

Cross-presenting dendritic cells are required for control of *Leishmania major* infection

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Leishmania major infection induces self-healing cutaneous lesions in C57BL/6 mice. Both IL-12 and IFN- γ are essential for the control of infection. We infected Jun dimerization protein p21SNFT (*Batf3*^{-/-}) mice (C57BL/6 background) that lack the major IL-12 producing and cross-presenting CD8 α ⁺ and CD103⁺ DC subsets. *Batf3*^{-/-} mice displayed enhanced susceptibility with larger lesions and higher parasite burden. Additionally, cells from draining lymph nodes of infected *Batf3*^{-/-} mice secreted less IFN- γ , but more Th2- and Th17-type cytokines, mirrored by increased serum IgE and *Leishmania*-specific immunoglobulin 1 (Th2 indicating). Importantly, CD8 α ⁺ DCs isolated from lymph nodes of *L. major*-infected mice induced significantly more IFN- γ secretion by *L. major*-stimulated immune T cells than CD103⁺ DCs. We next developed CD11c-diphtheria toxin receptor: *Batf3*^{-/-} mixed bone marrow chimeras to determine when the DCs are important for the control of infection. Mice depleted of *Batf3*-dependent DCs from day 17 or wild-type mice depleted of cross-presenting DCs from 17–19 days after infection maintained significantly larger lesions similar to mice whose *Batf3*-dependent DCs were depleted from the onset of infection. Thus, we have identified a crucial role for *Batf3*-dependent DCs in protection against *L. major*.

Keywords: *Batf3* • CD8 α ⁺ dendritic cells • *Leishmania major*



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Introduction

The immune response to *Leishmania major* in mice has highlighted the relevance of Th1/Th2 cell differentiation in determining the outcome of infection in vivo. C57BL/6 mice develop self-healing lesions characterized by a Th1 cell response with high levels of IFN- γ secreted by CD4⁺ T cells, which activates the antimicrobial

properties of macrophages [1, 2]. In contrast, BALB/c mice develop nonhealing lesions associated with Th2- and Th17-cell immune responses with high levels of IL-4, IL-5, IL-13, and IL-17 [3–6]. The adaptive immune response is largely initiated by dermal and draining lymph node (dLN)-resident DC subsets [7].

DCs are antigen-presenting cells expressing a large number of costimulatory molecules that efficiently prime T cells. Several DC subsets have been described based on their origin, phenotypic characteristics, and location [8]. However, the role of the different subsets in *L. major* is debated due to the varying routes of administration, strain of parasite, and dosage of parasite inoculated that

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have been used in the studies [4]. Inflammatory monocyte DCs (iDCs) develop at the site of infection, and upon infection, migrate to the dLNs where they present *Leishmania* antigens to T cells [9–11]. In addition, there are three major subsets of skin DCs that encounter *L. major* parasite after infection; Langerin⁺ epidermal Langerhans cells and two subsets of dermal DCs (dDCs); CD11b⁺ CD103[−] and CD11b^{low} CD103⁺. Among the dDCs, CD11b⁺ DCs carry *Leishmania* antigen to the dLN and contribute to T-cell priming [12, 13]. CD11b^{low} CD103⁺ dDCs have been implicated in transporting *L. major* antigen [14] but their role in T-cell priming has not yet been determined. Importantly, several groups have addressed the kinetics of antigen presentation following *L. major* infection. The first wave of antigen presentation is concomitant with the immigration of dDCs, inflammatory DCs and Langerhans cells early after infection and finally the dLN-resident cells themselves present antigens in *L. major* infection models [15–17]. Interestingly, Kopf et al. identified a second wave of antigen presentation at 15–21 days post high-dose *L. major* infection in both susceptible BALB/c and resistant B10. D2 mice attributed to dLN-resident DCs [18].

Once the *L. major* antigens reach the dLN either in infected innate cells or as soluble antigen via the lymphatic ducts, the LN-resident DCs appear to play a major role in T-cell priming. In the dLN, there are two subsets of conventional DCs (cDCs): CD11b⁺ CD4^{+/−} and CD8 α ⁺ Langerin⁺ cells. It has been shown that MHC-II expression restricted to these two subsets is sufficient to control *L. major* infection [19]. The larger population of cDCs, CD11b⁺ DCs, can acquire soluble *L. major* antigen and efficiently mediate T cell-specific immune responses [18]. CD8 α ⁺ DCs are the least permissive to infection by *L. major* among DC subsets but are the most efficient producers of IL-12 [20]. Importantly, IL-12 is crucial for the development of protective Th1 CD4⁺ T-cell differentiation in *L. major* infection and additionally, using IL-12p40^{−/−} mice, it was shown that IL-12 was also required to maintain resistance to *L. major* [5, 21, 22]. CD8 α ⁺ DCs are functionally related to dermal CD103⁺ DCs. Both cell types have the ability to take up apoptotic cells via DEC205 and necrotic cells via Clec9A [23, 24], as well as to activate CD8⁺ T cells by cross-presentation of antigen [25, 26].

Recently the role of CD8 α ⁺ and CD103⁺ DCs following infection with other intracellular pathogens has been determined in mice lacking these two DC cell subsets. Batf3 (Jun dimerization protein p21SNFT) is a transcriptional repressor of AP-1-mediated proteins required for the development of CD8 α ⁺ cDCs and CD103⁺ dDCs. Batf3^{−/−} mice have normal repertoires of other subsets of DCs but lack the CD8 α ⁺ and CD103⁺ DCs particularly in the spleen [27, 28]. Following infection with *Toxoplasma gondii*, the absence of Batf3 renders the mice more susceptible to infection in an IL-12-dependent manner [29]. Contrarily, following *Listeria monocytogenes* infection, the splenic Batf3-dependent DCs transport bacteria from the marginal zone to the periarteriolar lymphoid sheaths thereby contributing to an exacerbated disease outcome [30]. These two opposing outcomes in Batf3^{−/−} mice in response to intracellular pathogens indicate that the absence of Batf3-dependent DCs can have distinct effects on the immune response depending on the pathogen.

Despite our current understanding of the immune response to *L. major*, there is no vaccine available. DCs can effectively regulate the immune response to this pathogen, making them attractive targets for modulating anti-*Leishmania* immunity. We therefore investigated the role of Batf3-dependent DCs following infection with *L. major*. We found an increased susceptibility to *L. major* in Batf3^{−/−} mice on the otherwise resistant C57BL/6 background. Enhanced susceptibility in these mice was accompanied by higher parasite burdens as well as a change in the cytokine profile of the dLN with reduced IFN- γ , increased IL-13, IL-4, IL-10, and IL-17. Importantly, among the two Batf3-dependent in vivo *L. major*-primed DCs, CD8 α ⁺ cDCs but not CD103⁺ DCs were able to induce IFN- γ production by T cells from infected mice in ex vivo restimulation assays. To identify when the Batf3-dependent DC subsets were exerting their anti-*Leishmania* effects, we developed mixed bone marrow chimeras (CD11c-DTR $\times$ Batf3^{−/−}) to begin depletion of the DC subsets at different time points postinfection and maintain DC subsets in a depleted state. We found that the depletion of Batf3-dependent DCs in mixed bone marrow chimeras during the second phase of *L. major* antigen presentation in vivo (17 days postinfection) resulted in a similar enhanced susceptibility as in Batf3^{−/−} mice. This effect was mediated by IL-12, as neutralization of this cytokine from day 17 of infection gave similar increased susceptibility. Depleting cross-presenting DCs from day 17–19 was sufficient to cause increased susceptibility. Taken together, our data strongly suggests that Batf3-dependent DCs can be targeted to effectively induce a protective response.

Results

Batf3^{−/−} mice on a C57BL/6 background display increased susceptibility to *L. major*

Batf3^{−/−} mice were infected with 3×10^6 *L. major* promastigotes in the hind footpad and lesion size was monitored over several weeks. In comparison to WT C57BL/6 mice, Batf3^{−/−} mice developed significantly larger lesion size after 6 weeks of infection while in C57BL/6 mice, the lesions decreased thereafter (Fig. 1A). To study whether the enhanced lesion size correlated with increased parasite burden, we determined the parasite load at 3, 6, and 10 weeks postinfection. Parasite burden was significantly higher by 3 weeks and increased at 6 weeks postinfection, when significant differences in the lesion size between Batf3^{−/−} and C57BL/6 mice began to appear (Fig. 1B). The peak of infection at 10 weeks corresponded to the peak of parasite load as well. Importantly, similar results were observed comparing subcutaneous (s.c. in footpad) and intradermal (i.d. in ear) infections (data not shown). We also observed the lesion size development upon infection with a lower dose of parasites in the ear pinna (2000 metacyclic promastigotes) and determined the parasite load after 35 weeks of infection, which was past the peak of infection. Importantly, this dosage and route of administration also resulted in similar phenotype with an average lesion size that was transiently significantly larger in Batf3^{−/−} than their WT counterparts, accompanied with

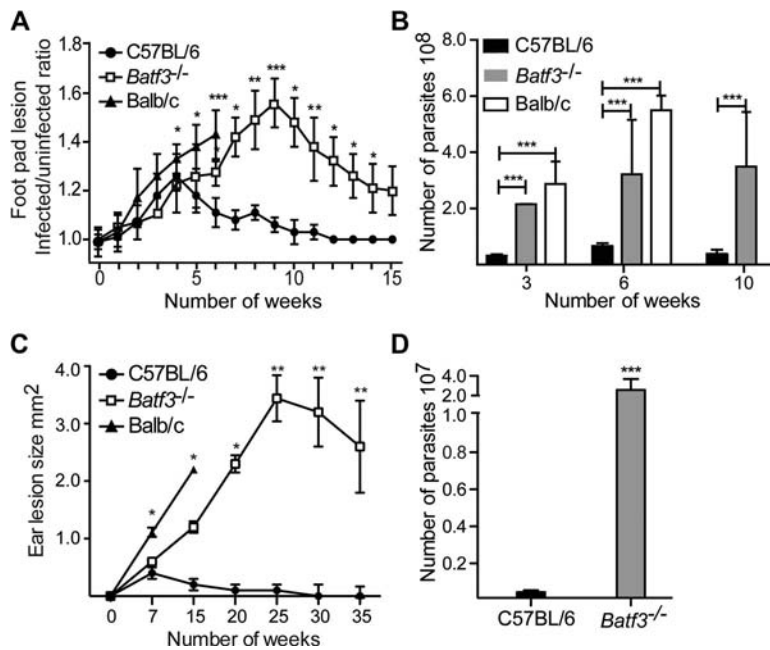


Figure 1. *Batf3*^{-/-} mice display an intermediate susceptibility to *L. major* infection. (A) C57BL/6 and *Batf3*^{-/-} mice were infected with 3×10^6 *L. major* promastigotes in the footpad and monitored for lesion size growth over 15 weeks. (B) Parasite load at the site of infection at 3, 6, and 10 weeks of infection as determined by the limiting dilution assay for the mice shown in (A). (C) Mice were infected with 2000 metacyclic parasites in the ear dermis and lesion size was followed at the indicated time points. (D) Parasite burden was determined at 35 weeks postinfection for the mice shown in (C). Data are from a single experiment representative of 2–3 independent experiments with $n = 6$ per experimental group and are shown as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's *t*-test).

higher parasite burdens (Fig. 1C and D). Heterozygous *Batf3*^{+/-} mice developed lesions similar to C57BL/6 mice (Supporting Information Fig. 1). Taken together, our data suggests that the absence of *Batf3*-dependent DC subsets in C57BL/6 mice compromises their ability to have an effective anti-*Leishmania* response. As both doses of parasites resulted in similar transient susceptibility, we thereafter used s.c. inoculation with 3×10^6 stationary promastigotes to investigate the immune response to *L. major* in *Batf3*^{-/-} mice.

L. major-infected *Batf3*^{-/-} dLNs have significantly higher numbers of cells including some DC subsets

We next assessed the composition of the dLN in *L. major*-infected mice at 3, 6, and 10 weeks postinfection. Mice were sacrificed at various time points and T cells, B cells, iDCs, CD11b⁺, CD8 α ⁺ as well as plasmacytoid DCs (pDCs) were identified using the gating strategy depicted in Fig. 2A. Infected *Batf3*^{-/-} mice had significantly higher absolute number of dLN cells at 6 and 10 weeks postinfection (Fig. 2B), a feature also observed in *L. major*-susceptible BALB/c mice (data not shown). Since differences in lesion size became significant 6 weeks postinfection, we analyzed the dLN cellular composition at this time point. There were no significant differences in the percentage and absolute numbers of CD4⁺ or CD8⁺ T lymphocytes (CD3⁺) in the dLN (Fig. 2C and D). In contrast, while the percentage of B cells (B220^{hi}) was similar between the different strains of mice (Fig. 2C), the absolute number was significantly higher in *Batf3*^{-/-} mice compared with C57BL/6 mice (Fig. 2D). Notably, as expected, the frequency of CD8 α ⁺ DCs was lower in *Batf3*^{-/-} mice compared with WT controls (Fig. 2E and F). *Batf3*^{-/-} mice showed similar frequency of CD11b⁺ and pDCs but higher absolute numbers relative to

C57BL/6 mice, also in agreement with the relative differences in dLN swelling. Together, the significantly enlarged dLNs of infected *Batf3*^{-/-} mice compared with C57BL/6 mice, and the increase in absolute number of B lymphocytes, CD11b⁺ cDCs and pDCs indicates an altered immune response in *Batf3*^{-/-} mice.

Batf3^{-/-} dLN cells produce less IFN- γ and more IL-4, IL-13, IL-10, and IL-17

Since IFN- γ production by CD4⁺ T cells is required to control parasite burden during *L. major* infection, we determined the IFN- γ production profile of T cells from the dLN cells of infected mice at 3, 6, and 10 weeks postinfection. We analyzed secretion of IFN- γ in ex vivo dLN cells that were restimulated with PMA and Ionomycin of *Batf3*^{-/-} mice and compared it with that of *L. major*-infected C57BL/6 mice. While CD4⁺ T cells from *Batf3*^{-/-} mice exhibited a tendency toward lower IFN- γ at other time points, this difference was significant only at 6 weeks postinfection (Fig. 3A). The supernatant from UV-treated *L. major*-restimulated dLN cells from *Batf3*^{-/-} infected mice also displayed lower IFN- γ production compared with dLN cells from WT mice at 3 weeks postinfection (Fig. 3B). The observed transient decrease in IFN- γ suggests that lower levels of IFN- γ likely do not account for the peak of infection seen at 10 weeks and that there are other cytokines that may contribute to the increased lesion size in *Batf3*^{-/-} mice.

We thus analyzed cytokine production from dLN cells from mice infected 3, 6, and 10 weeks postinfection that were restimulated in vitro for 72 h with UV-treated *L. major* promastigotes. The levels of IL-4, IL-10, IL-13, and IL-17 in the supernatant were then determined. IL-4, which acts synergistically with IL-13, was elevated significantly at 6 weeks postinfection in *Batf3*^{-/-} mice and was still higher at 10 weeks postinfection (Fig. 3C). IL-13, IL-10,

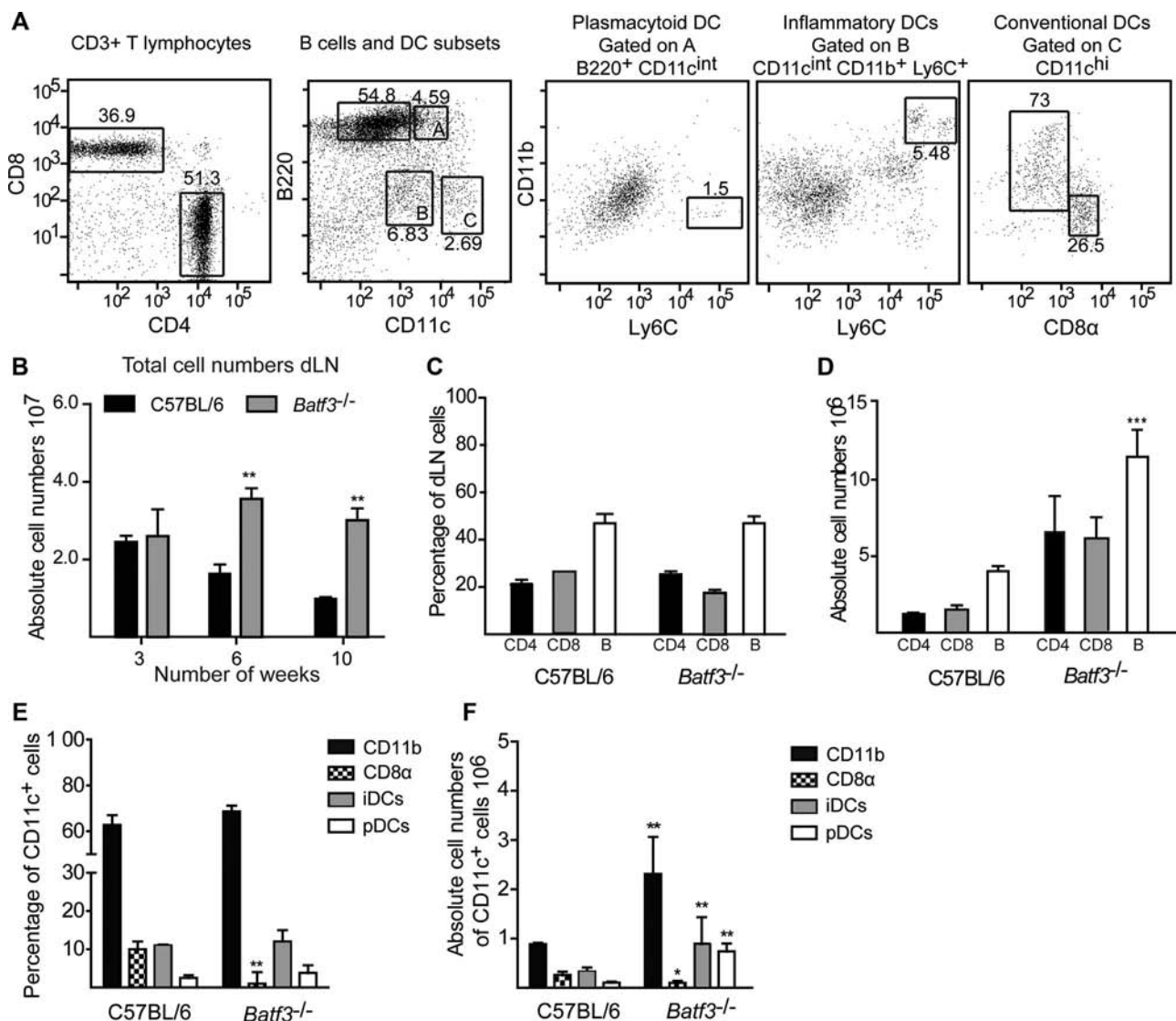


Figure 2. The dLN cell composition in *L. major*-infected *Batf3*^{-/-} mice displays altered cell populations. C57BL/6 and *Batf3*^{-/-} mice were infected with 3×10^6 *L. major* promastigotes in the footpad and dLNs were isolated at 3, 6, and 10 weeks postinfection. The gating strategies used to identify the various cell populations are shown in (A). (B) The absolute number of cells in dLNs at 3, 6, and 10 weeks postinfection is shown. Six weeks postinfection, the percentage and absolute cell numbers of (C, D) T and B lymphocytes and (E, F) the percentage and absolute number of CD11c⁺ DC subsets in the dLNs were determined. Data are shown as the mean + SD of four mice per group. The data are from a single experiment representative of two independently performed experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (Student's *t*-test).

and IL-17 were significantly higher in *L. major*-infected *Batf3*^{-/-} mice at 6 and 10 weeks postinfection in comparison with infected C57BL/6 mice (Fig. 3D–F). No IL-5 was detectable in the supernatants (data not shown). Altogether, the data from the cytokine profile revealed a weaker Th1 cell response accompanied by an enhanced production of Th2-type cytokines, Th17-type, and regulatory cytokines in *L. major*-infected *Batf3*^{-/-} mice.

As an alternative potential source of IL-10, we analyzed the frequency of CD4⁺ CD25⁺ FoxP3⁺ regulatory T (Treg) cells at 6 weeks postinfection, since it was after this time point that parasite burdens continued increasing in *Batf3*^{-/-} mice in contrast to C57BL/6 mice. At this time point, infected *Batf3*^{-/-} mice had a

significantly higher proportion and absolute number of Treg cells in their dLNs compared with their WT counterparts (Supporting Information Fig. 2A and B). Taken together with the high levels of IL-10 from restimulated dLN cells, this data suggests that the increased frequency of Treg cells could contribute to parasite persistence in *Batf3*^{-/-} mice.

We also determined the titers of *Leishmania*-specific immunoglobulin 1 (IgG1) (Th2-cell indicator) and IgG2a (Th1 cell indicator) in infected mice. We found that at 3, 6, and 10 weeks postinfection; *Batf3*^{-/-} mice had a significantly higher ratio of *Leishmania*-specific serum IgG1/IgG2a compared with C57BL/6 mice (Fig. 3G). Additionally, total serum IgGE was also elevated

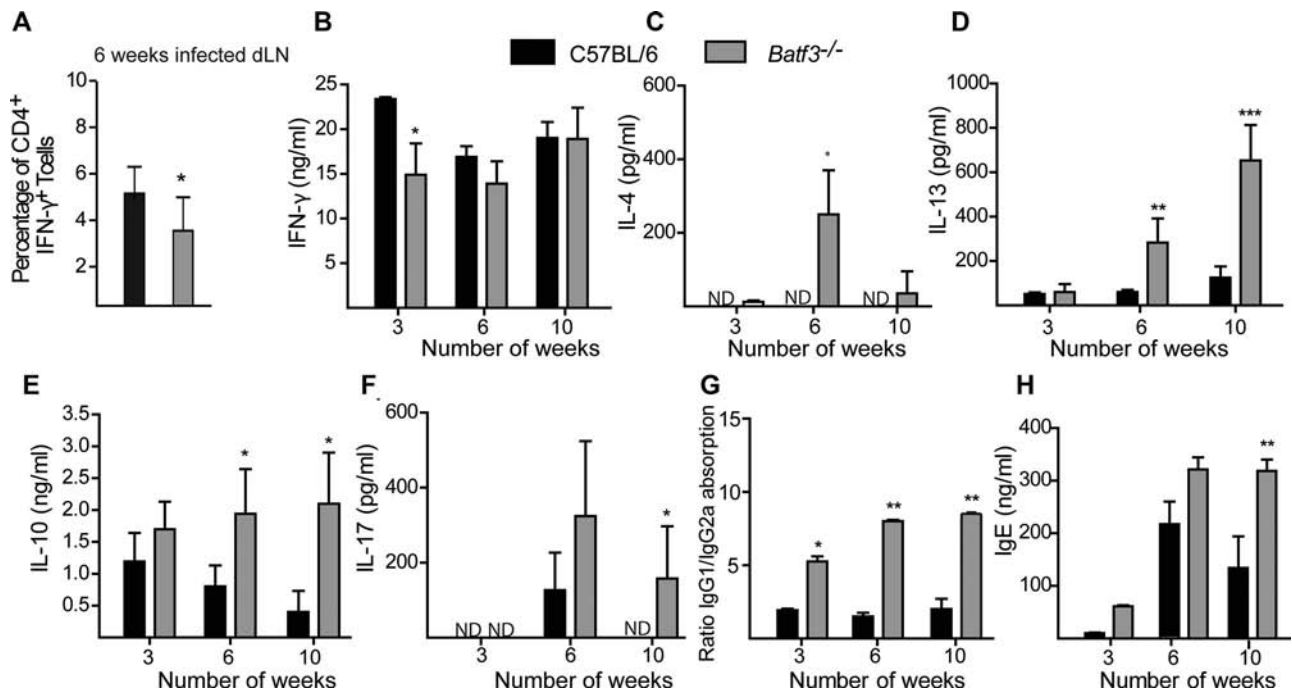


Figure 3. CD4⁺ T cells in the dLNs of *L. major*-infected *Batf3*^{-/-} mice produce transiently less IFN-γ. C57BL/6 and *Batf3*^{-/-} mice were infected with 3×10^6 *L. major* promastigotes 6 weeks prior to dLN cell isolation and restimulated in vitro for 4 h with PMA and ionomycin in the presence of Brefeldin-A. (A) Flow cytometry on CD3⁺ CD4⁺ T lymphocytes was performed as shown in Figure 2 (A) to determine the percentage of CD4⁺ IFN-γ⁺ T cells. (B–F) dLN cells were also restimulated with UV treated *L. major* in vitro for 72 h and the supernatant was analyzed for the levels of (B) IFN-γ, (C) IL-4, (D) IL-13, (E) IL-10, and (F) IL-17. (G and H) Serum from infected mice was tested for (G) the ratio of *L. major* specific IgG1 to IgG2a levels and (H) total IgE by ELISA. Data are shown as the mean + SD of four mice per group pooled from two independently performed experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. ND—Not detected (Student's *t*-test).

in infected *Batf3*^{-/-} mice (Fig. 3H). This data is in line with the in vitro restimulated dLN cytokine profile, with an enhanced Th2-type cytokine balance in *L. major*-infected *Batf3*^{-/-} mice.

CD8α⁺ DCs are more potent inducers of IFN-γ production by T cells ex vivo than are CD103⁺ DCs

To distinguish between the two *Batf3*-dependent subsets responsible for the Th1-cell response in WT C57BL/6 mice, we sorted CD8α⁺ DCs and CD103⁺ DCs from the dLNs of mice 4 weeks postinfection and compared their ability to induce IFN-γ production by in vivo-primed T cells after infection in vitro. DCs were isolated based on previously described surface markers: CD103⁺ dDCs (MHCII^{hi} CD11c^{int} CD11b^{low} CD103⁺) and CD8α⁺ DCs (MHCII^{hi} CD11c^{hi} CD8α⁺ cDCs) [26] (Fig. 4A). We isolated T cells from the same dLNs of mice 4 weeks postinfection and cocultured them with the different DC subsets, with and without the addition of soluble *Leishmania* antigen (SLA) for 72 h. Importantly, in the absence of added antigen, IFN-γ was detected in T cells exposed to CD8α⁺ DCs while no IFN-γ was detected with CD103⁺ DCs (Fig. 4B). Additionally, in the presence of SLA, CD8α⁺ induced 2.5-fold more IFN-γ compared with CD103⁺ DCs (Fig. 4B). Intracellular cytokine staining also displayed higher IFN-γ levels in CD4⁺ T cells cultured with SLA and CD8α⁺ DCs (Fig. 4C). No difference in the ability to affect proliferation of T cells was detected

and comparable low levels of IL-2 were observed in the presence of both types of DCs (data not shown). Thus, our results indicate that among the two *Batf3*-dependent DC subsets, CD8α⁺ DCs are superior in inducing production of IFN-γ by *L. major*-primed T cells ex vivo.

Cross-presenting DCs are required for a resistant Th1 response later than 17 days postinfection

Having established that *Batf3*-dependent DCs are required for resistance to *L. major* infection, we then addressed the time point at which these cells were exerting their anti-*Leishmania* effects. For this purpose we developed mixed bone marrow chimera mice in which we could begin depletion of *Batf3*-dependent DCs at different time points postinfection and keep them depleted throughout the course of infection.

We reconstituted lethally irradiated *Batf3*^{-/-} mice with a 1:1 ratio of bone marrow cells from CD11c-diphtheria toxin receptor (DTR) and *Batf3*^{-/-} mice (Fig. 5A). CD11c-DTR mice have the human diphtheria toxin (DT) receptor under the control of the CD11c promoter and upon administration of DT these mice are depleted of all CD11c-expressing DCs. Thus, when chimeric mice were administered DT, they were depleted of all subsets of DCs sensitive to DT (including *Batf3*-dependent DCs from CD11c-DTR

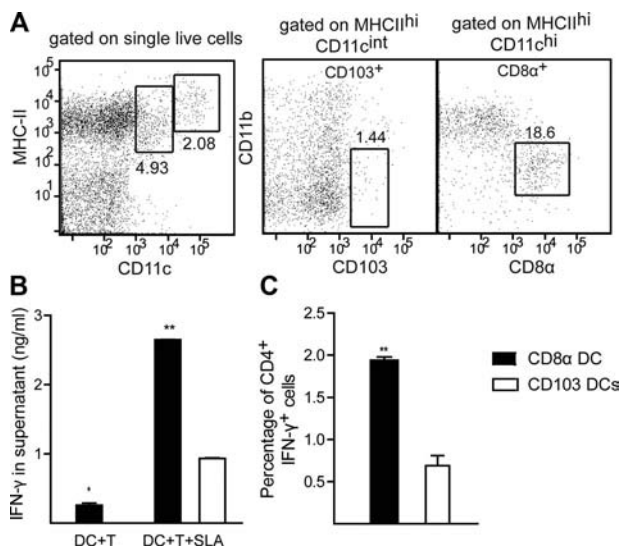


Figure 4. CD8α⁺ cDCs⁺ are potent inducers of IFN-γ from CD4⁺ T cells. (A) dLN cells from *L. major*-infected mice 4 weeks postinfection were pooled and DC populations were identified based on their surface markers. Flow cytometry plots are representative of two independent experiments. Cells were cocultured with primed-T cells purified by MACS. (B) Supernatants were collected after 72 h either in the presence or absence of SLA and analyzed for the presence of IFN-γ by ELISA. (C) IFN-γ production was assessed by intracellular flow cytometry in CD4⁺ T cells from the same animals. (B, C) Data are shown as the mean ± SD of eight mice pooled from two experiments performed in triplicates. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (Student's *t*-test).

bone marrow cells) and maintained all other subsets of DCs (from *Batf3*^{-/-} bone marrow cells) (Fig. 5B).

To control for chimera production, we also transferred C57BL/6 bone marrow cells into C57BL/6 mice and *Batf3*^{-/-} bone marrow cells into *Batf3*^{-/-} recipient mice, respectively (Fig. 5C). We compared the lesions in these mice to those in WT C57BL/6 and *Batf3*^{-/-} nonchimeric mice that were infected at the same time, and observed a similar increased susceptibility in the *Batf3*^{-/-} control mice (Fig. 5D). In groups that were administered DT, the toxin was administered every 3 days after the first injection throughout the 16 weeks of infection (Fig. 5E).

Subsequently, we set up groups of mixed bone marrow chimeras that differed by the starting time point of DT injections before or following *L. major* infection. To mimic the setup of *Batf3*^{-/-} mice, in one of the control groups we began depletion from day -1 (now lacks *Batf3*-dependent DC from the start, expected to become susceptible like *Batf3*^{-/-} mice). In the experimental groups we targeted depletion of *Batf3*-dependent cells during the first early wave of DC migration in the dLN (day 4) or second wave (day 17) of antigen presentation. Importantly, this time point correlates with the establishment of a resistant Th1 cell response following high-dose infection in WT mice (reviewed in [1]) and correlates with the second wave of antigen presentation [18]. We observed that following *L. major* infection, as expected, the group that was not administered DT (i.e. WT phenotype) developed healing lesions that were comparable to those of WT C57BL/6 mice. Contrarily, chimeras that received DT from

day -1 of infection developed an enhanced susceptible phenotype with larger lesions that took longer to resolve, similar to the chimera formation controls mentioned previously (Fig. 5F). Significantly, mice administered DT 17 days postinfection also displayed similar enhanced susceptibility (Fig. 5G). We also administered DT to chimera mice beginning 4 or 8 days postinfection and observed an enhanced lesion size (data not shown). Both DT-administered groups that were depleted of *Batf3*-dependent DCs displayed enhanced susceptibility and this corresponded to higher parasite burden in these mice 16 weeks postinfection (Fig. 5H). Taken together, the data from the mixed bone marrow chimera suggests that the removal of *Batf3*-dependent DC subsets during *L. major* infection 17 days postinfection, at a time corresponding to the establishment of a protective Th1 cell response, is detrimental to lesion healing.

In order to confirm the observation that the removal of *Batf3*-dependent DCs after 17 days of infection leads to enhanced susceptibility; we targeted IL-12 production and cross-presentation as potential mechanisms. We administered 500 μg of anti-IL-12 antibody (C17.15) to WT C57BL/6 mice twice a week to block the protective effects of IL-12 from 17 days until 10 weeks postinfection. A previous study has shown that the cross-presenting subset of DCs, particularly CD8α⁺ DCs, undergoes selective apoptosis upon administration of cytochrome c [31]. Due to the short in vivo half-life, we administered 5 mg horse cytochrome c subcutaneously, three times a day to WT C57BL/6 mice between days 17 and 19 postinfection with *L. major*. While anti-IL-12 was administered throughout the course of infection after 17 days, only a transient depletion from 17 to 19 days postinfection was performed by the administration of cytochrome c. We found that both groups of experimental mice displayed transient increased susceptibility compared to nontreated infected mice (Fig. 5I). These results highlight the requirement towards a protective immune response of cross-presenting, *Batf3*-dependent DCs from days 17–19 post-*L. major* infection.

Discussion

Experimental *L. major* infection is a useful tool to analyze the contribution of different DC subsets in the innate and adaptive T-helper cell immune response. Using *Batf3*^{-/-} mice, we demonstrate that the presence of *Batf3*-dependent DCs is critical for resistance to *L. major* infection.

Batf3^{-/-} mice developed exacerbated lesions compared with resistant WT C57BL/6 mice. The transient susceptibility observed in *Batf3*^{-/-} mice correlated with higher parasite burdens. We ruled out the possibility of the phenotype being affected by the dosage of parasite since the transient susceptibility was also observed following injection with a low dose of parasites, at a dose that is closer to the dose injected by the sandfly. The inability of susceptible mice to control parasite growth most likely results from the suppression of macrophage leishmanicidal activity resulting from the high levels of IL-13 and to a lesser extent IL-4 cytokines, which was reflected in the altered cytokine balance in the dLN.

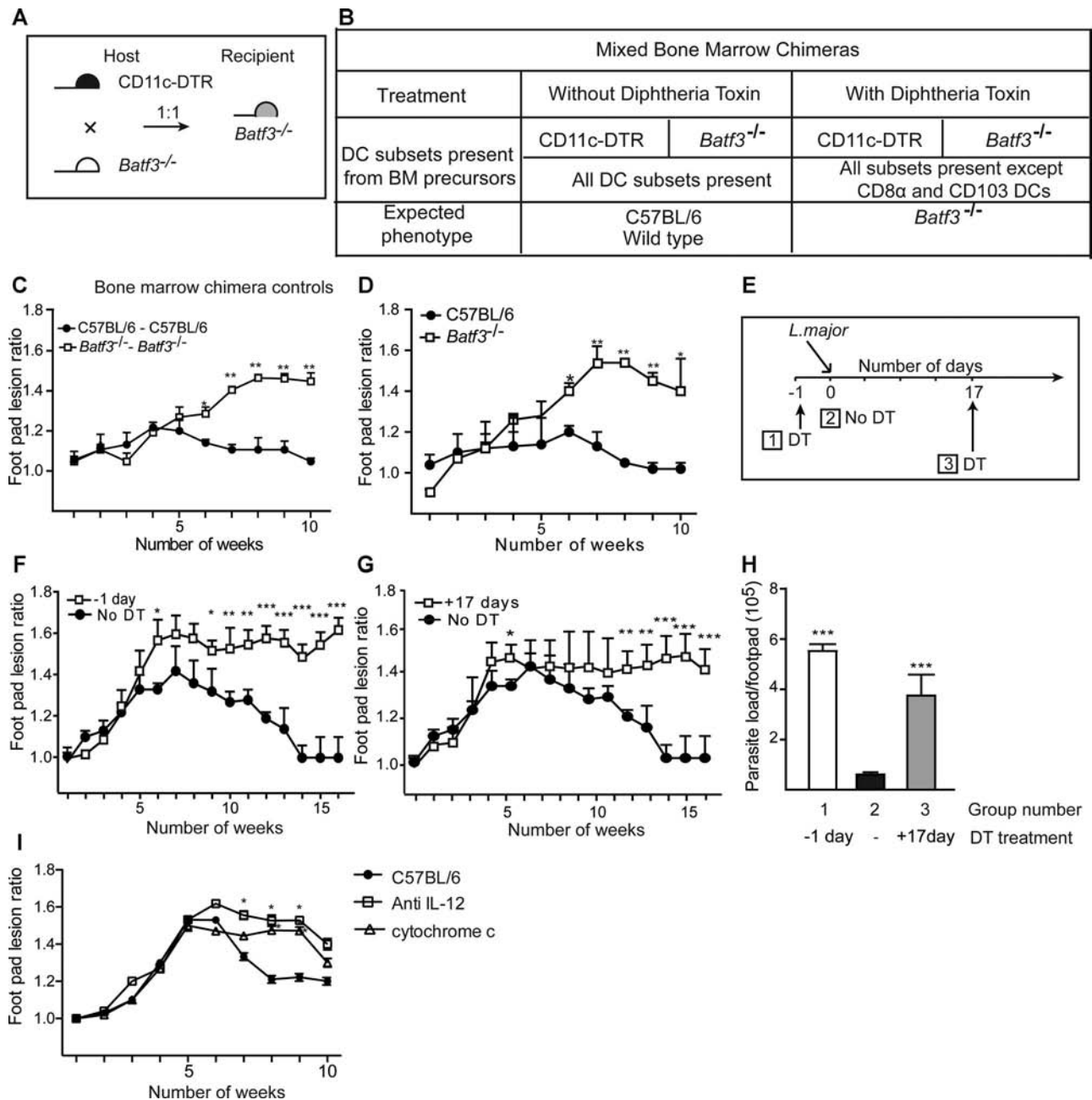


Figure 5. The absence of *Batf3*-dependent DCs adversely affects the establishment of a *L. major*-resistant response. (A) Experimental setup and generation of bone marrow chimeras. (B) The expected phenotypes in the mixed bone marrow chimeric mice with or without the administration of diphtheria toxin (DT). (C, D) Mice were infected with 3×10^6 *L. major* promastigotes and lesion size was monitored over several weeks. (E) Experimental setup for (F and G). DT administration was performed every 3 days and was continuous during the course of infection. Starting at (F) day -1 prior to infection and (G) 17 days postinfection, lesion size was monitored in control mice and mice administered DT. (H) Parasite burdens for the different groups were determined at 16 weeks postinfection. (I) C57BL/6 mice were infected with 3×10^6 *L. major* promastigotes and administered either anti-IL-12 (C17.15, twice a week until 10 weeks) beginning 17 days postinfection or cytochrome c between 17 and 19 days of infection (three times a day between days 17 and 19) and lesion size was measured. (C, D, F–I) Data are shown as mean $\pm$ SD of four samples representative of two independent experiments. For (C–I) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's *t*-test).

Following *L. major* infection, IL-13 was previously shown to contribute to the susceptibility since WT resistant C57BL/6 mice overexpressing IL-13 are susceptible [32]. The Th17 cell differentiation was also altered in *Batf3*^{-/-} mice since higher levels of IL-17 were found in dLN of *Batf3*^{-/-} mice compared with C57BL/6

mice. IL-17 in C57BL/6 mice does not appear to control the infection [6], while in the absence of IL-10, IL-17 was shown to contribute to enhanced susceptibility in these mice by inducing increased neutrophil infiltration at the site of parasite inoculation [33]. In addition, IL-10 has been implicated in maintaining a low

number of parasites in C57BL/6 mice following lesion resolution and *Batf3*^{-/-} mice dLN have elevated levels of this cytokine [34, 35].

Recently, effector CD4⁺ T cells have been identified as important producers of early IL-10 and vaccination with DCs suppressed this effect [36]. *Batf3*^{-/-} mice had elevated levels of IL-10 at 3 weeks postinfection as well as in later time points. CD4⁺ CD25⁺ Treg cells can also secrete IL-10 following *L. major* infection. However, the relative importance of IL-10 secretion by FoxP3⁺ Treg cells following *L. major* inoculation depends on several factors including the mouse strain and the parasite substrain inoculated (reviewed in [4]). C57BL/6 mice depleted of Treg cells developed similar lesion size to their WT counterparts [37]. However, the IL-10 secreted by Treg cells was critical for the maintenance of nonsterile immunity after the peak on infection [38]. In *Batf3*^{-/-} (C57BL/6 background) mice, enhanced number of Treg cells and higher levels of IL-10 secretion by dLN cells is observed, however further studies will be needed to understand their role in the observed susceptibility.

The transient enhanced susceptible phenotype observed in *Batf3*^{-/-} mice can be attributed to multiple factors. Previous studies have reported that shortly after infection or induced inflammatory conditions there can be a compensatory repopulation of CD8 α ⁺ and CD103⁺ DCs in *Batf3*^{-/-} mice [39–41]. Following *L. major* infection, only a minor repopulation was observed in *Batf3*^{-/-} mice 6 weeks postinfection, and the lesion size continued to increase thereafter. This minor repopulation was not sufficient to induce the differentiation of protective Th1 cells. The subsequent decrease in lesion size starting 10 weeks postinfection could however be explained by a repopulation of a low number of Batf-3-dependent cells.

Importantly, we identified which Batf3-dependent DC subtype contributes the most to the increased susceptibility phenotype observed in *L. major* infected *Batf3*^{-/-} mice. Ex vivo isolation of infected dLN DCs cocultured with primed T cells enabled us to identify the superior IFN- γ -induction capability of CD8 α ⁺ DCs in the presence or absence of added antigen compared with the dermal CD103⁺ cells. However, it is important to note that in the presence of added antigen, the CD103⁺ DCs did induce IFN- γ , thereby highlighting their intact antigen presenting capabilities. This is in accordance with a previous study wherein the Langerin⁺ dDCs were required for an effective early CD8⁺ T-cell response perhaps by cross-presentation [42]. Additionally, in that study, Langerin⁺ CD8 α ⁺ DCs are also likely to be depleted thereby implicating cross-presenting subsets in CD8⁺ T-cell-priming early in *L. major* infection. Taken together, this data suggests that both the subsets of DCs are involved in anti-*Leishmania* effects as migratory dDCs were shown previously to transfer antigens to CD8 α ⁺ DCs in the dLN and subsequently present them to T cells [15].

We addressed the timing of the contribution of the CD8 α ⁺ DCs following *L. major* infection by developing mixed bone marrow chimeras (CD11c-DTR $\times$ *Batf3*^{-/-}) in which we could deplete CD8 α ⁺ and CD103⁺ cells in *Batf3*^{-/-} recipient mice at specific

time points and maintain the depleted state over the course of infection. *L. major* antigen presentation in the dLN postinfection is thought to occur in two waves: the first wave within 1 day and the second wave commencing at days 15–21 [18]. The second wave of antigen presentation coincides with the establishment of Th1 effector cells, enabling WT mice to heal after infection [1]. Removal of Batf-3-dependent DCs at this critical time point rendered the mice incapable of controlling the infection. Their lesions harbored higher pathogen load and this correlated with higher serum of IgE levels (data not shown). The parasite load in mice depleted prior to infection was higher than the mice depleted 17 days postinfection although this did not reach significance in the mixed chimera experiment suggests that CD8 α ⁺ DCs could still play a role in the early phase of infection as well. This is in addition to other subsets of DCs and some innate cells that have been identified as sources of IL-12 in C57BL/6 mice early after *L. major* infection [21]. Collectively, the data suggest that Batf-3-dependent DCs contribute to Th1 cell differentiation to mice after the initial wave of antigen presentation and thus play a role later in infection immunity (reviewed in [43]).

Further, we analyzed if the distinct properties of Batf-3-dependent DCs namely IL-12 production and cross-presentation were possible mechanisms for the phenotype observed in *Batf3*^{-/-} mice. Previously, it was shown that the blocking of IL-12R using monoclonal antibodies at the beginning of infection until 24 days postinfection, resulted in exacerbated disease progression in the otherwise resistant C57BL/6 mice while using IL-12p40^{-/-} mice, it was determined that IL-12 played a role not only early after infection but also for the maintenance of resistance [44, 45]. In addition, in a vaccination model with LACK DNA, IL-12 was shown to be required at the initiation, maintenance and the effector phases of immune response to *L. major* [46]. Thus, using the same time window identified in the mixed bone marrow chimera model, we found that the mice with neutralized IL-12 17 days postinfection developed transiently enhanced lesion sizes compared to nontreated mice. The second possible contribution of Batf3-dependent DCs is perhaps cross-presentation. However, so far it has been shown that priming of T cells occurs via a TAP-independent pathway. Also, a more recent study identified that *Leishmania* parasites evade host immunity by directly cleaving the SNARE-VAMP8 pathway thereby inhibiting cross-presentation [47, 48]. We found that the removal of cross-presenting DCs by the administration of cytochrome c at the time window of 17–19 days postinfection resulted in a transient exacerbation of disease compared with WT mice.

In conclusion, our study highlights the requirement of Batf-3-dependent DCs in controlling cutaneous *L. major* infection in mice. These data open up the opportunity to further study the in vivo ability of these cells to induce IFN- γ by T cells. This will have important implications in vaccine design, as targeted vaccine delivery to Batf-3-dependent DCs could potentially be used to induce a robust immunization against infection.

Materials and methods

Mice

Wild type C57BL/6 and BALB/c mice were purchased from Harlan and Charles River, respectively. Additional tenth generation backcrossed C57BL/6 *Batf3*^{-/-} mice were purchased from Jackson Labs [27]. Mice were age and sex-matched for each experiment and were generally between 6 and 15 weeks old. All mice were maintained according to the institutional guidelines at the University of Lausanne, Biochemistry Department.

Parasites and infections

L. major LV39 stationary phase promastigotes were used in all experiments unless noted otherwise. The isolates were derived from infected DBA mice footpads wherein the egressed parasites were filtered and maintained at 26°C in complete M199 medium (10% fetal bovine serum, 4% HEPES, 1 U/mL penicillin, and 100 µg/mL streptomycin). For infections, the parasites were counted and injected subcutaneously (3×10^6) in PBS in the hind footpad of mice for most experiments. For parasite burden determination, infected mice footpads were surgically removed and homogenized in complete M199 and limiting dilution assay was performed as described previously [49]. For intradermal infections, 2×10^3 metacyclic promastigotes were injected into the dermis in a final volume of 10 µL. Experiments were performed three times with 25 mice in total. For parasite determination, the limiting dilution assay was performed with 1×10^6 homogenized ear cells.

Isolation and analysis of dLN cells and tissues

dLNs from infected mice were isolated at different times after infection and digested in medium containing 1 mg/mL collagenase-D and 40 µg/mL of DNAase at 37°C for 15 min. For experiments analyzing intracellular cytokines, 1×10^6 cells were stimulated with 50 µg/mL of PMA, 500 µg/mL Ionomycin, and 1 µg/mL of Brefeldin A for 4 h at 37°C and surface stained. Cells were then fixed with 4% PFA for 30 min at room temperature followed by permeabilization overnight at 4°C with Saponin buffer (PBS, 3% FCS, 0.5% Saponin). Staining for IFN-γ (clone XMG1.2) and IL-4 (clone 11B11) was then performed at 4°C for 30 min. Isotype controls included Rat IgG1 (clone eBRG1) and Rat IgG2a (eBR2a). For footpad homogenization, infected foot pads were surgically removed and homogenized in HBSS for 30 min. The cells were then filtered using 40 µm filter and suspended for parasite count determination. For ear cell isolation, infected ears were surgically removed and homogenized in cDMEM containing 0.2 mg of Liberase TL (Roche) for 2 h. The cells were then washed in cDMEM and the supernatants used for parasite count determination.

Flow cytometry

Cells were incubated with antibodies in the presence of Fc receptor block 24G2 solution in FACS buffer (PBS, 0.5 M EDTA, and 2% FCS). Surface staining was done for 30 min at 4°C. The following antibodies were purchased from Ebioscience FITC-anti CD11b [50], PE-anti CD3e (145-C11), allophycocyanin-anti Ly6C (HK1.4), Alexa 700-anti MHC-II (M5/114), PeCy7-anti CD11c (N418), PerCP-Cy5.5-anti CD8a, allophycocyanin-Cy7-anti CD4 (GK1.5), E-fluor 450-anti B220 (RA3-6B2), PerCP-cy5.5- anti Dec-205, PeCy7- anti IFN-γ, and PE-anti IL-4.

ELISA

ELISA kits for IL-4, IFN-γ, IL-5, IL-13, IL-17, and IL-10 were purchased from E-bioscience and performed as per manufacturer's instructions. Serum IgG1, IgG2, and IgE were determined using ELISA kits from Bethyl Inc.

Anti-IL-12 and cytochrome c administration

Five hundred microgram of anti-IL-12 (C17.15) was administered intraperitoneally in a final volume of 500 µL biweekly for 7 weeks after 17 days of infection. Horse cytochrome c (Roche) was prepared as per manufacturer's instructions in PBS and three injections daily for a period of 3 days (days 17–19 postinfection inclusive) was given subcutaneously.

Bone marrow chimera generation and DT administration

Recipient 8-week-old C57BL/6 and *Batf3*^{-/-} mice were lethally irradiated at 9.6 Gy while BALB/c mice were irradiated at 7.5 Gy, respectively. Twenty-four hours later, bone marrow cells from donor 6-week-old CD11c-DTR and *Batf3*^{-/-} mice were mixed in 1:1 ratio and injected retro-orbitally into recipient mice. A total of 10×10^6 cells were injected. Mice were then bled 8 weeks after injection of cells and monitored for chimerism using real-time PCR on genomic DNA with primers detecting the deleted *Batf3* gene using albumin for comparison (forward deleted exon 1 and 2 mutants—5' GATCGACCTTTGAATGTGTGA 3', Reverse 5' GATACAGGCTGCTGATCTGAG 3'). Mice were also infected with *L. major* at 3×10^6 parasites in the hind footpad. Lesion size was monitored over 15 weeks. In order to maintain the depletion of all CD11c-DTR sensitive cells, mice of the relevant groups were administered with DT at 4 ng/g body weights every three days throughout the course of infection starting at day -1, 4, 8, or 17 of infection.

Ex vivo DC culture

C57BL/6 mice were infected with 3×10^6 LV39 *L. major* in the footpad and dLN cells were isolated 4 weeks after infection.

CD11c⁺ cells were then sorted (BD FACS Aria) on the basis of the expression of CD11c, MHCII, CD103, and CD8 α . Subsequently, a negative selection for both CD4⁺ and CD8⁺ T cells were purified from dLNs of the same mice using the MACS Pan T cell isolation kit II (Miltenyi 130–095–130) and stained with proliferation dye Efluor 670 (Ebioscience 65–0840–85). Sorted DCs and T cells were cocultured for 5 days in the ratio of 1:15 with or without 20 μ g/mL of *L. major* freeze thaw isolate. Supernatant was collected for cytokine estimation using ELISA and cells were collected for intracellular IFN- γ after stimulation with PMA, Ionomycin in the presence of Brefeldin A.

Statistical analysis

Data was analyzed using SigmaPlot (Sistat). Statistical analysis using the two-tailed Student's *t*-test was performed where indicated. Differences were considered significant at *p* < 0.05.

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Abbreviations: Batf3: Jun dimerization protein p21SNFT · CDC: conventional DC · dDC: dermal DC · dLN: draining lymph node · DT: diphtheria toxin · DTR: diphtheria toxin receptor · IgG: immunoglobulin · SLA: soluble *Leishmania* antigen

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Cross-presenting dendritic cells are required for control of *Leishmania major* infection

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Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision – 9 December 2013

Dear Prof. Acha-Orbea,

Please accept my sincere apologies for the prolonged delay in processing the review of your manuscript ID ej.201344242 entitled "CD8 α + Dendritic Cells are Required to Control *Leishmania major* Infection", but there was a severely delayed report. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication.

You should also pay close attention to the editorial comments included below. *In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.*

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If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Laura Soto Vazquez

On behalf of Prof. Bernard Malissen

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Reviewer: 1

Comments to the Author

In this manuscript, the authors examine the role of Batf3-dependent DC subsets in controlling L.major infection. Although this study is potentially of great interest, there are a number of concerns that preclude publication of the manuscript.

Major points

- Batf3^{-/-} mice lack both resident CD8⁺ DCs and migratory CD103⁺ DCs. In the manuscript, the authors emphasize the major role of CD8⁺ DCs, however they do not properly address the contribution of CD103⁺ DCs. If the authors wish to conclude that CD8⁺ DCs are more important than CD103⁺ DCs, they need to provide additional evidence, especially in vivo (the authors could use mice deficient for CD103⁺ DCs). If not, the authors need to modulate their conclusions and change the title of the manuscript.
- A key reference is missing : Priming of CD8⁺ and CD4⁺ T cells in experimental leishmaniasis is initiated by different dendritic cell subtypes.

Brewig N, Kissenpfennig A, Malissen B, Veit A, Bickert T, Fleischer B, Mostböck S, Ritter U. J Immunol. 2009 Jan 15;182(2):774-83.

In this article, the authors have examined the role of Langerin⁺ DCs in L.major infection, using exactly the same settings as the authors. Because Langerin⁺ DCs correspond to Langerhans cells and CD103⁺ DCs,

Peer review correspondence

the results shown in this article are particularly relevant to the present study. The authors need to cite this article and discuss the results shown in the light of their own data.

- The analysis of CD103+ DCs shown in figure 4 need to be performed again. The gating strategy does not allow proper isolation of CD103+ DCs (the cells that are present in the gate are scattered and do not form a defined population). Migratory DCs have high MHC II molecule expression : the MHC II^{high} CD11c^{int} gate set by the authors actually contains a lot of cells with lower MHC II expression, while the cells with the highest expression were left out. I would suggest the following gating strategy : first a broad gate on CD11c⁺ MHC II⁺ cells, then a CD11b vs CD8 staining : CD8⁺ CD11b⁻ cells are resident CD8⁺ DCs, finally on CD11b⁻ CD8⁻ cells a staining CD103 vs CD205 : the CD103⁺ CD205⁺ cells are migratory CD103⁺ DCs.
- in figure 5, it seems that the "17 days" condition was performed only once (according to the legend). If this is the case, this experiment needs to be repeated. Although it is a heavy experiment, it is not acceptable to publish the results of an experiment performed only once.

Minor points

- the number of mice and independent experiments is not indicated in the legend of figure 2, figure 3 and supplementary figures
- in the summary, the authors state that Batf3^{-/-} mice are incapable of controlling the infection, however in the text they write "increased susceptibility" and in the legend "intermediate susceptibility". This is confusing. In figure 1A, results show that Batf3^{-/-} mice can control the infection in part, because the lesion size reduces after 10 weeks, therefore the statement that the mice can not control the infection seems too strong.

Reviewer: 2

Comments to the Author

Ashok et al. describe the effect of a Batf3-deficiency in a high and low dose model of murine L. major infection using gene knockout mice. Batf3-deficiency results in a lack of the CD103⁺ and the CD8a⁺ dendritic cell (DC) populations of which the latter are thought to be important for cross-presentation and for IL-12 production. The authors show convincingly that Batf3 deficient mice are less capable of controlling L. major infection after high dose infection and seem to lose control in the low dose infection model. They profiled LN cellularity as well as cytokine production and correlated this with infection outcome. Importantly, by generating bone marrow chimeras reconstituted with C11c promoterdriven Diphtheria toxin receptor transgenic, Batf3^{+/+} and batf3^{-/-} mixed donor bone marrow and by timed deletion of the batf3-proficient CD11c positive cells in these mice by Diphtheria toxin they show that batf3-dependent CD11c positive cells are required for infection control and healing even when starting depletion 17 days post-infection.

The study provides novel data and further support for a role for continuous stimulation of a protective immune response by certain DC subtypes.

The following specific points, however, deserve the authors' attention:

1. Title and Abstract last sentence (and p9 line 232-236): The claim that a crucial role of Batf3-dependent CD8a⁺ DC is demonstrated should be toned down. What the authors show is that continuous depletion of wt CD11c populations either 1 day before or 17 days after high dose L. major infection prevents healing in Batf3-deficient bone marrow chimeras with wt and Batf3^{-/-} bone marrow chimerism. In addition they show that CD8a⁺ DC isolated from infected mice present antigens to CD4 T cells and stimulate better IFN- γ release than a comparator population namely the also Batf3-dependent CD103⁺ DC population. Since neither experiments with transient depletion, nor experiments where in wt DC depleted mice were repopulated with CD8⁺ DC population were included the claim is only supported by inference.
2. Fig. 1D: A parasite load of greater 2×10^{10} is reported and should correspond to an ear lesion size of less than 3 mm². This is difficult to believe since 10^{10} packed parasite cells will already have a volume above 0.5 ml. Please check method.
3. Fig. 2: Why is the sum of CD4, CD8, and B cells only representing less than 50 % of the absolute number of LN cells counted (c.f. fig. 2B and D, respectively)? Similar inconsistencies are present when comparing fig. 2E with 2F (e.g. CD8a⁺ CD11c positive cells in B6 mice).
4. The details of the statistical tests used are insufficiently described to fully assess the results. For example, fig. 3 A which shows the transient but highlighted effect on IFN- γ , how many independent observations were made (what was n?) and was the test used one or two tailed? This information should be added and is particularly relevant for the results shown in fig. 3, fig. 4 (in this case only two independent observations are indicated in the legend and probably only one is shown, hence statistics is not really meaningful), and fig. 2 in supplementary data.
5. Discussion: p11 line 310: The view that IL10 secreted by Tregs is critical for the maintenance of immunity because sterile eradication of the pathogen is prevented has recently been challenged (see Schwarz et al. 2013). Please update.
6. p.10 line 267: lesions seem not to resolve at all (Fig. 5). Please comment.

Minor points:

1. p. 10 line 273: Text refers to a fig. 4H and to 16 weeks, however, it seems that fig. 5H is meant and there the time point mentioned is week 15. What is correct?

First Revision – authors' response – 27 December 2013

Reviewer 1:

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Major points:

1) "Batf3^{-/-} mice lack both resident CD8⁺ DCs and migratory CD103⁺ DCs. In the manuscript, the authors emphasize the major role of CD8⁺ DCs, however they do not properly address the contribution of CD103⁺ DCs. If the authors wish to conclude that CD8⁺ DCs are more important than CD103⁺ DCs, they need to provide additional evidence, especially in vivo (the authors could use mice deficient for CD103⁺ DCs). If not, the authors need to modulate their conclusions and change the title of the manuscript".

- We agree with the reviewer's assessment of our analysis regarding the use of the model Batf3^{-/-} mice that lack both the subsets of DCs, CD8 α ⁺ and CD103⁺. In the current state, the study does not rule out the in-vivo role for CD103⁺ DCs but does show ex-vivo studies suggesting the relative importance of CD8 α ⁺ DCs. We have thus re-framed our title to "Cross-presenting dendritic cell subsets are required to control *Leishmania major* infection". Additionally, we have stressed the role played by both subsets of DCs in the discussion and conclusion of the paper.

2) "A key reference is missing: Priming of CD8⁺ and CD4⁺ T cells in experimental leishmaniasis is initiated by different dendritic cell subtypes. Brewig N, Kissenpfennig A, Malissen B, Veit A, Bickert T, Fleischer B, Mostböck S, Ritter U. J Immunol. 2009 Jan 15;182(2):774-83".

- We thank the reviewer for this contribution. We have referred to this article in our discussion (line 350) and we have paid due attention to this paper since it highlights the role of Langerin⁺ dDCs in CD8 T cell priming particularly in the early phase of infection perhaps by cross-presentation. We addressed the possibility of Langerin-DTR also being depleted of CD8 α ⁺ DCs upon administration with DT and hence the phenotype can be affected by the lack of this subset as well. We have also discussed how the early DT administration (4 and 8 days post infection) in our model of mixed bone marrow chimera displays enhanced susceptibility which could be attributed to the role of Langerin⁺ dDCs in priming an early CD8 T cell response

3) "The analysis of CD103⁺ DCs shown in figures 4 need to be performed again. The gating strategy does not allow proper isolation of CD103⁺ DCs (the cells that are present in the gate are scattered and do not form a defined population). Migratory DCs have high MHC II molecule expression : the MHC IIhigh CD11c⁺ gate set by the authors actually contains a lot of cells with lower MHC II expression, while the cells with the highest expression were left out. I would suggest the following gating strategy : first a broad gate on CD11c⁺ MHC II⁺ cells, then a CD11b vs CD8 staining : CD8⁺ CD11b⁻ cells are resident CD8⁺ DCs, finally on CD11b⁻ CD8⁻ cells a staining CD103 vs CD205 : the CD103⁺ CD205⁺ cells are migratory CD103⁺ DCs"

To address this concern, we applied the suggested the gating strategy to our data: a first broad gate on CD11c⁺ MHC-II⁺ cells, then a CD11b vs CD8 α staining: CD8 α ⁺ CD11b⁻ (lymphoid tissue resident cells)

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then on CD11b- CD8- cells a staining SSC-A and CD103 as the dermal DCs. We found that this population overlapped with the one we had used for sorting. In addition, we have also performed this experiment twice using another gating strategy: a first broad gate on CD11chi and then CD11b- CD8α+ dLN cells and a second CD11cint gate followed by CD11b- CD103+ migratory DCs. Importantly, sorted populations from both experimental setups gave comparable results. We thank the reviewer for his consideration of the data in light of the multiple strategies that we used that gave comparable results to address this question.

5) "in figure 5, it seems that the "17 days" condition was performed only once (according to the legend). If this is the case, this experiment needs to be repeated. Although it is a heavy experiment, it is not acceptable to publish the results of an experiment performed only once"

- We thank the reviewer for the opportunity to explain the rationale behind performing this experiment. The data with depletion beginning at 4 days and 8 days was performed twice together with the controls. Instead of repeating the long chimera experiments and to avoid use of diphtheria toxins we treated mice with anti-IL12 antibodies from day 17 until the end or, alternatively, depleted the cross-presenting DC from day 17-19 post infection. We now include these results to substantiate the observation of the critical 17-day time point. Based on the increased susceptibility of the mice whose Batf-3 dependent cells were depleted 17 days post infection, we blocked IL-12 for the course of infection after 17 days of infection and in a second series of experiments, administered cytochrome C to deplete cross-presenting cells (i.e. CD8α+ and CD103+ DCs).

We show the following findings in WT C57BL/6 mice injected with L. major:

- (i) Anti-IL12 administration post 17 days of infection: the mice displayed a transient enhanced susceptibility similar to infected Batf-3-/- mice.
- (ii) Cytochrome C administration at days 17-19 post infection: the mice displayed a transient enhanced susceptibility albeit slightly less than that with anti-IL-12.

Taken together these data highlight the requirement of Batf-3 dependent cells at 17-19 days after infection. We would like to submit this data as part of Figure 5 and have discussed it likewise in the discussion. We thank the reviewer for his consideration in the light of this novel data supporting the study.

Minor Points:

- "The number of mice and independent experiments is not indicated in the legend of figure 2, figure 3 and supplementary figures "

We have duly incorporated this information in the legends of figure 2, 3 and the supplementary figures.

- "In the summary, the authors state that Batf3^{-/-} mice are incapable of controlling the infection, however in the text they write "increased susceptibility" and in the legend "intermediate susceptibility". This is confusing. "

We agree with the reviewer and have maintained 'increased susceptibility or enhanced susceptibility' throughout the text.

- "In figure 1A, results show that Batf3^{-/-} mice can control the infection in part, because the lesion size reduces after 10 weeks, therefore the statement that the mice can not control the infection seems too strong"

We agree with the reviewer that there is partial control of infection in our model and have thus rephrased it to 'Batf3^{-/-} mice display significantly increased susceptibility that is transient'.

Reviewer 2:

1. Title and Abstract last sentence (and p9 line 232-236): The claim that a crucial role of Batf3-dependent CD8a⁺ DC is demonstrated should be toned down. What the authors show is that continuous depletion of wt CD11c populations either 1 day before or 17days after high dose L. major infection prevents healing in Batf3-deficient bone marrow chimeras with wt and Batf3^{-/-} bone marrow chimerism. In addition they show that CD8a⁺ DC isolated from infected mice present antigens to CD4 T cells and stimulate better IFN- γ release than a comparator population namely the also Batf3-dependent CD103⁺ DC population. Since neither experiments with transient depletion, nor experiments where in wt DC depleted mice were repopulated with CD8⁺ DC population were included the claim is only supported by inference.

- We agree with the reviewer that the data shown is better represented with highlighting both subsets of Batf-3 dependent cells in the title and the abstract. To this end, we have changed the title, abstract and the text to indicate both subsets of DCs.

2. Fig. 1D: A parasite load of greater 2×10^{10} is reported and should correspond to an ear lesion size of less than 3 mm². This is difficult to believe since 10^{10} packed parasite cells will already have a volume above 0.5 ml. Please check method.

- We thank the reviewer for this observation and we have checked our data and methods and found the error. The calculations from the limiting dilution assay have been re-analyzed and parasite counts to the order of 10^7 were observed. We apologize for this error and have duly corrected it in the figure.

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3. Fig.2: Why is the sum of CD4, CD8, and B cells only representing less than 50 % of the absolute number of LN cells counted (c.f. fig. 2B and D, respectively)? Similar inconsistencies are present when comparing fig. 2E with 2F (e.g. CD8a+ CD11c positive cells in B6 mice).

- We have checked this data and we thank the reviewer for this observation in the error with Batf3^{-/-} 6-week lymphocyte counts and the dendritic cell counts of B6 mice. This information has been re-analyzed and corrected in Figure 2 accordingly.

4. The details of the statistical tests used are insufficiently described to fully assess the results. For example, fig. 3, A which shows the transient but highlighted effect on IFN- γ , how many independent observations were made (what was n?) and was the test used one or two tailed? This information should be added and is particularly relevant for the results shown in fig. 3, fig. 4 (in this case only two independent observations are indicated in the legend and probably only one is shown, hence statistics is not really meaningful), and fig. 2 in supplementary data.

- We agree with the reviewer that further information regarding the statistical analysis is required in the text and in the methods and have included this in the revised version of the manuscript. The T test performed is a two-tailed analysis in all the statistical analysis. The data in figure 3A is from 2 experiments with 4 mice each and the ELISA performed at the same time in duplicates. Fig 4A, the flow cytometry data is to represent the gating strategy used for the sorting in two independently performed experiments, performed once in duplicates and once in triplicates and the statistics is representing these two experiments together.

5. Discussion: p11 line 310: The view that IL10 secreted by Tregs is critical for the maintenance of immunity because sterile eradication of the pathogen is prevented has recently been challenged (see Schwarz et al. 2013). Please update.

We thank the reviewer for this reference and have included it in the discussion at the appropriate line indicated. In this study, mice deficient for T cell specific IL-10 had an early-enhanced inflammation and better parasite replication control. Particularly relevant to our study is the data that the effects of T cell specific IL-10 was suppressed by a DC based vaccination. We have discussed this paper in the light of T cells as an early source of IL-10 in the absence of DC subsets in Batf3^{-/-} mice.

6. p.10 line 267: lesions seem not to resolve at all (Fig. 5). Please comment.

- We thank the reviewer for the opportunity to explain our rationale for the proceeding experiment. The lesion size progression in the mixed bone marrow chimera mice progressed similar to that of controls in the experiment. This slower resolution might be due to side-effects of the toxin. To make the experiment

independent of the diphtheria toxin, we did a follow up study with the depletion of IL-12 at 17 days post infection and we were able to further narrow it down with short term treatment with cytochrome C that depletes cross-presenting cells, which was sufficient to confirm the requirement of Batf3-dependent DCs after 17 days of infection. We have presented this data as part of Figure 5 and also discussed its implications in the discussion part of the paper.

Minor points:

1. p. 10 line 273: Text refers to a fig. 4H and to 16 weeks, however, it seems that fig. 5H is meant and there the time point mentioned is week 15. What is correct?

We thank the reviewer for pointing out this mistake and we have corrected the manuscript to read "5H and 15 weeks".

Second Editorial Decision – 27 January 2014

Dear Prof. Acha-Orbea,

It is a pleasure to provisionally accept your manuscript entitled "Cross-presenting Dendritic Cells are Required to Control Leishmania major Infection" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Karen Chu

on behalf of Prof. Bernard Malissen

Dr. Karen Chu
Editorial Office

Peer review correspondence

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**Cross-presenting dendritic cells are required for control of *Leishmania major*
infection**

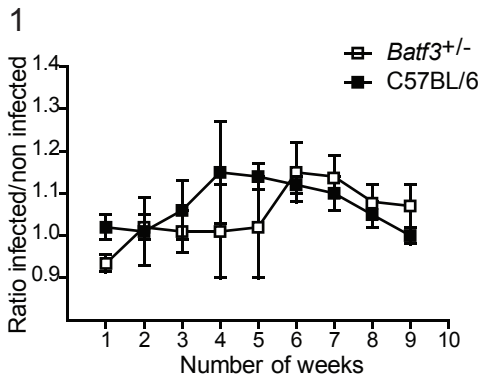


Figure 1: Lesion size progression in *Batf3*^{+/-} and C57BL/6 mice infected with *L. major*. Mice were infected with 3×10^6 *L. major* promastigotes in the hind foot-pad and monitored for lesion size growth. The experiment was performed 2 times with 5 mice per group. Data is mean $\pm$ SD (Student's t-test)

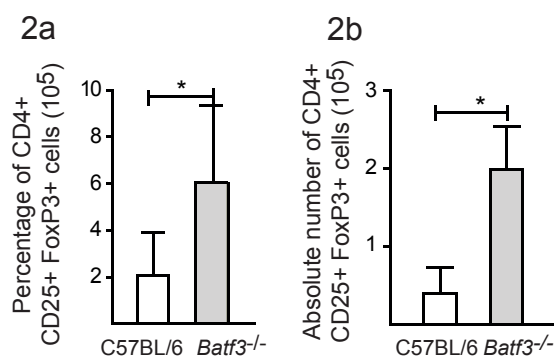


Figure 2: CD4⁺ CD25⁺ FoxP3⁺ Treg cells in *Batf3*^{-/-} and C57BL/6 mice 6 weeks post-infection by *L. major*. CD4⁺ CD25⁺ FoxP3⁺ cells were identified by flow cytometry in *Batf3*^{-/-} and C57BL/6 mice, 6 weeks post-infection and their (A) proportion and (B) absolute numbers in the dLN were determined. The experiment was performed 2 times with 5 mice per group. Data is mean + SD. *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t-test)