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BALB/c Mice Deficient in CD4⁺ T Cell IL-4Rα Expression Control *Leishmania mexicana* Load although Female but Not Male Mice Develop a Healer Phenotype

Karen J. Bryson¹, Owain R. Millington¹, Thabang Mokgethi¹, H. Adrienne McGachy¹, Frank Brombacher², James Alexander¹ *

¹Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, United Kingdom, ²Institute of Infectious Diseases and Molecular Medicine, Health Science Faculty, University of Cape Town, Cape Town, South Africa

### Abstract

Immunologically intact BALB/c mice infected with *Leishmania mexicana* develop non-healing progressively growing lesions associated with a biased Th2 response while similarly infected IL-4Rα-deficient mice fail to develop lesions and develop a robust Th1 response. In order to determine the functional target(s) for IL-4/IL-13 inducing non-healing disease, the course of *L. mexicana* infection was monitored in mice lacking IL-4Rα expression in specific cellular compartments. A deficiency of IL-4Rα expression on macrophages/neutrophils (in LysMcreIL-4Rα⁺/⁻/lox animals) had minimal effect on the outcome of *L. mexicana* infection compared with control (IL-4Rα⁺/⁺/lox) mice. In contrast, CD4⁺ T cell specific (LckcreIL-4Rα⁺/⁻/lox) IL-4Rα⁻/⁻ mice infected with *L. mexicana* developed small lesions, which subsequently healed in female mice, but persisted in adult male mice. While a strong Th1 response was manifest in both male and female CD4⁺ T cell specific IL-4Rα⁻/⁻ mice infected with *L. mexicana*, induction of IL-4 was manifested in males but not females, independently of CD4⁺ T cell IL-4 responsiveness. Similar results were obtained using pan-T cell specific (iLck creIL-4Rα⁺/⁻/lox) IL-4Rα⁻/⁻ mice. Collectively, these data demonstrate that upon infection with *L. mexicana*, initial lesion growth in BALB/c mice is dependent on non-T cell population(s) responsive to IL-4/IL-13 while progressive infection is dependent on CD4⁺ T cells responsive to IL-4.

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### Introduction

New world cutaneous leishmaniasis resulting from infection with *Leishmania mexicana* is under different genetic and immunoregulatory controls to those controlling *L. major* infection [1]. Also, unlike *L. major*, the majority of mouse strains are susceptible to *L. mexicana* infection [2–3]. As with the other *Leishmania* species, protective immunity against *L. mexicana* is the result of a STAT-4 dependent type-1 immune response, although this can be generated independently of IL-12 [4]. While the immunological pathways resulting in non-healing *L. major* infections in susceptible BALB/c mice remain somewhat controversial, IL-4 plays the major role in promoting non-healing *L. mexicana* infections in this mouse strain [5–7]. Thus, mice lacking IL-4 develop small lesions that heal while those lacking IL-4Rα fail to develop lesions [6]. This also indicates some input from IL-13 in the non-healing response to *L. mexicana* infection as IL-4 and IL-13 receptors share the IL-4Rα sub unit [6]. However, IL-4 and IL-13 are pleiotropic cytokines and numerous cell types of both the innate and adaptive immune responses produce these cytokines as well as express their receptors.

In order to better differentiate both the cellular sources and targets of IL-4/IL-13 initiating lesion growth and facilitating progressive non-healing disease, we have previously examined parasite growth in SCID mice reconstituted with IL-4⁻/⁻, IL-4Rα⁻/⁻, or wild type splenocytes [5–6]. These studies indicated that non-lymphocyte sources of IL-4/IL-13 may contribute to early lesion growth during *L. mexicana* infection. However, the non-healing disease phenotype was dependent on a lymphocyte source of IL-1-α and, in its absence, IL-4-deficient splenocyte-reconstituted SCID mice generated a healing response [5]. In addition, SCID mice reconstituted with IL-4Rα⁻/⁻ splenocytes demonstrated that initial lesion development was also dependent upon cells from this source responding to IL-4/IL-13 [6].

In order to better differentiate the specific role of IL-4/IL-13 responding cells from global effects in *vivo*, tissue specific IL-4Rα⁻/⁻ mice have been produced. So far macrophage/neutrophil specific (LysMcreIL-4Rα⁻/⁻/lox) [8] and CD4⁺ T cell specific (LckcreIL-4Rα⁻/⁻/lox) [9] IL-4Rα⁻/⁻ mice have been generated and the consequences for *L. major* infection studied. In contrast to susceptible BALB/c mice, BALB/c LysMcreIL-4Rα⁻/⁻/lox mice showed a significantly delayed disease progression after infection with *L. major*, concomitant with normal Th2 and type 2 antibody immune responses but with improved macrophage leishmanicidal activities [8]. These results suggest that alternatively activated macrophages were contributing to the susceptible phenotype in non-healer BALB/c mice. Furthermore T cell specific LckcreIL-4Rα⁻/⁻/lox BALB/c mice infected with *L. major* were significantly

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* E-mail: jalexander@strath.ac.uk
Author Summary

Leishmania species are parasites, transmitted by sandflies which are of extensive public health importance in the tropical and subtropical regions of the world. A large number of distinct Leishmania species cause cutaneous disease and the vast majority of studies utilize the causative agent of Old World cutaneous leishmaniasis, L. major. Other species, for example, L. mexicana, are associated with quite different patterns of disease following infection of mice when compared with L. major. Thus, while susceptible BALB/c mice deficient in the ability to respond to the cytokines IL-4/IL-13 are not protected against development of cutaneous leishmaniasis caused by L. major they are totally resistant to infection with L. mexicana. Here we describe the outcome of L. mexicana infection in BALB/c mice with cell-specific deletion of the receptor for IL-4/IL-13 on macrophages/neutrophils or T helper cells. Infections develop in both mutants but lesion growth is controlled only in T cell specific knockouts and female but not male mice heal. Male but not female T cell specific knockout mice achieve a strong IL-4/IL-13 response. This highlights the role of IL4/IL-13 in driving a non-healing phenotype and may in part explain why human males are more susceptible to this infection than females.

IL-4Rx+CD4+ T Cells Promote Leishmania mexicana

PCR genotyping as described [9–10]. The mice were maintained under specific pathogen free conditions. Animal experiments were performed in strict accordance with the UK Home Office Animal [Scientific Procedures] Act 1986 (licence number 60/3929) with approval by the University of Strathclyde Ethical Review Panel.

L. mexicana parasites and infection

L. mexicana (MYNC/BZ/62/M379) was maintained by serial passage of amastigotes inoculated into the shaven rumps of BALB/c mice. Amastigotes for use in infections were isolated from lesions and enumerated as described below. Two sites of infection were examined and either 5 × 10^4 at 50 μl were inoculated subcutaneously into the shaven base of the tail, or 2 × 10^4 L. mexicana amastigotes in a final volume of 25 μl were inoculated subcutaneously into the hind footpad. 6–8 week old male or female LysMcreIL-4Rxlox/lox and LckcreIL-4Rxlox/lox mice were used in each experiment, with age and sex matched cre negative IL-4Rxlox/lox littermates used as controls. The lesion diameter was measured using a sliding gauge micrometer at weekly intervals.

Enumeration of parasites

Lesions were excised from L. mexicana infected LysMcreIL-4Rxlox/lox, LckcreIL-4Rxlox/lox and IL-4Rxlox/lox mice and disrupted through a metal mesh with 5 μL of RPMI 1640 (Cambrex Bio Science Verviers, Belgium). The parasites were washed twice at 550g in RPMI and then enumerated using an improved Nauber haemocytometer. Alternatively parasite numbers were quantified by limiting dilution, as previously described [11].

Splenocyte stimulation and cytokine detection

Splenocytes were isolated from infected mice and cultured for 72 hours in 96-well plates (Corning-Costar, NY, USA) in the presence or absence of L. mexicana antigenic lysate, as previously described [6]. IFN-γ and IL-4 levels were detected in the supernatants by capture ELISA. Briefly the wells of Immulon 1B flat-bottomed microtitre plates (ThermoLabsystems, MA, USA) were coated with 50 μL of 1 μg mL^−1 purified anti-mouse IFN-γ antibody R4-6A2 (BD Biosciences) overnight at 4°C. Supernatants were then added to the individual wells and either 30 μL recombinant mouse IFN-γ (R&D Systems, Abingdon, UK) or recombinant mouse IL-4 (Genzyme, Cambridge, UK) added to individual wells in duplicate in a doubling dilution with a solution of pH 7.4 PBS supplemented with 10% v/v FCS (Harlan Sera-Lab Ltd., Crawley, UK), ranging from 20 ng mL^−1 to 0.01 ng mL^−1 (IFN-γ) or 2 ng mL^−1 to 0.0977 pg mL^−1 (IL-4). The plates were then incubated for 2 hours at 37°C. The bound cytokines were incubated with either biotinylated rat anti-mouse IFN-γ monoclonal antibody XMG1.2 or biotinylated rat anti-mouse IL-4 antibody BVD6-24G2 (both BD Biosciences) and detected with either conjugated streptavidin-alkaline phosphatase or conjugated streptavidin-horseradish peroxidase (BD Biosciences). The appropriate substrate was then added to the wells, p-nitrophenyl-phosphate (Sigma-Aldrich, Poole, UK) or tetramethylbenzidine in pH 5.5 sodium acetate buffer, containing 0.0005% hydrogen peroxide (BDH, Poole, UK). Finally the plates were read at an absorbance of 405 nm for IFN-γ or 450 nm for IL-4.

Detection of Leishmania mexicana specific -IgG1, -IgG2a and total IgE

L. mexicana specific-IgG1 and -IgG2a were detected in the plasma of infected mice by ELISA, as previously described [12].
Brieﬂy, Immulon 1B ﬂat-bottomed microtitre plates were coated with 100 µL of 10 µg ml⁻¹ Leishmania mexicana lyase (lyase preparation previously described [13] in PBS (pH 9.0) overnight at 4°C. Plasma samples were serially diluted in duplicate, followed by a 1 hour incubation at 37°C. Bound Leishmania speciﬁc antibodies were detected with a 1 hour incubation with horseradish peroxidase conjugated goat anti-mouse IgG1 or goat anti-mouse IgG2a (Southern Biotechnology Associates Inc., AL, USA). The substrate tetramethylbenzidine in pH 5.5 sodium acetate buffer, containing 0.003% hydrogen peroxide, was then added to the wells and, following colour development, the reaction stopped by the addition of 10% sulphuric acid, absorbance measured at 450nm using a SoftMax PRO (Molecular Devices, CA, USA) and the endpoint dilution was determined. Total IgE was detected by the addition of 10% sulphuric acid, absorbance measured at 492nm.

Flow cytometry
Draining lymph node cells were activated for 4 hours with 50 ng ml⁻¹ PMA and 500 ng ml⁻¹ Ionomycin (both Sigma-Aldrich) along with GolgiPlug (BD Biosciences). Following stimulation, cells were harvested and washed, resuspended in FACS Buffer containing Fc Block (2.4G2 hybridoma supernatant) together with the appropriate combinations of the following antibodies: CD4-APC, CD8-PerCP or B220-FITC (all from BD Biosciences). Intracellular cytokine staining was carried out using PE-conjugated anti-mouse IL-4 or IFN-γ with Cytotox/Cytoperm solution (all from BD Biosciences). Data was obtained using FACScanto (BD Bioscience) and analysed using FlowJo (Tree Star Inc., CA, USA).

Statistical analysis
Antibody analysis was performed using the Mann-Whitney U test and all other analysis used an unpaired Student’s t test.

Results
IL-4Rx signaling via macrophages/neutrophils plays little role in the susceptibility of BALB/c mice to L. mexicana
To compare the progression of L. mexicana lesion growth in LysMcreIL-4Rx⁻/lox and IL-4Rx⁻/lox littermate control and global IL-4Rx⁻/lox mice, animals were infected with 5x10⁶ amastigotes into the shaven base of the tail. While no discernible lesions were identiﬁed in infected global IL-4Rx⁻/lox mice, as previously demonstrated [6], rapidly growing non-healing lesions were observed in both LysMcreIL-4Rx⁻/lox and IL-4Rx⁻/lox (Figure 1A). Parasite burdens were also similar in LysMcreIL-4Rx⁻/lox, and IL-4Rx⁻/lox mice and signiﬁcantly higher (p<0.0001) than those recorded from global IL-4Rx⁻/lox animals (Figure 1B). In line with the non-healing progressive disease phenotype displayed by macrophage/neutrophil IL-4Rx⁻/lox mice, parasite antigen induced spleen cell IFN-γ production was similar to IL-4Rx⁻/lox mice and signiﬁcantly less (p<0.01) than that of global IL-4Rx⁻/lox mice (Figure 1C). Antigen induced splenocyte IL-4 production was similar in all 3 strains (Figure 1D), demonstrating once again that IL-4 induction can be independent of IL-4Rx signaling [6,14–16]. Further studies demonstrated that the close similarities in the disease phenotypes of LysMcreIL-4Rx⁻/lox and IL-4Rx⁻/lox mice were independent of site, dose of inoculum, and life cycle stage initiating infection (data not shown). These data suggest that the expression of IL-4Rx by a macrophage/neutrophil population is not important in determining susceptibility to L. mexicana infection.

IL-4Rx signaling via CD4⁺ T cells is essential for progressive non-healing disease following infection with L. mexicana
In subsequent studies, infection of CD4⁺ T cell speciﬁc (LckcreIL-4Rx⁻/lox) IL-4Rx⁻/lox mice with 5x10⁶ L. mexicana amastigotes into the shaven base of the tail resulted in control of the lesion growth observed in control IL-4Rx⁻/lox mice (wild-type equivalent). Interestingly, in one experiment using male mice, lesions in LckcreIL-4Rx⁻/lox mice did not heal completely, while experiments utilizing female mice fully resolved (Figure S1). Consequently, to conﬁrm and further investigate this apparent gender-dependent difference in control of L. mexicana infection male and female LckcreIL-4Rx⁻/lox, control IL-4Rx⁻/lox, and global IL-4Rx⁻/lox mice were infected in parallel with 5x10⁶ L. mexicana amastigotes into the shaven base of the tail (Figure 2A–D). While infected global IL-4Rx⁻/lox mice displayed a non-leision growth phenotype, lesion growth was progressive in control IL-4Rx⁻/lox mice (Figure 2A and B). These disease phenotypes were independent of gender. By comparison, L. mexicana infected female CD4⁺ T cell speciﬁc (LckcreIL-4Rx⁻/lox) IL-4Rx⁻/lox mice developed lesions which completely healed after 4–5 weeks (Figure 2A), while male CD4⁺ T cell speciﬁc (LckcreIL-4Rx⁻/lox) IL-4Rx⁻/lox developed lesions which failed to fully heal (Figure 2B). Indeed in agreement with lesion size parasite burdens up until week 6 were similar in both male LckcreIL-4Rx⁻/lox and IL-4Rx⁻/lox mice (Figure S2).

Parasite numbers at the termination of the study at week 12 were of a similar order of magnitude in both female and male control IL-4Rx⁻/lox mice while global IL-4Rx⁻/lox mice of both sexes were equally able to control infection with L. mexicana (Figure 2C and D). By contrast while infected CD4⁺ T cell speciﬁc (LckcreIL-4Rx⁻/lox) IL-4Rx⁻/lox mice of both sexes were able to signiﬁcantly control parasite growth (p<0.0001 and p<0.01 respectively for female and male mice), male mice (Figure 2D) were signiﬁcantly limited in this ability and had signiﬁcantly higher parasite burdens (p<0.001) than female mice (Figure 2C).
infected control IL-4Rα−/lox mice as early as week 6 post-infection (Figure 3C) it was not until week 12 post-infection that male LckcreIL-4Rα−/lox mice were producing significantly more IgG2a (p<0.025) than their infected control IL-4Rα−/lox counterparts (Figure 3D). Similar results were recorded in 3 separate experiments.

IL-4Rα signaling via CD4+ T cells promotes the induction of a Th2 response following infection with L. mexicana differentially in male and female mice

A clear dichotomy in antigen-induced splenocyte IL-4 production was identified between infected female and male LckcreIL-4Rα−/lox mice both compared with each other and compared with their wild-type equivalent counterparts (Figure 3C). Similar results were recorded in 3 separate experiments.

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to each other and intermediate between those induced in control IL-4Rα−/− mice and the absence of IgE in infected IL-4Rα−/− mice (Figure S3).

Anti-CD3 and ConA stimulation (Figure 5A and B) of spleen cells from 12 week infected mice demonstrated quite clearly that not only was IL-4 production from female LckcreIL-4Rα2/lox mice significantly less than that of female control IL-4Rα2/lox mice (**p < 0.003 for ConA and p < 0.01 for anti-CD3 respectively), but also significantly less than similarly treated male LckcreIL-4Rα2/lox mice (p < 0.02 for ConA and p < 0.05 for anti-CD3 respectively). Conversely male LckcreIL-4Rα2/lox mice splenocytes produced similar quantities of IL-4 to control male IL-4Rα2/lox mice with either treatment. Examination of draining inguinal lymph node cells indicated a significantly lower (p < 0.05) percentage of IL-4 and greater (p < 0.05) percentage of IFN-γ secreting CD4+ T cells in infected female but not male LckcreIL-4Rα2/lox mice compared with control sex-matched IL-4Rα2/lox mice (Figure 6 A–D).

**Discussion**

We have previously demonstrated that signaling via IL-4Rα plays the major role in the non-healing response of BALB/c mice following infection with *L. mexicana* [6] and that IL-4Rα−/− mice, unlike their wild-type counterparts that produce progressively growing non-healing lesions, display a non-lesion growth disease phenotype associated with an enhanced type-1 response. In the course of the present study using macrophage/neutrophil specific IL-4Rα−/− mice (LysMcreIL-4Rα2/lox) we failed to identify any significant role for IL-4Rα signaling via macrophages/neutrophils in the normal non-healing response of BALB/c mice infected with *L. mexicana*. In contrast, following early lesion growth, CD4+ T cell specific (LckcreIL-4Rα2/lox) IL-4Rα−/− mice were able to inhibit disease progression. However, while lesions in female CD4+ T cell specific (LckcreIL-4Rα2/lox) IL-4Rα−/− mice healed those in male CD4+ T cell specific (LckcreIL-4Rα2/lox) IL-4Rα−/− mice
Persisted. Furthermore, although both female and male CD4\(^+\) T cell specific (Lck\(^\text{cre}\)IL-4R\(^\alpha\)2/loxC) IL-4R\(^\alpha\)2/2 mice had significantly enhanced type-1 responses compared with IL-4R\(^\alpha\) intact (IL-4R\(^\alpha\)2/lox) mice, male CD4\(^+\) T cell specific (Lck\(^\text{cre}\)IL-4R\(^\alpha\)2/loxC) IL-4R\(^\alpha\)2/2 mice maintained strong type-2 responses compared with their female counterparts.

Although signaling via IL-4R\(^\alpha\) plays a significant role in the outcome of infection with Leishmania mexicana as well as L. major [6,8–9,15] the cell targets for IL-4/IL-13 activity and whether they promote or inhibit the disease process differ significantly between species.

Thus, while IL-4R\(^\alpha\) plays a significant role in the outcome of infection with L. mexicana as well as L. major [6,8–9,15] the cell targets for IL-4/IL-13 activity and whether they promote or inhibit the disease process differ significantly between species. Thus, while IL-4R\(^\alpha\) signaling via macrophages/neutrophils promotes early lesion growth in L. major infected BALB/c mice and macrophage/neutrophil specific (LysM\(^\text{cre}\)IL-4R\(^\alpha\)2/loxC) IL-4R\(^\alpha\)2/2 mice display delayed lesion growth [8], we have failed to identify any contributory role for IL-4R\(^\alpha\) signaling via macrophages/neutrophils during L. mexicana infection. The control of L. major early in infection in LysM\(^\text{cre}\)IL-4R\(^\alpha\)2/loxC mice has been identified as being due to enhanced macrophage microbicidal NO mediated activity in the absence of alternative macrophage activation. What may be critical in this regard is that L. amazonensis parasites, which belong to the “mexicana” complex of parasites, have been shown to be more resistant to macrophage-mediated control than L. major requiring higher levels of NO to induce killing [16–17]. Furthermore, recent evidence indicates that, unlike L. major, there is in fact enhanced replication of the amastigote stage of L. amazonensis in IFN-\(\gamma\)-stimulated murine macrophages [18], reportedly due to the induction of a novel L-arginine pathway independent of iNOS or host arginase [19]. In addition it has been demonstrated that arginase null-mutant L. mexicana have attenuated virulence in vitro and in vivo with the indication that the parasite arginase has a potential role in depleting host L-arginine available for iNOS activity [20–21]. Furthermore, the authors suggest that there could be different roles of arginase between L. mexicana and L. major as the Th2 response is blunted in animals.

Figure 3. IL-4R\(^\alpha\) deficiency in CD4\(^+\) T cells results in enhanced Th1 responses after L. mexicana infection. L. mexicana antigen induced (10 \(\mu\)g/ml) splenocyte IFN-\(\gamma\) (Figure 3A and B), and 6 and 12 week serum IgG2a levels (Figure 3C and 3D) produced from female (Figure 3A and C) and male (Figure 3B and D) IL-4R\(^\alpha\) intact (IL-4R\(^\alpha\)2/lox), CD4\(^+\) T cell specific (Lck\(^\text{cre}\)IL-4R\(^\alpha\)2/loxC) IL-4R\(^\alpha\)2/2, and global IL-4R\(^\alpha\)2/2 mice infected subcutaneously with \(5\times10^6\) amastigotes of L. mexicana. *\(p<0.05\), **\(p<0.01\) compared with IL-4R\(^\alpha\) intact mice. Representative of 4 separate experiments. doi:10.1371/journal.pntd.0000930.g003
infected with arginase null mutant \textit{L. mexicana} parasites while pharmacological inhibition of arginase during \textit{L. major} infection did not inhibit the Th2 immune response [22].

CD4\(^+\) T cell specific (Lck\(^{cre}\)IL-4R\(\alpha\)\(^2/lox\)) IL-4R\(\alpha\)\(^{-/-}\) mice are more resistant than global IL-4R\(\alpha\)\(^{-/-}\) mice to infection with \textit{L. major}, indicating that in the absence of a polarized Th2 response, there is a role for an IL-4/IL-13 responsive non-CD4\(^+\) T cell in early resistance to infection [9]. Conversely CD4\(^+\) T cell specific (Lck\(^{cre}\)IL-4R\(\alpha\)\(^2/lox\)) IL-4R\(\alpha\)\(^{-/-}\) mice are more susceptible than global IL-4R\(\alpha\)\(^{-/-}\) mice to infection with \textit{L. mexicana}, indicating a role for an IL-4/IL-13 responsive non CD4\(^+\) T cell population in early susceptibility. We have now studied the course of \textit{L. mexicana} infection in newly generated iLck\(^{cre}\)IL-4R\(\alpha\)\(^2/lox\) female and male mice that have IL-4R\(\alpha\) deleted on all T cell populations [23]. These produce the same disease and immunological phenotypes as CD4\(^+\) T cell specific (Lck\(^{cre}\)IL-4R\(\alpha\)\(^2/lox\)) IL-4R\(\alpha\)\(^{-/-}\) mice (data not shown). Consequently IL-4 responsive CD8\(^+\) T cells do not play a role in early susceptibility or the non-healing response following infection with \textit{L. mexicana}. Studies utilizing macrophage specific BALB/c IL-4R\(\alpha\)\(^{-/-}\) mice have demonstrated that IL-4/IL-13 operates through this population to enhance \textit{L. major} parasite growth via alternative macrophage activation [8] and consequently these are unlikely to be the population driving a Th1 response in CD4\(^+\) T cell specific (Lck\(^{cre}\)IL-4R\(\alpha\)\(^2/lox\)) IL-4R\(\alpha\)\(^{-/-}\) mice. However, IL-4 treatment of BALB/c mice prior to T cell priming has previously been demonstrated to instruct dendritic cells to produce IL-12 and facilitate a protective Th1 response against \textit{L. major} [24] and is required for protective type-1 responses to \textit{Candida} [25]. In addition IL-13 is able to prime monocytes for IL-12 production[26], which is also observed in listeriosis [27].

![Figure 4. IL-4R\(\alpha\) deficiency in CD4\(^+\) T cells results in decreased specific Th2 responses in female mice. \textit{L. mexicana} antigen induced (10 \(\mu\)g/ml) splenocyte IL-4 (Figure 4A and B), IL-5 (Figure 4C and D) and IL-10 (Figure 4E and F) produced from female (Figure 4A, C and E) and male (Figure 4B, D and F) IL-4Rx intact (IL-4R\(\alpha\)\(^{-/-}\)), CD4\(^+\) T cell specific (Lck\(^{cre}\)IL-4R\(\alpha\)\(^2/lox\)) IL-4R\(\alpha\)\(^{-/-}\), and global IL-4R\(\alpha\)\(^{-/-}\) mice infected sub-cutaneously with \(5 \times 10^6\) amastigotes of \textit{L. mexicana}. *\(p<0.05\), **\(p<0.01\) compared with IL-4Rx intact mice. Representative of 4 separate experiments. doi:10.1371/journal.pntd.0000930.g004

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T cells results in decreased non-specific Th2 responses in female mice. IL-4Rα deficiency in CD4+ T cells results in decreased non-specific Th2 responses in female mice. ConA and anti-CD3 induced (10 μg/ml) splenocyte IL-4 produced from female (Figure 5A) and male (Figure 5B) IL-4Rα intact (IL-4Rα+/lox), CD4+ T cell specific (LckcreIL-4Rα/lox) IL-4Rα−/−, and global IL-4Rα−/− mice infected sub-cutaneously with 5 × 10⁶ amastigotes of *L. mexicana* 12 weeks previously. *p < 0.05, **p < 0.01 compared with IL-4Rα intact mice. IL-4 production from female LckcreIL-4Rα/lox mice was significantly less than similarly treated male LckcreIL-4Rα/lox mice (p < 0.02 for ConA and p < 0.05 for anti-CD3 respectively). Results are mean±s.e. 5 mice. Representative of 2 separate experiments. doi:10.1371/journal.pntd.0000930.g005

Furthermore both IL-4 and IL-13 promote CD40L-induced IL-12 production by macrophages and dendritic cells [28]. This would indicate that dendritic cells may be the IL-4/IL-13 responsive cells facilitating protection against *L. major* in the absence of IL-1Rα responsive CD4+ T cells in BALB/c mice. As no distinct disease phenotype could be discerned in macrophage/neutrophil specific (Ly6McreIL-4Rα/lox) IL-4Rα−/− mice compared with IL-4Rα intact animals infected with *L. mexicana*, no IL-4/IL13 responsive non-T cell population can easily be ruled out in promoting early infection against this parasite. While a role for B cells and antibody production in the non-healing response to infection against this parasite. While a role for B cells and antibody production in the non-healing response to infection against this parasite. While a role for B cells and antibody production in the non-healing response to infection against this parasite.

Unlike the epidemiological and experimental reports on *L. major* and *L. tropica* which identify females as more susceptible. females are more resistant than males to cutaneous infection with *L. mexicana* (humans and mice) [5,12,31–33] and visceral leishmaniasis caused by *L. donovani* [34–35] or *L. infantum* (dogs) [36]. Female DBA/2 mice infected with *L. mexicana* develop much stronger Th1 responses, as measured by IFN-γ production, delayed-type hypersensitivity and IgG2a antibody levels, than similarly infected male mice [12,32]. Similarly, in humans infected with *L. mexicana*, females generally have increased Th1 responses as measured by DTH reactions and decreased Th2 responses as measured by IgE production than males [33]. The present studies using CD4+ T cell specific IL-4Rα−/− BALB/c mice have revealed a previously undetected, underlying male susceptibility to *L. mexicana* involving T cells. Thus, unlike female mice, male mice were unable to resolve infection and overall had a less polarized Th1 response and more polarized Th2 than their female counterparts. This was associated with IL-4 production independently of IL-4 signaling in male but not female CD4+ T cell specific (LckcreIL-4Rα/lox) IL-4Rα−/− BALB/c mice. The *L. mexicana* induced IL-4 producing Th2 phenotype in male LckcreIL-4Rα/lox BALB/c mice was not the result of differential Cre-mediated deletion efficiency of IL-4Rα in male mice as compared with female mice, as no CD4+ T cell population expressing IL-4Rα was detected from either gender (data not shown). While IL-4 production independently of IL-4 signaling has been observed in a number of immunological models previously [6,14–15] this is the first time a sex associated influence on this ability has been identified.
significant effect of sex hormones on CD4+ T cell function whereby infected male but not female CD4+ T cell specific IL-4Rα-/- mice can drive IL-4 production independently of IL-4Rα signaling.

Supporting Information

Figure S1 Female but not male T cell specific IL-4Rα-/- mice heal following L. mexicana infection. Mean lesion growth (Figure 1A and B) and male (Figure 1B) IL-4Rα intact (IL-4Rα+/lox), CD4+ T cell specific (LckcreIL-4Rα-/-lox) IL-4Rα-/-, and global IL-4Rα-/- mice infected sub-cutaneously with 5 x 10^6 amastigotes of L. mexicana. These are the results from 2 separate experiments carried out at different times the first using females (Figure 1A), the second using males (Figure 1B). While lesions healed in female CD4+ T cell specific (LckcreIL-4Rα-/-lox) mice they persisted in male CD4+ T cell specific (LckcreIL-4Rα-/-lox). Results are mean±s.e. Additional experiments utilised male and female groups infected in parallel. Found at: doi:10.1371/journal.pntd.0000930.s001 (0.20 MB TIF)

Figure S2 Similar parasite burdens in wild-type and T cell specific IL-4Rα-/- male mice 6 weeks post-infection. Mean lesion parasite burden ± s.e. at week 6 post-infection in male IL-4Rα intact (IL-4Rα+/lox), and CD4+ T cell specific (LckcreIL-4Rα-/-lox) IL-4Rα-/- mice infected sub-cutaneously with 5 x 10^6 amastigotes of L. mexicana. Found at: doi:10.1371/journal.pntd.0000930.s002 (0.11 MB TIF)
Figure S3 Less IgG1 production in infected female T cell specific IL-4Rα−/− mice. L. mexicana-specific IgG1 levels (Figure, 2A and B) and total IgE levels (Figure, 2C and D) in intact (IL-4Rα+/+), CD4 T cell specific (Lck−/−IL-4Rα−/−) IL-4Rα−/−, and global IL-4Rα−/− mice infected sub-cutaneously with $5 \times 10^6$ amastigotes of L. mexicana. *p<0.05, and ***p<0.001 compared with IL-4Rα intact mice. Representative of 4 separate experiments.

Found at: doi:10.1371/journal.pntd.0000930.s003 (0.38 MB TIF)

Author Contributions
Conceived and designed the experiments: FB JA. Performed the experiments: KJB ORM TM HAM JA. Analyzed the data: KJB ORM HAM JA. Contributed reagents/materials/analysis tools: FB JA. Wrote the paper: ORM FB JA.

References