natural re-infection (149). Leishmanization was abandoned in most countries because of logistical problems and safety concerns, due to some immunosuppressed individuals developing non-healing lesions (150). Interestingly, leishmanization can provide cross protection against the visceral form of disease, as leishmanized C57BL/6 mice infected with *L. major* were protected from heterologous visceral infection with *L. infantum*, associated with recruitment of IFN-γ-producing $Ly6C^+CD4^+$ T cells to both skin and visceral organs (151). Similarly, longitudinal studies in the Sudan indicated that people residing in an *L. major* endemic area were protected against VL caused by *L. donovani* (152, 153). In addition, CL caused by a *L. donovani* strain in Sri-Lanka provided cross protection against visceral disease (154). These findings suggest that leishmanization could be a strategy employed to increase protection against VL. However, a better understanding of the immunoregulatory mechanisms associated with this process is needed to fully exploit the positive aspects of leishmanization with improved safety.

STRATEGIES TO IMPROVE VACCINES

Although different *Leishmania* species cause a broad range of clinical symptoms, genetic analysis indicates a large degree of genomic conservation between species. Thus, it may be possible to generate broadly effective vaccines against different clinical diseases. However, despite many efforts, there is no effective, licensed vaccine to prevent human leishmaniasis. There is a major need for more efficacious and less toxic adjuvants and immune therapies for better vaccines for patients suffering from leishmaniasis. Studies in VL patients and experimental models (15, 155) indicate the rapid development of immunoregulatory networks following exposure to parasites, which raises questions about how these regulatory networks might influence subsequent immunity, particularly to vaccines. It is noteworthy that many vaccines tested in disease endemic regions have not performed as well as when tested in healthy volunteers. For example, the RTS,S/AS01 vaccine in children and infants affords 36 and 25% efficacy against clinical malaria, respectively (156), while a recent study showed that the efficacy of the same vaccine in healthy volunteers in CHMI studies was 52% (157). Similarly, BCG-mediated protection against pulmonary tuberculosis varies geographically and appears to be much less effective in areas with high incidence of previous infection with *M. tuberculosis* or sensitization with environmental *Mycobacteria* (158, 159). Although many reasons could account for the reduced efficacy of vaccines in disease endemic areas, these results suggest that the early establishment of potent, pathogen-specific immunoregulatory networks may be an important factor contributing to this problem (160). Treatment in the field of cancer has been revolutionized by immune checkpoint blockade strategies. These take advantage of the patients own immune system to recognize and kill cancer cells. Although many of the molecules being targeted by this approach were discovered in infectious diseases research, this approach has not been applied to reducing the burden of infection. Therefore, incorporating inhibitors of specific immune checkpoints into vaccine formulations may be one way to transiently reduce immune suppression to allow the generation of robust vaccine-mediated, antiparasitic immunity.

Both CTLA-4 and PD-1 blockade have been successfully used individually and in combination to treat cancer patients (161). Given that leishmaniasis is a chronic infection and shares several key immunoregulatory features with cancer, one strategy could be to "piggy back" on the success of immune checkpoint blockade drugs in cancer to either improve drug treatment protocols by making subsequent immunity more potent and long-lasting or enhance vaccine efficacy. However, it will be important to bear in mind that specific types and combinations of immune checkpoint blockade work best for particular types of cancer, and this is also likely to be the case with the spectrum of diseases caused by *Leishmania* species. Thus, careful consideration will need to be given to types of immune checkpoint blockade best suited to VL, CL, MCL, PKDL, or DCL because they are likely to differ in their outcomes.

STRATEGIES TO IMPROVE DRUG TREATMENT

Antimonial chemotherapy was the mainstay for VL treatment for many decades (162). However, parasite resistance against these drugs has developed, especially in the Indian subcontinent (163, 164). Therefore, these drugs are now mainly employed to treat VL in Africa, while drugs such as Amphotericin B, Ambisome, Miltefosine, and paromomycin have been introduced to treat VL in areas of antimonial drug resistance (164, 165). However, these drugs are not without problems, such as toxicity, high cost, potential development of parasite drug resistance, and prolonged treatment regimes [reviewed in Ref. (165)]. Recently, a single dose of Ambisome (lipid formulation of Amphotericin B) was found to be sufficient to successfully treat VL with low toxicity and has now been recommended as a choice of treatment in India subcontinent (166, 167). The oral drug miltefosin has also been used in combination with Amphotericin B. However, based on studies in preclinical models of leishmaniasis, there are concerns that even with combination therapy, drug resistance will develop (168). Further, these drugs do not cause sterile cure and parasites persist in the infected individuals after drug treatment (168–170). This is concerning because these persisting parasites may help promote transmission,

with people living in the same household being most at risk of infection (171, 172). Thus, not only should successful cure of disease be a goal of treatment, but lowering the burden of persisting parasites as far as possible is also desired if parasite transmission is to be minimized. However, when considering these goals, it will be important to remember that persistent parasites are also required to maintain concomitant immunity (170), so sterile cure of infected individuals may not necessarily be beneficial. Instead, it may be necessary to establish the level of parasite burden that is low enough to prevent parasite transmission, but at the same time, maintain concomitant immunity, and then try and achieve this through a combination of antiparasitic drug and immunomodulatory strategies.

Drug treatment works most effectively in association with the host immune system, and in particular, cell-mediated immune responses (164). Hence, understanding immunological changes during the course of infection and how these might be modulated to work best with drug is important. The use of biological molecules to stimulate cell-mediated immunity to help achieve therapeutic success has been tested in both preclinical and clinical studies on leishmaniasis (173). In an experimental model of leishmaniasis, treatment with recombinant IL-12 or anti-IL-10 receptor monoclonal antibody, along with pentavalent antimony (Sb^v), resulted in improved clearance of *L. donovani* parasites, compared with animals treated with drug alone (27, 174). In addition, human recombinant IFN-γ has been successfully used to accelerate antiparasitic and clinical responses when used with antimony treatment, and importantly, treat seriously ill VL patients with refractory disease (175–178). Many drugs used to treat VL not only kill parasites, but also promote host immunity. For example, antimonial drugs stimulate the generation of ROS and NO, while miltefosine and Ambisome induce the secretion of IFN-γ, TNF, IL-12, IL-6, and IL-1β from immune cells with a simultaneous decrease in anti-inflammatory cytokine production (179–182). Thus, combining immune-based therapy with conventional antiparasitic drugs is an obvious strategy to improve current treatment protocols.

Rather than supplement or block immune effector molecules, another approach to use host-directed therapy to improve drug treatment is to target intracellular signal transduction pathways. For example, it has been shown that *L. infantum* infection rapidly induces activation of phosphatidylinositol 3-kinase/Akt and extracellular signal-regulated kinase1/2 in bone marrow-derived dendritic cells (BMDDC), thereby limiting their maturation and pro-inflammatory cytokine secretion. The blockade of this pathway with wortmannin resulted in reduced infection rates of BMDDC (183). Similarly, the rapid activation of protein tyrosine phosphatase, such as SHP-1, by *Leishmania* is another important parasite evasion strategy, and administration of the SHP-1 inhibitor bpV-phen to mice infected with *L. major* and *L. donovani* promoted control of infection *via* induction of reactive nitrogen intermediates that would otherwise be repressed by parasite-activated SHP-1 (184, 185). Hence, small molecule inhibitors of key cell signaling pathways is another potential approach that could be used with current antiparasitic drug treatment protocols.

DEVELOPING STRATEGIES FOR RESOURCE POOR SETTING

Leishmaniasis is generally a disease associated with poverty (186) and as such, diagnosis, treatment, and hospitalization costs are an important consideration in disease control programs. In addition, drug development programs for leishmaniasis are often not a high priority for pharmaceutical companies. Furthermore, even if effective, high-cost drugs or vaccines are available, they are unlikely to be used without significant government or philanthropic subsidization. Hence, a practical challenge is to supply relatively cheap drugs in resource poor settings, and this will require the participation of regulatory bodies, as well as public and private sector partnerships. A successful example of this was the implementation of the wider use of Ambisome for VL treatment. This is normally a high-cost drug, but has been substantially reduced in cost for the distribution through the public sector agencies in developing countries, by an agreement negotiated between WHO and the manufacturer (187). However, other issues can complicate such arrangements. For example, the requirement of a reliable cold chain for Ambisome implementation can result in a failure to provide the drug or the use of drug stored under conditions not consistent with storage advice. Therefore, the introduction of new treatment regimes for diseases such as VL will need to consider multiple aspects of drug development, formulation, storage, and delivery. One approach for reducing development costs is to repurpose drugs already licensed for other indications, as was the case with Ambisome, which was first licensed as an anti-fungal drug. Another way of reducing cost is to develop cheaper small molecules rather than more expensive biologics to target parasites or host responses. In addition, as well as usual safety considerations, the stability of drugs in areas of unreliable cold-chain must be considered, as well as ease of manufacturing. Therefore, when considering strategies to promote host directed therapy, regardless whether this be targeting specific immune check points or stimulating microbicidal mechanisms, small molecules are likely to be more cost-effective than antibodies.

CONCLUDING REMARKS

Leishmaniasis has clear, unmet medical needs. These differ, depending on the disease in question. However, the host immune response to infection is a central component of each of these diseases, whether it is immune dysfunction in the case of VL and DCL or immune-mediated tissue pathology in the case of severe CL and MCL. Therefore, targeting these host responses, as is increasingly occurring in other chronic diseases, such as cancer and autoimmunity, offers promising new opportunities to either improve the efficacy of vaccine candidates or drug treatment protocols. Given the long time lines for vaccine development, the latter approach may have a greater impact in the short term. Furthermore, combining host-directed therapy with antiparasitic drug, offers the added advantage of further reducing parasite loads in treated individuals and improving long-term protective immunity. These outcomes will greatly benefit current disease elimination programs.

AUTHOR CONTRIBUTIONS

RK, SC, SN, SS, and CE wrote the paper. SN and SC prepared the figures and table.

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REVIEW
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Interleukin-4 Receptor Alpha: From Innate to Adaptive Immunity in Murine Models of Cutaneous Leishmaniasis

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The interleukin (IL)-4 receptor alpha (IL-4Rα), ubiquitously expressed on both innate and adaptive immune cells, controls the signaling of archetypal type 2 immune regulators; IL-4 and IL-13, which elicit their signaling action by the type 1 IL-4Rα/gamma common and/or the type 2 IL-4Rα/IL-13Rα complexes. Global gene-deficient mouse models targeting IL-4, IL-13, or the IL-4Rα chain, followed by the development of conditional mice and generation of important cell-type-specific IL-4Rα-deficient mouse models, were indeed critical to gaining in-depth understanding of detrimental T helper (Th) 2 mechanisms in type 1-controlled diseases. A primary example being cutaneous leishmaniasis, which is caused by the protozoan parasite *Leishmania major*, among others. The disease is characterized by localized self-healing cutaneous lesions and necrosis for which, currently, not a single vaccine has made it to a stage that can be considered effective. The spectrum of human leishmaniasis belongs to the top 10 infectious diseases according to the World Health Organization. As such, 350 million humans are at risk of infection and disease, with an incidence of 1.5–2 million new cases being reported annually. A major aim of our research is to identify correlates of host protection and evasion, which may aid in vaccine design and therapeutic interventions. In this review, we focus on the immune-regulatory role of the IL-4Rα chain from innate immune responses to the development of beneficial type 1 and detrimental type 2 adaptive immune responses during cutaneous *Leishmania* infection. We discuss the cell-specific requirements of the IL-4Rα chain on crucial innate immune cells during *L. major* infection, including, IL-4Rα-responsive skin keratinocytes, macrophages, and neutrophils, as well as dendritic cells (DCs). The latter, contributing to one of the paradigm shifts with respect to the role of IL-4 instructing DCs *in vivo*, to promote Th1 responses against *L. major*. Finally, we extend these innate responses and mechanisms to control of adaptive immunity and the effect of IL-4Rα-responsiveness on T and B lymphocytes orchestrating the development of $CD4^+$ Th1/Th2 and B effector 1/B effector 2 B cells in response to *L. major* infection in the murine host.

Keywords: interleukin-4 receptor alpha, interleukin-4/interleukin-13, murine cutaneous leishmaniasis, innate cells, adaptive cells

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INTRODUCTION

Human leishmaniasis, ranging from localized ulcerating lesions (cutaneous) to disseminated (mucocutaneous) and fatal infection (visceral), presents a global health concern, with over 12 million people currently infected and an additional 350 million humans at risk of infection and disease (1, 2). It is therefore not surprising that infection rates surpass 1.5 million new cases annually (3). Despite a concerted effort to develop a vaccine against the parasite, not a single candidate has been proven effective, and current therapeutic approaches are unable to achieve a sterile cure (2, 3). A thorough understanding of the mechanisms by which *Leishmania* spp. evade or exploit host immune mechanisms, to persist and establish disease, is paramount to identifying new and improved strategies for effective management of the disease. To address this, experimental models of cutaneous leishmaniasis (CL), which is the most common form of the disease, was established. In this model, disease is induced by infecting mice subcutaneously with *Leishmania major*. The *L. major* mouse model provided an excellent system for investigating the mechanisms underlying T helper (Th) 1 and Th2 cell differentiation relating to resistance and susceptibility to intracellular infection (4–6). This model, in global gene-deficient mice, established that the archetypal Th2 cytokines, interleukin-4 (IL-4) and interleukin-13 (IL-13), are susceptibility factors during *L. major* infection in BALB/c mice, counter-regulating a protective Th1 response, and induce their biological functions through a common receptor, the interleukin-4 receptor alpha (IL-4Rα) chain (7–9). However, IL-4Rα-deficient BALB/c mice remain susceptible to *L. major* infection in chronic stages (9, 10), indicating that IL-4/IL-13 may induce protective responses depending on which cell/s the IL-4/IL-13 ligand/s interact with during disease (11). Given the ubiquitous expression of the IL-4Rα signaling receptor on both innate and adaptive immune cells (12), cell-type-specific IL-4Rα-deficient mice were introduced to dissect the cell-specific roles of IL-4/IL-13 in CL. While these studies exemplified the role of IL-4Rα signaling on specific immune cells (10, 11, 13), it also questioned whether the Th1/Th2 paradigm of resistance/susceptibility to infection was in fact still relevant, considering that in certain disease settings, IL-4/IL-13 signaling essentially instructed a beneficial Th1 response (5, 11). Collectively, these reports highlight that the interplay between resistance and susceptibility to murine *L. major* infection involves a complex, dynamic interaction between the IL-4Rα chain and various innate and adaptive immune cells, with different clinical and immunological outcomes. In this review, we focus on the cell-specific requirements of the IL-4Rα chain signaling on crucial cells mediating innate and adaptive immunity to CL, relating primarily to *L. major* infection in mouse models.

THE IL-4Rα CHAIN: COMMON RECEPTOR FOR IL-4 AND IL-13 SIGNALING

Interleukin-4

Interleukin-4 plays a critical role in initiating and regulating Th2-type immune responses (14). In mice, IL-4 is a 14–19 kDa glycoprotein localized on chromosome 11, together with the genes for IL-5 and IL-13. During the innate immune response, evidence suggests that early IL-4-producers include basophils (15, 16), mast cells (17), eosinophils (18), natural killer (NK) T cells (19, 20), and innate-like skin keratinocytes (21). T and B lymphocytes orchestrating adaptive immunity, specifically $CD4^+$ Th2 cells (22), B effector 2 (Be2) B cells (23, 24), and γ/δ T cells (25), also secrete IL-4. Apart from regulating the differentiation of Th2 cells, IL-4 also controls immunoglobulin class switching in activated B cells, specifying human B cells to switch to the expression of IgE and IgG4 (26), while in mice, to IgE and IgG1, with the concomitant suppression of IgM, IgG2a, and IgG2b (27, 28). Moreover, alternatively activated macrophages are activated by IL-4 signaling through the IL-4Rα chain (29). Importantly, IL-4 inhibits inducible nitric oxide synthase (iNOS) expression thereby inhibiting IFN-γ-induced classically activated macrophages and induction of a type 1 response. As a whole, IL-4 counter-regulates the expression of IFN-γ (12) and increases the expression of MHC II molecules (30), co-stimulatory molecules CD80 and CD86 (31), and the IL-4 receptor (32). Reports have also indicated that dendritic cells (DCs) can respond to IL-4 *in vivo* and *in vitro* and become alternatively activated, in a manner similar to that described for alternatively activated macrophages, by upregulating multiple alternative activation markers such as mannose receptor and RELM-α (33). Moreover, although IL-4 has been shown to be the primary inducer of Th2 responses, studies have reported IL-4-independent Th2 differentiation, Th2 cytokine production, IL-4Rα signaling, and STAT6 regulation (34–41).

Interleukin-13

Murine IL-13 is an immunoregulatory cytokine with a molecular weight of 10–14 kDa (42), also localized on chromosome 11, together with the genes for IL-4 and IL-5. Similar to IL-4, murine IL-13 also promotes upregulation of MHC II antigens, co-stimulatory (CD80/CD86) and adhesion molecules. However, unlike IL-4, murine IL-13 has been shown not to affect Th2 differentiation, B cell switching or upregulation of the low-affinity IgE receptor (CD23), likely due to the absence of a functional IL-13 receptor on those cells in mice. By contrast, human B lymphocytes do respond to IL-13 (34). Innate mast cells, basophils, DCs, NK cells, activated $CD4^+$ Th2 cells, Be2 B cells, and NK T cells are IL-13-producers (34, 43–45). In fact, IL-13 is responsible for activating mast cells, modulating eosinophil function (42), and alternatively activating macrophages in conjunction with IL-4 (29). IL-13 also has immunosuppressive and anti-inflammatory effects on macrophages and monocytes, including suppression of pro-inflammatory cytokines and chemokines. In addition, nitric oxide (NO) production, along with antibody-mediated cytotoxicity, is inhibited by IL-13.

The IL-4 and IL-13 Receptor Complexes

The overlapping biological functions of IL-4 and IL-13 on certain cell-types could be partly attributed to the shared IL-4Rα component of natively distinct receptors (34). This theory was initially demonstrated in competitive studies in which treatment of mice with IL-4 antagonists or anti-IL-4Rα antibodies inhibited both IL-4- and IL-13-mediated responses (46–48). The IL-4Rα chain

(CD124) is a 140 kDa heterodimeric complex, serving as a common monomer in both the type 1 and type 2 receptor complexes (**Figure 1**). It is ubiquitously expressed in fairly low numbers on hematopoietic and non-hematopoietic cells (12). IL-4Rα interacts with the gamma common (γc) chain to form the type 1 IL-4 receptor and interacts with the 65–70 kDa IL-13-binding receptor alpha 1 (IL-13Rα1) chain to form the type 2 IL-4/IL-13 receptor (**Figure 1**) (12). The former is also shared by the receptors for IL-2, IL-7, IL-9, and IL-15. IL-4 binds the IL-4Rα chain with high affinity. By contrast, IL-13 binds the IL-13Rα1 chain with low affinity. However, when paired with the IL-4Rα chain, IL-13 binds the IL-13Rα1 chain with high affinity forming an active signaling unit (49). Expression of IL-13Rα1 is absent on human or murine T cells but constitutively expressed on B cells, epithelial cells and monocytes in both mice and humans (50, 51). In comparison, IL-13 shows a higher binding affinity for the α2 chain of the IL-13 receptor (IL-13Rα2), which is a 55–60 kDa protein (**Figure 1**). IL-13Rα2 was originally considered a decoy receptor for IL-13, devoid of signal transduction, since its short cytoplasmic domain was not reported to contain any binding motifs for signaling molecules (52). However, well-designed reports have demonstrated a signaling pathway for IL-13 through the IL-13Rα2 chain, which induces the production of TGF-β1 and mediates fibrosis (53). In addition to cell-surface localization, soluble forms of both IL-4Rα and IL-13Rα2 exist (**Figure 1**), which are capable of binding IL-4 and IL-13 with high affinity as non-signaling monomers. In doing so, the soluble receptors can act as competitive inhibitors of both IL-4 and IL-13 and modulate their effector responses (54, 55).

Mechanisms of IL-4 and IL-13 Signaling through the IL-4Rα Chain

Both IL-4 and IL-13 signal transduction *via* the IL-4Rα chain involves activation of the Janus-family kinases (JAK). JAK1 interacts with the IL-4Rα chain whereas JAK3 interacts with the γc chain and JAK2 with the IL-13Rα1 (**Figure 1**) (12, 57, 58). IL-4 engagement with the IL-4Rα chain results in tyrosine phosphorylation of the IL-4Rα chain itself as well as phosphorylation of STAT6 and insulin receptor substrate 2 (IRS-2) by JAKs, which then associates with the phosphoinositol-3 kinase (PI-3) (**Figure 1**) (12, 34). STAT6-deficient mice presented impaired IL-13-mediated functions, which confirmed that IL-13 also uses the JAK/STAT6 pathway for signal transduction (**Figure 1**) (34, 59). IL-13 signaling through the IL-13Rα2 requires initial engagement of the IL-13Rα1/IL-4Rα complexes in conjunction with TNF-α signaling, which increases surface expression of IL-13Rα2. IL-13 then binds to the IL-13Rα2 and, through

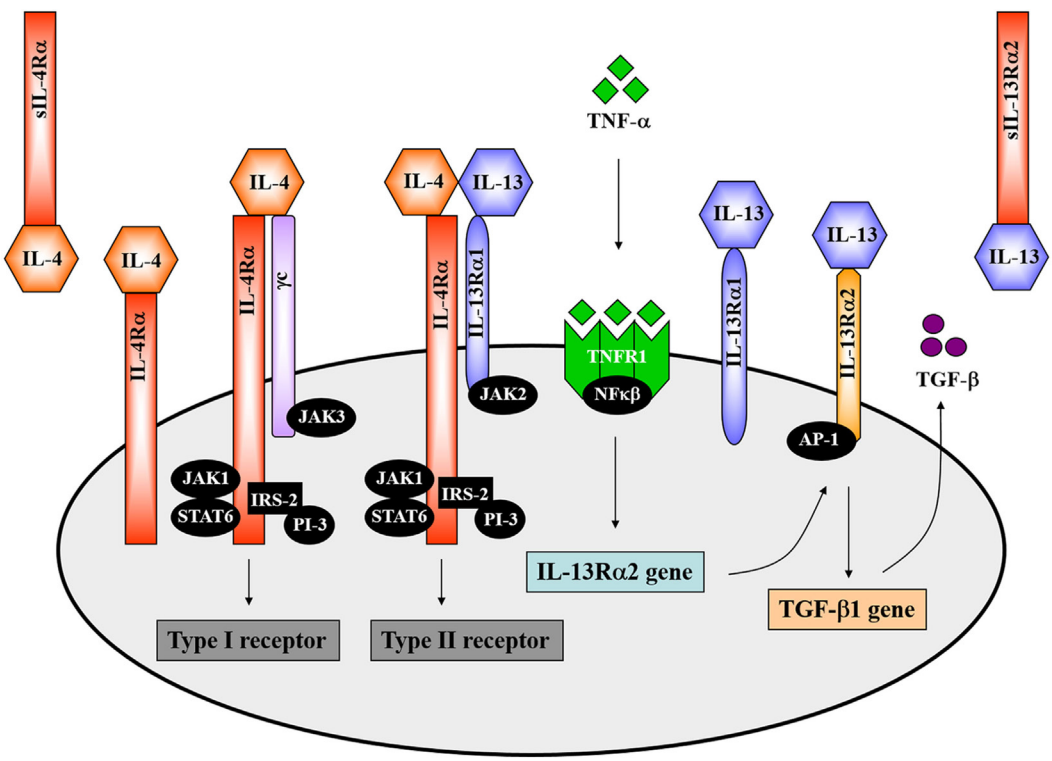

FIGURE 1 | The interleukin-4 (IL-4) and interleukin-13 (IL-13) receptor complexes. IL-4 interacts with the interleukin-4 receptor alpha (IL-4Rα) chain in combination with either the gamma common (γc) chain to form the type 1 receptor or with the IL-13-binding receptor alpha 1 (IL-13Rα1) to form the type 2 receptor complex. The IL-4Rα chain signals *via* the JAK1/STAT6 pathway. Definitively, JAK3 associates with the γc chain and JAK2 with the IL-13Rα1. IL-13 interacts with the type II receptor complex (through IL-13Rα1) or with the α2 chain of the IL-13 receptor (IL-13Rα2). A signaling pathway for IL-13 *via* IL-13Rα2 has recently been identified. TNF-α induces upregulation of IL-13Rα2 expression. IL-13 then binds to the IL-13Rα2, which activates AP-1 to induce gene expression and secretion of soluble TGF-β. Illustration adapted and redrawn from previous publications (12, 34, 42, 52, 56).

activation of the transcription factor AP-1, drives secretion of TGF-β (**Figure 1**) (52, 53).

CUTANEOUS LEISHMANISIS

Cell-Mediated Host Immune Responses to *L. major*

The *Leishmania* parasite has a complex digenetic life cycle alternating between two distinct stages; the promastigote form found in the female *Phlebotomus* sandfly vector and the amastigote form replicating within phagolysosomes of host macrophages and DCs. Ironically, macrophages (and DCs) being the primary immune cells involved in the eradication of *Leishmania* in a mammalian host, are the preferred host cells of the parasite (3, 11, 60, 61). Early pioneering studies focused on the modulation of cytokine signaling as a means to alter immune cell activation and Th cell differentiation. These studies were conducted in an attempt to understand the mechanisms by which *Leishmania* are able to survive and flourish in these hostile environments while also capitalizing on host defense mechanisms to favor the establishment of disease. These mouse models, involving experimental infection with *L. major*, established the Th1/Th2 paradigm of resistance/susceptibility to intracellular infection (5, 6). Upon infection with *L. major*, genetically resistant C57BL/6 mice develop a healing phenotype associated with an IL-12-driven protective Th1/type 1 immune response, upregulation of IFN-γ and classical activation of macrophages for subsequent killing of intracellular parasites by effector NO production (**Figure 2**) (13, 62–66). IL-18 augments IL-12 activity, both of which interact with the IL-12 receptor β2-chain (IL-12Rβ2) in activated Th1 cells (67) and protects against *L. major* infections (68). $CD8^+$ T cells and NK cells enhance this protective response by secreting IFN-γ that activates macrophages for parasite clearance. By contrast, the lack of healing in genetically susceptible BALB/c mice is associated with a Th2/type 2 response characterized by the secretion of IL-4, IL-5, IL-9,

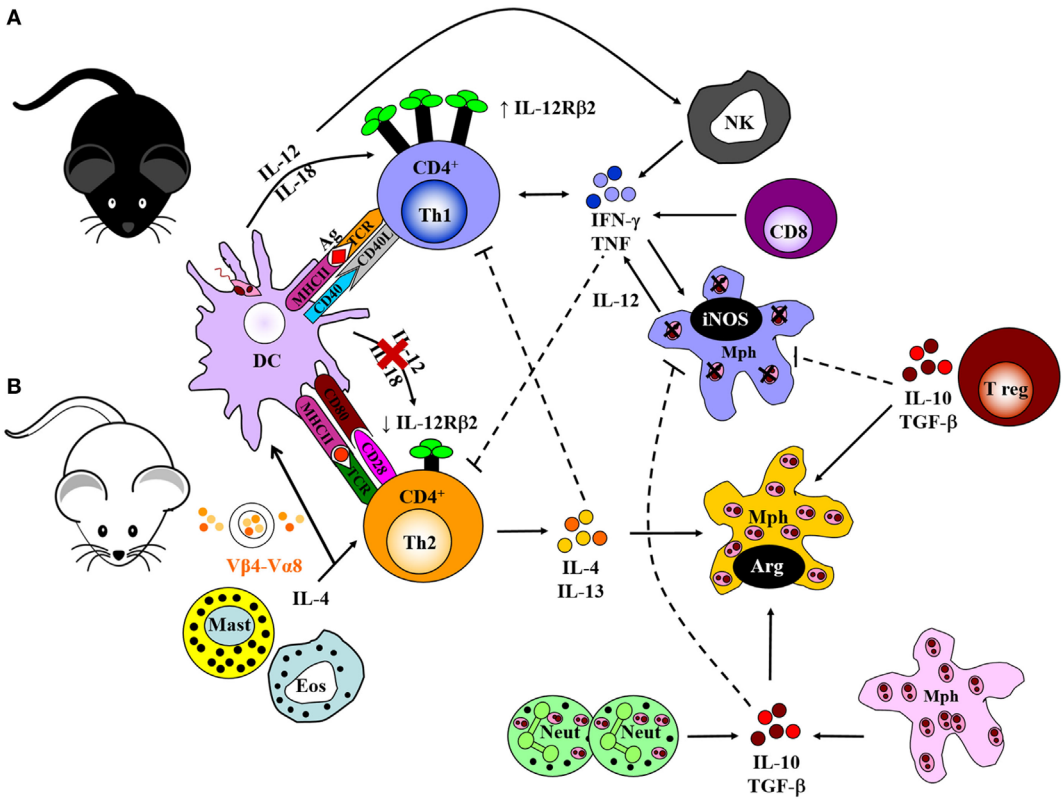

FIGURE 2 | Cell-mediated immune response to *Leishmania major* in resistant and susceptible mice. **(A)** Upon interaction with *L. major* parasites, activated dendritic cells (DCs) process and present parasite antigen and produce IL-12 and IL-18, which interact with the IL-12 receptor β2-chain (IL-12Rβ2) in activated Th1 cells. Together, a dominant Th1 response is generated by the production of IFN-γ and TNF-α. These cytokines upregulate the expression of inducible nitric oxide synthase (iNOS) and activate infected macrophages for intracellular killing. $CD8^+$ T cells and natural killer (NK) cells control infection by producing IFN-γ and effector killing by macrophages leading to parasite control as observed in C57BL/6 mice. **(B)** By contrast, parasite persistence in BALB/c mice is due to the failure of an IL-12-dependent redirection of the early Th2 response. This may be due to continued expression of early interleukin-4 (IL-4) from $Vβ4^+Vα8^+$ $CD4^+$ T cells, mast cells, and eosinophils at the inoculation site and draining lymph nodes. This delays secretion of IL-12, or loss of responsiveness to IL-12 due to reduced expression of the IL-12Rβ2 chain on activated Th2 cells. IL-4 and IL-13 suppress Th1 responses and inhibit intracellular killing by macrophages. This leads to arginase production by macrophages that favor parasite growth. Sustained neutrophil recruitment to the site of infection and regulatory T cells may promote susceptibility and inhibit Th1 responses by the production of IL-10 and TGF-β that inhibit activation of macrophages. Solid lines represent activation, and broken lines represent inhibition. Illustration drawn from previous publications (6, 75, 76).

IL-10, and IL-13, high type 2 anti-*Leishmania* antibody titers, and alternative activation of macrophages, which altogether promotes parasite survival and growth (**Figure 2**) (7, 8, 69–71). BALB/c mice present with lower levels of IL-12. Evidence suggests that this might be due to sustained IL-4 expression by $V\beta4^+V\alpha8^+$ $CD4^+$ T cells, mast cells and eosinophils at the inoculation site and draining lymph node (LN) (**Figure 2**), which delays secretion of IL-12. Alternatively, reduced expression of the IL-12Rβ2 chain on activated Th2 cells lead to a loss in responsiveness to IL-12. In addition, maintenance of inflammatory neutrophils at the site of parasite inoculation (72) and regulatory T cells (Tregs), constituting a source of IL-10, favors the persistence of parasites in leishmanial skin lesions (**Figure 2**) (73, 74).

The early IL-4 response to *L. major* is confined to $CD4^+$ T cells expressing a $V\beta4^+V\alpha8^+$ T cell receptor that recognizes the *Leishmania* antigen LACK (*Leishmania* homolog of receptors for activated C kinase) (14, 77), in addition to mast cells and eosinophils at the site of infection and draining LN. Both BALB/c and C57BL/6 mice secrete IL-4 early after infection; however, production of IL-4 is sustained in susceptible BALB/c mice and transient in resistant C57BL/6 mice (78). The resistant mice redirect the early Th2 response in an IL-12-dependent mechanism toward a beneficial Th1 response, whereas in susceptible mice, the Th2 response persists and dominates the disease outcome by suppressing effector mechanisms essential for parasite killing (6, 79).

IL-4/IL-13 and IL-4Rα Signaling in CL

Since both IL-4 and IL-13 share a common signaling pathway through the IL-4Rα chain, collectively modulating type 2 immunity, IL-4Rα-mediated mechanisms became our primary research interest, in cutaneous and visceral leishmaniasis (7–9, 80) and extending to acute schistosomiasis (81–83), nematode infections (84–86), and allergic airway disease (87, 88). Deletion of the IL-4Rα component impairs downstream signaling of both IL-4 and IL-13 responses *via* transcription factor STAT6 (**Figure 1**). This has been established in animal studies during *L. major* infection in which susceptible BALB/c mice deficient for IL-4 (7), IL-13 (8), IL-4Rα (9, 36), or STAT6 (89) were able to control disease progression. Initial control of *L. major* during the acute phase of infection in $IL\text{-}4^{-/-}$-deficient mice (7) and $IL\text{-}4R\alpha^{-/-}$-deficient (9) BALB/c mice is equivalent, with both strains of mice showing reduced footpad swelling (**Figure 3**), parasite loads and type 1 antibody responses (9). However, in contrast to $IL\text{-}4^{-/-}$ mice, $IL\text{-}4R\alpha^{-/-}$ mice developed progressive disease and necrotic footpad lesions during the chronic phase of infection (**Figure 3**). Thus, the absence of IL-13-mediated functions in $IL\text{-}4R\alpha^{-/-}$ mice implicated IL-13 as a susceptibility factor in chronic *L. major* infection (9). This was confirmed in IL-13 transgenic C57BL/6 mice, which developed a susceptible phenotype to acute leishmaniasis with impaired IL-12 and IFN-γ production, whereas IL-13-deficient BALB/c mice remained comparatively resistant (8, 90). This was attributed to IL-13 promoting susceptibility by activating alternative macrophages and suppressing secretion of NO, IL-12, and/or IL-18 (8, 90).

The role of IL-4 and IL-4Rα in *Leishmania* infection in mice is, however, controversial since contradictory reports have suggested that although IL-4 is important, it is not the sole mediator

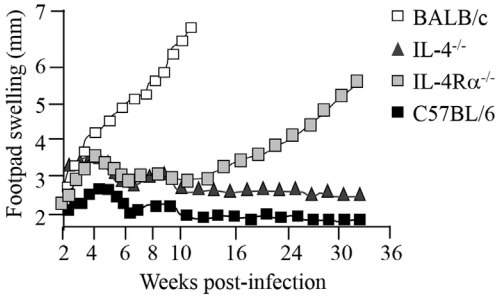

FIGURE 3 | Disease progression in *Leishmania major*-infected-$IL\text{-}4^{-/-}$ and $IL\text{-}4R\alpha^{-/-}$ mice. BALB/c mice deficient for interleukin-4 (IL-4) and interleukin-4 receptor alpha (IL-4Rα) were infected with *L. major* LV39 strain and footpad swelling monitored weekly as an indication of disease progression. Illustration prepared according to published reports (7, 9–11).

of susceptibility in BALB/c mice. While Kopf et al. and Mohrs et al. demonstrated that $IL\text{-}4^{-/-}$ and $IL\text{-}4R\alpha^{-/-}$ mice were able to control CL (7, 9) (**Figure 3**), Noben-Trauth et al. showed that $IL\text{-}4^{-/-}$- and $IL\text{-}4R\alpha^{-/-}$-deficient BALB/c mice remained susceptible to disease and could not contain parasites at the site of infection (37, 91). This discrepancy between the two studies and in general, the outcome of *L. major* infection, may be attributed to a variation of experimental factors, such as the parasite sub-strain used, level of parasite virulence and the embryonic stem cells used to create the knockout mice. Of recent interest, the complex interplay between commensal microbiota in host animals and their vicinity may also be a contributing factor (92). Important to note, despite the absence of IL-4 or IL-4Rα, Th2-cell development and Th2-related cytokines were significantly promoted, although in different Th1/Th2 ratios (7, 37, 91). Subsequently, studies sought to follow IL-4 expression and Th cell development *in vivo* in *L. major*-infected $IL\text{-}4^{-/-}$ and $IL\text{-}4R\alpha^{-/-}$ BALB/c mice (36, 38, 93). The results revealed that, despite a clear absence of IL-4/IL-13-mediated functions in $IL\text{-}4^{-/-}$ and IL-4Rα-deficient mice, unimpaired Th2 polarization and IL-4-producing $CD4^+$ T cells as well as other Th2-related cytokines were still present (7, 34, 36, 38). Accordingly, these pioneering studies confirmed that both IL-4-dependent and IL-4-independent factors contribute to the susceptibility phenotype in *L. major*-infected BALB/c mice. Together, these findings contradicted the idea that IL-4 is the sole regulator of susceptibility to *L. major* infection. The body of literature alternatively suggested that the combined action of IL-4/IL-13 heightens susceptibility to *L. major*, nonetheless, both cytokines may in fact act independently of each other to induce a non-healing response. More importantly, Th2 and type 2 immune responses may be induced independently of IL-4 and IL-13. Thus, at this stage 15 years ago, a definitive role for IL-4/IL-13 in progression of CL remained questionable.

Expression of the IL-4Rα chain reflects the pleiotropic nature of IL-4/IL-13 biology, as this receptor complex is endogenously expressed upon a diverse range of innate and adaptive immune cells in the host. In global gene-deficient mice, the target gene is deficient on all hematopoietic cells. Thus, IL-4- and IL-13-mediated functions at a cellular level remain uncharacterized.

Therefore, it is possible that the range of target cells interacting with IL-4 and IL-13, and their order of hierarchical effector function or importance in the host, may account for differential IL-4/IL-13-mediated mechanisms during cutaneous *Leishmania* infection (4, 5). The emerging principles of innate instruction of adaptive immunity further exemplify that IL-4Rα signaling on innate cells may contribute specific functions that shape the adaptive immune response and outcome of infection.

IL-4Rα SIGNALING ON SPECIFIC INNATE IMMUNE CELLS IN CL IN MICE

Cre/*lox*P Targeting to Generate Cell-Specific IL-4Rα-Deficient Mice

To determine the cell-specific requirements for the IL-4Rα and its ligands, in type 1 and type 2-controlled diseases, we established a second generation of knockout mice using conditional IL-4Rα-deficient mice, thus creating novel mice with a cell-specific deletion of the *il4rα* gene. This was achieved by the bacteriophage-derived Cre/*lox*P genetic recombination system under control of specific loci (**Figure 4**). In this system, cyclization recombinase (Cre) inserted downstream of a cell-specific promoter recognizes a pair of *lox*P sequences flanking the gene of interest (specifically, Exon 7 to Exon 9 of IL-4Rα). Cre-recombinase removes the intervening DNA by bringing the two *lox*P sites together (94). Transgenic Cre mice (Z^{cre}) are backcrossed to BALB/c or C57BL/6 for nine generations, then intercrossed with global IL-4Rα ($IL\text{-}4R\alpha^{-/-}$) BALB/c (9) mice to generate $Z^{cre}IL\text{-}4R\alpha^{-/-}$ BALB/c mice. Littermate mice are then subsequently intercrossed with floxed IL-4Rα ($IL\text{-}4R\alpha^{flox/flox}$) BALB/c mice (Exon 6 to Exon 8 flanked by *lox*P) (82) to yield cell-specific IL-4Rα-deficient mice (or $Z^{cre}IL\text{-}4R\alpha^{-/lox}$) (10, 82, 84). With this strategy, we increase the efficiency of Cre-recombination by reducing the LoxP substrate for Cre-recombinase by 50%, thereby avoiding early aberrant non-Mendelian Cre recombination (10, 82).

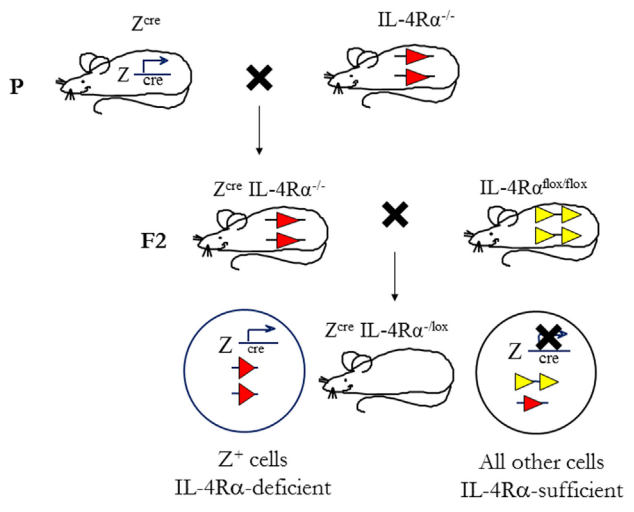

FIGURE 4 | Mouse breeding strategy to create cell-specific interleukin-4 receptor alpha (IL-4Rα)-deficient mice. $IL\text{-}4R\alpha^{flox/flox}$ BALB/c mice were intercrossed with transgenic mice expressing Cre-recombinase under control of a cell-specific promoter (Z^{cre}) and $IL\text{-}4R\alpha^{-/-}$ BALB/c mice to generate $Z^{cre}IL\text{-}4R\alpha^{-/lox}$ mice. The "loxed" IL-4Rα allele, yellow arrows; deleted allele, red arrows. Illustration adapted and redrawn from previous publications (10, 11, 82).

IL-4Rα-Responsive Innate-Like Skin Keratinocytes in Murine CL

In CL, the skin represents the site of primary infection between the sandfly, parasites and the mammalian host. It is therefore probable that this immunologic organ may provide decisive signals for the development of a Th response early after infection. In addition to 20 billion T cells (95), the skin also contains innate and innate-like cells such as keratinocytes, melanocytes, and Langerhans cells in the epidermis, and dermal DCs, plasmacytoid DCs, phagocytes as well as lymphoid cells in the dermis (96). Keratinocytes are the most abundant cells in the epidermal layer (~90%) capable of differentiating harmful pathogens from harmless commensal organisms, and directing an appropriate immune response against it. The latter is due to their expression of distinct pattern recognition receptors (PRRs) that recognize signature pathogen-specific molecular patterns. Biological activities of keratinocytes range from secretion of antimicrobial peptides to recruitment of host immune cells and notably, modulation of cytokine production (96–98). An immunomodulatory role for keratinocytes during *L. major* infection became evident when it was found that these cells secreted an array of cytokines, such as IL-12, TNF-α, IL-1β, and IL-6, within the first few hours after infection. The release of keratinocyte-specific inflammatory cytokines following *Leishmania* exposure implies the involvement of Toll-like receptor (TLR) activation on these cells since TLR recognition is often associated with the production of pro-inflammatory cytokines (99). Of interest however, besides the release of pro-inflammatory cytokines, IL-4 was also found to be produced by keratinocytes of both BALB/c and C57BL/6 mice (21). While the general consensus is that IL-4 acts as a canonical Th2 cytokine to induce a detrimental Th2/type 2 response, there have been studies demonstrating that early production of IL-4 at the site of infection may essentially drive a beneficial Th1 response, under the instruction of DCs secreting IL-12 (11, 100, 101). Importantly, direct uptake of *L. major* parasites by keratinocytes seems unlikely because of the subcutaneous infection and because keratinocytes have been reported not to take up *L. major in vitro* (21). Nevertheless, these reports suggested that keratinocytes may be the source of the early IL-4 that instructs DCs to drive a protective Th1/type 1 response. This is supported by the fact that keratinocytes express surface IL-4Rα predisposing the cells to possible autocrine stimulation by keratinocyte-derived IL-4 and IL-13, which may also influence DCs in a paracrine manner. Of note, Ehrchen et al. (21) did not identify strain-specific differences in the expression of IL-13 in the skin or specifically by keratinocytes. These cells can, however, secrete and signal IL-13 (97, 102). We therefore investigated if the autocrine action of IL-4 and IL-13, signaling on keratinocytes *via* the IL-4Rα complex, contributes to type 2 responses during CL. To address this, we generated keratinocyte-specific-IL-4Rα deficient ($KRT14^{cre}IL\text{-}4R\alpha^{-/lox}$) mice on a BALB/c

background using the cre/*lox*P system (**Figure 4**) under control of the keratinocyte 14 (*krt14*) locus. Considering that the strain of *Leishmania* initiating infection has a relevant influence on T cell-priming during infection, we incorporated experimental CL with *L. major* LV39 and IL81 strains by subcutaneous injection in the footpad. Surprisingly, despite minor immunological changes, KRT14^{cre}IL-4Rα$^{-/lox}$ mice on the BALB/c background developed non-healing disease similar to the littermate control mice following infection in the footpad. Moreover, the default Th2/type 2-driven cellular and humoral immune response characteristic of BALB/c mice, developed independently of IL-4Rα-responsive keratinocytes following *L. major* LV39 and IL81 infection in the footpad (**Figure 5A**) (submitted manuscript). Overall, our data and the reports above suggests that cytokine and chemokine-secreting skin keratinocytes might be involved in amplifying the *L. major*-induced inflammatory tissue signal by mobilizing leukocytes to the infection site following recognition of *L. major* parasites by keratinocyte-specific PRRs.

In contrast to our original hypothesis, this suggested that IL-4Rα-responsive keratinocytes may not play a role in regulating the default Th2 polarized response during *L. major* infection in BALB/c mice. Nevertheless, given that Ehrchen et al. (21) reported a higher secretion of keratinocyte-derived IL-4 in C57BL/6 mice, as opposed to BALB/c, this suggested that the absence of IL-4 on keratinocytes in C57BL/6 mice might have a pronounced effect on Th1 polarization. As above and in **Figure 4**, the cre/*lox*P system was therefore used to generate KRT14^{cre}IL-4Rα$^{-/lox}$ mice on a C57BL/6 background. The use of both *L. major* LV39 and IL81 strains, injected subcutaneously in the footpad and ear, once again revealed unexpected results. In the absence of IL-4Rα-responsive keratinocytes, C57BL/6 mice controlled the development of inflammatory lesions upon infection with *L. major* LV39 and IL-81, which correlated with reduced parasite burdens and the expansion of Th1/type 1 cellular and humoral immune responses (**Figure 5B**) (103). Collectively, the data obtained in both KRT14^{cre}IL-4Rα$^{-/lox}$ BALB/c and C57BL/6 mice mitigate an autocrine role for IL-4/IL-13 signaling on keratinocytes in the development of a non-healing Th2/type 2 or protective Th1/type 1 immune response, respectively, following experimental infection with *L. major* LV39 and IL-81 in mice.

IL-4Rα-Responsive Macrophages and Neutrophils in Murine CL

Following *Leishmania* infection in the skin, innate macrophages, neutrophils and DCs are recruited to the site of inoculation, which can become infected and as a result, have specific and important roles in shaping CD4^{+} Th cell-dependent immune responses to infection. Considering that each of these cell-types express the surface IL-4Rα complex, we investigated cell-specific requirements for the IL-4Rα and its ligands on macrophages, neutrophils, and DCs. Studies have demonstrated that *Leishmania*-derived molecules activate TLRs, such as TLR2, TLR4, and TLR9, on these professional phagocytes to initiate the innate response. However, the consequences of such activation are complex and depend on the nature of the TLR, the cell type, the parasite species, the timing in which these events occur and the cytokine milieu surrounding the infected cells (104). Concerning macrophages, it is now well-accepted that these cells can be activated by different stimuli, with IFN-γ leading to classically activated macrophages, while signaling of IL-4/IL-13, *via* the IL-4Rα complex, results in alternative macrophage activation. The former mediates secretion of pro-inflammatory cytokines and killing of intracellular *Leishmania*, while the latter favors growth and survival of the parasites. Importantly, iNOS-mediated NO production is counter-regulated by IL-4Rα-dependent mechanisms through depletion of L-arginine, the substrate utilized by iNOS, leading to arginase I-expressing alternatively activated macrophages.

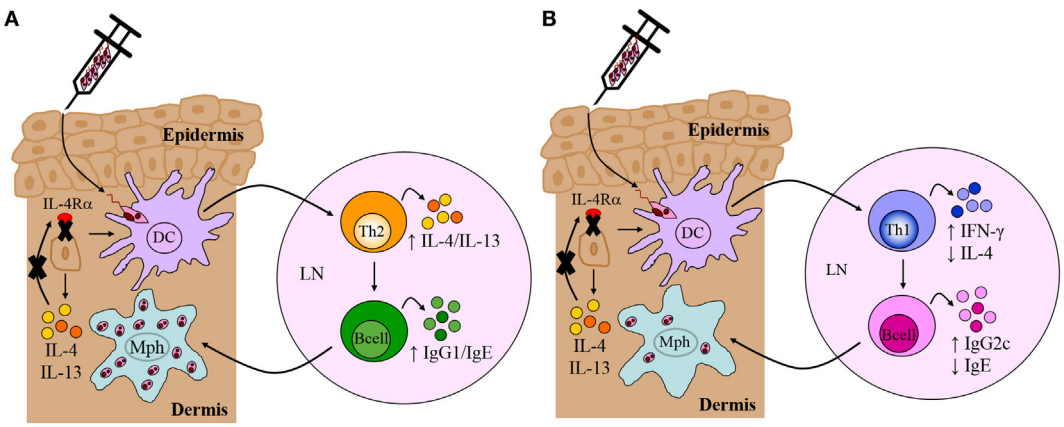

FIGURE 5 | Immune response in *Leishmania major*-infected BALB/c and C57BL/6 mice deficient for interleukin-4 receptor alpha (IL-4Rα)-responsive keratinocytes. **(A)** Th2/Type 2-mediated susceptibility in BALB/c mice deficient for IL-4Rα-responsive keratinocytes. KRT14^{cre}IL-4Rα$^{-/lox}$ BALB/c mice, generated by cre/*lox*P targeting, developed non-healing disease to experimental *L. major* LV39 and IL81 infection in the footpad, characterized by substantial interleukin-4 (IL-4)/interleukin-13 (IL-13) production and IgG1/IgE production, similar to littermate control BALB/c mice. Model prepared using information from submitted manuscript. **(B)** Th1/Type 1-mediated protective immunity in C57BL/6 mice deficient for IL-4Rα-responsive keratinocytes. Similar to control C57BL/6 mice, KRT14^{cre}IL-4Rα$^{-/lox}$ C57BL/6 mice, generated by cre/*lox*P targeting, controlled lesion development upon *L. major* LV39 and IL81 infection in the footpad or ear dermis, characterized by strong IFN-γ and IgG2c production and concomitantly reduced IL-4 and IgE (103). DC, dendritic cells; Mph, macrophage; LN, lymph node; Th1, T helper 1; Th2, T helper 2.

Therefore, we postulated that the absence of IL-4Rα-responsive macrophages at the site of infection would induce signals that lead to a polarized protective Th1 response due to the absence of alternatively activated macrophages. Accordingly, LysM^{cre}IL-4Rα$^{-/lox}$ mice on a susceptible BALB/c genetic background were generated, by cre/*lox*P targeting under control of the lysosome (*lysm*) locus (**Figure 4**), showing selective deficiency of IL-4Rα on macrophages and neutrophils.

In the context of *Leishmania* infection, neutrophils rapidly recruited to the site of inoculation (105) mediate persistence of parasites in skin lesions (72, 106). By contrast, it has also been shown that neutrophils mediate killing of intracellular *Leishmania* by neutrophil extracellular traps (107) or *via* activation of killing effector functions in macrophages through recruitment of TLR4 by neutrophil elastase (104, 108). However, the importance of type 2 signaling on macrophages and neutrophils during the early stages of *L. major* infection was undefined. The LysM^{cre}IL-4Rα$^{-/lox}$ BALB/c strain therefore provided an attractive model to interrogate the combined contribution of Th2/type 2 responses *via* the IL-4Rα on macrophages and neutrophils *in vivo* in progression of CL. Hölscher et al. (13) revealed that in the absence of IL-4Rα on macrophages and neutrophils, BALB/c showed a significant control of disease progression up to 13 weeks after infection with *L. major*, despite sufficient Th2 and type 2 immune responses similar to littermate controls (**Figure 6**). Delayed disease progression in *L. major*-infected LysM^{cre}IL-4Rα$^{-/lox}$ BALB/c mice was attributed to inhibition of alternative activation of macrophages and improved macrophage leishmanicidal activities due to NO production by classically activated macrophages (**Figure 6**). However, following 13 weeks of infection, LysM^{cre}IL-4Rα$^{-/lox}$ mice developed fulminant CL with increasing footpad swelling, accompanied by ulceration and necrosis concomitant with elevated parasite burdens requiring termination of the experiment by week 18. The action of macrophages and neutrophils appear to complement each other as both population of cells are rapidly recruited to the site of infection and capable of phagocytosing *Leishmania* parasites following TLR stimulation. Accordingly, TLR4 was shown to be required for efficient leishmanicidal activity as the absence thereof led to increased arginase-derived urea and reduced formation of NO, probably *via* the activity of iNOS. The latter requires IL-12 secretion by antigen-presenting cells (APCs), and this cytokine can be activated by TLR9 (109). Thus, once could speculate that enhanced TLR4/TLR9 activation might have contributed to early control of disease in LysM^{cre}IL-4Rα$^{-/lox}$ mice. Altogether, our study showed that IL-4Rα-mediated effects on macrophages and neutrophils seem to be involved in the development of early disease progression after *L. major* infection. The authors postulated that the transient effect of IL-4Rα-deficiency on macrophages/neutrophils after *L. major* infection may be that production of IL-4 and/or immunosuppressive cytokines by CD4^{+} T cells abolished the initial protective immune responses observed. Concurrently, an alternate hypothesis on IL-4Rα-dependent protective mechanisms emerged. Macrophage-derived IL-12 production was shown to be inhibited upon engagement of the IL-4Rα complex (82). Thus, Hölscher et al. (13) postulated that IL-4-mediated instruction of DCs may produce IL-12 (100), which indeed has been shown to promote resistance in *L. major*-infected BALB/c mice.

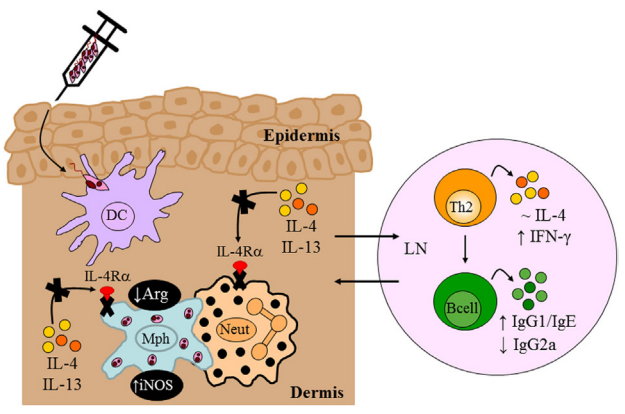

FIGURE 6 | Immune response in BALB/c mice deficient for interleukin-4 receptor alpha (IL-4Rα)-responsive macrophages/neutrophils. Despite delayed disease progression in the footpads of *L. major* LV39-infected LysM^{cre}IL-4Rα$^{-/lox}$ mice, unimpaired Th2/type II responses and reduced IgG2a were detected, similar to control BALB/c mice. The absence of IL-4Rα-responsive macrophages led to increased inducible nitric oxide synthase (iNOS) and reduced arginase, reflecting a shift to classically-activated macrophages likely as a consequence of increased IFN-γ compared to littermate control BALB/c mice. Illustration prepared from Hölscher et al. (13). DC, dendritic cells; Mph, macrophage; LN, lymph node; Th2, T helper 2.

IL-4Rα-Responsive DCs in Murine CL

Evidence from *in vitro* and *in vivo* studies began to contradict the detrimental role of IL-4 in susceptibility to *L. major* infection and the following observations proved critical: first, recombinant IL-4 was shown to promote IL-12 production by bone marrow-derived dendritic cells (BMDCs) upon stimulation with specific ligands; second, IL-4, not IL-13, enhanced the production of IL-12 *via* signaling through the type 1 IL-4R complex; third, exogenous IL-4-administered *in vivo* during the period of DC activation in a murine model of *L. major* infection upregulated DC-derived *il-12* transcripts leading to a protective Th1 response and healing in otherwise susceptible BALB/c mice, and fourth, administration of exogenous IL-4 *in vivo* during the period of T cell priming resulted in the default Th2 pathway and progressive disease in *L. major*-infected BALB/c mice. A follow-up study demonstrated that IL-4-mediated instruction was limited to DCs, excluding other APCs, and the mechanism responsible was inhibition of IL-10 by IL-4, leading to protective IL-12-driven Th1 responses (110). Altogether, the reports alluded to above portrayed IL-4 signaling on DCs as a double-edged sword in murine CL, emphasizing that the release of DC-derived IL-12 and the outcome of *L. major* infection in BALB/c mice depends on the timing of the IL-4Rα chain interacting with target cells. DCs are those innate cells that most acutely reflect innate control of adaptive immunity (111) as these sentinels of the immune system transition innate to adaptive immune responses owing to their proficient antigen-presenting function and migratory capacity (112).

To investigate a role for IL-4Rα-responsive DCs in resistance to *L. major* infection, we generated CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c

mice, in which dendritic-cell specific deletion of the *il4rα* gene was achieved by the Cre/*lox*P system under control of the *cd11c* locus (**Figure 4**). Infection studies with *L. major* LV39 and IL81 in the footpad revealed that IL-4-mediated instruction of DCs occurs *in vivo* with biological quantities of IL-4 acting on DCs to promote protective immunity. In the absence of IL-4Rα-responsive DCs, BALB/c mice became hypersusceptible to disease, when compared with littermate control mice, showing exacerbated lesion development and earlier tissue necrosis. This correlated with uncontrolled parasite replication at the infection site and a shift to Th2/type 2 cellular and humoral immune responses (**Figure 7**). Notably, CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c mice displayed a striking increase in parasite dissemination from the site of infection to the draining LN and peripheral organs, including the brain of infected animals. Collectively, the data appeared to suggest that impaired NO-mediated killing effector functions in IL-4Rα-unresponsive DCs (and macrophages) facilitated a safe haven for parasite multiplication and dissemination of parasites. Accordingly, secretion of IL-12 by IL-4Rα-deficient DCs was severely impaired whereas IL-10 production was increased thereby confirming the mechanism behind impaired DC instruction *in vivo*. Interestingly, efficient TLR ligation on DCs disposes a potent negative signal for Th2 cell development (113). Thus, an alternate mechanism for the heightened susceptibility in CD11c^{cre}IL-4Rα$^{-/lox}$ BALB/c might involve impaired TLR-mediated activation of DCs. In agreement, induction of BMDC-IL-12 following *L. major* infection was shown to be dependent on TLR9 activation (114). This might suggest a combined involvement of IL-4 and the TLR pathway in activating DC-derived IL-12 in this protective anti-*L. major* response. However, further research is required to elucidate a functional relationship.

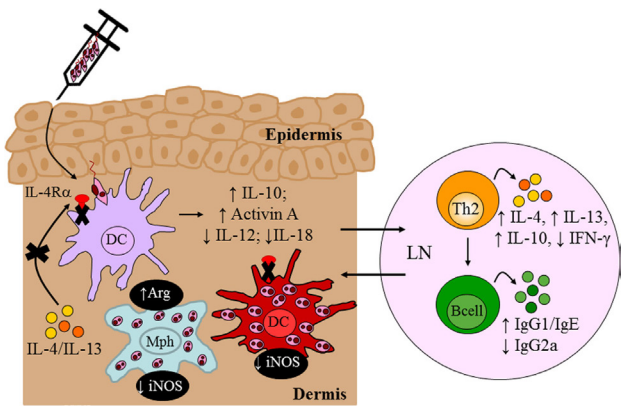

FIGURE 7 | Immune response in BALB/c mice deficient for interleukin-4 receptor alpha (IL-4Rα)-responsive DCs. Absence of interleukin-4 (IL-4)/interleukin-13 (IL-13) signaling on DCs led to hypersusceptibility in BALB/c mice upon *Leishmania major* LV39 and IL81 infection in the footpad, characterized by a shift to Th2/type II immune responses and alternatively activated macrophages stronger than that usually observed in littermate BALB/c mice. Expression of inducible nitric oxide synthase (iNOS) in DCs was also dramatically reduced with increased arginase (Arg) compared to littermate mice providing a safe haven for *L. major* growth, survival, and dissemination to peripheral organs. Illustration prepared from Hurdayal et al. (11). DC, dendritic cells; Mph, macrophage; LN, lymph node; Th2, T helper 2.

In a parallel study, we explored the efficacy of IL-4 as an adjuvant in DC-mediated vaccination as in the context of visceral leishmaniasis, IL-4 mediates protective immunity and has been shown to instruct successful chemotherapy and vaccination responses (80). Accordingly, IL-4Rα-deficient BMDCs, with reduced IL-12 and increased IL-10 secretion, failed to vaccinate BALB/c animals against acute leishmaniasis, while IL-4-sufficient BMDCs producing increased IL-12 and reduced IL-10 successfully immunized BALB/c mice against infection (115). These observations unveiled yet another paradigm, suggesting that *Leishmania* vaccines should potentially incorporate IL-4 as an adjuvant, rather than IL-12, to induce protective IFN-γ responses.

IL-4Rα SIGNALING ON SPECIFIC ADAPTIVE IMMUNE CELLS IN CL IN MICE

IL-4Rα-Responsive CD4$^+$ Th Cells in Murine CL

Recognition of *Leishmania*-specific molecular patterns by the PRRs of early innate immune cells provides the necessary signals that determine the choice of effector response in adaptive immunity, mediated primarily by T and B lymphocytes. Indeed, the central role of T cells during *L. major* infection was established early on (116); however, the contradictory roles of IL-4 (7, 91) questioned whether IL-4 counter-regulated a protective Th1 response to promote susceptibility to infection. In an attempt to reconcile these observations, we focused on specific abrogation of IL-4-responsive CD4$^+$ T cells by deletion of the IL-4Rα subunit in BALB/c mice (Lck^{cre}IL-4Rα$^{-/lox}$). In contrast to littermate control BALB/c mice, which developed ulcerating, necrotic lesions following infection with *L. major* LV39 and IL81, lack of IL-4-responsive CD4$^+$ T cells led to a healing phenotype similar to that of the resistant C57BL/6 mice (10). Resistance to *L. major* in Lck^{cre}IL-4Rα$^{-/lox}$ BALB/c mice correlated with early *il-12p35* mRNA transcription leading to increased IFN-γ production, elevated iNOS expression and enhanced memory responses, similar to C57BL/6 mice (**Figure 8**). In contrast to global IL-4Rα$^{-/-}$ mice, Lck^{cre}IL-4Rα$^{-/lox}$ BALB/c mice maintained chronic control of *L. major* infection. Collectively, the data demonstrated that CD4$^+$ T cell-specific IL-4Rα-mediated signaling drives susceptibility to *L. major* infection, altogether highlighting a protective role for IL-4/IL-13 signaling on non-CD4$^+$ T cells in *L. major*-infected BALB/c mice. Follow-up studies further demonstrated that iLck^{cre}IL-4Rα$^{-/lox}$ BALB/c mice, with IL-4Rα deficiency on all T cell populations (CD4$^+$, CD8$^+$, natural killer T, and γδ$^+$ T cells) were also able to resolve lesion development, correlating with reduced parasite replication, IFN-γ-mediated protective delayed type hypersensitivity responses and downregulated *L. major*-specific IgG1, similar to Lck^{cre}IL-4Rα$^{-/lox}$ and C57BL/6 mice (**Figure 8**). As expected, the non-healing littermate BALB/c control mice developed severe footpad swelling and increased parasite burdens (81). Taken together, these studies concluded that absence of IL-4Rα-responsive non-CD4$^+$, in addition to CD4$^+$ T cells, does not further affect transformation of BALB/c mice to a healer phenotype.

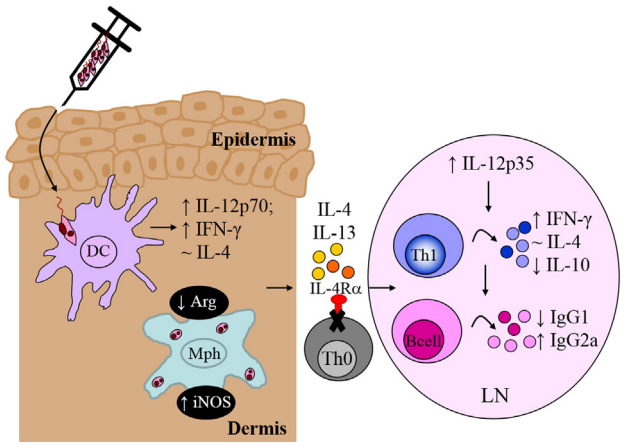

FIGURE 8 | CD4+ T cell-specific interleukin-4 receptor alpha (IL-4Rα)-deficient mice develop a polarized Th1/type I immune response. In the absence of interleukin-4 (IL-4)-responsive T cells, normally susceptible BALB/c mice developed a healing phenotype upon experimental infection in the footpad, characterized by increased IFN-γ and IgG2a and concomitantly reduced IL-10 and IgG1 compared to littermate BALB/c mice. Interestingly, CD4+ T cell-specific IL-4Rα-deficient mice maintained equivalent IL-4 production to control littermate BALB/c mice. Illustration prepared from Radwanska et al. (10). DC, dendritic cells; Mph, macrophage; LN, lymph node; Th0, naive T cell; Th1, T helper 1.

CD4+ Th17 and Tregs in CL in Relation to IL-4 and IL-4Rα Signaling

Apart from the Th1 and Th2 lineage of CD4+ T cells, naive CD4+ T cells may differentiate into additional lineages including CD4+ IL-17-producing cells (Th17) and Tregs. Th17 cells are characterized by the production of IL-17 and require retinoic acid-related orphan receptor gamma t, in addition to STAT3 and IL-23, for differentiation and maintenance (117). Various experimental models have linked the combined activity of Th17 cells, IL-17 and neutrophils to the pathogenesis of CL (118–120). A recent study indicated a link between IL-4 and the commitment of Th17 cells *via* the activation of IL-23 (121). In a model of cell-mediated inflammation, the authors demonstrated that IL-4 abrogates Th17 cells by selectively silencing IL-23 in APCs (121). Concurrently, IL-4 completely abrogates IL-23 to induce the IL-12-producing capacity of DCs. Altogether, the role of IL-4 and IL-4Rα signaling on this lineage of cells in CL would be an exciting avenue to investigate considering that in the study above, IL-12-dependent Th1 responses remained unaltered upon IL-4-mediated IL-23/Th17 silencing (121), and as already established, the former is needed for host protection to CL.

The ability for the host to maintain long-term immunity against *Leishmania* infection suggests that the parasite persists in an immune privileged site by a balance between CD4+ effector and Tregs that downregulate parasite-specific immunity (122). Differentiation of Tregs requires activation of forkhead box protein 3 (Foxp3) leading to the production of IL-10, TGF-β, and IL-4 (117, 123). In susceptible BALB/c mice, Tregs play a significant disease controlling role by regulating the biased Th2 response (ideally they suppress excessive Th2 responses) since the absence of CD4+CD25+ Treg cells dramatically increases IL-4 levels and exacerbates *L. major* infection (124, 125). In resistant C57BL/6 mice, CD4+CD25+ Tregs control protective Th1 responses by an IL-10-dependent mechanism mediating parasite persistence and latent infection (74). Of considerable interest, however, Pillemer et al. revealed that IL-4 signaling *via* the IL-4Rα–STAT6 axis was required to maintain Foxp3 expression in Tregs and promote their proliferation (123). Thus, while global depletion of Treg cells yielded informative and differing results in cutaneous disease, it provided little insight into the underlying mechanisms by which Tregs suppress or enhance Th2 responses *via* the IL-4/IL-4Rα axis (and the corresponding effects on Th1 immunity). The identification of Foxp3 as the crucial inducer of Tregs and our Cre/*lox*P technology enables us to provide further insight into this field. Consequently, we have generated BALB/c mice with a cell-specific deletion of the IL-4Rα chain on Tregs under control of the *foxp3* locus. Experimental studies in murine models are currently underway to elucidate the contribution of IL-4-signaling on Tregs in the infectious process caused by *L. major*.

IL-4Rα-Responsive B Cells in Murine CL

Apart from CD4+ T cells, independent researchers began to unravel the contribution of B lymphocytes in host protection or susceptibility to CL. Initial proof-of-concept studies provided evidence that B cells may play a role in susceptibility to infection with *L. major* (126, 127). Nevertheless, the use of mice genetically deficient for B cells (μMT or J_HD) demonstrated that B lymphocytes play a limited role in Th2-mediated susceptibility to *Leishmania* infection, as the absence of B cells in BALB/c mice did not critically alter disease outcome (128, 129). This conclusion changed dramatically when it became evident that B cells could also secrete cytokines that potentially modulate both pathologic or protective functions, independent of their antibody-secreting and antigen-presenting functions (130). This was further supported by several lines of evidence showing that B cells assist in regulating the quality and quantity of both primary and memory CD4+ Th cell responses (130). However, up until this point, the contribution of cytokine-producing B cells in the context of inflammation, infection, and autoimmunity, which were conventionally dependent on CD4+ T cells, was an unexplored aspect. A hallmark study employing both *in vitro* and *in vivo* methods characterized the subdivision of B cells into distinct cytokine-producing "effector" subsets. Defined effector B cells producing cytokines such as IFN-γ, IL-12p40, TNF-α, and IL-6 were termed B effector 1 (Be1) cells, whereas Be2 B cells secreted copious amounts of IL-2, IL-4, and IL-13 (23). The profiles of Be1 and Be2 cells closely resembled that of CD4+ Th1 and Th2 cells, which altogether led to a renaissance in this field of B cell biology. To date, cytokine-producing B cells have been reported in various models of parasitic and bacterial infection (23, 131, 132) and autoimmune diseases (133, 134). Collectively, the evidence provided by these studies were inclined to suggest that, in response to antigen, cytokine-producing B cells might function to initiate and/or maintain the magnitude and quality of CD4+ Th1/Th2-dependent immune responses.

Of interest to our team, it was reported that the differentiation of naive B cells into IL-4-expressing Be2 cells was critically dependent upon Th2 signals and the IL-4/IL-4Rα signaling pathway (24). This led to the generation of a novel mouse strain lacking IL-4Rα expression specifically on B cells, mb1^{cre}IL-4Rα$^{-/lox}$ BALB/c mice, generated by cre/*lox*P under control of the *mb1* locus (56). As murine lymphocytes are not responsive to IL-13, this mouse model provided an invaluable tool to investigate a role for IL-4-responsive B cells during infection with *L. major*. Infection studies with *L. major* LV39 and IL-81 in B cell-specific IL-4Rα-deficient BALB/c mice revealed a beneficial role for IL-4Rα-unresponsive B cells in host-protective immunity and concomitantly, a detrimental role for IL-4Rα-responsive B cells in the non-healing response to *L. major* (135). In the absence of IL-4 signaling on B cells, BALB/c mice effectively controlled progression of lesion development and parasite replication as a consequence of enhanced Th1/type 1 immunity and improved leishmanicidal effector functions (**Figure 9**), resembling a similar phenotype to CD4$^+$ T cell-specific IL-4Rα-deficient BALB/c (10). Mechanistic studies further revealed that the healing phenotype in mb1^{cre}IL-4Rα$^{-/lox}$ BALB/c mice was due to reduced *il-4* and increased *ifn-γ* transcripts in IL-4Rα-unresponsive B cells as early as Day 1 postinfection. Furthermore, mixed-bone marrow chimeras were instrumental in confirming that IL-4-producing B cells are crucial in driving the non-healing susceptible type 2 immune response characteristic of BALB/c mice during *L. major* infection (135). Recent evidence has indicated innate-like activation of B cells following recognition of foreign antigens (136, 137), *via* B cell receptor-independent mechanisms, leading to early B cell cytokine secretion. The efficient antigen-presenting function of B cells (138, 139) allows the cell to present the antigen it acquires and in lieu of its cytokine secretion, induce paracrine activation of neighboring cells, such as CD4$^+$ T cells. These reports and our current data appear to suggest a model in which early IL-4Rα-responsive B cells producing IL-4 after infection are capable of influencing early Th polarization toward detrimental Th2 responses that drive *L. major*-induced CL (135).

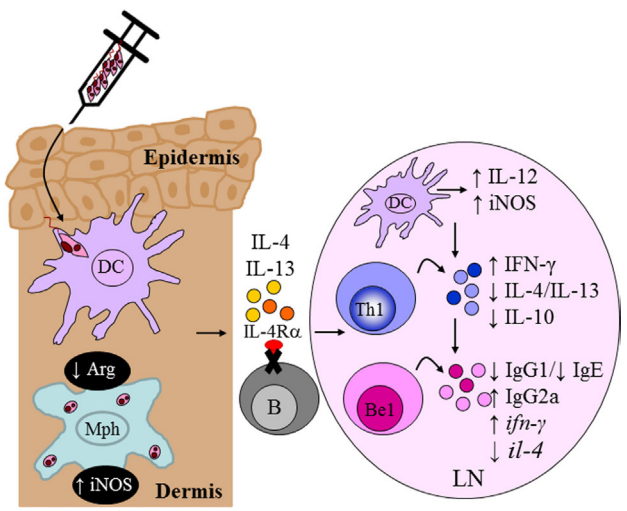

FIGURE 9 | Absence of interleukin-4 (IL-4)-responsive B cells transforms non-healer BALB/c mice to a healer phenotype. Mb1^{cre}IL-4Rα$^{-/lox}$ mice developed an IL-12-induced protective Th1/type I immune response compared to littermate control BALB/c mice following *Leishmania major* LV39 and IL81 infection in the footpad. IL-4Rα-deficient B cells secreted increased *ifn-γ* and reduced *il-4* transcripts, as early as day 1 postinfection, resembling a B effector 1 phenotype in comparison to IL-4Rα-sufficient B cells. This in turn led to improved macrophage leishmanicidal functions and control of parasite replication at the site of infection. Illustration prepared from Hurdayal et al. (135). DC, dendritic cells; Mph, macrophage; LN, lymph node; B, B cells; Be1, B effector 1 B cells; Th1, T helper 1.

CONCLUSION

Collectively, the development of conditional mice and generation of important immune cell-type-specific IL-4Rα-deficient mouse models have been critical in our understanding of Th2/type 2-mediated mechanisms in innate and adaptive immune cells during disease with the causative agent of CL, *L. major*. Our data show that the hierarchical importance of target cells interacting with the IL-4Rα and its ligands (IL-4 and IL-13) is a dynamic interaction, largely influenced by cytokines secreted in the vicinity of target and non-target cells, heterogenous CD4$^+$ T cell populations, autocrine versus paracrine signaling, the *Leishmania* species/strain initiating infection, and the timing of the immune response.

This is especially relevant when considering the full spectrum of human leishmaniasis and strain-specific roles of the IL-4Rα chain and its ligands. Visceral leishmaniasis, initiated by intravenous injection of *Leishmania donovani* amastigotes in mouse models, is quite idiosyncratic in this regard. Similar to *L. major* infection in BALB/c mice, protective immunity against *L. donovani* is dependent on IL-12-induced-IFN-γ production for classical activation of macrophages and NO-induced killing of intracellular parasites (140). However, early studies in both humans and mice demonstrated that control of visceral disease was independent of the differential production of Th1 and Th2-derived cytokines (141). Thus, the overall evidence suggested that the Th2 response did not counteract immunological control of visceral leishmaniasis (142, 143).

On the contrary, the introduction of gene-deficient mice revealed surprisingly protective roles for IL-4, IL-13 and IL-4Rα signaling, during primary *L. donovani* infection (144–146). In addition, the IL-4/IL-13 signaling cascade was reported to play a significant role in successful drug treatment with sodium stibogluconate (80, 145) and augmenting vaccination responses (147). These reports added immense value to our understanding of the Th2 response in remarkably, control of visceral leishmaniasis, improved chemotherapy and vaccination. However, it could not tell us which IL-4/IL-13-signaling cells were important in mediating protective immunity, in lieu of the global gene deficiency in these models. Consequently, current research within our team is aimed at unraveling IL-4 and IL-13 signaling on specific target immune cells during visceral leishmaniasis using our cell-type-specific IL-4Rα-deficient mouse models. Our initial studies have indicated that control of primary *L. donovani* infection, granuloma maturation, and chemotherapeutic efficacy is independent of IL-4Rα-responsive macrophages and neutrophils in BALB/c

mice (144). This raises intriguing questions regarding the modes of action of IL-4 and IL-13 on other cellular targets such as $CD4^+$ T cells, Treg cells, DCs, and B cells, which are currently under investigation.

AUTHOR CONTRIBUTIONS

RH and FB contributed equally to the design and writing of the present review.

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REVIEW
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Function of Macrophage and Parasite Phosphatases in Leishmaniasis

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The kinetoplastid protozoan parasites belonging to the genus *Leishmania* are the causative agents of different clinical forms of leishmaniasis, a vector-borne infectious disease with worldwide prevalence. The protective host immune response against *Leishmania* parasites relies on myeloid cells such as dendritic cells and macrophages in which upon stimulation by cytokines (e.g., interferon-γ) a complex network of signaling pathways is switched on leading to strong antimicrobial activities directed against the intracellular parasite stage. The regulation of these pathways classically depends on post-translational modifications of proteins, with phosphorylation events playing a cardinal role. *Leishmania* parasites deactivate their phagocytic host cells by inducing specific mammalian phosphatases that are capable to impede signaling. On the other hand, there is now also evidence that *Leishmania* spp. themselves express phosphatases that might target host cell molecules and thereby facilitate the intracellular survival of the parasite. This review will present an overview on the modulation of host phosphatases by *Leishmania* parasites as well as on the known families of *Leishmania* phosphatases and their possible function as virulence factors. A more detailed understanding of the role of phosphatases in *Leishmania*–host cell interactions might open new avenues for the treatment of non-healing, progressive forms of leishmaniasis.

Keywords: ***Leishmania*, macrophages, protein tyrosine phosphatase, signaling, exosome**

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INTRODUCTION

Epidemiology and Disease Development

Leishmaniasis is a worldwide prevalent infectious disease. Among all parasitic infections, leishmaniasis ranks second in mortality after malaria (1). Leishmaniasis is primarily encountered in tropical and subtropical countries, with an estimated number of 0.7–1 million new cases of cutaneous leishmaniasis (CL) and 50,000 to 90,000 new cases of visceral leishmaniasis accompanied by 20,000–30,000 deaths per year (World Health Organization[1] [WHO]). Due to the absence of an effective and well-tolerated vaccine, the lack of simple and efficient treatments and the strong correlation between disease and poor socioeconomic conditions, WHO has classified leishmaniasis as one of the 20 neglected tropical diseases worldwide.[2]

[1] http://www.who.int/mediacentre/factsheets/fs375/en/.
[2] http://www.who.int/neglected_diseases/diseases/en/.

Leishmaniasis is caused by kinetoplastid parasites of the Trypanosomatidae family that comprises monogenetic genera often found in insects or plants as well as several human pathogens of the heteroxenous genera *Trypanosoma* and *Leishmania* (*L.*). In nature, *Leishmania* parasites are transmitted by the bites of sand flies, which inject the extracellular, flagellated parasite stage (so-called infective or metacyclic promastigote) into the dermis of the skin. Once promastigotes are released into the blood pool of the skin wound, they are endocytosed by phagocytic cells (initially polymorphonuclear, neutrophilic granulocytes [PMNs], then blood monocytes, dendritic cells, and macrophages) in which they differentiate into intracellular, so-called amastigote *Leishmania* (2–4). Amastigotes contain only a stumpy rest of the flagellum within the flagellar pocket of the parasite. *Leishmania* amastigotes will multiply in non-activated phagocytic cells and are transported *via* the lymph and blood stream to various tissues and organs (e.g., draining lymph nodes, spleen, liver, bone marrow, healthy skin), depending on the host immune response and the *Leishmania* species involved. The life cycle of the parasite will be completed once a sand fly ingests amastigote-infected tissue phagocytes during its blood meal. Within the digestive tract of the vector, the parasites retransform into promastigotes, which develop from a non-infective procyclic into the infective metacyclic stage (5).

Depending on the affected tissues, leishmaniasis can be divided into three major forms: CL, mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL, also known as Kala-Azar) (6). Members of the subgenus *Leishmania* (e.g., *L. major*, *L. tropica*, *L. infantum*, *L. mexicana*, *L. amazonensis*) and the subgenus Viannia (*L. braziliensis, L. guyanensis, L. panamensis)* can cause cutaneous disease. CL is characterized by chronic papular, erythematous, plaque-like, or ulcerative skin lesions. These lesions ultimately heal in immunocompetent patients after months to years of infection (7). MCL symptoms develop especially after an infection with the South American species *L. braziliensis, L. panamensis* or *L. guyanensis*. This form of the disease is usually associated with a higher morbidity because of the destruction of mucocutaneous tissue sites such as the nose and nasal septum, the oropharynx or the palate (7). VL is the most severe form of the disease due to the systemic spread of the parasite and usually results from an infection with viscerotropic *L. infantum* strains (identical with *Leishmania chagasi*) or *Leishmania donovani*. VL, which is characterized by persistent fever, enlargement of liver and spleen, and pancytopenia resulting from secondary hemophagocytosis in the bone marrow, is lethal if untreated (8, 9). All these various disease developments are the result of a complex equation influenced by the arsenal of virulence factors of the parasite and the immune status of the host (10).

Innate Immune Response against *Leishmania* and Importance of Phosphorylation Events: A Brief Overview

To survive during the infection of mammalian hosts, *Leishmania* parasites must quickly adapt to their new and hostile environment. Expression of the stress response protein A2 specifically by *Leishmania* species causing VL is a good example of such adaptation. Indeed, A2 promotes heat shock resistance and thereby supports parasite survival and visceralization in inner and warmer organs (11). However, *Leishmania* parasites could not survive in their new host organisms without the development of additional strategies to escape the host defense mechanisms.

Phagocytic cells, such as neutrophils, dermal dendritic cells, and dermal macrophages, are the first immune cells encountered by the promastigotes injected into the skin by sand flies. In addition to being a primary source of antileishmanial effector molecules, they also represent the cellular niche chosen by *Leishmania* promastigotes to evade the cytotoxic humoral components (such as complement) and/or to differentiate into replicating amastigotes (12–14). Therefore, it is crucial to understand how *Leishmania* convert these microbicidal phagocytes into safe target cells.

Uptake of pathogens by phagocytes usually results in a proinflammatory innate immune response, which fosters their microbicidal activity and promotes the recruitment and activation of other immune cells. In the case of *Leishmania* parasites, PMNs are the first line of defense and exert protective effects *via* release of reactive oxygen species (ROS) and the formation of neutrophil extracellular traps. At the same time *Leishmania*-infected apoptotic PMNs also function as deactivating vehicles for the transfer of the parasites into macrophages and dendritic cells [reviewed in Refs. (2, 15, 16)]. Subsequently, macrophages and dendritic cells become activated by interferon (IFN)-γ and tumor necrosis factor (TNF) generated by natural killer cells and $CD4^+$ T lymphocytes, which leads to the expression of inducible or type 2 nitric oxide (NO) synthase (iNOS, NOS2) [reviewed in Ref. (17)]. iNOS-derived NO is not only a central antileishmanial effector molecule (18–21) but also paves the ensuing type 1 T helper (Th1) cell response [reviewed in Ref. (22)]. All cellular signaling pathways engaged in this response are tightly controlled and depend on numerous posttranslational protein modifications. Among them, versatile phosphorylation of molecules plays major and ubiquitous functions. It is therefore not a surprise that *Leishmania* parasites have been described to manipulate such a cardinal modification to attenuate the host immune response.

Phagocytosis is the first action undertaken by macrophages against transmitted *Leishmania*. However, maturation of the phagosome toward a lytic phagolysosome can be altered by one of the most abundant virulence factors produced by *Leishmania* promastigotes, the lipophosphoglycan (LPG). This surface molecule was found to target the phagosome membrane where it precluded the recruitment of the acidifying vesicular proton-ATPase (23) and the correct recruitment of protein kinase C (PKC) α (24). Other PKC isoforms, like PKCβ and PKCδ, also showed a disturbed activation during *Leishmania* infections (25–27). Like for PKCα, LPG impaired the proper activation of PKCβ resulting in an inhibition of ROS production (28). This important leishmanicidal mechanism of macrophages was also described as a target of another, intensely studied *Leishmania* virulence factor, the metalloprotease GP63 (also termed leishmanolysin or *Leishmania major* surface protease). The activity of PKC was attenuated by the proteolytic effect of GP63 leading to a diminished release of ROS (29). In this context, the role of mitogen-activated protein kinases (MAPKs), including p38, Jun N-terminal kinase (JNK), and extracellular signal-related kinases (ERK1/2), has been

investigated by several groups since they represent major players of the immune pathways present in myeloid cells. In early studies, an absence of MAPK activation during *Leishmania* infection was noted, which was a plausible explanation for the observed hyporesponsiveness of macrophages (30, 31). The ability of *Leishmania* to hinder ERK and p38 phosphorylation was linked to the inability of IFN-γ to induce TNF production in these cells (32). Likewise, the suppression of another proinflammatory cytokine, IL-12, by *L. major* might result from the inhibition of the PKCδ kinase. Mice deficient for PKCδ kinase were more sensitive to *L. major* infection than wild-type mice because of a reduced IL-12p40 and IL-12p70 release by macrophages and dendritic cells and an ensuing Th2-like immune response (33).

Protein kinases are not the only targets of *Leishmania* during infection. Inhibition of IL-12 production by activation of the phosphoinositide 3-kinase (PI3K) during *Leishmania* infection has been convincingly demonstrated using PI3K-deficient mice. These mice developed an improved Th1 response protecting them against *L. major* infection (34). Similarly, activation of the protein kinase RNA-activated (PKR) was important for the intracellular growth of *L. amazonensis* as it promoted the expression of anti-inflammatory cytokine IL-10 and subsequent deactivation of macrophages (35). Interestingly, PKR activation was not observed for all *Leishmania* species. In the case of *L. major*-infected macrophages, PKR activation was actively suppressed by the serine peptidase inhibitor ISP2 of the parasite; activation of PKR by exogenous poly I:C resulted in rapid parasite death due to the release of proinflammatory cytokines (36). Nevertheless, the disease-promoting role of IL-10 in both cutaneous and visceral leishmaniasis has been clearly documented (37–41). Similar to IL-12, its regulation depends on diverse pathways controlled by reversible phosphorylation events. PI3K and the serine/threonine-specific protein kinase AKT (also known as protein kinase B) were shown to be activated during macrophages infection by *L. donovani*. These kinase activities resulted in the inactivation of the glycogen synthase kinase-3β leading to the binding of an activating transcription factor, the cyclic AMP-responsive element-binding protein, to the IL-10 promoter (42).

Not only the production but also the response to proinflammatory cytokines is altered during *Leishmania* infections. JAK2, a tyrosine kinase binding to the receptor of IFN-γ, the key cytokine of protective Th1 cells, was deactivated by a host phosphatase following *Leishmania* infection (43, 44).

Altogether, these few examples illustrate the delicate balance between tightly controlled phosphorylation events in the host cells, which are necessary for initiating pathogen control mechanisms, and the ability of the parasite to subvert these reactions. Biochemically, the host-pathogen interaction is governed by kinases (which catalyze the phosphorylation) and by phosphatases, which hydrolyze phosphoester bounds. In the section above, we have briefly mentioned important host kinases involved in the interplay between infected cells and *Leishmania* parasites. In the following part of this review, the role of the phosphatases will be presented. We will focus not only on host phosphatases and how they are modulated by the parasite (e.g., by parasite proteases) but also on *Leishmania* phosphatases and their putative interference with immune cells functions (**Figure 1**).

HOST PHOSPHATASES

Protein phosphatases can be classified into two main families of enzymes depending on substrate specificity and catalytic signature. The serine threonine phosphatase (STP) family comprises three main groups: the phosphoprotein phosphatases (PPPs), the metal-dependent protein phosphatases (PPMs), and the aspartate-based phosphatases (F cell production phosphatase [FCP]/small carboxy-terminal domain phosphatase [SCP]) (45). The second family of phosphatases contains enzymes that are able to dephosphorylate phosphotyrosine residues (phosphotyrosine phosphatases [PTPs]), which are also categorized in four different groups depending on their modular organization and their substrate restriction (46) (see **Figure 2**).

Serine-Threonine Host Phosphatases Regulating the Outcome of *Leishmania* Infections

Immune cells responding to *Leishmania* infection rely on efficient signaling pathways to stimulate the production of leishmanicidal molecules or to initiate and support their migration, proliferation, and cytokine production. In general, these pathways are finely balanced between an activating signal transmitted by protein kinases and a stopping signal mediated by phosphatases. From the parasite's perspective, this balance needs to be shifted toward the phosphatase side to guarantee intracellular survival in host cells. Activation of host phosphatases following *Leishmania* infection has been described in macrophages (43) and dendritic cells (47) and helps to explain the reduced immune response observed during visceral leishmaniasis. In mice infected with *L. donovani*, lymphocytes were unresponsive to strong activating and proliferative stimuli like phorbol 12-myristate 13-acetate (PMA) and ionomycin. This defect could be reversed when PKC and ERK phosphorylation were restored by treating the lymphocytes with okadaic acid, an STP inhibitor specific for the heterotrimeric *STP protein phosphatase 2A (PP2A)* (48, 49). In *L. donovani*-infected macrophages activation of PP2A was triggered by host production of ceramide after parasite uptake which resulted in the dephosphorylation of AKT and the subsequent decrease of TNF secretion (50). In striking contrast to these findings are observations made with saliva from the sand fly vector *Phlebotomus papatasi*, which contains a non-proteinaceous inhibitor of the STPs *protein phosphatase 1 (PP1)* and PP2A. As PP1 and PP2A are required for the induction of the iNOS gene (51), the inhibitor present in the sand fly saliva impeded the production of NO by macrophages (52). Thus, both activation and inhibition of PP1 and PP2A can impair the defense against *Leishmania* parasites.

The mitochondrial phosphatase, *phosphoglycerate mutase 5* (*PGAM5*), which was recently described as an atypical, histidine-based STP (53), is another example for a host-protective phosphatase. PGAM5, which acts downstream of receptor-interacting serine/threonine-protein kinase 1 (RIPK1), inhibited the intracellular replication of *Leishmania* (*L. infantum*, *L. major*,

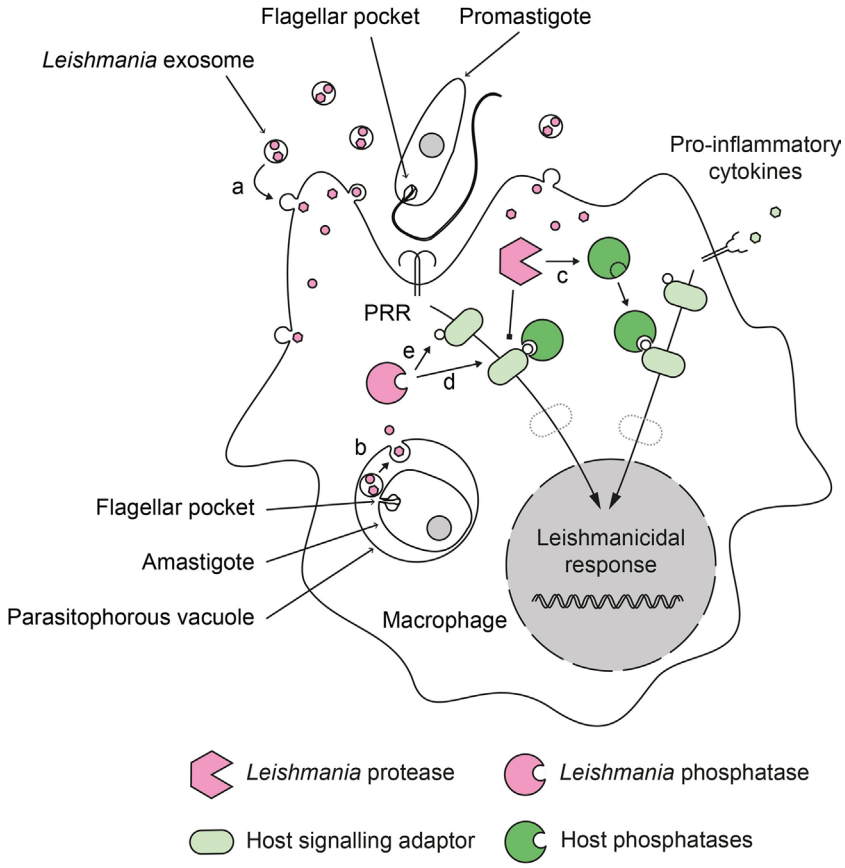

FIGURE 1 | Role of phosphatases in the deactivation of host cell during *Leishmania* infection. *Leishmania* parasites have developed a dual strategy to inactivate important signaling pathways in their host cell. During infection, promastigotes and amastigotes secrete exosomes containing virulence factors. These exosomes release their content into the infected cells after fusing with (a) cytoplasmic membranes (in the case of extracellular promastigotes) or (b) phagosomal membranes (in the case of intracellular amastigotes). Secreted *Leishmania* proteases can proteolytically activate host phosphatases (c) that subsequently down regulate activating cellular pathways. Secreted *Leishmania* phosphatases could synergize with host phosphatases (d) or act independently (e) to modulate the infected cell response.

L. amazonensis) in macrophages, presumably *via* induction of IL-1β and NO release. Mice deficient for PGAM5 showed a slight, but significant increase of the parasite load in the skin lesions of *L. amazonensis*-infected mice (54).

Phosphotyrosine Host Phosphatases Regulating the Outcome of *Leishmania* Infections

A first hint that replication and survival of *Leishmania* in phagocytes is dependent on this family of phosphatases came from the use of inhibitors. *In vitro*, macrophages treated with a PTP inhibitor, the peroxovanadium compound bpV(phen), showed an improved control of *L. donovani* infection, mediated by an increased production of NO (55). Inhibition of ERK2 by a still unknown phosphatase was suggested to account for a similar phenotype seen in a *L. major*-infected human macrophage-like cell line (56). Treatment of macrophages with a related PTP inhibitor, sodium orthovanadate, enhanced the phagocytosis of *Leishmania* promastigotes and thereby allowed a safe entry of the parasite into its cellular niche (57, 58). These *in vitro* results were corroborated by *in vivo* experiments with *L. major*-infected BALB/c mice, in which the PTP inhibitors bpV(phen) and pbV(pic) led to partial parasite control *via* induction of iNOS activity (59). Thus, the activities of PTPs seemed to support intracellular parasite growth.

Dual-Specificity Phosphatases (DUSPs)

The therapeutic effect of PTP inhibitor treatments was in line with observations made during *in vivo* treatment of *L. donovani*-infected mice with okadaic acid, a STP PP2A inhibitor (see above). The comparative analysis of both inhibitors in macrophages revealed that PP2A synergistically inhibited the activity of the MAPK ERK1/2 together with a phosphatase belonging to the PTP family, the DUSP6, also known as MAP kinase phosphatase (MKP) 3 (60). The inhibition of MAPKs downstream of PKCζ following *Leishmania* infection led to a decreased iNOS expression (60). In the same study, the role of DUSP1, also termed MKP1, was analyzed. Activation of DUSP1 downstream of PKCε resulted in the inhibition of p38 MAPK (60). A host-damaging role for these two DUSPs during visceral leishmaniasis was also

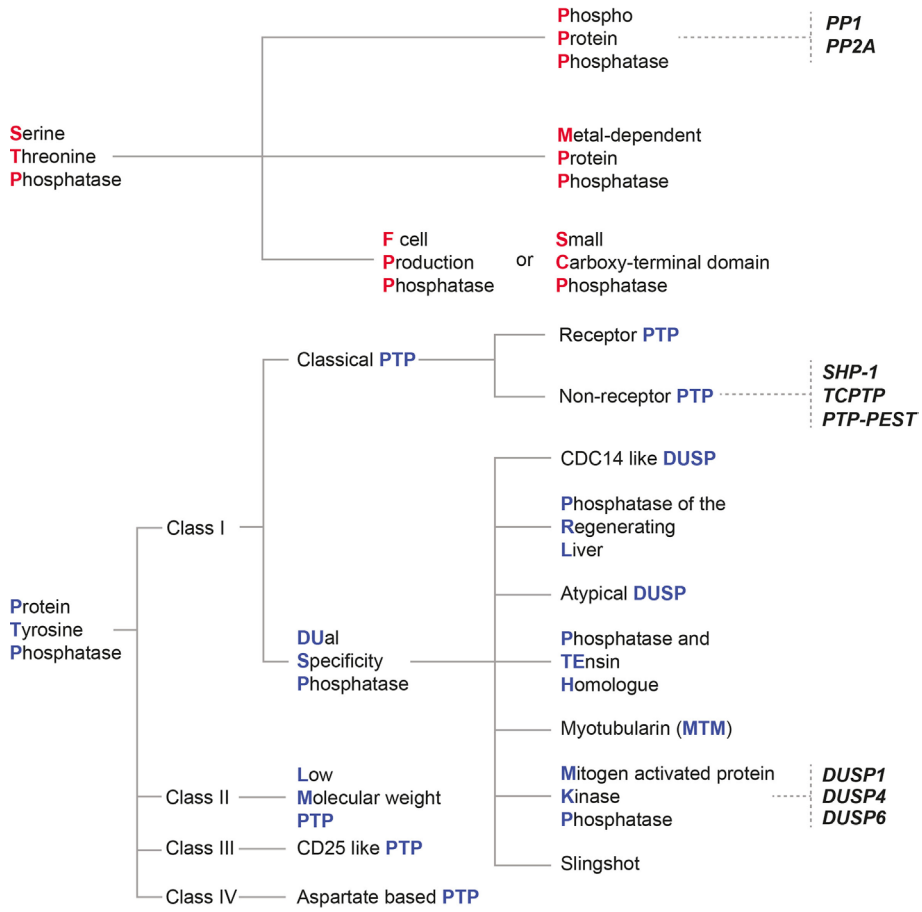

FIGURE 2 | Classification of mammalian protein phosphatase families and their relevance for *Leishmania* infection. Protein phosphatases are classified depending on their structural features and their substrate specificity. The two main families of protein phosphatases and their respective subfamilies are depicted here. Serine threonine phosphatases are marked with red initials, whereas protein tyrosine phosphatases are given with blue initials. Host protein phosphatases which were already shown to affect the outcome of *Leishmania* infections are written in bold and italic black letters.

demonstrated in *L. donovani*-infected BALB/c mice treated with 18β-glycyrrhetinic acid (GRA), a compound derived from the medicinal plant licorice (*Glycyrrhizza glabra*). Treatment with GRA caused a shift of the immune response from a detrimental Th2 response to a protective Th1 response (61). At the molecular level, the shift was explained by a decreased DUSP1 and DUSP6 expression and the concomitant activation of mitogen- and stress-activated protein kinase 1 (MSK1), which ultimately led to the production of leishmanicidal NO (62).

Findings with *L. major*-infected macrophages, however, indicated that the activity of DUSP6 is not always detrimental for the outcome of *Leishmania* infections. In *L. major*-infected macrophages activated by anti-CD40, siRNA-mediated inhibition of DUSP6 led to ERK activation, enhanced production of IL-10, and reduced expression of IL-12 and iNOS, whereas inhibition of DUSP1 caused p38 activation and increased generation of IL-12 and iNOS. Consequently, *in vivo* overexpression of DUSP6 or pharmacological inhibition of DUSP1 both ameliorated the course a cutaneous *L. major* infection (63). This functional dichotomy between DUSP6 and DUSP1 has also been reported in the context of macrophage coinfections with the immunomodulatory bacterium *Mycobacterium indicus pranii* and *L. donovani*. The protective effect of this bacterial infection relied on a toll-like receptor (TLR) 4-dependent increase of DUSP6 and simultaneous decrease of DUSP1. Whereas DUSP1 inhibition led to the activation of p38, the production of NO and the release of IL-12, the activation of DUSP6 blocked the ERK1/2 pathway and the expression of IL-10 and arginase-1 (Arg1). Overall, *M. indicus pranii* conferred protection against visceral leishmaniasis by reciprocal regulation of DUSP1 and DUSP6 and a shift toward a Th1 response (64).

A phenotype similar to the one of DUSP6 was also observed for DUSP4 (syn. MKP2). Mice deficient for the *dusp4* gene were more susceptible to a cutaneous infection with *L. mexicana* than wild type mice. This was due to an enhanced JNK and p38 phosphorylation in macrophages that increased the expression of

Arg1 and was associated with a disease-promoting Th2 immune response (65, 66).

Src Homology Region 2 Domain-Containing Phosphatase-1 (SHP-1)

In macrophages infected with *L. donovani*, a PTP activity identified as *Src homology region 2 domain-containing phosphatase-1* (*SHP-1;* also known as protein tyrosine phosphatase non-receptor type 6 [PTPN6]) was rapidly activated and induced hypo-responsiveness to IFN-γ by directly interacting with the kinase JAK2 recruited to the IFN-γ receptor (31, 43, 44). The confirmation that SHP-1 played a key role in the pathogenicity of leishmaniasis came from infection experiments with viable motheaten (me^v) mice that are deficient for this phosphatase (67). Me^v mice were largely protected against *L. major* infection, most likely because of an increased NO production by their phagocytes (68) and an enhanced secretion of proinflammatory cytokines like TNF, IL-1β, and IL-6 (69). Another group, however, did not observe an impact of the SHP-1 deficiency in me^v mice on the course of *L. major*-infection and questioned a selective role of SHP-1 in macrophages as SHP-1 is also regulating T and B cell responses (70). It is also important to point out that even in the absence of SHP-1 activity different species of *Leishmania* (*L. major*, *L. braziliensis*, *L. mexicana*, and *L. donovani*) caused degradation and reduced nuclear translocation of STAT1 in primary mouse macrophages (71). This finding indicates that at least part of the suppression of the JAK2/STAT1 pathway in *Leishmania*-infected macrophages and the subsequent reduced release of cytokines and NO in response to IFN-γ is SHP-1 independent.

Infection of SHP-1-deficient macrophages also revealed the importance of this phosphatase for the downregulation of MAPK activity after IFN-γ (31, 44). Control of MAPK by SHP-1 was proposed to be partially responsible for the inhibition of iNOS during IFN-γ stimulation (44, 72). However, involvement of individual MAPK seemed to differ depending on the stimuli used to trigger iNOS expression during *Leishmania* infection. Although SHP-1 negatively regulated ERK1/2 and JNK after IFN-γ stimulation (72), this phosphatase boosted the activity of ERK1/2 after CD40 stimulation, which promoted the secretion of IL-10 and simultaneously inhibited p38 MAPK activation (73).

During *Leishmania* infection, SHP-1 did not only alter the macrophage responses to proinflammatory stimuli originating from T cell such as IFN-γ and CD40 ligand. Signaling pathways triggered by pathogen recognition receptors (PRRs), e.g., TLR4, were also disturbed. In mouse bone marrow-derived macrophages, infection with different *Leishmania* species (*L. major*, *L. mexicana*, *L. donovani*) caused rapid activation of SHP-1, which bound to a kinase tyrosyl-based inhibitory motif (KTIM) of the interleukin 1 receptor-associated kinase 1 (IRAK-1) and thereby blocked the intrinsic kinase activity of IRAK-1. The inactivation of IRAK-1 by SHP-1 was associated with the inability of IRAK-1 to detach from the TLR4 adaptor MyD88 and to attach to the downstream signal transducer TRAF6 so that the LPS-induced macrophage responses (e.g., release of NO and TNF) were severely impaired (74). According to the authors of this study KTIMs are also present in other targets of SHP-1, such as ERK1/2, p38, and JNK, and therefore offer a molecular explanation for the multiple effects of SHP-1 (74). A SHP-1 mediated hypo-responsiveness to LPS was also observed in human macrophages infected by *L. donovani* (75).

Two recent studies demonstrated that SHP-1 also modulates the signaling downstream of PRRs of the lectin receptor family during leishmaniasis. C-type lectin receptor Mincle expressed by dendritic cells recognized a still unknown ligand present on the surface of *L. major*. Ligation of the Mincle receptor by *L. major* impaired the activation of dendritic cells due to recruitment of SHP-1, which converted the canonical immunoreceptor tyrosine-based activation motif (ITAM) of the FcRγ-chain signaling component of the receptor into an inhibitory motif (ITAMi). In accordance with these *in vitro* findings Mincle-deficient mice developed less severe skin lesion after infection with *L. major* (76). Likewise, I-type lectin receptors Siglec-1 and -5 expressed by macrophages were described to recognize sialylated ligands on the surface of *L. donovani* parasites. Engagement of Siglec-1 facilitated the uptake of the parasites by the macrophages, whereas ligation of Siglec-5 led to a reduced production of leishmanicidal ROS and NO accompanied by a shift toward a Th2 response. The latter resulted from the recruitment of SHP-1 to the Siglec-5 receptor promoting here again the deactivation of the immune cell (77).

Together, these data illustrate that phosphatases are critical regulators of the immune response against *Leishmania* parasites. Parasite-driven activation of PTPs, notably SHP-1, clearly impairs the effector function of macrophages [reviewed in Ref. (78)]. On the other hand, host cells have developed strategies to counteract the evasion mechanisms of *Leishmania* parasites. As an example, natural resistance-associated macrophage protein 1 (NRAMP-1) inhibited the function of PTPs, either *via* a direct interaction between PTPs and the metal cation (Fe^{2+}) transported by NRAMP-1 or by oxidation of the phosphatases resulting from NRAMP-1/iron-dependent ROS-formation (79). However, in the arm race between host and parasite, *Leishmania* parasites were shown to again antagonize this ROS-mediated inhibition of host PTPs in macrophages by inducing the expression of the uncoupling protein 2 (UCP2), a negative regulator of mitochondrial ROS generation. This led to lower ROS production resulting in an enhanced host PTP activity (80).

Evidence for the Activation of Host Phosphatases by *Leishmania* Virulence Factors

As summarized above, infection of macrophages with *Leishmania* leads to the induction of phosphatase activities, especially SHP-1, which help the parasite to create a safe niche for its replication. Phosphatase activation can be the outcome of normal cell signaling pathways triggered by cytokines and/or PRRs. Alternatively it is possible that microbial virulence factors released inside the host cell directly stimulate phosphatases such as SHP-1. The first evidence that *Leishmania* could do so came from pull-down assays with *L. donovani* extracts and recombinant SHP-1. In these experiments, the *Leishmania elongation factor 1α* (*EF-1α*) was identified as an interactor of the host phosphatase. *Leishmania*

EF-1α secreted by intraphago(lyso)somal parasites reached the cytosol of the infected macrophages. Purified *Leishmania* EF1α directly bound to SHP-1 and induced its activity (81), whereas mammalian EF-1α was unable to do so, presumably because of an additional hairpin loop at its surface (82). The secretion of EF-1α by intracellular *Leishmania* amastigotes during macrophage infection was later confirmed and attributed to the exosomes released by the parasites (83).

Production of exosomes by *Leishmania* appears to be the main secretory pathway used by the parasite to export its virulence factors inside and outside the host cells (83, 84). The important contribution of exosomes to the pathogenicity of *Leishmania* was supported (a) by their increased release after a temperature shift mimicking the transit from the sand fly vector (26°C) to the human host (37°C) (85) and (b) by their immunomodulatory effects on innate immune cells (86, 87).

In addition to EF-1α, other proteins contained in *Leishmania* exosomes (83) were found to activate SHP-1. The *fructose-1,6-biphosphate aldolase* was also secreted by *L. donovani* during macrophage infection and could directly interact and activate *in vitro* the phosphatase activity of SHP-1 (88). Unfortunately, the molecular mechanism of SHP-1 activation by both virulence factors, EF-1α and the aldolase, is still unknown.

One of the best studied virulence factors of *Leishmania* is the zinc *metalloprotease GP63*. This multifunctional virulence factor is the most abundant C-terminal glycosyl-phosphatidyl-inositol-anchored protein associated with the cell surface of *Leishmania* promastigotes, but is found in limited quantities also inside the parasite (89). On the surface of *Leishmania* parasites, GP63 increased the resistance of promastigotes to complement-mediated lysis by rapidly converting C3b into the inactive complement fragment iC3b (90). iC3b opsonized the parasite and facilitated its silent phagocytosis *via* the CR3 receptor (CD11b/CD18), which synergized with the fibronectin receptor that directly binds GP63 (91). Importantly, GP63, like EF-1α and the aldolase, was also secreted by promastigotes inside exosomes (83, 92). In the cytosol of infected cells, multiple targets of GP63 protease activity have been identified. These include (a) the cytoskeleton adaptors p130Cas (93), cortactin (93) and MARCKS-related protein (94), all of which are degraded by GP63; (b) the translational regulator mTOR (95), which is cleaved by GP63 leading to repression of macrophage translation; (c) PKC, which is also cleaved by GP63 leading to the attenuation of PKC activity (29); (d) transcription factors c-Jun (96) and NF-κB p65/RelA fragment (97), which are inactivated or activated by GP63-mediated proteolysis, respectively; and (e) the host phosphatases SHP-1, PTP1B and TCPTP which are proteolytically activated by GP63 (98). As already discussed before, SHP-1 is a key regulator of many different signaling pathways, including the TLR (74, 75), lectin receptor (76, 77), the MAPK (72, 73, 93) and the IFN-γ-triggered JAK/STAT pathways (44, 68) (**Figure 3**). It is worth noting that SHP-1 was not the only host phosphatase targeted by GP63 and shown to modulate the activity of JAK2. PTP1B was also proteolytically activated by GP63. Interestingly, whereas under resting conditions both SHP-1 and PTP1B were bound as full-length enzymes to JAK2, in *L. major*-infected macrophages the GP63-activated fragment of PTP1B and the uncleaved (i.e., inactive) SHP-1 prevailed, indicating that PTP1B might have a predominant role compared to SHP-1 in the deactivation of JAK2 (98). The importance of PTP1B activation for parasite survival was further underlined by the increased resistance to *L. major* seen in PTP1B-deficient mice (98).

Two further host phosphatases have been described to be proteolytically activated by GP63 during *Leishmania* infection: TCPTP (92, 93, 98) and PTP-PEST (93). However, their functional relevance for the infection has not been tested so far. Moreover, it seemed that the proteolytic activation of these phosphatases by GP63 was species-specific. GP63-mediated proteolysis was only observed after infection with *L. major*, *L. donovani*, and *L. mexicana*, but not with *L. tarentolae* and *L. braziliensis* (93).

Another protease, the *cysteine proteinase b* of *L. mexicana* (LmCPb) was proposed to synergize with GP63 to cleave PTP1B. This protease seemed to also participate in the degradation of important transcription factors such as NF-κB, AP-1, and STAT-1 (99). Intriguingly, PTP1B activation only occurred during infection of macrophages by promastigotes, but not by amastigotes (99), unlike SHP-1 that is cleaved after infection with either of both parasite stages, and despite the higher expression of LmCPB in amastigote (100). The authors suggested that amastigotes did not need to target PTP1B, whose regulatory functions overlap with SHP-1, because amastigotes were already more adapted to their niche than promastigotes.

LEISHMANIA PHOSPHATASES

The data summarized in the preceding section illustrated that activation of host phosphatases by *Leishmania* is essential for their infection of mammalian organisms. However, *Leishmania* phosphatases might be equally relevant for the development of the parasite and its survival during the infection process. In the following, we will review the *Leishmania* protein phosphatases already characterized in the context of the parasite life cycle. Next, the role of extracellular acid phosphatases (AcPs) of *Leishmania* will be discussed and compared to a newly described secreted *Leishmania* phosphatase which appears to contribute to the virulence of the parasite.

Phosphatases Involved in the Life Cycle of *Leishmania* Parasites

Recent technical advances in mass spectrometry allowed systematic analysis of protein phosphorylation in multiple biological contexts. In case of *Leishmania*, studies have initially focused on the differentiation of promastigotes into amastigotes, especially because only a few genes are differentially regulated during this adaptive process, making posttranslational modification a good candidate to regulate this transformation (101). Axenic differentiation of *L. donovani* promastigotes into amastigotes revealed the presence of 627 proteins phosphorylated on 1,614 residues with a majority modified on serine (80%) and threonine (19.4%) and only a few on tyrosine (0.4%). Among them, only 27% of the modifications were present in both developmental stages, whereas 35 and 38% of the phosphorylation were restricted to promastigotes or amastigotes, respectively (102). A refined analysis of

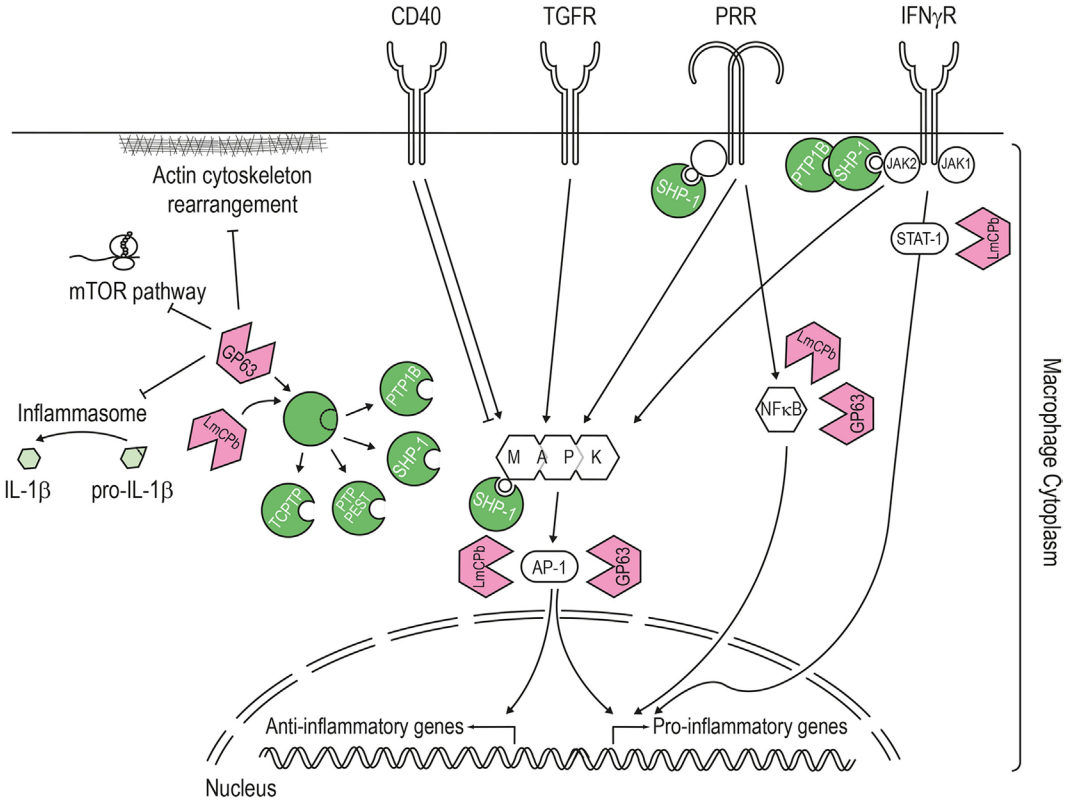

FIGURE 3 | Pathways targeted by the *Leishmania* virulence factors. LmCPb and GP63 proteases are *Leishmania* virulence factors secreted *via* the exosome route and found inside the host cell cytoplasm where they can alter numerous signaling pathways. The best studied *Leishmania* protease is GP63, which proteolytically cleaves and activates host phosphatases (such as PTP1B, PTP-PEST, TCPTP, and SHP-1) or the NF-κB subunit p65/RelA. Once activated, SHP-1 downregulates the macrophage response to interferon-γ by interacting with the kinase JAK2, modulates the pathways induced by pathogen recognition receptors (e.g., toll-like and lectin receptor) and also targets signaling kinases such as mitogen-activated protein kinase. In addition, *Leishmania* proteases contribute to this general downregulation of the host cell response by degrading proteins of the actin cytoskeleton, mTOR (leading to repression of host cell translation), transcription factors (like STAT1 or AP-1), and components of the NLPR3 inflammasome (thereby blocking the generation of IL-1β).

the kinetic of differentiation suggested that promastigotes sensed an environmental signal triggering a phosphorylation pathway and resulting in *Leishmania* transformation (103). Like for the stress response protein A2 expressed by *Leishmania* species causing VL (11), gene ontology analysis of the phosphoproteome of *Leishmania* pinpointed the critical role of the heat shock response during differentiation (104). Obviously, parasites need to adapt to the temperature increase experienced during the inoculation into the skin. Indeed, experiments using parasites deficient for heat shock protein (Hsp)70/Hsp90-organizing protein (Hop) (also termed stress-inducible protein 1), a protein belonging to a chaperone complex, proved the importance of phosphorylation in the regulation of the heat shock response (105).

The fine tuning of all these adaptive pathways as well as the metabolic equilibrium of *Leishmania* parasites requires a balanced activation of phosphatases and kinases. Bioinformatic analyses identified 199 protein kinases (106) and 88 protein phosphatases (107) in the genome of the *L. major* species Friedlin. Among the kinases, a few have been identified and functionally characterized. Mainly, the role of MAPK was described in different pathways responsible for sensing environmental changes and ranged from flagellar control (108) to arginine deprivation (109). Regarding protein phosphatases, their characterization is still patchy as we will see in the following paragraphs (**Tables 1** and **2**) [reviewed in Refs. (107, 110)].

Leishmania Protein Serine/Threonine Phosphatases

Leishmania major, the model species used for this review, encodes 58 genes for serine/threonine phosphatases (STP) which are members of the three different groups of enzymes within this family: the PPP group (30 genes), the PPMs (15 genes), and the aspartate-based phosphatases (FCP/SCP; 13 genes) (see **Table 1**). These enzymes are highly conserved in the different *Leishmania* species (Table S1 in Supplementary Material).

Within the *PPP-group*, the eight *Leishmania* phosphatases that belong to the PP1 subgroup have not yet been biochemically and/or functionally characterized. Surprisingly, five of them are encoded by genes organized in tandem on chromosome 34. This organization suggests a common regulation of the expression of the different isoforms. PP2A phosphatases are also not yet fully characterized, but their increased expression has been linked to resistance against antibiotics such as paromomycin (111). Additional information on their functions in *Leishmania* parasites could be derived from studies of orthologs in other kinetoplastid.

TABLE 1 | Serine threonine phosphatases of *Leishmania major*.[a]

Family			*L. major* gene	Characterized *Leishmania* gene	Reference
STP	PPP	PP1	LmjF.15.0220		
			LmjF.28.0690		
			LmjF.31.2630		
			LmjF.34.0780		
			LmjF.34.0790		
			LmjF.34.0800		
			LmjF.34.0810		
			LmjF.34.0850		
		PP2A	LmjF.25.1320		
			LmjF.28.2670		
		PP2B	LmjF.26.2530		
			LmjF.36.1980		
		PP4	LmjF.32.3040	LdBPK_323230.1	(111)
		PP5	LmjF.18.0150		(112)
		PP6	LmjF.34.4190		
		PP7 (PPEF)	LmjF.12.0660	LinJ.12.0610	(113, 114)
		ApaH-like(ALPH)	LmjF.22.1600		
			LmjF.17.0580		
		Shewanella-like	None		
		kSTP (pseudo)	LmjF.05.0100		
			LmjF.09.0470		
			LmjF.12.0050		
			LmjF.13.1510		
			LmjF.13.1570		
			LmjF.22.1490		
			LmjF.24.0270		
			LmjF.26.2100		
			LmjF.34.2770	LinJ.34.2610	(115)
			LmjF.36.2050		
			LmjF.29.0440		
			LmjF.30.3280		
	MPP (PP2C)		LmjF.14.0900		
			LmjF.15.0170		
			LmjF.36.0530		
			LmjF.32.1690		
			LmjF.27.2320		
			LmjF.36.1230		
			LmjF.30.0380		
			LmjF.25.2060		
			LmjF.06.0900		
			LmjF.25.0750	LinJ.25.0780	(85, 116)
			LmjF.31.1320		
			LmjF.34.2500	LinJ.34.2310	(114)
			LmjF.34.2510	LinJ.34.2320	(114)
			LmjF.27.1180		
			LmjF.34.0730		
	FCP SCP		LmjF.26.0160		
			LmjF.34.3920		
			LmjF.32.0100		
			LmjF.25.2030		
			LmjF.24.0290		
			LmjF.34.1250		
			LmjF.35.2620		
			LmjF.36.6780		
			LmjF.35.3520		
			LmjF.27.2180		
			LmjF.29.2400		
			LmjF.35.0190		
			LmjF.03.0890		

[a]*Phosphatases identified by mass spectrometry in the secretome of Leishmania parasites are marked in red (83–85, 92).*

STP, serine threonine phosphatase; PPP, phosphoprotein phosphatase; MPP, metal-dependent phosphatase; FPP, F cell production phosphatase; SCP, small carboxy-terminal domain phosphatase; PP, protein phosphatase; PPEF, protein phosphatases with a E-helix/F-helix (EF)-hand (PPEF); kSTP, kinetoplastid specific STP.

TABLE 2 | Tyrosine phosphatases of *Leishmania major.*[a]

Family					*L.* major gene	Charact. gene	Ref.
PTP	Class I		Receptor		Nd		
		Classical PTP	Non-receptor PTP	Eukaryotic like	LmjF36.5370 LmjF36.2180	LdBPK_365610.1	(117)
				Kinetosplastid	LmjF32.0640		
			eDUSP PRL		LmjF16.0230 LmjF16.0250		(118)
			eDUSP CDC14		LmjF09.0420		
			Atypical DUSP	LRR-DUSP	LmjF28.0170		
				Kinatase	LmjF34.2190		
				ANK-DUSP	Nd		
				STYX	LmjF21.0700		
				MKP-like	LmjF27.1840		
				Lipid-like phosphatases	LmjF04.0560 LmjF25.0570 LmjF33.2840 LmjF22.0250		(119)
		DUSP	PTEN	Eukaryotic-like	LmjF34.1430		
				Kinetosplastid	LmjF25.0230		
			MTM		LmjF20.1480 LmjF12.0320		
			Kinetoplastid DUSP		LmjF03.0510 LmjF05.0220 LmjF08.0100 LmjF09.0550 LmjF13.0770 LmjF27.2210 LmjF28.0790 LmjF35.4650 LmjF32.1010 LmjF04.0840 LmjF05.0720		
			MAKP phosphatase		Nd		
			Slingshot		Nd		
	Class II	LMPTP/ArsC reductase			LmjF01.0200		
	Class III	CDC25/ACR2 reductase			LmjF32.2740		(120–123)
	Class IV	Asparate based PTP EyA			Nd		

[a]*Phosphatases identified by mass spectrometry in the secretome of Leishmania parasites are marked in red (83–85, 92).*
PTP, protein tyrosine phosphatase; DUSP, dual specificity phosphatase; PRL, phosphatase of the regenerating liver; STYX, inactive STYX pseudo-phosphatase; MKP, mitogen activated protein kinase phosphatase; PTEN, phosphatase and tensin homologs; MTM, myotubularin; LMPTP, low-molecular -weight PTP; ArsC red/ACR2, arsenate reductase.

PP1 and PP2A phosphatases of *Trypanosoma* parasites participate in amastigote transformation (110) and, interestingly, share the highest homology with *Leishmania* orthologs (Table S1 in Supplementary Material).

Regarding the calcineurin-like phosphatase subgroup (PP2B), a calcium- and calmodulin-dependent phosphatase activity was purified from *L. donovani* parasites but their respective genes have not yet been sequenced (124). In *L. major* two genes, *LmjF26.2530* and *LmjF36.1980*, might account for this activity (**Table 1**) as they encode proteins carrying a calcineurin A catalytic domain and the three canonical regulatory domains: the calcineurin B (CnB) binding domain, the calmodulin (CaM) binding domain and an autoinhibitory domain (possibly missing in *LmjF36.1980*) (125). Noteworthy, the ortholog found in *T. cruzi* does not contain the last two domains which likely causes a substantial functional difference (125). Interestingly, *Leishmania* CnB (*LmjF.21.1630*), the regulatory subunit of calcineurin A, was shown to control the temperature adaptation of *L. major*. Promastigotes deficient for CnB failed to differentiate into amastigotes and were completely avirulent in macrophages and during *in vivo* infection (126).

The PP7-like phosphatase, encoded by the gene *LmjF.12.0660*, showed a strong homology to protein phosphatases with a E-helix/F-helix (EF)-hand (PPEF) that were identified in sensory cells of higher eukaryotes. However, LmPPEF did not bind calcium because its EF-hand domains appeared degenerated and the functionality of its N-terminal calmodulin binding domain was not ascertained (113). Due to a N-myristoylation modification, LmPPEF appears to be membrane-bound and localized in the vicinity of the kinetoplast and the flagellar pocket (113), an active secretory site, which might explain its detection in the exosome content of promastigotes (83, 92).

PP5 phosphatases showed some similarities with PP7 proteins, but instead of calcium-dependent regulatory domains they carry

N-terminal tricopeptide repeat (TPR) domains, which can mediate protein–protein interaction and autoinhibition. The PP5 phosphatase encoded by *L. major* (*LmjF18.0150*) has been biochemically characterized. Mutation analysis of the recombinant protein confirmed its phosphatase activity and the autoinhibitory role of the TPR domains (112). Functional data on this protein are scarce but suggested its involvement in stress response as also seen with the *T. brucei* homolog (110). Expression of the unique PP5 gene in *L. infantum* correlated with increased resistance to antimony (114).

Regarding the eukaryotic-like PP6 phosphatases or the eukaryotic phosphatases homologous to the bacterial enzyme diadenosine tetraphosphate hydrolase ApaH (ApaH-like phosphatases [ALPHs]), only few data exist on their biochemical or physiological role. In *Trypanosoma brucei*, ALPH1 was shown to have all features of an mRNA decapping enzyme (127).

The last subgroup of the PPPs comprises kinetoplastid-specific proteins (kPPP) that carry a mutation in their catalytic site. These pseudo-phosphatases (kSTP pseudo) show some similarities to plant and fungal PPPs. They likely add an extra level of regulation to the kinase/phosphatase activities by stably binding to phosphorylated substrates and shielding them against other phosphatase activities (110). These adaptations to numerous substrates combined with the absent catalytic activity might explain the higher divergence observed between kPPP from *Leishmania* and *Trypanosoma* (Table S1 in Supplementary Material). Expression of such pseudo-enzyme, encoded by the gene *LinJ.34.2610*, has been negatively correlated with the resistance of *L. infantum* to antimony treatment (115).

Regarding the 15 phosphatases of the *PPM (or PP2C) group*, their biological functions have not yet been unraveled, but their dependency on divalent manganese or magnesium cations has been validated in *L. donovani* and *L. infantum/L. chagasi* promastigotes (116, 128). In case of *L. infantum/chagasi*, a 42 kDa PP2C-like phosphatase named LcPP2C, encoded by the gene *LinJ.25.0780*, was biochemically characterized in promastigotes and amastigotes (116). Interestingly, the ortholog of this protein was secreted by *L. mexicana* along with 71 other proteins detected (84, 85). However, whether this enzyme contributes to the modulation of the host cell signaling (e.g., *via* activation of the host phosphatases SHP-1 and PTP-1B) by the *L. mexicana* secretome, was not investigated (85). Other PP2C-like proteins have been detected in *L. mexicana* promastigotes but they still lack functional characterization (129). Moreover, expression of two additional PP2C genes, *LinJ.34.2310* and *LinJ.34.2320*, was recently linked to methotrexate resistance of *L. infantum* (114).

Altogether, only 6 of the 58 STP genes have been partly characterized despite the suggested crucial role in amastigote transformation (**Table 1**). More research on this phosphatase family could therefore open new avenues toward the development of alternative therapeutic approaches.

Leishmania Protein Tyrosine Phosphatases

In additions to STPs, 31 putative protein tyrosine phosphatases (PTPs) are encoded in the *L. major* genome (**Table 2**). All of them share a common catalytic site surrounded by a cysteine and an arginine (CX_5R). They are well conserved among *Leishmania* species and are divided into four classes (Table S1 in Supplementary Material). *Class I PTPs*, which form the largest group, contain enzymes with either proven PTP activity or a structural organization characteristic for PTPs, whereas classes II and III, represented by a single member each, might not convey such activities. Class IV enzymes were not detected in the genome of *Leishmania* parasites.

Proteins of the *class II PTPs* are homologous to low-molecular-weight (LMW) PTPs found in human or bacteria. However, the protein encoded by the gene *LmjF.01.0200* carries a mutated catalytic cysteine as revealed during our own sequence analyses, suggesting that it is probably inactive and might therefore act, similar to the kPPPs, as substrate trapping molecule. Another degenerated LMW PTP-like enzyme is encoded by the gene *LTRL590_050007600.1*, whose expression correlated to antimony resistance in *L. tropica* field isolates (130).

Regarding the *class III PTPs*, the enzyme named LmACR2 has been initially characterized in *L. major* as an arsenate (As)/antimony (Sb) reductase despite containing a canonical PTP catalytic site. The antimony reductase activity of LmACR2 converted Sb(V), present in Sb-containing antileishmanial drugs (e.g., sodium stibogluconate), into Sb(III) which is the active microbicidal molecule. Overexpression of LmACR2 in promastigotes supported this observation as parasites became more sensitive to antimonials (120). However, metalloids like Sb are not commonly encountered by *Leishmania* parasites in nature. Therefore, the evolutionary pressure to develop such activity should have been low. Thus, the concomitant PTP activity of LmACR2 detected *in vitro* might reflect the true physiological function of this enzyme (121, 122).

Most of the PTPs expressed in *Leishmania* parasites belong to the *class I PTPs* that can be further divided into two subgroups of enzymes. The first subgroup consists of classical PTPs homologous to human PTP1B. However, in *Leishmania* and other kinetoplastid parasites, this group does not contain any receptor-PTP usually found in higher eukaryotes. Among the three non-receptor-PTPs encoded by *Leishmania* parasites, only the eukaryotic-like LdPTP1 was functionally characterized. The deletion of the gene encoding for this enzyme, *LdBPK_365610.1*, resulted in complete loss of amastigote transformation during infection of macrophages with *L. donovani* promastigotes and in a dramatic decrease of the parasite virulence *in vivo* (117). The molecular mechanism underlying this drastic phenotype has not yet been elucidated. However, the study of the TbPTP1 ortholog in *Trypanosoma brucei* revealed that this family of phosphatase could control the integration of environmental differentiation signal (131, 132).

The second subgroup of class I PTPs comprises dual specificity phosphatases (DUSP) (**Table 2**). These phosphatases are characterized by a substrate spectrum that extends beyond phosphotyrosine residue (e.g., dephosphorylation of serine and threonine residues). Some of these DUSPs are even described as lipid phosphatases:

(a) Myotubularins (MTMs) were shown to target phosphoinositides like PI(3)P or $PI(3,5)P_2$ in mammals, and therefore control endocytosis and membrane trafficking (133). In

Leishmania parasites, the MTM group consists of only two genes (instead of the 16 encoded by the human genome making it the largest group of human DUSP). *Leishmania* MTMs are characterized by their very large size with up to 3,245 amino acid (as in the case of *LmjF.12.0320*), which is due to long N-terminal extensions. However, their function has not been yet studied in trypanosomatids.

(b) Two *Leishmania* genes encode still uncharacterized phosphatase and tensin homologs (PTEN) that are well known to target PI(3,4,5,)P_3 in mammals. Whereas one gene is homologous to kinetoplastid PTENs, the other one shares homology with eukaryotic phosphoinositide phosphatases. Interestingly, the latter subgroup is not represented in all Trypanosomatidae, as it is missing in *T. brucei* (Table S1 in Supplementary Material).

(c) A group of four *Leishmania* DUSPs encodes lipid-like phosphatases that have similarities with bacterial virulence factors. *LmjF.22.0250* was identified as a close homolog of the secreted PIP phosphatases MptpB from *Mycobacterium tuberculosis* (119) and LipA from *Listeria monocytogenes* (134). Strikingly, *LmjF.22.0250* is the only protein phosphatase gene that is not shared by all *Leishmania* species or strains as it was absent from the genome of *L. panamensis* MHOM/COL/81/L13 and *L. braziliensis* MHOM/BR/75/M2903, but present in *L. braziliensis* MHOM/BR/75/M2904 (Table S1 in Supplementary Material). As this phosphatase was not found to be secreted, the absence of *LmjF.220250* might be compensated by the other three members of this group of lipid-like phosphatases encoded in the genome of *Leishmania*. Nonetheless, lipid phosphatases might contribute to the parasite virulence, because one of them, *LbrM.25.21.80* was among the 100 genes most abundantly expressed by *L. braziliensis* in skin lesions of humans with CL (135).

In addition to these phospholipid phosphatases, DUSPs dephosphorylating proteins on tyrosine, serine or threonine residues were also identified in the *Leishmania* genomes:

(a) Three *Leishmania* genes encode eukaryotic-like DUSPs (eDUSPs). These include a homolog of the human CDC14 phosphatase that was described to control mitosis by targeting cyclin dependent kinase (136). However, this crucial function has not been yet documented in trypanosomatids. The two genes homologous to phosphatase of the regenerating liver (PRL) will be discussed later in the context of their importance for the virulence of *L. major*.

(b) In addition to the lipid-like phosphatases discussed above, the group of atypical DUSPs encodes for (i) a kinetoplastid MAP kinase-like phosphatase (MKP) lacking a functional rhodanese homology domain, (ii) a leucine-rich repeat (LRR) DUSP, (iii) a kinatase having LRR domains and a pseudokinase domain, and (iv) an inactive STYX pseudophosphatase. None of these enzymes has yet been characterized in Trypanosomatidae.

(c) The remaining *Leishmania* DUSPs belong to the group of still uncharacterized kinetoplastid-specific DUSPs.

Although the large majority of the *Leishmania* DUSPs have so far not been functionally studied in *Leishmania*, it is interesting to note that two of them (LmjF.16.0230 and LmjF34.2190) were secreted in exosomes by *Leishmania* parasites (83, 92) (**Table 2**). These two DUSPs along with the six STPs also detected in exosomes (i.e., LmPPEF, LcPP2C, two PP1, and two kSTPs) represent a small pool of *Leishmania* phosphatases that might directly interact with phosphorylation cascades inside host cells during infection.

Leishmania Membrane and Secreted AcPs

Until this year, direct modulation of host responses by *Leishmania* phosphatases had only been documented for AcPs. These phosphatases do not belong to the classical STP or PTP families because of the unique composition of their catalytic site that is based on histidine residues. In the literature, histidine phosphatases are usually not discussed along with other protein phosphatases as their substrate specificity has not been ascertained. AcP activities have been described against various substrates, including glycerophosphate, fructose-1,6-diphosphate (137, 138), phosphoinositides (139), pyrosphosphate (140), and phosphoproteins (138, 141).

A search for histidine phosphatases in the genome of six species of *Leishmania* pathogenic for humans (*L. major*, *L. mexicana*, *L. braziliensis*, *L. panamensis*, *L. infantum*, *L. donovani*) using their signature motif (Interpro accession reference for the histidine phosphatase superfamily: IPR000560) revealed that each *Leishmania* genome contained six or seven genes of this family. Extending our search to pathogenic *Trypanosoma* species (*T. brucei* and *T. cruzi*) revealed that this family of phosphatase is well conserved among the Trypanosomatidae. These genes are distributed in two main groups with four to five genes encoding membrane acid phosphatase (MAcP; also known as ectophosphatases) and two to three genes encoding secreted acid phosphatases (SAcP) (**Figure 4A**; Table S2 in Supplementary Material).

Membrane Acid Phosphatases

All MAcPs share the same structural organization starting with a signal peptide at the N-terminus followed by the catalytic domain containing the histidine residues and a transmembrane domain at the C-terminus (**Figure 4B**). Phylogenetically, MAcPs are organized in four distinct groups (**Figure 4A**, groups I–IV), all represented by one MAcP gene sequence derived from each of the six analyzed *Leishmania* genomes. Interestingly, an additional MAcP gene, *LinJ.36.6740*, could be detected in the genome of *L. infantum*. Despite being more related to SAcP and being substantially shorter than all other MAcP (only 35 kDa instead of usually around 60 kDa), this phosphatase harbors a signal peptide and a putative transmembrane domain at its end, illustrating the genetic plasticity of *Leishmania* parasites in which diverging paralogous genes are common. Gene divergence was also seen in the MAcP gene of *L. donovani*. The MAcP protein of the *L. donovani* strain MHOM/SD/62/1S-CL2D (148) carried a transmembrane domain, which was found to be replaced by a not-functionally defined C terminal sequence of 91 amino acids in the MAcP gene of the *L. donovani* strain BPK282A1 (149). Comparing the

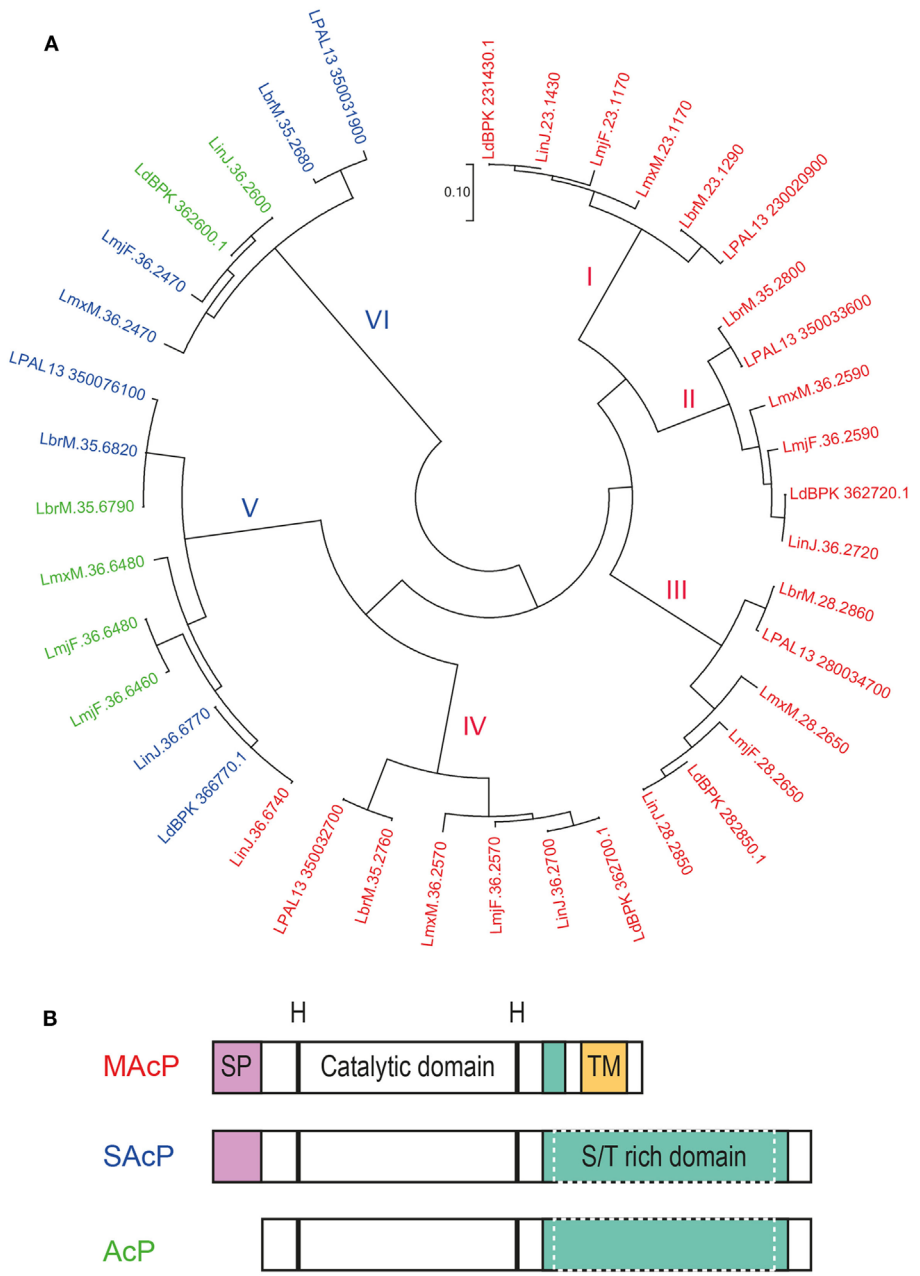

FIGURE 4 | Acid phosphatases (AcPs) of *Leishmania* parasites. AcP, secreted by *Leishmania* parasites or expressed on their surface, belong to the family of histidine phosphatases. **(A)** Phylogenetic comparison between histidine phosphatases of *L. major* Friedlin, *L. infantum* JPCM5, *L. donovani* BPK282A1, *L. mexicana* MHOM/GT/2001/U1103, *L. braziliensis* MHOM/BR/75/M2904, *L. panamensis* MHOM/COL/81/L13. Available gene sequences were extracted from the TriTrypDB Kinetoplastid Genomics Resource database (IPR: 000560) and were used to construct a phylogenetic topology with the minimum evolution method. The evolutionary distance (scale) is measured in number of base substitutions per site. The evolutionary history was inferred using the Minimum Evolution method (142). The evolutionary distances were computed using the Maximum Composite Likelihood method (143) and are in the units of the number of base substitutions per site. The minimum evolution tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (144) at a search level of 1. The Neighbor-Joining algorithm (145) was used to generate the initial tree. The analysis involved 39 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 409 positions in the final data set. Evolutionary analyses were conducted in MEGA7.0.18 (146). Protein sequences of the respective genes were analyzed for the presence of a putative signal peptide (SP) and transmembrane domain (TM) with the Phobius software (147). Genes are labeled in red for the membrane AcP (MAcP) containing a signal peptide (SP) and transmembrane domain (TM), in blue for the secreted AcP (SAcP) containing only a SP and in green for the phosphatases lacking both domains. **(B)** Schematic organization of the protein domains of *Leishmania* AcP. Membrane and secreted AcP have a SP at their N-terminus followed by a catalytic domain based on histidine residues. Next, AcP harbor a serine/threonine(S/T)-rich domain that varies in length and can be significantly larger in SAcPs. Finally, MAcPs harbor a TM close to their C-terminus. Some AcPs (green in panel A) are devoid of SP and TM but can harbor long S/T-rich domains.

published sequences, the variation between these *Leishmania* strains was not related to frameshifts or aberrant stop codons but might result from a more complex evolutionary process.

Numerous studies aimed to characterize the function of MAcPs as they represented the first enzymatic activities identified on the surface of *Leishmania* promastigotes (150). Having been transported *via* the classical intracellular secretion pathway, it was an expected finding that the MAcPs were phospho-glycosylated (151). Interestingly, expression of MAcP varied during the different life stages of *Leishmania* parasites. In *L. major*, MAcP expression increased during metacyclogenesis of promastigotes and correlated with the translocation of the protein from the cytoplasm to the membrane (152). Increased expression of some of the MAcPs was also detected during metacyclogenesis and differentiation into amastigotes following macrophage infection (153, 154). Most recently, MAcPs were found to be differentially expressed in clinical isolates of the same *Leishmania* species (155). A MAcP of *L. tropica* (LTRL590_280034100, annotated as LmjF.28.2650) was described as one of the 30 most diversely expressed genes in a collection of 14 clinical isolates of *L. tropica* from different geographic areas. This might indicate that the parasite adapts the level of extracellular phosphatase activity depending on the specific infection situation. Functionally, early reports suggested that these ectophosphatases altered the response of neutrophils (156). Partially purified MAcP activity from *L. donovani* specifically interfered with the production of ROS by neutrophils after stimulation with the tripeptide *N*-formylmethionine-leucyl-phenylalanine (fMLP), whereas activation by PMA was resistant to MAcP activity (157). Other authors proposed that a phosphoinositide phosphatase activity of MAcP might play a role in this inhibition, because the phagocyte NADPH oxidase (Phox) relies on these signaling molecules for its activation (139). In accordance with this hypothesis, overexpression of LdMAcP by *L. donovani* promastigotes resulted in an increased survival during *in vitro* infection of a mouse macrophage cell line (158). However, deletion of the MAcP gene of *L. mexicana* (*LmxM.36.2570* [LmxMBAP]) raised doubts about an essential role of this class of phosphatases for the virulence of *L. mexicana* parasites; LmxMBAP-deficient parasites were as virulent as wild-type parasites during *in vitro* infection of macrophages or *in vivo* infection of susceptible BALB/c mice (159). However, a definitive statement on the function of MAcPs is still not possible because of potential compensatory mechanisms as *Leishmania* parasites carry a minimum of four MAcP genes.

Secreted Acid Phosphatases

Secreted acid phosphatases are among the most abundant proteins secreted by *Leishmania* parasites (137, 160). However, a few *Leishmania* species, such as *L. major*, were shown to express very little SAcP as promastigotes (161). This difference could be due to the number of genes coding for truly secreted phosphatases. Our *in silico* analysis of the three *L. major* histidine phosphatases devoid of transmembrane regions revealed that only one, *LmjF.36.2470*, might have a functional signal peptide (Table S2 in Supplementary Material), whereas other *Leishmania* species such as *L. panamensis*, *L. braziliensis*, *L. mexicana*, and *L. donovani* can express more than one intact SAcP. The phylogenetic analysis also allowed the classification of SAcPs in two different families that are substantially divergent. One group of phosphatases (**Figure 4A**, group VI) branched out early from the histidine phosphatase family but is highly conserved across species, while the other group (**Figure 4A**, group V) is more closely related to MAcPs but is also more diverse. In members of group V, a signal peptide is sometimes absent, the enzymes can be significantly shorter than other orthologs and truncation could occur in the catalytic domain. This is the case for the two putatively non-secreted phosphatases of *L. major*, *LmjF.36.6460*, and *LmjF.36.6480*, indicating that the function of these two genes probably differs from their orthologs in case they are expressed.

For parasites expressing large amounts of SAcP, such as *L. donovani*, soluble phosphatase activity could be detected all along their differentiation from promastigotes to amastigotes (162). Interestingly, SAcP activity was also found in the cytosol of macrophages after 24 h of infection (11). However, the exact molecular identity of the phosphatase was not determined and this family of enzymes was also not detected inside exosomes that represent the main transit route of *Leishmania* cargo toward host cell cytosol. Like MAcPs, SAcPs are phospho-glycosylated proteins (163, 164). This modification relies on the same family of enzymes responsible for the LPG biosynthesis as a deletion of *lpg2* gene, but not *lpg1*, abolished the phospho-glycosylation of SAcPs in *L. donovani* (165). Immunoprecipitation of SAcP suggested that the glycosylation pattern might increase during the parasite differentiation into amastigotes (11, 162). In addition to this posttranslational modification, SAcPs can form filamentous polymers. This quaternary structure has been extensively described for *L. mexicana*, but, surprisingly, was not seen with SAcPs from *L. donovani*, which remained mono- or oligomeric and non-filamentous (166). In case of *L. mexicana*, the filament formation occurred in the flagellar pocket of promastigotes (167) to create homo- or hetero-polymers of two phosphatases, LmSAP1 and LmSAP2. The genes encoding these two SAcPs are tandemly arranged and nearly identical, only differing by the addition of a long stretch of serine-threonine repetitions after the catalytic domain of LmSAP2 (168). This stretch is the site of intense glycosylation but does not interfere with the formation of the filament backbone constructed around the catalytic domains of LmSAPs (169). Remarkably, the published sequences of LmSAPs derived from *L. mexicana* MNYC/BZ/62/M379 could not be fully retrieved from the *L. mexicana* MHOM/GT/2001/U1103 strain that was sequenced, illustrating again the high plasticity of *Leishmania* genomes. An ortholog of the LmSAPs, *LmxM.36.6480*, has even a higher number of serine-threonine repetitions and seems to lack a functional signal peptide. In general, our search of the different *Leishmania* genomes showed that all SAcPs share a similar architecture but the size of the serine/threonine rich domain varies. This probably reflects the need to adapt the level of phospho-glycosylation to the different environments required by each parasite species.

Comparative genetic analysis of two different isolates of *L. major* from patients with contrasting severity of disease indicated that the hypo-virulent strain carried a mutated version of SAcP (170), suggesting a role of SAcP in virulence. Interestingly, a similar study looking at the genetic differences between two

strains of *L. donovani* isolated from patients with cutaneous or visceral leishmaniasis, respectively, did not report a link between *Leishmania* AcPs and virulence (171). The function of SAcP in the virulence *Leishmania* has been directly addressed with parasites lacking the genomic locus encoding the two LmSAPs. Deletion of this locus abolished the virulence of *L. mexicana* promastigotes as they failed to transform into amastigotes during macrophage infection (172). However, this phenotype was independent of the LmSAPs because phenotypic complementation was achieved by adding back the MAPK gene that was identified in the intergenic region between the two LmSAP genes (172).

Together, these results demonstrate that the exact contribution of MAcPs and SAcPs to maintain the life cycle of *Leishmania* parasites is still unknown. First, the lack of a phenotype in a *Leishmania* mutant deficient for an AcP does not exclude its functional relevance, because compensation between different genes of each group could occur. Second, MAcP could also compensate for SAcP deficiency and *vice versa*. Finally, a role for these phosphatases also needs to be considered during sand fly infection by promastigotes. To experimentally address these hypotheses, the nature of the AcP substrate needs to be defined. If, however, these enzymes are primarily responsible for providing sufficient inorganic phosphate to the parasite as it has been proposed by Freitas-Mesquita et al. (140), it will be difficult to dissect the functional role of the different classes of AcPs and/or to overcome compensatory mechanisms.

Leishmania Phosphatase LmPRL-1 As a Secreted Virulence Factor

Recently, our laboratory has characterized a new dual specificity phosphatase, LmPRL-1, secreted by *L. major* during macrophage infection (118). The LmPRL-1 gene, *LmjF.16.0230*, is organized in tandem with a paralogous gene, *LmjF.16.0250*, which codes for LmPRL-2. Both PTPs represent the only two members of the family of the *phosphatase of the regenerating liver* (*PRL*) within *L. major* (**Table 2**). Human PRLs are well studied and are thought to participate in the control of various processes such as cell proliferation, differentiation and motility (173). Remarkably, both LmPRLs are highly conserved in all sequenced *Leishmania* species indicating that they might play a crucial role in the life of the parasite.

Structurally, we verified that *Leishmania* PRL-1, like its mammalian homolog, has two important characteristics. First, its cysteine-based catalytic site was shown to be under the control of a neighboring regulatory cysteine. Under oxidative conditions, this cysteine formed a transient inactivating disulfide bridge with the catalytic cysteine. Second, a third cysteine at the C-terminus could be farnesylated. This posttranslational modification controlled the correct subcellular localization inside promastigotes. During metacyclogenesis, LmPRL-1 was constantly expressed by promastigotes. Its level tended to decrease after amastigotes transformation in macrophages and also showed a weaker expression in amastigotes isolated from footpad lesions of *L. major*-infected BALB/c mice. Strikingly, during the first days of macrophage infection LmPRL-1 reached the cytoplasm of its host cells through the exosome route. There, LmPRL-1 appeared to support the intracellular replication of the parasite in primary mouse macrophages (118). We currently do not know the molecular mechanism by which LmPRL-1 promotes *L. major* virulence. One possibility is that LmPRL-1 has an intrinsic regulatory effect on parasite development and/or transformation. As secreted LmPRL-1 is detectable in host cell cytosol, it is also plausible to assume that LmPRL-1 modulates host cell signaling pathways by targeting phosphoproteins, interfering with the normal function of host PRLs, or altering the activity of other secreted virulence factors (**Figure 5**). We currently address these possible mechanisms in studies with LmPRL-1-deficient *L. major* parasites.

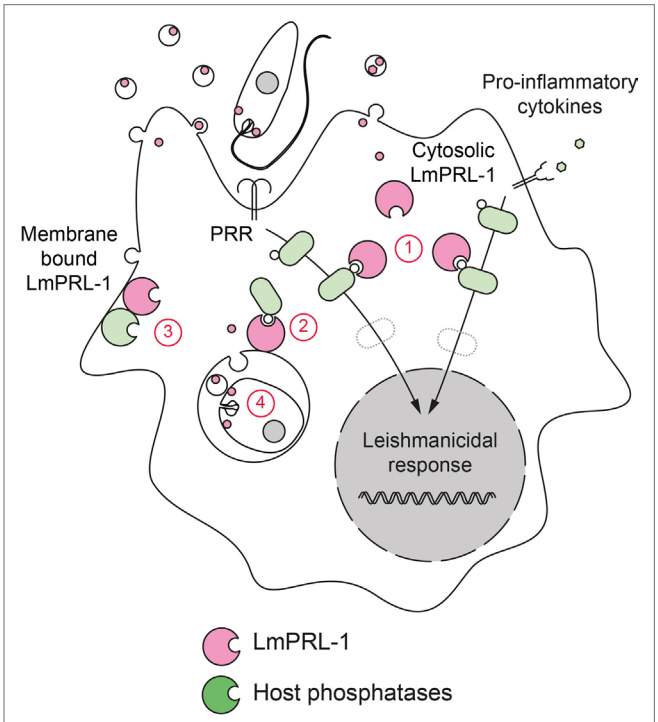

FIGURE 5 | Hypotheses on the virulence mechanism of LmPRL-1. The phosphatase LmPRL-1 is secreted by *Leishmania major* promastigotes and amastigotes during macrophages infection. Once secreted, LmPRL-1 is localized at the membrane of the parasitophorous vacuole (PV) and/or in the cytosol of the infected macrophage. The observed support of intracellular parasite survival by LmPRL-1 could result from an inhibitory effect on important signaling pathways that otherwise promote the microbicidal response of the host macrophage (1). Other pathways not directly related to the innate immune response, but contributing to the general fitness of the macrophage and its function as host cell niche for the parasite could also be targeted by LmPRL-1 (2). Putative targets of LmPRL-1 are the host PRL proteins. Mammalian PRL activity relies on the formation of PRL trimers. LmPRL-1 might modulate the activity and function of its mammalian orthologs by physically interacting with them (3). Finally, a parasite-autonomous, intrinsic prosurvival effect of LmPRL-1 also needs to be envisaged (4).

CONCLUDING REMARKS

Host–pathogen interactions taking place during leishmaniasis rely on complex antagonistic and synergistic molecular interplays some of which we have summarized in this review.

Leishmania parasite survival and disease development depend on the fitness of the host immune reaction. To counteract the generation of a microbicidal host response, *Leishmania* parasites have developed efficient strategies to extinguish or impede the proinflammatory signaling pathways triggered by their detection. Proteases secreted by the parasite, such GP63 and LmCPb, represent important components to achieve this goal and finally escape the deadly production of leishmanicidal compounds. Among the targets of these virulence factors, host protein phosphatases, especially SHP-1, are of particular relevance. Once proteolytically activated, these host phosphatases can directly inhibit the signaling pathways that are required for an effective anti-*Leishmania* host response (**Figure 3**).

While the parasite-driven activation of host phosphatases appears to be the *modus operandi* of choice for *Leishmania* to impede host immune responses, an additional strategy has been postulated. Already in the 1980s, the discovery of SAcP activities suggested that *Leishmania* parasites could use its own secreted enzymes to manipulate its host. However, until now a link between SAcP and *Leishmania* virulence has not yet been ascertained, probably because of the redundancy between the different orthologous gene encoded by each *Leishmania* genome. Only recently, our study of the *Leishmania* dual specificity phosphatase of the PRL family, LmPRL-1, provided the first evidence for a phosphatase secreted by *Leishmania* that promotes the virulence of the parasite during macrophage infection (**Figure 5**). As LmPRL-1 is secreted within exosomes, it is accompanied by a miscellaneous pool of proteins and small RNAs (174). Among these exosomal proteins, five other phosphatases have been detected that require further characterization with respect to their possible role as virulence factors of the parasite and their participation in the modulation of phosphorylation events in host cells.

In the past, the mechanism of activation of host phosphatases by *Leishmania* has been extensively studied by several research groups and thereby led to the identification of some of the signaling pathways that are targeted by the parasite. The efforts should now focus on the biochemical and functional characterization of new *Leishmania* virulence factors that directly interfere with phosphorylation events in host cells. In addition, our knowledge of host and *Leishmania* phosphatases that was mainly acquired in mouse models, still requires to be tested in clinical settings in order to establish whether differential expression of *Leishmania* phosphatases contributes to the severe courses of infection seen in mucocutaneous or visceral leishmaniasis.

AUTHOR CONTRIBUTIONS

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01838/full#supplementary-material.

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Nivolumab Enhances *In Vitro* Effector Functions of PD-1$^+$ T-Lymphocytes and *Leishmania*-Infected Human Myeloid Cells in a Host Cell-Dependent Manner

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Functional impairment of T-cells and a concomitant augmented expression of programmed death-1 (PD-1) have been observed in visceral leishmaniasis patients, as well as in experimental models for visceral and cutaneous leishmaniasis. The PD-1/PD-1-ligand (PD-1/PD-L) interaction negatively regulates T-cell effector functions, which are required for parasite control during leishmaniasis. The aim of this study was to elucidate the impact of the PD-1/PD-L axis in a human primary *in vitro* infection model of *Leishmania major* (*Lm*). Blocking the PD-1/PD-L interaction with nivolumab increased T-cell proliferation and release of the proinflammatory cytokines TNFα and IFNγ during the cocultivation of *Lm*-infected human monocyte-derived macrophages (hMDMs) or dendritic cells (hMDDC) with autologous PD-1$^+$-lymphocytes. As a consequence *Lm* infection decreased, being the most pronounced in hMDDC, compared to proinflammatory hMDM1 and anti-inflammatory hMDM2. Focusing on hMDDC, we could partially reverse effects mediated by PD-1 blockade by neutralizing TNFα but not by neutralizing IFNγ. Furthermore, PD-1 blockade increased intracellular expression of perforin, granulysin, and granzymes in proliferating CD4$^+$-T-cells, which might be implicated in reduction of *Lm*-infected cells. In all, our data describe an important role for the PD-1/PD-L axis upon *Lm* infection using a human primary cell system. These data contribute to a better understanding of the PD-1-induced T-cell impairment during disease and its influence on immune effector mechanisms to combat *Lm* infection.

Keywords: *Leishmania*, programmed death-1, programmed death-1 ligand 1, programmed death-1 ligand 2, nivolumab, human macrophages, human dendritic cells, T-cells

INTRODUCTION

The parasitic disease leishmaniasis is still endemic in 97 countries, causing up to 30,000 deaths annually, a number potentially increasing due to climate changes and global warming (1). A prerequisite for controlling *Leishmania* infection is a strong adaptive immune response. Based on experimental mouse models, it is widely accepted that disease susceptibility is associated with IL-10 and IL-4 producing T-helper 2 (T_H2) cells, whereas a strong T-helper 1(T_H1)-mediated IFNγ production promotes healing by inducing leishmanicidal nitric oxide in the *Leishmania*-harboring

cells (2). In human leishmaniasis this T_H1/T_H2 dichotomy does not always hold true and the resulting T-cell response strongly depends on the *Leishmania* strain and the immune status of the host (3–6). In addition, *in vitro* data from cutaneous Leishmaniasis patients show parasite control to be mediated rather by IFNγ-induced reactive oxygen species (ROS) then by nitric oxide (7, 8). Macrophages and dendritic cells, the final host cells of *Leishmania* parasites, play an important role in the initiation of the adaptive immune response. Several *in vitro* studies demonstrated *Leishmania*-naive healthy human donors to possess a natural T-cell response against live parasites, antigen extracts or specific components of different *Leishmania* strains (9–16). This early MHC class II dependent T-cell response was shown to dampen *Leishmania* parasite burden in autologous human macrophage/T-cell cocultures (11). The activation of $CD8^+$- and $CD4^+$-T-cells is regulated by various signals such as costimulatory molecules, which can either positively or negatively influence T-cell priming.

The coinhibitory receptor programmed death-1 (PD-1, CD279), which is a member of the B7-CD28 family, is expressed on activated T-cells and B-cells. Upon association with its ligands PD-L1 (CD274) or PD-L2 (CD273), which are expressed on, e.g., macrophages and dendritic cells, T-cell activation is suppressed by inhibition of CD28 signaling (17). The role of the PD-1/PD-L axis in T-cell exhaustion, a functional impairment of T-cells, is very well studied in the field of cancer and in chronic infections such as HIV, HCV, or lymphocytic choriomeningitis virus (LCMV) (18–20). Recent publications indicate that the PD-1/PD-L pathway may play a similar role in *Leishmania* infection (21–24). In the canine and mouse model of visceral leishmaniasis, PD-1/PD-L-mediated T-cell exhaustion together with an impaired phagocyte function was observed. Blocking the PD-1/PD-L interaction in these models partially rescued effector functions of exhausted T-cells, which resulted in a lower parasite burden (21, 23). In splenic aspirates of visceral leishmaniasis patients an anergic/exhausted $CD8^+$ T-cell phenotype plus an augmented expression of PD-1 was found (24). Nevertheless, functional data regarding the involvement of the PD-1/PD-L axis in human leishmaniasis is scarce.

In this study, we aimed to define a role for the PD-1/PD-L axis during *Leishmania* infection of human primary myeloid and lymphoid cells. By using a newly established autologous *in vitro* model consisting of functionally impaired $PD\text{-}1^+$-T-lymphocytes, three potential *Leishmania major* (*Lm*) host cell types and the cancer therapeutic anti-PD-1 antibody nivolumab, we could demonstrate that PD-1 blockade reinvigorated T-cell effector functions. Depending on the type of parasitized human primary myeloid cell, the magnitude of T-cell-mediated parasite elimination varied. Focusing on dendritic cells, we found PD-1 blockade-mediated effects to be partly TNFα dependent. Furthermore, PD-1 blockade enhanced almost exclusively *Lm*-induced proliferation of $CD4^+$ and not $CD8^+$ T-cells. Moreover, an increased expression of cytolytic T-cell effector molecules was detected, which are likely to be implicated in reduced parasite survival.

In all, our study gives insight into the role of the PD-1/PD-L axis during *Leishmania* infection of primary human cells. This information may be useful for the development of immunotherapeutic strategies targeting leishmaniasis.

MATERIALS AND METHODS

Lm Parasites

Leishmania major (MHOM/IL/81/FEBNI) wild-type and transgenic parasites (dsRED) were cultured as described (11, 25, 26).

Human Peripheral Blood Mononuclear Cells (PBMCs)

Human PBMCs were isolated from buffy coats (DRK-Blutspendedienst Hessen GmbH, 506838) from blood donations by healthy German adults without known exposure to *Leishmania* parasites. PBMC isolation was performed as described previously (11). Up to 96–99% pure monocytes (Impurities: 1–4% lymphocytes) were isolated by $CD14^+$ MACS selection (Miltenyi, 130-050-201). By the use of different cytokines, monocytes were differentiated in complete medium (CM; RPMI1640, 10% FCS, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM HEPES) into pro-inflammatory human monocyte-derived macrophages type 1 (hMDM1) (10 ng/mL human GM-CSF; Leukine®, sargramostim, Bayer HealthCare), anti-inflammatory human monocyte-derived macrophages type 2 (hMDM2) (30 ng/mL human M-CSF; R&D Systems), or human monocyte-derived dendritic cells (hMDDC) (5 ng/mL GM-CSF; 10 ng/mL human IL-4, Gibco®, PHC0045) for a period of 5 days at 37°C, 5% CO_2 as described (27). $CD14^-$ cells or peripheral blood lymphocytes (PBLs), respectively, were seeded in six-well plates (1×10^6 cells/mL) and stimulated with 0.5 µg/mL phytohemagglutinin (PHA) (Oxoid, R30852801) in CM for 6 days.

Infection of Human Primary Macrophages or Dendritic Cells

Human monocyte-derived macrophages or dendritic cells were detached, counted and seeded in 1.5 or 2 mL microcentrifuge tubes. For infection, stationary-phase *Lm* promastigotes (wild-type or dsRED parasites) were added at a multiplicity of infection (MOI) of 10. After 24 h of incubation at 37°C, 5% CO_2, extracellular parasites were removed by centrifugation of microcentrifuge tubes and washing steps with CM. (Non-) infected hMDM/hMDDC were analyzed by flow cytometry or used in the CFSE-based proliferation assays (see below).

CFSE-Based Proliferation Assay

The hMDM or hMDDC, which still contain 1–4% lymphocytes, were stained prior to *Lm* infection, using 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (Sigma, C1157) as described previously (11). PHA-stimulated autologous PBLs ($PBLs^{PHA}$) were labeled with CFSE and coincubated with the (non-) infected CFSE-labeled hMDM/hMDDC, at a ratio of 5:1. The anti-PD-1 fully human IgG4 (nivolumab, Opdivo®, Bristol-Myers Squibb) was used for PD-1 blocking experiments at a final concentration of 0.625 µg/mL. For neutralization of

cytokines 20 µg/mL anti-IFNγ (clone B27, Biolegend®), 20 µg/mL anti-TNFα (infliximab, Remsima®, Mundipharma) or 20 µg/mL Isotype Control (MOPC-21, Biolegend®, data not shown) were used. After 5 days of coculture at 37°C, 5% CO_2, supernatants were frozen at −80°C and cells were collected. After immunostaining, proliferation ($CFSE^{low}$-T-cells) and infection (*Lm* dsRED$^+$ hMDM or hMDDC) were analyzed by flow cytometry. $CD4^+$ and $CD8^+$ $PBLs^{PHA}$ were separated from autologous $PBLs^{PHA}$ using $CD4^+$ and $CD8^+$ MACS Isolation (130-045-101, 130-045-201, Miltenyi) before the cocultivation step.

Flow Cytometry

For flow cytometric analysis of human primary cells, at least 0.3×10^6 cells were labeled with fluorescently labeled antibodies (Table S1 in Supplementary Material) and corresponding isotype controls as defined by the manufacturer. Intracellular proteins were labeled by prior fixation (4% paraformaldehyde) and permeabilization (0.5% saponin) of cells. Intranuclear transcription factors Tbet and GATA3 were labeled by using eBioscience™ Foxp3/Transcription Factor Staining Buffer Set. To label specifically nivolumab, a goat antihuman IgG-Fc polyclonal Fab_2 R-PE (Dianova; 109-116-098) was used. Upon analyzing, at least 10,000 events (human cells) were recorded using a BD LSR II flow cytometer (BD Bioscience, Heidelberg). Data were analyzed by FlowJo software (Treestar).

ELISA

TNFα and IFNγ levels were analyzed in the supernatants by using Human TNF-alpha DuoSet ELISA (DY008) or Human IFN-gamma DuoSet ELISA (DY285) from R&D systems according to the manufacturer's protocol plus a TECAN® Infinite F50® microplate reader.

Statistical Analysis

Samples were tested for normal Gaussian distribution using D'Agostino-Pearson omnibus normality test. In case of normally distributed paired samples a parametric paired *t*-test was performed. Otherwise, the Wilcoxon signed-rank test was used. All calculations were done using Graph-Pad Prism version 7. A value of $P < 0.05$ was considered statistically significant. Pearson's *r* correlations were also calculated by Graph-Pad Prism version 7. $r \geq 0.7$ indicates a positive correlation; $r \leq -0.7$ indicates an inverse correlation.

RESULTS

PD-1 Ligand Cell Surface Expression on Macrophages and Dendritic Cells Is Differently Modulated by *Leishmania* Infection

To assess whether PD-1/PD-Ligand (PD-1/PD-L) interactions can influence *Lm* infection of human myeloid cells, we first determined cell surface expression of PD-L1 and PD-L2 after *Lm* infection. For this purpose, we generated $CD14^+CD163^-$ proinflammatory macrophages (hMDM1), $CD14^+CD163^+$ anti-inflammatory macrophages (hMDM2), or $CD1a^+CD14^-$ dendritic cells (hMDDC) as described previously (27). We analyzed all three host cell types, as their individual roles in human *Lm* disease are insufficiently clarified. The cell surface density of both PD-L1 and PD-L2 was quantified by flow cytometry. Therefore, the relative fluorescence intensity (RFI) was measured as the ratio of the mean fluorescence intensity of specific markers to the mean fluorescence intensity of isotype controls. Low levels of PD-L1 were detected on hMDM1 (RFI: 1.42 ± 0.28) and hMDM2 (RFI: 1.21 ± 0.23). After *Lm* infection, expression of both ligands significantly increased (hMDM1: RFI: 2.01 ± 1.09 and hMDM2: RFI: 1.78 ± 0.41, respectively) (**Figure 1A**). Interestingly, basal surface expression of PD-L1 on hMDDC (RFI: 2.13 ± 0.37) was higher compared to hMDM1 or hMDM2, which however did not increase upon *Lm* infection (RFI: 2.33 ± 0.36) (**Figure 1A**). Focusing on PD-L2 expression, also a low basal surface expression was observed on hMDM1 (RFI: 1.91 ± 0.88), hMDM2 (RFI: 1.90 ± 0.74), and hMDDC (RFI: 3.27 ± 2.36), respectively. During *Lm* infection, PD-L2 surface expression significantly increased on hMDM1 (RFI: 2.78 ± 1.44) (**Figure 1A**). However, surface expression levels of PD-L2 on hMDM2 and hMDDC did not differ in presence or absence of *Lm* infection. Taken together, we show that PD-L1 and PD-L2 are expressed on all three host cell types and their expression is partially upregulated by *Lm* infection, which is a prerequisite to modulate the PD-1/PD-L axis by anti-PD-1 blockade.

PHA Treatment Mimics T-Cell Exhaustion As Determined by Surface Expression of Various Marker Proteins

In the chronic LCMV mouse model, where T-cell exhaustion was initially defined, persistent antigen stimulation leads to T-cell exhaustion, which is characterized by a stepwise upregulation of several inhibitory molecules like PD-1, PD-L1, 2B4, lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and CD160 (28, 29). To mimic an exhausted phenotype, we stimulated PBLs with PHA for 6 days. In contrast to CD3/CD28 stimulation, PHA stimulation has been reported to lead to an expansion of functionally impaired T-cells (30). After 6 days of PHA stimulation, we observed that most of the B-cells and NK-cells were overgrown by T-cells shown by the significant increase of CD3 positivity (96.3 ± 1.58%), compared to the unstimulated control (80.9 ± 5.59%) (**Figure 1B**). To assure that the T-cells were not functionally impaired by senescence, characterized by downregulation or loss of CD28, we additionally analyzed expression of this costimulatory molecule. CD28 expression tended to be higher in PHA-stimulated T-cells (RFI: 15.65 ± 10.06) compared to the untreated control (RFI: 12.7 ± 5.81) (**Figure 1B**). Therefore, we excluded T-cell senescence. Next, we investigated surface expression of several T-cell exhaustion markers on T-cells of PHA-prestimulated PBLs ($PBLs^{PHA}$). Compared to the unstimulated control, PHA-prestimulated T-cells expressed the coinhibitory molecules PD-1 (RFI: 1.87 ± 0.55; 7.41 ± 2.50), PD-L1 (RFI: 4.09 ± 1.42; 7.52 ± 2.18), 2B4 (RFI: 4.27 ± 1.71; 9.70 ± 3.71), LAG-3 (RFI: 1.66 ± 0.34; 2.95 ± 0.35), and TIM-3 (RFI: 1.47 ± 0.16; 28.73 ± 18.65) to a significantly higher degree

(**Figure 1B**). CD160 expression was not affected by PHA stimulation (RFI: 1.17 ± 0.07; 1.45 ± 0.56) (**Figure 1B**). Due to the high expression of several inhibitory molecules, we considered T-cells of PBLs^{PHA} to resemble exhausted T-cells. Thus, we subsequently used PBLs^{PHA} (containing PD-1$^+$ T-cells) in autologous cocultures with the three *Lm* host cell types (PD-L1$^+$ and PD-L2$^+$) to investigate how T-cell effector functions are affected by nivolumab treatment.

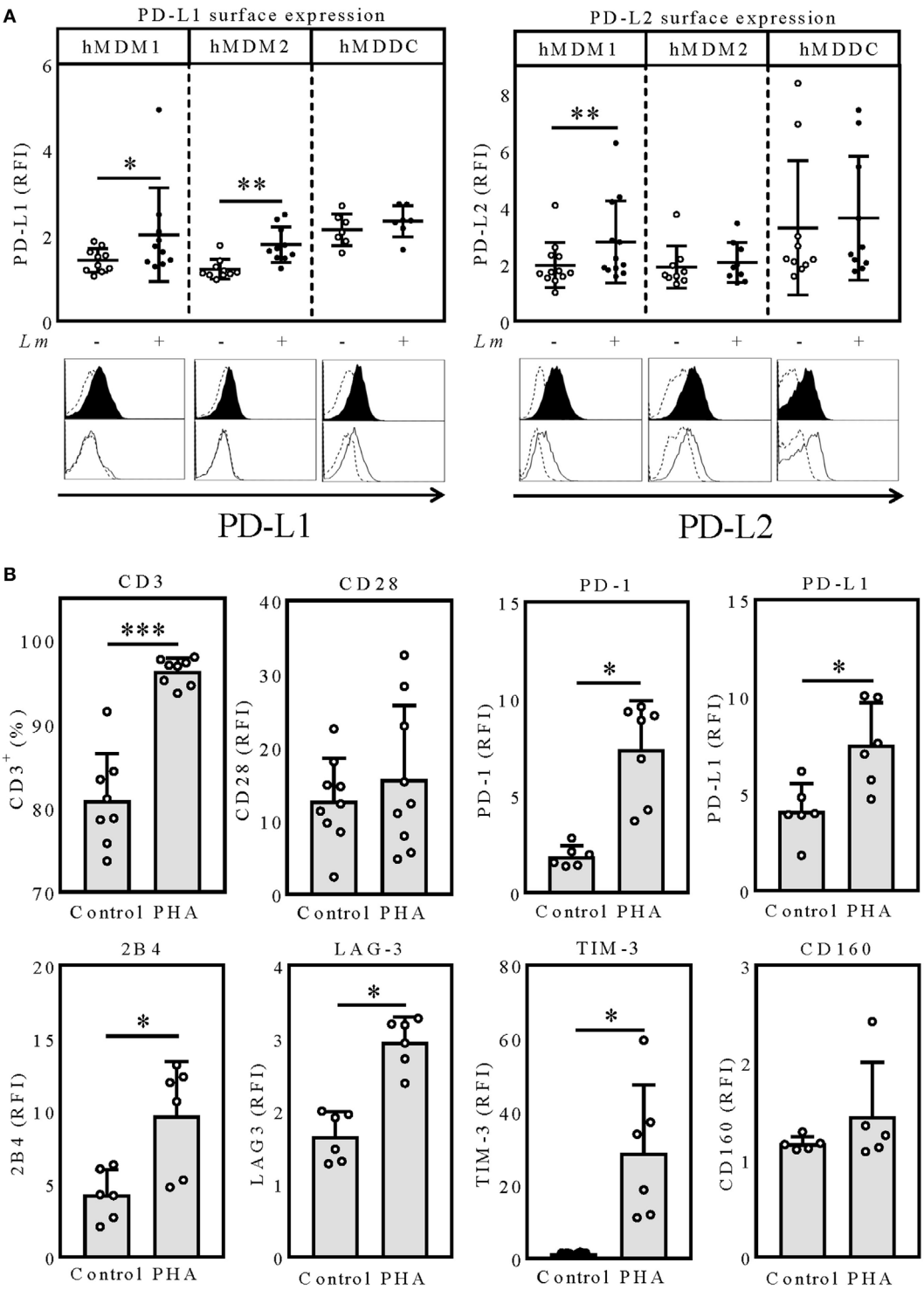

FIGURE 1 | Continued

FIGURE 1 | PD-L1/PD-L2 surface expression on hMDM and hMDDC after *Lm* infection. **(A)** hMDM1, hMDM2, or hMDDC were incubated with/without *Lm* (MOI 10) for 24 h and expression levels of PD-L1 or PD-L2 were determined by flow cytometry, respectively. hMDM and hMDDCs were gated using their FSC/SSC properties. Data are presented as mean ± SD RFI (ratio of the mean fluorescence intensity of specific markers to the mean fluorescence intensity of isotype controls). Representative histograms for a surface marker staining of PD-L1 and PD-L2 on infected (black filled histogram) or non-infected (black non-filled histogram) hMDMs and hMDDCs, compared to the respective isotype (black dashed line) control. Statistics were calculated by Wilcoxon matched-pairs signed rank test, $P < 0.05$ is considered statistically significant (*$P < 0.05$; **$P < 0.01$). At least three independent experiments were performed ($n = 7–9$). Expression of exhaustion marker after PHA-stimulation of PBLs. **(B)** PBLs were incubated for 6 days with/without PHA (0.5 µg/mL) and expression levels of the indicated surface molecules were determined *via* flow cytometry. T-cells were gated *via* their FSC/SSC properties and CD3. Data are presented as RFI (ratio of the mean fluorescence intensity of specific markers to the mean fluorescence intensity of isotype controls) or percentage of $CD3^+$ cells. Statistics were calculated using the parametric paired *t*-test (CD3, CD28) or Wilcoxon matched-pairs signed rank test; $P < 0.05$ is considered statistically significant (*$P < 0.05$; **$P < 0.01$). At least three independent experiments were performed ($N = 6–9$). hMDM1, proinflammatory human monocyte-derived macrophages type 1; hMDM2, anti-inflammatory human monocyte-derived macrophages type 2; hMDDC, human monocyte-derived dendritic cells; *Lm*, *Leishmania major*; PD-L1, programmed death-1 ligand 1; PD-L2, programmed death-1 ligand 2; MFI, mean fluorescence intensity; PHA, phytohemagglutinin; PBL, peripheral blood lymphocytes; LAG-3, lymphocyte-activation gene 3; TIM-3, T-cell immunoglobulin and mucin-domain containing-3.

PD-1/PD-L Blockade-Induced T-Cell Proliferation and *Lm* Infection Rate Are Dependent on the Initial Host Cell Phenotype

First, we addressed, whether T-cell effector functions of $PBLs^{PHA}$ can be reinvigorated by the therapeutic anti-PD-1 antibody nivolumab and whether this is influenced by the type of antigen presenting cell. Therefore, we compared hMDM1, hMDM2 and hMDDC, respectively, as *Lm* antigen presenting cells in cocultures with autologous $PBLs^{PHA}$. Cocultivation of *Lm*-infected hMDM1 with $PBLs^{PHA}$ significantly increased T-cell proliferation (12.32 ± 9.49%), compared to the uninfected control (4.63 ± 3.33%) (**Figure 2A**). This indicates that PHA-prestimulated T-cells have residual effector functions. Furthermore, by blocking PD-1, T-cell proliferation (25.96 ± 15.07%) was significantly enhanced (**Figure 2A**). Concomitantly, we observed a significant reduction in *Lm* infection in the sample cocultivated with PBL^{PHA} (44.62 ± 6.87%) compared to infected hMDM1 only (69.66% ± 5.07%), which consequently was reduced to a stronger extent upon PD-1 blockade (39.47 ± 9.48%) (**Figure 2A**). In cocultures of *Lm*-infected hMDM2 with autologous PBL^{PHA}, no significant difference in T-cell proliferation was observed (3.84 ± 1.74%) compared to the uninfected control (3.21 ± 2.05%) (**Figure 2B**). However, PD-1 blockade enhanced T-cell proliferation significantly in the infected sample (9.71 ± 5.13%) (**Figure 2B**). Consistently, *Lm* infection rate of hMDM2 in the presence of $PBLs^{PHA}$ was not decreased significantly (49.33 ± 10.90%) compared to infected hMDM2 only (63.49 ± 23.31%). Surprisingly, no significant effect on *Lm* infection could be detected when PD-1 was blocked (45.99 ± 8.94%), even though T-cell proliferation was increased (**Figure 2B**). Compared to hMDM1 and hMDM2 *Lm*-infected hMDDC induced the highest T-cell proliferation in the $PBLs^{PHA}$ coculture (17.71 ± 10.73%) and, after PD-1 blockade, enhanced T-cell proliferation the most (49.30 ± 13.81%) (**Figure 2C**). Consequently, less *Lm*-infected hMDDCs were observed in the presence of $PBLs^{PHA}$ (42.02 ± 9.17%) and PD-1 blockade dampened the infection rate of hMDDCs the most (24.68 ± 8.15%), compared to hMDM1 and hMDM2 (**Figure 2C**). PD-1 blockade in the absence of $PBLs^{PHA}$ did not influence *Lm* infection rate in hMDM1, hMDM2 or hMDDC (Figure S1 in Supplementary Material). Furthermore, we showed that PD-1 is blocked on T-cells throughout the whole coculture experiment, indicating that the used amount of nivolumab is sufficient (Figure S2 in Supplementary Material). Our data demonstrate that T-cell function is enhanced by PD-1 blockade, however, the extent of the effects strongly depends on the *Lm* host cell phenotype.

TNFα and IFNγ Release Is Strongly Enhanced after PD-1 Blockade in the *Lm*-Infected hMDDC Coculture

The proinflammatory cytokines TNFα and IFNγ play a critical role in shaping the immune response against *Leishmania* infection (31). Thus, we analyzed TNFα and IFNγ levels in the coculture supernatants by ELISA. PD-1 blockade significantly increased TNFα levels in the infected hMDM1 samples (216.60 ± 114.90 pg/mL; 88.57 ± 52.52 pg/mL) (**Figure 2D**). Also IFNγ release was augmented by PD-1 blockade in the infected hMDM1 samples (80.61 ± 59.73 pg/mL; 16.95 ± 25.68 pg/mL) (**Figure 2D**). TNFα levels in the hMDM2 samples were similar to the hMDM1 samples. In contrast to hMDM1, IFNγ was barely detectable in the PD-1-blocked infected hMDM2 sample (17.62 ± 24.18 pg/mL) (**Figure 2E**). Regarding *Lm*-infected hMDDC, PD-1 blockade significantly increased TNFα levels in the supernatants (374.50 ± 156.60 pg/mL; 167.20 ± 89.77 pg/mL). IFNγ levels in the supernatant of *Lm*-infected hMDDC were low (25.58 ± 25.54 pg/mL), but highly enhanced by PD-1 blockade (288.40 ± 103.20 pg/mL) (**Figure 2F**). This suggests that effects induced by PD-1 blockade may be mediated by TNFα and IFNγ. To investigate this idea, we performed a correlative analysis and neutralized both cytokines.

TNFα but Not IFNγ Neutralization Partially Reversed Effects of Nivolumab Treatment

To assess whether cytokine release correlates with T-cell proliferation or *Lm* infection rate, we performed a correlative analysis. To this end, we focused on *Lm*-infected hMDDC, because these host cells displayed the strongest effects mediated by PD-1 blockade in the coculture experiment (**Figures 2C,F**). We only used the datasets of infected samples in presence and absence of nivolumab (**Figure 3A**). TNFα release correlated with T-cell proliferation ($r = 0.90$). TNFα release tendentially correlated

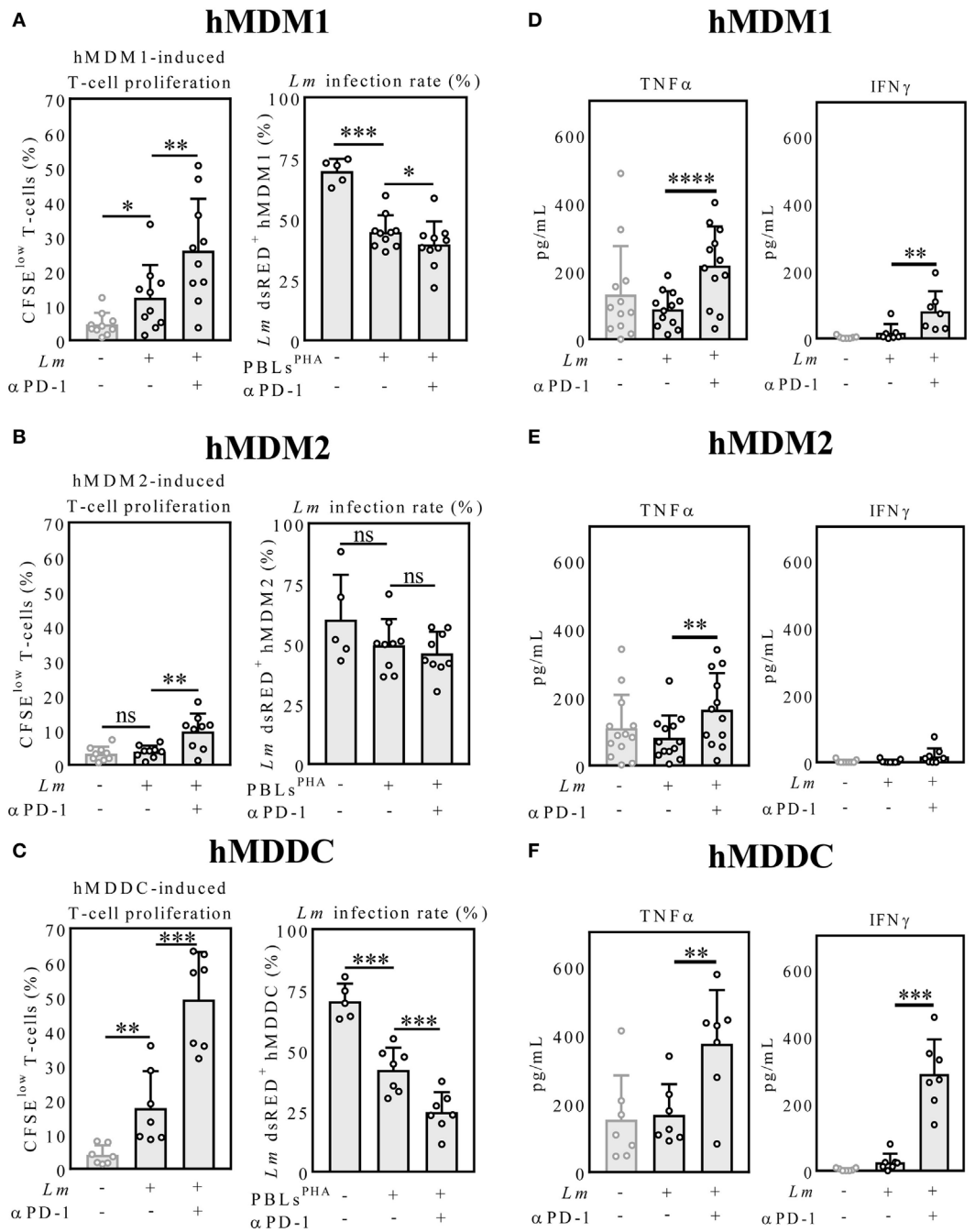

FIGURE 2 | Host cell phenotype-dependent T-cell proliferation, parasite killing and cytokine release upon PD-1 blockade. **(A–C)** CFSE-labeled autologous PBLs^{PHA} were cocultivated with *Lm*-infected host cells and blocked with nivolumab (αPD-1). After 5 days, cells were harvested and analyzed by flow cytometry (hMDM/hMDDC gated *via* their FSC/SSC properties, T-cells additionally *via* CD3). Data are presented as mean percentage of CFSE^{low} proliferating T-cells ± SD or *Lm* dsRED-infected hMDDC ± SD, respectively. **(D–F)** IFNγ and TNFα were measured in the coculture supernatants by ELISA as described. Data are presented as mean pg/mL ± SD. For statistics, a parametric paired *t*-test was performed, $P < 0.05$ is considered statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). At least three independent experiments were performed ($N = 5$–13). hMDM1, proinflammatory human monocyte-derived macrophages type 1; hMDM2: anti-inflammatory human monocyte-derived macrophages type 2; hMDDC, human monocyte-derived dendritic cells; *Lm*, *Leishmania major*; PD-1, programmed death-1; PBL, peripheral blood lymphocytes; TNFα, tumor necrosis factor α; IFNγ, interferon-γ; CFSE, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester.

inversely with infection rate ($r = -0.63$). There was a positive correlation between IFNγ release and T-cell proliferation ($r = 0.83$). Also infection rate tendentially correlated inversely with IFNγ release ($r = -0.79$). To investigate a causal connection between increased TNFα/IFNγ release and T-cell proliferation/parasite survival, we neutralized TNFα and IFNγ. Neutralization of IFNγ

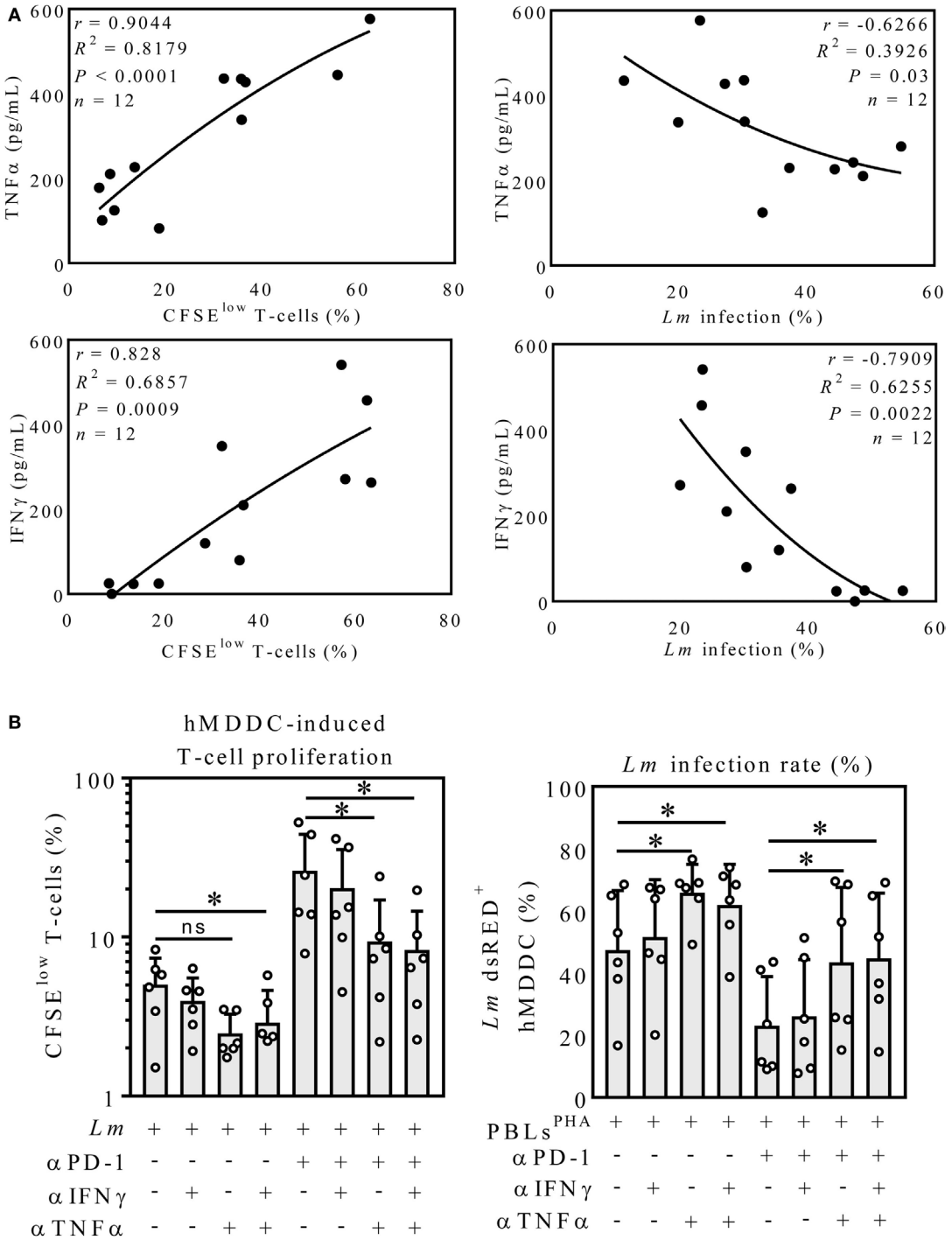

FIGURE 3 | Neutralization of TNFα but not IFNγ partially reversed PD-1 blockade-mediated effects. **(A)** TNFα- and IFNγ-release correlates with T-cell proliferation. All *Lm*-infected hMDDC samples cocultured with PBL^{PHA} in presence or absence of anti-PD-1 (αPD-1) were considered (TNFα and IFNγ: $n = 12$) and Pearson correlation coefficients (r), the coefficient of determination (R^2) plus the significance level (P) were calculated for the indicated datasets. $r \geq 0.7$ = positive correlation; $r \leq -0.7$ = inverse correlation. **(B)** CFSE-labeled autologous $PBLs^{PHA}$ were cocultivated with *Lm*-infected hMDDC, nivolumab (αPD-1), IFNγ neutralizing antibody (αIFNγ) and TNFα neutralizing antibody (αTNFα) as indicated. After 5 days, cells were harvested and analyzed by flow cytometry (hMDM/hMDDC gated *via* their FSC/SSC properties, T-cells additionally *via* CD3). Data are presented as mean percentage of $CFSE^{low}$ proliferating T-cells ± SD or *Lm* dsRED-infected hMDDC ± SD, respectively. For statistics, a parametric paired *t*-test was performed, $P < 0.05$ is considered statistically significant, $^*P < 0.05$, $^{**}P < 0.01$. At least three independent experiments were performed ($N = 5$–6). hMDDC, human monocyte-derived dendritic cells; *Lm*, *Leishmania major*; PD-1, programmed death-1; PBL, peripheral blood lymphocytes; TNFα, tumor necrosis factor α; IFNγ, interferon-γ; CFSE, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester.

in the infected sample had no significant effect on T-cell proliferation (**Figure 3B**). Neutralization of TNFα tendentially reduced T-cell proliferation compared to *Lm* infection only (2.48 ± 0.80%; 5.02 ± 2.36%). Simultaneous neutralization of both cytokines reduced T-cell proliferation significantly (2.89 ± 1.74%) compared to the untreated control. PD-1 blockade increased T-cell proliferation (26.18 ± 18.11%). Neutralization of IFNγ did not decrease PD-1 blockade-induced T-cell proliferation (20.29 ± 15.08%), whereas anti-TNFα treatment had a significant reducing effect (9.37 ± 7.72%). Simultaneous neutralization of both cytokines did not enhance this effect (8.30 ± 6.25%). In the absence of anti-PD-1 antibody, *Lm* infection rate (47.42 ± 19.11%) was not increased in the IFNγ neutralized sample (51.55 ± 18.48%). Interestingly, TNFα neutralization highly increased parasite survival (65.85 ± 9.31%), which was not further enhanced by additional neutralization of IFNγ (61.87 ± 13.05%). Infection rate in hMDDCs decreased in the presence of anti-PD-1 (23.12 ± 15.78%). Neutralizing single or both cytokines revealed the same effect, as observed for the samples without nivolumab. Again, only TNFα neutralization significantly increased parasite survival (43.40 ± 24.17%) (**Figure 3B**). Blocking with an isotype control did not alter proliferation or parasite survival (data not shown). Even though the correlative analysis pointed toward a role for IFNγ in T-cell proliferation and parasite killing, only neutralization of TNFα partially reversed PD-1 blockade-mediated effects. On the other hand, TNFα neutralization already improved parasite survival in the absence of PD-1 blockade, indicating that TNFα might act independently of PD-1.

PD-1 Blockade Shifts *Lm*-Induced CD4$^+$-T-Cells toward a T_H1- or T_H1/T_H2 Phenotype

We sought to examine the T-cell response after PD-1 blockade in more detail. Therefore, we characterized the proliferating CFSE^{low} and the non-proliferating CFSE^{hi} CD4$^+$ and CD8$^+$-T-cells in the hMDDC coculture (**Figure 4A**). T-cells were composed of ~60% CD4$^+$ cells and ~40% CD8$^+$ (uninfected control); in the PD-1 blocked *Lm*-infected sample almost exclusively CD4$^+$-T-cells proliferated (33.89 ± 17.95% CFSE^{low} CD4$^+$; 8.52 ± 5.73% CFSE^{low} CD8$^+$) (**Figure 4A**). We confirmed this finding in a coculture experiment using purified CD4$^+$ PBLs^{PHA} or CD8$^+$ PBLs^{PHA} and detected T-cell proliferation only in the presence of CD4$^+$ PBLs^{PHA} (**Figure 4B**). Hence, PD-1 blockade mainly enhanced effector functions of CD4$^+$ T-cells. As mentioned earlier, in the classical mouse model of leishmaniasis the induction of a T_H1 response leads to healing, whereas induction of T_H2 cells promotes disease (2). In human leishmaniasis such a T_H1/T_H2 dichotomy is not that evident (5, 6). Thus, we examined in our model whether the T_H1-specific transcription-factor Tbet and the T_H2-specific transcription-factor GATA3 are differentially expressed upon PD-1 blockade. The representative dot-blots on the left of **Figure 4C** indicate differential intra-nuclear expression of Tbet and GATA3 in presence or absence of nivolumab in the *Lm*-infected sample. Compared to the untreated infected sample, a significantly higher number of Tbet$^+$ (13.06 ± 8.35%; 26.06 ± 9.46%) and Tbet$^+$/GATA3$^+$ (8.68 ± 9.22%; 14.41 ± 11.33%) proliferating CD4$^+$ T-cells was measured in the presence of nivolumab, whereas the percentage of GATA3$^+$ T-cells was tendentially lower (18.77 ± 16.68; 12.68 ± 10.54) (**Figure 4C**). We conclude that PD-1 blockade shifts *Lm*-induced CD4$^+$ T-cells more toward a T_H1- or T_H1/T_H2 phenotype. The higher percentage of T_H1 T-cells might be implicated in the improved parasite killing.

Intracellular Expression of Perforin, Granzymes, and Granulysin (GNLY) Is Increased in PD-1-Blocked *Lm*-Induced CD4$^+$ T-Cells

CD4$^+$ T-cells can release an extensive repertoire of cytolytic effector molecules, used either to help or to initiate apoptosis of a target cell (32). To examine, whether PD-1 blockade increases expression of such cytolytic effector molecules, we analyzed intracellular expression of perforin (PRF1), granulysin (GNLY), granzyme A (GZMA), and granzyme B (GZMB) in CFSE^{low}CD4$^+$-T-cells in the hMDDC coculture using flow cytometry (**Figure 4D**). Intracellular expression of PRF1 was significantly higher (1.13–4.26-fold increase) in the proliferating CD4$^+$-T-cells after PD-1 blockade relative to the untreated infected sample (**Figure 4D**). Like for PRF1, we measured a higher GZMA (1.53–8.17-fold increase), GZMB (1.31–4.3-fold increase) and GNLY expression (1.01–1.93-fold increase) in the infected samples when PD-1 was blocked (**Figure 4D**). To conclude, we determined intracellular PRF1, GNLY, GZMA, and GZMB to be increased in the *Lm*-induced proliferating CD4$^+$ T-cells upon PD-1 blockade. This strongly suggests that reduced parasite survival upon PD-1 blockade is mediated in part by PRF1, GZMA, GZMB, and GNLY.

DISCUSSION

The function of the coinhibitory PD-1/PD-L pathway and the phenomenon of T-cell exhaustion have been well defined in the chronic LCMV mouse model (18, 28, 33). Moreover, T-cell exhaustion was demonstrated to occur in many human diseases caused by viruses (34, 35), bacteria (36), fungi (37), cancer (38, 39) and protozoan infections including leishmaniasis (24, 40, 41). Remarkably, effector functions of exhausted T-cells can be reinvigorated by blocking the PD-1/PD-L interaction, thereby reducing LCMV loads in the chronic LCMV mouse model (18). This principle has been extended to other applications, like human cancers (42) and animal infection models (21, 23). Experimental animal models of leishmaniasis indicate that the use of PD-1/PD-L blocking antibodies might be beneficial for treatment of chronic forms of leishmaniasis (21–23).

Previously we reported that *Lm*-infected macrophages generated from monocytes of *Leishmania*-naive German blood donors induce a MHC class II-dependent T-cell response in autologous *in vitro* cocultures. This early T-cell response reduced parasite load in the infected macrophages (11, 27). PD-1/PD-L interactions are not prominent during the early T-cell priming phase, but they regulate the T-cell response during the effector phase (43). During chronic infections and cancer, PD-1/PD-L interactions play an important role in induction of T-cell exhaustion (44).

To investigate PD-1/PD-L interactions in *Lm* infection of human myeloid and lymphoid cells, we used a newly established

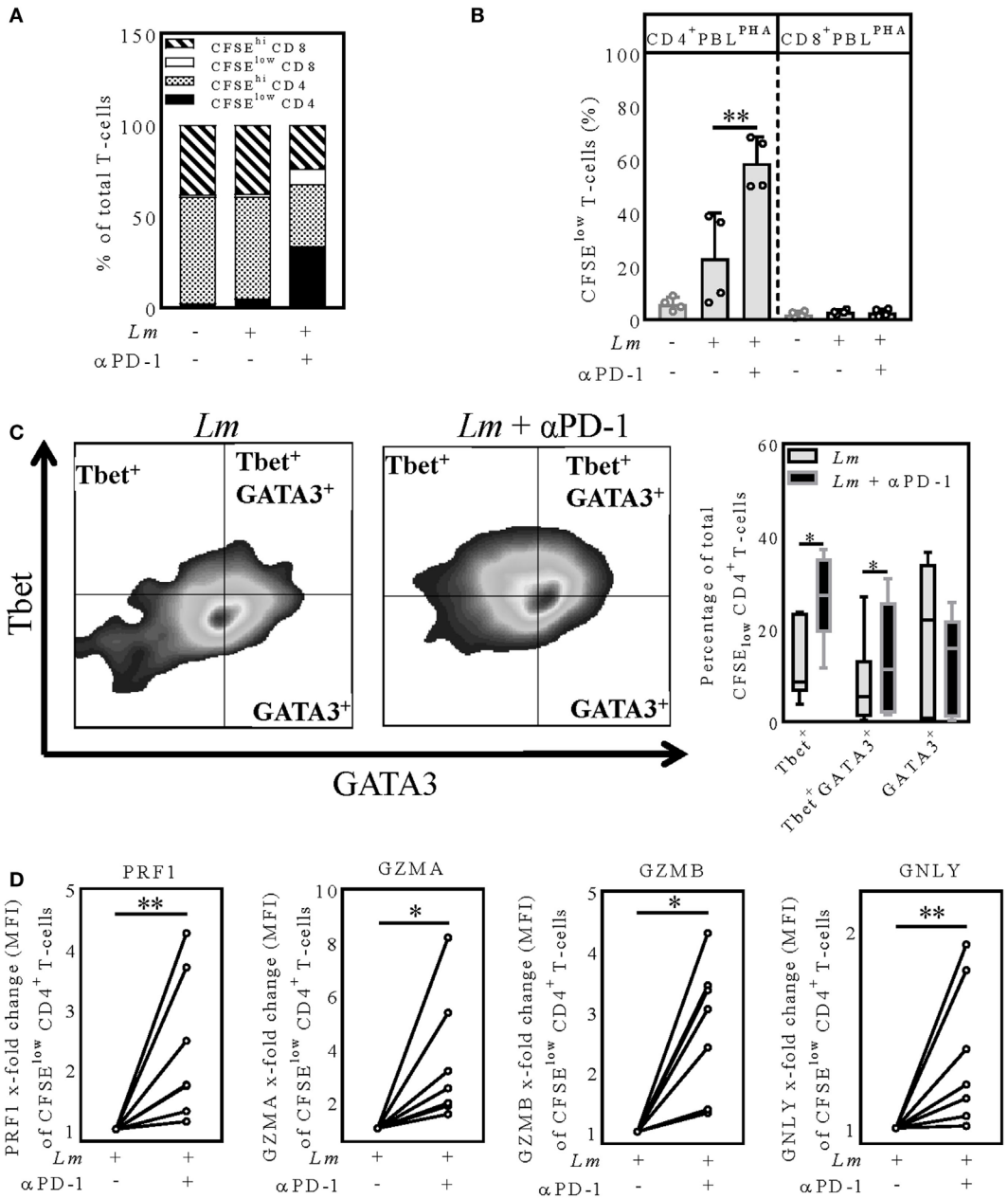

FIGURE 4 | PD-1 blockade shifts proliferating CD4+-T-cells toward a T_H1 or T_H1/T_H2 phenotype and increases expression of cytolytic effector molecules **(A,C,D)** CFSE-labeled autologous $PBLs^{PHA}$ were cocultivated with *Lm*-infected hMDDC (MOI = 10) and nivolumab (αPD-1). After 5 days, cells were harvested, immunostained **(A)** (CD3, CD4, CD8) **(C,D)** (CD3, CD4, PRF1, GZMA, GZMB, GNLY, Tbet, GATA3) and analyzed by flow cytometry. T-cells were gated *via* their FSC/SSC properties and the labeled antibodies. Data are presented as **(A)** percentage of total T-cells, **(B,C)** mean ± SD percentage or **(D)** mean ± SD *x*-fold change (MFI) relative to the untreated infected sample. At least two independent experiments were performed ($N = 4–8$). **(B)** CFSE-labeled autologous CD4+ or CD8+ $PBLs^{PHA}$ were cocultivated with *Lm*-infected hMDDC and nivolumab (αPD-1). After 5 days, cells were harvested and analyzed by flow cytometry. T-cells were gated *via* their FSC/SSC properties and CD3. At least two independent experiments were performed ($N = 4$). Statistics were calculated **(B)** using the parametric paired *t*-test or **(C,D)** the Wilcoxon matched-pairs signed rank test; $P < 0.05$ is considered statistically significant ($^*P < 0.05$; $^{**}P < 0.01$). hMDDC, human monocyte-derived dendritic cells; *Lm*, *Leishmania major*; PHA, phytohemagglutinin; PBL, peripheral blood lymphocytes; PRF1, perforin; GZMA, granzyme A; GZMB, granzyme B; GNLY, granulysin; Tbet, T-box transcription factor; GATA3, GATA-binding protein 3; CFSE, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester; MFI, mean fluorescence intensity.

in vitro model. In this model, we mimicked exhausted T-cells by stimulating PBLs with PHA prior to cocultivation with autologous *Lm*-infected host cells. PHA is a mitogen used for polyclonal activation and expansion of T-cells (45). Furthermore, it is reported to lead to functionally impaired T-lymphocytes in contrast to CD3/CD28 stimulation (30). We confirmed

PHA-stimulated T-cells express high levels of PD-1, PD-L1, LAG-3, TIM-3, and 2B4, and thus resemble exhausted T-cells.

In the current study, we found the anti-PD-1 antibody nivolumab to enhance T-cell effector functions and reduce parasite survival differently depending on the *Lm* host cell phenotype that was initially infected. Our data suggest *Lm*-infected hMDM2 not to be target of PD-1/PD-L-mediated inhibition, whereas PD-1/PD-L interactions strongly inhibit T-cell effector functions on *Lm*-infected hMDDC. A reason for this might be that both PD-1 ligands are higher expressed on hMDDC compared to hMDM2. Furthermore, antigen-presenting dendritic cells are much more potent in inducing T-cell responses compared to macrophages. Arnold et al. compared differently polarized monocyte-derived macrophages and dendritic cells in their ability to induce autologous T-cells by using mycobacterial purified protein derivate as recall antigen or keyhole limpet hemocyanin as a primary antigen for naive T-cell responses. In this context dendritic cells (hMDDC) induced the highest levels of T-cell proliferation to both antigens, whereas LPS + IFNγ-treated macrophages (comparable to hMDM1) were less effective in inducing antigen-specific T-cell responses followed by IL-4-treated macrophages (comparable to hMDM2) (46).

Focusing on hMDDC, we could partially reverse effects of PD-1 blockade by neutralization of soluble TNFα. Chiku et al. demonstrated elevated levels of TNFα and a concomitant decrease of parasite load after PD-1/PD-L blockade in the canine model of visceral leishmaniasis (47).

Furthermore, we found that neutralization of IFNγ, which was induced after PD-1 blockade in the hMDDC samples, had no significant effect on T-cell proliferation or parasite load. IFNγ is reported to mediate resistance to *Lm* in mice by inducing iNOS expression (2). In human myeloid cells iNOS (or NOS2) expression and function is controversially debated (48, 49). *In vitro* generated human myeloid cells are unable to express functional iNOS (NOS2) because the essential iNOS cofactor BH4 is missing (48). In contrast to mice, ROS play an important role in parasite control during human Leishmaniasis. IFNγ was shown to induce ROS in *Leishmania*-infected human monocytes, which dampened overall parasite load (8). Furthermore, ROS-dependent parasite control was rather evident in monocytes from CL patients than in monocytes from healthy individuals (7). Thus, it could be that IFNγ-induced ROS production in *Lm*-infected hMDDC was to low to see significant effects of IFNγ neutralization on infection rate in our *in vitro* model. One reason for why we did not observe significant differences in T-cell proliferation might be the general increased survival of T-cells upon IFNγ. However, IFNγ does not increase the number of antigen-specific T-cell divisions. This was shown, e.g., for murine OVA-specific $CD4^+$-T-cells (50).

In our *in vitro* model, preferentially $CD4^+$-T-cells proliferated upon *Lm* infection and nivolumab treatment. In the classical mouse model of *Lm*, induction of T_H1 response leads to resistance whereas the induction of a T_H2 response promotes disease (2). Human cutaneous leishmaniasis patients with moderate disease symptoms show a balanced T_H1/T_H2 response, whereas an imbalance of T_H1/T_H2 is associated with disease severity (51). Experiments with blood of prostate and advanced melanoma cancer patients revealed that PD-1 blockade augments T_H1 responses und suppresses T_H2 responses (52). By analyzing intranuclear expression of the T_H1-specific Tbet and T_H2-specific GATA3, we found PD-1 blockade to shift *Lm*-induced $CD4^+$ T-cells more toward a T_H1 or T_H1/T_H2 phenotype, respectively. Thus, the higher abundance of T_H1 T-cells and their effector functions may be implicated in reduction of *Lm* infection.

Specific killing of intracellular parasites in a concerted action of perforin, granzymes, and granulysin (expressed by T-cells) with minimal collateral damage to the host cell was demonstrated in transgenic mouse models (53). PD-1 blockade is described to increase perforin, granzyme B, and granulysin expression in T-cells of tuberculosis and cancer patients (54). Focusing on *Lm*-induced $CD4^+$ T-cells, we detected increased intracellular levels of perforin, granzyme A and B, and granulysin after PD-1 blockade. This suggests that cytolytic molecules might contribute to the reduction of *Lm* in hMDDC.

Altogether, our data suggest how the PD-1/PD-L axis could modulate *Lm* host cells and $CD4^+$ T-cells in patients suffering from chronic forms of leishmaniasis. So far, the focus of research groups is mostly on $CD8^+$ T-cell exhaustion, which was observed in diffuse cutaneous (40) and visceral human leishmaniasis (24). In case of the cutaneous forms of human leishmaniasis, $CD8^+$ T-cells have a dual role (55) but their contribution in resolving primary cutaneous *Leishmania* infection might be negligible (56, 57). $CD4^+$ T-cells activate leishmanicidal functions of infected macrophages and dendritic cells. In our experiments infected dendritic cells benefit the most from PD-1 blockade, as this strongly enhanced $CD4^+$ T-cell effector functions and parasite killing. Additionally we found increased levels of maturation markers on *Lm*-infected dendritic cells after PD-1 blockade (Figure S3 in Supplementary Material). To induce a strong cell-mediated immunity, e.g., after vaccination, adequate maturation of dendritic cells is important. In animal models, it was demonstrated that vaccination using *L. mexicana* LPG induced PD-1/PD-L2 expression on several immune cells in a dose dependent fashion (58). Blocking the PD-1/PD-L interaction could be a valuable approach to enhance efficacy of *Leishmania* vaccine candidates. Dendritic cell-based immunotherapy in combination with antimonials has been shown to significantly reduce parasite burden in experimental models of visceral leishmaniasis (59, 60). This approach might also benefit from PD-1 checkpoint inhibitors.

Collectively, by using a limited reductionist approach the present work provides new insights regarding the PD-1/PD-L axis in *Lm* infection of primary human cells and its consequence for adaptive immunity. Further experiments using material obtained from chronic leishmaniasis patients can contribute to a better understanding of PD-1 blockade-mediated effects.

AUTHOR CONTRIBUTIONS

CF, PC, ZW, GR, and GVZ contributed to conception and design of the study; CF, KA, and GANN performed and analyzed experiments;

CF wrote the first draft of the manuscript; all authors contributed to manuscript revision, read, and approved the submitted version.

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Absence of Tumor Necrosis Factor Supports Alternative Activation of Macrophages in the Liver after Infection with *Leishmania major*

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The absence of tumor necrosis factor (TNF) causes lethal infection by *Leishmania major* in normally resistant C57BL/6J (B6.WT) mice. The underlying pathogenic mechanism of this fatal disease has so far remained elusive. We found that B6.WT mice deficient for the *tnf* gene (B6.TNF$^{-/-}$) displayed not only a non-healing cutaneous lesion but also a serious infection of the liver upon *L. major* inoculation. Infected B6.TNF$^{-/-}$ mice developed an enlarged liver that showed increased inflammation. Furthermore, we detected an accumulating monocyte-derived macrophage population (CD45^{+}F4/80^{+}CD11b^{hi}Ly6C^{low}) that displayed a M2 macrophage phenotype with high expression of CD206, arginase-1, and IL-6, supporting the notion that IL-6 could be involved in M2 differentiation. In *in vitro* experiments, we demonstrated that IL-6 upregulated M-CSF receptor expression and skewed monocyte differentiation from dendritic cells to macrophages. This was countered by the addition of TNF. Furthermore, TNF interfered with the activation of IL-6-induced gp130-signal transducer and activator of transcription (STAT) 3 and IL-4-STAT6 signaling, thereby abrogating IL-6-facilitated M2 macrophage polarization. Therefore, our results support the notion of a general role of TNF in the inflammatory activation of macrophages and define a new role of IL-6 signaling in macrophage polarization downstream of TNF.

Keywords: ***Leishmania major*, liver, tumor necrosis factor, monocytes, IL-6**

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INTRODUCTION

The infectious disease leishmaniasis has been identified as a "neglected tropical disease" by the WHO and affects a significant number of people worldwide. The infection is caused by members of the intracellular protozoan parasite genus *Leishmania* spp. (1) and occurs when parasites are transmitted to their human hosts by *Phlebotomus* spp. or *Lutzomyia* spp. sandfly vectors during their blood-meal (2). Depending on the specific parasite species and the immune response of the host, infection with *Leishmania* spp. results in a variety of clinical manifestations that can range from self-healing skin lesions to progressive and ultimately fatal infections of visceral organ such as the spleen and liver (3). In the mammalian host, the parasites reside in their amastigote form within macrophages and dendritic cells (DCs) (4). The experimental model of cutaneous leishmaniasis, which is based on a subcutaneous inoculation with isolates of the species *Leishmania major* features

a strong genetic dichotomy. Mice of a C57BL/6 background (B6.WT) display a localized, self-healing infection characterized by interferon-γ (IFN-γ) production, while mice of the BALB/c background respond to infection with a preferential production of Th2 cytokines, such as IL-4, IL-10, and IL-13. These mice succumb to the infection after a progressive course of disease and a marked visceralization of the pathogen. This genetic dichotomy as a basis of resistance or susceptibility was instrumental in the development of the T helper (Th)1/Th2 paradigm (1).

The early expression of these respective cytokines has a profound effect on the activation state of macrophages, the predominant host cells of these pathogens. The T cell and NK cell cytokine IFN-γ triggers a classical activation that equips macrophages located in skin and local draining lymph node with mechanisms such as an upregulation of inducible nitric oxide synthase (iNOS) that allow an effective response to the challenge (5). In contrast, the cytokines IL-4, IL-10, and IL-13 result in an alternative activation in macrophages (6) that allows them to contribute to tissue remodeling and wound healing (7). While these cytokines have clearly accepted general roles in the establishment of an immune response to pathogens the proinflammatory cytokine tumor necrosis factor (TNF) has been seen as a cofactor that had an important but limited role in the immune defense (8). Nevertheless, infection experiments using *L. major*, specifically the BNI substrain, and other intracellular pathogens demonstrated a strong protective effect of TNF since *L. major* BNI infected TNF-deficient mice succumbed rapidly to the parasites despite a strong Th1-type response (9, 10). Recent observations have allowed an insight in the underlying deficiencies that cause this susceptibility. It could be demonstrated that in the *L. major* model TNF is necessary to prevent an ill-timed accumulation of alternatively activated macrophages concurrently to classically activated macrophages thus indicating a new and unique role for TNF (11). In the absence of TNF an elevated accessibility of arginase-1 (Arg-1) promoter and enhancer structures permitted a hyper-expression of Arg-1 and caused a subsequent lack of nitric oxide (NO) production presumably due to competition between the two enzymes iNOS and Arg-1, that depleted their common substrate L-arginine (12).

This mechanistic model of TNF-dependent restriction of alternative activation and the consequences for a host that lacks TNF has been established in skin and draining lymph nodes of *L. major* BNI infected mice (12). Other organs such as spleen and liver also show significant reproduction of parasites which is not detectable in TNF-positive hosts. The immune response in these organs has not yet been investigated in more detail and we hypothesized that we would also find increased alternative activation of macrophages. By analyzing *L. major* BNI infection in the liver, we found a comparatively low iNOS expression in B6.TNF$^{-/-}$ macrophages and an accumulation of a myeloid population that exhibited an alternatively activated-like macrophage phenotype, with high expression of Arg-1, CD206 and IL-6. In an *in vitro* assay, we demonstrated that bone marrow-derived DCs treated with IL-6 increased the expression of M-CSFR and generated less CD11c^{+} cells, while adding TNF reinstated CD11c^{+} cell generation and concurrently inhibited M-CSFR expression. Furthermore, we could show that both TNF and IL-6 had a regulatory effect on M2 macrophage differentiation which depends on modulation of mIL-6/gp130/signal transducer and activator of transcription (STAT) 3 or IL-4-STAT6. These findings of our study are emphasizing again that expression of TNF is critical to preventing a spread of parasites to visceral organs.

RESULTS

Progressive Liver Infection by *L. major* BNI in B6.TNF$^{-/-}$ Mice

Infection of C57BL/6 mice that lack an expression of TNF with *L. major* BNI results in a progressive course of disease and visceralization while B6.WT mice contain the infection and recover spontaneously (10). In our infection experiments, a significant lesion was observed in both B6.WT and B6.TNF$^{-/-}$ mice from day 21 after infection. While the footpads swelling remained moderate in B6.WT mice and subsided after day 35, B6.TNF$^{-/-}$ mice showed a progressively increasing footpad swelling (**Figure 1A**). Additionally, in B6.TNF$^{-/-}$ mice the infection spread to visceral organs such as the liver resulting in a mild hepatomegaly (**Figure 1B**) and a significant increase in liver weight (**Figure 1C**). Parasites in the liver were detected from day 21 after infection increasing in number to more than 3×10^5 parasites per gram liver tissue at day 42 postinfection (p.i.), while the liver of infected B6.WT mice remained essentially parasite free (**Figure 1D**).

Discrete Inflammatory Foci in the Liver of B6.TNF$^{-/-}$ Mice during Cutaneous Leishmaniasis

In both B6.WT and B6.TNF$^{-/-}$ mice normal, uninfected liver tissue consists of hexagonal hepatocytes radiating from the region of the central vein toward the periphery. From day 35 after infection, a point in time coinciding with a significant increase of liver weight and parasitic burden (**Figures 1B,C**), abnormal liver structures such as swelling of hepatocytes and diffusely infiltrating inflammatory cells could be detected in *L. major* BNI-infected B6.TNF$^{-/-}$ mice (**Figure 2A**). Additionally, inflammatory foci appeared almost exclusively in infected gene-deficient mice (**Figure 2A**). The generic macrophage marker CD68 was used to determine the phenotype of the cell focus (**Figure 2B**). Weak hepatic expression of CD68 was observed in B6.WT and B6.TNF$^{-/-}$ control groups, which was upregulated at day 42 after infection compared to its B6.WT counterpart. Cells that were CD68^{+} were mainly detected in areas around the borders of inflammatory foci. Taken together, these results suggested that infiltration of inflammatory cells and hepatic inflammation were significantly elevated in B6.TNF$^{-/-}$ mice, compared to the corresponding B6.WT mice. Interestingly, the number of inflammatory foci was significantly higher in B6.TNF$^{-/-}$ mice as compared to that in B6.WT mice (**Figure 2C**).

Cytokines Levels in Serum of B6.WT and B6.TNF$^{-/-}$ Mice after Infection

Cytokine levels in the serum are an indication of the type of immune response to *L. major* BNI (9). The titers of monocyte chemoattractant protein (MCP)-1, IL-6, IFN-γ, IL-10, TNF, and

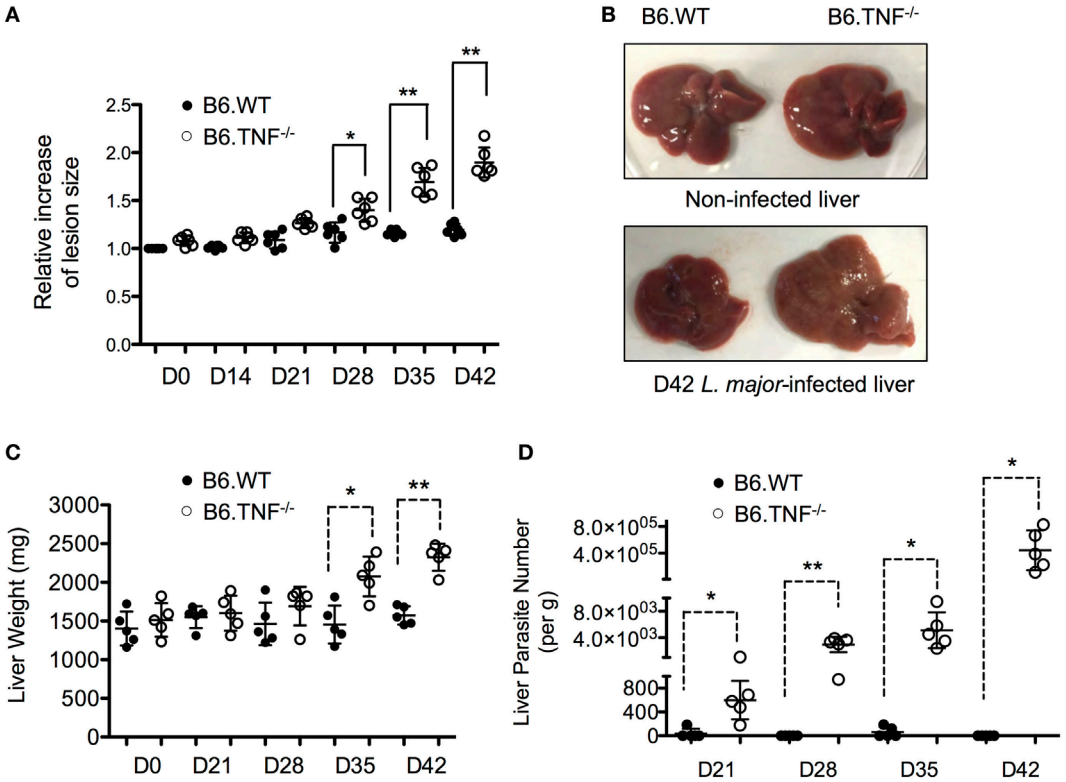

FIGURE 1 | Liver enlargement and increased parasite burden in B6.TNF$^{-/-}$ mice. While the lesion and liver size and weight remained initially identical between B6.WT and B6.TNF$^{-/-}$ genotypes it increases significantly in footpads **(A)** and liver **(B,C)** of B6.TNF$^{-/-}$ mice 42 days after *L. major* BNI infection. Six B6.WT and B6.TNF$^{-/-}$ mice were used to determine lesion size **(A)**, and five mice were used to determine liver weight at each time point **(C)**. Error bars represent the mean ± SD from one representative of three independent experiments. The *p*-values were calculated using a two tailed Mann–Whitney *U*-test (*$p < 0.05$, **$p < 0.01$). **(C)** The number of viable parasites in the liver tissue of B6.WT and B6.TNF$^{-/-}$ mice was determined by limiting dilution analysis **(D)**. The mean parasitic burden in the liver tissue of five mice from one representative experiment is shown. One circle represents one animal (black: B6.WT; white: B6.TNF$^{-/-}$). The results were confirmed by the other two independent replications. All data are represented as mean ± SD. Significance was calculated using a two tailed Mann–Whitney *U*-test (*$p < 0.05$, **$p < 0.01$).

IL-12 (p70) were determined in the serum of *L. major* BNI-infected B6.WT and B6.TNF$^{-/-}$ mice using a cytokine bead array (CBA) and compared to uninfected controls.

Serum levels of the proinflammatory cytokines MCP-1, IL-6, and IFN-γ increased significantly in B6.TNF$^{-/-}$ mice over the course of disease (**Figure 3**). High levels of MCP-1 were detected in the B6.TNF$^{-/-}$ mice after day 28 after infection (**Figure 3A**) indicating that *L. major* BNI-induced inflammation increased the potential to recruit monocytes. IL-6 was found to be increased by 17 times at the same point in the course of infection and 9 times at day 35 and day 42 after infection when compared to infected B6.WT mice (**Figure 3B**). Finally, a high concentration of IFN-γ is characteristic for a Th1-type immune response in both genotypes and was determined to validate our data. Similar to previously published results (9), B6.TNF$^{-/-}$ mice showed a significant increase of IFN-γ (**Figure 3C**) compared to that in B6.WT (day 28 after infection: mean ± SD, 269.45 ± 163.56 versus mean ± SD, 3.50 ± 1.49; day 35 after infection: mean ± SD, 668.39 ± 424.14 versus mean ± SD, 3.50 ± 2.46; day 42 after infection: mean ± SD, 766.03 ± 622.3 versus mean ± SD, 6.14 ± 4.11). Serum concentration of IL-10 (limit of detection 17.5 pg/ml) and IL-12p70 (limit of detection 10.7 pg/ml) remained below the detection limit for the assay in more than half of the serum samples throughout the course of infection (data not shown). TNF was only observed in B6.WT mice (data not shown).

A Monocyte-Derived Macrophage (Mo-M) Population Accumulates in the Liver of *L. major* BNI-Infected B6.TNF$^{-/-}$ Mice

A strong accumulation of inflammatory myeloid cells has been described in skin and draining lymph node in *L. major* BNI infection (11). Furthermore, it has been demonstrated that TNF is necessary for classically activated macrophage phenotype (12). Therefore, we analyzed the quantity and composition of infiltrating inflammatory cells in the liver of B6.WT and B6.TNF$^{-/-}$ mice using comprehensive flow cytometric analysis with two different panels over the course of infection.

Three distinct subsets were characterized based on the expression of CD11b, Ly6C, CD45, and F4/80 as described previously (13). The liver-resident Kupffer cells (KCs) were defined as CD45^{+}F4/80^{+}CD11b^{-}Ly6C^{-} population. Recruited

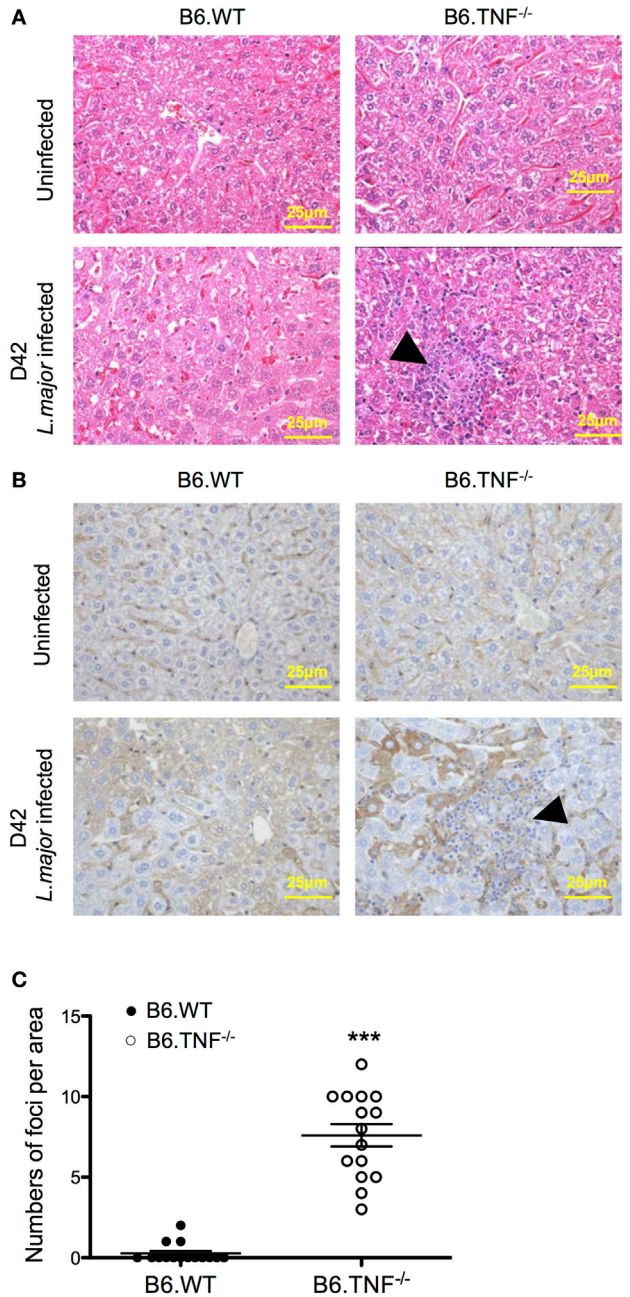

FIGURE 2 | Liver morphology of B6.WT and B6.TNF$^{-/-}$ mice. **(A)** Representative H&E-stained liver sections of 15 B6.WT and B6.TNF$^{-/-}$ mice are shown before infection and at day 42 after infection. B6.TNF$^{-/-}$ mice show an increased infiltration of inflammatory cells and the presence of inflammatory foci (arrowhead; magnification 400×). **(B)** Liver tissue from B6.WT and B6.TNF$^{-/-}$ mice (uninfected and 42 days after infection). At day 42 after infection, CD68 immunostaining is markedly increased in B6.TNF$^{-/-}$ mice compared to the B6.WT group. Bar = 25 μm for all the pictures. **(C)** The number of inflammatory foci per area is shown. The *p*-values were calculated using two tailed Mann–Whitney *U*-test (***$p < 0.001$).

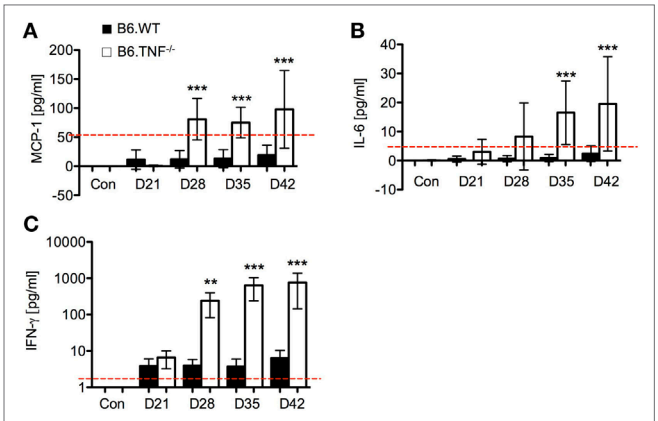

FIGURE 3 | Cytokine secretion as measured by cytokine bead array using flow cytometry. Serum levels of **(A)** monocyte chemoattractant protein-1 (MCP-1), **(B)** IL-6, and **(C)** interferon-γ (IFN-γ) were measured over the course of *Leishmania major* infection between B6.WT and B6.TNF$^{-/-}$ mice. The red dashed lines represent the limit of detection for each cytokine (IL-6 5 pg/ml, MCP-1 52.7 pg/ml, and IFN-γ 2.5 pg/ml). Concentrations were expressed and compared with the basal level found in B6.WT and B6.TNF$^{-/-}$ mice without infection. Each value represents the mean of three independent experiments and each experiment was performed in five mice. Error bars denote SD. The *p*-values were calculated using two tailed Mann–Whitney *U*-test (**$p < 0.01$, ***$p < 0.001$).

inflammatory monocytes (Mo) which differentiate to Mo-M and potentially to inflammatory DCs (Mo-DC) were defined as CD45$^+$F4/80$^+$CD11b^{low}Ly6C^{hi}. Finally, Mo-M displayed the phenotype of CD45$^+$F4/80$^+$CD11b^{hi}Ly6C^{low} (13–15) (#or gating strategy and flow cytometric identification of subpopulations see Figure S1 in Supplementary Material).

The number of KCs was not significantly different between the genotypes over the course of a *L. major* BNI infection except for day 21 after infection. At this point in time, the CD45$^+$F4/80^{hi}CD11b$^-$Ly6C$^-$ population was around two-fold higher in B6.WT mice as compared to B6.TNF$^{-/-}$ mice (**Figures 4A,B**). The number of inflammatory monocytes increased in correlation with the footpad swelling irrespective of the genotype. At day 42, the infiltrating monocytes started to decrease (B6.WT) or reached a plateau (B6.TNF$^{-/-}$) (**Figures 4A,C**). At the same time, Mo-M which represented a small population in B6.WT mice were elevated significantly in B6.TNF$^{-/-}$ mice (**Figures 4A,D**). To analyze the specific role of TNF in the accumulation of Mo-M, we employed TNF-competent mice of the BALB/c strain which are highly susceptible to *L. major* BNI infection and display progressive visceralization (Figures S2A,B in Supplementary Material). This control experiment showed a Mo-M population comparable to B6.WT mice and indicated that the accumulation of the Mo-M population depends on TNF.

Infection leads to a strong increase of CD11c$^+$iNOS$^+$TNF$^+$ inflammatory DCs (here termed Mo-DC) at the infection site. These cells are also derived from inflammatory monocytes and act as effector and antigen-presenting cells. Therefore, we investigated whether the absence of TNF affects the differentiation process of Mo-DCs during *L. major*-BNI induced liver infection and we examined the expression of CD11c based on CD45$^+$CD11b^{hi}Ly6C^{hi} population. Mice of both genotypes had a very distinct CD11c$^+$ CD11b^{hi} Ly6C^{hi} population of comparable size. In B6.TNF$^{-/-}$ mice the populations displayed a comparatively

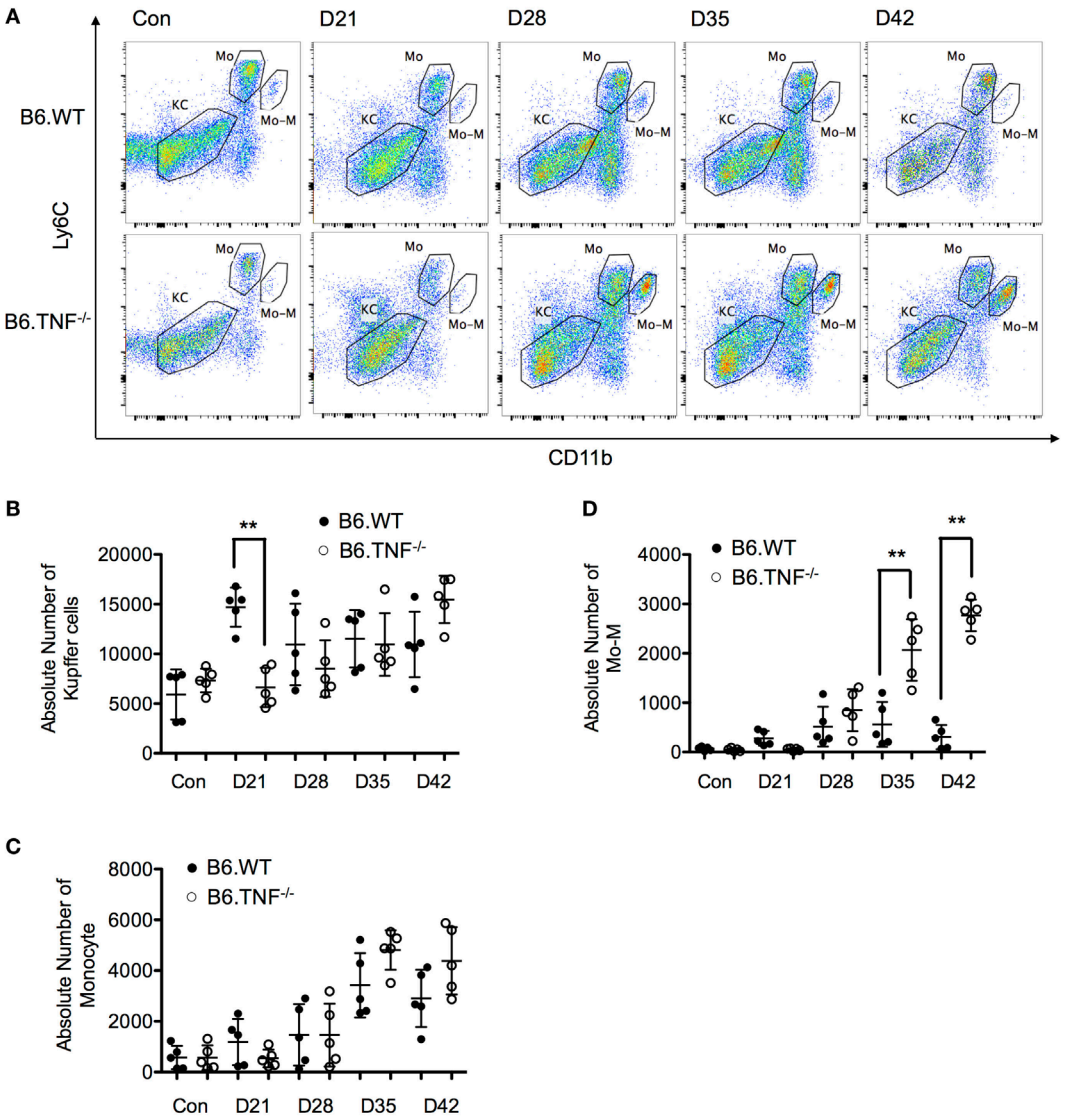

FIGURE 4 | Analysis of resident and inflammatory myeloid populations in the liver in *Leishmania major* infected B6.WT and B6.TNF$^{-/-}$ mice. **(A)** Flow cytometry analysis was used to demonstrate the presence of three different liver macrophage populations based on the markers CD45, F4/80, CD11b, and Ly6C from B6.WT and B6.TNF$^{-/-}$ mice and to analyze the changes of these populations over the course of *L. major* BNI infection. Kupffer cells (KCs) were defined as CD45^{+}F4/80^{+}CD11b^{-}Ly6C^{-}, inflammatory monocytes (Mo) as CD45^{+}F4/80^{+}CD11b^{low}Ly6C^{hi} and monocyte-derived macrophages (Mo-M) as CD45^{+}F4/80^{+}CD11b^{hi}Ly6C^{low}. A representative staining is shown. Quantification by flow cytometry of the total populations of **(B)** KC, **(C)** Mo, and **(D)** Mo-M from five B6.WT and B6.TNF$^{-/-}$ mice in the course of *L. major* BNI infection is shown. Each error bar represents means ± SD from one experiment. Results were confirmed by two independent experiments. The *p*-values were calculated using two tailed Mann–Whitney *U*-test (**$p < 0.01$).

lower expression of CD11b, Ly6C, and CD11c than B6.WT mice (Figures S3A,B in Supplementary Material), which could indicate differences of inflammatory status or maturity of Mo-DCs.

The Mo-M Population in TNF$^{-/-}$ Mice Display an Alternatively Activated Phenotype with High IL-6 Expression

As shown above, B6.TNF$^{-/-}$ mice fail to clear the parasites from the liver and display an accumulation of a TNF-specific, unique Mo-M accumulation. To further investigate this Mo-M population in B6.TNF$^{-/-}$ mice during murine leishmaniasis, we followed the previously used gating strategy and combined both CD11b^{+}Ly6C^{+} populations since CD11b^{high}Ly6C^{low} are missing in B6.WT mice (**Figure 5A**). We characterized the phenotypes using intracellular flow cytometry of the markers IL-6, CD206 and, as control for the quality of the sort, IFN-γ. The intracellular antigens were depicted against SSC (**Figure 5B**). Corresponding to the increased IL-6 secretion in serum of infected B6.TNF$^{-/-}$ mice, an increased level of IL-6 expression was detected in liver macrophages. Although IL-6 is regarded as proinflammatory cytokine, it could be involved in the establishment of a Th2

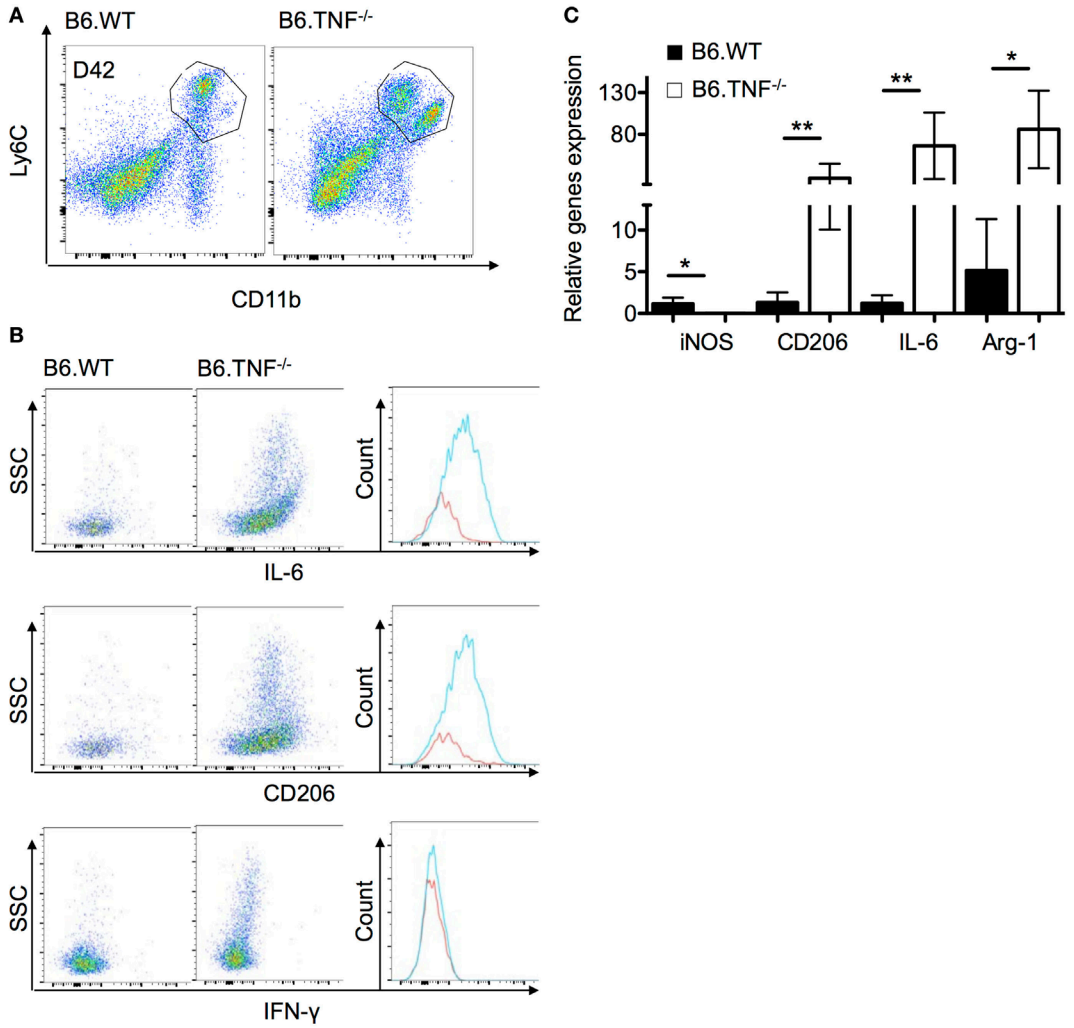

FIGURE 5 | Phenotypic characterization of monocyte-derived macrophages in the liver of B6.TNF$^{-/-}$ mice. **(A)** Gating strategy used in these experiments. **(B)** The expression of IL-6, CD206, and interferon-γ were investigated in the combined population from B6.WT and B6.TNF$^{-/-}$ mice at day 42 p.i. using flow cytometry. **(C)** Gene expression of IL-6, CD206, inducible nitric oxide synthase, and arginase-1 relative to β-actin expression in the combined population of B6.WT and B6.TNF$^{-/-}$ mice at d42 p.i. Each error bar represents the means ± SD from five mice in one experiment, and results were confirmed by further two independent experiments. The *p*-values were calculated using a two tailed Mann–Whitney *U*-test (*$p < 0.05$, **$p < 0.01$).

response which in turn, could modulate the activation pathway of the macrophage differentiation. Additionally, the macrophage mannose receptor CD206, was strongly upregulated. As expected, there was no difference in the presence of IFN-γ between B6.WT and B6.TNF$^{-/-}$ because IFN-γ is not produced by Mo or Mo-M. In summary, we found SSC, IL-6 and CD206 increased in the combined Mo and Mo-M of B6.TNF$^{-/-}$mice, indicating that this population comprises are large proportion of alternatively activated macrophages in B6.TNF$^{-/-}$ mice during *L. major* BNI infection. The detection of SSC^{high} cells in B6.TNF$^{-/-}$ mice was striking and indicates a marked presence of cells with high granularity that could represent infected cells.

Previously, it had been shown that a CD11b^{hi} Ly6C^{low} myeloid population harbored a markedly increased number of parasites in skin and draining lymph nodes (11). Because infected cells with a large burden of parasites are fragile and difficult to detect using flow cytometry, we sorted the distinct Mo and Mo-M populations from B6.WT and B6.TNF$^{-/-}$ mice and conducted a Romanowsky stain (Diff-Quik). There was no distinctive visible difference between Mo and Mo-M cells. In B6.WT mice there were no visible parasite associated with macrophages. In contrast, in both CD11b^{+}Ly6C^{hi} and CD11b^{+}Ly6C^{low} macrophage populations isolated from B6.TNF$^{-/-}$ mice parasites could be detected inside and on the surface of macrophages (Figure S4 in Supplementary Material).

To further analyze the Mo-M population in the liver during *L. major* BNI infection, we isolated these cells and characterized their phenotype with regard to the gene expression of these marker molecules using qPCR. We confirmed our flow cytometry results and showed that IL-6 and the alternative activation

markers including Arg-1 and CD206 were significantly higher expressed in B6.TNF$^{-/-}$ mice as compared to B6.WT mice. In contrast to previous results, in the liver iNOS expression was decreased significantly in B6.TNF$^{-/-}$ mice (**Figure 5C**).

Macrophage Activation Is Correlated with Parasitic Burden in Liver

The regulation of myeloid iNOS expression after challenge *in vivo* is complex and influenced by factors such as pathogen, genetic background and cytokine environment. Therefore, we examined the response of CD11b^{+} cells to *L. major* BNI in the livers of B6.WT and B6.TNF$^{-/-}$ mice (**Figures 6A,B**). Mice from both genotypes showed a strong presence of CD11b^{+} cells at day 42 after infection while *L. major* amastigotes could only be detected directly in B6.TNF$^{-/-}$ mice (**Figures 6A,B**). The absence of *L. major* in the livers of B6.WT mice was correlated with a strong iNOS expression, while in the absence of TNF, iNOS expression was decreased (**Figure 6A**) similar to the situation in susceptible BALB/c mice (Figure S2A in Supplementary Material) while CD206 expression was increased and associated with a strong presence of *L. major* (**Figure 6B**). Thus, these results suggested a presence of alternatively activated macrophages during leishmanial infection in the liver in B6.TNF$^{-/-}$ mice.

TNF and IL-6 Modulate the Process of Monocyte Differentiation to Macrophage or DC *In Vitro*

One of the cytokines which was upregulated significantly in the liver is IL-6 (compare **Figure 3**). The presence of IL-6 has been shown to modulate the differentiation of monocytes and skew the developmental outcome to macrophages rather than DC, which can be reversed by TNF (16). The observed overexpression of IL-6 in B6.TNF$^{-/-}$ mice could facilitate the increased presence of alternatively activated macrophages in the liver. To test this hypothesis, we decided to use mouse bone marrow-derived macrophages to analyze their differentiation in the context of TNF and IL-6 *in vitro*. Bone marrow cells of B6.WT mice were cultured with IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) resulting in 64.2% CD11b^{+}CD11c^{+} DC. The addition of IL-6 at day 3 of culture reduced the generation of cells with this

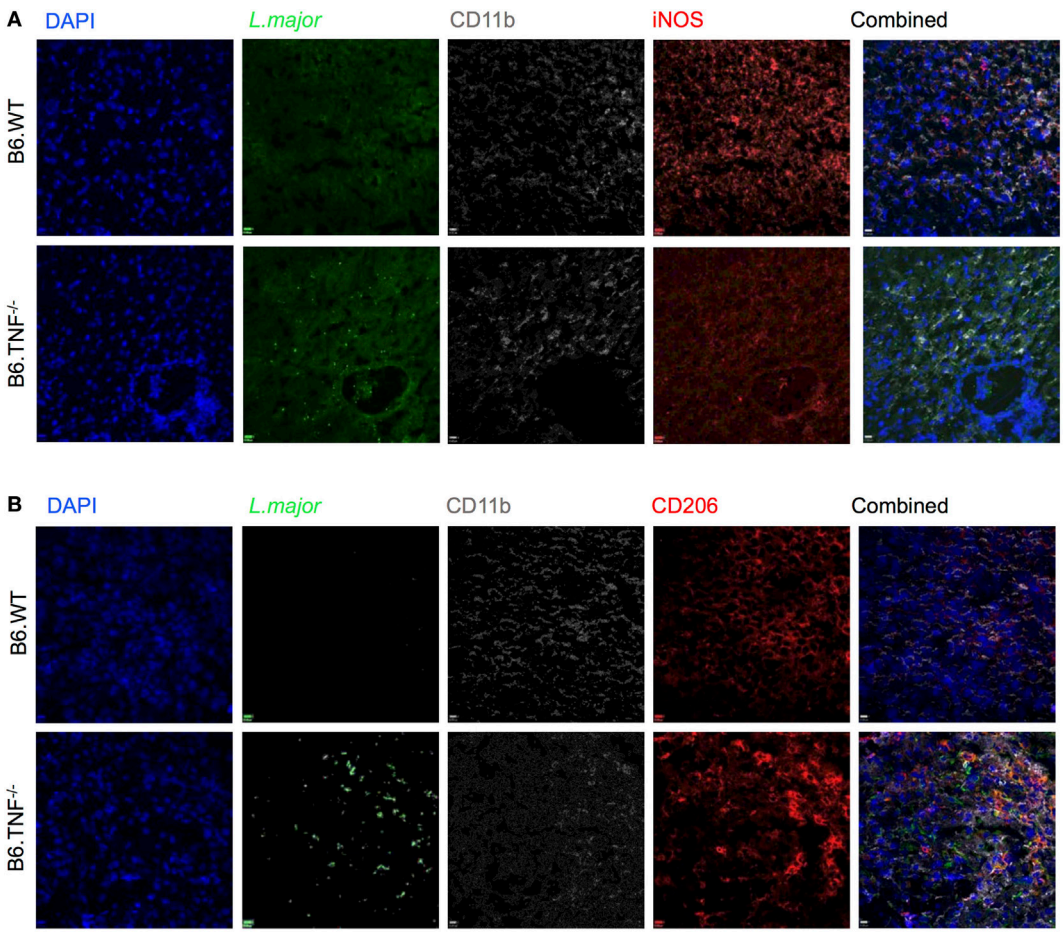

FIGURE 6 | Inducible nitric oxide synthase (iNOS) and CD206 expression in B6.TNF$^{-/-}$ mice. Immunofluorescence staining of **(A)** CD11b, iNOS, and *Leishmania major* and **(B)** CD11b, CD206, and *L. major* in liver tissue of B6.WT (upper panel) and B6.TNF$^{-/-}$ mice (lower panel) 42 days after infection. Single color staining is shown for *L. major* (green), CD206 and iNOS (red), CD11b (gray), and DAPI (blue). The single colors have been merged. The figure represents one of three independent experiments.

phenotype to 51% (**Figure 7**). In contrast, culturing cells in the presence of a combination of IL-6 and 50 ng/ml TNF allowed the expression of CD11c to be restored to previous levels, indicating TNF reversed the differentiation process from monocytes to DCs which was inhibited by IL-6 (**Figures 7A,B**). The combination of GM-CSF and IL-4 potentially caused ectodomain shedding of the M-CSF receptor, inhibiting the differentiation of macrophages from monocytes (17). Thus, we next examined the level of M-CSFR expression when treated with IL-6 and TNF using flow cytometry, immunofluorescence and qPCR (**Figures 7B,C**). Adding IL-6 resulted in an increased level of M-CSFR expression compared to the control group which was consistent with the increased accumulation of DCs (**Figure 7A**). After an additional exposure to TNF, IL-6-treated DCs showed a markedly reduced M-CSFR level (**Figure 7A**). Using immunofluorescence DCs showed increased expression of M-CSFR when treated with IL-6 alone, but its CD11c expression was reduced compared to the control group (**Figure 7B**). An exposure of cells to IL-6 and TNF-α together reduced the expression of M-CSFR and CD11c was elevated. These results were confirmed using qPCR (**Figure 7C**).

A Regulatory Balance of TNF and IL-6 Mediate Alternative Macrophage Polarization

Since TNF skewed IL-6-driven differentiation from macrophage to DC, we next examined whether TNF and IL-6 also affected alternative activation as represented by comparing F/80 and CD206 expression patterns. Treatment of the three different types of macrophages with IL-6 alone did not change M0 and M1 phenotype, based on the expression of $F4/80^+CD206^+$ compared to the control group (**Figure 8A**). However, a significant increase of $F4/80^+CD206^+$ population was observed after IL-4 treatment (**Figures 8A,B**). An analysis using qRT-PCR also revealed increased expression of CD206 and Arg-1 mRNA, and a reduced iNOS mRNA expression (**Figure 8C**), which indicated IL-6 only interfered with IL-4-induced alternative activation. Furthermore, when applied as cotreatment with IL-6 and TNF, M0 and M1 were still not affected based on the parameters assessed, compared to IL-6-treated group. The size of the $F4/80^+CD206^+$ population as well as the expression of CD206 and Arg-1 mRNA were significantly reduced with increased concentration of TNF but the iNOS mRNA expression was upregulated in the presence of TNF-α and IL-6 (**Figure 8C**).

Effect of a Blockade of the Interaction of TNF and IL-6

To further dissect the regulatory effect between TNF-α and IL-6 in M2 macrophage activation, we used a human TNF-receptor:Fc fusion protein (Enbrel) which is a TNF inhibitor with human/mouse cross-reactivity (18). After 8 days in M-CSF-containing medium the bone marrow derived undifferentiated macrophages expressed IL-6 (**Figures 9A,G**). Additional exposure to IL-4 for 48 h induced macrophages from both B6.WT and $B6.TNF^{-/-}$ mice to display CD206 (**Figures 9B,H**). The level of IL-6 was largely unchanged in B6.WT macrophages but strongly upregulated in $B6.TNF^{-/-}$ macrophages. Interestingly, macrophages pretreated with increasing concentrations of Enbrel (5, 10, 25, 50 μg/ml) and then exposed to IL-4 not only upregulated the expression of CD206 but also increased the level of IL-6 (**Figures 9C–F**). Together, these data showed that IL-6 was associated with IL-4-driven alternative macrophage activation. The presence of TNF inhibited this process as indicated by a downregulation of Arg-1 and CD206. In contrast, blockade of TNF was facilitating a CD206 and IL-6 expression, supporting recent data obtained in $TNF^{-/-}$ mice (12) and suggesting a balancing effect between TNF and IL-6 in alternative macrophage activation.

The Regulatory Effect of the Balance between TNF and IL-6 Affects gp130/STAT3 and IL-4/STAT6 Signaling

Using qPCR, we examined the expression of the receptor molecules specific for IL-6, IL-6 receptor (IL-6R), and gp130, to determine which signaling pathway is active after stimulation with TNF and IL-6 in M2 macrophages. There was no significant change of IL-6R mRNA while gp130 was increased significantly in the IL-6-treated group but its expression was reduced when cells were additionally exposed to TNF (**Figure 10A**). A Western Blot analysis of IL-6R and gp130 showed an upregulation of these molecules in the presence of IL-6 and a significant downregulation by TNF (**Figures 10B–D**). Further analysis of the levels of STAT3 and 6 demonstrated that the protein expression of these transcription factors was unchanged *per se* but that the level of phosphorylation decreased in the presence of TNF (**Figures 10B,E,F**).

MATERIALS AND METHODS

Mouse Strains

C57BL/6J mice (B6.WT), BALB/c mice (both Jackson Laboratories, Bar Harbor, ME, USA) and C57BL/6J mice genetically deficient for the *tnf* gene ($B6.TNF^{-/-}$) (19) were used at 6–12 weeks of age. All mice were kept under specific pathogen-free conditions at the animal facilities of the Menzies Institute for Medical Research, Hobart, Australia. Infection was performed with sex- and age-matched B6.WT and $B6.TNF^{-/-}$ mice. Animal care and experiments were approved by the animal ethics committee of the University of Tasmania, Hobart, Australia (Animal Ethics Number: A13934 and A13935).

Culture of *L. major* and Infection

The virulent *L. major* strain BNI (MHOM/IL/81/FE/BNI) was kindly provided by Prof. Christian Bogdan (Institute of Microbiology, Hygiene and Immunology, Erlangen, Germany). The infectivity of the parasites was maintained by passage through the tissue of susceptible BALB/c mice as described (20). Prior to infection, the parasites were cultured *in vitro* in Novy-MacNeal-Nicolle blood agar slants in RPMI 1640 medium (Thermo Fisher Scientific, VIC, Australia) containing 10% rabbit serum (Applied Biological Products Management, SA, Australia), penicillin/streptomycin, nonessential amino acids, and 10 mM HEPES (Thermo Fisher Scientific) as described (10, 20). Mice were injected with 3×10^6 stationary-phase *L. major* promastigotes bilaterally into the skin of the hind footpads. The progress

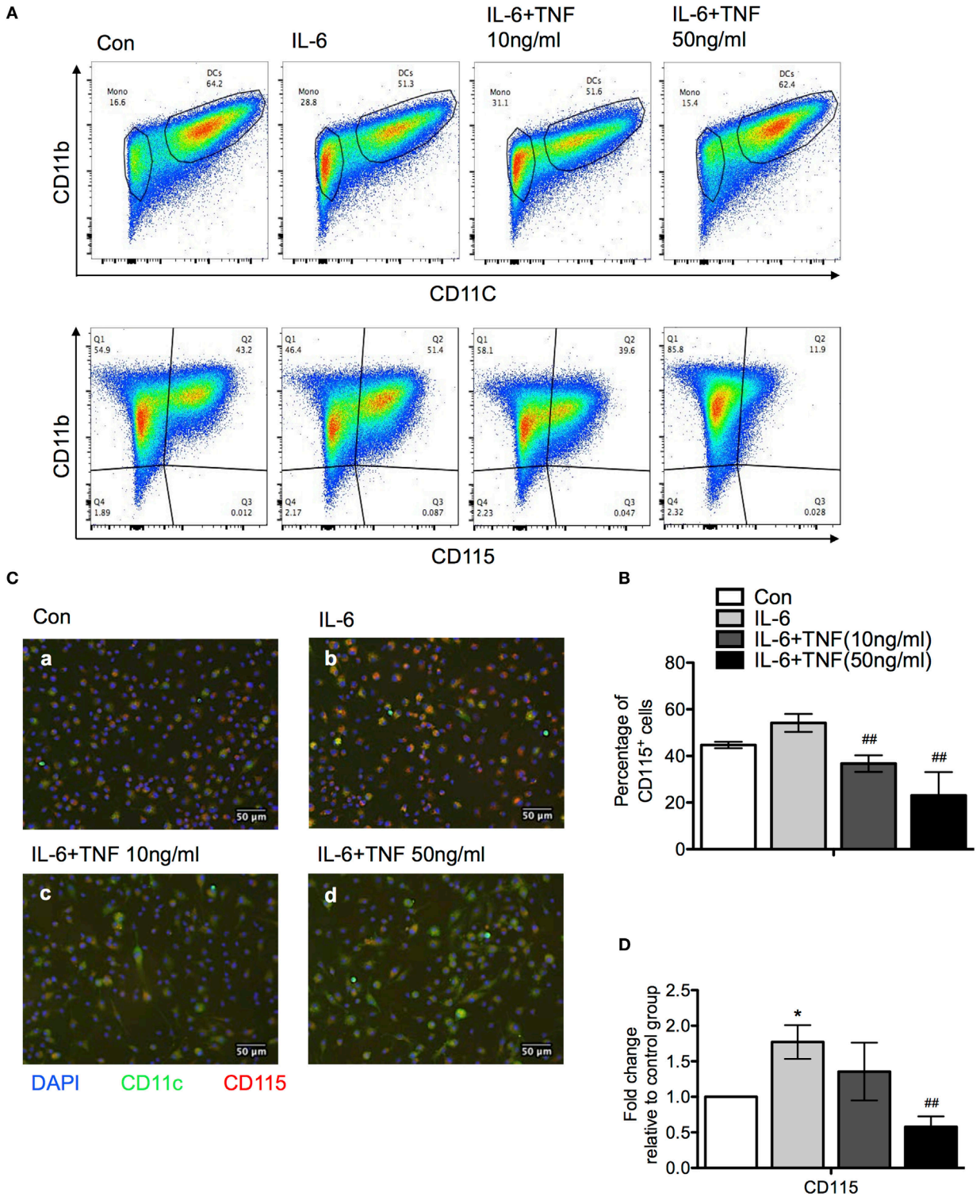

FIGURE 7 | The effect of tumor necrosis factor (TNF) and IL-6 in monocyte differentiation. **(A)** Bone marrow monocytes were cultured for 4 days with granulocyte macrophage colony-stimulating factor and IL-4. IL-6 and/or TNF were added after day 4. At day 7, differentiation was monitored using the expression of CD11b, CD11c, and M-CSFR (CD115) using flow cytometry. A representative result is shown. **(B)** Quantification the results of a CD11b versus M-CSFR (CD115) staining of three independent differentiation experiments. The *p*-values were calculated using one-way ANOVA (*$p < 0.05$). **(C)** Immunofluorescence photographs represent sections of cells from each culture condition labeled for M-CSFR (red) CD11c (green) and DAPI (blue). **(D)** qPCR data revealed M-CSFR gene expression in cells treated with IL-6 and TNF. The results are representative of three independent experiments. Means ± SD were calibrated to median values of three experiments. The *p*-values were calculated using one-way ANOVA and Tukey's comparison test (*$p < 0.05$ and $^{\#\#}p < 0.01$).

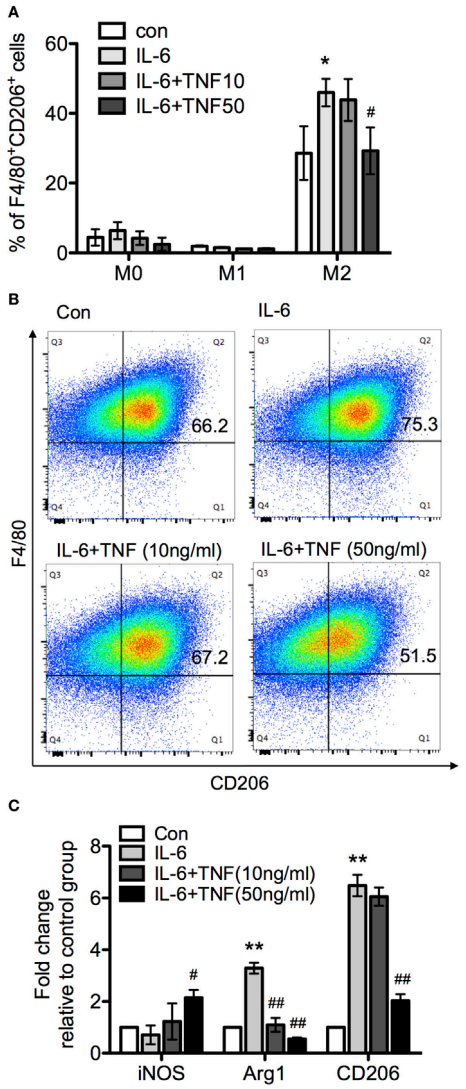

FIGURE 8 | The effect of tumor necrosis factor (TNF) and IL-6 in macrophage differentiation. Bone marrow-derived macrophages were cultivated in the presence of macrophage colony-stimulating factor and harvested after 8 days. Subsequently, macrophages were exposed to LPS and interferon-γ or IL-4 for 24 h to generate M1 and M2 macrophages. The upregulation of the marker molecules F/80 and CD206 was quantified. Unchanged macrophages were considered as M0 phenotype. **(A)** M0, M1, and M2 macrophages were incubated with IL-6 or IL-6/TNF, and differentiation was analyzed. All the data are presented as means ± SD after normalization to control group values of three experiments. The *p*-values were calculated using one-way ANOVA and Tukey's comparison test (*$p < 0.05$ when compared to the control group, and $^{\#}p < 0.05$ when comparing to the IL-6-treated group). **(B)** Flow cytometry of cells from each culture condition using the macrophage markers F4/80 and CD206. **(C)** Quantification of the expression of inducible nitric oxide synthase (iNOS), arginase-1 (Arg-1), and CD206 in M2 macrophages treated with IL-6 and TNF using qPCR. Results are representative of three independent experiments, and presented as mean ± SD normalized with regard to the control group. The *p*-values were calculated using one-way ANOVA and Tukey's comparison test (**$p < 0.01$ when compared to control group, and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ when compared to the IL-6-treated group).

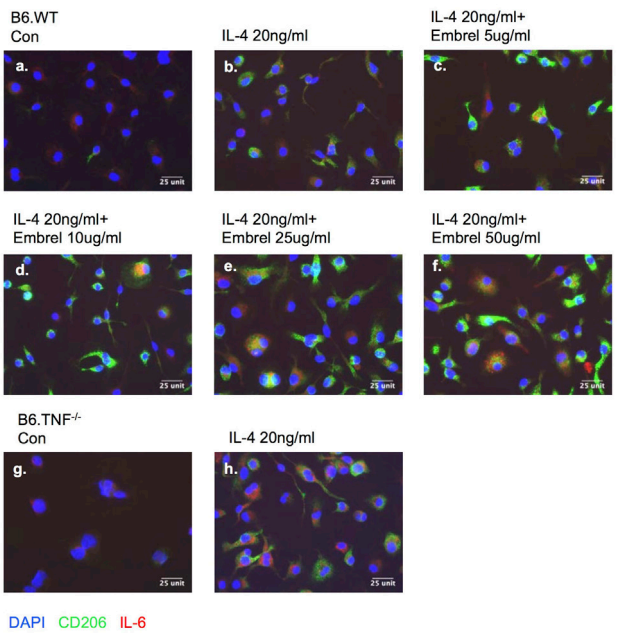

FIGURE 9 | Blocking tumor necrosis factor skews macrophage differentiation. Bone marrow-derived macrophages from B6.WT **(A–F)** and B6.TNF **(G,H)** mice were cultured with IL-4 and different concentrations of etanercept (Enbrel). Immunofluorescence photographs represent cells from each culture condition labeled for IL-6 (red), CD206 (green), and DAPI (blue). One of three independent experiments is shown.

of the infection was monitored by measuring the footpad swelling (lesion size) using a metric caliper (10).

Limiting dilution experiments were performed to determine the parasite burden in the infected liver. Single cell suspensions were prepared in supplemented Schneider's media (Thermo Fisher Scientific) and serial dilutions (threefold) were pipetted across a 96-well plate with 12 replicates in an end-point titration. The plates were incubated for 10–14 days at 27°C before the number of *Leishmania*-positive wells were determined using both a light microscope Olympus CKX31 (Olympus, VIC, Australia) and a spectra Max/M2 Microplate reader (Molecular Devices, Sunnyvale, CA, USA). The parasitic burden was calculated as described (10).

CBA Assay

Mouse whole blood was collected by cheek bleeding. Mouse serum was stored at −80°C for further use. Cytokine titers of IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 were determined in serum using the mouse inflammation CBA kit (BD Biosciences, NSW, Australia) following the manufacturer's instructions. Samples were acquired on a BD FACSCanto II using FACSDiva 6.1 software and analyzed with FCAP Array version 1.0 software (BD Biosciences).

Liver Mononuclear Cell Isolation

Mice were sacrificed by CO_2 and then perfused slowly *via* the ascending aorta with 30 ml PBS and EDTA (Thermo Fisher

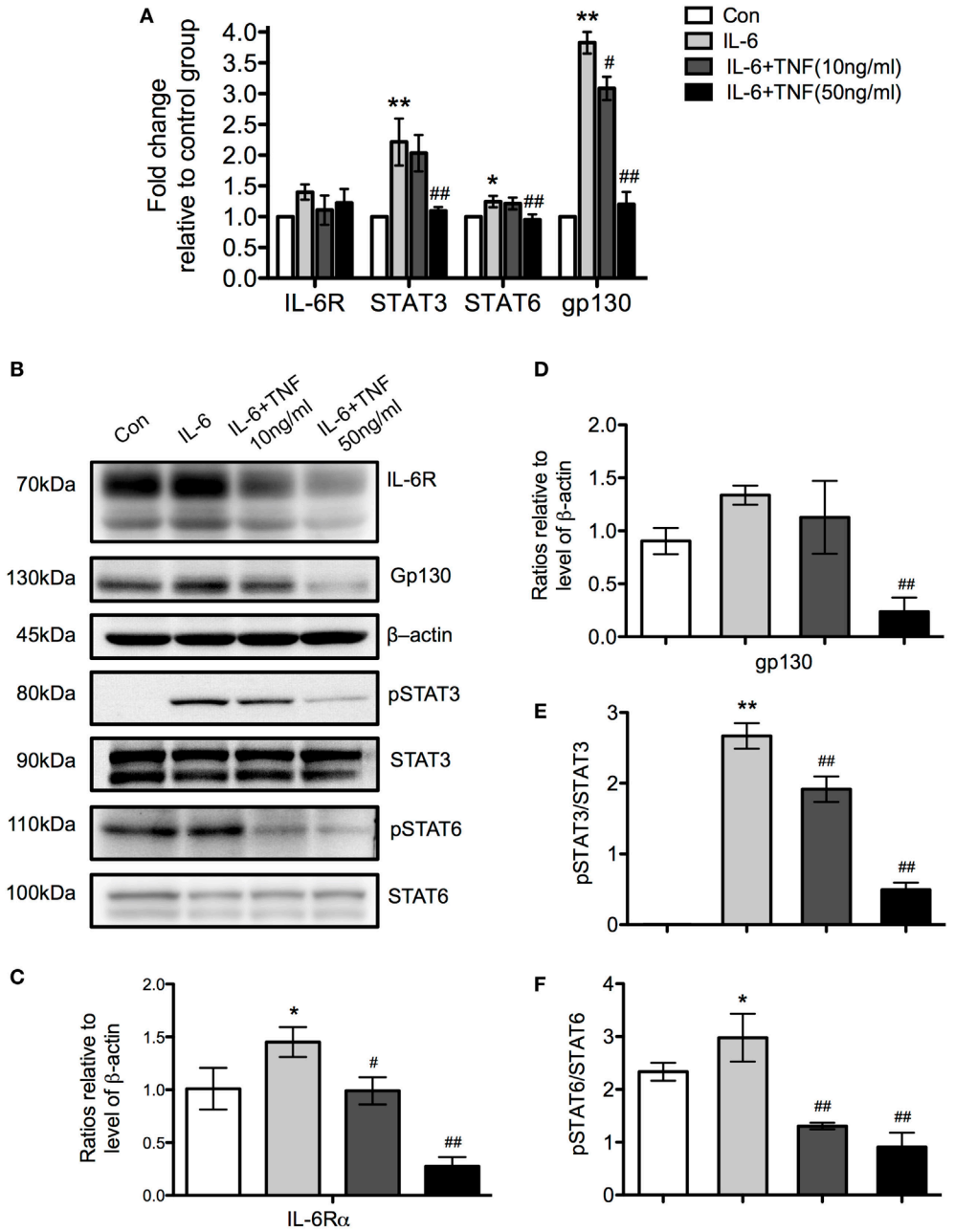

FIGURE 10 | The regulatory effects of tumor necrosis factor (TNF) and IL-6 affect the expression of gp130 and IL-6R and the phosphorylation of signal transducer and activator of transcription (STAT) 3 and STAT6. **(A)** qRT-PCR analysis of different signaling molecules in M2 macrophages treated with IL-4 and TNF. Results were normalized to the control group and represent means ± SD of three experiments. Statistical analysis was performing using ANOVA with Tukey's posttest, and the results were considered significant with a $^{*}p < 0.05$, $^{**}p < 0.01$ when compared to the control group, and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ when compared to the IL-6-treated group. **(B)** Western Blot analysis of IL-6R, gp130 and b-actin as loading control. Furthermore, the transcription factors signal transducer and activator of transcription (STAT) 3 and STAT6 were analyzed as phosphorylated and non-phosphorylated proteins. One of three experiments is shown. **(C,D)** Changes of IL-6R and gp130 relative to β-actin are shown. **(E,F)** The ratio of as phosphorylated and non-phosphorylated STAT3 and 6 is depicted. Results were calibrated to the control group value and represent means ± SD of three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ when compared to the control group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ when compared to the IL-6-treated group. One-way ANOVA analysis with Tukey's postcomparison.

Scientific). Livers were then removed and stored as required. Isolation of liver mononuclear cell was achieved by cutting the organ in small pieces and then digesting it in Hank's Balanced Salt solution (Thermo Fisher Scientific) containing collagenase II (100 U/ml, Thermo Fisher Scientific) and DNase I (1 U/μl, Sigma-Aldrich, NSW, Australia) for 30 min at 37°C shaking at 200 rpm. The suspension was filtered through a 100 μm strainer (Thermo Fisher Scientific) to remove tissue debris. Cells were

resuspended in PBS/BSA and mononuclear cell were isolated using a Histopaque 1083 gradient (Sigma-Aldrich). The gradient was initially centrifuged at 80 g for 3 min followed by centrifugation at 1,400 *g* for 15 min at 4°C. The cells at the interface were harvested, resuspended in 10 ml PBS and centrifuged at 600 *g* for 10 min at 4°C. The pellet was resuspended in PBS and the cell number determined in a Neubauer hemocytometer (Australian Scientific, NSW, Australia).

Generation and Tissue Culture of Bone Marrow-Derived Macrophages and DC

BM cells were flushed from femur and tibia of uninfected B6.WT and B6.TNF$^{-/-}$ mice and cultured in RPMI 1640 media (Thermo Fisher Scientific) supplemented as described with penicillin/streptomycin, nonessential amino acids, and 10 mM HEPES (Thermo Fisher Scientific) and either 10% of L929 tissue culture supernatant containing macrophage colony stimulating factor (M-CSF) for 7 days (21) or 10% tissue culture supernatant of GM-CSF-transfected X63-AG8 cells for 10 days (22). Recombinant mouse IFN-γ (20 ng/ml; Peprotech, Lonza, VIC, Australia) and IL-4 (10 ng/ml; Peprotech) were added into the medium for 24 h to differentiate M1 and M2, respectively. For the final 24 h, recombinant mouse IL-6 (10 ng/ml, Peprotech), TNF (Peprotech) were added. In blocking experiments increasing concentrations of a human TNFR:Fc (Enbrel; Amgen, North Ryde, NSW, USA) were added for 4 h before the addition of IL-4.

Flow Cytometry and Cell Sorting

Multicolor flow cytometry was performed following an established protocol (23). Cells were stained first for surface marker expression with rat antimouse CD45 (Biotinylated; 30-F11; BD Biosciences), rat antimouse Ly6C (FITC; clone HK 1.4; BioLegend, WA, Australia), rat antimouse F4/80 (APC-Cy7; clone BM8; eBioscience, VIC, Australia), and rat antimouse CD11b (PerCP-Cy5.5; clone M1/70; BD Biosciences). For intracellular flow cytometry the cells were fixed with FOXP3 Fix/Perm buffer and permeabilized with FOXP3 Perm buffer (BioLegend) according to the manufacturer's protocol. Intracellular proteins were targeted with rat antimouse CD206 (PE; clone C068C2; BioLegend), rat antimouse IL-6 (PE; clone MP5-20F3; BD Biosciences), rat antimouse IFN-γ (PE; clone XMGI-2; BD Biosciences), rabbit anti-*L. major* [clone V121 (11)], and mouse antimouse Arg-1 (PE; polyclonal antiserum; R&D Systems, Sydney, NSW, Australia). Streptavidin conjugated to V500 (BD Biosciences) was used to reveal biotinylated primary mAbs. Cells were acquired on a BD FACSCanto II flow cytometer using BD FACSDiva version 6.1.3 (BD Biosciences) and analyzed with FlowJo software version 10.1(Tree Star Inc., Ashland, OR, USA). For flow cytometric cell sorting, two populations defined by CD45$^+$F4/80$^+$CD11b$^+$Ly6C^{low} and CD45$^+$F4/80$^+$CD11b$^+$Ly6C^{hi} were sorted using a Beckman Coulter Astrios MoFlo. For liver DC marker comparison, CD11c (PE-Cy7; clone HL3; BD Biosciences) was used. Bone marrow-derived cells were stained with CD11b (FITC; clone M1/70; BD Biosciences), CD11c (PE-Cy7; clone HL3; BD Biosciences), F4/80 (APC-Cy7; clone BM8; eBioscience), CD206 (PE; clone C068C2; BioLegend), and M-CSFR (APC; clone AFS98; Biolegend) as experiments required.

Immunohistochemistry

Liver tissue specimen was fixed in formalin and embedded in paraffin. Histological sections of 4 μm thickness were stained with hematoxylin and eosin using a standard protocol. The histopathological changes before and after *L. major* infection were observed using a Leica DM2500 (North Ryde, Australia). To assess the degree of inflammation, three representative inflammatory foci were imaged at 100× magnification. The number of inflammatory foci in each image was quantified and the average for each of the three representative areas per animal was then calculated.

For immunohistochemical staining, tissue sections were deparaffinized in xylene and rehydrated. The antigens were retrieved in 10 mmol/l sodium citrate buffer (pH 9.0) for 10 min at 100°C and then cooled to room temperature before being stained. Endogenous peroxidase activity was quenched by treatment with 3% H_2O_2 in methanol for 10 min. The sections were blocked in protein block (X0909, Dako, VIC, Australia) for 30 min at room temperature and then incubated with a primary mAb to CD68 (ab31630, Abcam, VIC, Australia) for 1 h at 37°C. Immunoreactivity was visualized with diaminobenzidine (Dako) using the Envision system (Dako) according to the manufacturer's protocol. The nuclei were lightly counterstained with hematoxylin solution. A negative control was prepared using the same staining procedure but was not incubated with the abovementioned primary antibodies. Images were obtained using an Olympus BX53 microscope (Olympus), and semiquantitative analysis was conducted using Image-Pro Plus software (ImageJ, USA).

Flow cytometrically sorted cells were centrifuged on slides in a Cytospin™ 4 Cytocentrifuge (Thermo Fisher Scientific) at 500 *g* for 10 min and underwent microscopical analysis after air-drying and staining with Diff-Quik reagent.

Immunofluorescence

Liver tissue samples were dissected and rapidly frozen in Tissue-Tek optimal cutting temperature medium (VWR, QLD, Australia) in liquid nitrogen vapor and stored at −80°C. Sections (8 μm) were cut using a cryotome (Thermo Fisher Scientific), air-dried, and fixed in acetone at −20°C. Prior to staining, sections were rehydrated in PBS/1% BSA for 60 min. Sections were incubated for 60 min with a biotinylated or purified antibody, washed three times with PBS/BSA and labeled for 60 min with a secondary reagent. The primary antibodies and the second labeling reagents are described in **Table 1**. Sections were mounted with polyvinyl alcohol mounting media with DABCO (Sigma-Aldrich) to prevent fading. Anti-*Leishmania* antibodies (clone V121, MHOM/IL/67/Jericho II) were purified from rabbit serum (a generous gift from Emanuela Handman, WEHI, Australia) (11). Immunofluorescence images were visualized using UltraView Spinning disk confocal microscope with Velocity Software (Perkin Elmer, MA, USA). All the images were processed using ImageJ version 1.50i (ImageJ, USA).

Quantitative Real-Time PCR

RNA was extracted from sorted liver macrophages and from tissue culture by using Tri-Reagent (Sigma-Aldrich) and RNA

TABLE 1 | Primary and secondary antibodies used in confocal microscopy.

	Host	Target	Fluorochrome	Clone	Supplier
Primary antibodies	Rabbit	*L. major*	–	V121	M. Mack, Regensburg
	Rat	CD11b	Biotin	M1/70	BD Biosciences
	Mouse	iNOS	–	6/iNOS/NOS Type II	BD Biosciences
	Rat	CD206	–	MR5D3	BD Biosciences
	Rat	IL-6	Biotin	MP5-32C11	BioLegend
Secondary reagents	Rat	Streptavidin	Alexa Fluor 546	–	Thermo Fisher Scientific
	Donkey	Rat IgG	Alexa Fluor 647	–	Thermo Fisher Scientific
	Goat	Rabbit IgG	Alexa Fluor 488	–	Thermo Fisher Scientific
	Goat	Mouse IgG	Alexa Fluor 647	–	Thermo Fisher Scientific

isolation mini kit (Bioline, Alexandria, Australia) according to the manufacturer's instructions. RNA was stored in RNase-free water at −80°C. The QuantiTect Reverse Transcription Kit (Qiagen, Melbourne, VIC, Australia) was used to reverse-transcribe up to 1,000 ng total RNA. cDNA (2 µL) was amplified by Quantitative real-time PCR on the Rotor-Gene Q qPCR instrument (Qiagen) with 10 µl reactions using the SensiFAST™ SYBR No-Rox Kit (Bioline). The appropriate oligonucleotide primers were listed in **Table 2**, and the reaction was performed under the following conditions: samples were heated at 95°C for 3 min and amplified with 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 30 s. Reactions were performed in duplicate and gene expression levels were normalized to β-actin. Relative gene expression between samples was calculated using the $2^{-\Delta\Delta Ct}$ calculation method.

Immunoblotting

Cells were lysed in RIPA lysis buffer (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 10 mmol/l phenylmethylsulfonylfluoride, 1 mmol/l EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) for 30–40 min on ice. Protein concentrations were determined using Pierce™ BCA protein assay kit (Thermo Scientific). Proteins were resolved by SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore, VIC, Australia). Membranes were blocked for 1 h at room temperature in 5% skim milk in 0.1% TBS/0.1% Tween20 and then incubated overnight with rabbit polyclonal antibodies to IL-6Rα (Sino Biological, Beijing, China), gp130 (R&D Systems), phospho-STAT6 (R&D Systems) and phospho-STAT3 (Cell Signaling Technology), STAT-3 (Cell Signaling Technology, QLD, Australia), and goat polyclonal antibodies to STAT6 (R&D systems). β-Actin (Abcam) was used as loading control.

Membranes were incubated with appropriate antigoat or antirabbit secondary antibodies (Santa Cruz, QLD, Australia) for 1 h at room temperature. Membranes were washed, incubated with Western Lightning Plus Enhanced Chemiluminescence Solution (PerkinElmer, Woodbridge, ON, Canada) for 1 min and exposed to Amersham Imager 600 (GE Healthcare Life Sciences, NSW, Australia) for 5 s to 10 min. For the analysis of the ratio of phosphorylated/non-phosphorylated transcription factors blots were stripped using mild conditions (Thermo Fisher Scientific) and reprobed with the appropriate antibodies. The density of the specific bands was quantified using Image J software (ImageJ, USA).

TABLE 2 | Primers used for qPCR characterizing monocytes.

Gene	Forward primer	Reverse primer	Product size (bp)
Arg-1	ATGGAAGAGAC CTTCAGCTAC	GCTGTCTTCCCA AGAGTTGGG	224
iNOS	GGAATCTTGGA GCGAGTTGT	CCTCTTGTCTTT GACCCAGTAG	99
CD206	TGCAAAGCTATA GGTGGAGAGC	ACGGGAGAAC CATCACTCC	164
IL-6	AGTTGCCTTCTT GGGACTGA	TCCACGATTTC CCAGAGAAC	159
CD115	TCATTCAGAGCC AGCTGCCCAT	ACAGGCTCCCA AGAGGTTGACT	560
STAT3	CAAGCCTTTCC TGACAGAGG	AGACAATGTC CTCACTGCCC	221
STAT6	CATCTGAACCG ACCAGGAACT	CTCTGTGGGG CCTAATTTCCA	135
IL-6R	TGGGACCCGAG TTACTACTT	TGGATGACGCAT TGGTACTG	110
β-Actin	AGAGGGAAATCG TGCGTGAC	CAATAGTGATGAC CTGGCCGT	138

Statistical Analysis

Statistical evaluation of liver weight, limiting dilution, RT-PCR, and immunohistochemical analysis results were presented as mean ± SD, as appropriate. Results were analyzed using GraphPad prism 5 software (Graphpad Software, San Diego, CA, USA) by Mann–Whitney tests for samples with unknown and potentially disparate variances, or by one-way ANOVA followed by *post hoc* analysis with Tukey's test with $p < 0.05$ accepted as a level of statistical significance.

DISCUSSION

Tumor necrosis factor is a pleiotropic cytokine originally named after its proposed tumoricidal effects that has since been identified as a central effector cytokine with a broad range of biological activities such as induction of cell death, modification of cell migration, and regulation of DCs differentiation *in vitro* (24). Interestingly, its presence has been shown to be irreplaceable for effective immune responses to the bacterial or parasitic intracellular pathogens such as *Mycobacterium tuberculosis*, *Listeria monocytogenes*, or *L. major* but the underlying mechanisms that lead to this susceptibility are still not clear (10, 25–27).

After deletion of the *tnf* gene, normally resistant B6.WT mice are unable to control a cutaneous infection with *L. major* BNI (10). They develop a progressive infection that ultimately spreads to visceral organs including the liver. In our detailed analysis of this organ during *L. major* infection, we found that after day 21 after infection the size in B6.TNF$^{-/-}$ mice was enlarged significantly and increasing numbers of viable *L. major* parasites could be detected while B6.WT controls essentially remained parasite free. The progressive infection that was caused by the absence of TNF was accompanied by a strong increase in the proportion of Mo-Ms that showed clear signs of alternative activation. The marked expression of IL-6 prompted us to analyze the interaction of IL-6 and TNF during macrophage differentiation and the signaling pathways *via* IL-6Ra, gp130, and STAT3 and 6 *in vitro*. Our results point to a role of IL-6 facilitating macrophage differentiation downstream of TNF.

Macrophages, while acting as a major reservoir for *L. major* parasites (28), are also potent effector cells that kill parasites *in vivo* with a strong production of NO (29, 30). In the skin and draining local lymph nodes, the tissues predominantly analyzed in *L. major* infection, resident macrophages are initially relevant for the immune response but are quickly outnumbered by inflammatory monocyte derived DCs (31–33). In the liver, which constitutes a major target organ of a visceralized *L. major* infection in immune-incompetent mice, the inflammatory infiltration of monocytic cells has not yet been addressed in detail. In our study, we defined tissue resident KC, recruited inflammatory monocytes (Mo) and the cell types Mo differentiate into, Mo-DCs and Mo-M according to their expression of the marker molecules CD45, F4/80, CD11b, and Ly6C (13–15) and analyzed the dynamic of these cell populations in response to *L. major* infection in the absence of TNF. Fate-mapping has demonstrated that liver-resident F4/80^{+} KC are derived from embryonic cells and self-maintain independently from hematopoietic input under non-inflammatory steady state conditions (34). These cells have been shown to have an anti-inflammatory function that is abrogated by inflammation (35). Only hours after inoculation with *L. monocytogenes,* KC become infected and rapidly undergo necroptosis, thereby triggering the recruitment of monocytes (13). Unsurprisingly, in the slower moving *L. major* infection we did not see a significant decline of the numbers of KC nor did we detect a significant genotype-dependent changes between B6.WT and B6.TNF$^{-/-}$ during the course of infection (with the exception of day 21 after infection). Interestingly, while the number of liver Mo increased in correlation with the lesion size but independent of the mouse genotype, the population of Mo-M saw a significant increase in TNF-deficient mice but was hardly detectable in B6.WT mice. Since Mo-M in the liver differentiate out of the population of Mo in response to inflammatory signals (13) this observation could point to an absence of these signals in B6.WT mice due to an infection that is contained in skin and draining LN or potentially, to a contributing role of TNF in the differentiation (12).

Monocytes are present in form of steady-state precursors in the peripheral circulation and are recruited to organs such as the liver during inflammation (34). Here they differentiate to Mo-M and Mo-DCs in response to inflammatory cues. In the experiments presented here, the population of Mo increased after day 35 p.i. in line with the increased footpad swelling in both groups of mice. Numbers of Mo began to decrease at day 42 p.i. in B6.WT indicating that during the resolution phase of infection the recruitment of Mo ceased. However, in B6.TNF$^{-/-}$ mice Mo continued to be recruited and to differentiate into Mo-M resulting in a strong presence of this cell type in the late phase of infection. Like TNF-deficient mice the highly susceptible BALB/c mice fail to control leishmaniaisis. However, Mo-M are not found in *L major* infected BALB/c mice, which supports the notion that an accumulation of Mo-M is due specifically to the absence TNF. In previous experiments, a similar Mo-M population was observed in the skin and the draining lymph node of B6.TNF$^{-/-}$ mice during *L. major* BNI infection. It exhibited a phenotype that was CD11b^{+}Ly-6C^{low}CCR2^{low}iNOS^{low} and harbored a large number of parasites (11). In further detailed mechanistic analyzes it could be demonstrated that these cells coexpressed iNOS with high levels of CD206 and Arg-1, which indicated an M2-like phenotype (12). This finding was seemingly contradicting a long line of publications that showed that TNF was supporting the expression of iNOS. The role of TNF in the induction of iNOS and the effector molecule NO during the innate immune response to *L. major* was investigated initially in *in vitro* models (36). It could be shown that it activated macrophages and synergized with IFN-γ to induce effector functions (8, 37). However, in more complex *in vivo* infection models, the well-defined role of TNF in the iNOS-inducing cytokine network determined *in vitro* became controversial (38) while the central effector role of NO remained undisputed (29, 30). A *L. major* infection of a TNFR1-negative mouse strain showed that in absence of this proinflammatory signaling pathway these mice developed persistent lesions but controlled the pathogen (38). Further investigations, using TNFR2- and TNFR1/2-deficient mice clearly demonstrated that the TNFR2 signaling pathway lacked a clear inflammatory function while the outcome of the infection of mice deficient for both TNFR1 and TNFR2 was similar to the TNFR1-negative strain (39). Unexpectedly, it became clear that the expression of iNOS was sustained in these mice (39). Finally, infection experiments using genetically pure TNF-negative C57BL/6 mice resulted in a progressive and ultimately fatal, infection (10) despite a strong Th1 response which was characterized by a hyper-expression of IFN-γ and the presence of iNOS (9, 11). The differences in the published clinical outcomes of these infection experiments were probably due to variations of the genetic background of the parasite strains (40) in combination with a contaminating presence of congenic regions (41) in the genomes of the TNFR1- and 2-deficient mice (39).

Recently, the apparent contradiction of the presence of iNOS in TNF-deficient mice and their concurrent susceptibility to *L. major* BNI infection could be explained with the observation that TNF caused a direct suppression of Arg-1 expression and of other molecules associated with an alternative activation of myeloid cells. In TNF-negative mice the number of Arg1^{+} cells was increased in skin and draining LN and in the absence of TNF a coexpression of Arg-1 and iNOS could be detected. Since both enzymes share L-arginine as substrate a coexpression in macrophages caused competition for the substrate. Consequently, loss

of TNF significantly reduced the production of NO, resulting in fatal leishmaniasis (12). Interestingly, in this model the CD11b$^+$ Arg1$^+$ cells isolated from skin and draining LN of *L. major* BNI-infected B6.WT and B6.TNF$^{-/-}$ mice were predominately coexpressing CD11c$^+$ (12) and had therefore a phenotype that had been described earlier in the leishmanial model (42). This aspect was not addressed in the present study but it should be noted that the concept of inflammatory versus alternative activation can also be observed in DC (43). Taken together, our conclusive detection of M2 macrophages in the liver has strengthened the concept that the M2-suppressing role of TNF is not organ- or tissue-specific and, together with the observation of M2-like cells in the spleen of *L. monocytogenes* infected TNF-deficient mice, supports the notion of a fundamental, yet so far undescribed biological activity of this cytokine (12, 44, 45).

In the present study, we confirmed that IFN-γ was detectable at a higher level in B6.TNF$^{-/-}$ mice than in B6.WT mice (9). Similarly, MCP-1 (46), a pivotal cytokine implicated in recruitment and activation of monocytes (47) was overexpressed in the serum of TNF-deficient mice after *L. major* BNI infection. However, most interestingly, we could show that the major proinflammatory cytokine IL-6 was significantly overexpressed. Historically, after it had been demonstrated that these cytokines were involved in the acute phase reaction (48) this pleiotropic cytokine has been grouped with IL-1 and TNF as a classical proinflammatory mediator that appears early in the immune response (49). The interplay of TNF and IL-6 has been addressed in *in vitro* experiments using DC and macrophages. An activation of TNF-negative bone marrow derived DC resulted in the secretion of a decreased amount of IL-10 but IL-6 production remained unchanged (50). An infection with *L. major* or *L. donovani* with subsequent activation with LPS resulted in a strong IL-6 expression (51). Finally, IL-6 can downregulate the expression of proinflammatory cytokines including TNF (52) and it has been described to skew the differentiation of monocytes to macrophages (53). This action can be reversed by the additional presence of TNF (16) and this activity is caused by opposite effects of these cytokines on M-CSF receptor expression and internalization. One major activities of IL-6 could potentially be important *in vivo* during the immune response to *L. major* BNI. The presence of IL-6 has been reported to block the differentiation of regulatory T cells (Tregs) and to support the generation of IL-17$^+$ Th cells (Th17) (54). However, an infection of mice which were genetically deficient for IL-6 with *L. major* demonstrated an effective and protective antileishmanial response (55) despite originally having been described to have an impaired antibacterial, antiviral, and acute phase response (56); a more detailed analysis of the adaptive and the innate branches of the immune system could not detect major deficiencies in these mice (57).

In the classical IL-6 signaling pathway, IL-6 signal transduction requires the formation of a trimer consisting of ligand, the IL-6 receptor (IL-6R) α-chain and the signal transducing membrane glycoprotein gp130. IL-6 binds to membrane-bound IL-6R (mIL-6Rα) which recruits its signaling component gp130. This combination contributes to the plethora of pro- and anti-inflammatory functions (58). Unlike the ubiquitously distributed gp130, mIL-6Rα is expressed exclusively on the surface of hepatocytes and some myeloid and lymphoid cell populations such as monocytes and T cells (59). However, most proinflammatory functions of IL-6 are mediated through a second IL-6-dependent signaling pathway *via* the soluble IL-6R in the IL-6 trans-signaling pathway which can trigger IL-6-mediated response in cells negative for mIL-6R (58).

In our study, stimulation with IL-6 increased the expression of mIL-6R and gp130 but the additional presence of TNF caused a rapid downregulation of these receptor molecules and a significant reduction of STAT3 and 6 phosphorylation. In the context a *L. major* infection of TNF-competent animals, the role of IL-6 in the determination of the outcome of macrophage differentiation seems to be negligible (57). This changes however, in the absence of TNF. Now a strong expression of IL-6 contributes significantly to the dysregulation that leads to a fatal presence of M2 macrophages in skin (12), lymphoid organs and visceral organs such as the liver. Potentially our finding could have ramifications for the meanwhile ubiquitous anti-TNF and IL-6 therapy in human chronic inflammatory disorders. Therefore, the complex regulatory interactions of IL-4, IL-6, and TNF in macrophage differentiation need to be investigated at the molecular level in more detail (12, 16, 53).

Taken together, we show that in the absence of TNF an infection with *L. major* BNI quickly spreads to the liver. The infection causes a strong infiltration of monocytes and leads to the presence of an accumulating number of Mo-Ms which display an M2 phenotype, express IL-6 and harbor parasites which contributes to the fatal outcome of leishmaniasis in these mice. Furthermore, we show that in the absence of the M1 determining cytokine TNF, anti-inflammatory function of IL-6 can dominate the differentiation of monocytes and provide a first insight that this cytokine can contribute to the skewing of monocytes to a M2 phenotype.

ETHICS STATEMENT

Animal care and experiments were approved by the animal ethics committee of the University of Tasmania, Hobart, Australia (Animal Ethics Numbers: A13934 and A13935).

AUTHOR CONTRIBUTIONS

SH: designed experiments, carried out experiments, acquired data, analyzed data, and edited the manuscript. CM and JD: carried out experiments, analyzed data, and edited the manuscript. WW: edited the manuscript and revised the final version. AL: designed experiments, edited the manuscript, and revised the final version. HK: conception of the project, designed experiments, analyzed data, wrote the manuscript, edited the manuscript, and revised the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00001/full#supplementary-material.

FIGURE S1 | Macrophages derive from recruited monocytes. Flow cytometric gating strategy and analysis of the three distinct subsets of CD45$^+$ F4/80$^+$ liver macrophages after *L. major* BNI infection.

FIGURE S2 | Analysis by immunofluorescence and flow cytometry in *L. major*-infected BALB/c mice. **(A)** Immunofluorescence staining of CD11b (gray), iNOS (green), *L. major* (red), and DAPI (blue) in liver tissue of BALB/c mice postinfection. Results represent one of three biological repeats. **(B)** Flow cytometric analysis revealed the changes of three different CD45$^+$F4/80$^+$ liver macrophage populations based on identical gates as in **Figure 3** over the course of *L. major* BNI infection.

FIGURE S3 | The expression of CD11c on monocytic cells from B6.WT and B6.TNF$^{-/-}$ mice. **(A)** Flow cytometric analysis of liver Ly6C$^+$CD11b$^+$ cells obtained from *L. major* BNI-infected B6.WT and B6.TNF$^{-/-}$ mice. The populations of Ly6C$^+$CD11b$^+$ cells were gated and the CD11c expression of these cells is shown in a representative example. **(B)** Quantification by flow cytometry of the expression of CD11c on the Ly6C$^+$CD11b$^+$ cells upon *L. major* BNI infection. The data represent the median of mean intensity of fluorescence (MIF) of CD11c expression by Ly6C$^+$CD11b$^+$ cells upon *L. major* BNI infection. Results represent means ± SD, $n = 15$. $^*p < 0.05$ comparing to B6.WT group, two-tailed Mann–Whitney *U*-test.

FIGURE S4 | The morphology of Mo and Mo-M from B6.WT and B6.TNF$^{-/-}$ mice. **(A)** Representative Romanowsky (Diff-Quik) stain of liver sections are shown ($n = 3$ per group). After day 42 p.i., *L. major* BNI parasites were found inside and outside of the cells (arrowheads) in the liver of B6.WT and B6.TNF$^{-/-}$ mice (magnification 400×).

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Monocyte-Derived Signals Activate Human Natural Killer Cells in Response to *Leishmania* Parasites

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Activated natural killer (NK) cells release interferon (IFN)-γ, which is crucial for the control of intracellular pathogens such as *Leishmania*. In contrast to experimental murine leishmaniasis, the human NK cell response to *Leishmania* is still poorly characterized. Here, we investigated the interaction of human blood NK cells with promastigotes of different *Leishmania* species (*Leishmania major*, *Leishmania mexicana*, *Leishmania infantum*, and *Leishmania donovani*). When peripheral blood mononuclear cells or purified NK cells and monocytes (all derived from healthy blood donors from Germany without a history of leishmaniasis) were exposed to promastigotes, NK cells showed increased surface expression of the activation marker CD69. The extent of this effect varied depending on the *Leishmania* species; differences between dermotropic and viscerotropic *L. infantum* strains were not observed. Upregulation of CD69 required direct contact between monocytes and *Leishmania* and was partly inhibitable by anti-interleukin (IL)-18. Unexpectedly, IL-18 was undetectable in most of the supernatants (SNs) of monocyte/parasite cocultures. Confocal fluorescence microscopy of non-permeabilized cells revealed that *Leishmania*-infected monocytes trans-presented IL-18 to NK cells. Native, but not heat-treated SNs of monocyte/*Leishmania* cocultures also induced CD69 on NK cells, indicating the involvement of a soluble heat-labile factor other than IL-18. A role for the NK cell-activating cytokines IL-1β, IL-2, IL-12, IL-15, IL-21, and IFN-α/β was excluded. The increase of CD69 was not paralleled by NK cell IFN-γ production or enhanced cytotoxicity. However, prior exposure of NK cells to *Leishmania* parasites synergistically increased their IFN-γ release in response to IL-12, which was dependent on endogenous IL-18. $CD1c^+$ dendritic cells were identified as possible source of *Leishmania*-induced IL-12. Finally, we observed that direct contact between *Leishmania* and NK cells reduced the expression of CD56 mRNA and protein on NK cells. We conclude that *Leishmania* activate NK cells *via* trans-presentation of IL-18 by monocytes and by a monocyte-derived soluble factor. IL-12 is needed to elicit the IFN-γ-response of NK cells, which is likely to be an important component of the innate control of the parasite.

Keywords: *Leishmania*, natural killer cells, monocytes, innate immunity, human cutaneous and visceral leishmaniasis

Abbreviations: DC, dendritic cell; NK, natural killer; IFN, interferon; ILC, innate lymphoid cell; ft-lysate, freeze–thaw lysate; PBMC, peripheral blood mononuclear cell; Pfa, paraformaldehyde; Th, T helper lymphocytes; TW, transwell.

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INTRODUCTION

Natural killer (NK) cells are members of the innate lymphoid cells (ILCs), which do not express rearranged antigen receptors and are characterized by an absent or only slow clonal expansion. Based on their ability to rapidly release the T helper (Th) 1 signature cytokine interferon (IFN)-γ upon stimulation, NK cells belong to the type 1 ILCs. However, in contrast to other ILC1s NK cells are developmentally dependent on eomesodermin (eomes), require interleukin (IL)-15 instead of IL-7 for cell survival, and kill virally infected or tumor cells by exocytosis of cytotoxic granules [reviewed in Ref. (1)].

As a first sign of activation mouse and human NK cells upregulate the expression of surface CD69, a type II C-type lectin absent from resting NK cells (2–5). CD69 is a costimulatory molecule which is able to enhance NK cell effector functions (6). Depending on the activation signal NK cells can also produce soluble mediators other than IFN-γ including pro- [e.g., tumor necrosis factor (TNF)] or anti-inflammatory cytokines (e.g., IL-10), growth factors (e.g. granulocyte-monocyte colony-stimulating factor), and chemokines (e.g., CCL2-5 and CXCL8) [reviewed in Ref. (7)]. Based on these properties, mouse and human NK cells exert various immunoregulatory functions and contribute not only to the antitumor response, but also to the defense against viruses, bacteria, fungi, and parasites (8–15).

To acquire full effector capacity NK cells require priming by cytokines and accessory cells such as dendritic cells (DCs) (16–19). Cytokines that activate human NK cells include IFN-α/β (20, 21), IL-1 (22), IL-2 (23), IL-12 (24), IL-15, IL-18 (25), IL-21 (26), and IL-27 (27). In most cases, a combination of at least two cytokines is needed to achieve a full NK cell response. In addition, NK cells can also be activated by ligation of pattern recognition receptors such as toll-like receptors (TLRs) or by differential engagement of activating and inhibitory NK cell receptors [reviewed in Ref. (7, 28)]. One of the NK activating receptors, NKp46 [*syn.* natural cytotoxicity triggering receptor (NCR)1] represents the most specific NK cell marker in mammalian organisms (29). Besides NKp46, CD56, also known as neural cell adhesion molecule 1 (NCAM1), is commonly used to define human NK cells as $CD56^{+}CD3^{-}$ cells. In humans, the two main NK cell effector functions, cytotoxicity and cytokine production, have been associated with two distinct NK cell subsets: $CD56^{bright}CD16^{-}$ NK cells that predominate in lymphatic tissues and are specialized in IFN-γ secretion, and $CD56^{dim}CD16^{+}$ NK cells that are mainly present in peripheral blood and show cytotoxic activity (30, 31). However, dependent on the mode of activation, both NK cell subpopulations may also exhibit the "non-specialized" NK cell effector function (32–34). The function of CD56 on NK cells is largely unknown, but published data indicate a relationship between the height of CD56 expression and the degree of activation (35).

Leishmania are protozoan parasites with a dimorphic cell cycle. The flagellated, promastigote form of *Leishmania* is transmitted by the bites of sand flies. In the mammalian host, the promastigotes are endocytosed by phagocytic cells and transform into the aflagellated stage (amastigotes) that replicates within phago(lyso) somes (36). Depending on the *Leishmania* species and strain and the immune response and genetic background of the host, infections can be asymptomatic, lead to self-healing or chronic cutaneous leishmaniasis (CL; e.g., *Leishmania major*, *Leishmania mexicana*) or non-healing, progressive mucocutaneous leishmaniasis (e.g., *Leishmania braziliensis*), or can cause visceral leishmaniasis (VL; *Leishmania infantum* and *Leishmania donovani*) due to systemic spreading of the parasites (37). Experimental animal models of CL and VL led to the identification of the key immune mechanisms required for the control of infection, which include the generation of IL-12 and TNF, the expansion of IFN-γ-producing $CD4^{+}$ and $CD8^{+}$ T cells and the induction of antileishmanial effector pathways such as inducible nitric oxide synthase (iNOS). By contrast, induction of macrophage-deactivating cytokines such as IL-10 and transforming growth factor (TGF) β as well as overshooting production of Th2 cytokines were associated with disease progression [reviewed in Ref. (38–40)]. Many of the above-mentioned mechanisms also hold true in human leishmaniasis, as biopsies of chronic CL lesions and leukocytes of VL patients displayed high IL-10 and TGFβ content, whereas cells of cured patients produced IL-12 and IFN-γ (41–46).

Natural killer cells were found to participate in the innate control of *Leishmania* in infected mice but were not essential for generating a Th1 response and ultimate healing of the disease [reviewed in Ref. (13)]. During later stages of VL, mouse NK cells showed adverse effects and inhibited protective immunity in an IL-10-dependent manner (47). The protective function of NK cells in murine leishmaniasis is largely due to their release of IFN-γ and subsequent stimulation of iNOS-dependent killing of parasites, as they were not able to recognize *Leishmania*-infected host cells as targets for direct cytolysis *in vitro* and *in vivo* (48). During the early phase of infection, NK cell activation in *Leishmania*-infected mice required DC- and TLR9-dependent production of IL-12, T cell-mediated release of IL-2, and the presence of IL-18 (18, 49, 50). In *L. major* infections of mice, IFN-α/β was necessary for full NK cell activation (51). *Leishmania* parasites failed to directly activate mouse NK cells (18).

Several observations argue for a protective role of NK cells also in human leishmaniasis. These include (a) a reduced NK cell number in the blood of patients with acute VL that was restored after successful chemotherapy; (b) the influx of NK cells into lesions of CL patients, who showed suppressed NK cell cytotoxicity during active disease, but positive response to treatment (52–54); and (c) a reduced number, TLR expression and IFN-γ and TNF-production by NK cells in patients with diffuse as compared with localized CL due to *L. mexicana* infection (55, 56). Unlike to murine NK cells, mechanisms of human NK cell activation are less clear. Some studies claimed indirect stimulation of human blood NK cells by accessory cells releasing cytokines after contact to *Leishmania* (57–59). Other reports suggested direct activation of NK cells by *Leishmania* in a lipophosphoglycan (LPG)/TLR2-dependent or LPG-independent manner (60, 61) or even excluded a NK cell IFN-γ response in *Leishmania*- or *Leishmania* antigen-stimulated peripheral blood mononuclear cells (PBMCs) (62, 63).

To define the activation signals required for a human NK cell effector response to *Leishmania* parasites and to address the question whether there are differences between *Leishmania* species, we performed cocultures of human PBMCs or highly purified

cell populations from healthy German volunteers with four different *Leishmania* species and analyzed the NK cell response. The data obtained show that NK cells cannot be directly activated by *Leishmania* promastigotes but require cytokine signals from monocytes.

MATERIALS AND METHODS

Leishmania Parasites

Promastigotes of the following *Leishmania* species and strains were used: *L. infantum* MHOM/DE/98/LUB1 [isolated in our laboratory from bone marrow (BM) of a German patient with VL] (64), *L. infantum* MHOM/DE/2012/VA21737 (isolated in our laboratory from BM of a German patient with VL), *L. infantum* MHOM/DE/2014/VA20763 (isolated in our laboratory from the skin lesion of a Croatian patient with CL), *L. infantum* MCAN/ES/2010/BON (isolated in our laboratory from peripheral blood of a Swiss dog with VL), *L. major* MHOM/IL/1981/FEBNI (isolated from the skin lesion of an Israeli patient with CL) (65), *L. mexicana* MNYC/BZ/1962/M379 [isolated from a vesper rat (ATCC® 50156™); kindly provided by Sigrid Roberts, Hillsboro, OR, USA] and *L. donovani* (MHOM/SD/1962/1S-CL2D clonal line LdBob; originally isolated from a Sudanese patient with VL; kindly provided by Steve Beverley, St. Louis, MO, USA) (66). In case of *L. infantum,* the strain MHOM/DE/98/LUB1 was used unless otherwise stated. For all strains, aliquots of a promastigote culture (derived from amastigotes isolated from experimentally infected mice) were frozen after only two to three *in vitro* passages for expansion. All experiments were performed with freshly thawed aliquots of these promastigotes which were grown at 28°C/5% CO_2/95% humidified air in modified Schneider's *Drosophila* insect medium as described (67) for a maximum of six *in vitro* passages. For fixation of promastigotes, parasites were incubated for 10 min in 4% paraformaldehyde (Pfa) at room temperature (RT) followed by three washes with PBS. Freeze–thaw (ft) lysates of *Leishmania* promastigotes were generated by four cycles of freezing at −80°C and thawing at RT.

PBMC Preparation and Purification of Different Cell Populations from the Blood

Mononuclear cells from EDTA-anticoagulated human peripheral blood (PBMCs) of healthy human volunteers living in Erlangen and without any history of leishmaniasis were isolated using density centrifugation (1.077 g/ml Biocoll, Biochrom). For generation of autologous plasma, blood was first centrifuged, and the resulting plasma supernatant (SN) was heat inactivated (56°C, 30 min) and filtered, while the remaining cell pellet was resuspended in PBS to proceed with PBMC preparation. Different cell populations of PBMCs including $CD3^+CD56^-$ T cells, $CD19^+$ B cells, $CD56^+CD3^-$ NK cells, $CD14^+$ monocytes, $CD14^{high}CD16^-CCR2^{high}$ classical monocytes, $CD14^{low}CD16^{high}CCR2^-$ non-classical monocytes, and $CD14^{high}CD16^{low}CCR2^+$ intermediate monocytes were purified by cell sorting using the FACS Aria II instrument (BD Biosciences). Purification of $CD1c^+$ DCs was performed as described with slight modifications (68). Briefly, leukocyte reduction cones retrieved from anonymous healthy adult donors were used as source for PBMCs. $CD1c^+$ DCs were then enriched with the EasySep Pan-DC Pre-Enrichment Kit (Stemcell Technologies) and isolated by cell sorting using an FACS Aria II (BD Bioscience) as $CD3^-CD14^-CD19^-CD20^-CD56^-$HLA-$DR^+CD1c^+CD11c^+$ cells. All sorted cell populations showed a purity of >96%. For immunofluorescence analysis, monocytes were purified by negative selection using the Monocyte Isolation Kit II (Miltenyi Biotech) following the manufacturer's instructions.

Culture and Stimulation of Cells

Human leukocytes with or without *Leishmania* promastigotes were cultured in 96-well plates (PBMCs: 5×10^5 cells/well, 200 µl; purified NK cells: $0.5–1.5 \times 10^5$/well depending on the total recovery, 200 µl), 48-well plates (PBMCs: 10^6 cells/well, total volume 500 µl; purified monocytes: 5×10^5/well, 500 µl), or in 24-well plates with a transwell (TW) insert (0.4 µm pore size; Corning, Wiesbaden, Germany; 5×10^5 NK cells/insert, $0.5–1 \times 10^6$ monocytes/bottom well) at 37°C and 5% CO_2/95% humidified air for 20 h using RPMI1640 (Gibco™ Life Technologies; ThermoFisher Scientific, cat. no. 21875-034) supplemented with 10 mM HEPES (ThermoFisher Scientific), 50 µM 2-mercaptoethanol (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (ThermoFisher Scientific), and 10% heat-inactivated autologous plasma. *Leishmania* promastigotes were added at different parasite/host cell ratios [multiplicity of infection (MOI) 0.2, 1, 5, 10, or 33]. Pfa-fixed *Leishmania* promastigotes or *Leishmania* freeze–thaw lysate were used in analogy to the MOI of viable parasites. When different purified leukocyte populations (e.g., NK cell and monocytes) were cocultured with *Leishmania* promastigotes, cell populations were used at the same ratios as present in non-separated PBMCs of this donor, unless otherwise stated. In some of the experiments, leukocyte/promastigote cocultures were incubated in the presence of specific blocking antibodies (Abs) against different cytokines [sheep-anti-IFN-α (1:350; 10,000 neutralizing units/ml) or sheep-anti-IFN-β antiserum (1:3; 1,000 neutralizing units/ml), obtained from the NIAID Repository, Braton Biotech Inc., Rockville, MD, USA; mouse-anti-IL-1β, 10 µg/ml, CRM56, eBioscience/ThermoFisher Scientific; mouse-anti-IL-2, 1 µg/ml, AB12-3G4, eBioscience/ThermoFisher Scientific; rat-anti-IL-6, 5 µg/ml, MQ2-13A5, BioLegend; mouse-anti-IL-12/IL-23p40, 20 µg/ml, C11.5, BioLegend; mouse-anti-IL-15, 1 µg/ml, ct2n, eBioscience/ThermoFisher Scientific; and mouse-anti-IL-18, 1.5 µg/ml, 125-2H, MBL] or the respective control sera or isotype control Abs. To verify the efficacy of the Ab-treatment, cells were stimulated with the appropriate recombinant cytokine. Cytokines/chemokines used were as follows: huIFN-α and huIFN-β (100 U/ml; NIAID Repository, Braton Biotech Inc., Rockville, MD, USA), rhuIL-1β (20 ng/ml, PeproTech), rhuIL-2 (200 U/ml; Chiron, Emeryville, CA, USA), rhuIL-4 (250 U/ml, PeproTech), rhuIL-6 (10 ng/ml, BioLegend), rhuIL-8 (10 ng/ml, BioLegend), rhuIL-12p70 (10 ng/ml, PeproTech), rhuIL-15 (12 ng/ml, PeproTech), rhuIL-18 (10 ng/ml, MBL), and rhuMIP1α (20 ng/ml, BioLegend). In addition, PBMCs or purified NK cells were stimulated with cell culture SNs (vol/vol 20–80%) of previous leukocyte/*Leishmania* cocultures of the same donor. In some cases, the SNs were incubated with blocking Abs to cytokines (see

above) for 1–2 h at 37°C and 5% CO_2/95% humidified air before being added to freshly isolated cells.

As a positive control for the stimulation of DCs resiquimod (R848, 5 µg/ml, InvivoGen), a TLR7/8 agonist, was used.

Cytokine Measurements in Cell Culture SNs

Commercial ELISA kits were used for determining the content of human IL-2, IL-6, IL-18, or IL-12p35/70 (eBioscience/ThermoFisher Scientific) and IL-10 or IL-12/IL-23p40 (BioLegend) in culture SNs. Multiplex ELISA was performed using the ProcartaPlex® Multiplex Immunoassay (Human Cytokine/Chemokine/Growth Factor Panel 1, 45 plex; eBioscience/ThermoFisher Scientific), which was analyzed with a MAGPIX® instrument and the xPONENT® software (eBioscience/ThermoFisher Scientific).

Flow Cytometry

For surface phenotyping and cell sorting of freshly isolated or cultured leukocytes, fluorochrome-labeled or biotinylated Abs against the following antigens were used (all from eBioscience/ThermoFisher Scientific, unless otherwise stated): CD3ε (OKT3, FITC, PerCP-Cy5.5, biotinylated), CD11b (ICRF44, V450, BD Biosciences), CD11c (3.9, PerCP-eflour®710), CD14 (61D3, FITC), CD16 (CB16, PerCP-eflour®710, eflour®450), CD19 (HIB19, PE, eflour®450), CD25 (BC96, PE-Cy7), CD56 (CMSSB, PE-Cy7, APC), CD69 (FN50, PerCP-Cy5.5, BioLegend), CD192/CCR2 (K036C2, PerCP-Cy5.5, PE-Cy7; BioLegend), and CD335/Nkp46 (9E2, PE, Miltenyi Biotech). Staining with biotinylated Abs was followed by incubation with fluorochrome (FITC or APC)-labeled streptavidin (BD Biosciences) to allow detection.

Staining of blood $CD1c^+$ DCs was done as described with minor modifications (68). Briefly, after enrichment of human DCs with the EasySep Pan-DC Pre-Enrichment Kit (Stemcell Technologies), cells were stained with fluorochrome-coupled Abs against CD1c (L161, APC/Cy7, BioLegend), CD3 (UCHT1, BUV395, BD Bioscience), CD11b (M1/70, Alexa Fluor 700, BioLegend), CD11c (3.9, PE/Cy7, BioLegend), CD14 (HCD14, Alexa Fluor 700, BioLegend), CD19 (HIB19, V450, BD Bioscience), CD20 (2H7, eflour®450), CD56 (5.1H11, Brilliant Violet 421, BioLegend), CD123 (6H6, BV605, BioLegend), CD141 (1A4, Brilliant Violet 711, BD Bioscience), CD303a (201A, PerCP-Cy5.5, BioLegend), and HLA-DR (L243, Brilliant Violet 510, BioLegend) for 30 min on ice.

The specificity of the stainings was verified by use of isotype control Abs. Cells were analyzed with an FACS Canto II system and Diva 6.1.2 (both BD Biosciences) and FlowJo 10.0.7 (FlowJo LLC, Ashland, OR, USA) software. DCs were analyzed with an FACS LSRFortessa™. For intracellular staining of IFN-γ, GolgiStop™ (1:1,500 µg/ml, BD Biosciences) was added during the final 6–10 h of cell culture to prevent secretion of cytokines. After staining of surface molecules, cells were fixed by Cytofix/Cytoperm™ (BD Biosciences), washed twice with a saponin-containing buffer, and stained for intracellular accumulated IFN-γ (α-huIFN-γ, 4S.B3, APC) (18).

Confocal Laser Scanning Fluorescence Microscopy (CLSFM) of Infected Monocytes

After coculture of untouched purified monocytes with *Leishmania* promastigotes (MOI 10) for 20 h, 2.5×10^5 cells in 30 µl were transferred to the marked reaction field of adhesion slides (Marienfeld Laboratory Glassware) prepared as recommended by the manufacturer. After cell adhesion, slides were washed twice in PBS buffer and cells were fixed with 4% Pfa. Fixed monocytes were either directly stained or additionally permeabilized with methanol (−20°C) before staining. For IL-18 staining, non-specific binding sites were blocked with PBS/2% BSA/10% normal goat serum and cells were stained with mouse-anti-IL-18 monoclonal Ab (125-2H, MBL) overnight at 4°C. As specificity control, the mouse-anti-IL-18 mAb was pretreated with rhuIL-18 (1.2 µg/ml, 30 min, 37°C). All Abs were diluted in PBS/0.5% BSA/0.5% normal goat serum. After washing with PBS/0.1% Tween Alexa Fluor 568-conjugated goat anti-mouse secondary Abs (ThermoFisher Scientific) were added for 30 min at RT. Cell nuclei were visualized by DAPI staining. Slides were mounted in Vectashield (Vector laboratories) and cover slips, dried in the dark for at least 12 h at 4°C, and analyzed by CLSFM (LSM700, Zeiss) using a 63× objective. Image processing was performed using the ZEN software 2009 (Zeiss).

Cytotoxicity Assay

Peripheral blood mononuclear cells (with the percentage of $Nkp46^+CD3^-$ NK cells determined by flow cytometry) were added to K562 tumor target cells in NK cell/target cell ratios of 20:1, 10:1, 5:1, and 2.5:1. A standard chromium-51 release assay was performed (48). Briefly, K562 tumor cells were labeled with ~150 µCi ^{51}Cr (Perkin-Elmer) for 90 min. Cocultures of effector and target cells were incubated in complete RPMI1640 medium containing 10% heat-inactivated fetal calf serum (Sigma-Aldrich, cat. no. F-7524, lot. no. 036K3397) for 4 h. The release of ^{51}Cr into the SNs was measured as counts per minute (cpm) using a TopCount NXT microplate gamma counter (Perkin-Elmer). Based on the spontaneous (target cells alone) and the maximum release (^{51}Cr-labeled cells directly added to the LUMA measurement plate) % specific lysis was calculated as $(cpm_{sample} - cpm_{spontaneous})/(cpm_{maximum} - cpm_{spontaneous}) \times 100$.

RNA Preparation and Quantitative RT-PCR

Total RNA was prepared with the RNeasy Mini Kit (Qiagen). cDNA synthesis and quantitative RT-PCR analysis were performed (49) using the following assays: NCAM1 (CD56) (Hs00941830_m1), GAPDH (Hs02758991_g1).

Statistical Analysis

Results were displayed as mean ± SEM or as median and were statistically analyzed by the Mann–Whitney *U* test using GraphPad Prism software v.4. Significant differences between unstimulated and stimulated samples were marked by asterisks, significant differences between stimulated samples by diamonds. Significant *p* values are indicated as follows: $^{*,\#}p < 0.05$; $^{**,\#\#}p < 0.01$; $^{***,\#\#\#}p < 0.001$.

RESULTS

NK Cells within PBMCs, but Not NK Cells Alone Upregulate CD69 in Response to *Leishmania*

To investigate whether *Leishmania* parasites themselves and/or host cell-derived factors activate human NK cells, PBMCs of healthy volunteers without history of leishmaniasis were cultured in the presence of promastigotes of different *Leishmania* species. Blood NK cells gated as NKp46$^+$CD3$^-$ viable single cells were analyzed for surface expression of the early activation marker CD69 by flow cytometry after 6, 12, and 20 h of incubation. As induction of CD69 was most prominent after 20 h (Figure S1 in Supplementary Material) and the viability of NK cells decreased thereafter, this time point was chosen for further analyses. Stimulation with promastigotes of all *Leishmania* species tested induced upregulation of CD69 on human NK cells in a large number of different blood donors (total of 36), most of which were tested several times in independent experiments (**Figure 1A**). The average induction of CD69$^+$ NK cells by *L. infantum* was lower than by *L. major*, *L. mexicana*, and *L. donovani*. There were no differences observed between viscerotropic and dermotropic human strains or a canine strain of *L. infantum* (**Figure 1B**). The percentage of CD69$^+$ NK cells increased with the parasite/host cell ratio used (**Figure 1C**). Fixed or lysed parasites still caused an induction of CD69 on NK cells, which, however, was tentatively or significantly reduced when compared with viable parasites (**Figure 1D**). By contrast, CD69 induction on NK cells was abolished when *Leishmania* promastigotes were separated from PBMCs by a membrane (pore size 0.4 µm) (**Figure 1E**).

Having seen that direct contact between *Leishmania* and NK cells or other cell types within the PBMC culture was necessary to upregulate CD69, we next investigated whether the parasite was able to directly activate human NK cells. To this end, highly purified CD3$^-$NKp46$^+$ NK cells as well as whole PBMCs of the very same donor were stimulated by promastigotes (**Figure 2A**). Whereas NK cells within the PBMC/*Leishmania* coculture readily upregulated CD69, purified NK cells failed to do so, irrespective of the *Leishmania* species used. From these

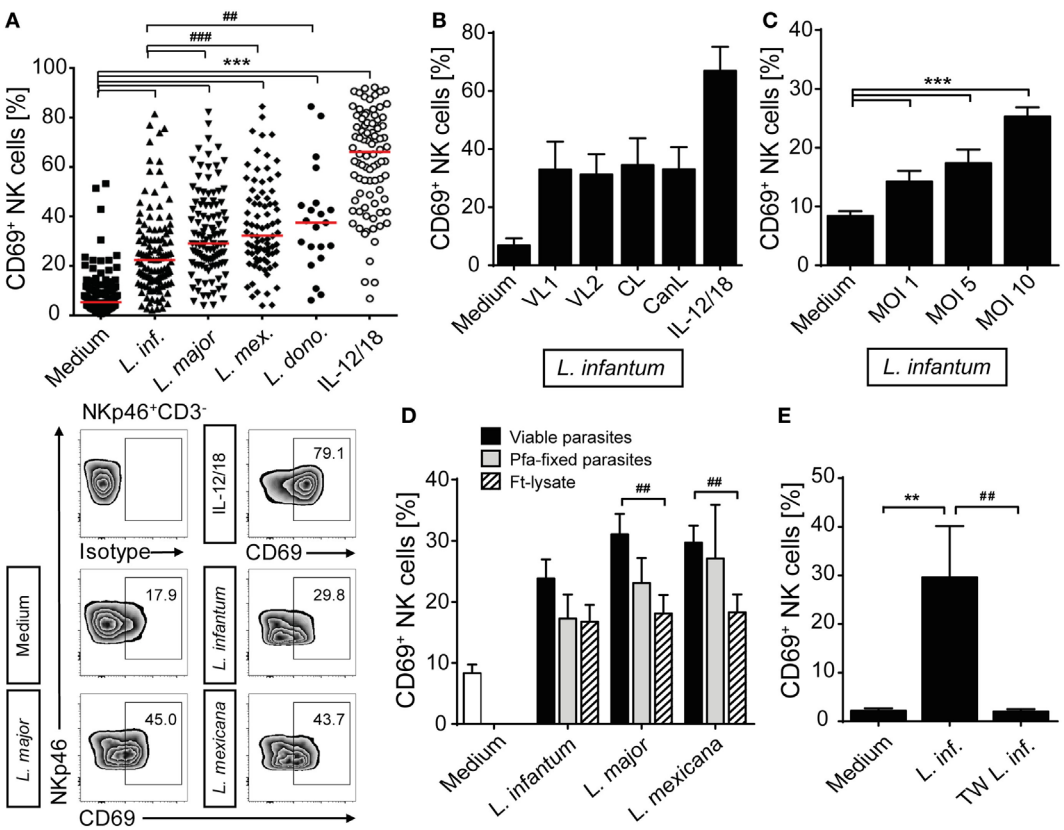

FIGURE 1 | CD69 is upregulated on natural killer (NK) cells after contact with *Leishmania*. Human peripheral blood mononuclear cells were cocultured for 20 h with *Leishmania* promastigotes of different species **(A)**, strains **(B)**, amounts **(C)**, or integrity **(D)**, and with or without host cell/parasite contact **(E)** or interleukin (IL)-12 and IL-18 (10 ng/ml) **(A,B)**, followed by surface expression analysis of CD69 on NKp46$^+$CD3$^-$ NK cells by flow cytometry. Unless otherwise indicated the multiplicity of infection (MOI) was 10. **(A)** Results of 129/117/107/83/21/88 blood samples for the six stimulations. Medians are indicated by red lines. FACS plots show results of NKp46$^+$CD3$^-$ NK cells of one representative experiment. **(B)** *Leishmania infantum* strains isolated from human patients with visceral leishmaniasis (VL) (VL1, VL2) or CL or a dog [canine leishmaniasis (CanL)]. Mean ± SEM of 9/9/9/9/7/9 donors. **(C)** Mean ± SEM of 119/43/17/119 donors for the four stimulations. **(D)** Mean ± SEM of 22/22/18/18 (viable), 8/9/6 [paraformaldehyde (Pfa)-fixed], and 17/15/14 [freeze–thaw lysate (Ft-lysate)] donors. **(E)** Mean ± SEM of six donors. Abbreviation: TW, transwell. $^{*,\#}p < 0.05$; $^{**,\#\#}p < 0.01$; and $^{***,\#\#\#}p < 0.001$ two-tailed Mann–Whitney *U* test.

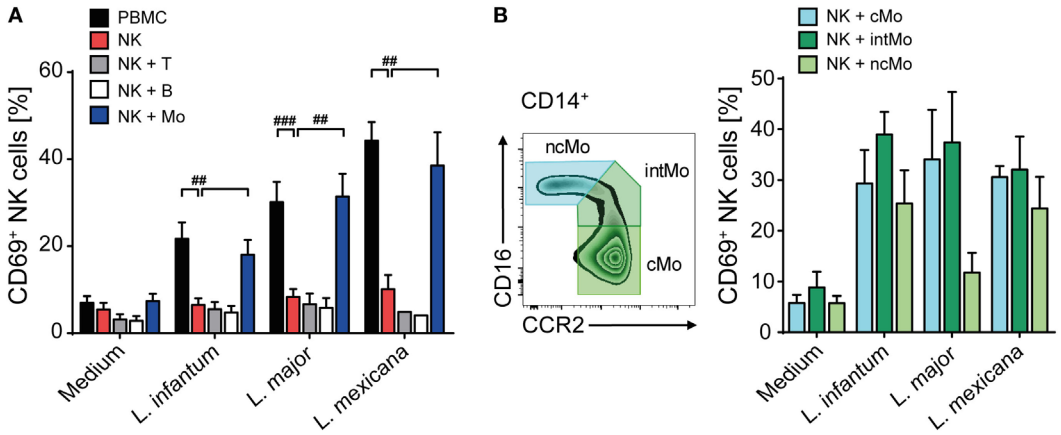

FIGURE 2 | Upregulation of CD69 on natural killer (NK) cells after coculture with *Leishmania* requires the presence of monocytes. **(A)** Peripheral blood mononuclear cells (PBMCs) or sorted NKp46$^+$CD3$^-$ NK cells with or without autologous, sorted CD3$^+$NKp46$^-$ T cells, CD19$^+$ B cells, or CD14$^+$ monocytes were cocultured with *Leishmania* spp. promastigotes (multiplicity of infection 10). **(B)** Using the surface markers CD16 and CCR2, classical, intermediate, or non-classical CD14$^+$ monocytes were sorted and cocultured with autologous sorted NK cells and *Leishmania* spp. promastigotes (MOI 33). After 20 h, surface expression of CD69 on NKp46$^+$CD3$^-$ NK cells was determined by flow cytometry. Mean ± SEM of **(A)** 15/14/14/7 (PBMC/NK + Mo), 15/14/14/4 (NK), and 7/7/6/2 (NK + T/NK + B) and **(B)** 5/5/4/4 donors for the four stimulations. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ two-tailed Mann–Whitney *U* test.

data we conclude that the upregulation of CD69 on NK cells in response to *Leishmania* is dependent on the presence of accessory cells.

Upregulation of CD69 on NK Cells Requires Infected Monocytes Trans-Presenting IL-18

To elucidate which additional cell population is needed to activate human NK cells in response to promastigotes, CD3$^+$Nkp46$^-$ T cells, CD19$^+$ B cells, and CD14$^+$ monocytes were sorted and added separately to a coculture of *Leishmania* and purified NK cells of the same donor. Addition of monocytes to the NK cell/parasite culture restored induction of CD69 on NK cells to a similar level as observed in whole PBMC cocultures, whereas addition of T or B cells did not support the expression of CD69 on NK cells (**Figure 2A**). As human CD14$^+$ monocytes are subdivided in classical (cMo, CD14^{high}CD16$^-$CCR2^{high}), intermediate (intMo, CD14^{high}CD16^{low}CCR2$^+$), and non-classical monocytes (ncMo, CD14^{low}CD16^{high}CCR2$^-$) (69), the three subpopulations were purified and evaluated for their capacity to induce CD69 on NK cells after a 20 h coculture with *Leishmania* and purified NK cells. All three types of monocytes were able to induce CD69 on NK cells in response to *Leishmania* (**Figure 2B**).

To define whether infected monocytes stimulated NK cells *via* a soluble factor or by a cell contact-dependent mechanism, a TW system was used. Whereas NK cells of a mixed NK cell/monocyte/*Leishmania* culture showed an increase in CD69 expression after 20 h of incubation, NK cells that were separated from infected monocytes by a membrane did not (**Figure 3**). Likewise, NK cells did not become activated in TW experiments, in which NK cells and *Leishmania* were separated from uninfected monocytes (two independent experiments, data not shown). Thus, direct contact between infected human monocytes and NK cells is essential to upregulate CD69 on NK cells.

In murine leishmaniasis NK cell activation is mediated by cytokines [reviewed in Ref. (13)]. We therefore hypothesized that this might also apply for human NK cells and screened for NK cell-activating cytokines that are trans-presented by myeloid cells to the respective receptor on NK cells without being necessarily secreted. As both IL-15 and IL-18 were reported to be trans-presented by human monocytes (70, 71), we tested whether they are involved in the induction of CD69. Using neutralizing Abs, we found that the expression of CD69 on NK cells in infected PBMCs was partially dependent on IL-18 (**Figure 4A**). In the case of NK cell/monocyte/*Leishmania* cocultures, a similar effect was observed, which, however, did not quite reach the level of significance (**Figure 4A**). By contrast, neutralization of IL-15 did not affect the expression of CD69 on NK cells (**Figure 4B**). Two observations argue for monocyte-mediated trans-presentation rather than secretion of IL-18: first, in most of the SNs of PBMC/*Leishmania*, NK cell/monocyte/*Leishmania* or monocyte/*Leishmania* cocultures IL-18 was not measurable by ELISA (detection limit was 20 pg/ml); only in few of them (mostly after *L. major* stimulation) low levels of IL-18 (≤500 pg/ml) were found (Table S1 in Supplementary Material). Second, IL-18 was visualized on the surface of purified monocytes which had been in contact with *L. major* for 20 h and were stained for IL-18 after fixation with Pfa without permeabilization (**Figure 5**). Permeabilization of infected monocytes intensified the IL-18 staining, because intracellular IL-18 became additionally detectable (**Figure 5**). Together, these data suggest that *Leishmania*-infected monocytes trans-present IL-18 to human NK cells.

A Heat-Labile Soluble Factor of Monocyte/*Leishmania* Cocultures Contributes to the Upregulation of CD69 on Human NK Cells

To investigate whether soluble factors released by infected monocytes are additionally involved in human NK cell activation, freshly isolated PBMCs or purified NK cells were incubated with culture SNs of *Leishmania*-stimulated (a) PBMCs, (b) purified NK cells (with or without monocytes), or (c) purified monocytes, all from the same blood donor. SNs of host cell-free *Leishmania* cultures were included as control. In the presence of SNs from previous PBMC/*Leishmania* or monocyte/*Leishmania* cultures, both NK cells within PBMCs and purified NK cells showed an upregulation of CD69, indicating that monocytes and/or *Leishmania* release a soluble factor after contact to each other that activates NK cells; SNs of promastigotes cultured without host cells had no effect (**Figure 6A**, upper and lower panels). The stimulatory effect of SNs derived from PBMC/*Leishmania* cocultures was concentration-dependent and, except for *L. mexicana*, as strong as direct stimulation of PBMCs by the parasite (**Figures 6B,C**). SNs of PBMCs incubated with dead parasites did not upregulate CD69 on NK cells (**Figure 6D**). The activity of the SNs was also lost, when they were boiled before addition to the PBMCs. By contrast, filtration (pore size 0.22 µm) did not influence their activity excluding host cell or parasite debris as stimuli (**Figure 6E**). Together, these data indicate that the NK cell stimulating activity of the SNs presumably results from a heat-labile protein released by infected monocytes.

Next, we analyzed the spectrum of cytokines and chemokines secreted by *Leishmania*-activated monocyte/NK cell cultures of three different donors. We focused on *L. major*, because SNs from *L. major*-stimulated cell cultures were on average most potent in upregulating CD69 on NK cells. Using a Procarta® Multiplex Immunoassay, substantial amounts of several cytokines and chemokines were measured in the SNs of all three tested individuals (Table S2 in Supplementary Material). In further experiments, we concentrated on those factors that were strongly induced and had already been linked to NK cell activation (IL-1β, IL-6, IL-8, IL-18, and MIP-1α) (72–78). In addition, IL-2, IL-12, IL-15, IL-21, and IFN-α/β, all known as NK cell stimulatory cytokines (7), were included in the analysis. IL-12p40 protein was detected in low amounts in the SNs of *Leishmania*-stimulated PBMCs but was absent when monocytes had been depleted (**Figure 6F**),

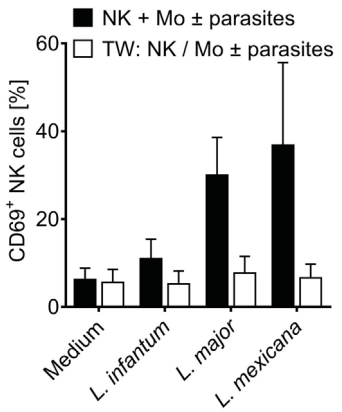

FIGURE 3 | CD69 induction on natural killer (NK) cells requires contact with infected monocytes. Sorted NKp46$^+$CD3$^-$ NK cells and autologous sorted CD14$^+$ monocytes were cocultured in the presence of *Leishmania* spp. promastigotes (multiplicity of infection 10 relative to the number of NK cells), either in one single well or in a transwell (TW) system in which the NK cells (in the insert) were separated from the monocytes and *Leishmania* (in the bottom well) by a membrane (pore size 0.4 µm). After 20 h, CD69 surface expression of NKp46$^+$CD3$^-$ NK cells was determined by flow cytometry. Mean ± SEM of 6/5/6/3 and 3/5/6/3 donors for the four stimulations.

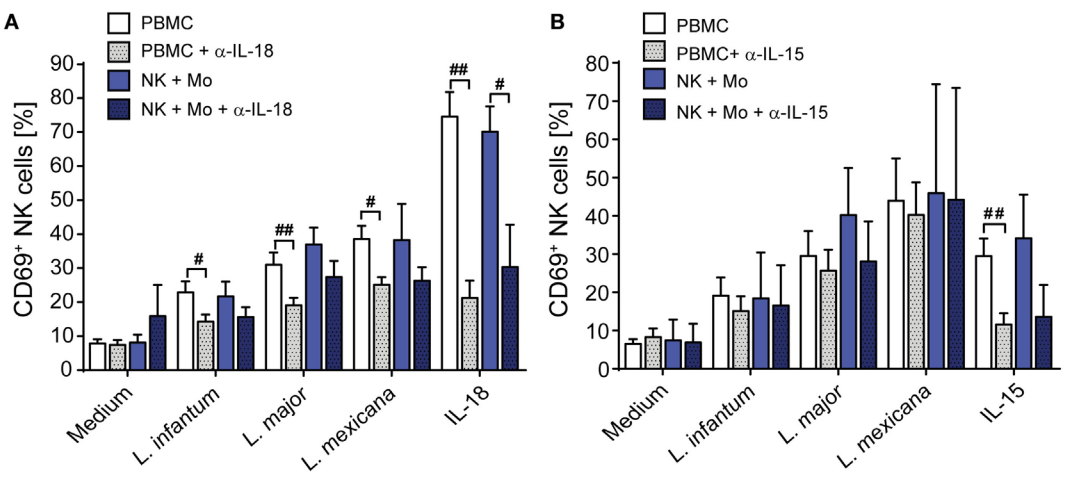

FIGURE 4 | Upregulation of CD69 on natural killer (NK) cells depends on interleukin (IL)-18. Peripheral blood mononuclear cells (PBMCs) or sorted NKp46$^+$CD3$^-$ NK cells ± autologous, sorted CD14$^+$ monocytes were cocultured with *Leishmania* spp. promastigotes (multiplicity of infection 10) in the presence or absence of neutralizing antibodies against **(A)** IL-18 (1.5 µg/ml) or **(B)** IL-15 (1 µg/ml). After 20 h, the CD69 surface expression of NKp46$^+$CD3$^-$ NK cells was determined by flow cytometry. Mean ± SEM of **(A)** 16/15/16/8/5 (PBMCs) or 8/8/8/5/6 (NK + Mo) donors and **(B)** 19/8/6/3/10 (PBMCs) or 2/2/2/2/2 (NK + Mo) donors for the five different stimulation conditions. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ two-tailed Mann–Whitney *U* test.

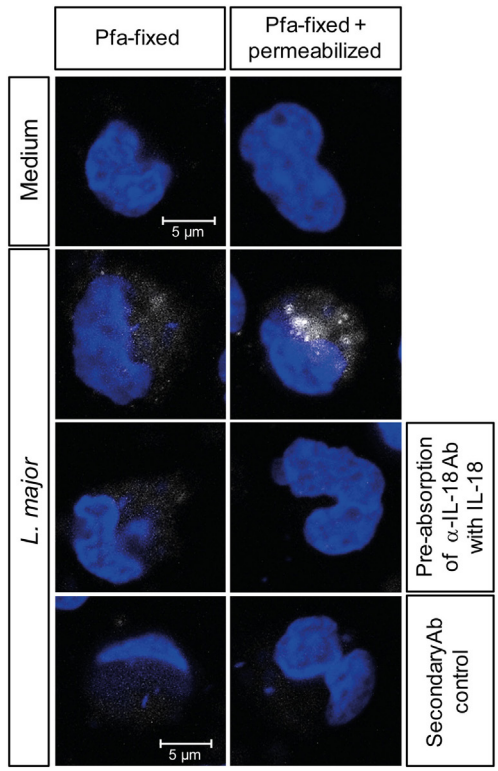

FIGURE 5 | Interleukin (IL)-18 is detectable on fixed, but non-permeabilized monocytes after exposure to *Leishmania*. Purified CD14$^+$ monocytes attached to an adhesion slide were incubated with or without *Leishmania major* promastigotes (multiplicity of infection 10) for 20 h. Thereafter, monocytes were either fixed with paraformaldehyde (Pfa) or fixed with Pfa and permeabilized with methanol, before being stained for IL-18 (white) and with DAPI (blue). As controls, the mouse-anti-IL-18 antibody (Ab) was pre-absorbed with rhuL-18, or the cells were incubated with the secondary Ab alone. Representative images of one of three independent experiments are shown.

indicating that it is released by *Leishmania*-triggered monocytes. The reason for the differential induction of IL-12p40 by the three *Leishmania* species (**Figure 6F**) tested is currently unknown, but is in line with previous observations that parasite species causing self-healing CL (*L. major*) elicit higher production of IL-12 as compared to parasite species associated with visceral disease (*L. donovani*) (79). Upregulation of CD69 on NK cells was clearly seen after stimulation of PBMCs with IL-2, IL-15, IL-18, or IFN-α/β, to a minor extent also with IL-1β and IL-6, but was not detectable following exposure of PBMCs to IL-8, IL-12, IL-21, or MIP-1α (**Figure 7A**). To determine whether one or several of the CD69-inducing cytokines represent the crucial NK cell-stimulatory component within the SNs of PBMC/*Leishmania* cocultures, SNs were preincubated with neutralizing Abs or respective isotype controls. We did not observe any alteration in NK cell CD69 expression upon addition of individual neutralizing Abs or combinations thereof, although the stimulatory effect of the respective recombinant cytokine was clearly abrogated by the Ab treatment (**Figure 7B**). We conclude that none of the CD69-inducing cytokines (IL-1β, IL-2, IL-12, IL-15, IL-18, and IFN-α/β) represents the soluble NK cell stimulating factor in supernatants of PBMC/*Leishmania* cocultures.

IL-12 Is Required to Elicit NK Cell IFN-γ Release in Response to *Leishmania* in PBMC or NK/Monocyte Cultures

Upregulation of CD69 is a first sign of NK cell activation but does not automatically entail the production of IFN-γ required for parasite control in mouse and human leishmaniasis (13). We therefore analyzed, whether IFN-γ was expressed by *Leishmania*-stimulated PBMCs. IFN-γ was neither detectable in culture SNs by ELISA (**Figure 8A**) nor in NK cells by intracellular cytokine staining (**Figure 8B**), whereas stimulation with IL-12/IL-18 elicited a clear IFN-γ response of NK cells (**Figures 8A,B**). Likewise, IL-12/IL-18, but not exposure to *Leishmania* enhanced the cytotoxic activity of NK cells (**Figure 8C**). Thus, stimulation of PBMCs by *Leishmania* was not sufficient to induce NK cell effector functions. Interestingly, stimulation of PBMCs with IL-12/18 and *Leishmania* promastigotes further increased NK cell cytotoxicity as compared with cells activated by IL-12/IL-18 alone (**Figure 8C**).

To determine whether exogenously added IL-12 and IL-18 differentially contributed to the NK cell effector response, PBMCs were cocultured with parasites in the presence of either IL-12 or IL-18. Whereas IL-18 was largely ineffective (**Figure 9A**), IL-12 and *Leishmania*, but not IL-12 alone, triggered the release of IFN-γ in PBMC cultures (**Figure 9B**). Titrating IL-12, a concentration of 300 pg/ml was sufficient to induce IFN-γ in the presence of *Leishmania* (**Figure 9C**). Blockade of IL-18 abolished the IL-12/*Leishmania*-induced IFN-γ response (**Figure 9D**).

In the mouse system, DCs are IL-12 producers during the early phase of *Leishmania* infection (18, 80). To investigate whether primary human DCs are capable to respond to *Leishmania* parasites by secreting IL-12, CD1c$^+$ DCs of human PBMCs were purified by cell sorting and cultured in the presence of *Leishmania* promastigotes. After 20 h, an average of 800 (±116) pg/ml IL-12p40 (mean ± SEM of four donors) was detected in the SNs of the DC cultures (**Figure 10**). By contrast, CD14$^+$ monocytes sorted in parallel did not release measurable amounts of IL-12p40 in response to *Leishmania* (**Figure 10**). Interestingly, when CD1c$^+$ DCs were incubated together with parasites and sorted monocytes and NK cells, the 20 h culture SNs contained an increased concentration of IL-12p40 (1,485 ± 213 pg/ml, mean ± SEM of four donors). Also, in the presence of all three host cells and *L. major* parasites low amounts of bioactive IL-12p70 (*ca.* 90 pg/ml) became detectable in two of four analyzed donors, whereas CD1c$^+$ blood DCs or monocytes alone or cultures of monocytes and NK cells failed to generate IL-12p70 in response to *Leishmania* (data not shown).

Taken together, these data demonstrate that the absent NK cell effector response in PBMC/*Leishmania* cocultures is most likely due to an insufficient IL-12 production that presumably results from the low number of DCs in human PBMCs (68). Once IL-12 is added (or released by DCs after contact with NK cells and monocytes), endogenous IL-18 (produced and trans-presented by monocytes) acts synergistically with the IL-12 to elicit the expression of IFN-γ in NK cells.

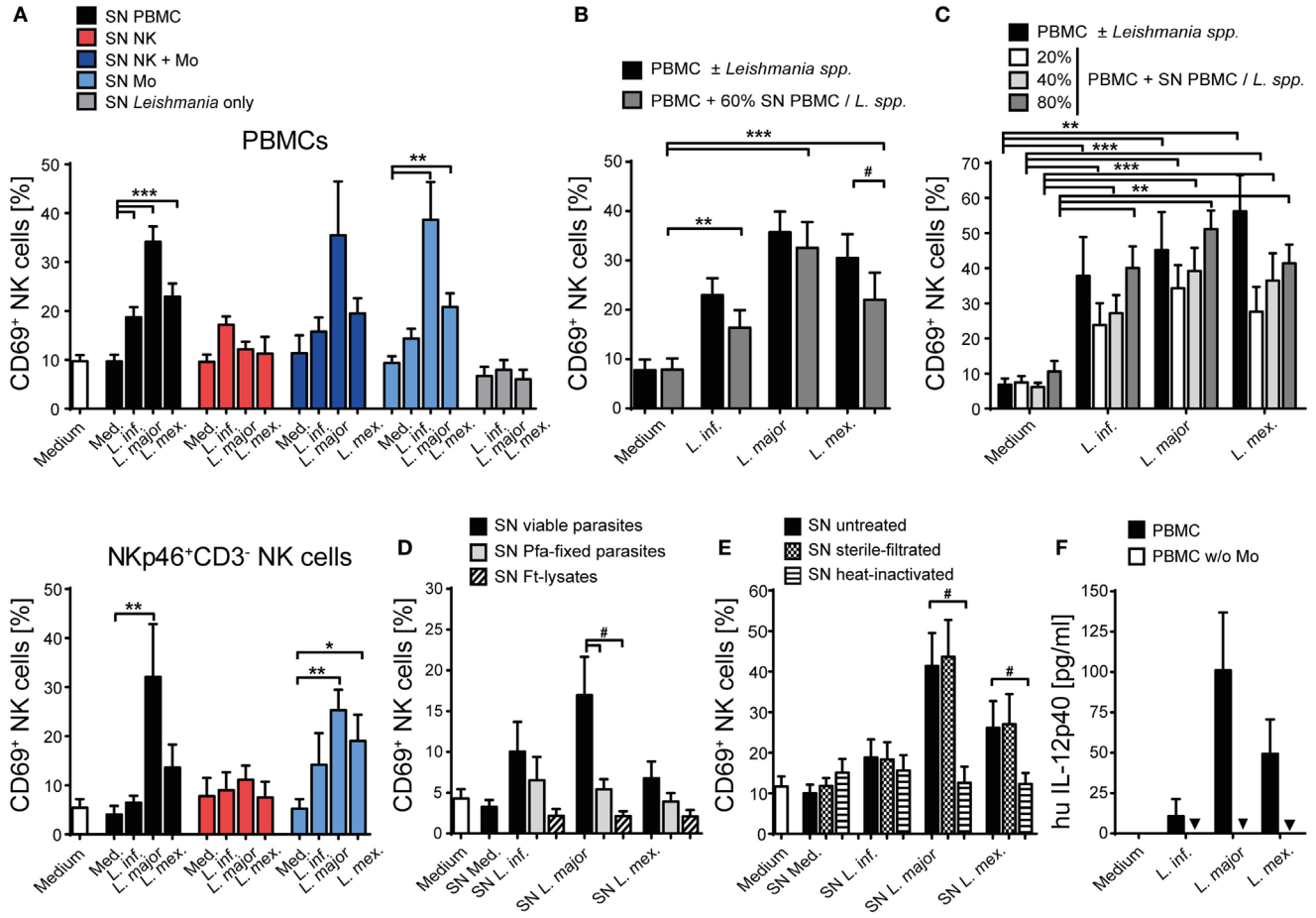

FIGURE 6 | CD69 on natural killer (NK) cells is induced by a heat-labile soluble factor derived from infected monocytes. Human peripheral blood mononuclear cells (PBMCs) [**(A)** (top panel), **(B–E)**] or purified NKp46$^+$CD3$^-$ NK cells [**(A)** (bottom panel)] were stimulated with supernatants (SN) of various cell-*Leishmania*-cocultures for 20 h. CD69 expression of NKp46$^+$CD3$^-$ NK cells was determined by flow cytometry. Unless otherwise indicated **(C)**, SNs were added **(A,B,D,E)** at a final concentration of 60% (vol/vol) and used untreated **(A–D)**, sterile filtered **(E)**, or heat-inactivated **(E)**. [**(A)** top panel] Mean ± SEM of 59/59/50/49 (SN PBMC), 4/3/4/3 (SN NK), 4/4/4/2 (SN NK + Mo), 4/4/4/4 (SN Mo), or 8/8/6 donors (SN *Leishmania* only). [**(A)** bottom panel] Mean ± SEM of 7/7/6/5 (SN PBMC), 3/3/2/3 (SN NK), or 6/6/6/6 (SN Mo) donors for the four stimulations. **(B)** Mean ± SEM of 24/23/20/15 donors for the four stimulations. **(C)** Mean ± SEM of 11/4/3/4 (*Leishmania* spp.), 9/9/7/8 (20% SN), 10/11/7/7 (40% SN), or 9/9/7/9 (80% SN) donors for the four stimulations. **(D)** Mean ± SEM of seven (SN viable), seven (SN pfa-fixed), and four [SN freeze–thaw lysate (ft-lysate)] donors. **(E)** Mean ± SEM of 10/10/8/10 (SN untreated), 9/9/7/9 (SN filtered), and 9/9/6/8 (SN heat-inactivated) donors for the four stimulations. **(F)** PBMCs or PBMCs depleted of monocytes were cocultured with *Leishmania* spp. promastigotes (multiplicity of infection 10) for 20 h. The concentration of interleukin (IL)-12p40 in culture SNs was determined by ELISA (values below detection limit are marked by triangles). Mean ± SEM of 6/6/5/5 (PBMC) and 6/5/5/6 (PBMC w/o monocytes) donors for the four stimulations. $^{*,\#}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ two-tailed Mann–Whitney *U* test.

Expression of CD56 on Human NK Cells Is Downregulated after Contact with *Leishmania*

When analyzing the activation of NK cells within human PBMCs or of sorted NKp46$^+$CD3$^-$ human NK cells after coculture with *Leishmania* promastigotes, we noticed that the surface expression of the NK cell marker CD56 (NCAM1), whose functional role is still unknown, was reduced. The downregulation of CD56 was dependent on the parasite/host cell ratio and the parasite species, with *L. infantum* and *L. mexicana* causing more pronounced effects than *L. donovani* or *L. major* (**Figures 11A,B**). The decrease of CD56 was observed in both the CD56^{bright} and CD56^{dim} NK cell population (data not shown; see also below in **Figure 14**, panel "medium" vs. "*L. infantum*"). Separation of NK cells and *Leishmania* using a TW culture system abolished the effect (**Figure 11C**), indicating that direct contact between NK cells and parasites was required. To exclude that *Leishmania* (products) occupy certain epitopes on NK cells and thereby prevent the detection of CD56, we tested different monoclonal Abs against human CD56 (clones CMSSB, HCD56, and MEM-188), all of which yielded similar results (data not shown). For *L. infantum*, the decrease in CD56 expression was not observed when fixed or lysed instead of viable parasites were used, whereas the downregulatory effect of *L. major* or *L. mexicana* promastigotes on CD56 was maintained even after lysis or fixation of the parasites (**Figure 11D**). A limited, but still significant suppression of CD56 was also seen with SNs from pure parasite cultures or from PBMC/*Leishmania* cocultures, but only

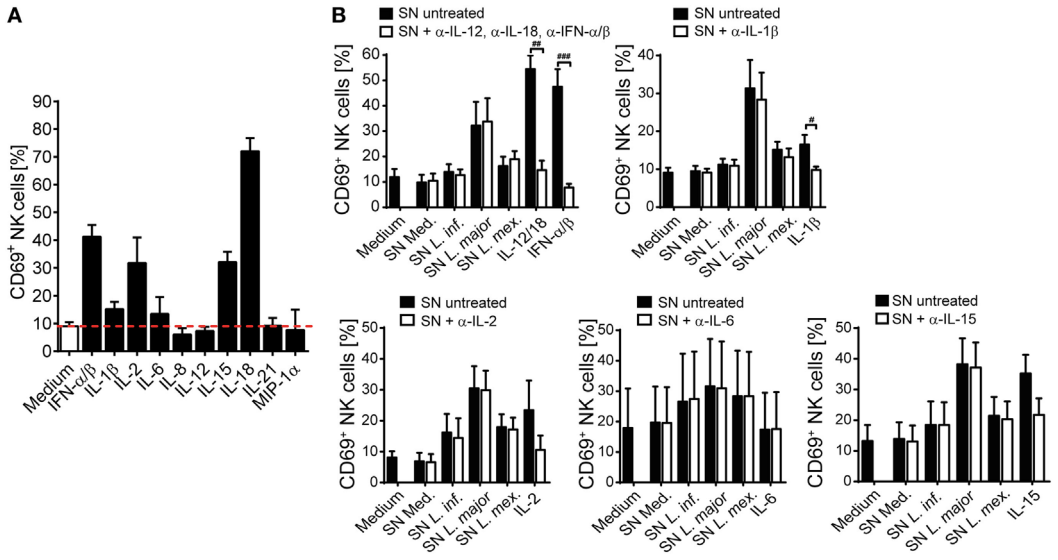

FIGURE 7 | CD69 upregulation on natural killer (NK) cells by supernatants (SNs) of peripheral blood mononuclear cell (PBMC)/*Leishmania* cocultures is maintained after neutralization of NK cell-activating cytokines. Human PBMCs were stimulated with different **(A)** recombinant cytokines or **(A,B)** SNs of PBMC/*Leishmania* cocultures of the respective donors for 20 h, followed by analysis of CD69 surface expression on NKp46$^+$CD3$^-$ NK cells by flow cytometry. **(B)** Before stimulation, the cytokines or SNs were pretreated with either one or several neutralizing antibodies (Abs) (37°C, 1–2 h). The concentrations of the cytokines and Abs were as described in Section "Materials and Methods." **(A)** Mean ± SEM of 57/57/17/11/12/8/19/8/7/3/3 donors for the 11 stimulations. **(B)** Mean ± SEM of 7/7/7/6/7/7/7 [SN + anti-interferon (IFN)-α/β, anti-interleukin (IL)-12, and anti-IL-18], 7/7/7/7/6/7 (SN + anti-IL-1β), 9/9/9/7/8/9 (SN + anti-IL-15), 5/5/5/5/5/5 (SN + anti-IL-2), or 4/4/4/4/4/4 (SN + anti-IL-6) donors for the different stimulations. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ two-tailed Mann–Whitney *U* test.

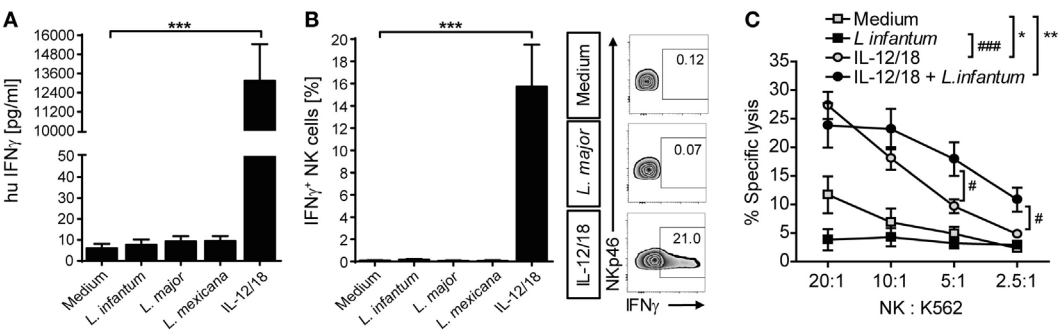

FIGURE 8 | Coculture of peripheral blood mononuclear cells (PBMCs) with *Leishmania* neither induced natural killer (NK) cell interferon (IFN)-γ response nor NK cell cytotoxicity. Human PBMCs were cocultured with *Leishmania* spp. promastigotes (multiplicity of infection 10) and/or interleukin (IL)-12 and IL-18 (10 ng/ml each) for 20 h. IFN-γ production was determined by **(A,B)** ELISA of cell culture supernatants or **(B)** flow cytometry of intracellular IFN-γ in NKp46$^+$CD3$^-$ NK cells. **(C)** NK cell cytotoxicity was determined by measurement of specific lysis of ^{51}Cr-labeled K562 tumor cells in a chromium-release assay. **(A)** Mean ± SEM of 76/71/55/46/74 donors for the five stimulations. **(B)** Mean ± SEM of 17/14/15/8/17 donors for the five stimulations; FACS plots show results of one representative donor. **(C)** Mean ± SEM of six (PBMC) or nine (PBMC + IL-12/18) donors. $^{*,\#}p < 0.05$; $^{**,\#\#}p < 0.01$; and $^{***,\#\#\#}p < 0.001$ two-tailed Mann–Whitney *U* test.

in the case of viable *L. infantum* and not with any of the other *Leishmania* species (**Figures 12A–C**).

As CD56 also exists in a soluble form which is directly secreted or released from the cell surface (81, 82), we considered the possibility that *Leishmania* parasites induce CD56 shedding by NK cells. However, the concentration of soluble CD56 detected in SNs of purified NK cell/*Leishmania* spp. cocultures was comparable to the amount of sCD56 found in SNs of NK cell cultures without parasites (**Figure 13A**). Instead, exposure of sorted Nkp46$^+$CD3$^-$ NK cells to *Leishmania* caused a significant reduction of CD56 mRNA in a dose-dependent manner (**Figure 13B**). Thus, *Leishmania*-induced transcriptional suppression of CD56 mRNA rather than shedding of CD56 surface protein accounts for the decrease in CD56$^+$ NK cells after contact with *Leishmania*.

Finally, we addressed the question, whether the *Leishmania*-induced down-modulation of CD56 influences NK cell cytokine responsiveness. Therefore, PBMCs were simultaneously exposed to *Leishmania* and IL-12/18. As under these conditions the IFN-γ

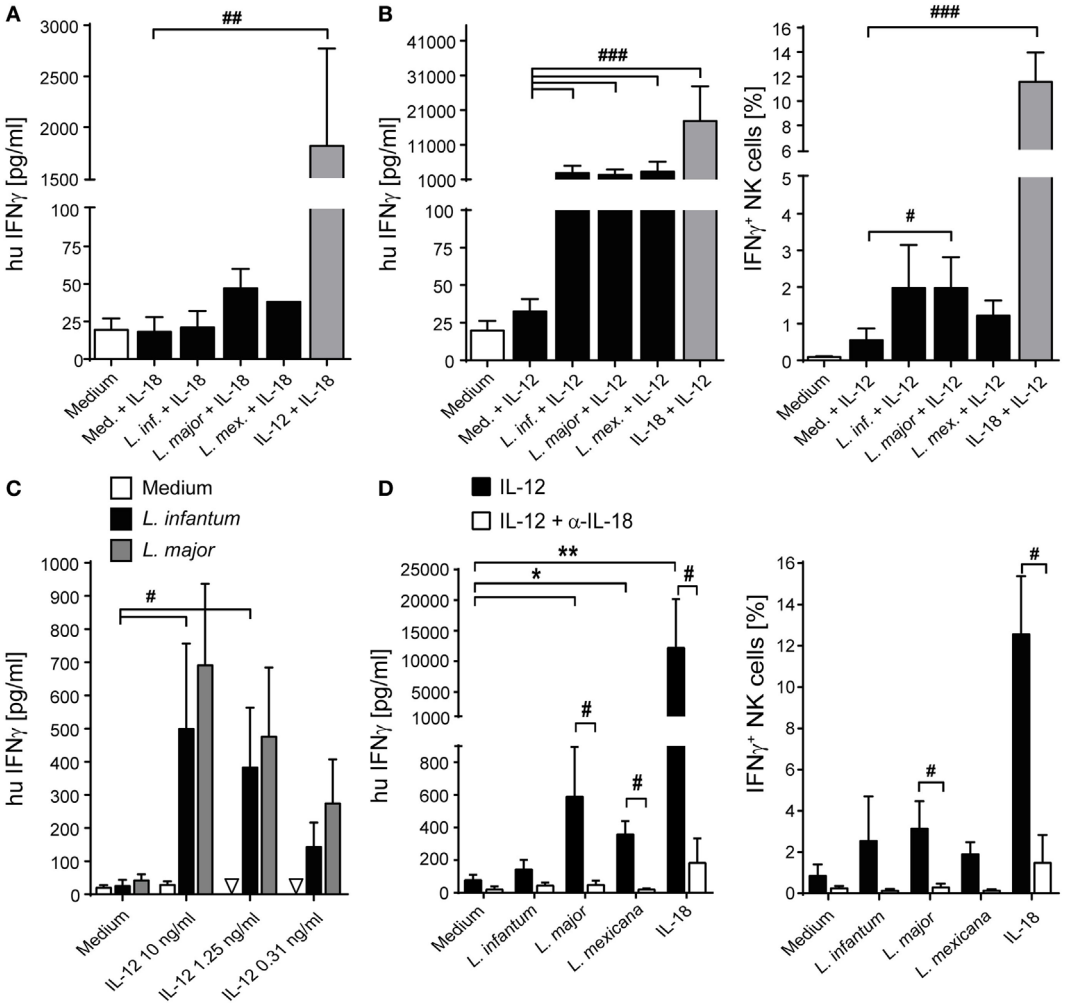

FIGURE 9 | Effect of exogenous interleukin (IL)-12 and IL-18 on interferon (IFN)-γ production of natural killer (NK) cells in peripheral blood mononuclear cell (PBMC)-*Leishmania* cocultures. Human PBMCs were cocultured with *Leishmania* spp. promastigotes (multiplicity of infection 10) in presence or absence of exogenous IL-12 and/or IL-18 (10 ng/ml each, or as indicated) and neutralizing IL-18 antibody (1.5 µg/ml) for 20 h. Thereafter, IFN-γ production was measured either by ELISA of cell culture supernatants [**(A,B)** (left graph), **(C,D)** (left graph)] or by flow cytometry of intracellular IFN-γ in NKp46^{+}CD3^{-} NK cells [**(B)** (right graph), **(D)** (right graph)]. Values below detection limit are marked by triangles. **(A)** Mean ± SEM of 6/6/6/3/1/6 donors for the six stimulations. **(B)** Mean ± SEM of 22/22/22/21/18/22 (ELISA, left panel) or 9 (ICS, right panel) donors. **(C)** Mean ± SEM of 6/6/3 donors for the three stimulations (medium, *Leishmania infantum*, and *Leishmania major*). **(D)** Mean ± SEM of six (ELISA, left panel) or five (ICS, right panel) donors. $^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ two-tailed Mann–Whitney *U* test.

production by NK cells was higher as with cytokine stimulation alone and CD56$^{\text{dim/negative}}$ NK cells turned out to be strong IFN-γ producers (**Figure 14**), we conclude that a lack of CD56 does not hamper NK cell activation by IL-12/IL-18.

DISCUSSION

Human blood NK cells will be in contact with *Leishmania* promastigotes during the first hours of natural infection, as the sand flies vectors regurgitate the parasites into a blood pool generated by laceration of skin capillaries. Previous analyses of the effector responses of human blood NK cells to *Leishmania* parasites yielded controversial results and did not identify the host-derived signals required for NK cell activation [reviewed in Ref. (13); see discussion below]. In this study, we investigated the effect of different parasite species and strains and aimed to define the cellular and humoral prerequisites for *Leishmania*-induced NK cell activation. We used blood NK cells of volunteers from a non-endemic area that were incubated with *Leishmania* promastigotes for 20 h in the presence of 10% autologous plasma, thus mimicking the early phase of infection and the microenvironment of a naïve host during primary infection.

CD69 and Activation of Human Blood NK Cells by *Leishmania*: Monocyte Contact Dependent vs. Soluble Signals

Our experiments revealed that efficient upregulation of the early activation marker CD69 on *Leishmania*-stimulated NK cells required (a) cell–cell contact between NK cells and monocytes

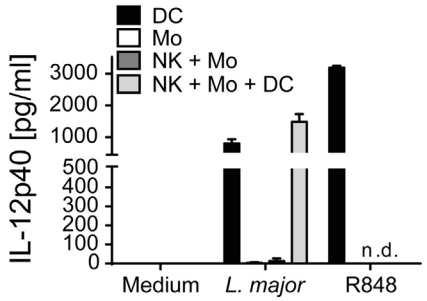

FIGURE 10 | Blood CD1c$^+$ dendritic cells (DCs) but not monocytes are a source of interleukin (IL)-12p40 in response to *Leishmania major*. Human blood CD1c$^+$ DCs, CD14$^+$ monocytes and NKp46$^+$CD3$^-$ natural killer (NK) cells were sorted and stimulated by *L. major* promastigotes (multiplicity of infection 10) for 20 h either alone or in combinations. R848 (5 μg/ml) was used as control. IL-12p40 content of the cell culture supernatants was measured by ELISA. Mean ± SEM of 4/4/3 donors for the three stimulations.

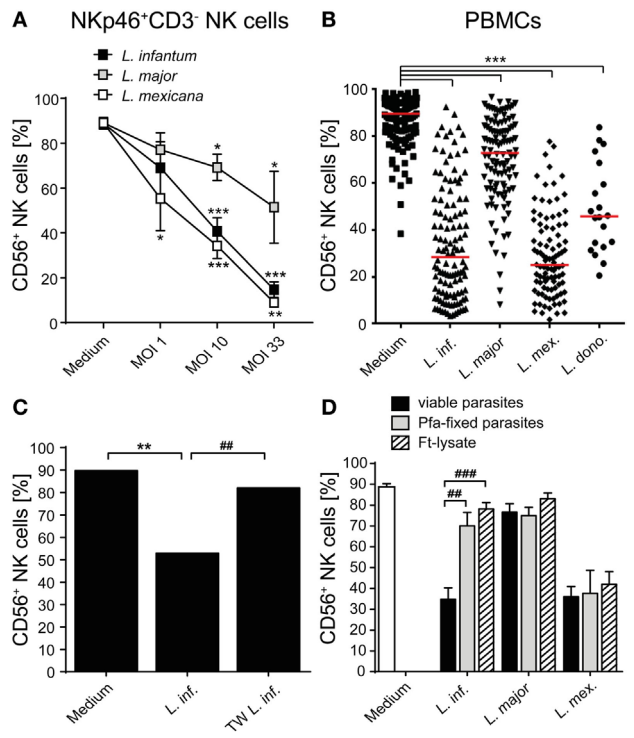

FIGURE 11 | CD56 surface expression on natural killer (NK) cells is suppressed after contact with *Leishmania* promastigotes. **(A)** Purified NKp46$^+$/CD3$^-$ NK cells or **(B–D)** human peripheral blood mononuclear cells (PBMCs) were cocultured with viable **(A–D)**, paraformaldehyde (Pfa)-fixed **(D)**, or freeze–thaw-lysed **(D)** *Leishmania* spp. promastigotes [multiplicity of infection (MOI) 10 unless otherwise indicated] for 20 h. CD56 surface expression on NKp46$^+$/CD3$^-$ NK cells was measured by flow cytometry. **(A)** Mean ± SEM of 17/16/12/12 donors for the four stimulations. **(B)** Mean ± SEM of 132/121/114/97/20 donors for the five stimulations. **(C)** Mean ± SEM of 6/6/6 donors for the three stimulations [transwell (TW)]. **(D)** Mean ± SEM of 22 (medium), 22/17/18 (untreated and viable parasites), 8/9/6 (Pfa-fixed parasites), or 17/14/14 [freeze–thaw lysate (ft-lysate)] donors. $^{*,\#}p < 0.05$; $^{**,\#\#}p < 0.01$; an $^{***,\#\#\#}p < 0.001$ two-tailed Mann–Whitney *U* test.

and (b) a soluble, heat-labile factor released by infected monocytes. This two-signal model is based on the observations that on the one hand upregulation of CD69 was prevented following physical separation of NK cells and monocytes, whereas on the other hand native, but not heat-treated SNs of cocultures of viable *Leishmania* promastigotes with monocytes were able to induce CD69 expression on NK cells. The findings were true for all *Leishmania* species tested [*L. infantum* (dermotropic and viscerotropic strains), *L. major*, *L. mexicana*, and *L. donovani*], although the extent of CD69 upregulation varied between the numerous donors analyzed and differed significantly between *Leishmania* species.

Several results obtained in this study strongly support the idea that IL-18 trans-presented by infected monocytes to NK cells constitutes the contact-dependent signal: (i) IL-18 was detectable on the surface of non-permeabilized monocytes after exposure to *Leishmania*; (ii) culture SNs of infected monocytes contained little or no IL-18; and (iii) neutralizing Abs to IL-18 largely prevented CD69 induction on NK cells when added directly to PBMC/*Leishmania* cultures, but did not abolish the CD69-inducing activity of SNs from previous PBMC/*Leishmania* cultures. The available data, however, do not formally exclude the possibility that pro-IL-18, which lacks a secretory leader sequence, is nevertheless locally released into synapses between NK cells and monocytes *via* directed exocytosis of secretory lysosomes as described for NK/DC interactions (83) and that anti-IL-18 is able to access and neutralize IL-18 in such a scenario.

With respect to the soluble factor, which is heat-labile and awaits further characterization, the NK cell-activating cytokines IFN-α/β, IL-1β, IL-2, IL-6, IL-8, IL-12, IL-15, IL-18, IL-21, and MIP-1α were excluded to account for the CD69-upregulating effect of the culture SNs. At this stage, we cannot rule out that the soluble factor is a *Leishmania*-derived protein that is only released by infected monocytes [e.g., *via* exosomes (84)], but not by the parasite itself, as SNs of pure *Leishmania* cultures had no effect on CD69 expression of NK cells. Considering that culture SNs of *Leishmania*-infected monocytes remained active after passage through a 0.22 μm sterile filter, it was unexpected that NK cell activation was completely prevented when monocytes and *Leishmania* were separated from NK cells by a membrane with 0.4 μm pore size using a TW system. A plausible explanation is that in case of the culture SNs the starting concentration of the unknown factor is much higher (as it accumulated over 20 h), whereas in the TW setting the factor is newly produced and only slowly builds up.

CD69 and Activation of Human Blood NK Cells by *Leishmania*: Direct vs. Indirect Stimulation

The observation that myeloid cells were necessary to activate human NK cells by *Leishmania* is in line with our observations in murine leishmaniasis (18, 49, 80). There are also a few previous studies, in which human blood NK cells alone failed to respond to *Leishmania* antigen (57) and required the presence of adherent PBMCs (58, 59); however, in these reports only proliferation and cytokine production of NK cells, but not their expression of CD69

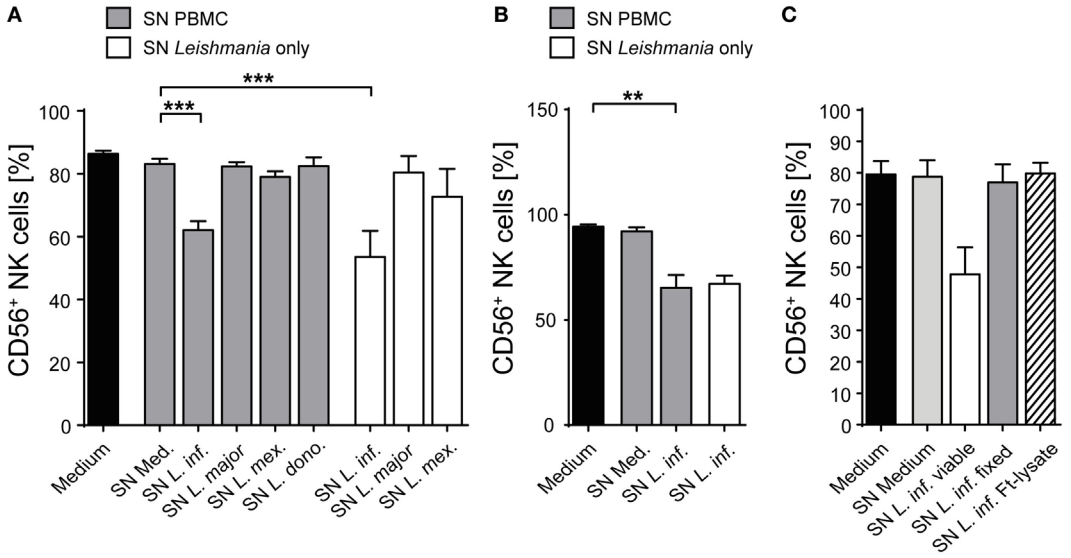

FIGURE 12 | Supernatants (SNs) from peripheral blood mononuclear cell (PBMC)/*Leishmania infantum* cocultures downmodulate CD56 expression on natural killer (NK) cells. SNs from PBMC/*Leishmania* cocultures (multiplicity of infection 10) or from pure *Leishmania* cultures were used at a concentration of 60% (vol/vol) to stimulate **(A,C)** PBMCs or **(B)** purified NKp46$^+$/CD3$^-$ NK cells for 20 h. CD56 surface expression on NKp46$^+$/CD3$^-$ NK cells was determined by flow cytometry. **(A)** Mean ± SEM of 61 (medium), 59/59/53/50/15 (SN PBMC/*Leishmania*-coculture), or 8/8/6 (SN *Leishmania* only) donors. **(B)** Mean ± SEM of eight (medium), eight (SN PBMC/*Leishmania*-coculture), or two (SN *Leishmania* only) donors. **(C)** Mean ± SEM of 8/8/8/7/5 donors for the five stimulations. $^{**}p < 0.01$; and $^{***}p < 0.001$ two-tailed Mann–Whitney *U* test.

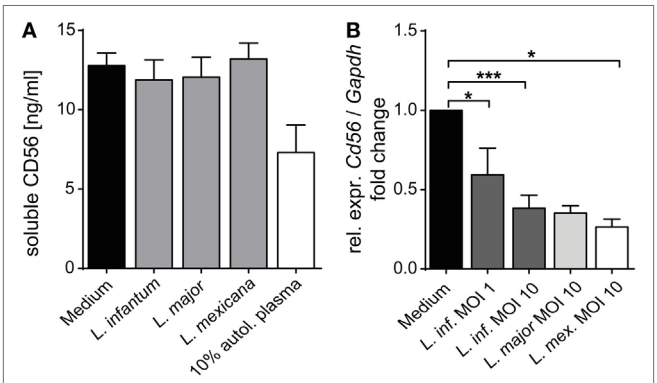

FIGURE 13 | Mechanism of *Leishmania*-induced reduction of surface CD56 on natural killer (NK) cells. **(A)** Purified human NKp46$^+$CD3$^-$ NK cells were cocultured with *Leishmania* promastigotes [multiplicity of infection (MOI) 10] for 20 h. The concentration of soluble CD56 in the cell culture supernatants was determined by ELISA. Mean ± SEM of 9/7/6/7/3 donors for the five stimulations. **(B)** Purified human NKp46$^+$CD3$^-$ NK cells were cocultured with *Leishmania* promastigotes at different parasite/host cell ratios for 20 h. mRNA expression of CD56 was quantified by TaqMan RT-PCR. The expression was normalized against the endogenous control (huGAPDH), and the fold change was calibrated to the respective medium value. Mean ± SEM of 8/3/8/2/5 donors for the five stimulations. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ two-tailed Mann–Whitney *U* test.

were analyzed. On the other hand, our current results clearly contrast with earlier findings that viable or dead promastigotes of *Leishmania aethiopica*, *L. mexicana*, and *L. donovani* directly triggered IFN-γ release by sorted NK cells (60, 61). In one of these studies purified *Leishmania* LPG was claimed to directly bind to TLR2 on NK cells (61), whereas Nylen et al. (60) found that LPG-deficient *L. mexicana* mutants were as potent as wild-type parasites in activating NK cells. Notably, the same group later failed to recapitulate accessory cell-independent NK cell activation by *Leishmania* using two different strains of *L. major* (85). Possible explanations for the lack of direct NK cell stimulation by *Leishmania* in our hands are (a) a higher degree of NK cell purity [>96 vs. 90% (60)], (b) a shorter stimulation period [20 vs. 48 h (60)], and/or (c) the use of blood cells from donors of a non-endemic vs. endemic area (61).

The variable degree of monocyte-dependent NK cell activation we have seen with the four tested *Leishmania* species might reflect species-specific differences in the monocyte/parasite interaction. For example, *L. infantum* promastigotes were less efficiently phagocytosed by human monocytes than *L. major* promastigotes (86), whereas twice as many *L. major* promastigotes were needed to achieve the same infection rate in monocytes as with *L. donovani* (87). Thus, the differential uptake of *Leishmania* parasites by monocytes exactly correlates with our results on the upregulation of CD69 on NK cells (*L. donovani* > *L. major* > *L. infantum*). The possibility that differences in CD69 induction are indeed determined by the infection rate of monocytes is further supported by three observations. First, higher parasite/host cell ratios were associated with an increased percentage of CD69$^+$ NK cells in the culture. Second, fixed parasites, which are morphologically intact and therefore likely engage phagocytosis-accelerating receptors (36), were more potent in upregulating CD69 than parasite lysates. Third, non-classical monocytes, which tentatively showed the weakest effect on CD69 induction, were reported to exhibit low phagocytic activity (88).

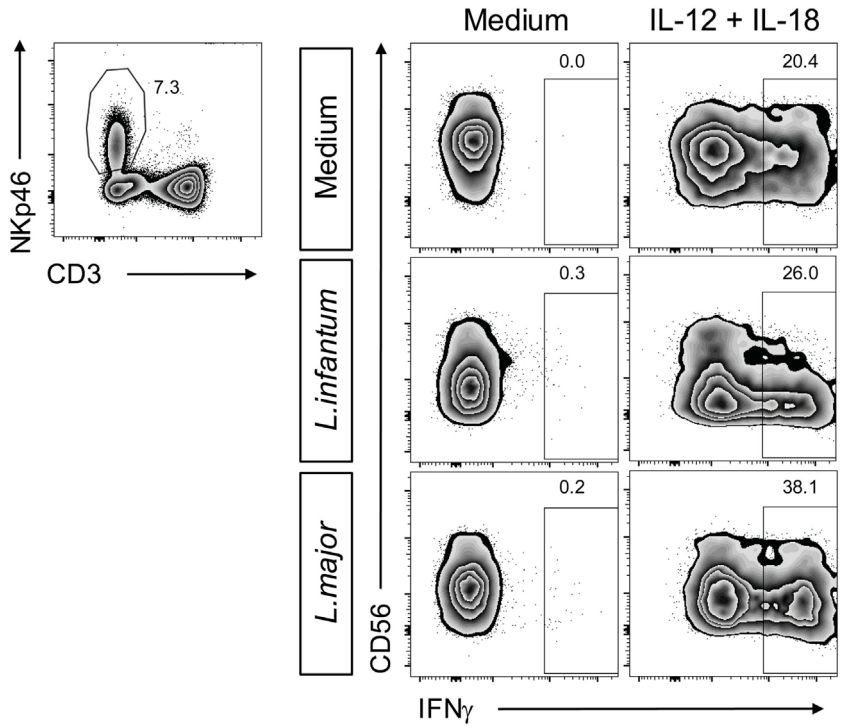

FIGURE 14 | Suppression of natural killer (NK) cell CD56 expression by *Leishmania* does not affect cytokine-induced interferon (IFN)-γ production. Human peripheral blood mononuclear cells were stimulated by *Leishmania* promastigotes (multiplicity of infection 10) in the absence or presence of interleukin (IL)-12 and IL-18 (10 ng/ml each) for 20 h. Intracellular IFN-γ in NKp46$^+$CD3$^-$ NK cells was measured by flow cytometry. Results of one of two donors analyzed are shown.

NK Cell Effector Response upon Stimulation by *Leishmania*

Despite induction of CD69 neither NK cells within *Leishmania*-stimulated PBMCs nor purified NK cells cocultured with infected monocytes produced IFN-γ or showed an upregulation of cytotoxic activity. However, the parasites acted as costimulus by augmenting IL-12/IL-18-induced NK cytotoxicity in PBMC cultures. Furthermore, NK cells within PBMC/*Leishmania* cultures were capable to secrete IFN-γ following addition of exogenous IL-12 (≥300 pg/ml). This result is in accordance with previous observations reporting a lack of IFN-γ production by NK cells in pure PBMC/promastigote cocultures (62, 63) or in human NK/DC cocultures after neutralization of IL-12 (89). Furthermore, it is known that activation of NK cell effector responses frequently requires cooperation between cytokines (e.g., IL-12 and IL-2 or IL-15; IL-2 and IL-15; IL-12 and IL-18) (25, 49, 90–92). Especially IL-18 was shown to prime NK cells to become responsive to IL-12 (16, 83, 93). Our results on IL-12- and *Leishmania*-mediated induction of IFN-γ secretion by human NK cells represents a further example of the cooperative interaction between IL-12 and endogenously generated IL-18 and confirm our findings in the mouse (18, 49).

Interleukin-12 production in pure PBMC/*Leishmania* promastigote cocultures was ineffective. Infected monocytes produced only low amounts of IL-12 during the 20 h culture period, and DCs, which can release IL-12 in response to *Leishmania* (79, 94), are rare in PBMCs (68). Primary CD1c$^+$ blood DCs stimulated by *L. major* promastigotes secreted IL-12p40 without any further maturation signal. In contrast to previous work (94), the sorted CD1c$^+$ DCs reacted to promastigotes and not only to amastigotes. Coculture of CD1c$^+$ DC with monocytes, NK cells and *L. major* even led to the detection of IL-12p70. As CD1c$^+$ DCs are also present in the skin (95), the primary site of *Leishmania* infection, they likely become activated during the early immune reaction and might contribute to NK cell activation *in situ* by release of IL-12. Also, in secondary lymphoid organs DCs were reported to colocalize with NK cells (89). Thus, close interaction between NK cells, DCs, and infiltrating monocytes during *Leishmania* infection appears plausible also in other organs such as spleen and liver, which are main targets of the parasite in VL.

Suppression of CD56 by *Leishmania*

Direct contact of *Leishmania* promastigotes with NK cells caused reduction of CD56 mRNA and protein. While the decrease in CD56 mRNA was comparable for *L. major*, *L. infantum*, and *L. mexicana*, surface CD56 was less strongly downregulated by *L. major* as compared with *L. infantum* and *L. mexicana*. Differential secretion or shedding of CD56 was excluded. However, as NK cells are able to export CD56 in exosomes (96), the divergent regulation of CD56 protein might result from *Leishmania* species-specific induction of CD56$^+$ exosomes and their release by NK cells.

Reduction of CD56 on human NK cells in response to *L. major* was previously observed by Lieke et al. (85). Compared with our results the effect was much stronger, which might reflect the use of different *L. major* strains. Lieke et al. postulated that the *Leishmania* surface protease gp63 was critical for the suppression of CD56 protein on NK cells (85). However, this hypothesis remains questionable, as in NK/*Leishmania* cocultures gp63-deficient parasites lacked the downregulation of CD56 only after 24 h, but not after 120 h of coculture when the effect of wild-type and knockout parasites was comparable. As gp63 is produced by all *Leishmania* species, it is unlikely to account for the differential regulation of CD56 by the *Leishmania* species tested in our study.

Contact-dependent reduction in CD56 surface expression on human NK cells in response to pathogens is not restricted to *Leishmania* parasites, but was also observed in cocultures of NK cells with *Aspergillus* spp. (97), suggesting that it might be a more general evasion mechanism. Our knowledge of the function of CD56 expressed by human NK cells is still limited. Several publications showed upregulation of CD56 upon activation of human NK cells; conversely, downregulation of CD56 was reported when NK cells had been exposed to an immunosuppressive milieu (35). It is not yet clear whether CD56 only represents a marker of an activated cell state or is directly involved in immune effector functions. Recently, CD56 was found to play a role in NK cell maturation, as it facilitated migration of NK cells on stromal cells (98). Also, homophilic interactions between CD56 molecules on $CD56^+$ NK cells and $CD56^+$ target cells triggered NK cell cytotoxicity (99). Viral infections and autoimmune diseases were reported to give rise to dysfunctional CD56-negative NK cells, which showed reduced activity even after stimulation with cytokines (35). In our study, however, NK cell IFN-γ release in response to activating cytokines and *Leishmania* parasites was not impaired, despite the downregulation of CD56.

ETHICS STATEMENT

The work with human cells from normal donors had been approved by the Ethics Committee of the Friedrich-Alexander-University (FAU) Erlangen-Nürnberg (approval no. 112_12 B and 185_12 B). Informed written consents were obtained in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: US, HM, DD, and CB. Performed the experiments: HM, HS, and LH. Analyzed the data: HM, HS, LH, DD, CB, and US. Wrote the paper: CB, HM, and US.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00024/full#supplementary-material.

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Type I Interferon Signaling Is Required for CpG-Oligodesoxynucleotide-Induced Control of *Leishmania major*, but Not for Spontaneous Cure of Subcutaneous Primary or Secondary *L. major* Infection

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We previously showed that in mice infected with *Leishmania major* type I interferons (IFNs) initiate the innate immune response to the parasite at day 1 and 2 of infection. Here, we investigated which type I IFN subtypes are expressed during the first 8 weeks of *L. major* infection and whether type I IFNs are essential for a protective immune response and clinical cure of the disease. In self-healing C57BL/6 mice infected with a high dose of *L. major*, IFN-α4, IFN-α5, IFN-α11, IFN-α13, and IFN-β mRNA were most prominently regulated during the course of infection. In C57BL/6 mice deficient for IFN-β or the IFN-α/β-receptor chain 1 (IFNAR1), development of skin lesions and parasite loads in skin, draining lymph node, and spleen was indistinguishable from wild-type (WT) mice. In line with the clinical findings, C57BL/6 IFN-$\beta^{-/-}$, IFNAR1$^{-/-}$, and WT mice exhibited similar mRNA expression levels of IFN-γ, interleukin (IL)-4, IL-12, IL-13, inducible nitric oxide synthase, and arginase 1 during the acute and late phase of the infection. Also, myeloid dendritic cells from WT and IFNAR1$^{-/-}$ mice produced comparable amounts of IL-12p40/p70 protein upon exposure to *L. major in vitro*. In non-healing BALB/c WT mice, the mRNAs of IFN-α subtypes (α2, α4, α5, α6, and α9) were rapidly induced after high-dose *L. major* infection. However, genetic deletion of IFNAR1 or IFN-β did not alter the progressive course of infection seen in WT BALB/c mice. Finally, we tested whether type I IFNs and/or IL-12 are required for the prophylactic effect of CpG-oligodesoxynucleotides (ODN) in BALB/c mice. Local and systemic administration of CpG-ODN 1668 protected WT and IFN-$\beta^{-/-}$ mice equally well from progressive leishmaniasis. By contrast, the protective effect of CpG-ODN 1668 was lost in BALB/c IFNAR1$^{-/-}$ (despite a sustained

suppression of IL-4) and in BALB/c IL-12p35$^{-/-}$ mice. From these data, we conclude that IFN-β and IFNAR1 signaling are dispensable for a curative immune response to *L. major* in C57BL/6 mice and irrelevant for disease development in BALB/c mice, whereas IL-12 and IFN-α subtypes are essential for the disease prevention by CpG-ODNs in this mouse strain.

Keywords: *Leishmania major*, type I interferon, interferon-alpha/beta, innate immunity, cutaneous leishmaniasis

INTRODUCTION

The term "type I interferons" is used for a multigene family of cytokines that in the mouse comprises 14 interferon (IFN)-α genes encoding proteins and single genes of IFN-β, IFN-κ, and IFN-ε (1). All type I IFNs signal *via* a common IFN-α/β-receptor (IFNAR) complex, which consists of two chains (IFNAR1 and IFNAR2) (2). Type I IFNs were originally described for their antiviral activity, which is due to the induction of genes that degrade mRNA, impair protein synthesis, help to sequester viral nucleocapsids, induce cytoplasmic viral nucleic acid detecting receptors, or amplify the IFN response (3–5). Since then it has become clear that type I IFNs also have numerous immunomodulatory functions, which include both activating and inhibitory effects on macrophages, dendritic cells (DCs), natural killer (NK) cells, T lymphocytes, and B cells (5–8). Therefore, it was not surprising to see that in mice exposed to certain non-viral pathogens (bacteria, protozoa, fungi, or helminths) or microbial products a deficiency of IFN-α/β signaling or the application of type I IFNs positively or negatively influenced the outcome of the infection [reviewed in Ref. (9–11)]. To date, there is little information available on the expression and differential activities of the type I IFN subtypes during non-viral infections *in vivo*. In general, IFN-β is assumed to play a master role, as in many cell types it induces and amplifies the expression of IFN-α genes and thereby dominates the entire type I IFN response (12, 13); however, IFN-β-independent production of IFN-α has also been described (14, 15).

Leishmania promastigotes are flagellated protozoan parasites that under natural conditions are transmitted to mammalian organisms by the bite of sand flies. Infections can lead to cutaneous, mucocutaneous or visceral disease depending on the parasite species and strain, the infection inoculum, and the immune response of the host. The experimental infection of different inbred strains of mice with *Leishmania* (subgenus *Leishmania*) *major* (in the following abbreviated as *L. major*) has proven to be a useful model for self-healing vs. non-healing cutaneous leishmaniasis and for the analysis of the components of the immune system that are required for parasite control and resolution of the infection. Previous studies showed that interleukin (IL)-12, IFN-γ, tumor necrosis factor (TNF), inducible or type 2 nitric oxide synthase [iNOS (NOS2)], and CD4$^+$ type 1 T helper (Th1) cells are essential for overcoming an infection with *L. major* (16–18).

With respect to type I IFNs, earlier results pointed to a protective function in mouse *L. major* infections. First, *in vitro* simultaneous exposure of mouse macrophages to purified IFN-α/β and *L. major* promastigotes led to expression of iNOS and subsequent killing of intracellular amastigotes (19). Similarly, human mononuclear phagocytes acquired antimicrobial activity against *L. major* amastigotes after stimulation with IFN-β (20). Second, short-term neutralization of IFN-α/β immediately before cutaneous infection with *L. major* strongly reduced the expression of iNOS protein, the activation of NK cells, the expression of IFN-γ mRNA, and the containment of the parasites at days 1 and 2 of infection in the skin and draining lymph node (dLN) (21). Third, prophylactic treatment of otherwise non-healing BALB/c mice with low doses of recombinant mouse IFN-β protected the majority of the mice from progressive disease (22). However, so far it has not been analyzed which type I IFN subtypes are expressed during the course of *L. major* infection. Furthermore, it is unknown whether endogenous type I IFNs are required for the control of primary or secondary *L. major* infections in self-healing C57BL/6 mice or for the previously reported (23) immunoprophylactic effect of CpG-oligodesoxynucleotides (ODN) in BALB/c mice. Finally, it has never been investigated whether type I IFN signaling contributes to the susceptibility of *L. major*-infected BALB/c mice as suggested by the disease-aggravating role of type I IFNs observed in infections with other *Leishmania* species (24–26).

In this study, we were able to address these issues with the help of recombinant mice that were deficient for IFN-β (IFN-β$^{-/-}$ mice) or type I IFN signaling (IFNAR1$^{-/-}$ mice) and that were thoroughly backcrossed to the C57BL/6 or BALB/c background.

MATERIALS AND METHODS

Mice

Wild-type (WT) C57BL/6 and BALB/c mice were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany). IFNAR1$^{-/-}$ mice, which were originally generated on a 129/SvEv background (27), were backcrossed to C57BL/6 for more than 20 generations (B6.IFNAR1$^{-/-}$) by one of us (UK) at the Paul-Ehrlich-Institute, Langen, Germany. Breeding pairs of IFNAR1$^{-/-}$ mice (27) backcrossed to BALB/c for seven generations (28) were kindly provided by Daniel Portnoy (University of California, Berkeley, USA) and were then used for further backcrossings to BALB/c background (BALB/c.IFNAR1$^{-/-}$) for

Abbreviations: BM, bone marrow; BM-DC, bone marrow-derived dendritic cell; (c, m) DC, (conventional or myeloid) dendritic cell; iNOS (NOS2), inducible (or type 2) nitric oxide synthase; NK, natural killer; pDC, plasmacytoid dendritic cell; SN, supernatant.

four generations using speed congenic technology (29). IFN-$\beta^{-/-}$ mice (12) were backcrossed to C57BL/6 (B6.IFN-$\beta^{-/-}$) or BALB/c background (BALB/c.IFN-$\beta^{-/-}$) for 12 or 15 generations, respectively. IL-12p35$^{-/-}$ mice backcrossed to C57BL/6 background for 11 generations were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). Breeding pairs of IL-12p35$^{-/-}$ mice (30) backcrossed to BALB/c background for five generations were generously supplied by G. Alber (University of Leipzig, Germany).

Wild-type and knockout mice were age- and sex-matched in the experiments. All mice were kept under specific pathogen-free conditions in the facilities of (a) the Institute of Medical Microbiology and Hygiene at the University Hospital Freiburg, (b) the Microbiology Institute at the University Hospital Erlangen, or (c) the Franz-Penzoldt-Zentrum for Animal Research, Friedrich-Alexander-University of Erlangen-Nürnberg. The infection experiments were approved by the governmental animal-welfare committees of the Regierungspräsidium Freiburg, Germany and of the Government of Middle Franconia, Ansbach, Germany.

Parasites and Infection

The origin of the *L. major* strain MHOM/IL/81/FEBNI was described before (31, 32). Promastigotes were maintained *in vitro* in RPMI1640 plus 10% FCS on Novy–Nicolle–MacNeal (NNN) rabbit blood agar slants for a maximum of six passages. Fresh *L. major* promastigotes were derived from amastigotes that were isolated from non-ulcerated skin lesions of infected BALB/c mice (33). For *in vitro* expansion, *L. major* promastigotes were transferred from the NNN-cultures into complete Schneider's *Drosophila* insect cell medium [Genaxxon Bioscience; supplemented with 10% (v/v) heat-inactivated FCS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.27 mM L-asparagine, 0.55 mM L-arginine, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 2% (v/v) normal human urine in modification of previous protocols (34, 35)] and grown to stationary phase. Mice were infected with 3×10^6 stationary phase *L. major* promastigotes (derived from NNN-cultures) in 50 µl of PBS subcutaneously (s.c.) into one or both hind footpads. For control purposes, mice were injected with PBS alone in some experiments. Footpad swelling was determined once or twice weekly with a metric caliper (in mm; Kroeplin, Schlüchtern, Germany). The relative footpad thickness increase was calculated in relation to the other footpad (in unilateral infection experiments) or the footpad thickness before infection (in bilateral primary infection experiments or after secondary infection).

Quantification of Parasite Burdens

Tissue parasite burdens were determined by limiting dilution analysis using twofold, threefold, or fivefold dilution steps and 12 replicates per dilution in complete Schneider's *Drosophila* medium (see above) as described before (33, 36). The statistical analysis was performed with the L-Calc™ (StemSoft Software, Vancouver, BC, Canada) or ELIDA software, which analyses data by applying the Poisson distribution and by the χ^2 test (37). Statistical significance was assumed when 95% confidence intervals did not overlap.

Treatment of Mice with CpG-ODNs

Following a published protocol (23), BALB/c mice (WT, IFN-$\beta^{-/-}$, or IFNAR1$^{-/-}$) were treated twice with 10 nmol CpG-ODN 1668 or 2216 (Thermo Electron, Ulm, Germany), i.e., 2 h before and 10 h after the infection with *L. major* promastigotes. At each time-point, half of the dose (5 nmol) was given s.c. at the site of infection in 40 µl PBS (20 µl per footpad in experiments with bilateral infection), while the other half was administered intraperitoneally (i.p.) in a volume of 500 µl PBS. Alternatively, the total applied dose of CpG-ODN was reduced to 10 nmol (with 5 nmol given s.c. at 2 h before infection and 5 nmol injected i.p. at 10 h after infection), which was equally effective in protecting WT BALB/c mice. Control mice received PBS alone.

Gene Expression Analysis

Excised organs and tissues were directly stored in RNAlater reagent (Qiagen, Hilden, Germany) for at least 24 h. Organs were then homogenized in a Mixer Mill MM 200 (Retsch, Haan, Germany) before extracting total RNA using TRIZOL reagent (Life Technologies Invitrogen, Darmstadt, Germany). Contaminating genomic DNA was removed with DNase (DNAfree, Life Technologies Ambion®). Presence of genomic DNA was excluded by performing a PCR reaction with 1 µl of the RNA sample as template and primers for mouse β-actin (*sense*: 5′-CACCCGCCACCAGTTCGCCA-3′; *antisense*: 5′-CAGGT CCCGGCCAGCCAGGT-3′). Five to ten micrograms of RNA were reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Life Technologies Applied Biosystems). For quantitative PCR, 100 ng of each cDNA was analyzed in triplicates using the ABI PRISM HT7900 system (Life Technologies Applied Biosystems) and the following gene-specific assays (TaqMan Gene Expression Assays; Life Technologies Applied Biosystems): mIFN-α2 (Mm00833961_s1), mIFN-α4 (Mm00833969_s1), mIFN-α5 (Mm00833976_s1), mIFN-α6 (Mm01258374_s1), mIFN-α9 (Mm00833983_s1), mIFN-α11 (Mm01257312_s1), mIFN-α12 (Mm00616656_s1), mIFN-α13 (Mm00781548_s1), mIFN-α14 (Mm01703465_s1), mIFN-β (Mm00439546_s1), mIFN-γ (Mm00801778_m1), mIL-2 (Mm00434256_m1), mIL-4 (Mm00445259_m1), mIL-10 (Mm00439616_m1), mIL-12p35 (Mm00434165_m1), mIL-12p40 (Mm00434170_m1), mIL-13 (Mm00434204_m1), mIL-15 (Mm00434210_m1), mIL-18 (Mm00434225_m1), IL-23p19 (Mm00518984_m1), murine iNOS (NOS2) (Mm00440485_m1), and murine arginase 1 (Arg1) (Mm00475988_m1). The gene for mouse hypoxanthine guanine phosphoribosyl transferase 1 (mHPRT-1; Mm00446968_m1) was used as an endogenous control for quantification of mRNA levels. All mRNA levels were determined in duplicates or triplicates with the help of the SDS 2.3 Software (Life Technologies Applied Biosystems®). Relative expression levels were calculated using the following formula: relative expression $= 2^{-(\mathrm{Ct(Target\ Gene)}-\mathrm{Ct(Endogenous\ Control)})} \times f$, with $f = 10^4$ as an arbitrary factor.

DCs and Stimulation by *Leishmania* and CpG-ODNs

Bone marrow (BM) was isolated from hind legs of naïve WT, IL-12p35$^{-/-}$ or IFNAR1$^{-/-}$ mice after anesthesia and subsequent cervical dislocation. Femoral and tibial bones were opened on both sides under sterile conditions, and bone marrow cells were flushed out with PBS using a 27G hollow needle.

Bone marrow-derived conventional (or myeloid) DCs (cDCs or mDCs) were differentiated by culturing 6×10^6 BM cells in RPMI1640 medium [containing 2 mM L-glutamine, 10 mM HEPES, 50 µM 2-mercaptoethanol, 100 U/ml penicillin G, 100 µg/ml streptomycin, 10% (v/v) FCS, and 10% (v/v) culture supernatant (SN) of Ag8653 myeloma cells transfected with a murine GM-CSF expression plasmid (38)]. BM cells were cultured for 7 days in 60 cm^2 culture dishes with initially 10 ml of medium, before on days 3 and 6, 10 ml fresh medium was added. mDC cultures contained 60–80% CD11b$^+$CD11c$^+$ mDC on day 7 and were purified as immature CD11b$^+$CD11c$^+$CD86$^-$ cells by flow cytometric cell sorting (MoFlo) (purity of >99%).

Bone marrow-derived plasmacytoid dendritic cells (pDCs) were generated from total BM cells in the presence of Flt3 ligand (39). After incubation in red blood cell lysis buffer for 5 min, cells were washed twice with 20 ml PBS. Bone marrow cells were cultured in 5 ml RPMI1640 [containing 2 mM L-glutamine, 1× non-essential amino acids, 1 mM sodium pyruvate, 100 µg/ml kanamycin, 50 µM 2-mercaptoethanol, 10% (v/v) FCS, and 50 ng/ml rmFlt3L (R&D Systems, Wiesbaden, Germany)] for 7–8 days at 2×10^6 cells/ml in 25 cm^2 cell culture flasks. At day 4, 2.5 ml of the culture medium was exchanged against fresh medium with 25 ng/ml Flt3L. After 7–8 days, 10–20% of the cells were B220$^+$CD11b^{int}CD11c$^+$, and pDCs were further purified by MoFlo sorting gating on B220$^+$CD11b^{int}CD11c$^+$ cells (purity >99%).

For ELISA and IFN-α/β bioassay studies, MoFlo™-sorted pDC and mDC were cultured in 96-well culture plates (10^5 cells/well in 250 µl) using the respective pDC or mDC culture medium without growth factors. Cells were stimulated for 48 h with CpG-ODN 2216 (1 µM), CpG-ODN 1668 (1 µM), LPS (200 ng/ml), or *L. major* promastigotes (stationary growth phase; multiplicity of infection 3). SNs were harvested and stored at −20°C.

Cytokine Measurements

Interferon-α/β levels were determined with an L929/vesicular stomatitis virus-protection assay using triplicates and serial two-fold dilutions of the culture SNs (21). Purified mouse IFN-α/β and a neutralizing sheep-anti-IFN-α/β antiserum (provided by I. Gresser, Institute Curie, Paris) were used as a standard or to ascertain that all antiviral activity in the SNs was due to IFN-α/β. The content of TNF (eBiosciences, sensitivity 40 pg/mL), IL-12p40, or IL-12p70 (BD Biosciences, sensitivity 40 pg/mL) was measured by ELISA.

Statistics

Statistical significance was analyzed using the non-parametric Mann–Whitney test. A p value <0.05 was considered significant.

RESULTS

L. major Infection Leads to Differential Expression of Type I IFNs

In a previous study, we reported the expression of IFN-α/β protein in skin lesions of C57BL/6 × 129/SvEv mice at 24 h after infection with *L. major*. The immunohistological analysis was restricted to this early time point and based on the use of a polyvalent anti-IFN-α/β antibody, which did not allow for differential detection of type I IFN subtypes (21). To obtain a more detailed view on type I IFN expression during the course of experimental cutaneous leishmaniasis (days 1–56), we performed quantitative mRNA expression analyses for several type I IFN family members in the skin lesions of C57BL/6 mice subcutaneously infected with *L. major*. Within 24–48 h of *L. major* infection the relative IFN-β mRNA expression level increased by a factor of 4.7 from 0.52 (±0.10) in uninfected mice to 2.45 (±0.34) in infected ones (mean ± SEM of seven independent experiments with two to six samples; $p < 0.001$ Mann–Whitney test). From week 1 of infection onward, the IFN-β mRNA remained on a high expression level until the end of the observation period (day 56), when the footpad lesions had already started to resolve (**Figure 1A**). Considerably weaker and more transient was the upregulation of IFN-α4 and IFN-α5 mRNA, which returned to baseline levels within 3–4 weeks of infection. IFN-α11 and especially IFN-α13 mRNA were constitutively expressed in the skin of naïve mice. Following infection, both IFN-α subtypes initially decreased but returned to normal levels after 6–7 weeks of infection (**Figure 1A**).

In the dLN, we also observed a temporary upregulation of the IFN-α4 and IFN-β mRNA expression in response to *L. major*. Unlike to our findings in the skin, however, the baseline IFN-α13 mRNA levels in the popliteal lymph nodes of naïve C57BL/6 mice were much lower and clearly increased during the first 4 weeks of infection (**Figure 1B**). In the spleen of *L. major*-infected C57BL/6 mice, the mRNA expression levels of the above-mentioned type I IFN subtypes remained largely constant throughout the infection period (data not shown). With respect to IFN-α6, IFN-α12, and IFN-α14, hardly any changes were observed in the three analyzed tissues, or mRNA expression was not detectable at all (data not shown).

To exclude that the injection procedure itself accounts for the early induction of type I IFNs in the skin and dLN, we compared PBS-injected (day 2) and *L. major*-infected C57BL/6 mice (day 2) with naïve C57BL/6 mice (day 0) for their expression of IFN-α5 and IFN-β mRNA. Injection of PBS alone already caused a slight, but significant induction of type I IFNs at both tissue sites. However, in the presence of *L. major*, the expression levels were significantly higher than in PBS-treated mice (Figure S1 in Supplementary Material).

Taking these data together, we conclude that an infection with *L. major* triggers a specific and primarily local type I IFN response with differential regulation of IFN-β and IFN-α subtypes.

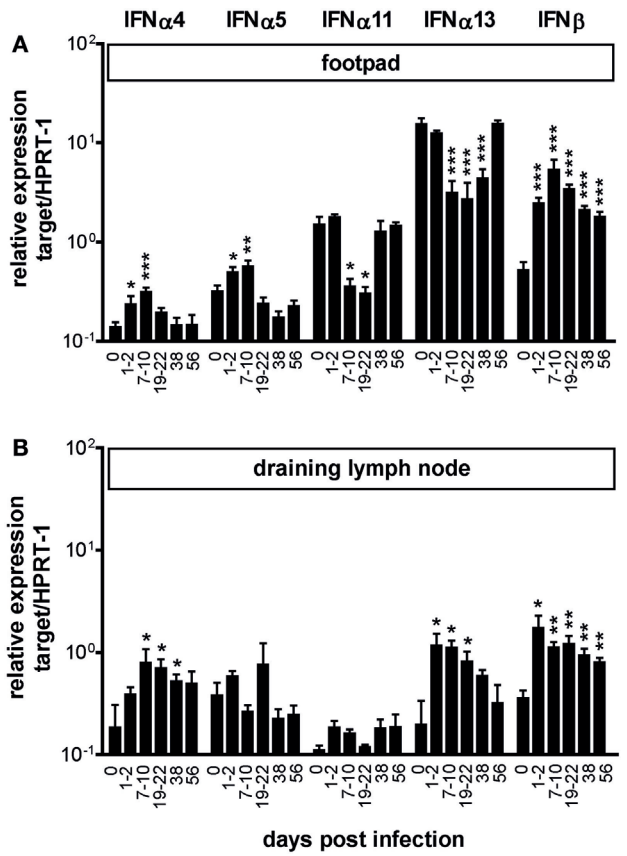

FIGURE 1 | Expression of type I interferon (IFN) subtypes in *Leishmania major*-infected C57BL/6 mice. C57BL/6 wild-type mice were infected with 3×10^6 stationary phase *L. major* promastigotes into the hind footpads or treated with PBS. The mRNA expression of type I IFN subtypes was analyzed in the footpad tissues **(A)** and in the draining lymph nodes **(B)** at different time-points after infection using quantitative RT-PCR (day 0 are naïve mice). Results are shown as mRNA levels (mean ± SEM) normalized to the endogenous control HPRT-1 from four independent experiments (three mice per group and time point, respectively). Asterisks represent the respective significance values compared with uninfected mice (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$; Mann–Whitney test).

Type I IFNs Are Dispensable for the Control of *L. major* in Self-healing C57BL/6 Mice

The infection-dependent regulation of type I IFN expression led us to investigate a possible function of IFN for the outcome of *L. major* infections. In the light of the strong induction of IFN-β mRNA (**Figure 1**) and its known amplifying effect on the expression of other type I IFNs (40), we first investigated the course of *L. major* infection in C57BL/6 IFN-$\beta^{-/-}$ mice. IFN-$\beta^{-/-}$ mice controlled the clinical infection as efficiently as the respective WT mice (**Figure 2A**). Accordingly, the tissue parasite burdens in the skin lesions, dLNs, and spleens from WT and IFN-$\beta^{-/-}$ mice were comparable (**Figure 2B**). Also, the time course of the mRNA expression of cytokines (IFN-γ, IL-12p35, IL-12p40, IL-4, and IL-13) and effector pathways (iNOS, arginase 1) that determine the quality of the anti-*Leishmania* immune response were virtually superimposable (**Figure 3**, solid squares vs. open circles).

The previous notion that IFN-β governs the entire type I IFN response has recently been challenged (14, 15, 41). To test the impact of the entire type I IFN family on an infection with *L. major*, we resorted to C57BL/6 mice that lack a functional type I IFN receptor. When C57BL/6 IFNAR1$^{-/-}$ were infected with *L. major*, the clinical course of infection (**Figure 2A**), the parasite burden (**Figure 2B**), the cytokine mRNA expression pattern (IFN-γ, IL-12p35, IL-12p40, IL-4, and IL-13) at the site of infection and in the dLN, and the ability to mount a strong and persistent iNOS response along with a transient upregulation of arginase 1 (**Figure 3**, solid squares vs. open triangles) were indistinguishable in C57BL/6 WT and IFNAR1$^{-/-}$ mice. After resolution of the skin swelling, we did not observe any clinical relapses in the mutant mice during an observation period of up to 241 days. Notably, when we infected IFNAR1$^{-/-}$ mice [generated on a 129Sv/Ev background (27)] that had been backcrossed to C57BL/6 for only 6 instead of 20 generations, the size of the cutaneous lesions in the IFNAR1$^{-/-}$ mice was significantly smaller than in the respective WT controls (data not shown). This observation presumably reflects incomplete backcrossing. Unlike to C57BL/6 mice that lack a mature and functional natural resistance-associated macrophage protein (NRAMP1^S), 129Sv/Ev mice carry a fully functional NRAMP1 protein (NRAMP1^R) and usually develop only minor skin lesions following *L. major* infection (42).

As type I IFNs have been described to rescue activated or memory T cells from apoptosis and to increase the longevity of these cells (43–45), we considered the possibility that IFN-$\beta^{-/-}$ and IFNAR1$^{-/-}$ might only show a phenotype during secondary infection with *L. major*. We therefore challenged C57BL/6 WT and IFNAR1$^{-/-}$ mice, which had healed a primary subcutaneous infection with *L. major*, by injection of the same parasite inoculum into the contralateral footpad. As expected based on earlier reports (46, 47), the secondary skin lesions of C57BL/6 WT mice were less severe and healed more rapidly than during the primary infection. Although there was a tendency for a disease aggravation in IFNAR1$^{-/-}$ mice, the differences in lesion development (**Figure 4A**) and tissue parasite burden (**Figure 4B**) between C57BL/6 WT and IFNAR1$^{-/-}$ mice were not significant in three independent experiments.

From these data, we conclude that both the control of a primary and of a secondary infection with *L. major* can occur independently of type I IFN signaling.

Type I IFNs Are Dispensable for *L. major*-Induced Production of IL-12 by C57BL/6 Myeloid DCs

The unaltered expression of IL-12p35 and IL-12p40 seen in *L. major*-infected C57BL/6 IFNAR1$^{-/-}$ mice as compared to C57BL/6 WT mice (**Figure 3**) contrasts with a previous *in vitro* study in which type I IFN activity was required for optimal IL-12 expression by mouse bone marrow-derived dendritic cells (BM-mDCs) after stimulation with toll-like receptor (TLR) 3

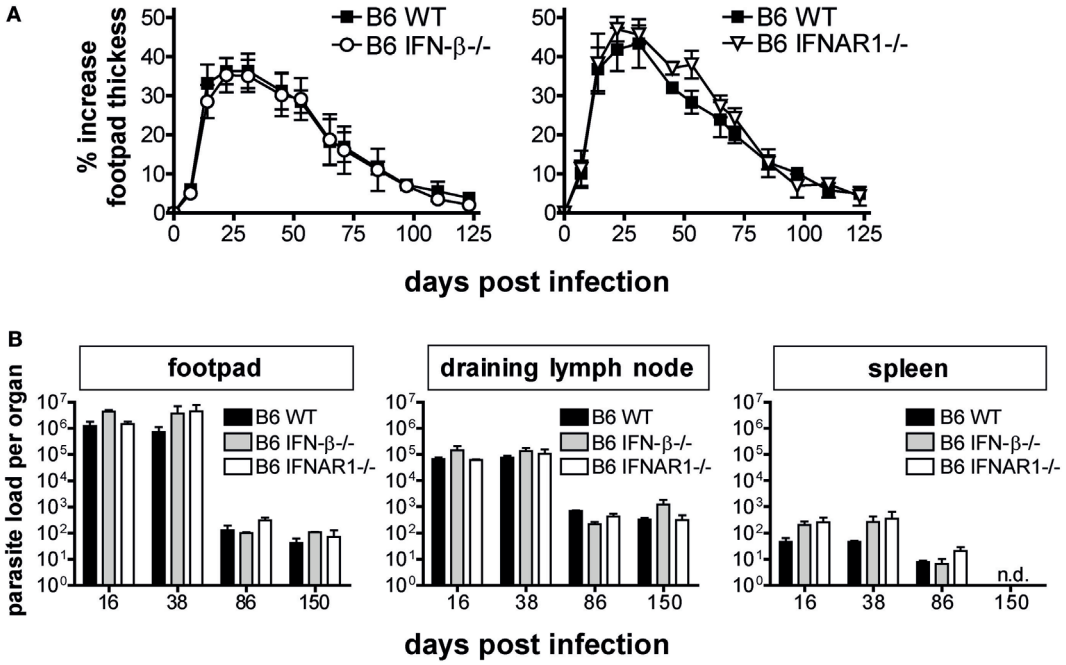

FIGURE 2 | Interferon (IFN)-β and IFNAR signaling are dispensable for the control of primary infection with *Leishmania major* in C57BL/6 mice. **(A)** Development of footpad lesions in C57BL/6 wild-type (WT) vs. IFN-$\beta^{-/-}$ vs. IFNAR1$^{-/-}$ mice after infection with 3 × 10^6 stationary phase *L. major* promastigotes into both hind footpads. The mean (±SEM) of the relative footpad thickness increase of 4 independent experiments with 9–12 mice per group is shown. **(B)** Parasite burden in the footpads, draining lymph nodes, and the spleens of C57BL/6 WT vs. IFN-$\beta^{-/-}$ vs. IFNAR1$^{-/-}$ mice. At the indicated time-points, three mice per group were analyzed for their parasite load in different organs by limiting dilution assays. The mean results (±SEM) of one representative out of four independent experiments **(A)** are presented. n.d., Not detectable.

plus TLR7 or TLR4 plus TLR7 agonists (48). We therefore tested whether the production of IL-12p40/p70 and IFN-α/β by BM-mDC in response to *L. major* promastigotes, a process that is triggered by TLR9 (15, 49), is dependent on endogenous IFNAR signaling. The TLR9 agonists CpG-B ODN 1668 and CpG-A ODN 2216 were used as control stimuli and mouse BM-pDC as control cells. In experimental leishmaniasis, mDCs are the key source of IL-12 (50–52) but weak producers of IFN-α/β (15), whereas pDCs generate considerably less IL-12, but copious amounts of IFN-α/β in response to *Leishmania* parasites (15). Both the IL-12 and the IFN-α/β production by BM-mDCs remained unaltered in the absence of IFNAR, irrespective of the stimulus used (**Figure 5A**). By contrast, in pDCs IFNAR signaling not only positively regulated the *Leishmania*- or CpG-induced production of IFN-α/β confirming our previous data (15), but was also necessary for maximal IL-12 release (**Figure 5B**).

From these data, we conclude that the *L. major*-induced production of IL-12 by mDCs, but not by pDCs, is fully preserved in the absence of type I IFN signaling.

IFN-β or IFNAR1 Deficiency Does Not Alter the Course of Infection in Non-Healing BALB/c Mice

In the light of the multiple suppressive functions of type I IFNs on DCs, macrophages, NK cells, and T cells [reviewed in Ref. (5–8)] and previous reports on disease-aggravating effects of type I IFNs in mouse and human infections with South American *Leishmania* species (24–26), we considered the possibility that type I IFNs might contribute to the non-healing pathology of cutaneous *L. major* infection observed in BALB/c mice. Infection of BALB/c IFN-$\beta^{-/-}$ or IFNAR1$^{-/-}$ mice, however, revealed that the clinical development of the skin lesions and the tissue parasite burden were not significantly different from WT BALB/c mice (**Figures 6A,D**), despite the rapid induction of various type I IFNs (i.e., IFN-α2, IFN-α4, IFN-α5, IFN-α6, and IFN-α9) at the site of infection in BALB/c WT mice (see **Figure 7**, upper panel, PBS/*L. major* vs. PBS/PBS). Thus, type I IFNs are unlikely to account for the lack of parasite control in BALB/c mice.

The CpG-ODN Induced Protection of BALB/c Mice Is Preserved in the Absence of IFN-β, but Lost in IFNAR1 Deficiency

Finally, we addressed the question, whether the previously described protection of BALB/c mice from progressive cutaneous leishmaniasis following a prophylactic treatment with the CpG-ODN 1668 (23) is also observed when the type I IFN system is functionally impaired or blocked. As expected (23), injection of CpG-ODN 1668 before and shortly after infection prevented ulcerative skin lesions and restored parasite control in otherwise non-healing BALB/c WT mice. This was also the case in BALB/c IFN-$\beta^{-/-}$ mice (**Figures 6A,B**). Equivalent results were obtained with CpG-ODN 2216 (data not shown). In BALB/c

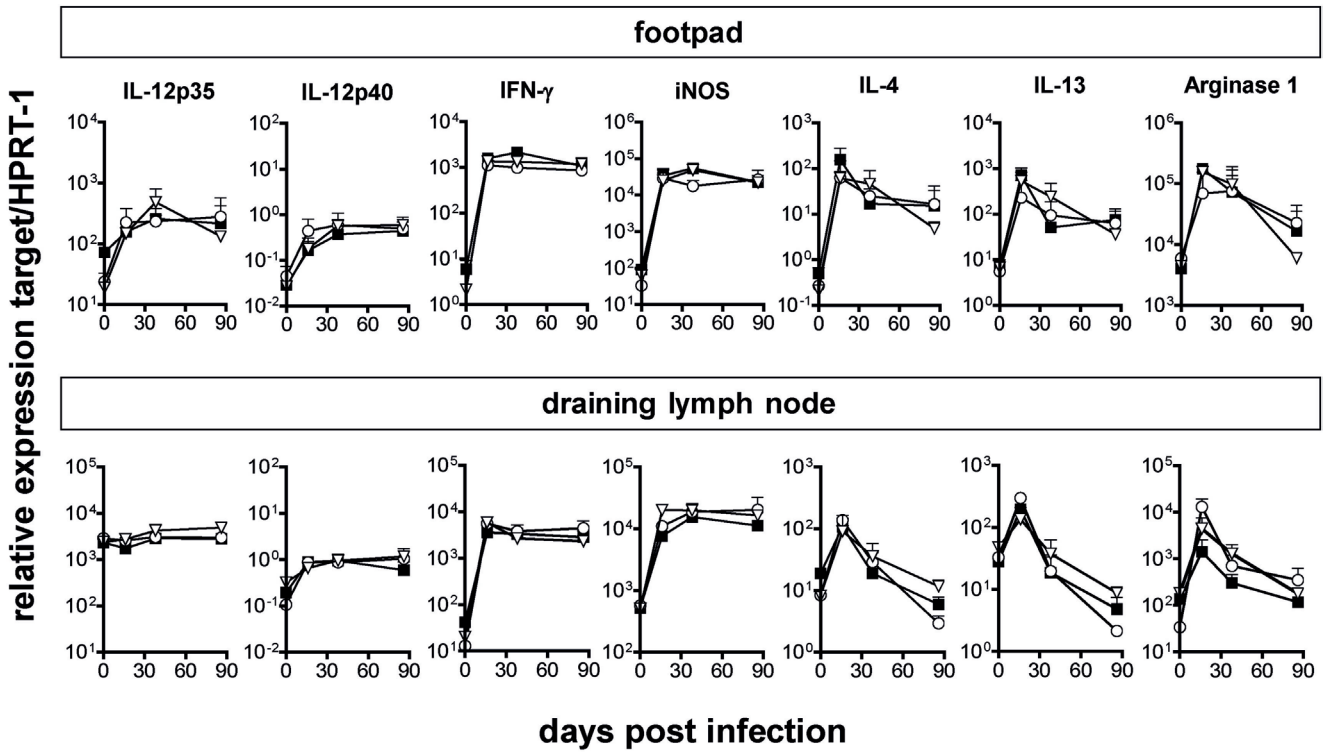

FIGURE 3 | Comparable mRNA expression of cytokines and effector pathways in C57BL/6 wild-type (WT), IFN-β$^{-/-}$, and IFNAR1$^{-/-}$ mice during *Leishmania major* infection. Total RNA was isolated from footpad tissue or draining popliteal lymph nodes of WT (solid squares), IFN-β$^{-/-}$ (open circles), and IFNAR1$^{-/-}$ mice (open triangles) and reverse transcribed. Gene expression levels were determined by quantitative RT-PCR analysis using assays for the respective genes. Expression levels were calculated relative to the expression level of the endogenous control gene (HPRT-1). Results are mean expression levels from three mice per group and time point with error bars indicating SDs. The results of one of two experiments are shown.

IL-12p35$^{-/-}$ mice (**Figures 6A,B**) and in BALB/c IFNAR1$^{-/-}$ mice (**Figures 6C,D**), however, the protective effect of CpG-ODN 1668 was lost. From these data, we conclude that IFNAR1 signaling (notably by IFN-α) and IL-12 are required for the immunoprophylactic activity of CpG-ODNs, whereas IFN-β is dispensable for the protective effect of CpG-ODNs.

To further ascertain the cooperation of type I IFNs and IL-12, we investigated whether the intracutaneous and intraperitoneal application of CpG-ODN 1668 indeed leads to a simultaneous upregulation of type I IFNs, IL-12, and possibly other cytokines (IFN-γ and TNF) that are known for their macrophage activating and protective effects in murine cutaneous leishmaniasis (53–56). In the footpads of *L. major*-infected WT BALB/c mice from three independent experiments, CpG-ODN 1668 led to a significant increase of IFN-γ, IL-12p35, TNF, and iNOS mRNA expression at 36 h after infection (i.e., 26 h after the last CpG-ODN 1668 injection) (**Figure 7**, upper panel). As seen in a previous study (49), IL-12 p40 mRNA was undetectable in whole organ RNA preparations from skin lesions, because at this early time point of infection the expression of IL-12 is restricted to a small number of DCs. With the notable exception of IFN-α13, CpG-ODN 1668 also enhanced the expression of type I IFNs in *L. major*-infected BALB/c WT mice, although the level of significance was only reached for IFN-β (**Figure 7**, upper panel). In *L. major*-infected BALB/c IFN-β-deficient mice, the stimulatory effect of CpG-ODN-1668 on the expression of IFN-α subtypes (α2, α4, α5, and α9), cytokines (IFN-γ, IL-12p35, and TNF), and iNOS expression was mostly maintained, whereas in IFNAR1-deficient BALB/c mice the upregulation of IFN-α subtypes, IFN-β, IFN-γ, IL-12p35, and iNOS was absent (**Figure 7**, middle and lower panel).

Finally, we tested whether *L. major*-infected BALB/c WT mice and IFNAR1$^{-/-}$ mice treated with PBS or CpG-ODN 1668 differed in their expression of IL-4 and IFN-γ mRNA at day 23 or day 24 of infection, when the lesions of CpG-ODN 1668-treated WT mice were strikingly smaller than of CpG-ODN 1668-treated IFNAR1$^{-/-}$ mice (**Figure 6C**). As shown in **Figure 8**, treatment with CpG-ODN 1668 insignificantly upregulated the expression of IFN-γ mRNA in BALB/c WT mice. By contrast, in BALB/c IFNAR1$^{-/-}$ mice, IFN-γ mRNA was strongly reduced as compared with WT mice and was not rescued by CpG-ODN 1668 treatment. In both strains of mice, CpG-ODN 1668 caused a strong suppression of IL-4 mRNA (**Figure 8**). Consequently, the IFN-γ/IL-4 mRNA ratio was approximately fourfold lower in CpG-ODN 1668-treated BALB/c IFNAR1$^{-/-}$ mice as compared with CpG-ODN 1668-treated BALB/c WT mice.

Taken together, we conclude that CpG-ODN 1668 prevented progressive disease in BALB/c WT mice by (a) boosting the early

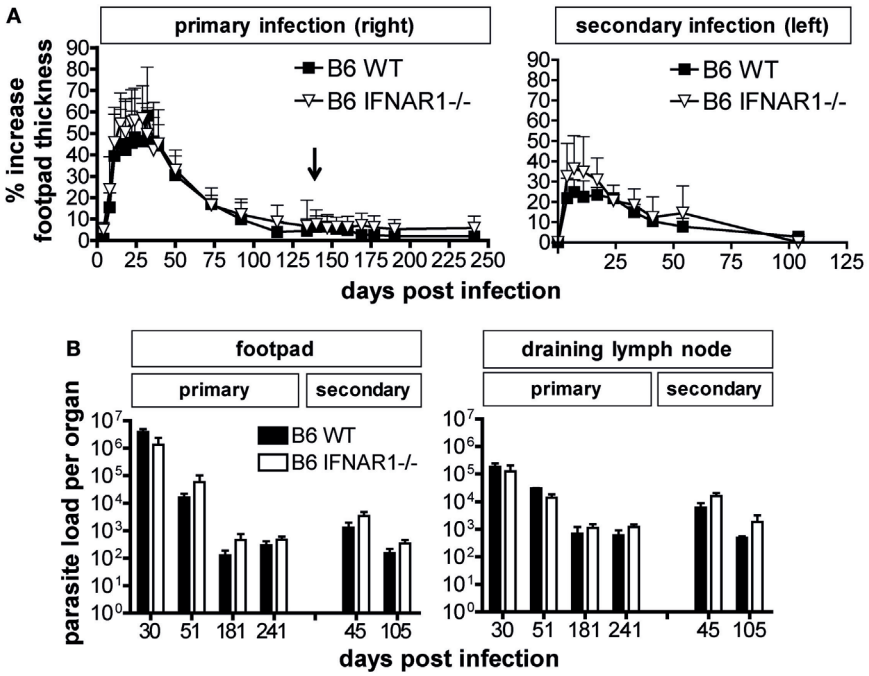

FIGURE 4 | Comparable control of secondary *Leishmania major* infection in C57BL/6 wild-type (WT) and IFNAR1$^{-/-}$ mice. For primary infection, mice were injected subcutaneously with 3×10^6 stationary phase *L. major* promastigotes into the right hind footpad. At day 136 (indicated by ↓), i.e., after healing of the primary skin lesion, mice were reinfected with an identical parasite inoculum into the left hind footpad. **(A)** Clinical course of infection in C57BL/6 WT vs. IFNAR1$^{-/-}$ mice. The mean (±SD) of the relative footpad thickness increase during primary (right footpad) and secondary infection (left footpad) is shown. One of three independent experiments with 12–18 mice per group is presented. In panel **(B)**, the tissue parasite burden in the right and left footpad and draining lymph node at various time points after primary (left) and secondary infection (right) is depicted (please note the different time scale of the abscissas). At the indicated time points, three mice per group were analyzed by limiting dilution assays. The mean results (±SEM) of one representative out of three independent experiments are shown.

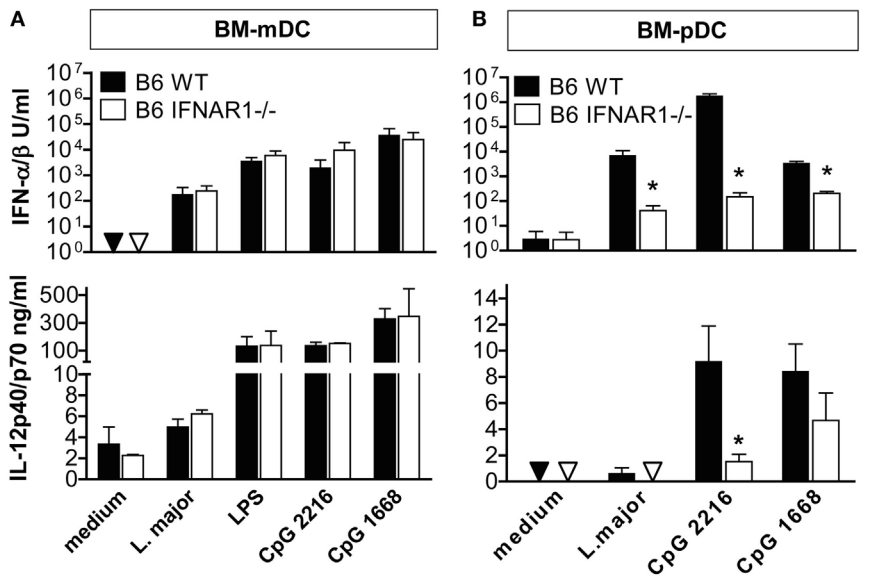

FIGURE 5 | IFNAR signaling affects the *Leishmania major*-induced production of interferon (IFN)-α/β and interleukin (IL)-12 in BM-plasmacytoid dendritic cells (pDCs), but not bone marrow-derived dendritic cells (BM-mDCs). CD11b$^+$CD11c$^+$CD86$^-$ sorted BM-mDCs and B220$^+$CD11b^{int}CD11c$^+$ sorted BM-pDCs were cultured for 48 h with *L. major* promastigotes (multiplicity of infection = 3), 200 ng/ml LPS, 1 μM CpG-oligodesoxynucleotides (ODN) 2216 or CpG-ODN 1668. Culture supernatants were analyzed for IFN-α/β (vesicular stomatitis virus bioassay with L929 fibroblasts) and IL-12p40/p70 content (ELISA). Triangles depict values below the detection limit of the assays. **(A)** BM-mDCs, **(B)** BM-pDCs. Mean results (±SEM) of three to four independent experiments are shown. Asterisks depict significant differences between wild-type (WT) and IFNAR1$^{-/-}$ cells (*$p < 0.05$; Mann–Whitney test).

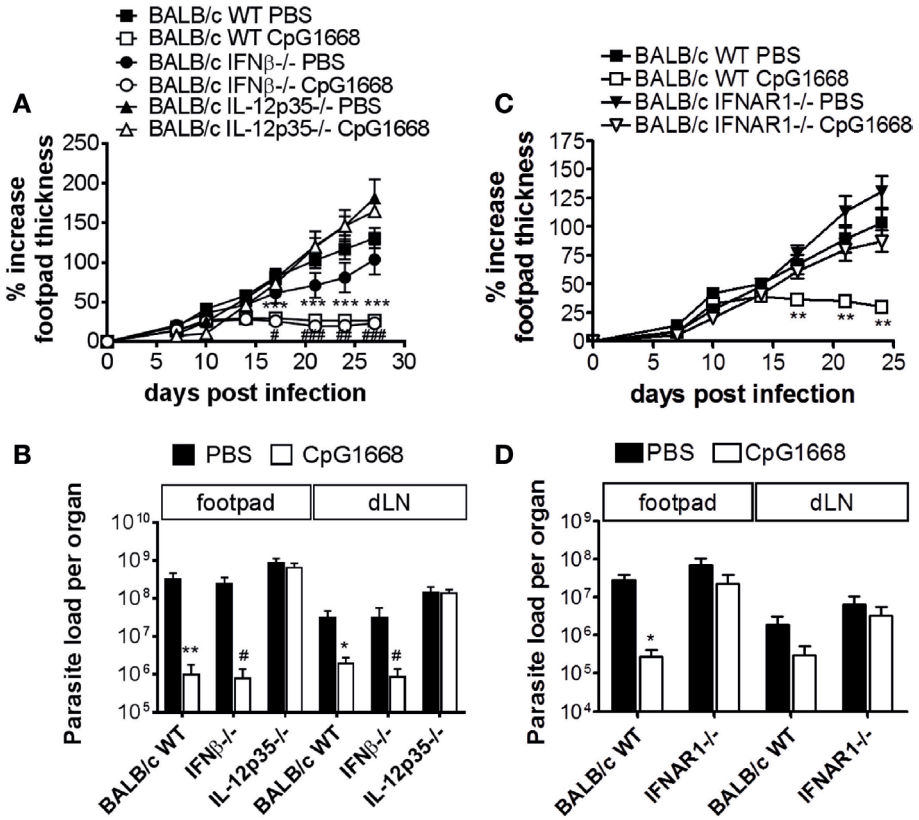

FIGURE 6 | Course of *Leishmania major* infection in untreated or CpG-oligodesoxynucleotides (ODN) 1668-treated BALB/c wild-type (WT), interferon (IFN)-β$^{-/-}$, IFNAR1$^{-/-}$, and interleukin (IL)-12p35$^{-/-}$ mice. Mice were infected with 3 × 10^6 stationary phase *L. major* promastigotes into both hind footpads. 2 h before infection, 5 nmol CpG-ODN 1668 was administered subcutaneously at the site of infection, and 10 h postinfection additional 5 nmol CpG-ODN 1668 was injected intraperitoneally. **(A,C)** Development of footpad lesions in untreated vs. CpG-ODN 1668-treated BALB/c WT vs. IFN-β$^{-/-}$ vs. IL-12p35$^{-/-}$ or BALB/c WT vs. IFNAR1$^{-/-}$ mice. The relative footpad thickness increase (mean ± SEM) of two to three independent experiments with three to five mice per group is shown. **(B,D)** Parasite burden in the footpads and draining lymph nodes of untreated vs. CpG-ODN 1668-treated BALB/c WT vs. IFN-β$^{-/-}$ vs. IL-12p35$^{-/-}$ or BALB/c WT vs. IFNAR1$^{-/-}$ mice at day 27 (B) or day 24 (D) after infection. Mean results (±SEM) of two to three independent experiments with three to five mice per group are shown. Significant differences by Mann–Whitney test between PBS- and CpG-treated BALB/c WT mice (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$) or PBS- and CpG-treated IFN-β$^{-/-}$ mice ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$; and $^{\#\#\#}p < 0.001$) are indicated.

expression of iNOS and several protective cytokines (IFN-α subtypes, IFN-γ, IL-12p35, and TNF) and (b) by downregulating IL-4, which—except for the effects on TNF and IL-4—required an intact IFNAR signaling. Therefore, CpG-ODN 1668 failed to convey protection in BALB/c IFNAR1$^{-/-}$ mice.

DISCUSSION

During infections of mice with bacteria, protozoa, or fungi, endogenously produced type I IFNs either supported or impeded the control of the pathogens and the survival of the animals (57–78). In this study, we found that the self-healing course of infection seen in *L. major*-infected C57BL/6 WT animals was fully preserved in C57BL/6 mice lacking the IFN-β-gene or a functional type I IFN receptor (IFNAR1$^{-/-}$). In genetically susceptible BALB/c mice, which develop progressive skin lesions after cutaneous infection with *L. major* and ultimately succumb to visceral disease, a deficiency of IFN-β or IFNAR also did not alter the clinical outcome of the infection. However, the protective effect of a treatment of otherwise non-healing BALB/c mice with CpG-containing ODNs was completely abolished in the absence of type I IFN signaling. These results raise important questions on the regulation and function of type I IFN expression during different infections and *in vivo* conditions.

Type I IFN Expression during Non-Viral Infections

Very few studies have addressed the expression of type I IFNs in response to pathogens other than viruses *in vivo*. Several groups measured IFN-α/β bioactivity or IFN-β protein in the serum of mice infected with bacteria or protozoa, but quantitative type I IFN subtype analyses *in vivo* during the course of infection are lacking [reviewed in Ref. (6, 10, 11)]. In the C57BL/6 *L. major* infection model studied here, we found a rapid and striking induction of IFN-β mRNA in the infected skin, to a lesser degree also of IFN-α4 and IFN-α5 (**Figure 1**). Based on results obtained with viral infections of cell lines, IFN-β and IFN-α4

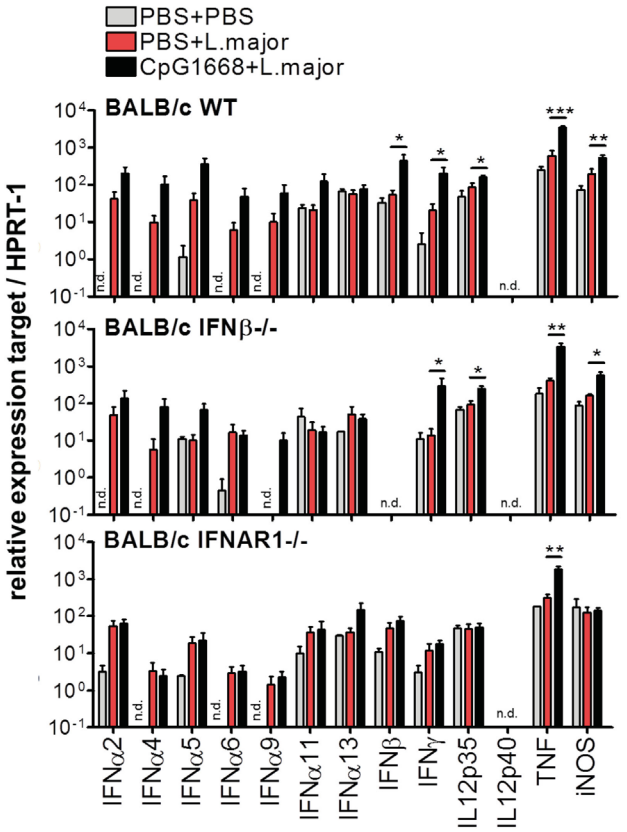

FIGURE 7 | Effect of CpG-oligodesoxynucleotides (ODN) 1668 treatment on the cytokine mRNA expression in the footpad of *Leishmania major*-infected mice. BALB/c wild-type (WT), interferon (IFN)-β$^{-/-}$ and IFNAR1$^{-/-}$ mice were infected with 3 × 10^6 stationary phase *L. major* promastigotes into both hind footpads or injected with PBS as control. 2 h before infection, 5 nmol CpG-ODN 1668 was administered subcutaneously at the site of infection, and 10 h postinfection additional 5 nmol CpG-ODN 1668 was injected intraperitoneally. Control mice received PBS. Total RNA was isolated from footpad tissue 36 h p.i. and reverse transcribed. Gene expression levels were determined as described in legend of **Figure 3**. Results are mean expression levels (±SEM) from three independent experiments with two to three mice per group. Significant differences by Mann–Whitney test between PBS- and CpG-treated infected BALB/c WT, IFN-β$^{-/-}$, or IFNAR1$^{-/-}$ mice (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$) are indicated. n.d., Not detectable.

(and presumably also IFN-α5) act as immediate-early IFNs that are elicited by activation of the transcription factor interferon-regulatory factor (IRF)-3 and IRF-7 and account for the subsequent amplification of the type I IFN response *via* crosslinking of IFNAR (41). IFN-α13 has been originally described as an IFN-α subtype that is expressed in mouse fibroblast cell lines even in the absence of a viral stimulus or an IFN-α/β priming signal (79). In this study, we found that the constitutive expression of IFN-α13 (and of IFN-α11) in naïve mice is subject to negative regulation following *L. major* infection. Looking at the entire course of infection, IFN-α13 and IFN-β are reciprocally regulated and expressed, which suggests that both subtypes fulfill different functions. In the human type I IFN system, there is evidence for a functional diversity of IFN-α/β subtypes [reviewed in Ref. (80)]. The underlying structure–function relationships are only beginning to emerge (81, 82). In the mouse system, we are still largely lacking important tools (recombinant proteins, subtype-specific antibodies, and knockout mice) to investigate the function of the different IFN-α/β subtypes.

Function of Endogenous Type I IFN during Non-Viral Infections

IFNAR1$^{-/-}$ mice infected with group B streptococci, *Streptococcus pneumoniae*, *Escherichia coli* (65), *Trypanosoma cruzi* (61, 62), *Plasmodium yoelii* (74), *Plasmodium berghei* (83), *Pneumocystis carinii* (84), or *Cryptococcus neoformans* (64) all showed a significantly enhanced pathogen load and/or reduced survival compared with the respective WT control mice. The protective effect of type I IFN seen in these models is contrasted by studies on other infectious pathogens, in which type I IFN signaling was either clearly associated with reduced pathogen control, striking tissue damage and increased mouse mortality [e.g., *Listeria monocytogenes* (28, 58, 59, 69), *Mycobacterium tuberculosis* (60, 63, 71, 85), *Staphylococcus aureus* (67), and *Candida albicans* (72)] or without strong impact on the course of infection at all [e.g., *Legionella pneumophila* (86)]. These findings strongly suggest that the diverse immunomodulatory activities of type I IFNs are either beneficial or detrimental for the host, depending on the eliciting infectious agent. Even within one pathogen species (i.e., *T. cruzi*, *M. tuberculosis*, and *P. berghei*) opposing functional roles of type I IFNs (mediating resistance vs. susceptibility) have been described depending on the pathogen strain and infection dose (62, 70), the genetic mouse model used (71, 77), or the time points of infection analyzed (73, 83).

A similar complexity of the activity of type I IFNs is also seen in experimental cutaneous leishmaniasis, where the infection site, the parasite inoculum, and the parasite species and strain are known to affect the immune response and course of infection [reviewed in Ref. (17, 18)]. As shown in the present analysis of *L. major*-infected C57BL/6 mice, parasite and disease control was unaffected in the absence of IFNAR signaling. At first sight, this result was unexpected considering the striking impact of type I IFNs during the NK cell phase of *L. major* infection (days 1 and 2) (21) and the sustained expression of certain type I IFNs during the course of infection documented here for the first time (**Figure 1**). However, the non-essential role of type I IFNs in this model is most likely due to the fact that the swiftly starting production of IFN-γ by NK cells and recruited CD4$^+$ T cells [reviewed in Ref. (87)] makes type I IFNs rapidly dispensable as inducers of iNOS, also because IFN-γ is considerably more potent than type I IFNs in triggering this antileishmanial effector mechanism (88). In addition, at least *in vitro* the production of high amounts of type I IFNs in response to *L. major* parasites appeared to be restricted to pDCs (15), which represent a minor cell population *in vivo*, whereas the more abundant mDCs and macrophages released considerably smaller or very low amounts of IFN-α/β, respectively (21, 89) (**Figure 5A** vs. **Figure 5B**). Furthermore, the production of IL-12 by mouse mDCs, which is crucial for eliciting a Th1 response, was completely independent of IFNAR1 signaling (**Figure 5A**, lower panel), in contrast to recent findings

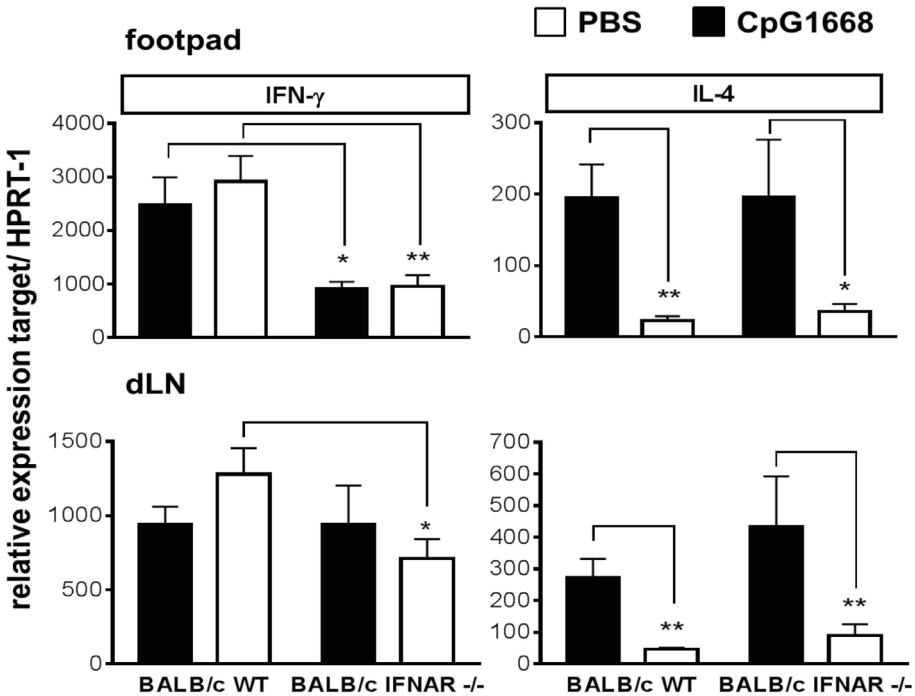

FIGURE 8 | Interferon (IFN)-γ and interleukin (IL)-4 mRNA expression in the footpads and draining lymph nodes (dLN) of *Leishmania major*-infected BALB/c wild-type (WT) and IFNAR1$^{-/-}$ mice treated with CpG-oligodesoxynucleotides (ODN) 1668. BALB/c WT and IFNAR1$^{-/-}$ mice were infected with 3×10^6 stationary phase *L. major* promastigotes into both hind footpads. 2 h before infection, 5 nmol CpG-ODN 1668 was administered subcutaneously at the site of infection, and 10 h postinfection additional 5 nmol CpG-ODN 1668 was injected intraperitoneally. Control mice received PBS (same volume). Total RNA was isolated from footpad and dLN tissue at day 23 or day 24 of infection and reverse transcribed. Gene expression levels of IFN-γ and IL-4 were determined as described in legend of **Figure 3**. Results are mean expression levels (±SEM) from two independent experiments with three mice per group. Significant differences by Mann–Whitney test (*$p < 0.05$ and **$p < 0.01$) are indicated.

with human DCs (90). Finally, *L. major* parasites impeded the expression of various type I IFN subtypes in macrophages (89) and were reported to produce a mammalian casein kinase 1-ortholog, which modestly decreased the expression of IFNAR1 on the surface of mouse BM macrophages and human DCs (91). All these factors might limit the expression and/or function of type I IFNs in *L. major*-infected C57BL/6 mice.

In *L. amazonensis*-infected 129Sv mice, which develop non-healing, progressive skin lesions, deletion of IFNAR1 was associated with a markedly attenuated clinical course of infection and parasite load. Absence of IFNAR1 led to an increased recruitment and death of neutrophils, which upon interaction with macrophages facilitated the killing of parasites (25). By contrast, in *L. mexicana*-infected mice, another model for non-healing cutaneous leishmaniasis, the lesion development and parasite burden were comparable in WT and IFNAR$^{-/-}$ mice on a mixed 129Sv/C57BL/6 background, with only a transient defect of the production of IFN-γ and IL-10 upon *in vitro* restimulation of lymph node T cells (92). Strains of *Leishmania (Viannia) guyanensis*, which carried high amounts of *Leishmania* RNA virus-1 (LRV1) and elicited metastatic skin lesions, activated macrophages for the release of much higher levels of IFN-β compared with non-metastatic parasite strains that lacked the virus (93). IFNAR1$^{-/-}$ mice infected with LRV1-positive strains of *L. V. guyanensis* developed markedly attenuated skin lesions compared with WT control mice, demonstrating that in chronic non-healing cutaneous leishmaniasis exuberant amounts of type I IFNs (triggered by the activity of the *Leishmania* RNA virus) are counterprotective (26). In line with these observations, exogenous IFN-β impeded the killing of *Leishmania (Viannia) braziliensis* or *L. amazonensis* by human macrophages *via* induction of superoxide dismutase 1 and subsequent degradation of O_2^- (24, 94). By contrast, the quantities of type I IFNs generated in BALB/c mice infected with *L. major* were not sufficient to impair the immune response as revealed by the unaltered course of infection in BALB/c IFNAR1$^{-/-}$ mice.

Finally, in mouse visceral leishmaniasis caused by *Leishmania donovani*, the parasite burden in liver and spleen was reported to be unaffected in the absence of IFNAR signaling, but original data were not presented in this publication (95). The reduced parasite control observed in *L. donovani*-infected mice deficient for either the interferon-regulatory factor-5 (IRF-5) or IRF-7, both of which control type I IFN expression, was attributed to an impaired generation of Th1 cells and IFN-γ and a defective induction of iNOS (96, 97).

Mechanism of CpG-Induced Protection

The finding that CpG-ODN 1668 conferred protection equally well in *L. major*-infected BALB/c WT and IFN-β$^{-/-}$ mice, but was ineffective in both BALB/c IFNAR1$^{-/-}$ and BALB/c

IL-12p35$^{-/-}$ mice, suggested that IFN-β is either irrelevant or that its function can be fully compensated by IFN-α subtypes and/or that IFNAR signaling might affect the CpG-induced expression of protective cytokines other than IFN-α subtypes. Indeed, in IFN-β$^{-/-}$ mice, the CpG-induced upregulation of certain IFN-α subtypes (α2, α4, α5, and α9) and of IFN-γ mRNA was completely preserved (**Figure 7**, middle panel). By contrast, in IFNAR1$^{-/-}$ mice CpG-ODN 1668 failed to boost the mRNA expression of most IFN-α subtypes as well as of IFN-β, IFN-γ, and IL-12p35 (**Figure 7**, lower panel), whereas the CpG-ODN 1668-mediated suppression of IL-4 was maintained in the absence of IFNAR1 (**Figure 8**). IFNAR signaling is known to amplify the expression of type I IFNs (especially IFN-α) in a positive feedback loop (12–15). Thus, CpG-ODN 1668 is likely to protect BALB/c mice from non-healing disease *via* the induction of IFN-α, IFN-γ, and IL-12 and the ensuing expression of iNOS.

In summary, this study shows that several members of the type I IFN family are prominently expressed and regulated during *L. major* infection. Although type I IFNs are dispensable for the spontaneous cure of primary or secondary *L. major* infections in self-healing mice, their disease-preventive activity can be readily revealed during CpG-ODN-induced protection of otherwise non-healing BALB/c mice. However, previous reports on other non-viral infections illustrated that the function of type I IFNs clearly varies with the pathogen (also within the genus *Leishmania*) and ranges from resistance-mediating to pathology- and disease-promoting. This needs to be considered when type I IFNs are applied for the treatment of chronic viral infections, autoimmune diseases, or malignancies in patients with persistent bacterial or parasitic infections.

ETHICS STATEMENT

Animal housing and experimental studies were approved by the local or governmental authorities in Freiburg and Ansbach.

AUTHOR CONTRIBUTIONS

US, JL, NJ, SH, and HS designed, performed, and analyzed experiments, interpreted data, and prepared the figures. TM and HS performed and analyzed experiments. UK and SW provided mice and helped to interpret data. CB designed experiments, interpreted the data, and wrote the manuscript. All the authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00079/full#supplementary-material.

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Hypoxia, Hypoxia-Inducible Factor-1α, and Innate Antileishmanial Immune Responses

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Low oxygen environments and accumulation of hypoxia-inducible factors (HIFs) are features of infected and inflamed tissues. Here, we summarize our current knowledge on oxygen levels found in *Leishmania*-infected tissues and discuss which mechanisms potentially contribute to local tissue oxygenation in leishmanial lesions. Moreover, we review the role of hypoxia and HIF-1 on innate antileishmanial immune responses.

Keywords: hypoxia-inducible factor 1, leishmaniasis, macrophages, oxygen, *Nos2*, hypoxia

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INTRODUCTION

Low oxygen (O_2) environments are a key feature of infected and inflamed tissue. Several lines of evidence demonstrate that oxygen levels of afflicted tissues are much lower than these currently used in standard cell culture experiments and, in general, correspond to values below 4% O_2 [reviewed in Ref. (1, 2)] Low oxygen levels are able to incapacitate oxygen-dependent antimicrobial effector enzymes such as the phagocytes oxidase or inducible NO synthase which both require oxygen as cosubstrate in order to produce their antimicrobial reactive oxygen species (ROS) and reactive nitrogen species (RNS) [reviewed in Ref. (1, 2)]. Moreover, hypoxia is not only a state of reduced availability of oxygen but also in addition induces a transcriptional response, which is governed by the transcription factors (TFs) hypoxia-inducible factor (HIF)-1 and HIF-2. Both TFs belong to the basic helix–loop–helix-PAS family of TF, consisting of HIF-1α or HIF-2α and its dimerization partner aryl hydrocarbon receptor nuclear translocator (ARNT) [reviewed in Ref. (3)]. Prolyl-hydroxylase domain (PHD) enzymes play a key role in the regulation of HIF-1α and HIF-2α since oxygen is a critical substrate for the PHD enzymes. Under conditions of ample oxygen, they hydroxylate HIF-α subunits that target HIF-α in a von Hippel-Lindau tumor suppressor-dependent manner to proteasomal degradation [reviewed in Ref. (4–6)].

Subsequent studies revealed that HIF-1α is not only involved in adaption of cells to low oxygen environments but that this TF is also stabilized upon infectious and inflammatory stimuli under conditions of ample oxygen as well. Furthermore, HIF-1α is required for inflammatory responses of innate immune cells *in vitro* and *in vivo* [reviewed in Ref. (1, 7–11)]. Mechanistically, normoxic, inflammatory HIF-1α stabilization is closely linked to nuclear factor (NF)-κB activation (12, 13), and involves transcriptional and posttranslational signaling events (14–16). Altogether, these findings demonstrate that hypoxic and inflammatory responses are intertwined.

Therefore, there is a growing interest to uncover the impact of hypoxia and HIF-1α in infectious diseases and its impact on host–pathogen interaction [reviewed in Ref. (1, 2, 17–21)]. In this review, we will summarize the evidence of hypoxia and the TF HIF-1α and its impact on innate immune responses directed against infection with *Leishmania major*, *Leishmania amazonensis*, and *Leishmania donovani,* which are able to cause cutaneous, mucocutaneous and systemic (visceral) diseases, respectively (**Table 1**).

TABLE 1 | Role HIF-1α in mononuclear phagocytes in Leishmaniasis.

Leishmania species	Type of disease	Tissue tropism	Role of HIF-1α in mononuclear phagocytes	
			In vitro	*In vivo*
L. major	Cutaneous leishmaniasis	(Local) skin	Parasite control	Induction of *Nos2* Cutaneous control of parasites
L. amazonensis	Mucocutaneous leishmaniasis	Skin with diffuse chronic progression	Parasite survival	Unknown
L. donovani	Visceral leishmaniasis	Spleen, bone marrow, liver	Parasite survival	Induction of MDSC Propagation of parasites

OXYGEN LEVEL IN LEISHMANIAL LESIONS

In guinea-pigs, intravenous challenge experiments with guinea-pig adapted *Leishmania enrietti* demonstrate that skin tissue predisposes to the development of leishmanial lesions (22). Given that skin tissue is known to display low oxygen levels (23–25), these data suggest that low oxygen micro-environments might provide a safe haven for *Leishmania* parasites (22). Araújo et al. assessed lesional tissue oxygen levels with an immunohistochemical method in cutaneous *L. amazonensis* infection (26). For that purpose, they injected a 2-nitroimidazol derivative into *L. amazonensis* infected mice. Upon injection, these compounds are enriched in tissues with very low oxygen tensions and form adducts that can be visualized after staining with adduct-specific antibodies (27, 28). Using this technology, they found that in *L. amazonensis*-induced lesions very low oxygen tensions are present (26).

To the best of our knowledge, there are no data available on tissue oxygen levels after infection with *L. donovani* in liver tissue. Since it is known that steep oxygen gradients exist in the liver (27), it is very likely that *L. donovani*-infected liver tissues display low oxygen levels as well. Using 2-nitroimidazol based techniques to visualize hypoxic tissues, Hammami et al. revealed that in *L. donovani*-infected spleens, very low tissue oxygen prevail (29).

Although staining of hypoxic areas with 2-nitroimidazol-derivatives allows for identification of severely hypoxic tissue, this method does not provide quantitative data and is not suitable for continuous recording of lesional tissue oxygen levels (25). Alternative methodologies to quantitatively assess tissue oxygen levels rely on polarographic oxygen sensors or imaging of tissue oxygenation by using oxygen-dependent luminescence quenching (25). For instance, non-invasive luminescence-based oxygen imaging using sensor foils can be used to quantitatively assess tissue oxygenation in a non-invasive manner over time (25). Using this technology in a mouse model of self-healing cutaneous leishmaniasis, Mahnke et al. observed that oxygen tension in leishmanial skin lesions displayed oxygen levels around ~2.8% O_2 when the lesions reached their maximum size (30). Resolution and healing of the lesion was paralleled by an increase of tissue oxygenation (30). Altogether, these findings indicate that low oxygen levels prevail in *Leishmania*-infected tissue.

Potential Factors Regulating Tissue Oxygen Levels in *Leishmania*-Infected Tissues

The mechanisms that drive low oxygen levels in *Leishmania* infected tissue are, however, largely unknown. Recent studies demonstrate that influx of granulocytes results in increased O_2 demand which ultimately results in low tissue oxygen levels (31). Low tissue oxygenation in *L. major*-infected tissue might result from either (i) infection-induced disturbance of local tissue perfusion or (ii) an increased local O_2 consumption by the infectious agent and/or infiltrating immune cells (**Figure 1**). However, the contribution of infiltrating granulocytes, monocytes, NK cells, and T cells on tissue oxygen levels in *Leishmania* infected tissues is unexplored. Moreover, *Leishmania* might interfere with inflammation induced angiogenesis. For instance, *Leishmania* is able to scavenge angiogenic factors such as VEGF-A. Thereby *Leishmania* might interfere with inflammation-associated angiogenesis and disturb local oxygen supply (32). Nevertheless infection with *L. major* eventually triggers a vascular endothelial growth factor (VEGF)-A/VEGF receptor (VEGFR)-2-dependent proliferation of endothelial cells (33). Underscoring the potential important role of VEGF-A/VEGFR-2 signaling, Araújo and Giorgio suggest that VEGF-A levels upon infection might predict the outcome of *L. amazonensis*-infection. They recorded higher VEGF-A levels in healer mice compared to non-healer mice (34). Since VEGF-A is a known HIF-1α target (35) and HIF-1α is present in *L. amazonensis*- and *L. major*-infected lesions in humans and in preclinical models (26, 33, 34, 36), it is possible that HIF-1α plays a role in infection-induced proliferation of the endothelial cells. However, to the best of our knowledge, this has not been tested in *L. major* and *L. amazonensis* mouse models yet. In addition to maintaining oxygenation of infected tissue, endothelial cell proliferation might curtail *L. major* induced-inflammatory responses (37). The activity of endothelial NO synthase plays an important role in endothelial cell mediated anti-inflammatory activity (38–40). Endothelial cell mediated anti-inflammatory activity might reduce the influx of immune cells and/or their O_2-consumption, thereby increase tissue oxygen levels and facilitate subsequent resolution of disease. Since endothelial NO synthase expression is linked to HIF-1α-accumulation (41), endothelial HIF-1α might promote anti-inflammatory properties in endothelial cells as well. Further studies, however, are required to investigate this issue. Earlier findings demonstrate that the "myeloid cell differentiation antigen carcinoembryonic antigen-related cell adhesion molecule 1"

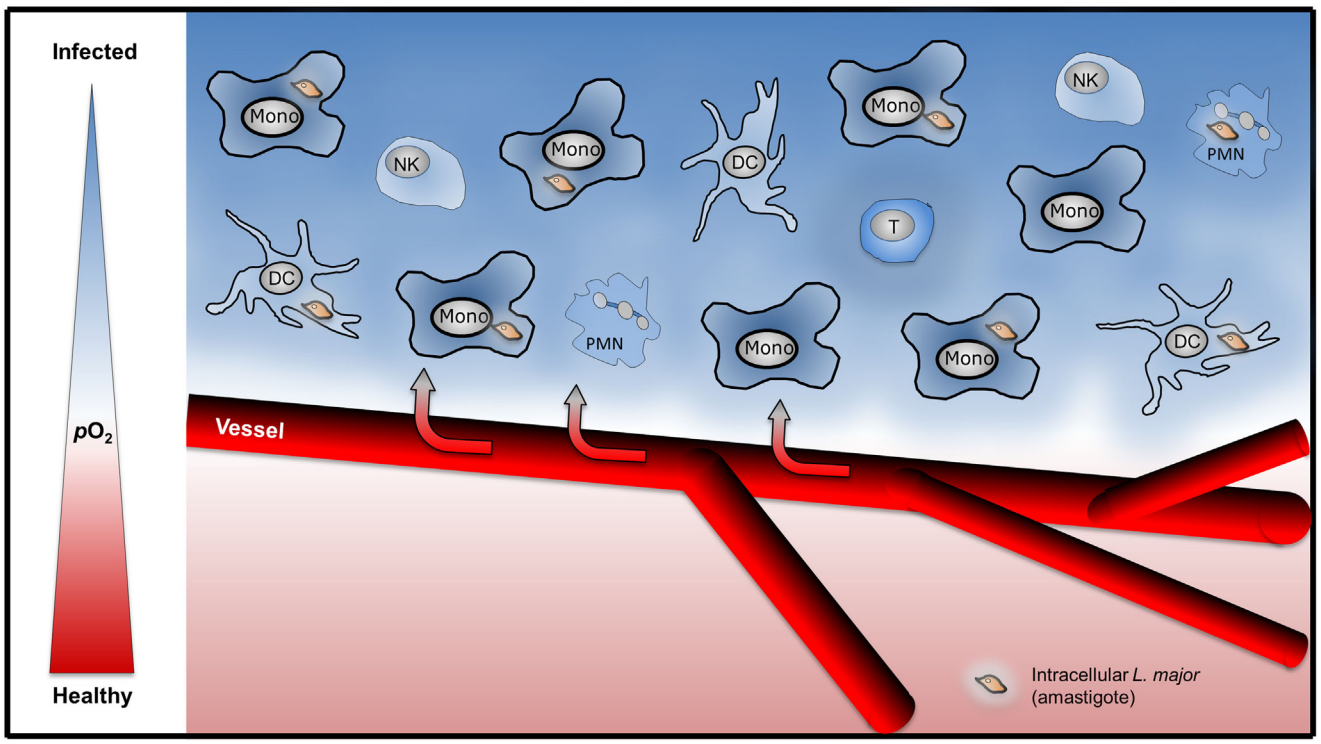

FIGURE 1 | Infiltration of immune cells might drive low tissue oxygenation in *Leishmania major*-infected skin. Upon *L. major*-infection, infiltration of granulocytes [polymorphonuclear granulocyte (PMN)], monocytes (Mono), dendritic cells (DC), NK cells, and T cells might induce increased local consumption of O_2 resulting in hypoxic tissue O_2 levels.

(CEACAM1) is involved in angiogenesis and antileishmanial control (42). Whether CEACAM1-dependent signaling affects VEGF-A/VEGFR and/or HIF-1α signaling in this state of affair is unknown. Further investigations are required to understand the interplay of inflammation-triggered angiogenesis, enhanced O_2 demand in inflamed tissues, and its impact on overall tissue oxygenation in leishmaniasis.

IMPACT OF HYPOXIA ON HOST–PATHOGEN INTERACTION

Internalization of *Leishmania* and Hypoxia

After transmission of *Leishmania* by its vector, myeloid cells are the main host cell [reviewed in Ref. (43–45)]. Hypoxic conditions are known to modulate the phagocytic/endocytic uptake of mononuclear phagocytes (46, 47). However, to the best of our knowledge, the impact of oxygen levels below 4% O_2 on *Leishmania* uptake or internalization is unexplored.

Polymorphonuclear Granulocytes (PMN), Hypoxia, and *Leishmania*

Polymorphonuclear granulocytes are quickly attracted to the site of infection. Dependent on the *Leishmania* species and the host's genetic background, they play a rather detrimental role in cutaneous leishmaniasis. PMN are inefficient in killing promastigotes and undergo apoptosis which hampers the proper activation of recruited dendritic cell (DC) [reviewed in Ref. (44, 48)]. In general, PMN are relatively short-lived cells, whose survival, however, is prolonged in NF-κB- and HIF-1α-dependent manner under hypoxic conditions. Moreover, hypoxia induces the production of macrophage inflammatory protein-1β which further facilitates neutrophil survival (49). In line with this, Monceaux et al. demonstrated recently that anoxic conditions prolong PMN survival (50). Therefore, hypoxia most likely favors influx and lifespan of PMN in leishmaniasis as well. Since these cells are not able to eliminate *Leishmania*, this might ultimately prolong and/or exacerbate the disease. However, to the best of our knowledge, there is no detailed analysis on the specific role of hypoxia on PMN–*Leishmania* interaction.

DC, Hypoxia, and *Leishmania*

Upon *Leishmania* exposure, DC phagocytose the parasite, become activated and establish an antileishmanial T cell response and thereby critically contribute to the resolution of disease [reviewed in Ref. (51)]. For instance, priming and induction of *Leishmania*-specific T_H1 response results in secretion of IFN-γ which is required for antileishmanial immunity [reviewed in Ref. (44)]. In addition, even after resolution of disease, DC still contain viable parasites which are able to maintain antigen-specific T cell responses against *L. major*. This contributes to the long term protection against the parasites [reviewed in Ref. (51)]. Although, there is evidence that oxygen levels impact on DC functions, there are to the best of our knowledge only

limited reports on the contribution of hypoxia on DC-mediated establishment of antileishmanial T cell immunity. Hypoxia might impact on DC viability (52), migratory capacity (53), maturation, and antigen presentation (54–56).

NK Cells, Hypoxia, and *Leishmania*

In early phase of *Leishmania* infection, NK cells are activated by IL-12 and IL-18. These activated NK cells contribute to parasite control through production of IFN-γ [reviewed in Ref. (57)]. NK cells respond to hypoxia by downregulation of surface markers NKp46, NKp30, NKp44, NKG2D, and CD16, decreased cytotoxic and antiviral activity, and reduced IFN-γ expression (58–60). In contrast to these findings, Krzywinska et al. observed no mitigated IFN-γ secretion by NK cells under hypoxia (61). However, the impact of hypoxia on NK-mediated antileishmanial defense is unexplored and requires further studies.

Monocytes/Macrophages, Hypoxia, and *Leishmania*

A few days after *L. major* and *L. donovani* infection, recruited monocytes are highly abundant in afflicted tissues (62, 63). Inflammatory monocytes are able to upregulate their ROS production in the early stage of infection with *L. major* and *L. donovani* (64, 65). However, in inflammatory monocytes infected with *L. major*, ROS production does eliminate the parasites (63). Moreover, *L. donovani*, for instance, has the ability to overcome the ROS-mediated apoptosis of host macrophages *via* induction of suppressor of cytokine signaling proteins (66). It is established that IFN-γ and TNF are two key cytokines that empower macrophages (67–69) and monocytes (63) to clear *Leishmania*. In the mouse system this results in a robust induction of the antileishmanial effector enzyme, the inducible or type 2 NO synthase (iNOS or NOS2). A high level of NO production is prerequisite of the cutaneous defense against *Leishmania major* [reviewed in Ref. (70, 71)]. Moreover, in mouse models of visceral leishmaniasis, *Nos2* expression is important for control of *L. donovani* in the liver (72), and contributes to the containment of *L. donovani* (73) in the spleen.

For induction of NOS2 and production of high level of leishmanicidal NO, costimulation of macrophages with TNF plus IFN-γ is required (63, 68, 72, 74–78). Low oxygen conditions, however, impair NO production of activated macrophages because the inducible NO synthase activity critically hinges on the availability of O_2 as a substrate (79–81). Accordingly, under oxygen conditions below 4% O_2 NO production of activated macrophages is diminished (30). *L. major* possesses a globin-coupled heme containing oxygen sensor soluble adenylate cyclase which allows for its adaption to hypoxic conditions (82). Hence, while *L. major* is able to adjust to hypoxia, low oxygen conditions impair the NO-dependent antileishmanial potential of macrophages resulting in impaired clearance and survival of *L. major* in activated macrophages under low oxygen conditions. However, leishmanicidal activity is restored when the cells are reoxygenated (30). These data demonstrate that local tissue oxygen levels—at least transiently—do not match the macrophages' O_2 demand to fight against *L. major*.

In visceral leishmaniasis, parasites express high tissue tropism toward monocytes in the spleen and bone marrow. *L. donovani*, e.g., induces expansion of hematopoietic stem cell-like mononuclear cells from bone marrow which serve as host cells for the parasite (62). Compared to the role of *Nos2* in the defense against visceral leishmaniasis in the liver (72), the contribution of *Nos2* in the control of parasite load in the spleen in visceral leishmaniasis is less pronounced. While there was no induction of *Nos2* in spleen macrophages upon infection of hamsters with *L. donovani* (83), *Nos2* expression, nevertheless, contributed to the parasite-containment in the spleen of *L. donovani* and *L. infantum*-infected mice (73, 84). In preclinical models of visceral leishmaniasis, the impact of local oxygen levels on *Nos2*-mediated antileishmanial defense and NO production in the spleen and liver is, however, to the best of our knowledge unexplored. It is tempting to speculate that low oxygen levels found in *L. donovani*-infected spleen tissue (29) incapacitate the *Nos2*-dependent antileishmanial defense in the spleen and, thus provide a safe niche for persistence and proliferation of *L. donovani*.

In contrast to *L. major*, exposure of *L. amazonensis*-infected macrophages to O_2 levels of 5% and 1% O_2 resulted in enhanced clearance of intracellular parasites (85, 86). These findings suggest that either (1) NO-independent mechanisms are involved in control of *L. amazonensis* under hypoxic conditions and/or (2) *L. amazonensis* is not able to adjust to low oxygen conditions. For instance, the divergent effect of hypoxia on the survival of members of the genus *Leishmania* in mononuclear phagocytes under hypoxia might be due to different metabolic requirements and O_2-demands of the respective *Leishmania* species investigated. While it is known that *L. major* and *L. donovani* are able to adjust to hypoxic conditions by increasing their rate of glycolysis (87, 88), to the best of our knowledge detailed investigations on the intermediary metabolism of *L. amazonensis* upon exposure to low oxygen conditions are lacking.

MONONUCLEAR PHAGOCYTES, HIF-1α, AND ANTILEISHMANIAL IMMUNITY

In addition to low oxygen conditions, various pathogens and pathogen-derived molecules are able to induce HIF-1α even in the presence of ample oxygen [reviewed in Ref. (1, 8–17)]. Inflammatory HIF-1α accumulation under normoxic conditions critically hinges on NF-κB activation (12, 13). HIF-1α transactivation potential, however, is highly contextual. For instance, hypoxic and inflammatory HIF-1α activation, both, result in activation of glycolytic HIF-1α-target genes while only inflammatory HIF-1α activation results in robust induction of inflammatory target genes such as *Nos2* (89).

Leishmania and Stabilization of HIF-1α in the Presence of Ample Oxygen

Currently, there is no consensus on whether *Leishmania* on its own is able to induce HIF-1α accumulation under normoxic conditions (**Table 1**). Infection of mouse macrophages *in vitro* with *L. amazonensis* and *L. donovani* triggers HIF-1α accumulation under normoxic conditions without requiring any additional

inflammatory signals (90, 91). Blockade of this *Leishmania*-induced HIF-1α accumulation with cadmium chloride or RNAi results in impaired *Leishmania* recovery under normoxic conditions (90–93). In contrast to these findings and in contrast to stimulation with *Toll*-like receptor ligands [reviewed in Ref. (1, 8, 9)] or infection with various pathogens such as *S. pyogenes* (35, 94), *M. tuberculosis* (95), *Histoplasma capsulatum* (96), infection of mouse macrophages with *L. major* does not induce HIF-1α accumulation on its own but requires exogenous stimulation by IFN-γ and/or LPS (36). In line with this, Hammami et al. demonstrated that upon *L. donovani*-infection HIF-1α accumulation in CD11c$^+$ mouse mononuclear phagocytes *in vivo* requires interferon regulatory factor-5-dependent inflammatory signaling (97). This suggests that *L. donovani* infection on its own is not sufficient to induce HIF-1α accumulation in mouse mononuclear phagocytes *in vivo* as well (97).

The different potential of various *Leishmania* species to induce HIF-1α accumulation in mononuclear phagocytes is unclear. HIF-1α accumulation in mononuclear cells might, for instance, help *Leishmania* to fuel its metabolic needs (98, 99). Mechanistically, the differential ability of certain *Leishmania* species to interfere with NF-κB activation (100–102) might underlie the divergent ability of different *Leishmania* species to block infection-associated inflammatory HIF-1α-accumulation under normoxic conditions. However, further studies are required to assess this issue.

Role of HIF-1α Expression in Mononuclear Phagocytes

Earlier findings demonstrate a requirement of HIF-1α for NOS2-induction (89, 94, 103–105). In line with this, LPS/INFγ-coactivated HIF-1α-deficient macrophages displayed a diminished NOS2-dependent production of NO under normoxic conditions and impaired killing of *L. major in vitro*. Conditional targeting of HIF-1α in macrophages revealed that HIF-1α-activation is required for antileishmanial defense against cutaneous *L. major*-infection under normoxic conditions (36). This was paralleled by a blunted induction of NOS2 in lesional myeloid cells (36). However, it remains unclear whether in addition to *Nos2* other HIF-1α-regulated targets are involved in cutaneous containment of *Leishmania* as well.

While HIF-1α in mononuclear phagocytes is protective in *L. major*-induced cutaneous leishmaniasis, HIF-1α expression in these cells emerged as detrimental factor in visceral leishmaniasis. Hammami et al. used CD11c Cre-deleter mice to target HIF-1α in Cd11c$^+$ mononuclear phagocytes and to test the contribution of HIF-1α in a *L. donovani*-induced preclinical model of visceral leishmaniasis (29). In line with an inhibitory role of HIF-1α in DC-mediated T cell responses (106), Hammami et al. provide evidence that in *L. donovani*-infected mice HIF-1α in CD11c$^+$ cells limits the expansion of short lived effector CD8$^+$ T cells and thereby exacerbates disease at early time points after infections (29). In a recently published follow-up study, they demonstrate that HIF-1α in CD11c$^+$ cells limits the frequency and numbers of granulocyte–monocyte progenitors and inflammatory monocytes. Moreover, HIF-1α in CD11c$^+$ cells enhances the inhibitory function of splenic mononuclear phagocytes while this TF is not involved in tissue neovascularization and splenomegaly (29). Of note, Hammami et al. demonstrate that HIF-1α negatively impacts on the expression of *Nos2* in *L. donovani*-infected mononuclear phagocytes. Discrepancies of this finding to the published literature on the requirement of HIF-1α on *Nos2*-expression and NO production might be caused by the different context of HIF-1α stabilization (29). This issue, however, demands further detailed investigation. Overall, the data suggest that HIF-1α in CD11c$^+$ cells favors a myeloid-derived suppressor cell-like (MDSC) behavior of these cells. These findings were paralleled by an impaired antileishmanial control in the bone marrow of *L. donovani*-infected mice at late time points (29).

Taken together, these findings demonstrate that HIF-1α expression in CD11c$^+$ mononuclear phagocytes favors development of visceral leishmaniasis in spleen and bone marrow, while HIF-1α in LysM$^+$ myeloid cells supports defense against cutaneous *L. major* infection (**Table 1**). These findings underscore that the role of HIF-1α in myeloid cells is highly dependent on the respective context. HIF-1α is known to promote proinflammatory function of mononuclear phagocytes (35, 36, 94, 95, 105). However, the very same TF is able to induce regulatory and immunosuppressive functions in mononuclear phagocytes as well (107–109). Therefore, it is very likely that the differential context of HIF-1α stabilization in visceral and cutaneous leishmaniasis impacts on the functional consequence of HIF-1α activation. Further studies are needed to uncover the distinct signals that ultimately result in differential HIF-1α-dependent innate immune cell function.

AUTHOR CONTRIBUTIONS

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Dectin-1 Positive Dendritic Cells Expand after Infection with *Leishmania major* Parasites and Represent Promising Targets for Vaccine Development

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Resistant mouse strains mount a protective T cell-mediated immune response upon infection with *Leishmania* (L.) parasites. Healing correlates with a T helper (Th) cell-type 1 response characterized by a pronounced IFN-γ production, while susceptibility is associated with an IL-4-dependent Th2-type response. It has been shown that dermal dendritic cells are crucial for inducing protective Th1-mediated immunity. Additionally, there is growing evidence that C-type lectin receptor (CLR)-mediated signaling is involved in directing adaptive immunity against pathogens. However, little is known about the function of the CLR Dectin-1 in modulating Th1- or Th2-type immune responses by DC subsets in leishmaniasis. We characterized the expression of Dectin-1 on CD11c$^+$ DCs in peripheral blood, at the site of infection, and skin-draining lymph nodes of *L. major*-infected C57BL/6 and BALB/c mice and in peripheral blood of patients suffering from cutaneous leishmaniasis (CL). Both mouse strains responded with an expansion of Dectin-1$^+$ DCs within the analyzed tissues. In accordance with the experimental model, Dectin-1$^+$ DCs expanded as well in the peripheral blood of CL patients. To study the role of Dectin-1$^+$ DCs in adaptive immunity against *L. major*, we analyzed the T cell stimulating potential of bone marrow-derived dendritic cells (BMDCs) in the presence of the Dectin-1 agonist Curdlan. These experiments revealed that Curdlan induces the maturation of BMDCs and the expansion of *Leishmania*-specific CD4$^+$ T cells. Based on these findings, we evaluated the impact of Curdlan/Dectin-1 interactions in experimental leishmaniasis and were able to demonstrate that the presence of Curdlan at the site of infection modulates the course of disease in BALB/c mice: wild-type BALB/c mice

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treated intradermally with Curdlan developed a protective immune response against *L. major* whereas Dectin-1$^{-/-}$ BALB/c mice still developed the fatal course of disease after Curdlan treatment. Furthermore, the vaccination of BALB/c mice with a combination of soluble *L. major* antigens and Curdlan was able to provide a partial protection from severe leishmaniasis. These findings indicate that the ligation of Dectin-1 on DCs acts as an important checkpoint in adaptive immunity against *L. major* and should therefore be considered in future whole-organism vaccination strategies.

Keywords: Curdlan, β-glucan, Dectin-1, cutaneous leishmaniasis, adaptive immunity, dendritic cells, T helper 1 and T helper 2 cells

INTRODUCTION

Cutaneous leishmaniasis (CL) is a vector-borne parasitic infectious disease encountered in tropical and subtropical regions of the world (1). The causative agents are flagellated protozoans of the genus *Leishmania (L.)*, which are inoculated into the skin during the blood meal of female sandflies. Different clinical manifestations in humans, ranging from a self-limiting cutaneous infection to disseminating visceral leishmaniasis (VL), are described with respect to the transmitting vectors and *Leishmania* species (2).

Comparable to the course of disease in humans, *L. major* parasites can develop cutaneous manifestations in C57BL/6 and BALB/c mouse models (3). The infection of inbred mice with stationary phase promastigote *Leishmania* parasites allowed the examination of basic mechanisms, resulting in innate and adaptive T cell-mediated immunity (3).

It is known that *L. major* parasites require phagocytic cells for replication and spreading within the host (4). In this regard, neutrophils and macrophages play a pivotal role as host cells for the initial survival and spreading of parasites. However, macrophages produce leishmanicidal molecules after appropriate activation by certain T helper (Th) 1 cytokines such as IFN-γ (3, 5) and become effector cells during the host response against *L. major*. Thus, healing of CL in C57BL/6 mice is associated with a pronounced expansion of IFN-γ-secreting Th1 cells (6), whereas susceptibility of BALB/c mice is associated with a diminished IFN-γ, but increased IL-4-production in high-dose infection models (5, 7, 8). Therefore, CD4$^+$ T cells are crucial key players in modulating antigen-specific immunity in leishmaniasis (3).

It is mostly accepted that Th1- and Th2-effector cells derive from a common CD4$^+$ T cell precursor (9). Several stimuli have been reported to influence the pathway of maturation of these CD4$^+$ T cell progenitors (10). Using CD4$^+$ T cells, transgenic for a unique TCR alpha/beta receptor, it was proven that distinct cytokines such as IL-12 and IL-4 are crucial for the polarization of Th1 and Th2 cells (5, 10). Th1 cell differentiation takes place after *Leishmania*-specific T cells have been primed sufficiently by antigen-presenting cells in skin-draining lymph nodes (SDLNs) (11). Based on the current model it must be assumed that Langerin$^-$ dermal dendritic cells (dDCs) are pivotal for the induction of the protective Th1-mediated immune response against *L. major* in C57BL/6 mice (12–14). Of note, Langerin$^+$ epidermal Langerhans cells are dispensable for the generation of protective immunity in experimental leishmaniasis (13–16).

T cell-mediated immunity against *L.* major parasites is a multistep process whereby mature DC subsets are pivotal to start the adaptive immune response within SDLNs (12, 14). Maturation of DCs is accompanied by high expression of chemokine receptor (CCR) 7, paving the way to the SDLN and costimulatory molecules crucial for DC/T-cell communication (17). Certain cytokines such as TNF-α and IL-6 are known to support this process (17, 18). Additionally, pattern recognition receptors (PRRs) binding pathogen components are also potent activators of DC maturation (19). Especially toll-like receptors that represent the most popular PRRs (20), recognize pathogen-associated molecular patterns derived from various pathogens, including viruses, bacteria, fungi, parasites, and protozoa, and are crucial for innate immune mechanisms (21).

The C-type lectin receptors (CLRs) also belong to PRRs and recognize predominantly carbohydrates and non-carbohydrates through mechanisms that are still not fully understood (22). The CLR Dectin-1 recognizes β-glucans (22–25) that naturally occur in the cell wall of fungi (23). In addition to pathogen-derived factors, endogenous *N*-glycans present on the surface of tumor cells also represent potential Dectin-1 ligands and trigger tumor killing by NK cells (26).

Mouse Dectin-1 is expressed by various immunologically relevant cells belonging to the adaptive and innate immune system. Preferentially, monocytes, macrophages, neutrophils, and a subset of T cells were described to be positive for Dectin-1 (27). There is growing evidence that engagement of Dectin-1 on DC subsets is crucial for T-cell polarization in experimental mouse models (28, 29). In humans, Dectin-1 is widely expressed by all monocyte populations as well as by macrophages, DC, neutrophils, eosinophils, and B cells (27, 30). In line with these findings, it was shown that the function of human Dectin-1 is equivalent to that of mouse Dectin-1 (30). Thus, CLRs in general and Dectin-1 in particular might be considered as important checkpoints for adaptive immune responses.

It could be demonstrated that Dectin-1$^{-/-}$ macrophages from C57BL/6 mice show a slightly reduced capacity for phagocytosis of *L. infantum* parasites *in vitro* (31). Thus, Dectin-1 might be involved in the formation of parasitophorous vacuoles (32). In line with these findings, it is important to mention that infected macrophages from C57BL/6 show an enhanced expression of Dectin-1 after infection with *L. amazonensis in vitro* (33).

Consequently, the pronounced Dectin-1 expression by infected myeloid cells might potentiate the uptake of parasites and favors the spreading of the obligatory intracellular parasites during the first stage of innate immunity. An interaction of Dectin-1 with parasite-derived carbohydrates was not identified so far. Nevertheless, β-glucan can activate infected macrophages from BALB/c mice to control the replication of *L. donovani* parasites *in vitro* (34, 35). Additionally, it was shown that NK cells can also be activated by *Aureobasidium*-derived soluble branched (1,3–1,6)-β-glucan that results in enhanced cellular immunity against *L. amazonensis* parasites in BALB/c mice (36). The scientific evidence, that β-glucan can modulate innate immune mechanisms against *L. major* parasites at the site of infection, is still pending.

Dectin-1 signaling is also discussed to be crucial in directing adaptive T cell-mediated immune responses. Thus far, it is known that Dectin-1 ligation by fungal components triggers Th1- and Th17-mediated immune responses against fungi (37–41). Accordingly, Dectin-1 deficiency results in impaired T cell-mediated immunity and loss of control of fungal infection (42). Long before Dectin-1 was described as a receptor for β-glucans, these glucose polysaccharides were used as adjuvants for immunization and systemic therapies of VL in BALB/c and C57BL/6 mice (43–47). In line with this, Ghosh et al. were able to efficiently treat BALB/c mice infected with *L. donovani* by multiple intraperitoneal (i.p.) applications of the linear β-glucan Curdlan, which induced Th17-mediated adaptive immunity and macrophage activation (34). Most of the studies investigating the effect of β-glucans were carried out using VL-causing *L. donovani* parasites. However, one study is published demonstrating that multiple systemic applications (i.p. and i.v.) of β-glucan after infection of BALB/c mice with *L. major* parasites blocked lesion development or parasite spreading in normally susceptible BALB/c mice (48). Whether Dectin-1 is responsible for the observed immunological phenomenon has not been shown until now. Furthermore, quantification and characterization of Dectin-1$^+$ DCs in experimental leishmaniasis and in patients suffering from CL are missing.

In this study, we investigated the potential impact of β-glucan and of Dectin-1 on DC physiology and subsequent modulation of T-cell immunity. Here, we were able to demonstrate an expansion of Dectin-1$^+$ DCs in experimental leishmaniasis as well as in patients suffering from CL. Additional studies revealed that intradermal application of *L. major* parasites in combination with Curdlan changes the course of leishmaniasis: BALB/c mice treated with Curdlan developed a protective immune response against *L. major*, whereas Dectin-1$^{-/-}$ BALB/c mice still suffered from a fatal course of disease after Curdlan treatment. Based on these data, it appears that Dectin-1/Curdlan interactions *per se* are sufficient to modulate Th-cell differentiation. Further *in vitro* studies were performed to explore the cellular mechanisms. One important finding was the change in the phenotype and functionality of infected DCs triggered by Curdlan. They increase the expression of Dectin-1 and costimulatory molecules and become potent antigen-presenting cells, capable of accelerating the expansion of *L. major*-specific T cells.

The results presented in this article support the view that Dectin-1$^+$ DCs represent promising targets for modulating adaptive T cell-mediated immunity and should therefore be considered in future whole-organism vaccination strategies.

MATERIALS AND METHODS

Human Samples

7–10 mL of peripheral blood samples were collected from patients suffering from CL and Ethiopian healthy controls (EHCs) living in Oromo and Amhara regions of Ethiopia. The sample collection was permitted based on the local ethical committee (allowance number PO25/08, Addis Ababa, Ethiopia) and the national health research ethics review committee (approval number 310/227/07, Addis Ababa). Written informed consent was obtained from the participants of this study. CL was confirmed by positive parasite cultures and PCR analysis as described elsewhere (49). CL patients were excluded from the study if they show one of the following criteria: younger than 18 and older than 55 years, chronic lesions (more then 6 months), positive for HIV, clinical evidence for coinfections, and drug intake.

Mice

Female wild-type BALB/c and C57BL/6 mice (Janvier Labs, Le Genest St. Isle, France) were kept at the animal facility of the University of Regensburg under pathogen-free conditions. Dectin-1$^{-/-}$ mice on BALB/c background [kindly provided by G.D. Brown, University of Aberdeen (50)] were bred and maintained under conventional animal housing conditions. All experiments and animal housing were performed according to the guidelines for the care and use of experimental animals. The animal work was approved by the local veterinary authorities of the district government based on the European guidelines and national regulations of the German Animal Protection Act (approval no. AZ 54-2532.105/11). Female animals between 6 and 12 weeks of age were used for experiments.

Parasites and Infection of Mice

Virulent *L. major* parasites (MHOM/IL/81/FE/BNI) were propagated *in vitro* in blood agar cultures as described previously (51). Stationary phase promastigotes from the third to seventh *in vitro* passage were harvested, washed four times, and resuspended in PBS. Mice were infected *via* intradermal injection of 3×10^6 stationary phase promastigotes in 30 µL into the hind footpads. The increase in lesion size was monitored weekly by measuring the footpad thickness with a metric caliper (Kroeplin Schnelltaster, Schlüchtern, Germany). The increase in footpad thickness (%) was determined as described elsewhere (52).

Curdlan Application

Curdlan (WAKO Chemicals GmbH, Neuss, Germany) was dissolved in sterile PBS to a concentration of 50 µg/µL. 3×10^6 stationary phase promastigotes were resuspended in 30 µL of Curdlan solution and injected intradermally into the hind footpads. Alternatively, soluble *L. major* antigens [SLA; (15)]

corresponding to 3×10^6 stationary phase promastigotes were used. The course of disease was monitored as described earlier.

Quantification of *L. major*-Specific IgG Subtypes

Serum from naïve mice and from mice infected with *L. major* parasites was prepared at the corresponding time point and thereafter stored at −20°C until use. Detection of *L. major*-specific IgG_1 (Invitrogen, Darmstadt, Germany), IgG_{2a} (BD Pharmingen, Heidelberg), and IgG_{2c} (Jackson ImmunoResearch, Hamburg Germany) isotypes were performed as described earlier (53). The results were standardized by calculation of relative ELISA units (REU). REU were determined by the formula: OD_{450} serum (infected mice)/OD_{450} serum (naïve mice).

Delayed-Type Hypersensitivity (DTH) Reaction

Three weeks after *L. major* infection, SLA (corresponding to 3×10^6 parasites) was injected in a volume of 20 µL in the foreleg. As a control, 20 µL of PBS was injected in the contra lateral foreleg. The increase in swelling was measured with a metric caliper (Kroeplin Schnelltaster) and referred to the control foreleg. The swelling was measured with a metric caliper (Kroeplin Schnelltaster) after 24, 48, and 72 h.

Generation of Bone Marrow-Derived Dendritic Cells (BMDCs)

Bone marrow-derived dendritic cells were generated as previously described (54). Bone marrow cells from C57BL/6 or BALB/c mice were seeded in 10 cm BD Tissue culture dishes at a density of 2×10^6 per dish in 10 mL of RPMI medium supplemented with 10% fetal calf serum (FCS; PAN Biotech GmbH), penicillin–streptomycin, 50 µM β-ME, and 10% GM-CSF containing supernatant harvested from Ag8653 myeloma cells transfected with the gene encoding murine GM-CSF (kindly provided by B. Stockinger, NMRI, Mill Hill, London, UK). BMDCs were harvested on day 10 for T cell proliferation experiments.

Generation of Bone Marrow-Derived Macrophages (BMDMs)

Mouse BMDMs were generated from female BALB/c mice and cultured for 7 days in hydrophobic Teflon bags (FT FEP 100C Dupont, American Durafilm, Holliston, MA) as described earlier (55, 56). For killing experiments, BMDMs were pulse-infected with *L. major* promastigotes at a 1:30 ratio for 4 h as described earlier (56). After infection, extracellular promastigotes were removed by washing with PBS and cultured in the absence or presence of Curdlan (50, 100, and 250 µg/mL) or LPS/IFN-γ (10 and 20 ng/mL, respectively). After 72 h, BMDMs were fixed, stained, and analyzed microscopically using Diff-Quick staining (Medion Diagnostics, Düdingen, Switzerland) for the determination of the percentage of infected cells and the number of parasites per infected cell. LPS (*Escherichia coli* O111:B4) was purchased from Sigma-Aldrich (Taufkirchen, Germany). Recombinant murine IFN-γ was purchased from eBioscience (Frankfurt, Germany).

Nitrite Accumulation

Nitrite accumulation in the supernatants was determined as an indicator for NO activity after stimulation of BMDMs using the Griess reaction as described earlier (56).

Labeling of *Leishmania* Parasites with CFSE

Stationary phase promastigotes from the third to seventh *in vitro* passage were harvested, washed four times, and resuspended in PBS. After lysis of the remaining erythrocytes, parasites were labeled by incubation in 1 µM CFSE staining solution as described earlier (57).

Leishmania-Specific T Cell Proliferation Assay

C57BL/6 and BALB/c mice were infected with *L. major* parasites as described earlier. Ten days post infection, SDLNs were removed, and T cells were enriched untouched *via* MACS separation columns (Miltenyi Biotec, Bergisch Gladbach) according to the manufacturer's guidelines. Single-cell suspensions were labeled with PE-conjugated CD11b, CD11c, and B220 antibodies (Abs) and subsequently detected with anti-PE Beads (Miltenyi Biotec) in order to deplete myeloid cells and B cells. The purified T cells were labeled with CFSE as described earlier (15). 2×10^5 CFSE-labeled T cells were seeded in 96-well round-bottom plates (Nunc) to 2×10^4 BMDCs that have been primed for 24 h with SLA (as an equivalent of 5 parasites: 1 BMDC) or *L. major* parasites (10 parasites: 1 BMDC) in the presence or absence of Curdlan (50 µg/200 µL). 72 h after coculture, the cells were analyzed by flow cytometry. The proliferation index of samples was calculated according to the formula: (number of proliferating cells after stimulation)/(number of proliferating cells in the absence of stimulation). The proliferation index of stimulated samples was then normalized to that of unstimulated BMDC/T-cell cultures. Supernatants were collected and stored at −80°C for cytokine analysis.

CLR-Fc Fusion Protein Staining of Parasites

The CLR-Fc fusion proteins (Dectin-1, CLEC-9a, and MGL-1) were prepared as described previously (58). After blocking (PBS/10% FCS/10% mouse serum), *L. major* parasites were washed three times in PBS. To analyze interactions with CLRs, parasites were incubated with 20 µg/mL of CLR-Fc fusion proteins diluted in lectin binding buffer (50 mM HEPES, 5 mM $MgCl_2$, and 5 mM $CaCl_2$, in dH_2O, pH 7,4) at 4°C for 1 h. After three washing steps with PBS, CLR-Fc binding to *L. major* was detected with a FITC-conjugated goat anti-hFc Ab (Dianova, Hamburg, Germany). Parasite DNA was detected with 6-diamidino-2-phenylindole obtained from Sigma Aldrich, as described earlier (59). After mounting with PermaFluor (Thermo Scientific, Dreieich, Germany), the sections were analyzed using Axio Imager.M1 (Zeiss, Jena, Germany) equipped with high-sensitivity gray scale digital camera (AxioCam MRm, Zeiss). Separate images were collected for each section, analyzed, and merged afterward (acquisition software: Zeiss AxioVision 4.6.3). Final image processing for

illustrations was performed using Adobe Photoshop Elements (Adobe Systems GmbH, Munich, Germany).

Flow Cytometry Analysis of Mouse Samples

Single-cell suspensions of tissues were generated as described elsewhere (60). The following Abs and reagents were used for phenotyping of single-cells: PE-labeled anti-mouse CD11c Ab (clone N418; eBioscience, Frankfurt, Germany), APC-labeled anti-mouse CD11b (clone M1/70, eBioscience, Germany), PE-labeled anti-mouse F4/80 (clone 521204, R&D), biotinylated anti-mouse Dectin-1 Ab (clone 2A11; Bio-Rad AbD Serotec GmbH, Puchheim, Germany), AlexaFluor647-labeled anti-mouse CD86 Ab (clone GL-1; BioLegend, San Diego, CA, USA), and FITC-labeled anti-BrdU Ab (clone B44; BD Pharmingen, Heidelberg, Germany). Depending on the experimental setup, the detection of biotinylated anti-mouse Dectin-1 was performed using streptavidin-conjugated to V500 (BD, Pharmingen, #561419), eFluor450 (eBioscience, #48-4317-82), Pacific Orange (Thermo Fisher, Munich, Germany), or PerCP (BD, Pharmingen, #554064). Ab specificity was verified using appropriate isotype controls and Dectin-1$^{-/-}$ mice (Figure S1 in Supplementary Material). Multicolor flow cytometry was performed as described previously (13). Mice were fed with BrdU-containing drinking water (0.8 mg/mL, Sigma, Deisenhofen, Germany) starting 3 days before the termination of the experiment. BrdU labeling was performed according to the manufacturer's instructions (BD Pharmingen, Heidelberg, Germany). After labeling of surface epitopes, the cells were fixed and permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen, Heidelberg, Germany) for 30 min on ice and afterward incubated for 10 min on ice with 0.01% Triton X-100 (SERVA, Heidelberg, Germany) in PBS/0.1% BSA (PAN, Aidenbach, Germany). For the detection of incorporated BrdU, the cells were treated with 30 µg DNAse (Sigma, Munich, Germany) for 1 h at 37°C. Labeling of cells with anti-BrdU Ab was performed for 20 min at room temperature. Cells were collected using BD™ LSRII Flow Cytometer (BD Biosciences, Heidelberg, Germany) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA). The determination of the total cell numbers in tissues was performed as described earlier (61).

Flow Cytometry Analysis of Human Samples

Surface antigens were detected on fresh cells using a modified version of the method described by Aldebert et al. (62). 7–10 mL of blood from patients and EHCs were taken with BD Vacutainer (BD Biosciences, UK) containing 170 IU of lithium heparin. 200 µL of whole blood cells was incubated for 20 min with FcR blocking reagent (Miltenyi Biotec GmbH, Germany). The staining was performed for 30 min at 4°C. The following Abs were used: FITC-labeled anti-lineage (Lin) panel (CD3, CD14, CD16, CD19, CD20, and CD56) (BD), PerCP-labeled anti-HLA-DR (R&D, clone L203), PE-labeled anti-CD123 (R&D, clone 32703), PE-Cy5.5-labeled anti-CD11c (BD, clone B-Ly6), and APC-labeled anti-Dectin-1 (R&D, clone 259931). Erythrocytes were lysed with BD FACS™ Lysing Solution (BD Biosciences), and the remaining white blood cells were washed and resuspended in FACS buffer (PBS buffer, pH 7.2 1% BSA fraction V, Roth, 8076.2), 0.01% NaN_3 (Sigma: S2002-25G), and 1% human serum. A total of 200,000 events were acquired and the positive population gated using the isotype controls for the different fluorochromes. Data were acquired with a FACS Canto flow cytometer (BD Bioscience). Data analysis was performed with FlowJo 8.8.6.

Gene Expression Analysis by Quantitative RT-PCR

$CD4^+$ T cells were sorted with the $CD4^+$ Cell isolation Kit® (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's specification. Following cell sorting, the $CD4^+$ cells were lysed in 350 µL Buffer RA1 (Macherey-Nagel, Düren, Germany) supplemented with 3.5 µL β-ME. Total RNAs were isolated using the NucleoSpin® RNA kit (Macherey-Nagel), following the manufacturer's instructions. RNAs were quantified by spectrophotometry and 170 ng of RNAs were used for cDNA synthesis using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, München, Germany), as recommended by the manufacturer. Quantitative PCR was performed on a RotorGene Q (Qiagen, Hilden, Germany) using a two-step PCR program (95°C 15 s, 60°C 60 s; 40 cycles). 20 µL quantitative PCR reactions were performed using 0.3–0.6 µL of cDNA template (corresponding to 2.5–5 ng RNA equivalent) and a self-made master-mix containing SYBR Green I and HotStarTaq DNA Polymerase (Qiagen). Data were normalized to GAPDH mRNAs and expressed as relative mRNA levels, as previously reported (63, 64). Forward (fwd) and reverse (rev) mouse-specific quantitative PCR primers were as follows: GAPDH, 5′-AGCTTGTCATCAACGGGAAG-3′ (fwd) and 5′-TTTGATGTTAGTGGGGTCTCG-3′ (rev); GATA, 5′-CCAAGGCACGATCCAGCACAGA-3′ (fwd) and 5′-GGCCGACAGCCTTCGCTTGG-3′ (rev); IFNG, 5′-AGGTCAACAACCCACAGGTCC-3′ (fwd) and 5′-GATTCCGGCAACAGCTGGT-3′ (rev); IL-4, 5′-ACAGGAGAAGGGACGCCAT-3′ (fwd) and 5′-GAAGCCCTACAGACGAGCTCA-3′ (rev); IL-17A, 5′-AACTCCCTTGGCGCAAAAG-3′ (fwd) and 5′-GAGAGTCCAGGGTGACGTGG-3′ (rev); STAT4, 5′-CCTGGGTGGACCAATCTGAA-3′ (fwd) and 5′-CTCGCAGGATGTCAGCGAA-3′ (rev); STAT6, 5′-CCCCAACAAACTTCTCATCCA-3′ (fwd) and 5′-TTTGGCGTTGTTGTCTTGGTT-3′ (rev); and T-BET, 5′-ACCAACAACAAGGGGGCTCT-3′ (fwd) and 5′-CTCTGGCTCTCCATCATTCACC-3′ (rev).

Quantification of *L. major* Parasites

To measure the parasite burden, genomic DNA was isolated using DNA purification solutions from QIAGEN (QIAGEN, Hilden, Germany). In brief, the cells were digested in cell lysis solution directly in the well. Protein was removed by adding protein precipitation buffer, and DNA was precipitated according to the manufacturer's instructions with 100% 2-propanol (Sigma Aldrich, Taufkirchen, Germany). The concentration of mouse β-actin-DNA was quantified by PCR (65). The *Leishmania* DNA concentrations in the same samples were determined using fluorescence resonance energy transfer real-time PCR with Leishmanial 18S ribosomal DNA sequences (66). The resulting

Leishmania DNA copy number was then divided by the copy number of β-actin-DNA to obtain the relative parasite units (RPU).

Quantification of Cytokines

Supernatants of cell cultures were analyzed with a FlowCytomix™ kit (IL-13, IL-1α, IL-22, IL-2, IL-21, IL-6, IL-10, IL-27, IFN-γ, TNF-α, IL-4, and IL-17) according to the manufacturer's (eBioscience, Thermo Fisher Scientific, Waltham, USA) specifications. Acquisition of probes was performed with a FACS Canto II (BD Bioscience).

Statistical Analysis

Statistical significance between groups were calculated by different tests: two-way ANOVA for comparing the footpad swelling of analyzed groups, non-parametric Mann–Whitney tests if no normal distribution (no Gaussian distribution) was determined, and two-tailed Student's *t*-test if normal distribution (Gaussian distribution) was determined. The calculation was performed using PRISM software (GraphPad, La Jolla, CA, USA). The corresponding p values are highlighted by stars only if $p < 0.050$.

RESULTS

Local Infection with *Leishmania* Parasites Results in Systemic Expansion of Dectin-1$^+$ DCs in Patients Suffering from CL

DCs are known to be important checkpoints for the generation of T cell-mediated protective immunity in experimental leishmaniasis (67). Based on the potential role of Dectin-1$^+$ DCs in adaptive immunity, we addressed the question whether a cutaneous infection with *Leishmania* parasites results in an expansion of Dectin-1$^+$ DCs.

In humans, it is difficult to analyze the dynamics of DC populations in tissues or secondary lymphoid organs. Therefore, we focused on the analyses of blood samples. DCs in the peripheral blood lack certain Lin-specific markers (68). Thus, Lin$^-$ leukocytes were used for further characterization of human DCs (**Figures 1A,B**). The Lin$^-$ population consists of HLA-DR$^-$ and HAL-DR$^+$ cells (**Figure 1B**). In accordance with other published data, the Lin$^-$ CD11c$^+$ cells are positive for HLA-DR and could be found within the Lin$^-$/HLA-DR$^+$ population (68, 69) (data not shown).

On the basis of the markers CD11c and CD123 (68, 70) myeloid DCs (mDC; CD11c$^+$/CD123$^{-/int}$) were dissected from plasmacytoid DC (pDC; CD11c$^-$/CD123^{bright}) subsets (**Figure 1C**). We could demonstrate that pDCs from EHCs and CL patients hardly express Dectin-1 (**Figure 1D**). The expression of Dectin-1 is predominantly restricted to mDCs (**Figure 1D**). Cutaneous infection with *Leishmania* parasites results in an increased frequency of Dectin-1$^+$-positive mDCs in the periphery compared to healthy controls (**Figure 1E**). Of note, the pDC subset does not show significant differences in Dectin-1 expression between EHCs and patients suffering from CL (**Figure 1F**). These data suggest that a local infection with *Leishmania* parasite results in the expansion of Dectin-1$^+$ mDC subsets in the peripheral blood of CL patients.

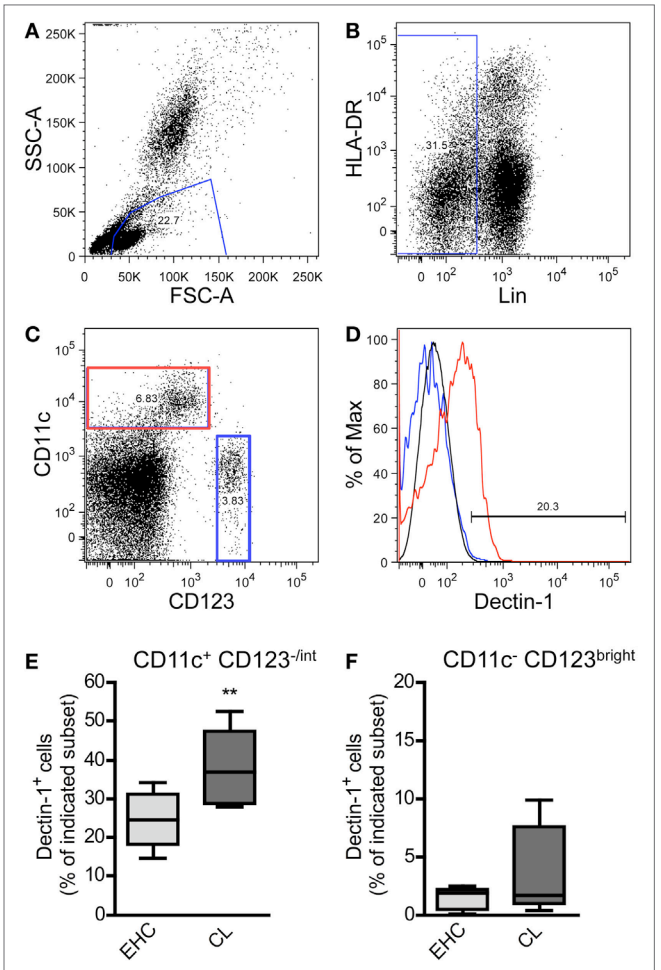

FIGURE 1 | Characterization of Dectin-1$^+$ DC subsets in blood samples from patients suffering from cutaneous leishmaniasis (CL). Peripheral blood samples from patients suffering from CL and Ethiopian healthy controls (EHCs) were stained for CD11c, Dectin-1, HLA-DR, CD123, and a lineage cocktail (anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20, and anti-CD56) abbreviated as Lin. The following gating strategy was used. **(A)** FSC and SSC plots were used to define the leukocyte population. **(B)** Lin$^-$ cells were selected for further analysis. **(C)** Lin$^-$ cells were differentiated into CD11c$^+$/CD123$^{-/int}$ and CD11c$^-$/CD123^{bright} populations displayed as dot plot. **(D)** The histogram plot indicates the expression of Dectin-1 on CD11c$^+$/CD123$^{-/int}$ (highlighted in red) and CD11c$^-$/CD123^{bright} cells (highlighted in blue). The black line represents the isotype control. **(E,F)** The frequency of Dectin-1$^+$ cells within the indicated subset is shown by box plot diagrams. Number of analyzed donors, $n = 5$. Data were analyzed using Student's *t*-test (**$p = 0.004$).

Local Infection with *Leishmania* Parasites Results in Systemic Proliferation of Dectin-1$^+$ DCs in BALB/c and C57BL/6 Mice

CD11c is hardly expressed by pDCs in mouse models (71, 72). Thus, most of the CD11c$^{+/bright}$ DCs belong to the subset of

conventional DCs including other mDC subsets (73, 74). In CL patients, Dectin-1$^+$ mDCs expand after infection. To prove the concept that a local infection also results in a systemic expansion of Dectin-1$^+$ mDCs, C57BL/6 and BALB/c mice were infected intradermally with *L. major* parasites. SDLNs, the site of infection, and the peripheral blood were analyzed for proliferating Dectin-1$^+$ CD11c$^{+/bright}$ DCs. Mice were administered the thymidine analog BrdU, as described in Section "Materials and Methods," to analyze cell proliferation *in vivo*. 13 days after infection, when clinical symptoms such as footpad swelling occur and *Leishmania*-specific T cell-mediated immunity is initiated (13, 60), Dectin-1 was measured on BrdU$^+$ (proliferating) and BrdU$^-$ (resting) CD11c$^+$ DCs (**Figures 2A,B**). Detailed quantification of Dectin-1$^+$ DCs within proliferating and resting CD11c$^+$ DCs revealed that both subsets express Dectin-1. However, the frequency of Dectin-1$^+$ CD11c$^+$ DCs is substantially higher within the proliferating subset compared to the resting CD11c$^+$ DCs of BALB/c and C57BL/6 mice (**Figures 2C,D**). Further analysis of the site of infection and SDLNs of infected BALB/c and C57BL/6 mice confirmed this result (Figures S2A,B in Supplementary Material). These data generated from mice reflect the results obtained in CL patients, indicating that a local infection with *Leishmania* parasites results in a systemic expansion of Dectin-1$^+$ DCs.

The Presence of Curdlan at the Site of Infection Results in an Adaptive Immune Response in Normally Susceptible BALB/c Mice

So far, only systemically (i.p. and/or i.v.) applied β-glucans have been used to treat BALB/c or C57BL/6 mice after infection (34, 43–48). No published data exist analyzing the effects of cutaneous application of β-glucan such as Curdlan in parallel to *L. major* infection. Given that Dectin-1$^+$ DCs expand at the site of infection and within SDLNs (**Figure 2**; Figure S2 in Supplementary Material), it seems feasible that a local stimulation of Dectin-1$^+$ DCs with Curdlan modulates the parasite-specific immune response. To test this hypothesis, BALB/c and C57BL/6 mice were infected with promastigote parasites suspended in PBS plus Curdlan. The course of infection was not substantially modulated in resistant C57BL/6 mice (Figure S3 in Supplementary Material). In contrast, normally susceptible BALB/c mice showed a resistant phenotype when the parasites were intradermally injected in combination with Curdlan (**Figure 3A**). BALB/c mice infected with *L. major* alone displayed the well-known severe course of disease (**Figure 3A**). An additional control experiment with Dectin-1$^{-/-}$ BALB/c was performed to confirm the specificity of the Curdlan/Dectin-1 interaction *in vivo*. Here, we were able to demonstrate that an application of Curdlan together with the parasites does not result in protective immunity against *L. major* parasites as shown in protected BALB/c wild-type mice (WT) (**Figure 3A**; Figure S4 in Supplementary Material). The experiments with non-healing BALB/c WT and Dectin-1$^{-/-}$ mice had to be terminated based on the severe ulcerated footpads.

C57BL/6 mice usually resolve a primary infection with *L. major*. Following resolution of the primary infection, they are immune to reinfection due to the generation of antigen-specific memory T cells (75, 76). To test whether Curdlan-protected BALB/c mice (**Figure 3A**) show classical signs of adaptive

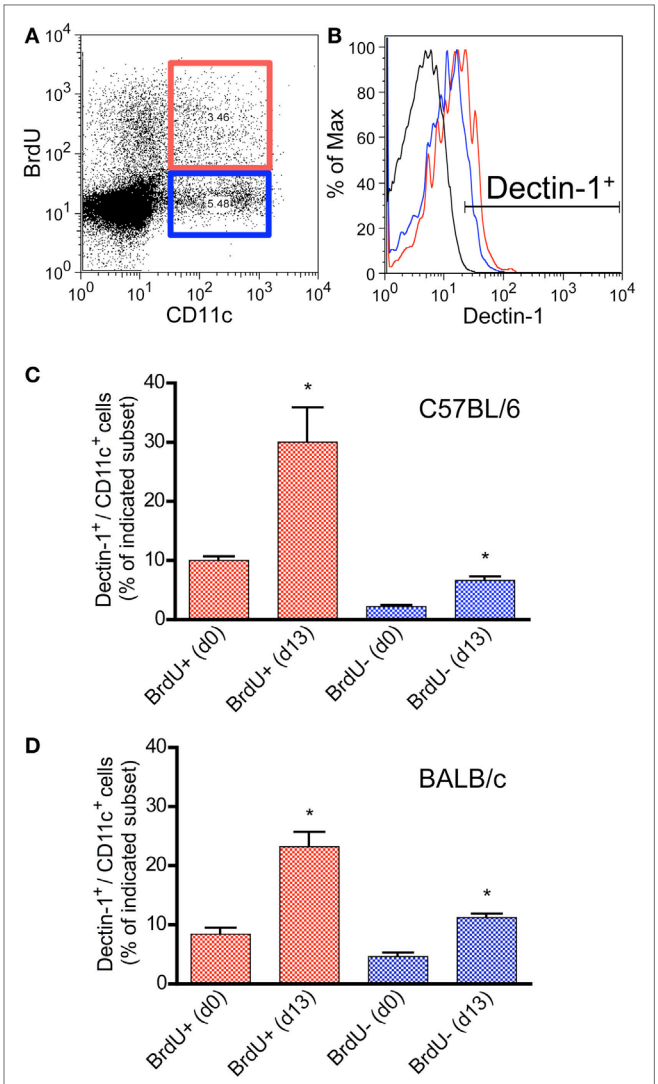

FIGURE 2 | Characterization of Dectin-1 expression on proliferating CD11c$^+$ DCs in blood samples of infected C57BL/6 and BALB/c mice. Mice were infected intradermally with *Leishmania major* parasites into the hind footpad, and the peripheral blood was analyzed 13 days after infection. Naïve mice served as controls (day 0). BrdU$^+$ was given 3 days before the analysis. Peripheral blood leukocytes were gated. **(A)** The dot plot diagram displays the CD11c and BrdU intensities. BrdU$^+$/CD11c$^+$ cells are highlighted by a red and BrdU$^-$/CD11c$^+$ by a blue gate. **(B)** Dectin-1 expression of the BrdU subsets is shown as histogram displaying isotype control in black, BrdU$^+$/CD11c$^+$ cells in red, and BrdU$^-$/CD11c$^+$ cells in blue line. **(C)** The frequencies of Dectin-1$^+$/CD11c$^+$ cells (blue bars, BrdU$^-$/CD11c$^+$; red bars, BrdU$^+$/CD11c$^+$) within the peripheral blood of C57BL/6 mice were analyzed. Pooled data from three different experiments are shown (mean ± SD). Data were analyzed using Student's *t*-test (**$p = 0.004$, ***$p = 0.0003$). **(D)** The frequencies of Dectin-1$^+$/CD11c$^+$ cells (blue bars, BrdU$^-$/CD11c$^+$; red bars, BrdU$^+$/CD11c$^+$) within the peripheral blood of BALB/c mice are indicated (mean ± SD). Pooled data from three different experiments were analyzed using the non-parametric Mann–Whitney test (*$p < 0.5$).

immunity, reinfection experiments were performed. BALB/c mice that had received Curdlan intradermally in the absence of parasites were used to exclude the possibility of an unspecific expansion of T cells potentially mediating protection. Resistant BALB/c mice, that had been infected with *L. major* parasites in the presence of Curdlan, were reinfected into the contra lateral footpad at day 131 after primary infection with a high-dose of parasites in the absence of Curdlan. At this time point, no clinical signs such as footpad swelling were detectable. BALB/c mice that have been treated intradermally only with Curdlan 131 days before the primary infection developed a severe course of disease, that is, comparable to infected BALB/c mice that were not pretreated (**Figure 3B**). In contrast, protected BALB/c mice, that resolved the primary infection, were able to rapidly control the second round of a high-dose infection with *Leishmania* parasites in the absence of Curdlan (**Figure 3B**). These mice now show

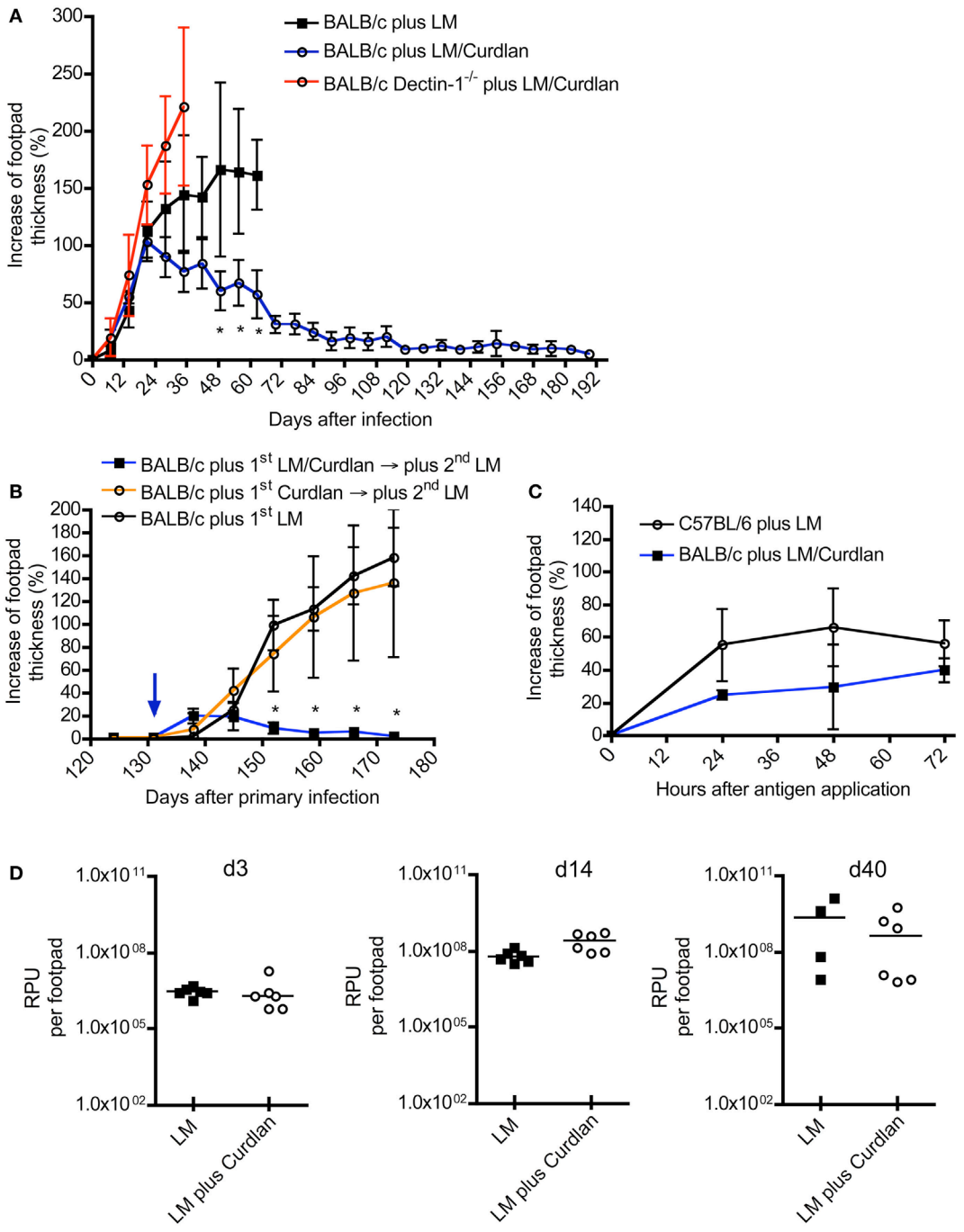

FIGURE 3 | Continued

FIGURE 3 | The presence of Curdlan at the site of infection results in an adaptive immune response in normally susceptible BALB/c. **(A)** BALB/c control mice were infected intradermally with *Leishmania (L.) major* into the right footpad (black line, black squares; 3×10^6 parasites/30 µL; $n = 19$), abbreviated as BALB/c plus LM. Another group of BALB/c mice was infected with 30 µL of mixture of 3×10^6 parasites and Curdlan ($c = 50$ µg/µL, blue line, black circles; $n = 16$), abbreviated as BALB/c plus LM/Curdlan. BALB/c mice deficient for Dectin-1 were also infected with 30 µL of a mixture of 3×10^6 parasites and Curdlan ($c = 50$ µg/µL, red line, black circles; $n = 5$), abbreviated as BALB/c Dectin-$1^{-/-}$ plus LM/Curdlan. Based on severe necrosis of the infected footpads, the groups BALB/c plus LM and BALB/c Dectin-$1^{-/-}$ plus LM/Curdlan had to be terminated at days 60 and 36, respectively. The *y*-axis depicts the increase in footpad thickness (mean ± SD). Data were analyzed using Student's *t*-test and highlighted if differences are significant (*$p < 0.05$; BALB/c plus LM compared to BALB/c plus LM/Curdlan). **(B)** Resistant BALB/c mice that had been treated with Curdlan in the presence of *L. major* parasites were reinfected at day 131 (blue arrow) after primary infection (abbreviated as BALB/c plus 1st LM/Curdlan → 2nd LM, blue line, black squares; $n = 3$). BALB/c mice that had been treated with Curdlan alone 131 days before (abbreviated as BALB/c plus 1st Curdlan → 2nd LM, orange line, black circles; $n = 3$) were also infected. Naïve BALB/c mice were infected (BALB/c plus 1st LM, black line, black circle; $n = 5$), too. The *y*-axis depicts the increase in footpad thickness (mean ± SD). Data (BALB/c plus 1st LM compared to BALB/c plus 1st LM/Curdlan → 2nd LM) were analyzed using two-way ANOVA (*$p < 0.05$). **(C)** A delayed-type hypersensitivity (DTH) response is shown, comparing C57BL/6 mice infected with 3×10^6 parasites (plus LM, black line, black circles; $n = 3$) with BALB/c mice infected with 3×10^6 parasites plus Curdlan (plus LM/Curdlan, blue line, black squares; $n = 3$). The DTH response was induced 217 days after primary infection as described in Section "Materials and Methods." The *y*-axis depicts the increase in footpad thickness (mean ± SD), the *x*-axis hours after antigen application. **(D)** Relative parasite units (RPU) representing the relative amount of parasites per footpad at days 3, 14, and 40 after infection are displayed. Infected BALB/c mice (LM, black squares) were compared with BALB/c mice that had been infected with parasites plus Curdlan (LM plus Curdlan, black circles). The mean (horizontal line) is shown. Each circle or square represents one analyzed footpad. Data were analyzed using Student's *t*-test (d3 $p = 0.779$, d14 $p = 0.062$, and d40 $p = 0.286$).

symptoms of an infection until the termination of the experiment 120 after the second exposition to *Leishmania* parasites in the absence of Curdlan.

Beside a reinfection, the DTH response is also an accepted indicator for the presence of an adaptive T cell-mediated immune response (75, 77). In contrast to resistant C57BL/6 mice, BALB/c mice hardly generate a measurable DTH reaction after a high-dose infection with *L. major* parasites (52). Using the high-dose model, we were able to demonstrate that Curdlan-protected BALB/c mice can indeed develop a DTH reaction that is comparable to that of resistant C57BL/6 mice (**Figure 3C**). Consequently, protected BALB/c mice mount an adaptive T cell-mediated immunity against *L. major* parasites that is accompanied by a clear reduction of the parasite load at the site of infection during the effector phase of experimental leishmaniasis (Figure S5 in Supplementary Material).

It needs to be mentioned that BALB/c mice can develop protective immunity against *L. major* if they have been infected with a low dose of parasites (8). Thus, we had to exclude that skin-homing macrophages eliminate the parasites after Curdlan contact shortly after infection. A milder course of disease would be the artificial consequence. In line with published data (31, 33), we were able to demonstrate that macrophages express Dectin-1 in the presence or absence of parasites (Figures S6A–D in Supplementary Material). However, Curdlan activation does not induce leishmanicidal mechanisms resulting in the elimination of *L. major* parasites (Figure S7 in Supplementary Material). In line with this finding, we were able to show that Curdlan-treated and control BALB/c mice show the same parasite burden at the site of infection during the early phase (days 3 and 14) of experimental leishmaniasis (**Figure 3D**). At day 40, after infection the beneficial effect of Curdlan treatment (the effect of Curdlan treatment on the parasite load at day 80 after infection, is shown in Figure S5 in Supplementary Material) is slightly indicated but not significant (**Figure 3D**).

In conclusion, we can demonstrate that the presence of Curdlan at the time of intradermal infection does not induce antigen-unspecific side effects but triggers the modulation of an adaptive T-cell response of BALB/c mice in a Dectin-1-dependent manner: they develop a resistant phenotype.

Cutaneous Curdlan Application Dampens the Th2 Response of Infected BALB/c Mice

The rapid clearance of *L. major* parasites after a high-dose reinfection and the positive DTH response of protected BALB/c mice (**Figures 3B,C**) supports the hypothesis that an adaptive Th1 response was generated. To assess that possibility, *Leishmania*-specific IgG subtypes, Th1- and Th2-indicating cytokines, and selected transcription factors were characterized.

Protective immunity to *L. major* infection does not depend on the production of specific Abs (78). However, *L. major*-specific IgG responses are an indication for the ability of the host organism to mount an antigen-specific immune reaction. In experimental leishmaniasis, BALB/c mice mount a Th2 response and produce predominantly IgG_1, whereas resistant C57BL/6 mice develop a Th1 response and an increased production of IgG_{2c} Abs (3, 79). An increase in parasite-specific IgG_{2c} is therefore an indicator for an ongoing protective Th1 response. In mouse strains with the *Igh1-a* allele, like BALB/c, the IgG_{2c} gene is deleted (80). Thus, we determined *Leishmania*-specific IgG_1 and IgG_{2a} subsets in sera of BALB/c mice that had been infected in the presence or absence of Curdlan. First, we analyzed mice that had not been treated with Curdlan. C57BL/6 and BALB/c mice generated *Leishmania*-specific IgG_1 25 days after infection (**Figure 4A**). In contrast, C57BL/6 mice produced a significantly higher amount of IgG_{2a} isotypes compared to susceptible BALB/c mice (**Figure 4B**). The infection in the presence of Curdlan led to an increase in *Leishmania*-specific IgG_{2a} in BALB/c mice (**Figure 4C**) that also provoked a higher IgG_{2a}/IgG_1 ratio of *Leishmania*-specific Abs (**Figure 4D**). Based on these data, we conclude that Curdlan treatment might dampen the development of a disease-promoting Th2 response in BALB/c mice.

In our study, we used a high-dose infection model and could demonstrate that Curdlan application results in protective immunity in BALB/c mice (**Figure 3**). It is important to mention that BALB/c mice do not necessarily develop a severe course of disease. In low-dose infection models, BALB/c mice develop a resistant phenotype, accompanied by the polarization toward a Th1-like phenotype (8). Thus, $CD4^+$ T cells from BALB/c mice can also differentiate into Th1 cells.

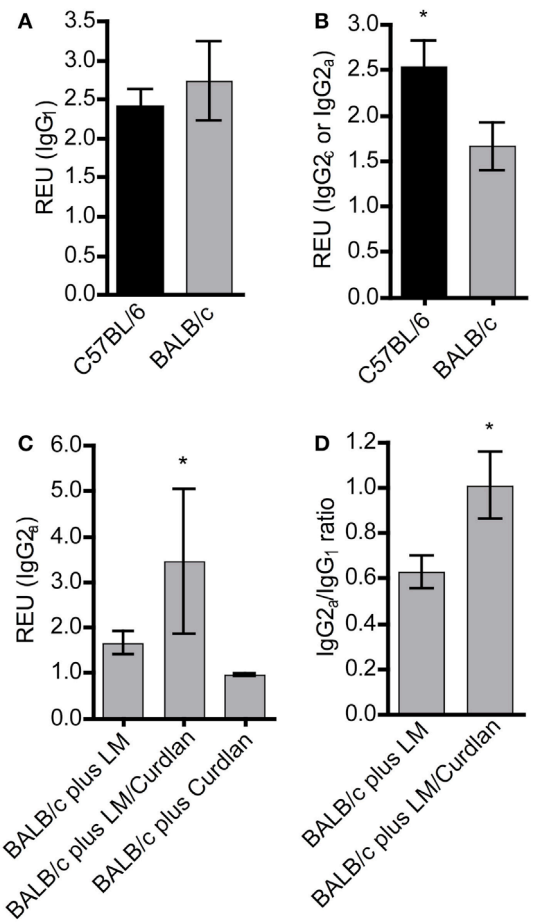

FIGURE 4 | The presence of Curdlan at the site of infection modulates the humoral immune response of BALB/c mice toward a pronounced production of *Leishmania*-specific IgG2a. C57BL/6 and BALB/c mice were infected with a volume of 30 µL PBS containing 3×10^6 *Leishmania (L.) major* (LM) parasites in the presence or absence of Curdlan ($c = 50$ µg/µL). Blood samples were collected 25 days after infection. *Leishmania*-specific IgG_1, IgG_{2a}, and IgG_{2c} in sera of infected mice were quantified by ELISA as described in Section "Materials and Methods." **(A)** Relative ELISA units (REU) of *Leishmania*-specific IgG_1 of infected BALB/c and C57BL/6 mice are depicted ($n = 3$). **(B)** REU of *Leishmania*-specific IgG_{2a} and IgG_{2c} of infected BALB/c and C57BL/6 mice were compared ($n = 3$; $^*p < 0.05$). **(C)** REU of *Leishmania*-specific IgG_{2a} of three groups are shown: infected BALB/c mice (plus LM), infected BALB/c mice in the presence of Curdlan (plus LM/Curdlan), and Curdlan-treated BALB/c mice (plus Curdlan) ($n = 3$; $^*p = 0.043$). **(D)** The ratio of *Leishmania*-specific IgG_{2a}/IgG_1 was calculated in BALB/c mice infected with *L. major* (plus LM) and BALB/c mice infected with *L. major* in the presence of Curdlan (plus LM/Curdlan) ($n = 3$; $^*p < 0.05$). Data were analyzed using the non-parametric Mann–Whitney test. The mean ± SD is displayed.

To address the question, whether protected BALB/c mice show a Th1 or Th2 profile, $CD4^+$ T cells were purified from SDLNs and analyzed by qRT-PCR. It is commonly accepted that activated $CD4^+$ T cells that receive IL-4 signaling upregulate GATA-binding protein 3 (GATA3) and become capable of producing Th2 cytokines (81, 82). Thus, GATA3 is regarded as an inducer for Th2-cell differentiation (83).

We measured the relative expression of GATA3 in $CD4^+$ T cells isolated from SDLNs of infected BALB/c mice. These qRT-PCR data revealed that Curdlan treatment results in a dampened expression of GATA3 at day 10 (Figure S8A in Supplementary Material), which is significantly reduced at day 28 (Figure S8C in Supplementary Material) after infection compared to control animals. Furthermore, four out of five Curdlan-treated BALB/c mice also showed a clear reduction in IL-4 expression within the pool of $CD4^+$ T cells (Figures S8B,D in Supplementary Material). Thus, a cutaneous application of Curdlan seems to dampen an early IL-4 production within SDLNs. Surprisingly, $CD4^+$ T cells isolated from SDLN of Curdlan-treated mice showed a transient trend toward a lower expression of the key transcription factor T-bet, known to define Th1 cells (84), and of the Th1 cytokine IFN-γ at day 10 after infection (Figures S8E,F in Supplementary Material). In contrast, at day 28 the expression of T-bet (Figure S8 in Supplementary Material) but not of IFN-γ (Figure S8H in Supplementary Material) was slightly increased in $CD4^+$ T cells of Curdlan-treated BALB/c mice.

A rich body of literature described the fact that Th-cell responses are characterized by the balance of Th1- and Th2-associated cytokines and not by the relative amount of single cytokines such as IFN-γ and IL-4 (85–90). Consequently, we calculated the Th1/Th2 ratio based on the relative expression levels of GATA3, IL-4, T-bet, and IFN-γ at days 10 and 28 after infection. As shown in **Figures 5A,B**, cutaneous Curdlan application was associated with a modulation of the IFN-/IL-4 ratios at day 10 and 28, indicating a shift toward a pronounced Th1 and a dampened Th2 response at day 28 after infection (**Figure 5C**). The IFN-/IL-4 ratios of BALB/c mice, infected in the absence of Curdlan showed no modification (**Figure 5D**). In line with this findings, the ratios of Th1-, Th2-driving transcription factors (T-bet/GATA3) was not substantial different at day 10 but significant enhanced at day 28 (**Figures 5E,F**), if mice had been treated with Curdlan. Additionally, we were able to demonstrate that this Curdlan-induced Th1 polarization increased from day 10 to day 28 after infection (**Figure 5G**). T-bet/GATA3ratios of BALB/c mice, infected in the absence of Curdlan showed no modification (**Figure 5H**). The Th1 polarization was also accompanied by a pronounced IL-17A mRNA expression of $CD4^+$ T cells (data not shown).

L. major-Harboring DCs Express Higher Levels of Dectin-1 and Start to Mature after Curdlan Stimulation

As shown earlier, Dectin-1^+ DCs expand at the site of infection and within SDLNs after infection with *Leishmania* parasites (**Figure 2**). A dermal exposure to Curdlan can protect BALB/c mice from severe leishmaniasis and induces T cell-mediated immune responses protecting from a high-dose reinfection (**Figure 3**). Cutaneous DCs are known to be crucial for the generation of adaptive immunity. These professional APCs capture parasites or parasite-derived antigens and are able to migrate to SDLNs for subsequent antigen presentation to T cells (14). To accomplish these tasks, DCs undergo a maturation program accompanied by the expression of surface molecules pivotal for

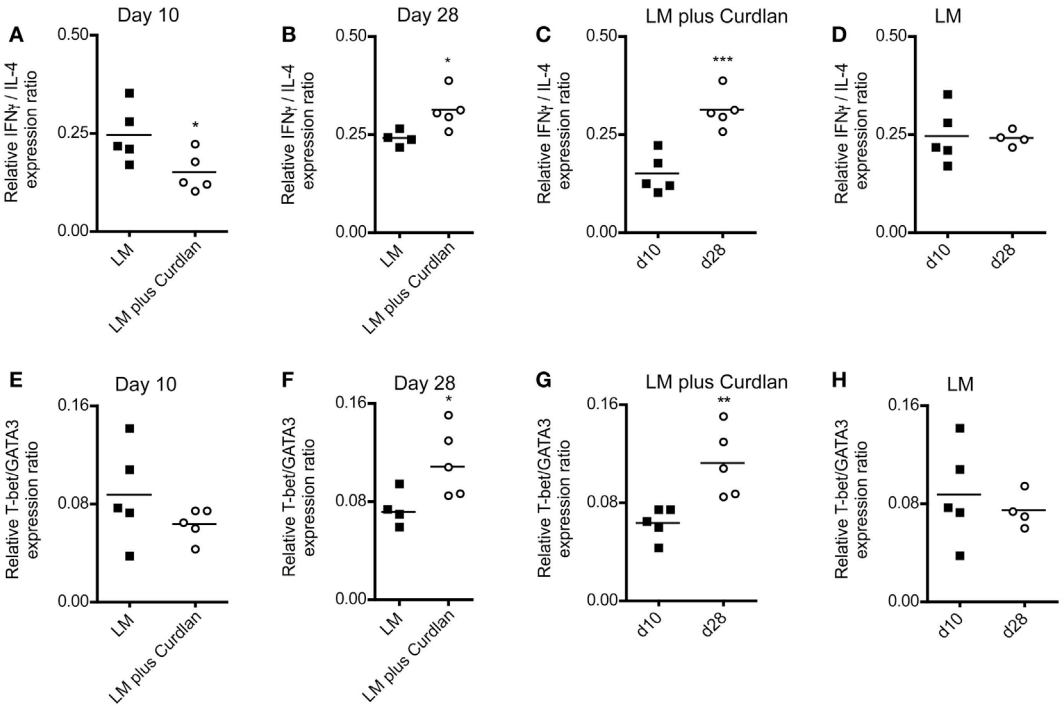

FIGURE 5 | Lymph node-resident CD4+ T cells from Curdlan-treated BALB/c mice show a shift toward an impaired T helper (Th) 2 response. BALB/c mice were infected with a volume of 30 µL PBS containing 3×10^6 *Leishmania (L.) major* (LM) parasites in the presence (abbreviated as LM plus Curdlan, $c = 50$ µg/µL) or absence of Curdlan (abbreviated as LM). CD4+ T cells were purified at days 10 and 28 after infection, and qRT-PCR was performed to determine the relative mRNA levels of target genes referred to GAPDH. The ratios of T-bet/GATA-binding protein 3 (GATA3) and of IFN-γ/IL-4 relative mRNA levels are shown. **(A,B)** The scatter plots depict the relative expression ratio of IFN-γ/IL-4 from the analyzed groups (LM and LM plus Curdlan) at day 10 [**(A)**, $^*p = 0.042$] and day 28 [**(B)**, $^*p = 0.032$]. **(C,D)** The scatter plots depict the relative expression ratios of IFN-γ/IL-4 from the analyzed groups in a time-dependent manner. **(C)** LM plus Curdlan ($^{***}p = 0.0007$) and **(D)** plus LM ($p > 0.05$). **(E,F)** The scatter plots depict the relative expression ratio of T-bet/GATA3 from the analyzed groups (LM and LM plus Curdlan). Day 10 [**(E)**, $p > 0.05$] and day 28 [**(F)**, $^*p = 0.048$] are shown. **(G,H)** The scatter plots depict the relative expression ratios of T-bet/GATA3 from the analyzed groups in a time-dependent manner. **(G)** LM plus Curdlan ($^{**}p = 0.008$) and **(D)** plus LM. Data were analyzed using Student's *t*-test. Each symbol represents an individual mouse, and the bars indicate the medians.

migration and of costimulatory molecules (17). Thus, we wanted to find out whether Dectin-1+ DCs get infected by parasites and if these parasite-harboring Dectin-1+ DCs undergo maturation after Curdlan treatment.

BMDCs were cocultured with CFSE-labeled promastigote parasites. This allows the differentiation of infected versus non-infected BMDCs subsets simultaneously (**Figure 6A**) (57). With this method, we could demonstrate that *L. major*+ BMDCs express a higher level of Dectin-1 compared to uninfected DCs (**Figures 6B,C**). The frequency of Dectin-1-positive BMDCs was also significantly higher in the infected BMDC population compared to BMDCs that were negative for *L. major* parasites (**Figure 6D**). Comparable to BMDCs, BMDMs show also a moderate increase in the frequency of Dectin-1+ cells and Dectin-1 expression intensity after exposition to *L. major* parasites (Figure S6 in Supplementary Material). This would suggest Dectin-1-mediated host pathogen interactions. Thus, we addressed the question whether pathogen-derived carbohydrates can interact with C-type lectins and especially with Dectin-1.

The CLRs DC-SIGN (CD209) and the mannose receptor C-type 1 (CD206) (91) have been shown to bind *Leishmania* antigens. However, it is unknown whether Dectin-1 recognizes *L. major*-derived molecules. With the help of selected CLR-Fc fusion proteins, we were able to demonstrate an interaction of *L. major* parasites with CLEC-9 and MGL-1 that recognizes f-actin (92) or galactose, respectively (93) (Figure S9 in Supplementary Material). However, a binding of Dectin-1-Fc fusion proteins to parasite structures could not be observed (Figure S9 in Supplementary Material). Consequently, even though myeloid cells such as macrophages and DCs are positive for Dectin-1 and show a higher Dectin-1 expression after parasite exposition, Dectin-1 alone is not crucial for the uptake of *L. major* parasites, because the parasites are negative for the Dectin-1 ligand. The fact that Dectin-$1^{-/-}$ phagocytes can still take up parasites supports that conclusion (31).

L. major parasites can be detected preferentially in BMDCs showing an immature phenotype (**Figures 7A,B**) and do not induce a maturation of infected cells (data not shown). Thus, parasites seem to inhibit the maturation of immature and Dectin-1^{high} DCs and thereby the subsequent migration to the SDLN (94, 95). The incubation of infected BMDCs with Curdlan is accompanied by a strong release of TNF-α and IL-6 and a maturation of infected BMDCs (**Figure 7C**; Figure S10 in Supplementary Material). This is of special interest, because TNF-α and IL-6 are known to be

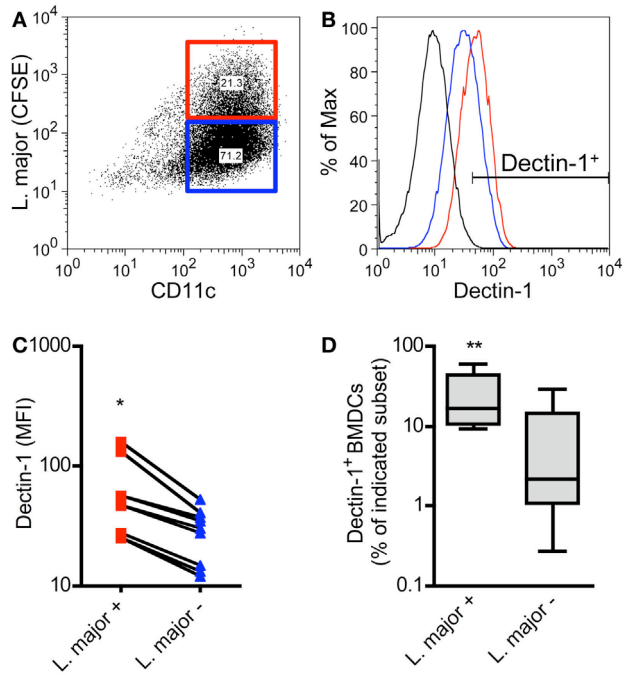

FIGURE 6 | Dectin-1 is expressed to a stronger degree by *Leishmania (L.) major* harboring bone marrow-derived dendritic cells (BMDCs) compared to non-infected BMDCs. BMDCs were infected with CFSE-labeled *L. major* promastigote parasites. After 24 h, BMDCs were harvested and characterized by flow cytometry analysis for the markers CD11c and Dectin-1. **(A)** The dot plot diagram depicts two populations of *L. major* harboring (*L. major* CFSE$^+$; red square) and not infected (*L. major* CFSE$^-$; blue square) CD11c$^+$ BMDCs. The gates were placed according to the isotype controls of not infected BMDCs. **(B)** The histogram visualizes the expression of Dectin-1 of infected (*L. major*$^+$; red line) and not infected (*L. major*$^-$; blue line) BMDCs. The black line depicts the isotype control. **(C)** The mean fluorescence intensity (MFI) of Dectin-1 expressed by *L. major*$^+$ (red squares) and not infected subsets (*L. major*$^-$; blue triangles) is shown. Each symbol represents an individual mouse. Pooled data of three independent experiments are included (*$p = 0.018$). **(D)** The frequency of Dectin-1$^+$ cells within the infected (*L. major*$^+$) and not infected (*L. major*$^-$) subsets is displayed as a box plot ($n = 5$ experiments, **$p = 0.0029$). Data were analyzed using the non-parametric Mann–Whitney test.

crucial for DC maturation and subsequent priming of T cells (17, 18). Based on the cytokine microenvironment, infected and non-infected Curdlan-treated BMDCs express high levels of CD86 and thus present a mature phenotype (**Figures 7D,E**). The stimulation of infected BMDCs with Curdlan also induces a strong release of T-cell growth factor IL-2 (Figure S10 in Supplementary Material). In association with the massive induction of costimulatory molecules such as CD86, Curdlan seems to represent an ideal adjuvant for efficient priming of *L. major*-specific T cells (96). In line with other published data, we showed that Curdlan activates BMDC maturation in a Dectin-1-specific manner (Figure S11 in Supplementary Material) (97, 98).

Additionally, we addressed the question whether Dectin-1 can be detected on Curdlan-activated BMDCs. In this context, we were able to demonstrate that immature BMDCs show in general a higher Dectin-1 expression compared to mature BMDCs (Figure S12A in Supplementary Material), and the detection of surface Dectin-1 is diminished after Curdlan stimulation (Figures S12B,E in Supplementary Material). The population of mature BMDCs, that is positive for *L. major* parasites, shows also a reduction in the Dectin-1 expression after stimulation with Curdlan (compare Q2, Figure S13 in Supplementary Material). BMDCs that have been exposed to parasites but do not harbor parasites, show no substantial reduction in Dectin-1 expression (compare Q2, Figure S13 in Supplementary Material).

In conclusion, we were able to demonstrate that Curdlan activation results in a maturation of infected BMDCs in a Dectin-1-specific manner that in turn might be crucial for the efficient priming of antigen-specific CD4$^+$ T cells.

Curdlan-Activated DCs Show a Pronounced Potential of Expanding *Leishmania*-Specific CD4$^+$ T Cells

The adjuvant effect of Dectin-1 ligation in T-cell immunity is undisputed, and it has been proven that Curdlan can polarize T-cell responses (99, 100). In line with these data, it has been shown that anti-Dectin-1 stimulation and the application of microparticulate β-glucan conjugates can potentiate an ovalbumin-specific CD4$^+$ T-cell response (101, 102). It was also described that Curdlan treatment can switch a Th2 to a Th1 response in tumor immunology (103). Last but not least, Dectin-1 ligation is discussed to be an important checkpoint in Th17-mediated immunity (28, 34, 38, 97, 104). However, the potential of Curdlan to accelerate the expansion of *L. major*-specific CD4$^+$ T cells has not been described yet. Thus, we isolated CD4$^+$ T cells from SDLN of infected BALB/c mice 10 days after infection. BMDCs were pulse-infected with *L. major* parasites and incubated in the presence or absence of Curdlan. The incubation of CD4$^+$ T cells with Curdlan-activated BMDCs resulted in a mild CD4$^+$ T cell proliferation (**Figures 8A,D**). Proliferation of CD4$^+$ T cells was substantially increased if BMDCs were pulsed with *L. major* parasites (**Figures 8B,D**). A pronounced enhancement of CD4$^+$ T cell proliferation was achieved by activating *L. major*-infected BMDCs with Curdlan (**Figures 8C,D**). Consequently, Curdlan is capable of triggering the antigen-specific expansion of CD4$^+$ T cells by infected BMDCs in a Dectin-1 specific manner (Figure S14 in Supplementary Material). Our data are in line with other experimental models showing that a Curdlan-induced priming of T cells depends on the Dectin-1 pathway (29). Curdlan also enhances the priming of antigen-specific T-cells by BMDCs exposed to *Leishmania* antigens (Figure S15 in Supplementary Material).

Supernatants of restimulated T cells were characterized by Multiplex analysis, to determine the expanded Th-cell subset. We could show that Curdlan treatment results in a 10-fold pronounced release of IFN-γ, IL-17, and IL-22 compared to control mice (Figure S16 in Supplementary Material). Thus, we conclude that Curdlan treatment induces a Th1 response in BALB/c mice that overlaps with a co-expression of Th17 cytokines (Figure S16 in Supplementary Material). Of note, the release of Th2 cytokines is not substantially modified (Figure S16 in Supplementary Material).

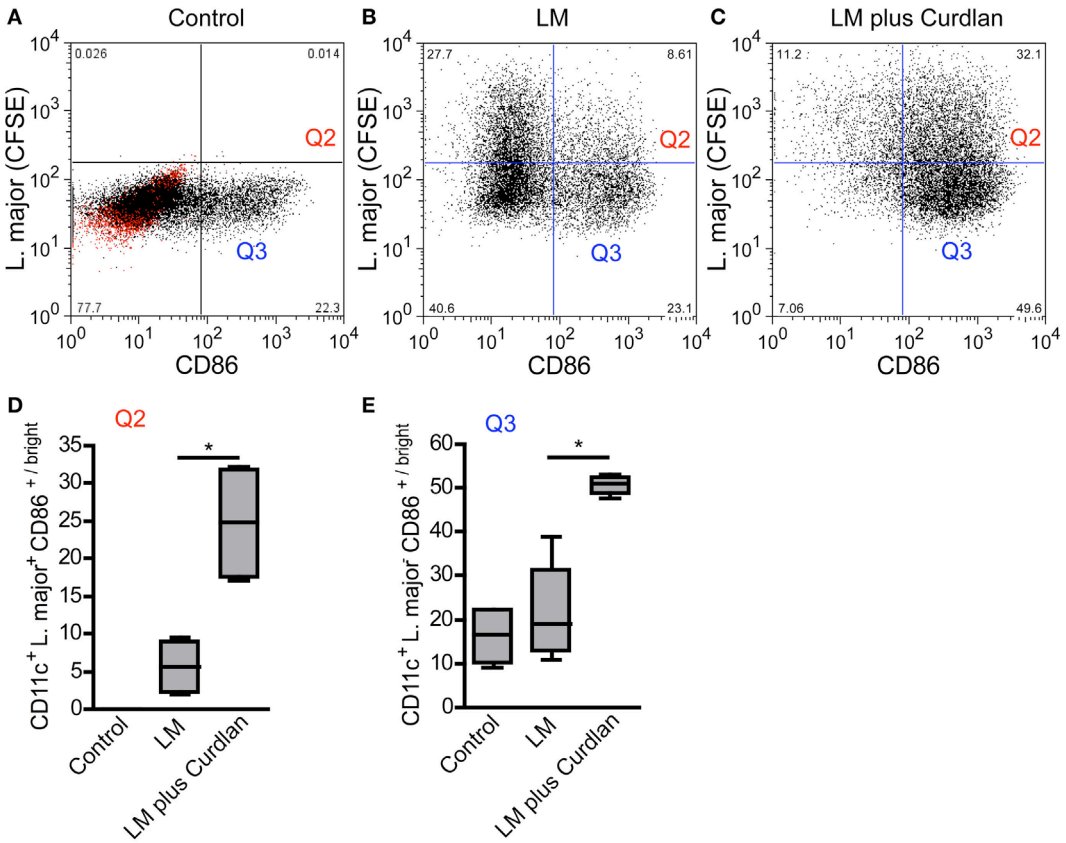

FIGURE 7 | *Leishmania (L.) major* (LM) harboring bone marrow-derived dendritic cells (BMDCs) mature after Curdlan stimulation. The effect of Curdlan on BMDC maturation was analyzed. 24 h after the Curdlan stimulation, BMDCs were harvested and characterized by flow cytometry using the markers CD11c and CD86. **(A)** The dot plot diagram depicts $CD11c^+$ BMDCs that had been cultured in the absence of Curdlan and CFSE-labeled *L. major* promastigote (control). The red population indicates the isotype control for the anti-CD86 antibody. The quadrants of interest are upper right (Q2, red; *L. major* $CFSE^+/CD86^{+/bright}$) and lower right (Q3, blue: *L. major* $CFSE^-/CD86^{+/bright}$). The quadrants were placed according to the isotype control and $CD11c^+$ BMDCs negative for CFSE-labeled parasites. **(B)** The dot plot diagram depicts the quadrants of interest of $CD11c^+$ BMDCs incubated with CFSE-labeled parasites (LM). **(C)** The dot plot diagram depicts the quadrants of interest of Curdlan stimulated $CD11c^+$ BMDCs cocultured with CFSE-labeled parasites (LM plus Curdlan). **(D)** The frequency of *L. major* $CFSE^+$ and $CD86^{+/bright}$ BMDCs (Q2) is shown. **(E)** The frequency of *L. major* $CFSE^-$ and $CD86^{+/bright}$ (Q3) BMDCs is depicted. The following culture conditions were compared: not infected, not stimulated BMDCs [Control, compare **(A)**], *L. major* harboring BMDCs [LM, compare **(B)**], and *L. major* harboring BMDCs stimulated with Curdlan [LM plus Curdlan, compare **(C)**]. The box plots **(D,E)** depict pooled data out of two independent experiments ($n = 4$). Data were analyzed using the non-parametric Mann–Whitney test [**(C,D)**, $^*p = 0.0286$].

A Mixture of *L. major* Antigens and Curdlan Induces a Milder Course of Experimental Leishmaniasis in BALB/c Mice

In combination with Curdlan, whole parasites might be used as a successful live "vaccine." This immunization protocol protects BALB/c mice upon challenge with a high-dose reinfection from severe leishmaniasis (compare **Figure 3**). Consequently, protected BALB/c mice must have developed an adaptive T-cell memory response capable of controlling and eliminating *L. major* parasites (75). We investigated whether vaccination of BALB/c mice with *L. major* antigens (SLA) in combination with Curdlan might also be a promising approach. We tested the adjuvant potential of Curdlan by vaccinating mice intradermally with a mixture of SLA and Curdlan. 28 days later, mice were challenged by a high dose (3×10^6) of *L. major* parasites into the contralateral footpad. Control mice that had received SLA in the absence of Curdlan developed a severe course of leishmaniasis that correlated with massive ulceration (compare **Figure 3**). These control groups were not protected, and the experiment had to be terminated at day 42 according to the animal health and welfare practices (**Figures 9A,B**).

These results again confirm the already published data that the simple administration of antigens does not induce protection of leishmaniasis in BALB/c mice (105). However, some BALB/c mice were partially protected after vaccination with SLA combination with Curdlan (**Figure 9A**). These mice developed a milder course of disease. Even though footpad swelling was still detectable, the ulceration of lesions was milder and substantially delayed (**Figure 9B**). Of note, a complete protection as achieved with living parasites could not be induced. The mice with delayed but ensuing mild ulcerations and constant footpad swelling were sacrificed during the monitoring period according

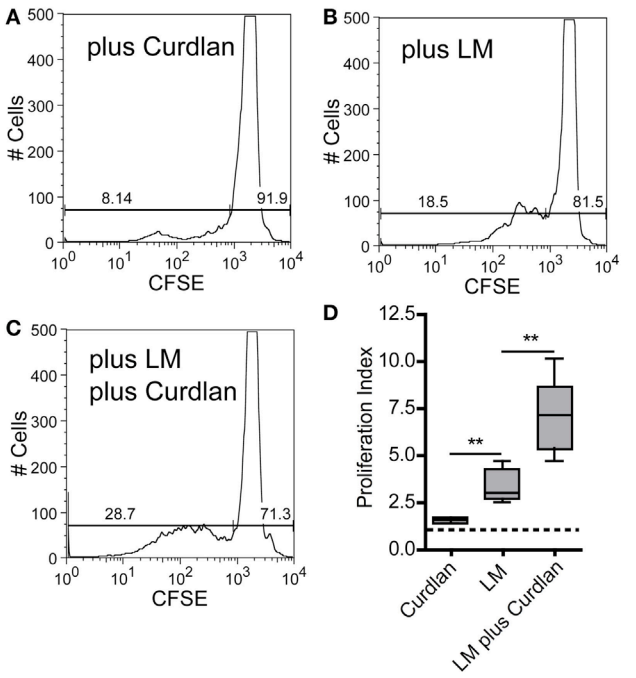

FIGURE 8 | Curdlan stimulation enhances the potential of parasite harboring bone marrow-derived dendritic cells (BMDCs) toward the expansion of *Leishmania*-specific CD4+ T cells. BALB/c mice were infected with a volume of 30 µL PBS containing 3×10^6 *Leishmania (L.) major* parasites. Ten days after infection, CD3+ T cells were isolated from skin-draining lymph nodes (SDLNs) and labeled with CFSE. BALB/c BMDCs were harvested at day 10 after *ex vivo* differentiation with GM-CSF and incubated for 24 h with *L. major* parasites (5:1) in the presence or absence of Curdlan (50 µg/200 µL). CFSE-labeled CD3+ T cells and BMDCs (10:1) were incubated for 72 h and analyzed by flow cytometry. The proliferative (left gate) and resting (right gate) CD4+ T cells are visualized by gates within the histogram plots. Following experimental approaches are displayed: **(A)** Curdlan activated but not infected BMDCs (plus Curdlan), **(B)** infected but not activated BMDCs (plus *L. major*), and **(C)** Curdlan-activated and infected BMDCs (plus *L. major;* plus Curdlan). The percentage of resting and proliferating cells is indicated inside the corresponding gates. **(D)** The proliferation index (described in detail in Section "Materials and Methods") is displayed as a box plot diagram. Pooled data from two independent experiments are shown. Data were analyzed using Student's *t*-test (Curdlan compared with LM, $^{**}p = 0.0018$ and LM plus Curdlan compared with LM, $^{**}p = 0.0058$; $n = 5$).

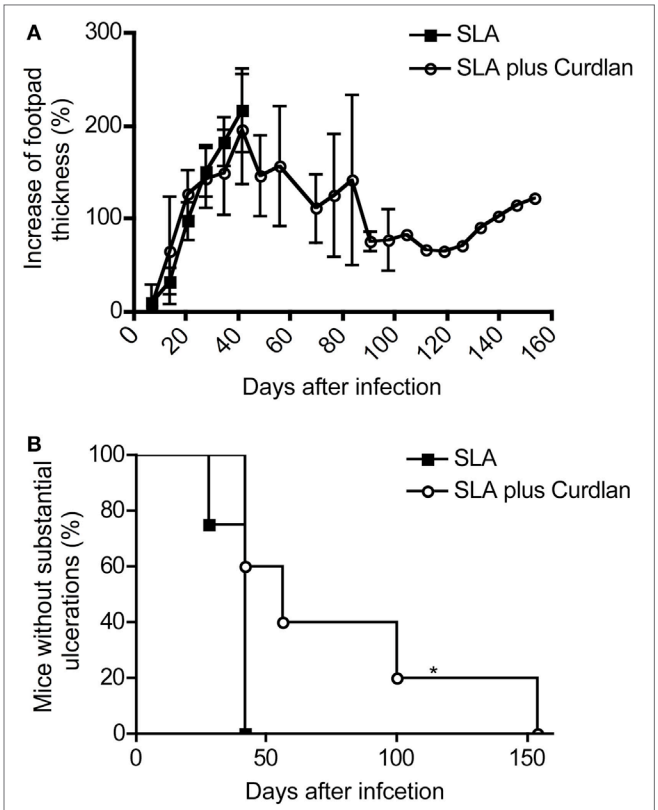

FIGURE 9 | Course of disease in vaccinated mice challenged with *Leishmania (L.) major*. Soluble *L. major* antigens (SLA, black square) or SLA plus Curdlan (black circle) treated were injected intradermally into the left footpad of BALB/c mice. The contralateral right footpad was used for the intradermal infection with 3×10^6 *L. major* parasites 28 days later. **(A)** The increase in footpad thickness after infection is shown (mean ± SD; $n = 11$ per group). **(B)** The number of mice that do not develop substantial ulcerations after intradermal infection into the contralateral hind footpad is depicted ($n = 11$; $^*p = 0.017$). Significances were calculated using a log-rank test.

to the guidelines for the care and use of experimental animals (**Figure 9B**). All analyzed lesions showing a delayed ulceration also exhibited a high parasite load (RPU $> 1 \times 10^8$).

DISCUSSION

Already in 1970s, β-glucans were used prophylactically or therapeutically in various infectious models of obligatory intracellular pathogens such as *Mycobacterium leprae* and *Plasmodium berghei* (106). Since β-glucans have no direct toxic effect on the named microbes, it has been suggested that a non-specific activation of the reticuloendothelial system was responsible for pathogen elimination (106). In experimental VL, β-glucans also elicited a strong but non-specific resistance against *L. donovani* in hamsters and BALB/c mice (107, 108). Other groups also demonstrated that an i.v. immunization with β-glucans combined with killed parasites induced a reduction in the parasite load in visceral organs of BALB/c and C57BL/6 mice (46, 109) and that CF-1 mice developed a milder course of VL, if they had been pre-treated s.c. or i.v. with β-glucans in combination with killed *L. donovani* parasites (43, 110). These immunization experiments demonstrate that a combination of dead parasites and β-glucans supports the development of a protective host response in VL. A therapeutic effect of the β-glucan Curdlan has also been reported in *L. donovani* (MHOM/IN/1983/AG83)-infected BALB/c mice, in association with the generation of Th17 and Th1 cytokines if systemically (i.p.) treated with Curdlan (34).

In contrast to VL mouse models, the biological impact of β-glucans seems to be heterogeneous in experimental CL. For instance, a systemic application (i.v.) of β-glucans was not sufficient to modulate the immune response of C57BL/6 mice infected with various *Leishmania* species such as *L. mexicana*, *L. braziliensis*, and *L. garnhami* (45). On the other hand, *L. major* (MHOM/IL/80/Fredlin)-infected BALB/c mice develop

a milder course of disease after multiple systemic applications (i.v. or i.p.) of β-glucans post infection (48). In line with the therapeutic potential of Curdlan, (i.p.) pretreatment with β-glucans dampened the spreading of *L. major* (HOM/SA/1983/SAYED) parasites in visceral organs of BALB/c mice (111). However, the effects of dermal applied β-glucans on the generation of adaptive T-cell responses had not been analyzed in detail.

In 2001, the CLR Dectin-1 was identified as the receptor for β-glucans (23). Subsequently, macrophages and professional antigen-presenting cells were identified as the most prominent cell subsets positive for Dectin-1 (112, 113). This finding raised the possibility that the activation of Dectin-1$^+$ DC subsets during acquisition of adaptive immunity might be modulated by Dectin-1-dependent pathways. Based on the current model, Langerin$^-$ dermal DCs are pivotal for the induction of the protective Th1-mediated immune response against *L. major* in experimental leishmaniasis (13, 15, 16). Thus, the precise conditioning of those DC subsets by Dectin-1-mediated signals might be crucial for the induction of adaptive immunity against *Leishmania* parasites. However, it is not known whether a cutaneous infection with *Leishmania* parasites causes an expansion of Dectin-1$^+$ DCs in humans or experimental models. Additionally, it was not clear whether Dectin-1 signaling results in a pronounced expansion and polarization of *L. major*-specific T cells.

We showed that Dectin-1$^+$ DC subsets expanded in patients suffering from CL. Consistent with other reports, we confirmed that Dectin-1 is expressed on peripheral blood mDCs of EHCs (27, 114). Furthermore, we were able to demonstrate that Dectin-1$^+$ peripheral blood mDCs expand in patients suffering from CL compared to EHCs. Given that such an expansion of DC subsets might also take place at the site of infection and in SDLNs of CL patients, Dectin-1$^+$ mDCs might represent promising targets for a Curdlan-based immunotherapy or vaccination strategy in humans. This is especially of interest since Dectin-1$^+$ mDCs are discussed to decrease disease-promoting Th2-type responses (115). In contrast to mDCs, the expression of Dectin-1 by human pDCs is controversially discussed. Unlike mouse Dectin-1, human Dectin-1 is also expressed on B cells (27). Consequently, an expression of Dectin-1 on lymphoid cell subsets is quite conceivable. Indeed, it was demonstrated by Western blot analysis that Dectin-1 is expressed by human CD303$^+$ (BDCA2) pDCs (116), whereas in another study CD303 (BDCA2)$^+$ pDCs were reported mostly negative for Dectin-1 (117). It can be concluded that the frequency of Dectin-1$^+$ DCs is higher within mDCs compared to pDCs. In line with these data, we could detect Dectin-1 on a small population of Lin$^-$/CD11c$^-$/CD123$^+$ pDCs within the blood of EHCs and patients suffering from CL. Nevertheless, this extremely small population did not expand after infection. This is important for a Dectin-1 ligand-based vaccination or therapy because pDCs are supposed to favor disease-promoting Th2-type CD4$^+$ T cell responses (118).

Infection with *Leishmania* parasites is associated with a pronounced myelopoiesis in mammalian hosts (119, 120). However, it has not been investigated so far whether this is also accompanied by an expansion of Dectin-1$^+$ DCs. We show that Dectin-1$^+$/CD11c$^+$ DCs expand within the blood of infected BALB/c and C57BL/6 mice, as in patients suffering from CL. CD11c is hardly expressed by pDCs in mouse models (71, 72). Therefore, most CD11c$^{+/bright}$ DCs belong to the subset of conventional DCs including other mDC subsets but not pDCs (73, 74). In addition, we found a clear increase in Dectin-1$^+$/CD11c$^{+/bright}$ DCs at the site of infection and SDLNs, proving the concept that Dectin-1$^+$ DCs represent promising targets for a Curdlan-based immunotherapy.

In vitro analysis was performed to characterize such a potentially beneficial impact of Curdlan/Dectin-1 interactions on DC-mediated priming of CD4$^+$ Th cells. To accomplish these tasks, DCs must undergo maturation to express surface molecules pivotal for migration such as CCR7 and costimulatory molecules (14, 17). Previous studies have already demonstrated that Curdlan can induce DC maturation (121, 122), whereas the biological effect of Curdlan on *Leishmania*-infected DCs was unknown. Parasite harboring DCs show an enhanced Dectin-1 expression but are immature. Curdlan activation resulted in the maturation of parasite-harboring DCs and the release of DC-activating cytokines. Subsequently, Curdlan-primed infected DCs were more potent in expanding antigen-specific T cells compared to DCs that had not been primed with Curdlan. Of note, Dectin-1$^{-/-}$ mice (on BALB/c background) were not protected from severe leishmaniasis by dermal Curdlan application. Additionally, Curdlan stimulation could neither mature BMDCs from Dectin-1$^{-/-}$ mice nor enhance the priming of antigen-specific T cells. These findings clearly demonstrate that Dectin-1 is exclusively responsible for the Curdlan-mediated effects *in vivo* and *in vitro*. These data are in line with the already published studies, demonstrating a Dectin-1-specific adjuvant effect of Curdlan on T cell priming in other experimental models (29, 98, 123). Consequently, Dectin-1 ligation can be considered as an important checkpoint for DC maturation and functionality in leishmaniasis.

We would like to point out that surface Dectin-1 is predominantly expressed by immature DC. This explains why infected DCs that in turn display an immature phenotype show a higher Dectin-1 mean fluorescence intensity (MFI) compared to uninfected DCs. Comparable to macrophages, Curdlan-exposed DCs respond with a reduction in the MFI of surface Dectin-1. Whether this reduction is due to the Dectin-1/Curdlan complex internalization, as described for macrophages, is not clear (124). Given the fact that mature DCs in general express lower amounts of surface Dectin-1, it cannot be excluded that maturation of DCs in general is accompanied by the downregulation of surface Dectin-1. In the light of our results, the data from Lima-Junior et al. deserves special consideration. The authors show an enhanced Dectin-1 MFI in *L. amazonensis* infected macrophages (33). Given the fact that infected and immature DCs display also a higher surface Dectin-1 MFI, it can be suggested that *Leishmania* parasites might favor preferentially Dectin-1$^+$ myeloid cells. Indeed, Dectin-1$^{-/-}$ phagocytes respond with a moderate reduction in the uptake of *L. infantum* parasites (31). However, we found that Dectin-1-Fc fusion proteins do not bind to *L. major* parasites. Thus, a *L. major*/Dectin-1 interaction can be excluded. This is of crucial importance in three aspects: first, *L. major* parasites do not induce Dectin-1 signaling-associated maturation of DCs, which might be considered as a smart escape mechanism, preventing DC maturation and subsequent T-cell activation.

Second, the higher frequency of infected Dectin-1-positive DCs is not the result of *L. major* Dectin-1 interactions, but might be an upregulation, based on so far not known mechanisms (33). Third, these data might explain the finding that Dectin-$1^{-/-}$ phagocytes can still phagocyte *L. infantum* parasites (31).

Apart from these items discussed earlier, Dectin-1^{+} mDCs might represent useful targets for the induction of a protective Th1-polarized $CD4^{+}$ T-cell response by β-glucans. Indeed, *in vivo* experiments revealed that a dermal application of Curdlan in the presence of living parasites induced a resistant phenotype in normally susceptible BALB/c mice. It is known that CLR signaling affects macrophage function in experimental leishmaniasis (31). Thus, we had to demonstrate in the high-dose infection model that the Curdlan-mediated effect on protective T-cell immunity was not the result of an early elimination of parasites at the site of infection by dermal macrophages. This would otherwise lead to a substantial reduction in parasite numbers shortly after high-dose infection. This is of crucial importance because low-dose infected BALB/c mice can mount a protective Th1 response (8, 125). Since the early parasite load at the site of infection was unaltered in the presence of Curdlan, we can exclude Curdlan-mediated early effects on *L. major* clearance by phagocytes. This finding was supported by the fact that Curdlan did not induce leishmanicidal mechanisms in infected macrophages *in vitro*. Hence, protection in Curdlan-treated BALB/c mice is not mediated by unspecific innate immune mechanisms, but an adaptive T cell-mediated response.

The ratio of Th1/Th2 cell and cytokines is crucial for efficient T cell-mediated immune responses and the outcome of leishmaniasis (86). From our data we can conclude that Curdlan treatment elicits a clear shift toward a protective Th1-mediated response in normally susceptible BALB/c mice. This conclusion is justified because Curdlan-treated BALB/c mice developed a resistant phenotype and show a clear DTH response that is associated with the generation of a T cell memory response, sufficient to protect BALB/c mice from a high-dose reinfection (75). The pronounced *Leishmania*-specific IgG2/IgG1 ratio supports also a manifested *Leishmania*-specific Th1 response (3). Furthermore, $CD4^{+}$ T cells isolated from SDLNs of Curdlan-treated infected BALB/c mice produced a lower level of IL-4 mRNA, but an increased level of cytokines such as IFN-γ and TNF-α after antigen-specific restimulation, further supporting a Th1 shift. These data are in line with previous studies, showing that early IL-4/IL-4R interactions promote Th2-cell polarization and impair the development of a Th1 response after infection with *L. major* (7, 85, 126–131). The enhanced release of IL-22 and IL-17 by $CD4^{+}$ T cells of Curdlan-treated mice needs a closer view. In mice, IL-17 and IL-22 are preferentially associated with Th17-cell differentiation (132). This is of interest, because the expressions of IL-17 and IL-22 are supposed to be crucial cofactors for *Leishmania* vaccine-induced immunity (133–136).

Successful vaccination programs against *Leishmania* parasites are still problematic. However, whole-organism vaccination strategies are most promising (137, 138). This implicates that pathogen-associated inflammatory responses at the site of infection might be crucial for the recruitment of appropriate immune cells and the differentiation of antigen-presenting cells, thus resulting in protective immunity (14, 139). Whole organisms in combination with Curdlan induce a protective immune response in normally susceptible BALB/c mice. The classical "vaccination" by *Leishmania* antigens in combination with Curdlan was able to dampen the severe progress of disease. However, full protection could not be achieved. The failure to mount a profound and solid adaptive immunity might be explained by the fact that *Leishmania* antigens do not persist at the site of infection and do not induce inflammatory immune responses, crucial for driving Th1-mediated adaptive immunity. In conclusion, we propose that whole-organism application in combination with Curdlan represents a promising strategy for the induction of *Leishmania*-specific immunity initiated by Dectin-1^{+} DCs.

ETHICS STATEMENT

Animals: all experiments and animal housing were performed according to the guidelines for the care and use of experimental animals. The animal work was approved by the local veterinary authorities of the district government based on the European guidelines and national regulations of the German Animal Protection Act (approval no. AZ 54-2532.1-05/11). Humans: the sample collection was permitted based on the local ethical committee (allowance number PO25/08, Addis Ababa, Ethiopia) and the national health research ethics review committee (approval number 310/227/07, Addis Ababa). Written informed consent was obtained from the participants of this study.

AUTHOR CONTRIBUTIONS

NZ acquired most of the data and analyzed the data. JJ and VS performed the macrophage experiments. MC performed the flow cytometry analysis in Ethiopia, AA, GZ, BL, MS, RW, AR, AW, and GB made substantial contributions to conception of the project and the article. GB and BL contributed materials essential for the work. UR designed the project and drafted and revised the manuscript critically for intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00263/full#supplementary-material.

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New Vaccine Formulations Containing a Modified Version of the Amastigote 2 Antigen and the Non-Virulent *Trypanosoma cruzi* CL-14 Strain Are Highly Antigenic and Protective against *Leishmania infantum* Challenge

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Visceral leishmaniasis (VL) is a major public health issue reported as the second illness in mortality among all tropical diseases. Clinical trials have shown that protection against VL is associated with robust T cell responses, especially those producing IFN-γ. The *Leishmania* amastigote 2 (A2) protein has been repeatedly described as immunogenic and protective against VL in different animal models; it is recognized by human T cells, and it is also commercially available in a vaccine formulation containing saponin against canine VL. Moving toward a more appropriate formulation for human vaccination, here, we tested a new optimized version of the recombinant protein (rA2), designed for *Escherichia coli* expression, in combination with adjuvants that have been approved for human use. Moreover, aiming at improving the cellular immune response triggered by rA2, we generated a recombinant live vaccine vector using *Trypanosoma cruzi* CL-14 non-virulent strain, named CL-14 A2. Mice immunized with respective rA2, adsorbed in Alum/CpG B297, a TLR9 agonist recognized by mice and human homologs, or with the recombinant CL-14 A2 parasites through homologous prime-boost protocol, were evaluated for antigen-specific immune responses and protection against *Leishmania infantum* promastigote challenge. Immunization with the new rA2/Alum/CpG formulations and CL-14 A2 transgenic vectors elicited stronger cellular immune responses than control groups, as shown by increased levels of IFN-γ, conferring protection against *L. infantum* challenge. Interestingly, the use of the wild-type CL-14 alone was enough to boost immunity and confer protection, confirming the previously reported immunogenic potential of this strain. Together, these results support the success of both the newly designed rA2 antigen and the ability of *T. cruzi* CL-14 to induce strong T cell-mediated

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immune responses against VL in animal models when used as a live vaccine vector. In conclusion, the vaccination strategies explored here reveal promising alternatives for the development of new rA2 vaccine formulations to be translated human clinical trials.

Keywords: visceral leishmaniasis, vaccine, *Trypanosoma cruzi* CL-14, *Leishmania infantum*, amastigote 2

INTRODUCTION

Visceral leishmaniasis (VL) is an infectious disease that affects mainly splenic and hepatic systems, being the second among the parasitic infections with highest mortality rates. This vector-borne tropical infection is caused by a flagellate protozoan of the genus *Leishmania spp* and affects more than 200,000 people per year (1). Failure to develop a potent human vaccine against this pathogen lies in the complexity of the immune response to the intracellular stage of parasite, given its previously reported defense mechanisms against innate and adaptive immunity (2).

The induction of a strong and long-lasting T helper type 1 (Th1) cellular immune response with high levels of IFN-γ production is a key feature, desired in ideal vaccine candidates. IFN-γ knockout mice fail to overcome infection with *Leishmania* species to which most immunocompetent mouse lines are resistant (3). In addition, IFN-γ is known to induce macrophage activation by increasing nitric oxide (NO) synthesis, leading to NO-mediated killing of intracellular pathogens (4).

The amastigote 2 (A2) proteins are mostly composed of a sequence of 10 amino acids that is repeated 40–90 times with molecular weights varying from 45 to 100 kDa (5). The A2 protein plays an important role in *Leishmania* survival in visceral organs, since the growth of A2-deficient amastigotes of *Leishmania donovani* into visceral organs was severely impaired when compared to A2-containing amastigotes (6). Similarly, the introduction of the *L. donovani* A2 gene into *Leishmania major* enhanced the ability of the latter to survive in visceral organs (7).

The recombinant A2 protein is recognized by human T cells (8) and has been reported as a vaccine candidate in different strains of mice using different vaccine formulations and delivery methods (9–11). In association with saponin, it was shown to induce protection against VL in beagle dogs (12). In all of these studies, protection was accompanied by a strong Th1 immune response with high levels of IFN-γ. In addition, successful vaccination against canine VL has been achieved in the previous years with the administration of Leish-Tec®, a commercial vaccine formulated with A2 (13, 14). However, due to its repeat-rich sequence, the expression and purification of recombinant A2 can be a laborious and time-consuming process. In this study, we optimized the A2 gene sequence for expression in *Escherichia coli*, including the entire non-repetitive domain of the protein and 10 repeated regions, in contrast with 40–90 repetitions from the original sequence.

Live vaccine vectors have been shown to improve T cell immune responses elicited by different antigens against several parasitic diseases (15, 16), including the A2 antigen (10). The *Trypanosoma cruzi* CL-14 non-virulent strain has been previously shown to be highly immunogenic in mouse models with production of high levels of IFN-γ and transient expansion of splenic $CD8^+$ T cells (17, 18). Moreover, a single injection of CL-14 has been shown to induce protection against lethal *T. cruzi* challenge using the highly virulent Y strain in BALB/c mice (19). To date, no studies have addressed whether immunization with CL-14 is enough to induce protection against VL challenge although both pathogens share a vast proteomic core and similar biology (20, 21). A previous study from our group tested the CL-14 strain as a vaccine vector, becoming a promising antigen delivery strategy to boost Th1 antigen-specific immune response (18).

To further explore the potential of A2 as a candidate antigen for human VL vaccines, we tested an optimized rA2 in combination with adjuvants approved for human vaccination. In addition, we generated stably transfected *T. cruzi* CL-14 expressing A2. Our data suggest that the newly designed rA2, formulated either with monophosphoryl lipid A (MPLA) or the synthetic oligodeoxynucleotides (ODNs) CpG B297, as well as the live vector *T. cruzi* CL-14 vaccine, can elicit robust antileishmaniasis immune responses. While the recombinant protein formulation has proven to be a good candidate to progress to human clinical trials, the live CL-14 vector vaccine may be a future alternative to achieve the prophylaxis of leishmaniasis and Chagas disease.

MATERIALS AND METHODS

Parasites

Epimastigote forms of non-pathogenic *T. cruzi* CL-14 were cultured in liver infusion tryptose medium supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 10,000 U/mL penicillin, and 10 mg/mL streptomycin (Gibco, USA) at 28°C in a biochemical oxygen demand (BOD) incubator. The transgenic parasites obtained were cultured in the same conditions with addition of 250 µg/mL geneticin (Gibco, USA) for selection of neomycin-resistant parasites. To perform immunization protocols, parasites were kept in stationary culture for 15–20 days, aiming to obtain metacyclic forms. Promastigotes of *Leishmania infantum* (MOM/BR/1970/BH46) were cultured in Schneider's Insect medium (Sigma Aldrich, USA) supplemented with 10% FBS, 10,000 U/mL penicillin, and 10 mg/mL streptomycin at 26°C in a BOD incubator.

Mice and Ethics Statement

Female BALB/c mice, 6- to 8-week-old mice were purchased from the Institute of Biological Sciences at Federal University of Minas Gerais (Belo Horizonte, Brazil). Mouse experiments were approved by and conducted according to animal welfare guidelines of the Ethics Committee of Animal Experimentation from Federal University of Minas Gerais, under the approved protocol number 73/2009.

Antigen Expression and Purification

To improve the A2 protein expression and purification protocols, without impairing the immunological outcome generated by

this antigen, we constructed an *E. coli* codon-optimized gene containing a selected nucleotide sequence spanning the entire coding region of the A2 protein. This improved gene contains 10, instead of the 40–90 repeated regions present in the original *L. infantum* A2 gene (XM_001465551) (22) and additional codons for a *C*-terminal 6× His tag. The synthetic gene was further cloned, through restriction enzyme digestion, into the pET9a vector to originate the plasmid pET9a24a-A2His, which was then transformed into *E. coli* C41 cells.

Protein expression was induced by IPTG. After induction, cells were harvested by centrifugation and lysed by sonication. The supernatant was then used for purification under denaturation conditions in a HisTrap column (GE Healthcare, UK) using the Äkta prime system. The purified protein was submitted to a second purification step using a HiTrap™Sepharose™ HP Ion Exchange column (GE Healthcare, UK). Finally, the purified fraction was loaded in an EndoTrap® HD column (Hyglos, DE) to remove endotoxins. The final purified protein was analyzed in Coomassie-stained SDS-PAGE, and its specific reactivity was assessed by western blot analysis using an anti-A2 monoclonal antibody, kindly provided by Dr. Greg Matlashewski.

Parasite Construction and Characterization

The codon-optimized A2 gene was isolated and cloned into the pROCKNeo plasmid using the *XhoI* and *XbaI* restriction sites. The resulting plasmid, pROCKNeoA2His (Figure S1 in Supplementary Material) was then used to transfect *T. cruzi* epimastigotes, as previously described (23). To achieve an integrative transfection into the parasite genome, the plasmids were linearized with *Not*I prior to electroporation. After transfection, the parasites were selected for neomycin resistance by addition of geneticin (G418) into culture medium. The presence of the A2 gene into the selected parasites was assessed by conventional PCR using: forward primer: HX1-5′ TTCTTCAAAATATGCAGCGGATT 3′ and reverse primer for A2: A2NOTRV-5′ TACCGCGGCCGCCTAGTGGTGATGG 3′.

Expression of the A2 protein in the recombinant *T. cruzi* CL-14 A2 parasite was assessed by both western blot and immunofluorescence analysis. For western blot analyses, the cell lysates of 2×10^7 epimastigotes of *T. cruzi* CL-14 or *T. cruzi* CL-14 A2 were submitted to SDS-PAGE and then transferred to a 0.45-μm nitrocellulose membrane (GE Healthcare, UK). To detect A2 expression by the *T. cruzi* CL-14 A2, we used a 1:100 dilution of anti-A2 monoclonal antibody. As the secondary antibody, we used anti-mouse anti-IgG horseradish peroxidase-conjugated antibody (Sigma Aldrich USA). The chemiluminescence reagent ECL Prime (GE Healthcare, UK) was used to reveal the reactions. The membranes were analyzed by an IS600 image system (GE Healthcare, UK).

For immunofluorescence analysis, 2×10^5 parasites were washed, fixed [phosphate-buffered saline (PBS) + 2% paraformaldehyde], loaded into a poly-L-lysine-coated glass slide, and incubated overnight at room temperature. The cells were then rehydrated and permeabilized with Triton X-100 0.2% in PBS. The monoclonal anti-A2 antibody was used as a primary antibody. Secondary antibody Alexa Fluor® 488 anti-mouse IgG (Thermo Scientific, USA) was used. After five wash steps, we added Vectashield containing DAPI to each spot (Vector Laboratories, USA). The glass slides were analyzed in a LSM Zeiss microscope.

Immunization Protocols and Challenge

For the immunization protocols, BALB/c mice ($n = 10$) were vaccinated with homologous prime-boost protocols. For the immunization groups receiving the recombinant protein, the following formulations were used: 10 μg of rA2 associated with 18 μg of CpG B297 (24) adsorbed in 30% (v/v) of Alum Rehydragel LV solution (Reheis, USA) or 10 μg of rA2 associated with 1.0 μg of MPLA (Butantan, Brazil). A total volume of 100 μL/mice was used as the dose. The negative control group received PBS. Both, protein and control group immunizations were done subcutaneously at the base of the tail, while the live vaccine groups received either 10^7 metacyclic forms of the CL-14 A2 or the CL-14 wild-type parasite, administered by intraperitoneal injections. For all protocols, the first dose was administered on the day 0, and each group was boosted 4 weeks later, with the same immunization dose.

For assessment of immunogenicity, mice ($n = 4$) were euthanized 21 days after the last vaccination dose. Serum samples and spleen of all groups were collected and used to assess, respectively, B and T cell immune responses elicited by the immunization protocols. Immunized mice ($n = 6$) were then infected subcutaneously, 21 days after the last vaccination dose, into the right hind footpad, with 1×10^7 stationary phase promastigotes of *L. infantum*. After challenge (30 days), the animals were euthanized; whole spleen was collected, individually processed, and used for parasite burden estimation. All experiments were performed twice, and representative results of each analysis are shown.

Immunological Assays

For IFN-γ and IL-10 production assays, 1×10^6 splenocytes from vaccinated mice were incubated at 37°C and 5% CO_2 for 48 h in the presence of 10 μg/mL rA2 or RPMI (non-stimulated control). The IFN-γ concentrations in the cell culture supernatants were determined using OptEIA™ Mouse IFN-γ ELISA Kit (BD, USA), according to manufacturer's instructions. OptEIA™ Mouse IL-10 ELISA Kit (BD, USA) was used to evaluate IL-10 concentration, according to manufacturer's instructions.

Antibody production was measured by enzyme-linked immunosorbent assay (ELISA), using rA2 protein as the coating antigen, at the concentration of 10 μg/mL. Serum from each immunized mouse was diluted 1:50, as established by a previous titration curve, and analyzed in duplicate. The reaction was developed using horseradish peroxidase-conjugated secondary antibodies (anti-IgG, IgG1, and IgG2a) (Zymed, USA) and substrate solution (Ortho-phenylenediamine + H_2O_2 diluted in citrate buffer pH 5,0). Optical densities were measured at 492 nm in the VersaMax microplate reader (Molecular Devices, USA).

To guarantee that effective immunization using *T. cruzi* CL-14 vector were achieved, we also evaluated the antibody response directed to the live vaccine vector by ELISA. For this purpose, total *T. cruzi* CL-14 extract was used as the coating antigen at a

concentration of 10 μg/mL, and the reaction was developed as described above.

Estimation of Parasite Load

For detection of *L. infantum* infection, 30 days after the challenge, animals were euthanized, and their spleens were harvested for parasite quantification by qPCR. DNA extraction of the analyzed organs was performed using the Illustra tissue and cells Mini Spin Kit (GE Healthcare, UK) according to the manufacturer's instructions. After extraction, samples were quantified in a NanoVue spectrophotometer (GE Healthcare, UK) and diluted with nuclease-free water to a final concentration of 10 ng/μL.

SYBR Green qPCR was performed, as previously described (25), with primers targeting the sequences of *L. infantum* KDNA (accession number: EU437407), namely, KDNA fw: 5' CCT ATT TTA CAC CAA CCC CCA GT 3' and kDNA Rv: 5' GGG TAG GGG CGT TCT GCG AAA 3' and also *Mus musculus* β actin gene (accession number: NM_007393.5) namely, actin fw: 5' CAG AGC AAG AGA GGC ATC C 3' and actin Rv: 5' TCA TTG TAG AAG GTG TGG TGC 3'. The qPCR amplification resulted in a 116-bp product for the kDNA primers and a 104-bp product for the actin β primers. Reactions were performed in a final volume of 20 μL, consisting of 1× MAXIMA SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA), 4 nM of each primer, and 50 ng of DNA template.

Standard curves were performed using eight serial dilutions of the target DNA, starting from 500,000–0.05 pg. All samples and negative controls were analyzed in duplicates for each run. Parasite loads were estimated as the number of *Leishmania* cells in 10^6 mouse cells. For determination of percentage of protection, the parasite loads in control PBS mice were considered as 100%. Reductions in parasite loads in vaccinated mice were then converted to percentage values.

Sequences Comparison

Sequence similarities between optimized A2 and proteins belonging to *T. cruzi* were accessed using BLASTp (NCBI, USA) analysis specific to *T. cruzi* strain CL Brener (taxid:353153) databank.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad, CA, USA). For data analysis and comparisons, we used one-way ANOVA followed by Tukey's multiple comparisons posttest. $p < 0.05$ was considered statistically significant ($^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$,).

RESULTS

Immunogenicity and Protection Conferred by rA2 Protein

The recombinant A2 antigen has been employed in several preclinical studies against leishmaniasis (9, 26) and is also commercially available as a canine vaccine against VL (13, 27). In this study, we developed an optimized A2 gene for expression in *E. coli*, including the entire non-repetitive domain of the protein and only 10 repeated regions, in contrast to 40–90 repetitions from the original sequence (**Figure 1A**). Therefore, this sequence has become more suitable for bacterial expression and should maintain the same immunogenicity as the original sequence. The protein obtained from *E. coli* expression followed by affinity purification is shown in the Coomassie-stained SDS-PAGE (left panel), while the anti-A2 specificity was confirmed by western blot analysis (right panel), both showing a band around 25 kDa, as expected for the optimized protein (**Figure 1B**).

To confirm the immunogenicity elicited by the optimized rA2, immunization protocols were performed as follows: BALB/c mice were immunized with PBS (control group) or rA2 in combination either with MPLA or CpG B297 plus Alum as adjuvant. To assess the humoral immune response, serum from immunized mice was subjected to ELISA anti-rA2 for IgG total, IgG1, and IgG2a antibody isotypes (**Figure 1C**). The data show that the rA2 protein, associated with either MPLA or CpG B297 adjuvants, was able to elicit a robust IgG total, IgG1, and IgG2a production, as previously observed with the original sequence (26).

Previous studies reporting vaccination strategies against *Leishmania* infection disclosed the importance of cell-mediated immunity for protection; specifically, the cellular responses based on the IFN-γ production. Therefore, by way of an ELISA assay, we measured the levels of IFN-γ present in the supernatants of splenocyte cultures from immunized mice after *in vitro* stimulation with rA2 or RPMI for 48 h. Results showed that the immunization with the rA2 antigen associated with MPLA or Alum/CpG led to high levels of IFN-γ (**Figure 1D**), when compared with PBS group under rA2 stimuli.

After the ability of the rA2-based vaccines to stimulate a Th1-biased T cell response had been confirmed, we tested if such immune response could induce protection against challenge with *L. infantum*. For this, parasite burden was evaluated in the spleen of mice in each immunization group 30 days after the challenge with 1×10^7 *L. infantum*. The results showed that the optimized rA2 antigen was capable of inducing a strong reduction of the parasite burden regardless of the adjuvant used (**Figure 1E**). However, these results support the potential use of the optimized rA2 antigen as a vaccine candidate for VL.

Stably Transfected CL-14 Parasites Express Recombinant A2 Proteins

Aiming to obtain transgenic *T. cruzi* CL-14 lines, we engineered an integrative plasmid with the vector pROCKNeo containing the coding sequence for A2 (Figure S1 in Supplementary Material). The pROCKNeo backbone contains sequences of homology to the abundantly prevalent locus of *T. cruzi* β-*tubulin* gene, allowing insertion of a multi-copy transgene into the genome of the parasite (23). Successful transfection of the recombinant plasmid into the parasite was confirmed *via* PCR using oligonucleotides flanking the A2 coding sequence. PCR results indicate that the constructs containing the A2 gene were efficiently inserted into (**Figure 2A**). Plasmids containing the construct (pROCKNeoA2) and the genomic DNA, derived from wild-type CL-14, were used, respectively, as positive and negative controls.

To confirm whether the neomycin-resistant CL-14 transfected parasites were expressing the recombinant protein, we

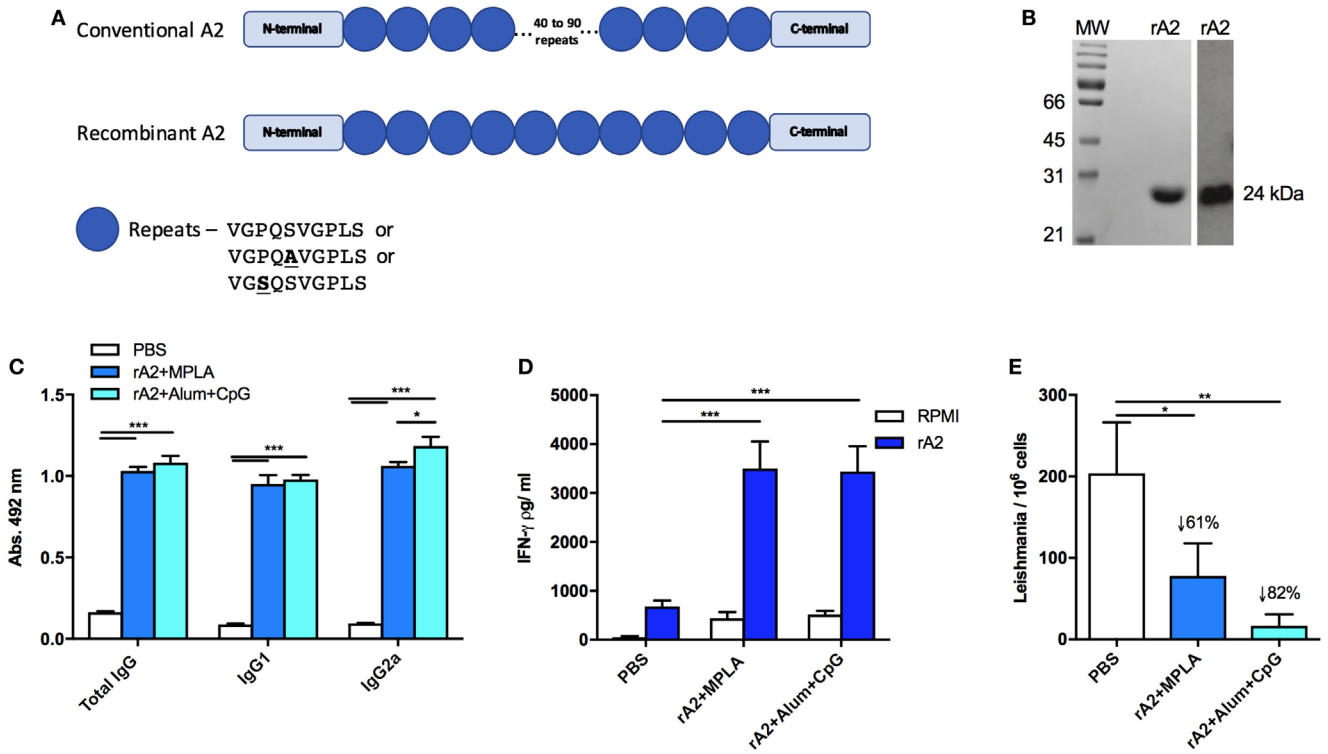

FIGURE 1 | Recombinant amastigote 2 (A2) confers immunogenicity and protection against *Leishmania infantum* challenge in BALB/c mice. **(A)** Schematic representation of the recombinant A2 protein compared with the original A2 sequence present in *Leishmania donovani*. Boxes represent the *C*- or *N*-terminal sequences, while the circles represent the repetitive amino acid sequences. Although the codon optimization was performed, it is not represented here. **(B)** Coomassie-stained SDS-PAGE of purified rA2 (left panel) and specific reactivity in Western blot with an anti-A2 monoclonal antibody (right panel). **(C)** Mice immunized s.c. with a homologous prime-boost protocol containing phosphate-buffered saline (PBS; negative control) or 10 μg of rA2 administered along monophosphoryl lipid A (MPLA) or Alum/CpG had their serum obtained 21 days after the last immunization dose. Serum samples were individually analyzed by enzyme-linked immunosorbent assay (ELISA) for total IgG, IgG1, or IgG2a, as previously described. **(D)** Mice immunized as previously described were sacrificed 21 days after the last immunization, and spleen cells were used for IFN-γ analyses by ELISA. For *in vitro* restimulation, it was used either rA2 or RPMI medium. **(E)** Mice immunized as previously described were euthanized 30 days after the challenge with *L. infantum*, and spleens were used for parasite loads' determination using qPCR assay. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Results are expressed as the mean and SEM values of duplicate assays using four to six different animals from each group individually analyzed.

used parasite extracts in western blot analysis. Recombinant A2 purified protein was loaded as a positive control, and the wild-type CL-14 extract was used as a negative control. The band profiles observed on the blots (**Figure 2B**) indicate that A2 is expressed only in neomycin-resistant parasites transfected with pROCKNeoA2. No expression was detected in the control group, represented by CL-14 WT. The A2 protein expressed by the transgenic CL-14 A2 parasites was further confirmed by immunofluorescence using monoclonal anti-A2 antibodies (**Figure 2C**). The last result supports the western blot data, whereby CL-14 WT was negative, and only the transfected parasites was positive for the A2 protein.

CL-14-Based Live Vaccine Induces Potent Immune Responses and Lead to Protection after *L. infantum* Challenge

After the expression of the rA2 by CL-14 A2 parasite had been confirmed, the ability of such vector to elicit specific humoral and cellular immune responses in mice was tested. For this purpose, we immunized mice with a prime/boost homolog protocol with the CL-14 A2, control wild-type CL-14 parasites, and also the rA2 protein along with Alum/CpG (Figure S2 in Supplementary Material). Negative control mice received only PBS.

With respect to the anti-rA2 humoral immune responses, mice immunized with rA2 + Alum/CpG showed a higher antibody response (IgG, IgG1, or IgG2a) when compared to mice immunized with the transgenic or control parasites (**Figure 3A**). Since mice immunized with the parental CL-14 or with the transgenic CL-14 A2 induced similar antibody levels to those observed in the control groups, we performed an ELISA using the total extract of *T. cruzi* CL-14. This assay was designed to confirm that the immunizations using these parasites were efficient. As seen in the **Figure 3B**, serum samples of animals immunized with the CL-14 parasites (transgenic or otherwise) were able to induce high levels of antibodies to the total extract of the CL-14. The same was not observed in the serum samples of animals immunized with PBS only or the recombinant protein formulations.

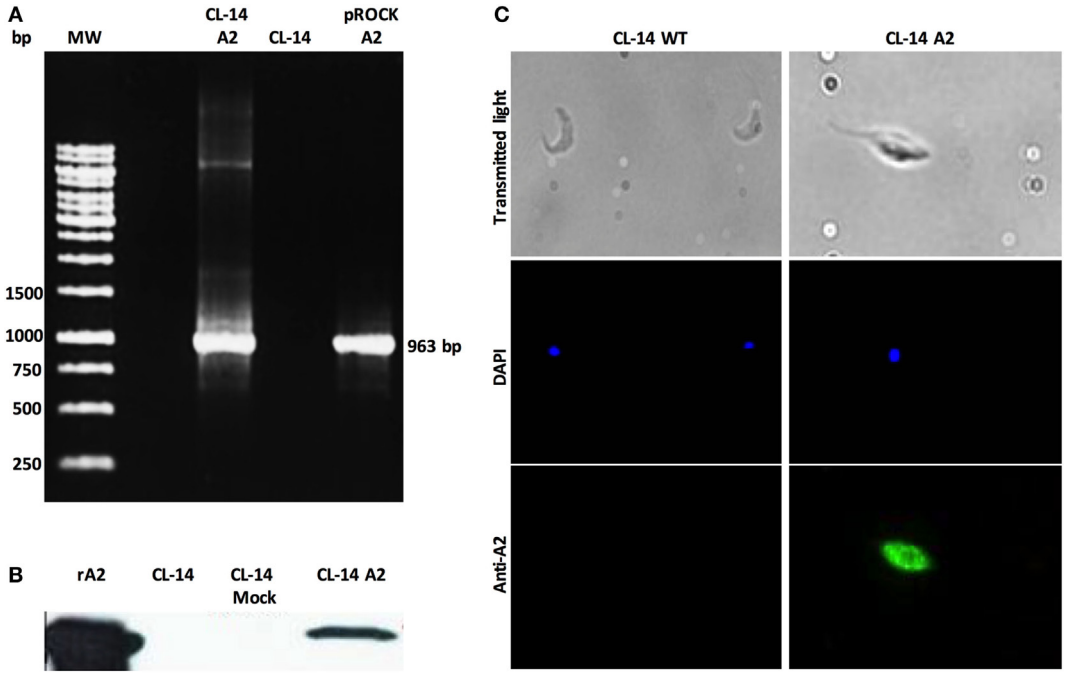

FIGURE 2 | Transgenic CL-14 parasites stably express recombinant amastigote 2 (A2). **(A)** Agarose gel electrophoresis of PCR products confirming specific amplification of the nucleotide sequences enconding the A2 (963pb) antigen by CL-14 A2 DNA extracts. As a positive control, plasmid DNA containing the nucleotide sequence-encoding rA2 was used (see Supplementary Material for plasmid maps). For negative control, we used untransfected CL-14 DNA extract. **(B)** Western blot analysis of transgenic CL-14 parasites lysates confirming the expression of 24 kDa A2. We used rA2 as a positive control. Monoclonal anti-A2 was used for detection of A2. **(C)** Immunofluorescence of fixed untransfected (CL-14 WT) and trasngenic CL-14 A2 parasites. 2×10^5 parasites were washed, fixed, and loaded into a poly-L-lysine-coated glass slide. DAPI shows nuclear staining, and the green channel shows protein localization detected by monoclonal anti-A2 with AlexaFluor 488 anti-mouse IgG as secondary antibody. The glass slides were analyzed in a LSM Zeiss microscope.

Considering that IFN-γ is the main cytokine involved in protection against *Leishmania*, we evaluated its production by spleen cells of mice vaccinated with the different formulations, using an ELISA assay, 21 days after the last immunization dose. When rA2 was used as a stimuli, the animals immunized with the respective recombinant protein associated with Alum/CpG or with the live vaccine (CL-14 A2) were able to produce increased levels of IFN-γ when compared to the control group (**Figure 3C**). Interestingly, immunization with the wild-type parasite CL-14 was also able to induce significantly increased production of IFN-γ. We also measured the levels of IL-10, an important cytokine related with parasite persistence in the host. Although increased IL-10 levels were detected after rA2 stimulation in all immunized groups, no significant differences were observed among them (**Figure 3D**).

Parasite loads in mice of all groups were determined in the spleen (**Figure 4A**) or liver (**Figure 4B**) by quantitative real-time PCR. The results revealed that mice immunized with rA2 + Alum/CpG (78%) as well as those immunized with control CL-14 (71%) and the transgenic parasite expressing A2 (78%) had a significant decrease in parasite burden when compared to the control group (PBS).

T. cruzi Proteins Share Similarity with rA2

Since animals immunized with CL-14 were able to produce elevated levels of IFN-γ when stimulated with rA2, we asked if this protein or its previous predicted epitopes would share sequence similarity with CL-14 proteins. Therefore, a BLASTp analysis was performed to address if the sequence covering most of the described B and T cell epitopes for A2 (SASAEPHKAAVDVGPLSVGPQSVGPLSVGPQAVGPLSV) (11) would have similarity with *T. cuzi* proteins. **Table 1** shows the BLASTp results of proteins that had similarities in the restricted epitope sequence.

DISCUSSION

To date, the best example of long-lasting protection against leishmaniasis has been achieved through a previous infection with the parasite *L. major* that usually causes local and benign cutaneous lesions, which may spontaneously heal, resulting in the induction of cellular immune responses (28). Based on this, a technique called leishmanization, consisting on the inoculation of infective material from cutaneous wounds, was used in the Middle East, to prevent cutaneous leishmaniasis (29, 30). The caveats of this procedure include safety issues and standardization. However, it indicates that a vaccine is feasible and suggests that chances of success are higher using a live vector strategy (28) although this issue may be eventually circumvented by the use of recombinant protein formulations.

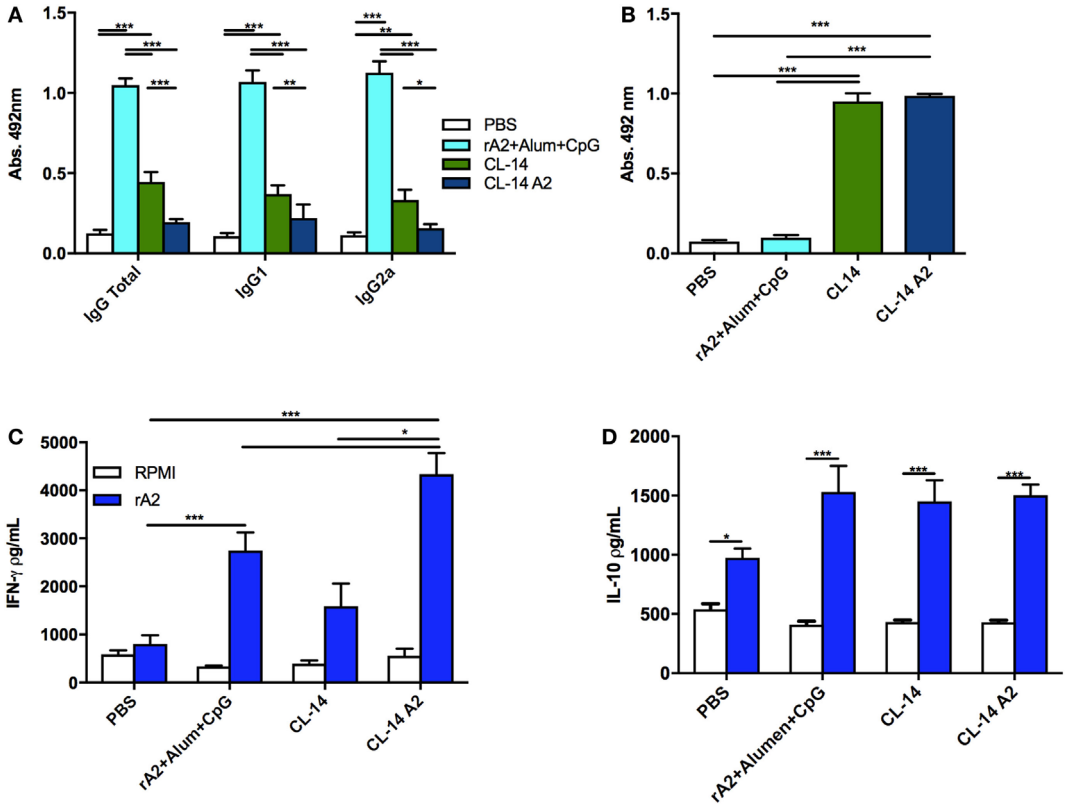

FIGURE 3 | Prime/boost immunization with CL-14 amastigote 2 (A2) induces an effective immune response in immunized BALB/c mice. **(A)** Specific antibody response (total IgG, IgG1, and IgG2a) induced by each immunization protocol was measured 21 days after the last immunization dose using serum samples of immunized mice. **(B)** Levels of total IgG against the live vaccine vector CL-14 were measured by enzyme-linked immunosorbent assay (ELISA) using serum samples of immunized mice. As a coating antigen, 10 μg of total CL-14 antigen extract was used. Mice immunized as previously described were euthanized 21 days after the last immunization dose. Spleen cells were cultured, and supernatants were used for IFN-γ **(C)** or IL-10 **(D)** analyses by ELISA. For *in vitro* re-stimulation, we used either RPMI or rA2. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Results are expressed as the mean and SEM values of duplicate assays using four to six different animals from each group individually analyzed.

Nonetheless, the development of clinically protective vaccines for VL remains a global challenge (31, 32). The majority of existing vaccines for bacteria and virus are based on the production of specific neutralizing antibodies (33, 34). Conversely, an efficient vaccine for intracellular parasitic infections, such as leishmaniasis, should elicit strong and long-lasting T cell-mediated immunity (35, 36). Therefore, the use of Th1-type adjuvants, including toll-like receptors (TLRs) agonists, as well as systems that prolong antigen exposure and mimic the infection are important strategies to promote and maintain a T cell-based protective response (37, 38).

Among the candidate antigens for second-generation vaccines against VL, A2 is a well-established molecule, employed in several preclinical leishmaniasis prevention protocols and, in combination with saponin, constitute the commercially available veterinary vaccine, Leish-Tec®, which is licensed on the Brazilian market since 2008 (13, 14, 27). Although extensively tested in dogs, the Leish-Tec® formulation cannot be directly transposed to human trials, since the adjuvant saponin, which induces lipid bodies and cross-presentation by DCs (39), is not approved for human use. Therefore, progress toward a human VL vaccine may require more specific and safe adjuvants, other than saponin (40). In addition, the original A2 sequence has significant drawbacks for production in industrial settings that may be circumvented. To improve recombinant expression and purification of this antigen, we developed an optimized gene coding for a shorter homolog of the A2 protein, which contains 10 repetitions, instead of the 40–90 repetitions from the original sequence. Codon optimization and the reduction of the repetitive sequences simplified the production process of the recombinant protein (41, 42). In addition, protein expression efficiency and yields were increased. These parameters are especially relevant in the context of quality control and high-scale production at industrial settings. Next, we tested the optimized A2 protein in different vaccine formulations to validate its immunogenicity, when associated with adjuvants more suitable for human use to human use. Our results indicated high levels of protection in BALB/c mice, by combining A2 with MPLA or CpG B297, corroborating previous results that indicate A2 as a promising candidate antigen, including those of preclinical trials in dogs or in Rhesus monkeys, a closer animal model to human VL (43). The combination of A2 with MPLA and ODN was based on prior evidence that these molecules are

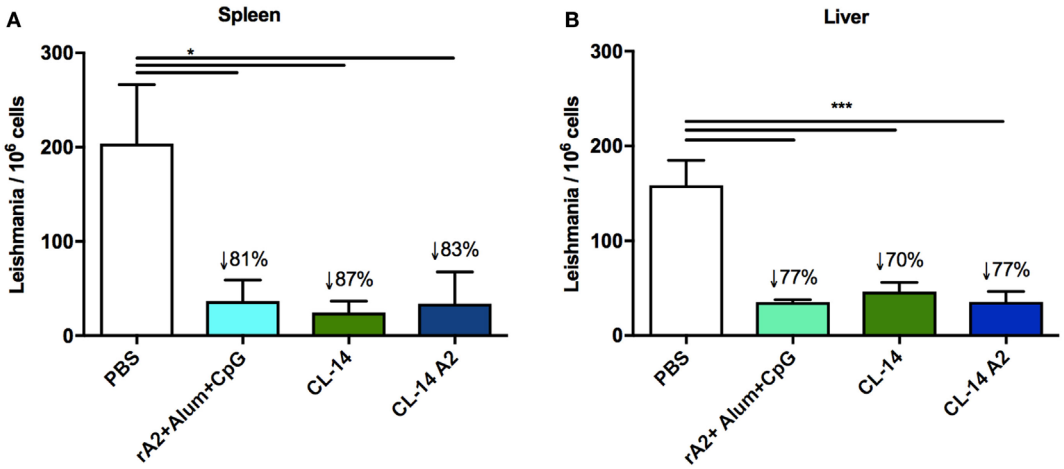

FIGURE 4 | Prime/boost immunization with CL-14 amastigote 2 (A2) induces protection in immunized BALB/c mice 30 days after the challenge animals were euthanized, and DNAs extracted from the spleen **(A)** or liver **(B)** were used for parasite burden using qPCR assay. The reduction percentage of parasite burden, based on the phosphate-buffered saline (PBS) group is shown for each group. *$p < 0.05$ and ***$p < 0.001$ analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Results are expressed as the mean and SEM values of duplicate assays using four to six different animals from each group individually analyzed.

TABLE 1 | Sequence comparison among rA2 and *Trypanosoma cruzi* CL Brener proteins.

T. cruzi proteins	Protein identity (%)	Query cover (%)	Similar sequences to A2 mapped epitopes: SASAEPHKAAVDVGPLSVGPQSVGPLSVGPQAVGPLSVGPQ
Transialidases (XP_808957)	32	91	SAEPEPTK--VDVGPASV
Transialidase (TcCLB.508247.90)	50	11	VDVASLSVG
Transialidase (XP_808957)	32	91	PKSAEP-KPAE
Hypothetical protein (TcCLB.506863.70)	80	80	LSI-PQSVGP
Hypothetical protein (XP_814399)	50	65	VSPLSAGPQ
Mucin TcMucII (TcCLB.510583.70)	77	47	ASAEPEHK-VDV
Amino acid permease (XP_814539.1)	24	54	VGVQVVGTLSVG
Zeta tubulin (XP_817160)	62	76	VAPQAVGELS
Surface protease GP63 (XP_804359.1)	58	70	AVGALNFGPQ

TLR4 and TLR9 agonists, respectively, with the ability to trigger Th1 responses (44). In addition, both TLRs are activated during *L. donovani* infection (45). Of note is the fact that the protection levels induced by the rA2/CpG B297 formulation against experimental VL were higher than those previously described in BALB/c mice immunized with formulations containing saponin, including the commercial Leish-Tec® vaccine (46). Although CpGs had emerged as good inducers of Th1 immune responses, the combined formulation with Alum increases the antigen/adjuvant depots, leading to an improved uptake by professional presenting cells, strengthening specific immune responses (47). CpG B297 is an ODN present in *T. cruzi* that is associated with enhanced Th1-type adjuvant activity, compared to other standard bacterial CpGs (24). More specifically, the rational selection of CpG B297 was founded on the recognition of this molecule by TLR9 homologs in both mice and human cells (24, 48). In contrast to MPLA, which is a bacterial-derived molecule, or saponin, which is purified from *Quillaja saponaria*, the synthetic nature of CpG B297 may circumvent problems generally associated with other adjuvants, including scale production and the lack of endotoxin or other contaminants, thus improving safety. Therefore, the use of CpG B297 molecule may more easily translate results of preclinical tests to human clinical trials, turning more feasible significant step toward a human vaccine.

The IFN-γ cytokine is the main Th1 marker associated with host protection in VL infection (49, 50). Induction of high levels of antigen-specific IFN-γ was detected for the groups receiving rA2, as well as CL-14 WT and transgenic CL-14 A2 vaccinated groups. Surprisingly, the group immunized with the CL-14 reference strain produced elevated levels of IFN-γ, when stimulated with A2. This intriguing result led us to search for sequence similarities among A2 predicted epitopes and *T. cruzi* proteins. Common epitopes for B cells are known among trypanosomatids and, depending of the transmission areas and the diagnostic test used, may hamper specific diagnosis between *T. cruzi* and *Leishmania* spp infections (51). Therefore, it is plausible that common T cell epitopes might be shared by these parasites, with different degrees of affinities for TCRs. Partial similarity was observed among the sequence SASAEPHKAAVDVGPLSVGPQSVGPV, which contains the A2 B and T cell $CD4^+$ and $CD8^+$ mapped epitopes (11), and short

amino acid sequences present in trans-sialidases, mucin, amino acid permease, GP63, and hypothetical proteins of *T. cruzi*, which may be potential T cell epitopes. Although these findings require further experimental support, they may explain why cells from CL-14 vaccinated animals produced IFN-γ upon rA2 stimulation. Among these proteins, members of the trans-sialidase superfamily are widely studied as antigen candidates for vaccination against Chagas disease since they lead to robust humoral and $CD8^+$ T cell-mediated immunity (52–55). In addition to these potential T cell epitopes, the *T. cruzi* CL-14 parasite has several immunomodulators, such as glycoinositolphospholipids and CpG ODNs (24, 56), explaining why the live vaccine possess intrinsic adjuvant properties.

Low levels of antigen-specific antibodies were observed for the CL-14 vaccinated groups. Other vaccine vectors that elicit Th1-biased responses favoring antigen presentation by MHC class I and activation of $CD8^+$ T cell, such as adenovirus or plasmid DNA, also lead to low antibody induction depending on the administration protocol (43). In contrast, the vaccination with the rA2 in combination with alum and CpG B297 led to high levels of antigen-specific total IgG, IgG1, and IgG2 antibodies. Generation of antibody-based response was expected for recombinant protein vaccines, as observed in previous studies testing these antigens (9–11, 13, 14, 57). In addition, aluminum salt adjuvants favor antibody production (58). Although questions remain about the mechanism of alum-driven immunomodulation (59), its combination with Th1 type adjuvants can result in both antibody production and increased IFN-γ levels. This pattern was observed in studies with cancer (18) and dengue virus (60) antigens in formulations containing alum plus CpG.

Immunization with CL-14 or recombinant CL-14 A2 parasites elicited a strong Th1-based cell immunity and displayed protection against a *L. infantum* challenge in BALB/C mice, suggesting CL-14 as a potential vaccine candidate for VL. Several factors justify the use of CL-14 as a live vaccine for leishmaniasis: (i) a great number of genes are shared by trypanosomatids; (ii) there is a similar intracellular biological cycle, favoring a CD8 T cell induction; (iii) *T. cruzi* has natural immunostimulatory molecules; and (iv) the long exposure time improves the generation of long-lasting immunity (18, 24, 48, 56). There are also other examples of leishmaniasis vaccine candidates based on non-pathogenic live parasites (28). Among them, *Leishmania tarentolae* is a non-pathogenic species to humans, also of particular interest. This lizard-infecting species is not able to persist long enough in mammalian host to cause infection, even in immunocompromised mice. However, this parasite alone is able to induce DC maturation, elicit Th1-type response, and induce partial cross protection against *L. donovani* challenge (61). Therefore, *L. tarentolae* has been tested as a vaccine vector and recombinant parasite expressing different heterologous antigens, including A2, which led to protection in mice (10, 62–64) and dogs (36). A major concern with live vaccination strategies is related to safety issues. For CL-14, this topic has been addressed many times. In tests with newborn mice, which are very susceptible to Chagas infection, no parasite was found in tissue or blood after inoculation of CL-14 (18, 65). In addition, in experiments with knockout mice, lacking important immunologic mechanisms, such as $CD8^+$ T cells, CL-14 was unable to generate disease (7). Regardless these safety issues, successful vaccination studies using live vectors have been also reported for other parasitic diseases such as malaria (66) and toxoplasmosis (67, 68). Noteworthy, the most promising malaria vaccine, which is in advanced clinical trial, is based on i.v. administration of radio-attenuated sporozoites (69). CL-14 was previously reported protective against Chagas disease and cancer, when expressing NY-ESO antigen (18, 24). Our results confirm the potential of live vaccination strategies for VL, specifically with the CL-14 model, increasing its range of target diseases. Nevertheless, even if the translation of this live vaccine candidate as a licensed human vaccine would not be feasible, the reported *T. cruzi* CL-14 data add important contribution to the vaccinology field, showing the potential of a multi-protozoa vaccine, which may favor future development of alternative vaccines for such complex diseases.

Together, our results support the success of the newly designed rA2 vaccine formulations and the *T. cruzi* CL-14 to induce strong T cell-mediated immune responses and protection against VL in animal models. Both vaccination strategies reveal promising alternatives for the development of new vaccine formulations for VL. On one hand, the CL-14 data expand the evidence that live vaccine vectors may be useful to induce cross protective responses and development of multiparasite vaccines; however, the A2 recombinant protein formulation is a more feasible vaccine to progress to human clinical trials.

ETHICS STATEMENT

Mice experiments were approved by and conducted according to animal welfare guidelines of the Ethics Committee of Animal Experimentation from Federal University of Minas Gerais under the approved protocol number 73/2009.

AUTHOR CONTRIBUTIONS

CJ and AF conceived the project and designed the experiments. AA, LM, DD, and FN were responsible for developing the experiments, data acquisition, analysis, and interpretation. AA, LM, DD, and CJ wrote the paper. LD was responsible for the rA2 expression optimization. RG made significant technical and conceptual contributions to the manuscript and data interpretation. All the authors provided intellectual content and approved the final version of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00465/full#supplementary-material.

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Conflict of Interest Statement: The author CJ, AF, RG, LD, and DD are holders of two patent deposit related to the present work. All other 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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Antigenicity of *Leishmania*-Activated C-Kinase Antigen (LACK) in Human Peripheral Blood Mononuclear Cells, and Protective Effect of Prime-Boost Vaccination With pCI-neo-LACK Plus Attenuated LACK-Expressing Vaccinia Viruses in Hamsters

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Leishmania-activated C-kinase antigen (LACK) is a highly conserved protein among *Leishmania* species and is considered a viable vaccine candidate for human leishmaniasis. In animal models, prime-boost vaccination with LACK-expressing plasmids plus attenuated vaccinia viruses (modified vaccinia Ankara [MVA] and mutant M65) expressing LACK, has been shown to protect against cutaneous leishmaniasis (CL). Further, LACK demonstrated to induce the production of protective cytokines in patients with active CL or cured visceral leishmaniasis, as well as in asymptomatic individuals from endemic areas. However, whether LACK is capable to trigger cytokine release by peripheral blood mononuclear cells from patients cured of CL due to *Leishmania infantum* (*L. infantum*) or induce protection in *L. infantum*-infected hamsters [visceral leishmaniasis (VL) model], has not yet been analyzed. The present work examines the *ex vivo* immunogenicity of LACK in cured VL and CL patients, and asymptomatic subjects from an *L. infantum* area. It also evaluates the vaccine potential of LACK against *L. infantum* infection in hamsters, in a protocol of priming with plasmid pCI-neo-LACK (DNA-LACK) followed by a booster with the poxvirus vectors MVA-LACK or M65-LACK. LACK-stimulated PBMC from both asymptomatic and cured subjects responded by producing IFN-γ, TNF-α, and granzyme B (Th1-type response). Further, 78% of PBMC samples that responded to soluble *Leishmania* antigen showed IFN-γ secretion following stimulation with LACK. In hamsters, the protocol of DNA-LACK prime/MVA-LACK or M65-LACK virus boost vaccination significantly reduced the amount of *Leishmania* DNA in the liver and bone marrow, with no differences recorded between the use of MVA or M65 virus vector options. In summary, the Th1-type and cytotoxic responses elicited

by LACK in PBMC from human subjects infected with *L. infantum*, and the parasite protective effect of prime/boost vaccination in hamsters with DNA-LACK/MVA-LACK and DNA-LACK/M65-LACK, revealed the significance of LACK in activating human and hamster immune responses and support LACK to be a valuable candidate for inclusion in a vaccine against human VL.

Keywords: vaccine, visceral leishmaniasis, *Leishmania*-activated C-kinase antigen, antigenicity, cytokines, hamster

INTRODUCTION

Leishmaniasis is one of the most neglected tropical diseases and has strong links with poverty (1). Visceral leishmaniasis (VL) is the most severe form; its annual incidence is 200,000–400,000 cases worldwide, and without treatment mortality is high (2). In South Asia and East Africa, VL is caused by *Leishmania donovani*, while in the Mediterranean, the Middle East and Latin America, the causal agent is *Leishmania infantum (L. infantum)*.

Visceral leishmaniasis treatment is based on pentavalent antimonials, oral miltefosine, liposomal amphotericin B, and paramomycin, but these have been associated with severe toxic side effects and increasing parasite resistance (3). The treatment of leishmaniasis is expensive ranging from US$ 30 to 1,500 for medication alone, which may further increase the poverty of affected individuals (1). While vaccination is the most cost-effective way of controlling infectious diseases (4), no vaccine against human VL exists. However, there are reasons for optimism that one or more safe and effective vaccines against leishmaniasis might be developed (3, 5, 6). A number of parasite antigens have been identified as candidates for vaccine development (5), including *Leishmania* analog receptor for activated C kinase (LACK) (7). *Leishmania*-activated C-kinase antigen (LACK) is a 36 kDa protein highly conserved across *Leishmania* species, and is expressed by both promastigotes and amastigotes (8). LACK antigen from *Leishmania braziliensis*, *Leishmania guyanensis*, and *Leishmania amazonensis* induces the production of IFN-γ and IL-10 in peripheral blood mononuclear cells (PBMC) from patients with cutaneous leishmaniasis (CL), and IL-10 in those of naïve individuals (7, 9–12). LACK drives the expansion of IL-4 secreting T cells (13), and for that reason LACK vaccination trials used approaches, like cytokines or DNA vectors, to redirect early IL-4 responses to a protective Th1 response (14, 15). In this sense, vaccinia virus is a strong adjuvant and delivery vector altogether, modifying IL-4 secretion of Vβ4 Vα8 CD4+ T cells (16).

Immunization with LACK-expressing plasmid DNA, with altered LACK peptides, or with the purified protein in the presence of IL-12, results in protection against CL in a mouse model, advancing the candidacy of LACK in the production of a vaccine against leishmaniasis (9). Heterologous immunization, i.e., priming with a DNA vector expressing LACK followed by a boost with non-replicating modified vaccinia virus Ankara (MVA) expressing LACK has proven to be an effective protocol in affording protection against *Leishmania major* infection in a murine model of CL, and against *L. infantum* infection in a canine model of VL (17, 18). Further, boosting with LACK-expressing replicative M65 vaccinia virus has been reported to protect against CL in a murine model (17). The protection conferred by heterologous prime/boost immunization involving a DNA vector expressing LACK, plus LACK-expressing M65 virus, however, has not been tested in any experimental model of VL. LACK-expressing M65 and MVA have both been reported to activate polyfunctional CD4+ and CD8+ T cells with effector memory phenotypes. However, prime/boost immunization involving a LACK-expressing pCI-neo plasmid plus LACK-expressing M65 preferentially induced CD4+ T cell responses, while the same plasmid plus LACK-expressing MVA preferentially induced a CD8+ T cell response (17). The replication competence of these viruses has also been described to play an important role in disease prevention (17). The preferential CD4+ T cell response of LACK-expressing M65 plus its replication competence suggest this virus, in combination with a priming LACK-expressing plasmid, might induce potent protection against VL.

With the eventual goal of producing an effective vaccine against human VL caused by *L. infantum*, the aim of this work was to examine the cellular immune response to the LACK protein induced in humans who have been in contact with *L. infantum,* and to assess the parasite efficacy of prime/boost immunization involving a LACK-expressing plasmid plus LACK-expressing MVA or M65 viruses in a hamster model of VL.

MATERIALS AND METHODS

Ethics Statement

Work involving human subjects was approved by the *Hospital de Fuenlabrada* Ethics and Research Committee (APR 12-65 and APR 14-64). All participants gave their written, informed consent to be included. Work involving animals was approved by the Research Ethics and Animal Welfare Committee of the *Instituto de Salud Carlos III*, and performed adhering to Spanish legislation on the protection of animals used for experimentation and other scientific purposes (Royal Decree 1201/2005 and Law 32/2007; this law is a transposition of Directive 86/609/EEC). One efficacy trial was performed in this study, and duplication of the animal experiments was not approved for the Research Ethics and Animal Welfare Committee.

Study Subjects

Peripheral blood samples were collected between 2013 and 2015 from 13 patients cured of VL (CVL) and 10 cured of CL (CCL) (with cure confirmed at 6 months after the end of treatment). The patients with VL had been treated with liposomal amphotericin B,

and those with CL with meglumine antimoniate. All were attended to at the *Hospital de Fuenlabrada*, Madrid. Blood samples were also collected from 90 healthy blood donors at the hospital blood bank. Among these, 18 showed a *Leishmania*-specific cell proliferative response when PBMC were stimulated *in vitro* with soluble *Leishmania* antigen (SLA) (SI > 2.1); these were defined as asymptomatic (ASYMP) (19) and selected for inclusion. Another 14 donors who showed no *Leishmania*-specific cell response were randomly selected for inclusion as endemic-area healthy controls (EC). Other classical assays, such as the leishmanin skin test (LST; no GMP commercial production approved in EU) and the SLA-based ELISA (very low sensitivity for *L. infantum* asymptomatic subjects), were not considered in this study for identification of asymptomatic infections. All patients plus the ASYMP and EC subjects lived in Fuenlabrada, a *L. infantum* post-outbreak area in Madrid (Spain).

Animals and Parasite Strain

Thirty two, 12-week-old, male golden hamsters (*Mesocricetus auratus*) were purchased from Janvier (France). All were housed in the animal facilities of the National Centre for Microbiology and randomly assigned to one of four experimental groups (see below).

Leishmania infantum promastigotes (MCAN/ES/98/LLM-724, JPC strain) were grown for 2 weeks in Novy-MacNeal-Nicolle (NNN) medium and RPMI medium (Gibco, UK) supplemented with 100 UI/ml of penicillin, 100 mg/ml of streptomycin, 2 mM L-glutamine, 2-mercaptoethanol, and 10% heat inactivated fetal calf serum (FCS) (Lonza, Spain).

Cells, Plasmids, and Viruses

The mammalian expression plasmid vector pCI-neo-LACK has been previously described (8). The empty pCI-neo plasmid (Promega, USA) was used as a control (plasmid-φ).

The viruses used in this study included the MVA strain used as a control (MVA-φ) and recombinant MVA and M65 vaccinia virus strains expressing the *L. infantum* LACK antigen at the viral HA locus (MVA-LACK and M65-LACK, respectively). MVA-LACK and M65-LACK were obtained by transfection with the pHLZ-LACK plasmid, produced by cloning the *L. infantum* LACK gene into the SmaI site of the pHLZ VACV insertion plasmid under the control of the synthetic early/late pE/L viral promoter and the hemagglutinin (HA) flanking sites. Transfection was achieved by infecting pHLZ-LACK plasmid-containing BSC-40 cells (for M65-LACK) or chick cells (for MVA-LACK) with MVA or M65 viruses and harvesting 48–72 h post-infection. Since pHLZ-LACK contains the *Escherichia coli* (*E. coli*) β-glucuronidase gene under the control of the p7.5 early/late viral promoter, β-glucuronidase-producing plaques were identified after the addition of X-Gluc to the agar. Recombinant viruses were plaque purified several times as previously described (20) (checked by PCR), grown in primary chicken embryo fibroblasts (CEF) (from pathogen-free 11-day-old eggs [Intervet, Spain]) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS in a humidified 5% CO_2 atmosphere at 37°C and purified by sedimentation after two sucrose-cushions. Viruses were titrated by immunostaining in CEF cells.

For inoculation in animals, pCI-neo-LACK plasmids and LACK-expressing viruses were diluted in endotoxin-free PBS.

Soluble *Leishmania* and LACK Antigens

Soluble *Leishmania* antigen was prepared from *L. infantum* promastigotes in the stationary phase of growth (MCAN/ES/98/LLM-724, JPC strain). Parasite cultures were centrifuged at 1,000 *g* for 20 min at 4°C. The pellet was then resuspended in lysis buffer (50 mM Tris/5 mM EDTA/HCl, pH 7), subjected to three cycles of freezing–thawing, sonicated, and further centrifuged at 27,000 *g* for 4 h at 4°C. The resulting supernatant was divided into aliquots and stored at −20°C. The protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, USA) following the manufacturer's recommendations.

Recombinant *L. infantum* LACK was expressed in *E. coli* BL21 pLysS (transformed with plasmid pRSET-B-LACK) as a fusion protein with an N-terminal histidine tag, and purified by affinity chromatography in a Ni^{2+} column. All bacteria were grown in the presence of ampicillin and LACK expression induced with isopropyl thio-β-D-galactoside (final concentration 0.5 mM). Cultures were centrifuged at 3,600 *g* for 15 min, resuspended in lysis buffer (8 M urea, 50 mM Tris-HCL pH 8, 500 mM NaCl), and incubated for 1 h with end-over-end mixing in a tube rotator at 4°C. After a further 30 min centrifugation at 14,000 *g*, the supernatant was incubated with 2 ml of equilibrated ProBond Nickel-Chelating Resin (Invitrogen, USA). The beads were then washed three times with wash buffer (8 M urea, 50 mM Tris-HCL pH 8, 500 mM NaCl, 10 mM imidazol) and elution performed using an increasing imidazol series (final concentration 200 mM). LACK protein for *in vitro* studies was kept in saline in PD10 Desalting Columns (GE Healthcare, USA). LPS levels were determined by the Limulus Amebocyte Lysate kit (Sigma-Aldrich, Heidelberg, Germany), and levels were equal to 0.2 endotoxin units per mg protein.

Quantification of Cytokines and Granzyme B in Supernatants From PBMC Stimulated With SLA and LACK

Peripheral blood mononuclear cells from study subjects were isolated from heparinized blood in a Ficoll–Hypaque gradient (Rafer, UK) and resuspended in RPMI 1640 supplemented with 10% FCS and 100 U/ml penicillin/streptomycin (Lonza, Sweden) as previously described (21). 2×10^5 cells/well were then distributed into 96-well plates and cultured for 5 days with supplemented RPMI 1640 medium either alone or with 10 μg/ml SLA or 25 μg/ml LACK at 37°C in a 5% CO_2 atmosphere. The supernatants were then collected and stored at −20°C until analyzed. IFN-γ, TNF-α, granzyme B, and IL-10 were quantified by flow cytometry using the BD Cytometric Bead Array Human Flex Kit (Beckton & Dickinson Bioscience, USA) as previously described (21). Data were analyzed using FCAP Array software v.3.0 (Beckton & Dickinson Bioscience, USA).

Immunization and Experimental Infection of Hamsters

Hamsters were randomly distributed into four experimental groups of eight animals each: (1) C-PBS—non-vaccinated controls;

(2) C-DNA—prime/boost vaccinated with plasmid-φ and MVA-φ; (3) MVA-LACK—prime/boost vaccinated with pCI-neo-LACK and MVA-LACK; (4) M65-LACK—prime/boost vaccinated with pCI-neo-LACK and M65-LACK.

The above priming injections were performed intramuscularly using 100 μg of plasmid-φ or pCI-neo-LACK as required in 100 μl final volumes of PBS. Four weeks later, the animals were boosted intramuscularly (2×10^7 PFU/hamster) with MVA-φ, MVA-LACK, or M65-LACK as required. All immunization protocols were repeated 4 weeks after the initial boost. The C-PBS control group was injected with 100 μl of PBS at each immunization point. Four weeks after the last reinforcement, all hamsters were inoculated with 2×10^7 promastigotes *via* the intracardiac route (22), and 4 months later anesthetized with isoflurane and sacrificed by cardiac puncture.

PBMC Isolation and Proliferation Assay in Hamsters

Blood was drawn from the hamsters at the time of sacrifice, and PBMC isolated using a Ficoll–Hypaque density gradient. 1×10^5 cells/well were plated on RPMI medium either alone or with 10 μg/ml SLA or 25 μg/ml LACK for 5 days. 5-bromo-2 deoxyuridine (BrdU) (25 μl, 10 μM) was added to each well for the last 18 h to examine lymphocyte proliferation using the BrdU Cell Proliferation Assay Kit (GE Healthcare Life Sciences, UK) according to the manufacturer's instructions. Results were expressed as stimulation indices, which represent the ratio between the mean absorbance of stimulated cells and that of unstimulated cells (22).

Immunoenzyme Assay

An aliquot of blood (with heparin) was also taken at the time of sacrifice to provide plasma samples *via* centrifugation at 2,000 *g* for 10 min. The plasma collected was then stored at −20°C until use. Maxisorp microtiter plates (Nunc, Denmark) were antigen-coated overnight with SLA (10 μg/ml) or LACK (25 μg/ml) in carbonate buffer (1 mM Na_2CO_3, 28 mM $NaHCO_3$, pH9.6) and blocked for 1 h at 37°C with 200 mL of 1% BSA and 0.1% Tween 20 in PBS. PBS containing 0.01% Tween 20 was used to wash the plates three times, which were then incubated for 30 min with 100 μl of plasma diluted 1:100 in buffer (0.1% BSA and 0.1% Tween 20 in PBS). Plates were washed and incubated for 30 min with 1:2,000 horseradish peroxidase-conjugated goat anti-hamster IgG (Abd Serotec, UK) for LACK ELISA, and 1:5,000 for SLA ELISA. o-Phenylenediamine dihydrochloride tablets (Sigma, Spain) were used as the enzyme's substrate; the reaction was stopped with 50 μl of 1 M H_2SO_4. The absorbance was measured at 492 nm in a Multiskan FC microplate photometer (Thermo Scientific, USA).

Histopathology

All 32 hamsters underwent a complete necropsy and samples of liver, spleen, kidney, and bone marrow were collected from each. Tissues were fixed in 10% buffered formalin, trimmed, processed, and embedded in paraffin wax following routine laboratory procedures, sectioned at 4 μm, and stained with hematoxylin-eosin for histopathological examination under the light microscope. Two samples of liver from the middle and lateral were processed. Two step sections 20 μm apart were obtained for examination from each liver sample. Similar step sections 20 μm apart from spleen, bone marrow, and kidneys were examined for each animal by a trained pathologist blinded for the experiment. Inflammatory and degenerative lesions in each tissue were qualitatively described and scored semi-quantitatively according to their severity as either non-existent (0), mild (+), moderate (++), or severe (+++). Mild was established when inflammatory infiltrates occupied less than 20% of the sections, moderate when inflammatory infiltrates occupied between 20 and 40% of the sections, and severe when inflammatory infiltrates occupied over 40% of the examined parenchyma. Additional specific features such as giant multinucleated cells and Schauman bodies were descriptively noted and recorded for each case.

DNA Isolation and Quantitative Real Time PCR

During necropsy, liver and spleen samples were homogenized in RPMI medium using a 40 μm stainless steel tissue grinder, and 1×10^6 cells used for total DNA isolation *via* traditional phenol/chloroform extraction and ethanol precipitation. Total DNA was resuspended in 100 μl of distilled water and quantified using a ND-1000 UV-V spectrophotometer (NanoDrop Technology, USA). *Leishmania* DNA was quantified by quantitative real-time PCR (qPCR) using a LightCycler high speed thermocycler and the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Spain), as previously described (22).

Statistical Analysis

Data were analyzed using the Mann–Whitney *U*-test. Significance was set at $p \leq 0.05$. The cut-off values for IFN-γ production after incubation with SLA or LACK were determined by calculating the area under the receiver operating characteristic (ROC) curve (AUC) along with the 95% confidence interval (CI). In the figures of the efficacy trial, each dot represents a single hamster, and the black line shows the group geometric mean. All calculations were performed using GraphPad Prism 7.0 software (GraphPad Software, USA).

RESULTS

LACK Induced a Significant Increase in TNF-α, IFN-γ, and Granzyme B Production in PBMC From *L. infantum* Cured and Asymptomatic Human Subjects

Leishmania-activated C-kinase antigen induced a significant increase in the secretion of IFN-γ and granzyme B by the PBMC from the CVL ($p \leq 0.001$), CCL ($p \leq 0.01$), and ASYMP subjects ($p \leq 0.0001$) over that produced by the EC subjects (**Figures 1A,C**). TNF-α production induced by LACK was significantly increased in the CCL ($p \leq 0.001$) and ASYMP subjects ($p < 0.01$) (**Figure 1B**). SLA stimulation led to significant increases in the production of IFN-γ, granzyme B, and TNF-α

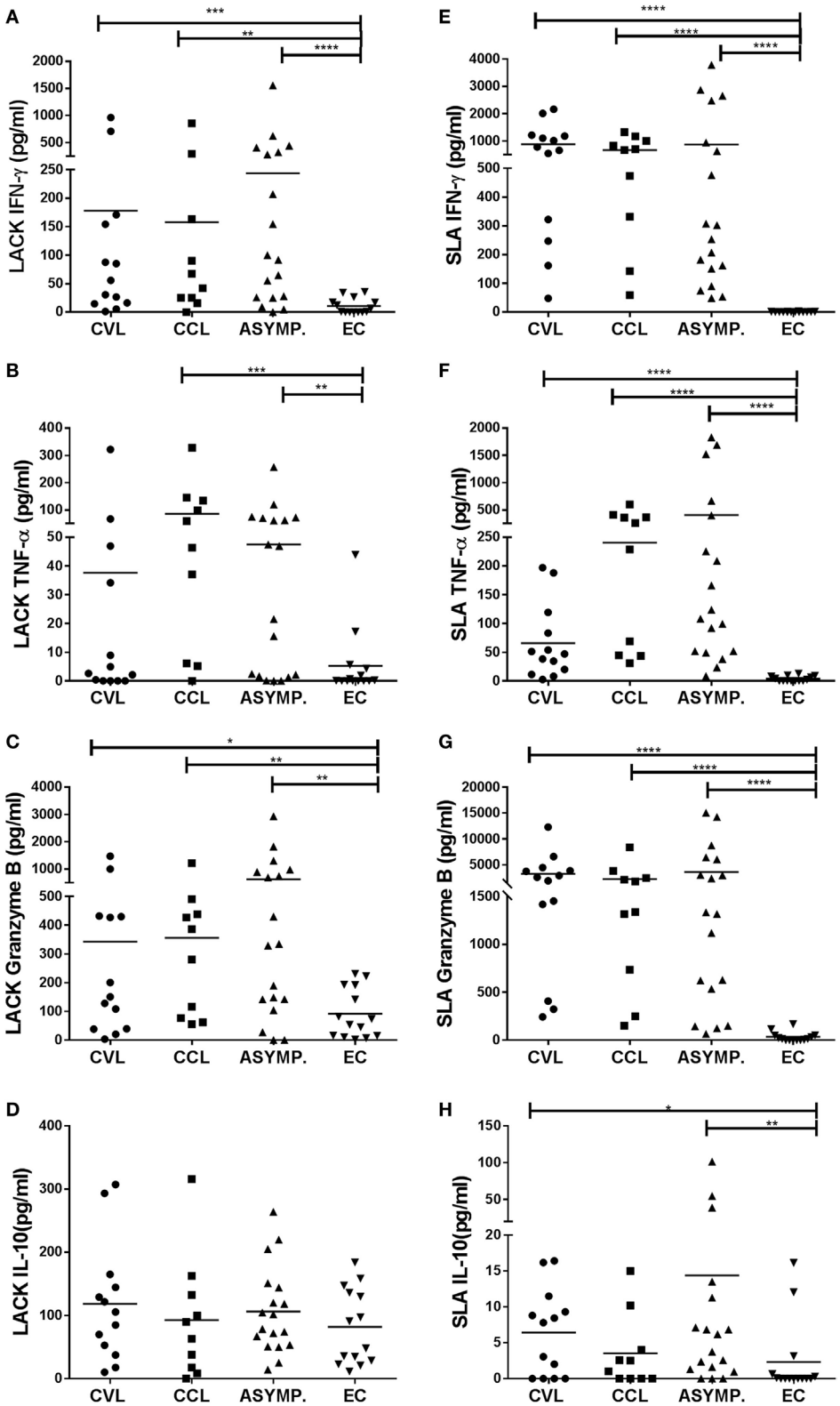

FIGURE 1 | Production of IFN-γ, TNF-α, granzyme B, and IL-10 in peripheral blood mononuclear cells stimulated with *Leishmania*-activated C-kinase antigen [**(A–D)** respectively], and with soluble *Leishmania* antigen [**(E–H)** respectively], in healthy controls (EC, $n = 14$), asymptomatic subjects (ASYMP, $n = 18$), patients cured of cutaneous leishmaniasis (CCL, $n = 10$), and patients cured of visceral leishmaniasis (CVL, $n = 13$). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

in the CVL, CCL, and ASYMP subjects ($p \leq 0.0001$ in all cases) (**Figures 1E–G**). IL-10 production was detected in all groups, including the controls, after LACK stimulation (**Figure 1D**). After SLA stimulation, PBMC from the CVL and asymptomatic subjects showed significantly greater IL-10 production compared to the EC subjects' cells (although levels were low) (**Figure 1H**).

LACK Was Recognized by a High Percentage of CVL, CCL, and Asymptomatic Subjects

Following SLA stimulation, the PBMC of all CVL, CCL, and ASYMP subjects showed increased production of IFN-γ compared to the EC subjects' cells. This cytokine was, therefore, chosen to determine the percentage of individuals that recognized LACK. IFN-γ was produced—and, therefore, LACK recognized—by 69% (9/13) of the CVL subjects, 80% of the CCL subjects (8/10), and 83% of the ASYMP subjects (15/18) (**Table 1**).

Hamsters Vaccinated With pCI-neo-LACK/MVA-LACK Had the Highest Anti-LACK IgG Titers

The MVA-LACK and M65-LACK groups produced greater amounts of anti-IgG antibodies against LACK than did the C-DNA control group ($p \leq 0.01$ and $p \leq 0.05$, respectively) (**Figure 2A**). The C-PBS control group also produced greater amounts ($p \leq 0.05$) of anti-LACK IgG antibodies than the C-DNA control group.

The MVA-LACK animals produced smaller amounts of anti-IgG antibodies against SLA than did the C-DNA control animals ($p \leq 0.05$) (**Figure 2B**).

TABLE 1 | Percentage of individuals with immune cellular memory against *L. infantum* who recognized *Leishmania*-activated C-kinase antigen and soluble *Leishmania* antigen.

Antigen	Cut-off	Se. (%)	Sp. (%)	AUC	% Recog. CVL	% Recog. CCL	% Recog. Asymp
LACK	21.17	78.05	78.5	0.85	69	80	83
SLA	25.44	100	100	1.00	100	100	100

Se, sensitivity; Sp., specificity; AUC, area under the curve; CVL, cured of visceral leishmaniasis; CCL, cured of cutaneous leishmaniasis; Asymp, asymptomatic individuals.

After Stimulation With SLA, Both Vaccinated Groups of Hamsters Showed Increased PBMC Proliferation

Following stimulation with LACK, no differences were seen between any of the groups in terms of PBMC proliferation (**Figure 3A**). Lymphoproliferation after SLA stimulation, however, was significantly greater in the MVA-LACK group compared to the C-PBS and C-DNA controls ($p \leq 0.05$). Proliferation was also greater in the M65-LACK group compared to the C-PBS group ($p \leq 0.05$) (**Figure 3B**).

Vaccinated Animals Showed Less Tissue Damage and Inflammation Than Control Group Animals

Inflammatory infiltrates were observed in the four groups and in all the examined organs with the exception of the kidneys. In the liver, inflammatory infiltrates were randomly distributed and varied in extension. The infiltrates were located surrounding portal triads and central veins. Inflammation was granulomatous in all cases, was frequently organized in granulomas, and consisted of macrophages, lymphocytes, plasma cells and neutrophils (**Figure 4**). Giant multinucleated cells and giant Langhans cells, many containing mineral concretions (Schauman bodies), were variably present regardless of the experimental group. Giant multinucleated cells, Langhans cells, and Schauman bodies were predominantly, though not exclusively, found in discrete granulomas (**Figure 4**). Amastigotes were very rarely observed within macrophages.

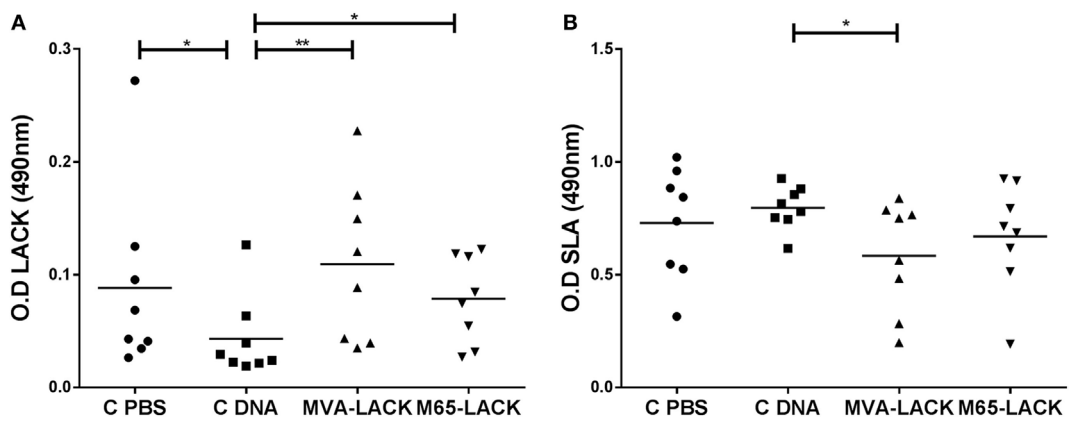

FIGURE 2 | IgG levels against *Leishmania*-activated C-kinase antigen (LACK) **(A)** and soluble *Leishmania* antigen (SLA) **(B)** in the plasma of prime-boost immunized, C-DNA, and C-PBS group hamsters later infected with *Leshmania. infantum*. *$p \leq 0.05$, **$p \leq 0.01$.

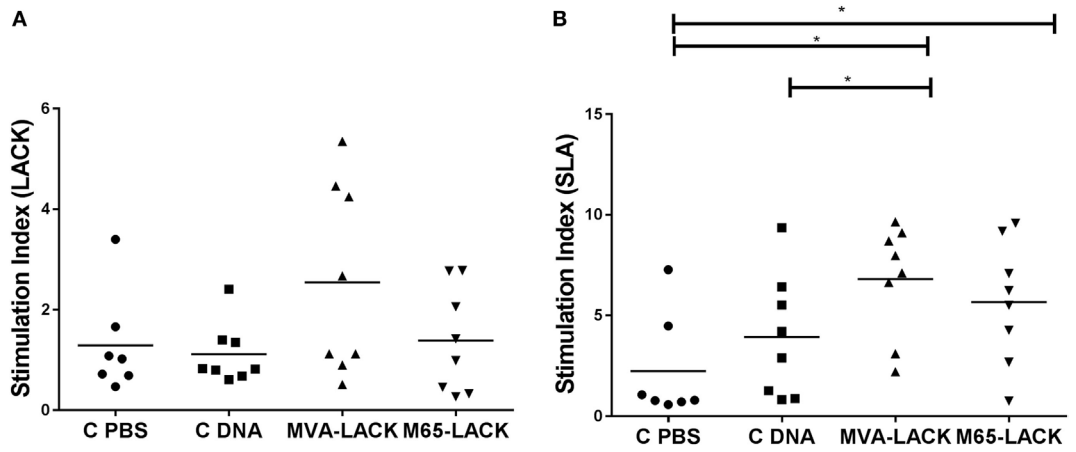

FIGURE 3 | Post-infection peripheral blood mononuclear cells stimulation indices for C-PBS, C-DNA, and prime-boost immunized group hamsters in response to *Leishmania*-activated C-kinase antigen **(A)** and soluble *Leishmania* antigen **(B)**. *$p \leq 0.05$.

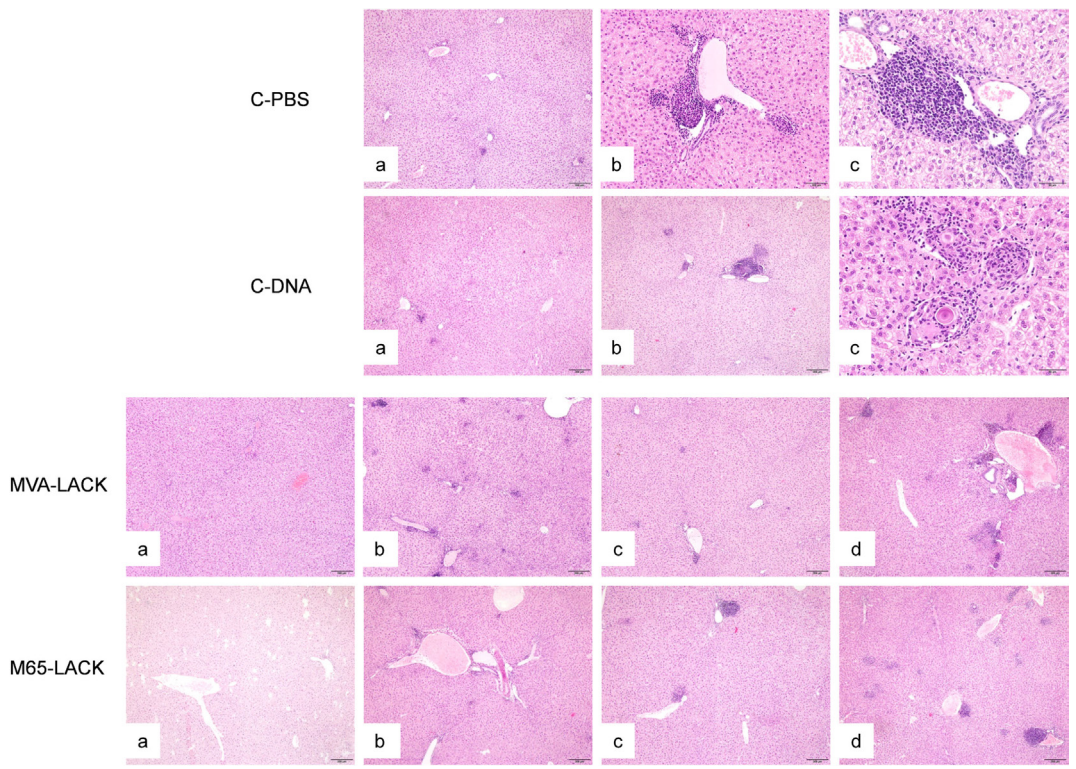

FIGURE 4 | Liver histopathology of hamsters in the C-PBS, C-DNA, MVA-LACK, and M65-LACK groups. C-PBS group— mild, multifocal granulomatous hepatitis (a), moderate (b), and severe, multifocal, periportal, granulomatous hepatitis with intralesional Shauman bodies (c). C-DNA group—minimal hepatic inflammation (a), moderate multifocal, periportal granulomatous hepatitis (b), and severe, multifocal to coalescing granulomatous hepatitis with intralesional Shauman bodies (c). MVA-LACK group—minimal hepatic inflammation (a), mild, multifocal granulomatous hepatitis (b), moderate, multifocal, granulomatous hepatitis (c), and severe, multifocal to coalescing granulomatous hepatitis (d). M65-LACK group—minimal hepatic inflammation (a), mild, multifocal granulomatous hepatitis (b), moderate, multifocal granulomatous hepatitis (c), and severe, multifocal to coalescing, periportal, granulomatous hepatitis (d).

The spleen and bone marrow also contained variable infiltrates of granulomatous inflammation. No giant cells or Schauman bodies were observed in either organ in any of the groups.

The severity of the inflammation varied among the infected animals (**Table 2**). Animals in the PBS group had more severe granulomatous hepatitis than animals in the MVA-LACK group

(**Figure 4**). The MVA-LACK group showed the least liver damage, followed by the M65-LACK group. A tendency to show inflammation in two (liver and spleen) or more organs was observed in both control groups.

Vaccination With pCI-neo-LACK/MVA-LACK and pCI-neo-LACK/M65-LACK Protected Hamsters Against *L. infantum*

The animals of the MVA-LACK group showed a significantly smaller liver amount of *Leishmania* DNA than the C-PBS and C-DNA control groups ($p \leq 0.01$), as did the M65-LACK animals ($p \leq 0.05$) (**Figure 5A**). The MVA-LACK and M65-LACK treatments also led to significant reductions in the bone marrow amount of *Leishmania* DNA compared to the C-PBS (both $p \leq 0.01$) and C-DNA control groups ($p \leq 0.001$ and $p \leq 0.01$, respectively) (**Figure 5B**). qPCR on spleen samples showed similar amount of *Leishmania* DNA among groups (data not shown).

TABLE 2 | Histopathological findings.

Observations	Tissue	Groups			
		C PBS	C DNA	MVA-LACK	M65-LACK
Injuries severity	Liver	0 (0/8) + (2/8) ++ (2/8) +++ (4/8)	0 (2/8) + (0/12) ++ (2/8) +++(4/8)	0 (2/8) + (1/8) ++ (3/8) +++ (2/8)	0 (1/8) + (1/8) ++ (4/8) +++ (2/8)
Multifocal to coalescing granulomatous infiltrates	Spleen	0 (6/8) + (2/8) ++ (0/8) +++ (0/8)	0 (4/8) + (0/8) ++ (3/8) +++ (1/8)	0 (6/8) + (1/8) ++ (1/8) +++ (0/8)	0 (5/8) + (1/8) ++ (2/8) +++ (0/8)
General inflammation	Spleen	0 (6/8) 1 (2/8)	0 (4/8) 1 (4/8)	0 (6/8) 1 (2/8)	0 (5/8) 1 (3/8)

Severity of histopathological findings: non-existent (0), mild (+), moderate (++), severe (+++), and general inflammation (1).
(n/n), number of animals with the given severity of the histopathological feature within each group.

DISCUSSION

The present results show that LACK recombinant protein is recognized by a high percentage of individuals with cellular immunity against the parasite, leading to the production of the Th1 cytokines (IFN-γ and TNF-α) and granzyme B. LACK protein is an immunodominant antigen of the parasite (12), that has previously been shown to induce the production of IFN-γ in lymphocytes from patients with active CL from *L. amazonensis*, *L. guyanensis*, and *L. braziliensis* (7, 12), as well as IFN-γ and TNF-α in asymptomatic individuals and patients cured of VL (23, 24). This is the first study in which PBMC from subjects cured of CL caused by *L. infantum* have been exposed to LACK. As in other groups of infected individuals, exposure to SLA and LACK elicited a Th1 response (production of IFN-γ and TNF-α) plus the production granzyme B. Although levels of LPS were residual (equal to 0.2 endotoxin units per mg protein), it is known that LPS-triggered cytokines produced by antigen-presenting myeloid cells contribute to the induction of T cell responses (25). For that reason, we cannot discard its potential adjuvant-like phenomenon in the present results (26).

Several studies have examined the capacity of *Leishmania* vaccine candidates to induce cytotoxic responses or to present epitopes restricted to HLA class I (9, 27–32). These showed that LACK-stimulation of PBMC induces the secretion of IFN-γ by CD8+ T cells in exposed individuals and patients with active CL (9, 31, 32). To our knowledge, this study is the first in which the LACK-induced production of granzyme B has been quantified in cured patients of CL and VL, and asymptomatic individuals from an *L. infantum*-endemic area. The cellular response seen in these subjects confirms that LACK induces specific cytotoxic responses in subjects with a cellular memory response against *L. infantum*.

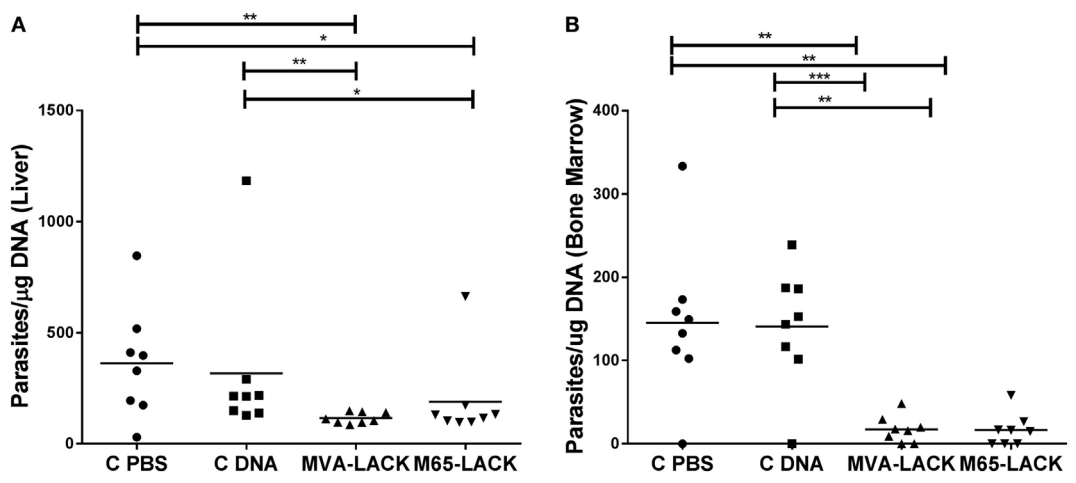

FIGURE 5 | Liver and bone marrow amount of *Leishmania* DNA in the liver **(A)** and bone marrow **(B)** as determined by qPCR. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

Following LACK-stimulation, the PBMC of all individuals—including those of the control groups—produced IL-10, as previously reported (7, 12, 23, 24). This production has been linked to a dominant Th2-type epitope lying between amino acids 157 and 173 (FSPSLEHPIVVSGSWDN) (12) of the antigen.

The significant recognition of LACK by the T receptors of the present cured and asymptomatic subjects, plus the ability of this protein to induce IFN-γ, TNF-α, and granzyme B (CD4+ and CD8+ T cell responses), indicates this antigen to be of potential use in vaccines against human leishmaniasis. However, the intrinsic ability of LACK to also induce IL-10 production and to expand IL-4-secreting T cells (13) illustrates the need to further optimize a LACK-based vaccine for the induction of a protective Th1 response. DNA vaccines involving the LACK antigen have returned promising results in murine models of *L. major* and *L. amazonensis* infection, as well as in a canine model of *L. infantum* infection (8, 17, 18, 33). In these earlier studies, the vaccines were applied under a heterologous immunization protocol, stimulating humoral, Th1-type, and cytotoxic immune responses.

The relevant effect observed in human PBMC from asymptomatic and cured patients of increase secretion of IFN-gamma and granzyme B in response to LACK, provided the rational to explore the significance of these findings in the hamster model of VL. Both the DNA-LACK/MVA-LACK and DNA-LACK/M65-LACK vaccines induced the production of anti-LACK antibodies beyond that seen in the C-DNA group. Immunization with both vaccines also induced a potent cellular response, as shown by the higher mean stimulation index to SLA observed in the immunized groups after experimental infection with *L. infantum* promastigotes. Vaccinia viruses are capable of inducing CD4+ and CD8+ T cell responses. In a mouse CL model, heterologous immunization with DNA-LACK/MVA-LACK has been reported to preferentially promote a CD8+ T cell-type response, whereas that generated by DNA-LACK/M65-LACK immunization is preferably of the CD4+ T cell type (17). In the present efficacy trial in hamsters, a similar immune response was observed in both the MVA-LACK and M65-LACK groups. As seen for the present response against VL in hamsters, Ramos et al. reported that dogs vaccinated with DNA-LACK/MVA-LACK showed greater lymphoproliferation after SLA-stimulation than did control animals. This specific immune response was related to the protection conferred by the vaccine in the canine model of *L. infantum* infection (18). In our study, the patent cellular response induced by both DNA-LACK/MVA-LACK and DNA-LACK/M65-LACK was associated with the control of the amount of *Leishmania* DNA observed in the liver and bone marrow. The extension and severity of the inflammatory infiltrates in the liver, observed histologically were not significant between groups, however. This could in part be explained because MVA-LACK and M65-LACK induce a more effective cellular response, as suggested by the higher lymphoproliferation values and lower parasite burdens, rather than a reduction in the quantity of the cellular response to the infection.

However, other authors report that immunization with plasmid DNA expressing the LACK antigen fails to induce any protection in mice infected with *L. infantum* or *L. donovani* (34, 35). The discrepancy with the present results is likely due to the fact that these earlier studies involved only homologous immunization with a LACK-expressing plasmid, while our studies were performed with heterologous combination of vectors, a protocol known to activate strong B and T cell immune responses to LACK antigen (31).

In conclusion, LACK is well recognized by the T cells from individuals cured of leishmaniasis, and by those of asymptomatic subjects living in a *L. infantum*-endemic area. Prime-boost vaccination with pCI-neo-LACK/MVA-LACK, and pCI-neo-LACK/M65-LACK, protected against *L. infantum* infection in a hamster model of VL. These results highlight the significance of LACK as immune activator and suggest that LACK should be considered in the formulation of a vaccine designed to induce a cellular response against VL in humans.

ETHICS STATEMENT

Work involving human subjects was approved by the Hospital de Fuenlabrada Ethics and Research Committee (APR 12-65 and APR 14-64). All participants gave their written, informed consent to be included. Work involving animals was approved by the Research Ethics and Animal Welfare Committee of the Instituto de Salud Carlos III, and performed adhering to Spanish legislation on the protection of animals used for experimentation and other scientific purposes (Royal Decree 1201/2005 and Law 32/2007; this law is a transposition of Directive 86/609/EEC).

AUTHOR CONTRIBUTIONS

LF, LS-S, CS, AI-M, MJ, and VA conducted the experiments. LF, CS, and AI-M acquired the data. LF, EC, MJ, and VA analyzed the data. EC, LS-S, ME, and JM designed the research studies. EC, LS-S, ME, and JM provided reagents. LF, EC, and JM wrote the manuscript. All authors have read and approved the final manuscript.

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