Deletion of IL-4 Receptor Alpha on Dendritic Cells Renders BALB/c Mice Hypersusceptible to *Leishmania major* Infection

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Abstract

In BALB/c mice, susceptibility to infection with the intracellular parasite *Leishmania major* is driven largely by the development of T helper 2 (Th2) responses and the production of interleukin (IL)-4 and IL-13, which share a common receptor subunit, the IL-4 receptor alpha chain (IL-4Rα). While IL-4 is the main inducer of Th2 responses, paradoxically, it has been shown that exogenously administered IL-4 can promote dendritic cell (DC) IL-12 production and enhance Th1 development if given early during infection. To further investigate the relevance of biological quantities of IL-4 acting on DCs during *in vivo* infection, DC specific IL-4Rα deficient (CD11ccreIL-4Rαlox/lox) BALB/c mice were generated by gene targeting and site-specific recombination using the cre/loxP system under control of the cd11c locus. DNA, protein, and functional characterization showed abrogated IL-4Rα expression on dendritic cells and alveolar macrophages in CD11ccreIL-4Rαlox/lox mice. Following infection with *L. major*, CD11ccreIL-4Rαlox/lox mice became hypersusceptible to disease, presenting earlier and increased footpad swelling, necrosis and parasite burdens, upregulated Th2 cytokine responses and increased type 2 antibody production as well as impaired classical activation of macrophages. Hypersusceptibility in CD11ccreIL-4Rαlox/lox mice was accompanied by a striking increase in parasite burdens in peripheral organs such as the spleen, liver, and even the brain. DCs showed increased parasite loads in CD11ccreIL-4Rαlox/lox mice and reduced iNOS production. IL-4Rα-deficient DCs produced reduced IL-12 but increased IL-10 due to impaired DC instruction, with increased mRNA expression of IL-23p19 and activin A, cytokines previously implicated in promoting Th2 responses. Together, these data demonstrate that abrogation of IL-4Rα signaling on DCs is severely detrimental to the host, leading to rapid disease progression, and increased survival of parasites in infected DCs due to reduced killing effector functions.

Introduction

*Leishmania* spp. are protozoan parasites that are transmitted by *Phlebotomus* spp. sandflies and can cause several forms of disease in humans, ranging from localized cutaneous lesions to visceral Leishmaniasis, where parasites invade internal organs such as the spleen and liver. The incidence of disease is approximately 1.5 million per annum for cutaneous Leishmaniasis, and 500 000 per annum for visceral Leishmaniasis, which is usually fatal if left untreated [1]. Currently there is no vaccine. To identify correlates of immune protection, which may aid in vaccine design and therapeutic strategies, experimental models of cutaneous Leishmaniasis have been established in which disease is induced by infecting mice subcutaneously with *L. major*. Susceptible BALB/c mice show progressive lesion development with dissemination of parasites to visceral organs, while resistant C57BL/6 mice are able to control infection and heal lesions [2–4]. Lack of healing in BALB/c mice is associated with a T helper (Th) 2 response characterized by secretion of interleukin (IL)-4, IL-5, IL-9 and IL-13 [3,5–8], high anti-*Leishmania* antibody titres [8,9] and alternative activation of macrophages [9,10]. In contrast, resistant C57BL/6 mice develop protective Th1 responses with production of IL-12 and IFN-γ, associated with classical activation of macrophages and killing of parasites by effector nitric oxide production [9,11–14]. IL-4 and IL-13, both of which signal through a common receptor chain, the IL-4 receptor alpha (IL-4Rα) are known to be important susceptibility factors in *L. major* infection [3,6,8,15,16]. 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...
Author Summary

Leishmaniasis is a parasitic infection caused by protozoan parasites of Leishmania species and is transmitted by the sandfly. Disease in humans ranges from localized cutaneous lesions to disseminated visceral Leishmaniasis. Mouse models of Leishmania major infection have demonstrated that a “healing” response in C57BL/6 mice requires the secretion of protective T helper (Th) 1 cytokines, including IFN-γ, which mediates parasite killing by inducing nitric oxide production. Conversely, “non-healer” BALB/c mice are unable to control infection and develop a Th2 immune response characterized by the production of IL-4 and IL-13 cytokines. Although IL-4 is the main inducer of Th2 responses, it has been shown that IL-4 can instruct dendritic cell (DC)-derived IL-12 production and Th1 development if administered during DC activation. To further investigate the role of DCs, a DC specific IL-4Rα-deficient mouse model was established. L. major studies demonstrated hypersusceptibility to infection and strikingly increased parasite loads in peripheral organs of mice lacking IL-4Rα on DCs. Moreover, increased parasite burdens were observed in host cells, including DCs, which showed reduced killing effector functions. In summary, this study demonstrates that IL-4Rα-mediated instruction of DCs occurs in vivo and is necessary to avoid rapid progression of disease in the host.

mice [17,18]. It appears that resistant mouse strains redirect the early Th2 response in an IL-12-dependent mechanism, while in susceptible mice the Th2 response persists and dominates the disease outcome by suppressing effector mechanisms needed for parasite killing [3].

While IL-4 is the primary inducer of Th2 responses [19], paradoxically it has also been shown that IL-4 promotes IL-12 production by bone marrow-derived dendritic cells (BMDCs) stimulated with CpG or LPS [20–23]. Furthermore, administration of 1 μg of recombinant IL-4 at 0 and 8 hours after infection with L. major led to increased IL-12 mRNA expression by dendritic cells (DCs) in vivo, promoted Th1 responses and rendered mice resistant to infection [21]. It has also been shown that global abrogation of IL-4Rα renders mice resistant to L. major only in the acute phase of infection, with mice continuing to develop necrotic footpad lesions during the chronic phase [15]. However, specific abrogation of IL-4Rα on CD4+ T cells does lead to resistance, indicating a protective role for IL-4Rα signalling on non-CD4+ T cells [24].

A candidate for this protective role may therefore be DCs. These sentinel of the immune system are specialized antigen-presenting cells, proficient at uptake of antigen, migration to the lymph nodes (LN) and activation of lymphocytes. Consequently, they play a critical role in the initiation and differentiation of the adaptive immune response [25,26]. To investigate the role of IL-4Rα signalling on DCs in resistance to Leishmania, CD11cCre/IL-4Rαlox/lox mice, deficient in IL-4Rα signalling on DCs, were generated and infected with L. major LV39 and IL81 strains. CD11cCre/IL-4Rαlox/lox mice were hypersusceptible to both strains of L. major, with increased footpad swelling and necrosis and substantially increased parasite burdens in peripheral organs, including the brain. Hypersusceptibility in CD11cCre/IL-4Rαlox/lox mice was associated with upregulation of Th2 responses, impairment in iNOS production by macrophages and inflammatory DCs and increased parasite loads in LN and spleen DCs. Therefore, it is clear that IL-4Rα signalling has important effects on DC phenotype during cutaneous L. major infection, and is necessary to avoid rapid disease progression in the host. This study therefore expands our knowledge on the role of dendritic cells during cutaneous Leishmaniasis and on the effects of IL-4Rα signaling on dendritic cells.

Results

Generation and characterization of CD11cCre/IL-4Rαlox/lox mice

Mice expressing cyclization recombinase (Cre) under control of the cd11c locus [27] were backcrossed to BALB/c for 9 generations, then intercrossed with global IL-4Rα (IL-4Rαlox/lox) BALB/c mice to generate CD11cCre/IL-4Rαlox/lox BALB/c mice. These mice were subsequently intercrossed with floxed IL-4Rα (IL-4Rαlox/lox) BALB/c mice (exon 6 to 8 flanked by loxP) [28] to generate CD11cCre/IL-4Rαlox/lox BALB/c mice (Figure 1A). CD11cCre/IL-4Rαlox/lox mice were identified by PCR genotyping (Figure 1B). Analysis of IL-4Rα surface expression on different cell types by flow cytometry demonstrated that IL-4Rα was efficiently depleted in DCs of the lymph nodes, spleen, skin and lungs, when compared with IL-4Rαlox/lox littermate controls and IL-4Rαlox/lox mice (Figure 1C). As expected CD11cCre/IL-4Rαlox/lox alveolar macrophages also had abrogated IL-4Rα surface expression. Other cell types such as T cells, B cells and macrophages had comparable IL-4Rα expression to IL-4Rαlox/lox littermate controls. Cre-mediated IL-4Rα deletion in DCs was confirmed at the genomic level by performing PCR for IL-4Rα exon 8 (absent in IL-4Rα-deficient cells) normalized to IL-4Rα exon 5 (present in all cells) using DNA from CD11cCre/MHCII+ DCs sorted from the spleens of naive mice (Figure 1D).

To assess functional impairment of DCs in CD11cCre/IL-4Rαlox/lox mice, we generated bone marrow-derived dendritic cells and stimulated them with LPS in the presence or absence of IL-4 or IL-13. IL-4 is known to enhance DC production of IL-12 in an IL-12 dependent manner, so called “IL-4 DC instruction” [21–23]. As expected, BMDCs derived from IL-4Rαlox/lox mice and BALB/c wildtype controls had significantly increased IL-12 production after the addition of IL-4 (Figure 1E). In contrast, LPS/IL-4 stimulated BMDCs derived from CD11cCre/IL-4Rαlox/lox mice or from global IL-4Rαlox/lox mice showed similar levels of IL-12 to those stimulated with LPS alone, with IL-4 having no effect. This demonstrates functional impairment of IL-4Rα signalling on DCs from CD11cCre/IL-4Rαlox/lox mice. In fact, after the addition of LPS alone, BMDCs with a functional IL-4Rα already showed a trend towards increased IL-12p40 levels, suggesting that endogenous levels of IL-4 found in the culture could influence these BMDCs. IL-13 did not increase levels of IL-12, confirming previous DC stimulation studies [22]. As previously reported [29], IL-4 and IL-13 had no significant effect on BMDC maturation, as shown by similar expression of MHCII, CD86, CD80, CD83 and CD40 (data not shown). Total yield of BMDCs per precursor cell seeded was similar in CD11cCre/IL-4Rαlox/lox mice and littermate controls and survival after maturation was not significantly different (data not shown).

CD11cCre/IL-4Rαlox/lox mice are hypersusceptible to acute L. major infection

In order to investigate the role of IL-4Rα signalling on DCs during cutaneous Leishmaniasis, CD11cCre/IL-4Rαlox/lox mice were infected subcutaneously with 2×10⁵ stationary phase metacyclic promastigotes of L. major LV39 [MRHO/SV/59/P; Figure 2A, 2B and 2C] or with a more virulent GFP-expressing L. major IL81 (MHOM/IL/81/FEBNI; Figure 2D, 2E and 2F) strains into the hind footpad. As previously shown [15,24], C57BL/6 mice and IL-4Rαlox/lox deficient BALB/c mice controlled lesion development
IL-4Rα Deficient DCs in Cutaneous Leishmaniasis

A

Deficient DCs in Cutaneous Leishmaniasis

B

CD11ccre × IL-4Rα−/−

CD11ccre IL-4Rα−/−

IL-4Rαlox/lox

CD11ccre IL-4Rαlox/lox

Dendritic cells

Alveolar macrophages

Other cells

C

Spleen DCs

LN DCs

Skin DCs

Lung DCs

Alveolar Mph

Peritoneal Mph

B cells

T cells

D

Spleen CD11c+MHCII+

Exon 8 / Exon5

E

IL-12p40

[ng/ml]

LPS

IL-4

IL-13

IL-4Rα−/−

CD11ccre IL-4Rα−/−

IL-4Rα−−/−

BALB/c

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Figure 1. Generation and characterization of CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> BALB/c mice. (A) IL-4R<sup>a+</sup> BALB/c mice were intercrossed with CD11c<sup>cre</sup> expressing and IL-4R<sub>a-/-</sub> mice to generate CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> BALB/c mice. (B) Genotyping of CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice. The deleted IL-4R<sub>a</sub> PCR is 471 base pairs, loxP is 450 base pairs (floxed) or 356 base pairs (wildtype) and CD11c<sup>cre</sup> specific is 517 base pairs. (C) IL-4R<sub>a</sub> surface expression was analyzed by flow cytometry from naïve IL-4R<sub>a</sub>-/lox (solid line), IL-4R<sub>a</sub>+/+ (dashed line) and CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> (grey tinted) mice. DCs were CD11c<sup>MHCII</sup> (SiglecF<sup>+</sup> in lungs), alveolar macrophages were CD11c<sup>Cre</sup>SiglecF<sup>+</sup>, peritoneal macrophages were F480<sup>+</sup>CD11b<sup>+</sup>, B cells were CD19<sup>+</sup>CD3<sup>-</sup> and T cells were CD3<sup>+</sup>CD19<sup>-</sup>. GM = geometric mean. (D) Genomic DNA was extracted from spleen DCs and IL-4R<sub>a</sub> exon 8 (deleted in IL-4R<sub>a-/-</sub> deficient cells) was determined by RT-PCR and normalized to exon 5 (present in all cells). (E) Bone marrow-derived DCs were stimulated with LPS in the presence of absence of IL-4 or IL-13 and IL-12p40 was measured in the supernatants 48 hours later. (*, p<0.05, **, p≤0.01. doi:10.1371/journal.ppat.1003699.g001

during acute infection with both <i>L. major</i> strains (Figure 2A and 2D), which correlated with low parasite numbers in infected footpads (Figure 2B and 2E) and draining lymph nodes (Figure 2C and 2F). Susceptible WT BALB/c and IL-4R<sub>a-/-</sub> littermate control mice developed progressive footpad swelling after infection with both strains (Figure 2A and 2D), with increased parasite burdens in the infected footpads (Figure 2B and 2E) and draining LN (Figure 2C and 2F). Hemizygous [IL-4R<sub>a-/-</sub>] mice had slightly reduced footpad swelling compared to BALB/c mice in IL81 infection. The greater virulence of IL81 is reflected in rapid disease progression, with footpad swelling and parasite burden reaching similar levels by 4 weeks to those obtained with LV39 in 8 weeks. Of importance, CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice were hypersusceptible to acute <i>L. major</i> infection compared to heterozygous littermate controls and BALB/c mice, showing considerably worsened disease progression when infected with either strain (Figure 2A and 2D), with earlier and dramatically larger footpad lesions, and development of early necrosis (Figure 2A and 2D). Increased disease progression was accompanied by significantly higher parasite numbers in the footpads (Figure 2B and 2E) and LN (Figure 2C and 2F) of infected animals. In addition, infection with a 10-fold lower dose of <i>L. major</i> LV39 also resulted in a hypersusceptible phenotype in CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice (Supplementary Figure S1 A-C). Histopathological analysis of CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> footpads at week 4 after infection with the virulent IL81 revealed severe destruction of epidermis, connective tissue and bone as a result of footpad necrosis, accompanied by increased inflammatory infiltrates and a high load of extracellular <i>L. major</i> amastigotes (Figure 2G). In contrast, infected footpads of IL-4R<sub>a-/-</sub> mice revealed moderate dermal inflammatory infiltrates with mostly intact epidermis, connective tissue and bone. Together, these data reveal that IL-4R signaling on DCs play an important role in host protection against acute <i>L. major</i> infection.

A shift towards Th2 responses in CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> BALB/c mice

Th1/Th2-type responses were investigated in CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice and controls during acute cutaneous leishmaniasis (IL81). Antibigen-specific restimulation of CD4<sup>+</sup> T cells sorted from the LN of infected mice and co-cultured with fixed antigen-presenting cells and soluble <i>Leishmania</i> antigen (SLA) revealed a significantly reduced IFN-γ response in CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice in comparison to the resistant C57BL/6 or IL-4R<sub>a</sub>-/lox strains as well as to the susceptible IL-4R<sub>a</sub>-/lox littermate controls (Figure 3A). Conversely, the levels of IL-4, IL-13 and IL-10 were significantly higher in CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice compared to IL-4R<sub>a</sub>-/lox and IL-4R<sub>a</sub>-/- and C57BL/6 mice (Figure 3B, 3C and 3D). The observed shift in cytokine responses was confirmed in LN cells, stimulated with anti-CD3 or SLA (data not shown) and systemically in the quality of <i>Leishmania</i>-specific antibody immune responses. Sera of week 4 infected mice revealed a predominant type 1 antibody response in IL-4R<sub>a-/-</sub> mice, as shown by elevated levels of <i>Leishmania</i>-specific IgG2a (Figure 3E). In contrast, CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice displayed a predominant type 2 antibody response shown by marked production of IgG1 and total IgE, which was significantly higher than that observed in littermate IL-4R<sub>a</sub>-/lox mice (Figure 3F and 3G). A shift towards Th2-type responses also occurred in CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice in a 10-fold lower dose <i>L. major</i> LV39 infection (Supplementary Figure S1 D-H).

As IFN-γ-induced nitric oxide synthase (iNOS) production by classically activated macrophages (caMphs) is a key control mechanism in <i>L. major</i> infection [14], the activation state of macrophages was determined in the infected footpad at week 4 after infection. Inflammatory macrophages (CD11b<sup>+</sup>MHCII<sup>+</sup> CD11c<sup>+</sup>) from CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice had significantly reduced iNOS expression compared to those of littermate IL-4R<sub>a</sub>-/lox control mice (Figure 3H). Conversely, expression of arginase 1, a marker of alternatively activated macrophages (aaMphs), was higher in macrophages of CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice (Figure 3I). This altered phenotype was confirmed in iNOS and arginase activity assays performed on total footpad cells stimulated with LPS (Figure 3J and 3K). Together, these results demonstrate a shift towards Type 2 responses and reduced macrophage effector functions in CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice.

CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice have increased parasite loads in peripheral organs

In <i>L. major</i> LV39 infection, parasites were present only in footpads and the draining lymph nodes at week 3, whereas by week 8 parasites had disseminated to the spleen and liver in both CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice and littermate controls (Figure 4A and 4B). parasite burdens were much higher in the organs of infected CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice, compared to littermate control mice. Moreover, in some CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice, but not in control mice, <i>L. major</i> parasitads had disseminated as far as the brain by week 8 after infection (Figure 4B). Similar disease progression was observed after infection with <i>L. major</i> IL81 (Figure 4C), where CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice already displayed noticeable splenomegaly at 4 weeks post infection (data not shown), and had strikingly increased parasite burdens in all organs analyzed, including the brain (Figure 4C). Histological analysis confirmed the increased presence of disseminated parasites in the spleen and liver of CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice (IL81, week 4), as shown by the high load of extracellular <i>L. major</i> amastigotes (spleen) and the prevalence of inflammatory foci and leishmanial bodies in mononuclear cells (liver) (Figure 4D). The presence of parasites in brains of perfused CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice (IL81, week 4) was also confirmed by confocal microscopy (Figure 4E). Parasites were not visible in the brains of littermate controls (data not shown). These results demonstrate a drastic increase in numbers of disseminated parasites in peripheral organs of infected CD11c<sup>cre</sup>IL-4R<sub>a-/-</sub> mice. Although it has been reported that dissemination could occur within hours after high-dose parasite inoculation [30], infection with GFP<sup>+</sup> <i>L. major</i> IL81 and analysis by flow cytometry demonstrated that GFP<sup>+</sup> parasites was not detectable in the spleen at 1 or 3 days post infection, whereas at
week 4 there was an increase in GFP+ cells compared to day 0 (Supplementary Figure S2).

IL-4Rα-deficient DCs are infected in LN and spleen at week 4 after L. major IL81 infection and have impaired killing effector functions

In order to determine if dendritic cells could harbor L. major parasites, GFP-expressing L. major parasites (IL81) were used to track infected cell populations in different organs by flow cytometry at different time-points (day 3, day 7 and week 4) after infection. Parasite replication occurred in GFP+ cell populations that were sorted and plated out for limiting dilution assays, indicating that GFP positivity was a good marker for viable parasites associated with cells (Supplementary Figure S3). At day 3 after GFP-L. major IL81 infection, plasmacytoid DCs (pDCs), macrophages and neutrophils had infiltrated the infected footpad (Figure 5A). By 4 weeks post infection, numbers of infiltrating cells had increased substantially, with conventional DCs (cDCs) also now present in high numbers (Figure 5B). The number of infiltrating cells was significantly higher in CD11ccreIL-4Rα-/-lox mice compared to IL-4Rα-/-lox mice (Figure 5B).

At early time point in FP, macrophages were infected with GFP+ Leishmania, with similar numbers in CD11ccreIL-4Rα-/-lox mice and littermate controls (Figure 5C). This was in contrast to the draining lymph node, where conventional and plasmacytoid DCs were infected, with higher numbers of DCs infected in CD11ccreIL-4Rα-/-lox mice compared to controls (Figure 5D). Similar results were obtained at day 7 post-infection (data not shown). At week 4 post infection, the footpad harbored a pool of infected cells, namely macrophages, cDCs and neutrophils (Figure 5E), while in the...
draining lymph node, the cDCs were still infected compared to the other cell types (Figure 5F). Again the number of infected DCs was significantly higher in CD11ccreIL-4Rα-/-lox mice (Figure 5E and 5F) compared to littermate controls. However, overall numbers of DCs infiltrating the LN at week 4 after *L. major* infection were similar in both CD11ccreIL-4Rα-/-lox mice and littermate control mice (data not shown), suggesting that differences in parasite killing and not DC migration were responsible for the increased number of infected DCs in CD11ccreIL-4Rα-/-lox mice.

Figure 3. T helper 2 immunity is enhanced in hypersusceptible CD11ccreIL-4Rα-/-lox mice in response to acute *L. major* infection. Experimental mice were infected subcutaneously with 2 x 10⁶ stationary phase metacyclic GFP-expressing *L. major* promastigotes into the hind footpad (A–D). At week 4 post infection, total CD4⁺ T cells from the draining lymph node were restimulated for 72 hrs with fixed APCs and soluble Leishmania antigen (SLA). The production of IFN-γ (A), IL-4 (B), IL-13 (C) and IL-10 (D) was determined by ELISA. (E–G) Antigen-specific IgG2a (E), IgG1 (F) and total IgE (G) antibody production was quantified from infected sera by ELISA. (H–I) Expression of iNOS and arginase 1 in total footpad cells. Total cells were isolated from footpads at week 4 after infection, surface-stained for CD11bhighMHCIIhighCD11c² macrophages followed by intracellular staining for iNOS (H) and arginase 1 (I). GM = geometric means. (J–K) Production of NO and arginase 1 in total footpad cells. Total cells were isolated from footpads at week 4 after infection and stimulated with 10 ng/ml LPS for 72 h. Production of NO was determined in cell supernatants (J) and cell lysates were assayed for arginase 1 production (K). A representative of two individual experiments is shown with mean values ±SEM. Statistical analysis was performed defining differences to IL-4Rα-/-lox mice as significant (*, p<0.05; **, p<0.01; ***, p<0.001).

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Infected DCs were also found in the spleen, with significantly increased numbers of infected cells in CD11c\textsuperscript{cre}IL-4R\textsuperscript{a-/-}lox mice compared to controls (Figure 6A). However, overall numbers of DCs infiltrating the spleen were also increased to a similar degree in both CD11c\textsuperscript{cre}IL-4R\textsuperscript{a-/-}lox mice and littermate controls at week 4 (data not shown), again suggesting that differences in parasite load were not solely due to changes in DC infection but rather to differences in overall immune responses.

**Figure 4. Impairment of IL-4R\textsuperscript{a} signaling in vivo results in increased *L. major* parasite loads in peripheral organs.** CD11c\textsuperscript{cre}IL-4R\textsuperscript{a-/-}lox and littermate mice were infected subcutaneously with 2 x 10\textsuperscript{6} stationary phase metacyclic *L. major* (*L. m* LV39) promastigotes into the hind footpad. Parasite load was determined by limiting dilution assay (LDA) of single-cell suspensions from homogenized footpad, lymph node, spleen, liver and brain at week 3 (A) and week 8 (B) after infection. Similarly, organs were harvested from mice infected with GFP-expressing *L. major* (*L. m* IL81) at week 4 after infection for limiting dilution assay (C). At the same time point, histopathology was analysed using formalin-fixed spleen and liver (D) stained with H&E and Giemsa, respectively (original magnification x100; asterisks indicate inflammatory foci and insets, arrows indicate amastigote parasites x800). Frozen brain sections (E) were stained with Hoechst nuclear stain (blue) and visualized by confocal microscopy for the presence of GFP-L. major amastigote parasites (original magnification x400). A representative of two individual experiments is shown with mean values ± SEM. Statistical analysis was performed defining differences to IL-4R\textsuperscript{a-/-}lox mice (*, p≤0.05, **, p≤0.01, ***, p≤0.001).

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killing and not DC migration were responsible for the increased parasite loads in CD11c creIL-4R a−/lox mice. Although it is well known that iNOS-mediated NO production in classically-activated macrophages drives intracellular killing of *L. major* parasites, a recent study has now implicated a population of iNOS + – producing inflammatory DCs in controlling *Leishmania* infection [31]. We therefore examined iNOS production by DCs in CD11c creIL-4R a−/lox and littermate control mice using intracellular FACS. In hypersusceptible CD11c creIL-4R a−/lox mice, a significantly reduced percentage of CD11c high MHCII high DCs produced iNOS compared to DCs from IL-4R a−/lox littermate control mice (Figure 6B). This was confirmed at the level of intracellular NO expression, which was also reduced in DCs from CD11c creIL-4R a−/lox mice (Figure 6C). Together, these data demonstrate that DCs from CD11c creIL-4R a−/lox mice have reduced NO killing effector functions, further explaining the increased parasite burdens in the DCs of these mice.

**IL-4Rα-deficient DCs have impaired DC instruction during infection in vivo**

Previous studies using BMDCs found that IL-4-mediated instruction results in reduced IL-10 production that is responsible for increased IL-12p40 production by DCs upon stimulation with IL-4 plus CpG or LPS [21,23]. To test whether endogenous amounts of IL-4 could mediate DC instruction in vivo, CD11c creIL-4R a−/lox mice and controls were infected with *L. major* IL81. At 4
weeks post infection, total LN cells were restimulated with SLA and cytokines were measured in the supernatant. Lymph node cells from infected CD11c<sup>cre</sup>IL-4Rα<sup>lox/lox</sup> mice produced significantly reduced IL-12p40 but increased IL-10 compared to those from IL-4Rα<sup>-/lox</sup> mice (*, p≤0.05, **, p≤0.01). doi:10.1371/journal.ppat.1003699.g006

Infection of CD11c<sup>cre</sup>IL-4Rα<sup>lox/lox</sup> mice with L. major LV39 and IL<sub>81</sub> revealed IL-4Rα<sup>+</sup> signaling on DCs to be highly important in protection against cutaneous Leishmaniasis. Compared to IL-4Rα<sup>-/lox</sup> littermate controls, CD11c<sup>cre</sup>IL-4Rα<sup>lox/lox</sup> mice showed dramatically worsened disease progression, with increased footpad swelling and necrosis, and significantly higher parasite burdens both locally and in visceral organs such as the spleen and liver. As expected, genetically resistant C57BL/6 mice effectively controlled infection, as did global IL-4Rα<sup>-/−</sup> mice, which have been shown to be resistant during the acute phase of L. major infection, with disease progression in the chronic phase only [15,24]. Progressive disease during L. major infection in BALB/c mice has been attributed to the predominance of Th2 cytokines and type 2 antibody immune responses [8,9,11], with a previous study by our laboratory showing that CD4<sup>+</sup> T cell specific IL-4Rα<sup>-/−</sup> deficient mice were highly resistant to L. major infection [24]. Analysis of CD4<sup>+</sup> T cell cytokine responses in CD11c<sup>cre</sup>IL-4Rα<sup>lox/lox</sup> mice revealed a decrease in IFN-γ accompanied by a marked increase in IL-10, IL-13 and IL-10, while increased secretion of IgG1 and IgE by B cells confirmed a shift towards a Th2-type immune phenotype. Aside from its role in instruction, IL-10 is known to be suppressive Th1-mediated effector functions [3,43]. In humans, IL-10 is strongly associated with persistent infection [44].

IFN-γ plays an important role in mediating protective immunity during L. major infection by classical-activating macrophages to induce nitric oxide synthase-mediated NO production for intracellular killing of parasites [9,14,45,46]. Latent Leishmaniasis is reactivated in chronically infected healthy C57BL/6 mice by inhibition of endogenous NOS-2, indicating that iNOS expression is crucial for the sustained control of L. major infection [9,31,47]. Induction of iNOS-mediated NO production is counter-regulated

Discussion

Understanding mechanisms of immune control in cutaneous Leishmaniasis is critical for the design of effective therapeutics and vaccines. Although several studies have clearly established that IL-4 is a key cytokine in the development of non-healing disease in BALB/c mice [8,19,34,35], apparently contradictory evidence also suggests that IL-4 has the ability to instruct protective Th1 responses [21,36–41]. The term “instruction theory” was coined when IL-4 was found to promote increased production of IL-12 by BMDCs [20–22]. IL-4, but not IL-13, enhances the production of IL-12 induced by pathogen products via signalling through the type 1 IL-4 receptor [21,22]. The mechanism behind instruction was found to be inhibition of IL-10 by IL-4, leading to higher levels of IL-12 and increased protective Th1 responses [23]. Several studies also indicate that IL-4 and IL-13 may play a role in promoting DC maturation [22,42]. However, most in vitro and in vivo studies on the effects of IL-4 and IL-13 on DCs have been conducted with exogenously administered IL-4 or IL-13, and thus the relevance of biological quantities of IL-4 signalling through IL-4Rα<sup>-/−</sup> on DCs during disease in vivo has not been demonstrated. To address these issues, dendritic cell-specific (CD11c<sup>cre</sup>IL-4Rα<sup>lox/lox</sup>) BALB/c mice were generated using the cre/loxP recombinase system under control of the cd11c locus. These mice were found to have abrogated IL-4Rα<sup>+</sup> expression on DCs and alveolar macrophages, with other cell types maintaining IL-4Rα<sup>+</sup> expression and functioning.

Infection of CD11c<sup>cre</sup>IL-4Rα<sup>lox/lox</sup> mice with L. major LV39 and IL<sub>81</sub> revealed IL-4Rα<sup>+</sup> signaling on DCs to be highly important in protection against cutaneous Leishmaniasis. Compared to IL-4Rα<sup>-/lox</sup> littermate controls, CD11c<sup>cre</sup>IL-4Rα<sup>lox/lox</sup> mice showed dramatically worsened disease progression, with increased footpad swelling and necrosis, and significantly higher parasite burdens both locally and in visceral organs such as the spleen and liver. As expected, genetically resistant C57BL/6 mice effectively controlled infection, as did global IL-4Rα<sup>-/−</sup> mice, which have been shown to be resistant during the acute phase of L. major infection, with disease progression in the chronic phase only [15,24]. Progressive disease during L. major infection in BALB/c mice has been attributed to the predominance of Th2 cytokines and type 2 antibody immune responses [8,9,11], with a previous study by our laboratory showing that CD4<sup>+</sup> T cell specific IL-4Rα<sup>-/−</sup> deficient mice were highly resistant to L. major infection [24]. Analysis of CD4<sup>+</sup> T cell cytokine responses in CD11c<sup>cre</sup>IL-4Rα<sup>lox/lox</sup> mice revealed a decrease in IFN-γ accompanied by a marked increase in IL-4, IL-13 and IL-10, while increased secretion of IgG1 and IgE by B cells confirmed a shift towards a Th2-type immune phenotype. Aside from its role in instruction, IL-10 is known to be a susceptibility factor for L. major infection, being produced at higher levels in susceptible BALB/c mice and capable of suppressing Th1-mediated effector functions [3,43]. In humans, IL-10 is strongly associated with persistent infection [44].
by IL-4/IL-13 and IL-4Ra, which promote the development of alternatively activated macrophages and arginase 1 production through depletion of L-arginine as a substrate for iNOS. Interestingly, IL-10 has also been shown to suppress intracellular killing of pathogens in macrophages by suppressing IFN-γ responses [48–50] and can induce an alternatively activated macrophage type phenotype in the absence of IL-4 and IL-13 [51]. Parasites such as *Leishmania* can utilize polyamines generated by arginase 1 activity for their own growth, making alternatively activated macrophages a favorable environment for their survival [52–54]. Accumulating reports have demonstrated a role for alternative macrophage activation and arginase 1 expression in influencing susceptibility to *L. major* infection [7,9,55,56]. LysM<sup>Cre</sup>IL-4Ra<sup>-/lox</sup> mice which lack IL-4/IL-13 induced alternative activation of macrophages were found to have increased resistance to infection [9], while neutralization of endogenous arginase 1 with N-hydroxy-nor-L-arginine leads to complete healing in BALB/c mice [55].

Macrophages from the footpads of CD11c<sup>Cre</sup>IL-4Ra<sup>-/lox</sup> mice were found to have reduced iNOS expression and increased arginase 1 expression compared to those from littermate control IL-4Ra<sup>-/lox</sup> mice, demonstrating a shift in macrophage effector function most likely as a consequence of increased IFN-γ and/or IL-10 [57] and RELM-α after administration of IL-4 [29]. In our study, the data suggest that IL-4Ra-independent alternative activation of DCs is also possible, as DCs from CD11c<sup>Cre</sup>IL-4Ra<sup>-/lox</sup> had decreased iNOS expression, possibly a consequence of reduced IFN-γ and/or increased IL-10 and activin A, and had higher parasite loads than those from littermate controls. Previous studies have revealed that iNOS-producing DCs constitute a major Th1-regulated...
within 24 hours from the site of infection in the footpad to the disseminated parasite loads in CD11ccreIL-4R mice is therefore likely to play a role in the uncontrolled parasite replication observed both in the footpad and at peripheral sites.

In susceptible BALB/c mice, L. major parasites can disseminate within 24 hours from the site of infection in the footpad to the popliteal lymph nodes, spleen, liver, lungs and bone marrow [30,59]. However, L. major parasites were not detected at early time points during IL81 infection (day 1 and day 3) but were detected at week 4, and were also detected at week 8 but not at week 3 during LV39 infection, suggesting that parasite dissemination may have occurred at a later stage of infection. Dissemination is inhibited by the administration of recombinant nation may have occurred at a later stage of infection.

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...es, such as increased migratory activity and increased expression of intracellular pathogens, phagocytes undergo phenotypical chang-
...nisms by which other pathogens, such as bacteria, invade the CNS has been frequently observed in visceral Leishmaniasis in both humans and dogs [64-67]. It has been suggested that parasites arrive in the CNS via infected leukocytes [65] and/or disruption to the blood brain barrier caused by inflammation [67]. Studying the mechanisms by which other pathogens, such as bacteria, invade the CNS may lend insights into Leishmania dissemination. Many intracellular organisms such as Mycobacterium tuberculosis, Listeria monocytogenes, Brucella spp. and Salmonella spp. appear to make use of the "Trojan-horse" mechanism, using phagocyte facilitated invasion for entry into the CNS [68]. After infection with intracellular pathogens, phagocytes undergo phenotypical changes, such as increased migratory activity and increased expression of adhesion molecules and proinflammatory cytokines, all of which could aid in dissemination and crossing of the blood-brain barrier [68,69]. Whether infected phagocytes are recruited to the CNS by specific or non-specific means is unknown [69]. In order to determine which cells were infected by L. major, mice were infected with GFP-IL81 parasites and cell populations containing parasites were identified by flow cytometry.

At day 3 and 7 after infection, macrophages harbored L. major in the footpad, while pDCs and cDCs were found to be infected in the lymph node. Similar to other reports, this indicates that DCs were responsible for transporting parasites to the lymph node [70]. At week 4, L. major parasites were still detected in macrophages in the footpads, as well as in DCs and neutrophils, but in the LN they were primarily found in DCs. The number of infected DCs in both footpad and LN was significantly higher in CD11c+/-IL-4R+/− mice. A previous study also reported that DCs were the primary infected cell population in the draining LN of L. major infected mice [70]. DCs were also infected with L. major parasites in the spleen, with CD11c+/-IL-4R+/− mice again showing a greater number of infected DCs. Numbers of DCs infiltrating the LN and spleen were equivalent in both CD11c+/-IL-4R+/− mice and littermate controls during infection. This suggests that the increased survival and/or growth of parasites in DCs, as a consequence of significantly reduced DC iNOS production, was responsible for the increase in infected cell numbers in CD11c+creIL-4R+/−/lox mice. Interestingly, a recent study found that infected DCs, which are monocyte-derived CD11b+/inflammatory DCs expressing Ly6C+, F4/80, Ly6G and iNOS, showed a unique ability to disseminate to peripheral sites in M. tuberculosis infection [71]. Furthermore, CD11b+Ly6C+ cells were found to be the principal phagocytic cells harboring L. monocytogenes in circulation [69,72]. We hypothesize that dendritic cells may therefore play a role in disseminating L. major parasites to peripheral sites and that their killing effector responses could be important in controlling disease.

The reduced Th1 and increased Th2 responses in CD11c+/-IL-4R+/−/lox mice suggests that instruction theory is relevant in vivo, and more importantly, that biological quantities of IL-4 acting through DCs can promote resistance to Leishmania infection. DCs from lymph nodes of CD11c+/-IL-4R+/−/lox mice produced more IL-10 and less IL-12 than those from IL-4R+/−/lox mice. Quantification of mRNA expression also revealed interesting differences in DCs from CD11c+/-IL-4R+/−/lox mice. Expression of the Th1-promoting genes for IL-12p40 and IL-18 was decreased compared to DCs from littermate control mice, while expression of the Th2-promoting genes for IL-23p19 and activin A were significantly increased. IL-23 production by DCs has been shown to promote Th17 [32], leading to increased neutrophils that enhance susceptibility to L. major by acting as Trojan horses [73]. Activin A is a pleiotropic cytokine belonging to the TGF-beta superfamily, and has previously been found to promote alternative activation of macrophages by inducing Arginase 1 and decreasing IFN-γ-induced expression of iNOS [33]. The absence of IL-4R+ signalling on DCs therefore appears to have a more complex influence on the dendritic cells than just affecting IL-12 production during cutaneous Leishmaniasis in vivo.

Dendritic cell instruction may not be restricted to Leishmaniasis, since other disease models have also demonstrated a protective role for IL-4. Experimental infections with Candida albicans in IL-4 deficient mice led to impaired development of Th1 responses [38], and a Th1 promoting effect of IL-4 has also been observed in autoimmunity [36,40,74], tumor immunity [39,75,76] and contact sensitivity reactions [41,77]. There is also evidence to suggest that IL-4 may promote Th1 development in humans, since both human and mouse DCs produce increased levels of bioactive IL-12 after stimulation with IL-4 [20]. A similar effect was observed in human peripheral blood mononuclear cells (PBMCs) treated with IL-4 plus lipopolysaccharide or Staphylococcus aureus [78]. Incorporating exogenous IL-4 as an adjuvant for enhancing strong Th1 responses could therefore be utilised to boost vaccine efficiency against cutaneous Leishmaniasis. Accordingly, parallel studies examining the efficacy of IL-4 as an adjuvant during BMDC-mediated vaccination against L. major, found that IL-4 instruction of DCs was critical in eliciting protective immune responses [79]. The role of IL-4R+ signalling on DCs in eliciting immunity to other intracellular pathogens is therefore of interest to vaccination strategies, and an exciting avenue to be explored.

Materials and Methods

Generation and genotyping of CD11c+creIL-4R+/−/lox BALB/c mice

CD11c+cre mice [27] were crossed with IL-4R+/−/lox BALB/c mice [28] and complete IL-4R+/− BALB/c mice [15] to generate hemizygous CD11c+/-IL-4R+/−/lox mice. Mice were backcrossed to a BALB/c background for 9 generations to generate CD11c+/-IL-4R+/−/lox BALB/c mice. Hemizygous littermate controls (IL-4R+/−) were used as controls in all experiments. Mice were genotyped as described previously [28]. All mice were housed...
in specific-pathogen free barrier conditions in individually ventilated cages. Experimental mice were age and sex matched and used between 8–12 weeks of age.

**Ethics statement**

This study was performed in strict accordance with the recommendations of the South African national guidelines and University of Cape Town of practice for laboratory animal procedures. All mouse experiments were performed according to protocols approved by the Animal Research Ethics Committee of the Health Sciences Faculty, University of Cape Town (Permit Number: 009/042). All efforts were made to minimize suffering of the animals.

**Analysis of IL-4Rα deletion efficiency**

Genomic DNA was isolated from spleen DCs (CD11c-MHCII) sorted using a FACS Vantage flow cytometer (BD Immunocytometry systems). Purity was determined by flow cytometry and checked by cytosin and staining with the Rapidiff Stain set (Clinical Diagnostics CC, Southdale, South Africa) and was at least 99%. A standard curve was prepared from serial 10-fold DNA dilutions of cloned IL-4Rα exon 5 and exon 8 DNA and RT-PCR was performed using the following primers; exon 5: forward 5′ CAGCGCACATTGTTTTT 3′ and reverse 5′ CA CAGTTCCATCTGGTAT 3′, exon 8: forward 5′ GTA CAGGGCAATTGTTTTT 3′ and reverse 5′ CTCGCGCGCA CTGACCCATCT 3′.

**Flow cytometry**

The following antibodies were used for flow cytometry: Siglec-F-PE, CD11c-APC, MHCII-APC, F480-PE, CD11b-FITC, CD3-FITC, CD19-PE, PDCA-APC, Siglec-H-PE, CD11b-PE, CD11c-PE, CD4-PerCP, CD8-PE, GR-1-PE, CD3-PerCP, anti-CD124-PE, rat anti-mouse IgG2a-PE, CD11c-biotin, CD103-biotin, CD124-biotin and rat anti-mouse IgG2a biotin with streptavidin-APC (all BD Bioscience, Erembodegem, Belgium) and MHCII-biotin with PerCP streptavidin (BD Bioscience). For intracellular cytokine staining, popliteal lymph node cells from infected mice were stained with anti-IL-10, anti-IL-12 and isotype controls (Biolegend, USA). Dendritic cells were stained with CD11c-PE-Cy7 (BD Bioscience) and MHCII-APC, fixed and permeabilized, and intracellular cytokines were stained with anti-IL-10, anti-IL-12 and isotype controls (BD Bioscience) (all PE-labelled). Cells were acquired on a FACS Calibur machine (BD Immunocytometry systems, San Jose, CA, USA) and data were analyzed using Flowjo software (Treestar, Ashland, OR, USA).

IL-4Rα responsiveness in bone marrow-derived dendritic cells (BMDCs)

BMDCs were generated from bone marrow progenitors of CD11c-IL-4Rα−/− and littermate control mice using 200 U/ml recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sigma-Aldrich) as previously described [80]. On Day 10, non-adherent cells were harvested and 5 × 10^5 BMDCs were stimulated with LPS (Sigma-Aldrich; 1 μg/ml) or Leishmania major IL-81 promastigotes (MOI: 10 parasites/cell) in the presence or absence of 1000 U/ml recombinant mouse IL-4 or IL-13 (IL-4/IL-13, BD Biosciences) for 48 h. Following incubation, levels of IL-12p40, IL-12p70 and IL-10 were measured in culture supernatants by ELISA as previously described [15].

**ELISAs**

Cytokines in cell supernatants were measured by sandwich ELISA as previously described [15]. For antibody ELISAs, blood was collected in serum separator tubes (BD Bioscience, San Diego, CA). Antigen-specific IgG1, IgG2a and IgG2b were quantified by ELISA, as previously described [15]. Detection limits were 5 ng/ml for IgG1 and IgG2b and 0.1 ng/ml for IgG2a and IgG3. Total IgE was determined as described [15]. The detection limits was 8 ng/ml for total IgE.

**Leishmania major infection**

*L. major* LV39 (MRHO/SV/59/P) and GFP-expressing *L. major* IL81 (MHOM/IL/81/FEBNI) (kind gift from Prof. Heidrun Moll, University of Wurzburg, Germany) strains were maintained by continuous passage in BALB/c mice and prepared for infection as described previously [15]. Anaesthetised mice were inoculated subcutaneously with 2 × 10^5 or 2 × 10^5 stationary phase metacyclic promastigotes into the left hind footpad in a volume of 50 μl of HBSS (Invitrogen). Swelling of infected footpads was monitored weekly using a Mitutoyo micrometer calliper (Brütsch, Zürich, Switzerland).

**Histology**

Footpads, spleens and livers were fixed in 4% formaldehyde in phosphate buffered saline and embedded in wax. Tissue sections were stained with either haematoxylin and eosin or Giemsa.

**Immunohistochemistry**

Following infection of mice with GFP-*L. major* IL81 parasites for 4 weeks, isolated brain tissue was immediately embedded in OCT (Tissue-Tek; Sakura, Zoeterwoude, Netherlands) medium. Prefixing of tissues was avoided to minimize background staining from the fixative. OCT-embedded brain tissue were cut into 10 μm frozen sections and mounted on 3-aminopropyltriethoxysilane-coated slides. Following acetone fixation of tissue, sections were stained with nuclear stain Hoechst. Coverslips were then mounted on sections using Mowiol 4–88 mounting medium (Calbiochem) with anti-fade (Sigma). Images were acquired and analyzed by Zeiss LSM 510 confocal microscope (Jena, Germany).

**Detection of viable parasite burden**

Infected and tissue cell suspensions were cultured in Schneider’s culture medium (Sigma). Prior to removal of mouse brain tissue for detection of parasite burden, animals were perfused with 20 ml sterile saline solution. Detection of viable parasite burden was estimated by two-fold limiting dilution assay as previously described [15].

**Antigen-specific restimulation**

CD4+ T cells were positively selected using anti-CD4 MACS beads (Miltenyi Biotec) according to the manufacturer’s instructions (purity >95%). Thy1.2-labeled splenocytes were T cell depleted by complement-mediated lysis to enrich antigen presenting cells (APCs). APCs were fixed with mitomycin C (50 μg/ml, 20 min at 37°C) and washed extensively in complete IMDM. A total of 2 × 10^5 purified CD4+ T cells and 1 × 10^5 APCs were cultured with SLA (30 μg/ml). After 72 h incubation at 37°C, supernatants were collected and cytokine production analysed as previously described [20].

**Isolation of footpad and spleen cells**

Muscle tissue was separated from infected footpads and digested in DMEM medium supplemented with Collagenase IV.
(Sign-man-Aldrich; 1 mg/ml) and DNase I (Sigma-Aldrich; 1 mg/ml) at 37°C for 60 min. Following incubation, single cell suspensions were isolated by straining through 70 µM cell-strainers. Spleen cells were isolated by pressing through 70 µM cell-strainers, red blood cell lysis was performed and white blood cells were washed and resuspended in 10% DMEM (Gibco).

Cell sorting
Total lymph node or footpad cells were labeled with specific mAbs and populations isolated by cell sorting on a FACS Vantage machine. Macrophages from the footpad were gated as CD11b<sup>high</sup>MHCII<sup>high</sup>CD11c<sup>−</sup> cells and DCs, macrophages, neutrophils and B cells from the lymph node were gated as CD11c<sup>high</sup>MHCII<sup>−</sup>, CD11b<sup>high</sup>MHCII<sup>high</sup>CD11c<sup>−</sup>, GR-1<sup>high</sup>-SSC<sup>high</sup>FSC<sup>high</sup>CD11c<sup>−</sup> and CD19<sup>−</sup>CD3<sup>−</sup>CD11c<sup>−</sup> cells, respectively. Cells were >98% pure and used for further analysis.

Quantitative RT-PCR
Dendritic cells were stained with specific mAb and sorted from the LN of infected mice. Total RNA was extracted from dendritic cells using TRI reagent (Applied Biosystems, Carlsbad, Calif) and mini-eclipse columns (Qiagen) according to the manufacturer’s protocol. cDNA was synthesized with Transcriptor First Strand cDNA synthesis kit (Roche), and real-time PCR was performed using Lightcycler FastStart DNA Master PLUS SYBR Green I mixture (Roche) on a Lightcycler 480 II (Roche). Primers for IL-12p40: forward 5’ GTGCCGAGTACACGTCGCCAC 3’ and reverse 5’ GTGCTTGGACACGGCAGCTTC 3’. IL-10: forward 5’ AGCCGGAGACAACTATGG 3’ and reverse 5’ CATTTCCGATAAGGTTGG 3’. IL-12: forward 5’ CAGCTTAAGGATGCCCTTG 3’ and reverse 5’ CTCAACAGTTTCTGATGCCA 3’. Values were normalized according to the expression of the housekeeping genes HPRT or rS12.

Nitric oxide synthase and arginase
Lymph node and footpad cells collected at week 4 after infection were restimulated with LPS (Sigma-Aldrich; 10 ng/ml). Supernatants were collected at 48 hours for quantification of nitric oxide synthase and arginase activity was measured in cell lysates [81]. Two individual experiments is shown with mean values ±SEM. Statistical analysis was performed defining differences to IL-4R<sup>−/−</sup> mice as significant (*, p<0.05; **, p<0.01; ***, p<0.001).

Statistics
Data is given as mean ± SEM. Statistical analysis was performed using the unpaired Student’s t test or 1-way Anova with Bonferroni’s post test, defining differences to IL-4R<sup>−/−</sup> mice as significant (*, p<0.05; **, p<0.01; ***, p<0.001) unless otherwise stated. (Prism software: http://www.prism-software.com).

Supporting Information
Figure S1 CD11c<sup>−</sup>IL-4R<sup>−/−</sup> mice are hypersusceptible to a 10-fold lower dose infection with L. major. Mice were infected with L. major LV39 (MRHO/SV/85/P) parasite strain. Footpad swelling was measured at weekly intervals in mice (5 per group) infected subcutaneously with a 10-fold lower dose of 2×10<sup>4</sup> stationary phase metacyclic L. major promastigotes into the hind footpad (A). “N” indicates necrosis or ulceration/mouse. Parasite burden was determined by limiting dilution of single-cell suspensions from homogenized footpads (B) and lymph nodes (C) at week 8 after infection. (D–F) Antigen-specific IgG2a (D), IgG1 (E) and total IgE (F) antibody production was quantified in sera by ELISA, at week 8 post infection. (G–H) Total cells from the draining lymph node were incubated for 72 hrs with no stimulation, αCD3 or soluble Leishmania antigen (SLA). The production of IFN-γ (G) and IL-4 (H) was determined by ELISA. A representative of two individual experiments is shown with mean values ±SEM. Statistical analysis was performed defining differences to IL-4R<sup>−/−</sup> mice as significant (*, p<0.05, **, p<0.01; ***, p<0.001).

Figure S2 Infiltration of GFP<sup>−</sup>L. major parasites in immune cell populations in spleen during infection in CD11c<sup>−/−</sup>IL-4R<sup>−/−</sup> mice. Mice were infected subcutaneously with 2×10<sup>6</sup> stationary phase metacyclic GFP-expressing L. major IL81 (MRHO/SV/85/P) strain into the hind footpad. At Day 0, Day 1, Day 3 and Week 4 after infection, total spleen cells were surface stained for dendritic cells (DCs)-CD11c<sup>high</sup>MHCII<sup>high</sup>, Macrophages (Mph-CD11b<sup>high</sup> MHCII<sup>high</sup>CD11c<sup>−</sup>) and neutrophils (Neut-GR1<sup>high</sup>CD11c<sup>−</sup>). The percentage of infiltrating GFP<sup>−</sup>-infected cells were determined by flow cytometry.

Figure S3 Viability of GFP<sup>−</sup>L. major parasites in immune cell populations during acute L. major IL81 infection by limiting dilution assay. Experimental mice were infected subcutaneously with 2×10<sup>6</sup> stationary phase metacyclic GFP-expressing L. major IL81 promastigotes into the hind footpad. At week 4 after infection, total lymph node cells were isolated and DCs (CD11b<sup>high</sup>MHCII<sup>high</sup>) macrophages (CD11b<sup>high</sup> MHCII<sup>high</sup>CD11c<sup>−</sup>) and neutrophils (GR1<sup>high</sup>CD11c<sup>−</sup>) were isolated by cell sorting on a FACS Vantage machine. Sorted cells were plated to determine viable parasite burden by limiting dilution assay in two-fold dilutions.

Figure S4 Intracellular IL-12 and IL-10 in lymph node DCs. Experimental mice were infected subcutaneously with 2×10<sup>6</sup> stationary phase metacyclic L. major IL81 promastigotes into the hind footpad. Total lymph node cells were incubated with PMA/Ionomycin/Monensin for 4 h at 37°C, then surface-stained for CD11b<sup>high</sup>MHCII<sup>high</sup> DCs followed by intracellular FACS staining for IL-12 and IL-10. Dot plots of percent cytokine producing cells are shown.

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Author Contributions
Conceived and designed the experiments: RH NEN FB. Performed the experiments: RH NEN MRB LS JCH SPP. Analyzed the data: RH NEN FB. Contributed reagents/materials/analysis tools: BR. Wrote the paper: RH NEN FB.
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