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Transgenic T cell-specific expression of CXCR3 enhances splenic and hepatic T cell accumulation but does not affect the outcome of visceral leishmaniasis

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Abstract

The outcome of visceral leishmaniasis, caused by parasite *Leishmania donovani*, depends on the recruitment of leishmanicidal Th1 cells. Chemokine receptor CXCR3, preferentially expressed by Th1 cells, is critical for migration of these T cells during infection. During chronic VL, there is a decrease in the presence of CXCR3-expressing CD4⁺ T cells in the spleen, which is associated with high parasitic burden in this organ. We therefore examined whether T cell-specific expression of CXCR3 in mice (CXCR3^{Tg}) would promote resistance to VL. *L. donovani* infected CXCR3^{Tg} mice showed increased accumulation of T cells in the spleens compared to WT littermates (CXCR3^{+/+}). However, CXCR3⁺ T cells from CXCR3^{Tg} mice showed low CD69 expression and these mice developed fewer granulomas. Additionally, both groups of mice showed similar cytokine profiles and parasitic burdens during the course of infection. In summary, although T cell-specific expression of CXCR3 promoted the accumulation of CXCR3-expressing T cells during *L. donovani* infection, this did not enhance resistance to VL.

Keywords

Leishmania donovani; Visceral leishmaniasis; CXCR3; Chemokine receptor; Transgenic; T cells; Granuloma; Cytokines

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Conflict of interest

The authors do not have a commercial or other association that might pose a conflict of interest

1. Introduction

Leishmania are intracellular, vector-borne parasites which cause the disease leishmaniasis. After transmission of parasites through the bite of infected sandflies, patients can remain asymptomatic or they may present with diverse clinical manifestations depending on the infective species and genetic susceptibility of the host [1]. Visceral leishmaniasis (VL), commonly caused by *Leishmania donovani*, is characterized by dissemination of parasites through the blood stream into the spleen, liver and bone marrow. Parasite metastasis and replication in macrophages induces severe immunopathology leading to hepatosplenomegaly which is often fatal to the host [1–3]. Despite progress in the development of anti-leishmanial drugs, VL remains the most severe clinical form of the disease in humans in terms of mortality and morbidity resulting in about an annual global burden of 0.2–0.4 million new cases [3].

Optimal outcome during *L. donovani* infection and clearance of parasites depend on the recruitment and activation of T cells in the spleen and liver [4]. Within the liver, the formation of organized granulomas and the generation of Th1 immune responses, characterized by IFN- γ production, activate macrophages to generate reactive oxygen and reactive nitrogen intermediates, mediating parasite killing. Similarly parasite control in the spleen is achieved by the presence of IFN- γ producing T cells, although other Th1-associated cytokines such as TNF- α promote the destruction of splenic architecture which is common during chronic VL infection [5,6]. CXCR3, a chemokine receptor expressed by activated Th1 cells, is important for T cell recruitment and generation of protective immune responses in various intracellular infection models. During *Listeria* infection, CXCR3-dependent homing of CD4⁺ T cells to the intestinal mucosa is critical for activation of inflammatory monocytes and for clearance of the bacteria [7]. In the context of cutaneous leishmaniasis (CL), our laboratory has demonstrated that CXCR3 is required for the trafficking of T cells and production of IFN- γ at the site of infection [8]. On the other hand, CXCR3 deficiency did not impede the generation of protective immune responses in the draining lymph node and visceral organs in CL and VL respectively [8,9]. Induction of the CXCR3 ligand, CXCL10, in the liver during *L. donovani* infection suggests that CXCR3 may be involved in the trafficking of Th1 cells during VL [10]. In order to better understand how CXCR3 is involved in the migration and generation of Th1 immune responses in a tissue-specific manner during VL, we characterized the expression of CXCR3 on T cells in *L. donovani* infected mice and employed a T cell specific-CXCR3 transgenic (CXCR3^{Tg}) mouse strain to investigate CXCR3-dependent immune responses upon infection with *L. donovani*.

2. Materials and methods

2.1. Animals

CXCR3 transgenic mice (CXCR3^{Tg}) were generated by the procedure described previously [11]. C57BL/6 CXCR3^{Tg} were backcrossed to WT BALB/c mice for 10 generations to generate BALB/c CXCR3^{Tg} mice and BALB/c wild-type littermates (CXCR3^{+/+}). Mice were maintained and bred at the Ohio State University animal facility according to animal protocols and University Laboratory Animal Resources (ULAR) regulations. The

experiments were performed using 8–10 week old sex-matched CXCR3^{Tg} and CXCR3^{+/+} mice according to the institutional guidelines for animal research.

2.2. Parasites and infections

Leishmania donovani (LV82 strain) was maintained as described previously by serial passage of amastigotes in Golden Syrian hamsters [12]. *L. donovani* amastigotes were isolated from the spleen of sick hamsters and experimental mice were injected with 10⁷ *L. donovani* amastigotes prepared in 100 µl volume by tail vein injection. Infected CXCR3^{Tg} and CXCR3^{+/+} mice were sacrificed at 60 days post-infection to evaluate parasite burdens and at 15, 40 and 60 days post-infection to evaluate cellular immune responses.

2.3. Parasite burden calculation

At 60 days post-infection, infected mice were sacrificed to harvest spleens and livers and parasite burdens were determined as described previously [12]. Briefly, organs were weighed and sectioned to prepare impression smears on microscopic slides. The smears were stained with Giemsa to calculate the number of amastigotes per thousand nucleated cells. The parasite loads were expressed in Leishman-Donovan Units (LDU) which was calculated as LDU = the number of amastigotes per 1000 nucleated cells × organ weight (in grams).

2.4. Flow cytometry

Flow cytometry was performed on single cell suspensions prepared from spleens of CXCR3^{Tg} and CXCR3^{+/+} mice as described previously [11]. Briefly, cells were blocked with normal mouse serum and incubated with conjugated antibodies against various cell surface markers including CD3, CD4, CD8, CXCR3 and CD69 (Biolegend). Samples were acquired on a BD FACS Calibur (BD biosciences) and data analysis was performed using FlowJo software (Tree Star Inc). During analysis, gating was performed based on the isotype controls for the corresponding antibodies. Surface marker expression on CD4⁺ T cells was analyzed by gating on CD3⁺ CD4⁺ T cells. Surface marker expression on CD8⁺ T cells was analyzed by gating on CD3⁺ CD8⁺ T cells.

2.5. T cell proliferation and cytokine ELISA

Harvested splenocytes were re-suspended in RPMI medium (supplemented with 10% FBS (Atlanta Biologicals), 1% Penicillin and Streptomycin, 1% HEPES and 0.1% β-mercaptoethanol) to prepare single cell suspensions, plated in 96-well plates in concentrations of 5 × 10⁶ cells/ml and stimulated with 20 µg/ml of freeze-thawed prepared *L. donovani* antigen for 72 h at 37°C. T cell proliferation was evaluated by alamarBlue reduction method. Briefly, at 60 h post-stimulation with *L. donovani* antigen, cells were incubated with 10% alamar blue (Life Technologies) for 12 h prior to measuring the absorbance at 570 nm and 600 nm using Spectramax Microplate reader and Softmax Pro software (Molecular Devices LLC). Absorbance values were used to calculate the rate of alamarBlue reduction and thereby, T cell proliferation using online alamarBlue colorimetric calculator (AbD Serotec). At 72 h, culture supernatants were also collected to determine concentrations of cytokines such as IFN-γ, IL-4, IL-6, IL-10, and IL-13 using anti-mouse

capture and detection antibodies (Biolegend) [11]. Recombinant mouse cytokines (BD Biosciences) were used as standards. Plates were read at an absorbance of 405 nm using Spectramax M3 microplate reader (Molecular Devices LLC) and cytokine concentrations were determined by extrapolation from the standard curves generated by Softmax Pro software (Molecular Devices LLC).

2.6. Histopathology

Tissue sections of liver and spleen were prepared from both the groups, stained by H&E and processed for histopathology. Liver granulomas were enumerated at each time point and livers from at least 4–5 individual mice were analyzed in each group.

2.7. Statistical analysis

All statistical analyses were conducted using Prism 5 (GraphPad software). Student's unpaired *t* test was employed to determine statistical significance of values obtained. The *p* values less than 0.05 were considered significant.

3. Results

3.1. CXCR3 expression in CD4⁺ and CD8⁺ T cells in livers and spleens of *L. donovani* infected BALB/c mice

CD4⁺ and CD8⁺ T cells are important for the development of protective immune response against visceral leishmaniasis [4,5]. CXCR3 expression on activated CD4⁺ and CD8⁺ T cells marks for type 1 anti-leishmania adaptive immunity. Therefore we evaluated the expression of CXCR3 on CD4⁺ and CD8⁺ T cells in the livers and spleens of wild type BALB/c mice infected with *L. donovani*. Compared to uninfected mice where we observed negligible numbers of CXCR3-expressing CD4⁺ T cells, *L. donovani* infected mice had significantly higher percentages of CXCR3-expressing CD4⁺ T cells in their spleens and livers peaking at 15 days post-infection. We also observed that livers of infected mice displayed significantly higher proportions of CXCR3⁺ CD4⁺ T cells compared to infected spleens at 15, 40 and 60 days post-infection (Fig. 1A). Likewise, the percentage of CXCR3⁺ CD8⁺ T cells in spleens and livers of infected mice was higher than uninfected mice, again peaking at 15 days post-infection. The difference between infected spleen and liver proportions of CXCR3⁺ CD8⁺ however was not as drastic. Livers of infected mice were observed to have only a slightly higher proportion of CXCR3⁺ CD8⁺ T cells (Fig. 1B).

3.2. Transgenic T cell-specific expression of CXCR3 increases the proportion of T cells in the spleens and livers of *L. donovani* infected mice

In order to determine whether enhanced expression of CXCR3 on T cells will promote resolution of VL infection, we generated CXCR3 transgenic mice (CXCR3^{Tg}) which show elevated expression of CXCR3 on all T cell populations. We infected CXCR3^{Tg} and their WT littermates (CXCR3^{+/+}) with *L. donovani* parasites and studied the progression of disease in these two groups of mice. First, we confirmed that T cells in CXCR3^{Tg} mice displayed higher expression of CXCR3 compared to T cells in CXCR3^{+/+} mice. As expected, we observed that both spleens and livers of CXCR3^{Tg} mice showed significantly higher percentages of CXCR3-expressing CD4⁺ T cells at 15, 40 and 60 days post-infection

compared to CXCR3^{+/+} controls (Fig. 2A and C). Similarly, there were significantly higher percentages of CXCR3⁺ CD8⁺ T cells in the spleens and livers of CXCR3^{Tg} mice compared to CXCR3^{+/+} mice at all time points (Fig. 2B and D).

We then evaluated whether transgenic expression of CXCR3 on T cells affected the recruitment of T cells during infection. At day 15, 40 and 60 post-infection, we observed that there was a significantly higher percentage of CD4⁺ T cells in the spleens and livers of CXCR3^{Tg} mice compared to CXCR3^{+/+} mice (Fig. 3A and C). Percentages of CD8⁺ T cells in the spleens of *L. donovani* infected CXCR3^{Tg} mice were higher compared to CXCR3^{+/+} mice at day 40 post-infection (Fig. 3B) while the proportion of CD8⁺ T cells were comparable in the livers of these two groups at this time point (Fig. 3D). It should be noted that at this 40 days post infection time point, infection of the spleen is still prevalent whereas the infection of the liver is mostly cleared. At 15 as well as 60 days post-infection, the proportion of CD8⁺ T cells were higher in both livers and spleens of CXCR3^{Tg} mice compared to CXCR3^{+/+} mice (Fig. 3B and D). Taken together, our results indicate that CXCR3^{Tg} mice have higher proportions of CXCR3-expressing CD4⁺ and CD8⁺ T cell populations in the spleens of VL infected mice.

3.3. Overexpression of CXCR3 in T cells does not alter the outcome of *L. donovani* infection

Based on our observations of the increased populations of CD4⁺ and CD8⁺ T cells during VL infection, we wanted to determine whether transgenic T cell-specific CXCR3 expression would alter the outcome of VL infection. We compared parasitic burdens in the livers and spleens of CXCR3^{Tg} and CXCR3^{+/+} mice. We observed that both groups of mice displayed similar splenic and hepatic parasitic loads during 15, 40 and 60 days post-infection (Fig. 4). While there was progressive increase in parasites in the spleens of both groups (Fig. 4A), the parasitic loads peaked during early stages of the infection in the livers of both groups of mice and declined at 40 and 60 days of infection (Fig. 4B). Therefore, our data indicates that transgenic expression of CXCR3 on T cells does not affect the outcome of *L. donovani* infection.

3.4. Transgenic expression of CXCR3 on T cells does not enhance the formation of hepatic granulomas in *L. donovani* infected mice

Formation of well-structured granulomas is an important feature of resistance to *L. donovani* infection [13]. We therefore studied whether transgenic T cell-specific expression of CXCR3 altered the formation of hepatic granulomas during VL. Interestingly, CXCR3^{+/+} mice displayed larger granulomas compared to CXCR3^{Tg} at days 40 and 60 post-infection (Fig. 5A). Additionally, we observed that CXCR3^{Tg} mice showed fewer mature and parasite free granulomas compared to WT counterparts at day 40 post-infection. No differences in numbers of these granulomas were observed at day 15 and day 60 post-infection between both groups of mice (Fig. 5B).

3.5. Effect of transgenic T cell-specific expression of CXCR3 on cytokine responses upon *L. donovani* infection

We compared cytokine produced by splenocytes from *L. donovani* infected CXCR3^{Tg} and CXCR3^{+/+} mice. We observed that both groups of mice showed similar cytokine profiles for IFN- γ (Fig. 6A), IL-10 (Fig. 6B), IL-13 (Fig. 6D) and IL-6 (Fig. 6E). Although CXCR3^{+/+} mice showed significantly higher levels of IL-4 at 40 days post-infection, there were no differences in IL-4 levels at 15 and 60 days post-infection (Fig. 6C). These results further corroborate our previous observations that transgenic expression of CXCR3 does not enhance the activation of T cells during *L. donovani* infection.

4. Discussion

Our study evaluated whether enhanced T cell-specific expression of CXCR3 promotes protection against VL. Our previous studies clearly indicated a critical role for CXCR3 in the development of a protective immune response against cutaneous leishmaniasis (CL). BALB/c mice, which displayed defective expression of CXCR3 on T cells, showed progressive cutaneous lesion formation when infected with *Leishmania major* [14]. Further, *L. major* infected CXCR3^{-/-} mice mounted a robust Th1 immune response but were unable to effectively recruit T cells into their lesions, thus leading to severe infection [8]. These results indicated that CXCR3 is important for trafficking of activated T cells into infection sites for the generation of leishmanicidal immune response. Surprisingly when CXCR3^{Tg} mice were infected with *L. major*, these mice developed larger lesions characterized by a Th2 skewed immune response [11]. In addition to highlighting the complex role for CXCR3 in progression of leishmaniasis, these studies suggest that resistance is dependent on which T cell subsets undergo CXCR3-mediated activation and home into the lymph nodes and sites of infection.

During *L. donovani* infection, it is known that chemokines such as CXCL10, a ligand for CXCR3, are upregulated in the liver suggesting that CXCR3-dependent migration could be critical for the trafficking of Th1 cells to the liver during VL [10]. Additionally, we observed that the proportion of CXCR3-expressing CD4⁺ T cells in the spleen is significantly lower compared to the liver of *L. donovani* infected mice (Fig. 1A) which inversely correlates with the high splenic parasite loads at later stages of VL infection (Fig. 4A). This led us to hypothesize that elevated T cell-specific expression of CXCR3 could promote the trafficking of Th1 cells to the spleen and enhance the clearance of parasites in this organ during *L. donovani* infection. Using mice that showed enhanced expression of CXCR3 on T cell populations, we evaluated the progression of disease and immune responses to VL. Despite elevated expression of CXCR3 on T cells (Fig. 2) and higher presence of T cells in spleens of CXCR3^{Tg} mice during infection (Fig. 3), there was no increase in CD69 expression on CD4⁺ and CD8⁺ T cells from these mice (Supplemental Fig. 1A and B). We also observed similar levels of T cell derived cytokines in the spleens of CXCR3^{Tg} and CXCR3^{+/+} mice (Fig. 6). This indicated that the increase in CXCR3 expression on T cells in the spleen did not lead to a concomitant increase in the activation status of these T cell populations nor further amplification of Th1 responses. These results are interesting in light of a previous report which indicates that CXCR3-expressing T cells stimulate such a Th1 inflammatory

cascade in a murine cerebral malaria model of infection [15]. Contrary to these findings, our study suggests that transgenic expression of CXCR3 on T cells reduces the activation status of these cells as demonstrated by the reduction of CD69 expression in CXCR3⁺ T cells in CXCR3^{Tg} mice (Supplemental Fig. 1). Such low levels of CD69 expression in T cells in CXCR3^{Tg} mice and thereby a likely decrease in their activation status may explain why we observe similar amounts of cytokine production in the spleens of both groups of mice despite the higher proportions of T cells in the CXCR3^{Tg} mice. A previous report by Chakravarty et al. demonstrates that in some infection models such as *M. tuberculosis* infections, CXCR3 adversely affects T cell priming and negatively regulates the expression of CD69 by T cells [16]. Hence it is possible that CXCR3 plays a minor inhibitory role in the activation of T cells during visceral leishmaniasis.

We also observed that overexpression of CXCR3 on T cells leads to a decrease in mature and parasite-containing granulomas (Fig. 5). This is surprising considering that CXCR3 deficient mice showed delayed granuloma formation upon infection with *L. donovani* [9]. The discrepancies in observations from these two models highlight the complex role for CXCR3 in immune responses against visceral leishmaniasis. Besides T cells, CXCR3 is also expressed by other immune cells such as macrophages which are critical for granuloma formation during VL [13,17,18]. Our transgenic mouse helped us evaluate the role of CXCR3 on T cell trafficking and function in visceral leishmaniasis. Future studies with similar transgenic models that are engineered to overexpress CXCR3 in immune cells such as macrophages could be employed to probe how CXCR3 regulates the migration and activity of these immune cells during VL.

In conclusion, our study shows that overexpression of CXCR3 on T cells has a negligible impact on the outcome of visceral leishmaniasis. This study enhances our understanding of the role of the chemokine receptor CXCR3 in leishmaniasis. In addition, this transgenic mouse model is shown to possibly have the potential to improve understanding of the role of CXCR3 in other infectious diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Contribution: SV, SO and ARS conceived and designed the study; SV, SO, GN, RS, EM and JS performed experiments and analyzed results; GN, SV, JS, SO, RS and ARS wrote the manuscript. All authors reviewed and approved the final manuscript. We would like to thank Dr. Cesar Terrazas and Jennifer Kimble for their assistance with designing experiments and proofreading the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cellimm.2016.08.016>.

Abbreviations

CD	cluster of differentiation
CXCR3	chemokine (C-X-C motif) receptor 3
CXCL10	chemokine (C-X-C motif) ligand 10
IL	interleukin
IFN-γ	interferon-gamma
LDU	Leishman-Donovan Units
Th1 cell	type 1 T helper cell
Th2 cell	type 2 T helper cell
WT	wildtype
VL	visceral leishmaniasis

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Fig. 1.

The proportion of CXCR3-expressing CD4⁺ and CD8⁺ T cells in spleens and livers of *L. donovani* infected mice is inversely correlated with parasitic burdens during VL. (A) Proportion of CXCR3-expressing CD4⁺ T cells and (B) proportion of CXCR3-expressing CD8⁺ T cells in the spleens and livers of *L. donovani* infected BALB/c mice intravenously infected with 10⁷ amastigotes. At 15, 40 and 60 days post-infection, cells from spleens and livers were harvested, blocked and stained with conjugated antibody for the respective surface molecules and proportion of cells expressing these surface molecules were determined by flow cytometry. Analyses were conducted by gating on (A) CD4⁺ T cells and (B) CD8⁺ T cells. The data represent mean $\pm$ S.E.M. of individual percentage values from three to five mice per group from two independent experiments with similar results. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 using unpaired *t* test.

Fig. 2.

Overexpression of CXCR3 on T cells enhances the proportion of CXCR3-expressing T cells in the spleens and livers of *L. donovani* infected mice. Proportion of CXCR3-expressing CD4⁺ T cells and CD8⁺ T cells in the spleens (A and B) and livers (C and D) of *L. donovani* infected CXCR3^{+/+} and CXCR3^{Tg} mice intravenously infected with 10⁷ amastigotes. Infected mice were sacrificed at the 15, 40 and 60 days of infection to harvest cells from spleens and livers. Cells were blocked and stained with conjugated antibody for the respective surface molecules and percentages of cells expressing these surface molecules were determined by flow cytometry. Analyses were conducted by gating on CD4⁺ or CD8⁺ T cells. The data represent mean $\pm$ S.E.M. of individual percentage values from three to five individual mice per group from two independent experiments with similar results. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ using unpaired *t* test.

Fig. 3.

Transgenic T cell-specific expression of CXCR3 enhances the proportion of splenic T cell populations during VL. Representative contour diagrams show the proportion of CD4⁺ T cells and CD8⁺ T cells in the spleens (A and B) and livers (C and D) of *L. donovani* infected CXCR3^{+/+} and CXCR3^{Tg} mice at 15 days post-infection. Complementary graphs display the level of CD4⁺ or CD8⁺ throughout the course of infection for the respective organs. Mice were intravenously infected with 10⁷ *L. donovani* amastigotes and cells from spleens and livers were harvested at 15, 40 and 60 days of infection. Cells were blocked and stained with

conjugated antibody for the respective surface molecules and percentages of cells expressing these surface molecules were determined by flow cytometry. Analyses were conducted by gating on CD4⁺ or CD8⁺ T cells. The numbers represent mean $\pm$ S.E.M. of individual percentage values from three to five individual mice per group from two independent experiments with similar results. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ using unpaired t test.

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Fig. 4.

Overexpression of CXCR3 on T cells does not affect splenic and hepatic parasite loads during visceral leishmaniasis. Parasite burdens in (A) spleens and (B) livers of CXCR3^{Tg} and WT mice infected with *L. donovani*. CXCR3^{Tg} and WT mice were infected with 10^7 *L. donovani* amastigotes and euthanized at day 15, 40 and day 60 post-infection to determine parasitic burdens in the spleens and livers. Data are expressed as mean LDU $\pm$ S.E.M. and represent values of three to five individual mice per group at each time point from two independent experiments with similar results. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ using unpaired *t* test.

Fig. 5. CXCR3^{Tg} mice showed defective granuloma formation during infection with *L. donovani*. (A) Histopathology of livers from *L. donovani* infected CXCR3^{Tg} and CXCR3^{+/+} mice. Infected mice were harvested at days 15, 40 and 60 post-infection to analyze granuloma formation in liver sections. Microscopic images of granulomas are at original magnifications, 400x. Data represent a single experiment of two experiments performed with three to five mice with similar results. (B) Liver sections from *L. donovani* infected CXCR3^{Tg} and CXCR3^{+/+} mice were scored for granuloma formation. Data represent the number of granulomas per ten high power fields at 200X magnification and are indicated as mean + S.E.M. from a representative experiment with three to five mice per group of two independent experiments with similar results. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 using unpaired *t* test.

Fig. 6.

Transgenic T cell-specific expression of CXCR3 does not alter cytokine responses of splenocytes upon *L. donovani* infection. Cytokine production (A–E) by splenocytes from CXCR3^{Tg} and CXCR3^{+/+} mice infected intravenously with 10^7 *L. donovani* amastigotes after 15, 40 and 60 days of infection. At respective time points, spleens were harvested to prepare single cell splenocyte suspensions. Splenocytes were stimulated with 20 μ g/ml *L. donovani* antigen for 72 h and cytokines (A) IFN- γ , (B) IL-10, (C) IL-4, (D) IL-13 and (E) IL-6 production were measured by ELISA. Data are indicated as mean of sample values + S.E.M. with three to five individual mice per group from two independent experiments with similar results. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ using unpaired *t* test.