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# Deletion of IL-4 Receptor Alpha on B Lymphocytes renders BALB/c Mice balance Th differentiation to Leishmania major Infection during the early stage of Infection.

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## ABSTRACT

B-lymphocytes are considered to play a minimal role in host defense against Leishmania major. 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). To investigate the relevance IL-4Rα mediated signalling in B Lymphocytes independently of non-B cells during the early stage in vivo infection, B-lymphocytes specific IL-4Rα deficient (MB-1-hCreIL4Rα$^{-/LOX}$) BALB/c mice were generated by gene targeting and site-specific recombination using the cre/loxP system under control of the MB-1 locus. DNA functional characterization through RT-PCR showed a balanced IL-4Rα expression on B-lymphocytes in MB-1-hCre IL4Rα $^{-/LOX}$ mice. We conclude from these data that the balanced expression shows that the Th differentiation doesn't happen in the early stage of infection and considered this an important host protective factor during early infection of cutaneous leishmaniasis.

## Indexing terms/Keywords

IL-4; transgenic mice; leishmaniasis; Th1 cells; Th2 cells.

## Academic Discipline And Sub-Disciplines

Immunology

## SUBJECT CLASSIFICATION

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## MATERIALS AND METHODS

### Mice

### Generation and genotyping of MB-1hCreIL-4Ra/lox BALB/c mice

Transgenic MB-1hCre mice [10] back-crossed to BALB/c for nine generations were intercrossed with IL-4Ra/ and IL-4Ralox/lox mice to generate MB-1hCreIL-4Ra-/lox BALB/c mice. WT littermates were used as controls in all experiments. Mice were genotyped as described previously [10]. All mice were housed in specific pathogen–free barrier conditions at the ICGEB, Cape town, South Africa and used in accordance with University ethical committee guidelines. All experimental mice were age and sex matched

### L. major infection

L. major LV39 (MRHO/SV/59/P) strain was maintained by continuous passage in BALB/c mice and cultured in vitro as described previously [11]. Mice were inoculated subcutaneously with 2 x $10^6$ stationary phase metacyclic promastigotes into the left hind footpad in a volume of 50 ll HBSS (Invitrogen). Swelling was monitored after the week of Innoculation using a Mitutoyo pocket thickness gauge (http: //www.mitutoyo. com).

### Detection of viable parasite burden

Infected organ cell suspensions were cultured in Schneider's culture medium (Sigma). Parasite burden was estimated according to a previously described limiting dilution method [11].

### Isolation of footpad and spleen cells

Muscle tissue was separated from infected footpads and digested in DMEM medium supplemented with Collagenase IV (Sigman-Aldrich; 1 mg/ml) and DNase I (Sigma-Aldrich; 1 mg/ml) at 37ºC for 60 min. Following incubation, single cell suspensions were isolated by straining through 40 mM cell-strainers. Spleen cells were isolated by pressing through 70 mM cell-strainers, red blood cell lysis was performed and white blood cells were washed and resuspended in 10% DMEM (Gibco).

### Quantitative RT-PCR

B-lymphocytes cells were stained with specific mAb and sorted from the Lymphnode of infected mice. Total RNA was extracted from B cells using Tri reagent (Applied Biosystems, Carlsbad, Calif) and mini-elute columns (Qiagen) according to the manufacturer's protocol. cDNA was synthesized with Transcriptor First Strand cDNA synthesis kit (Roche, France), and real-time PCR was performed by using Lightcycler Faststart DNA Master Plus SYBR Green I reaction mix (Roche Diagnostics, Meylan, France) on a Lightcycler 480 II (Roche). The 12-µl reaction mixture contained 1× LC FastStart DNA Master SYBR Green I, 2 mM MgCl2, 10 µM each primer, and 1.2 µl of template. Times and temperatures are shown in Table 1. For fluorescence signal acquisition, channel F1 was used and the gain was set at 5. For normalization of fluorescent data, the F1/1 ratio was applied. Primers for IFN-α: forward 5' GCTCTGAGACAATGAACGCT 3' and reverse 5' AAAGAGATAATCTGGCTCTGC 3', IL-12p35: forward 5' GATGACATGGTGAAGACGGCC 3' and reverse 5' GGAGGTTTCTGGCGCAGAGT 3', IL-10: forward 5' AGCCGGGAAGA-CAATAACTG 3' and reverse 5' CATTTCCGATAAGGCTTGG 3', IL-4: forward 5 CAGCTTAAGGATGCC-CAGGTT 3' and reverse 5' TCTCACAGTTTCTCGATGCCA 3'. Values were normalized according to the expression of the housekeeping genes rS-12.

### Statistics

Values are given as mean ± SD and significant differences were determined using Student's t test (Prism software, http://www.prism-software.com)

## INTRODUCTION

Phlebotomus spp acts a vector for the transmission of Leishmania spp. which cause cutaneous lesions to visceral Leishmaniasis, disease incidence is approximately 1.5 million per annum for cutaneous Leishmaniasis, and 500,000 per annum for visceral Leishmaniasis, become fatal if left unattended [1]. Currently there is no vaccine. Murine models of cutaneous Leishmaniasis have been established in non- healing in BALB/c mice is related to T helper (Th) 2 response characterized by secretion of interleukin (IL)-4, IL-5, IL-9 and IL-13 [2]. In contrast, healer C57BL/6 mice produce protective Th1 responses with production of IL-12 and IFN-α, associated with classical activation of macrophages and killing of parasites by effector nitric oxide production [3]. IL-4 and IL-13, both of which signal through a common receptor chain, the IL-4 receptor alpha (IL-4Ra) are known to be important susceptibility factors in L. major infection [4]. The healer and non-healer mice C57BL/6 and BALB/c mice secrete IL-4 early after infection however, production of IL-4 is sustained in susceptible BALB/c mice and transient in resistant C57BL/6 mice [5]. It appears that healer mouse strains redirect the early Th2 response in an IL-12-dependent mechanism, while in non-healer mice the Th2 response persists and dominates the disease outcome by suppressing effector mechanisms needed for parasite killing [2]. Though B-lymphocytes plays a minor role in the defence of BALB/c mice for Leishmaniasis major. Indeed, there is some evidence that B cells could play a role in the susceptibility to infection with L. major. First, anti-IgM-treated BALB/c mice control effectively their infection [6], and BALB/c Xid mice that lack the B1 B cell subset are more resistant to infection than controls [7]. Interestingly, B cells from susceptible BALB/c mice were shown to be better Th2 inducer than B cells from resistant C57BL/6 mice [8]. However, in contrast with these data, infection of mice genetically deficient in B cells with L. major generated conflicting

results. Indeed, both wild type and BALB/c MT mice were equally susceptible to infection with L. major and mounted a similar Th2 cell response [9]. Thus, given the different patterns of diseases obtained in B cell-deficient mice infected with L. major and the absence of clear evidence for a role of B cells in the susceptibility of BALB/c mice to infection, we analyzed in this study the susceptibility and T helper responses in BALB/c MT mice infected with L. major.

## RESULTS

Resistance to Acute and Chronic Leishmaniasis in MB-1hCreIL-4Rα/lox BALB/c mice controversy remains as to whether IL-4 [5] and/or IL- 4Ra signaling [11] are key components of susceptibility to L. major infection. Polarized Th2 cells certainly play a significant role in contributing to susceptibility [10]. To investigate the consequence of CD4+ T cell–specific IL-4Rα unresponsiveness in leishmaniasis, mice were infected subcutaneously with $2x10^6$ stationary phase metacyclic promastigotes of L. major LV39 (MRHO/SV/59/P). As expected, one week after infection wildtype resistant C57BL/6 mice developed nonhealing lesions were unable to control parasite burden with high parasite numbers in the footpads (Figure 1a) and lymph nodes (Figure 1b). IL-4Ra-/- mice showed lesser parasite load in the draining lymphnodes and footpad. However, as previously described [8], global IL-4Ra deficiency does not confer resistance to L. major infection, as the mice progressed to develop necrotic lesions in the chronic phase (Figure 1a). In contrast, MB-1hCreIL-4Ra-/lox mice were not able to resolve infection with lesion growth little higher when comparable with resistant wildtype C57BL/6 mice (Figure 1b).

### Balanced Type 1 and Type 2 responses in MB-1-hCre IL-4Ra -/lox of BALB/c Mice

IFN-α–driven iNOS production by macrophages is a key control mechanism in L. major infection [3][21]. We examined IFN-γ expression in MB-1-hCre IL4Rα $^{-/LOX}$ mice from the popliteal lymph node one week post infection, which shows IFN-γ production was equivalent with that of WT mice. The IL4Rα$^{-/-}$ mice produce more IFN-γ this may be due to absence of IL4Rα, which induces the IFN-α production. The MB-1-hCre IL4Rα$^{-/LOX}$ as they lack the IL4Rα may produce more IFN-α in later stage than IL4. Here y-axis indicates the number of copies of IFN-α mRNA/number of copies housekeeping gene rS-12 mRNA [Figure 2A]. IL-12 is a key protective cytokine involved in inducing protective responses following L. major infection. We examined IL-12 expression in MB-1-hCreIL4Rα$^{-/LOX}$ mice from the popliteal lymph node one week post infection. The IL-12p35 mRNA production was equivalent, when compared with that of WT mice. The IL4Rα$^{-/-}$produces less IL12p35 when compared with the MB-1-hCreIL4Rα$^{-/LOX}$ mice in the early stage of infection [Figure 2B].

IL-4 is a central cytokine for Th2 differentiation and previous studies have shown that B cells can produce IL-4 [3]. IL-4 and IL-13 share a common signaling pathway through the IL-4Rα chain [5], and so the role of this cytokines was studied in vivo in MB-1-hCreIL4Rα $^{-/LOX}$ mice from the popliteal lymph node which was one week post infection and compared with the wild type Balb/c mice. The production of the IL-4 in MB-1-hCreIL4Rα $^{-/LOX}$ mice is greater than that of the wildtype resistant C57BL/6 mice. The IL4 in wildtype resistant C57BL/6 mice was observed less when compared to MB-1-hCre IL4Rα$^{-/LOX}$ was evidenced from the study of [5] that no increase in IL-4 mRNA expression was observed in wildtype C57BL/6 resistant mice during the first 2 days of infection with L. major. The IL4Rα$^{-/-}$ Produces less IL4 than MB-1-hCre IL4Rα $^{-/LOX}$ mice may be due to absence of IL4Ra this reduces the IL4 production [9]. IL-10 is a potent anti-inflammatory cytokine, thus, we analyzed the effect of the IL-10 produced by MB-1-hCre IL4Rα $^{-/LOX}$ mice from the popliteal lymph node one week post infection. The level of IL10 in Balb/c WT mice was more than MB-1-hCre IL4Rα $^{-/LOX}$ in the early stage of infection with L.major. This result was demonstrated by that at early time-points during infection IL-10 production was significantly higher in the non-healer Th2 responder animals. Together, these data demonstrate that the early stage of infection (one week post infection) the production of the IFN-α, IL-4, IL12P35 and IL -10 is equal proportion.

## DISCUSSION

IL-4 and IL-13 share a common signaling pathway through the IL-4Rα chain and the combined role of both cytokines can be studied in vivo in IL-4Rα$^{-/-}$ mice. MB-1-hCre IL4Rα$^{-/LOX}$ mice will allow us to investigate the role of IL4 signalling specific on B cells, while maintaining IL4/IL13 mediated functions on non B cells. Here in the present study the analysis of all the data were mainly done by the RT PCR experiment by means of mRNA expression. In the initial stage of infection i.e one week post infection, due to the absence of IL4Rα especially to B cells resulted in consistently higher level of IFN-α secretion by the MB-1-hCreIL4Rα$^{-/LOX}$ mice compared with the wildtype resistant C57BL/6 mice. The IL4Rα$^{-/-}$ mice also produced higher IFN-α in the initial phase of infection. Increased IFN-α production correlated with protection against infection in MB-1-hCreIL4Rα$^{-/LOX}$ mice and wildtype resistant C57BL/6 mice mice. Together the results demonstrate that in the absence of IL4Rα signalling on the B cells leads to protective Th1 immune responses in cutaneous leishmaniasis result in effective macrophage activation and intracellular parasite elimination, during the initial stage of infection. This result was also confirmed by more IL12p35 mRNA production, which might have induced IFN-α production. L. major one week post infection induces a burst of IL-4 mRNA expression in the PLN cells of MB-1-hCreIL4Rα$^{-/LOX}$ mice. From MB-1-hCre IL4Rα$^{-/LOX}$ mice, we determined that early burst of IL-4 expression is IL-4Rα-independent IL-4 production. In contrast, infected IL4Rα$^{-/-}$ mice showed reduced IL-4 mRNA levels as that of WT mice this result correlates with [9] that the early IL-4 expression induced by L. major infection in BALB/c mice can be independent of signals mediated by IL-4Rα. IL-10 is a potent suppressor of macrophage activation, can abolish IFN-α/LPS–induced killing of L. major by macrophages, and can suppress development of DTH responses. IL10 production increased in BALB/c mice compared to WT mice and is a susceptibility factor for L.major infection. In contrast MB-1-hCreIL4Rα$^{-/LOX}$ mice and IL4Rα$^{-/-}$ mice produced higher IL10 compared to wildtype resistant C57BL/6 mice.

The results of L. major one week post infection in MB-1-hCreIL4Rα$^{-/LOX}$ mice shows the increased IL-10-secretion, early IL-12p35 mRNA induction, IL4 production, interferon-gamma production. These data demonstrate that in the early stage of infection with L. major IL4Ra independent IL4 production, subsequent IL-12 production leads to IFN-α production. This

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leads to the conclusion that in the early stage of infection (one week post infection) differentiation of $CD4^+$ T cells toward Th1 or Th2 cells is not taken, which shows Th1/ Th2 is in the balanced manner. But in the later stage of infection due to abrogation of IL-4R signalling in B cells will transform non-healer BALB/c mice to a healer phenotype. Furthermore, a beneficial role for IL-4Ralpha signaling in L. major infection will be revealed in which IL-4/IL-13-responsive non-B cells induce protective responses to MB-1-hCreIL4Rα$^{-/LOX}$ mice.

## ACKNOWLEDGEMENT

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## CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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## Legends to figures

**Figure 1** Mice were infected with L.major LV39 (MRHO/SV/5a/P0). Footpad swelling was measured after 1 week post infection (5 per group) subcutaneously with 2 x $10^6$ stationary phase metacyclic L.major promastigotes into the hind footpad 'N' indicates necrosis or ulceration/mouse. Parasite burden was determined by Limited Dilution Assay of cell suspension from homogenised footpad 1 week after infection as well as from poplitinial lymphnode

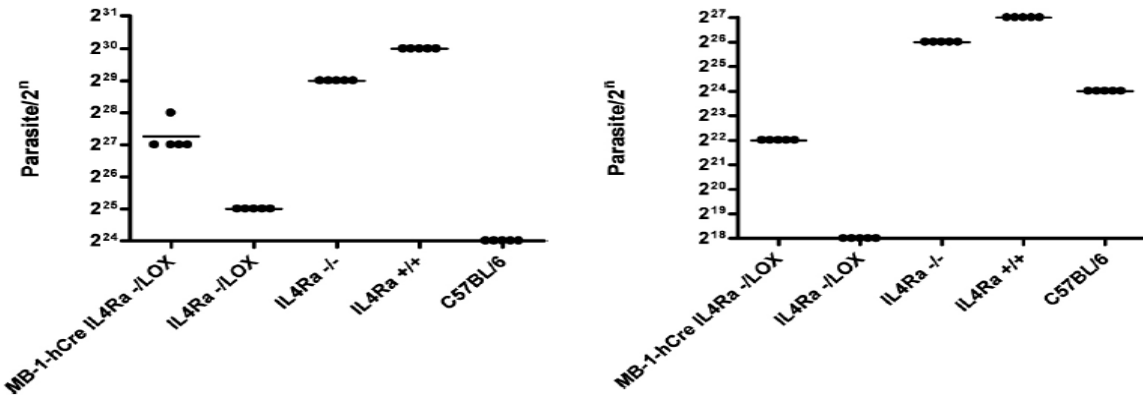

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**Figure 2** mRNA expression of IFNg, IL-12p35, IL-4, IL-10, was determined by real-time RT-PCR from sorted Lymphnode B cells. Expression was normalized against the housekeeping gene rs12.