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Batf3-dependent CD103$^+$ dendritic cells are major producers of IL-12 that drive local Th1 immunity against *Leishmania major* infection in mice

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The role of different DC subsets in priming and maintenance of immunity against *Leishmania major* (*L. major*) infection is debated. The transcription factor basic leucine zipper transcription factor, ATF-like 3 (Batf3) is essential for the development of mouse CD103$^+$ DCs and some functions of CD8α$^+$ DCs. We found that CD103$^+$ DCs were significantly reduced in the dermis of Batf3-deficient C57BL/6 mice. *Batf3*$^{-/-}$ mice developed exacerbated and unresolved cutaneous pathology following a low dose of intradermal *L. major* infection in the ear pinnae. Parasite load was increased 1000-fold locally and expanded systemically. Batf3 deficiency did not affect *L. major* antigen presentation to T cells, which was directly exerted by CD8α$^-$ conventional DCs (cDCs) in the skin draining LN. However, CD4$^+$ T-cell differentiation in the LN and skin was skewed to nonprotective Treg- and Th2-cell subtypes. CD103$^+$ DCs are major IL-12 producers during *L. major* infection. Local Th1 immunity was severely hindered, correlating with impaired IL-12 production and reduction in CD103$^+$ DC numbers. Adoptive transfer of WT but not IL-12p40$^{-/-}$ Batf3-dependent DCs significantly improved anti-*L. major* response in infected *Batf3*$^{-/-}$ mice. Our results suggest that IL-12 production by Batf3-dependent CD103$^+$ DCs is crucial for maintenance of local Th1 immunity against *L. major* infection.

Keywords: Adaptive immune response · Batf3 · Dendritic cells · IL-12 · *Leishmania major*

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Introduction

Leishmania major (*L. major*) intradermal infection in C57BL/6 mice mimics human cutaneous leishmaniasis. Infection is brought under control by an adaptive Th1 CD4$^+$ T-cell response after a few weeks of development of cutaneous lesions [1]. To trigger adaptive immunity, dendritic cells (DCs) should provide three signals to naive T cells: pathogen-derived peptides bound to MHC molecules, costimulation, and a polarizing signal that is mediated by soluble or membrane-bound factors [2]. Priming and maintenance of effector T cells against *Leishmania* require the coordinated action of different DC subsets [3, 4] but the overall contributions of these subsets is debated. Monocyte-derived DCs from the skin migrate to the draining LNs (dLNs) after uptake of the parasite and prime the generation of Th1 adaptive immunity [5]. Earlier reports showed that CD8α$^-$ Langerin$^-$ DCs form the basis of the protective immune response and that Langerhans cells and dermal DCs (dDCs) migrate poorly to LNs and play only a minor role in early CD4$^+$ T-cell activation [6, 7] and Langerhans cells play rather a negative role [8]. Infection of diphtheria toxin-treated

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Langerin-DTR mice revealed that early CD8$^+$ T-cell proliferation is affected by depletion of Langerin$^+$ dDCs, with the CD4$^+$ T-cell response dependent on Langerin$^-$ dDCs [9].

Basic leucine zipper transcription factor, ATF-like 3 (Batf3) is a transcription factor essential for the development of the CD103$^+$ subset of DCs [10–13]. In contrast, numbers of CD8α$^+$ conventional DCs (cDCs) in skin-dLNs are not significantly affected by *Batf3* deficiency in the C57BL/6 background, although they are partially impaired in function, for example CD8α$^+$ cDCs show deficient cell-associated cross-presentation [11–13]. *Batf3*$^{-/-}$ mice have been used to study the role of both DC subsets in several models of infection [10, 14–16].

Using a model of low dose intradermal (i.d.) infection with *L. major* in the ear pinnae [1], we show that Batf3 deficiency leads to an exacerbated and unresolved pathology, with a 1000-fold increase in local parasite load. A recent report has shown enhanced susceptibility of *Batf3*$^{-/-}$ mice to *L. major*, concomitant with a skewed cytokine production by T cells in the dLNs [17]. The authors associated this phenotype with a defective function of CD8α$^+$ cDCs, although the mechanism was not investigated in detail [17]. Our data extend this analysis and show that Batf3 deficiency does not affect priming of T-cell responses in the dLNs, which is directed by CD8α$^-$ Batf3-independent cDCs. We find that CD103$^+$ DCs are the main suppliers of IL-12 during *L. major* infection, which is impaired in *Batf3*$^{-/-}$ mice. Transfer of WT but not IL-12p40$^{-/-}$ Batf3-dependent DCs significantly improved anti-*L. major* responses in infected *Batf3*$^{-/-}$ mice. These data point to CD103$^+$ DCs as crucial providers of IL-12 for local maintenance rather than priming of Th1 immunity.

Results

Batf3$^{-/-}$ mice develop an exacerbated *L. major* cutaneous pathology with increased neutrophilia

To assess the role of Batf3-dependent DCs in generation of immunity against *L. major*, we monitored the cutaneous pathology induced by i.d. injection of 1000 *L. major* metacyclic promastigotes in *Batf3*$^{-/-}$ mice. These animals presented an exacerbated pathology that was established early from the 2nd week postinfection (p.i.) and maintained during the course of the infection, without apparent resolution (Fig. 1A and Supporting Information Fig. 1A and B). A similar pathology was provoked with a moderate dose of parasite (5×10^4), which was used in subsequent experiments (Supporting Information Fig. 1C).

One advantage of the i.d. ear model is the possibility to conduct local analysis of infection, parasite load, and the ongoing immune response. We found that infected WT mice readily controlled parasite load in the ear from the 3rd week p.i. (Fig. 1B). In contrast, *Batf3*$^{-/-}$ mice were unable to control local parasite load at any time point analyzed (Fig. 1B, left panel), resulting in

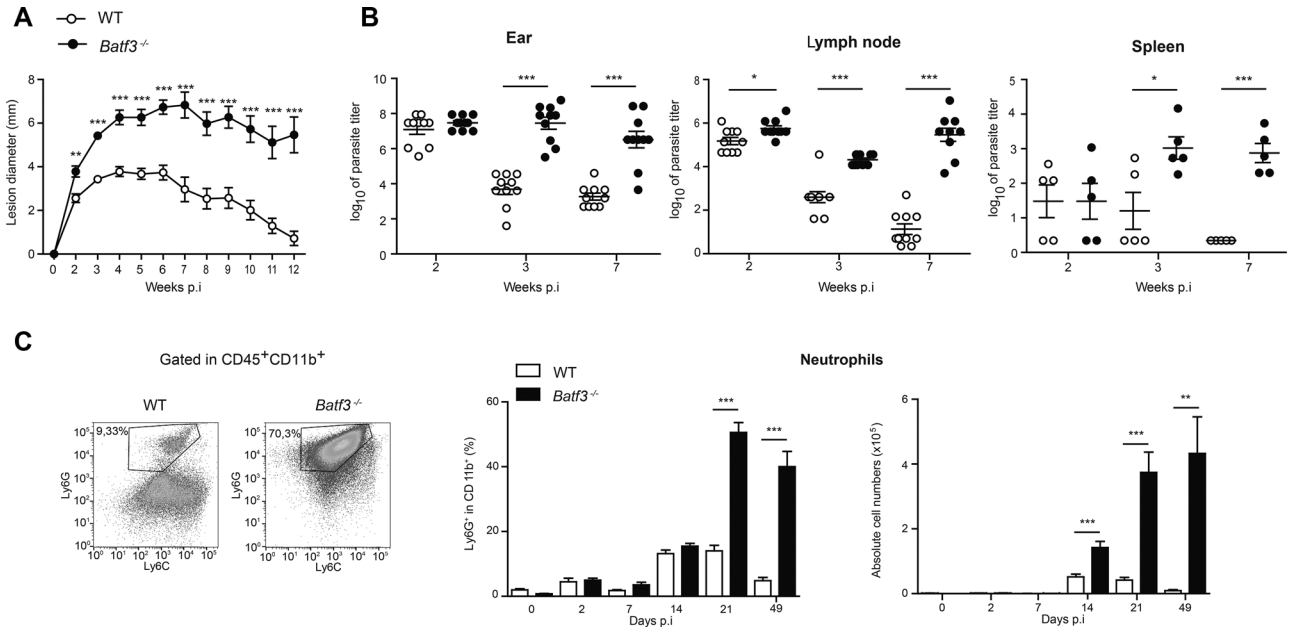

Figure 1. Batf3-deficient mice develop an exacerbated *L. major* cutaneous pathology with neutrophilia. (A) Pathology (the lesion diameter measured with a digital calliper) in WT and *Batf3*$^{-/-}$ mice was tracked for 12 weeks after i.d. infection in the ear pinnae with 1000 *L. major* parasites. Data are shown as arithmetic mean ± SEM of 20 samples and are from one experiment representative of three independent performed. (B–D) WT and *Batf3*$^{-/-}$ mice were i.d. infected in the ear with 5×10^4 *L. major* parasites. (B) Parasite load in the ear, dLNs, and spleen in WT and *Batf3*$^{-/-}$ mice at different time points p.i. Data are shown as arithmetic mean ± SEM (horizontal lines and whiskers) of individual data ($n = 5$ mice, each circle represents one sample) corresponding to one experiment representative of three performed. (C) Left: Representative plots showing analysis of myeloid cell infiltrates in the ear at day 21 p.i. Right: Analysis of frequency and absolute numbers of neutrophils (CD11b$^+$ Ly6G$^+$) in the ears at different time points p.i. Data are shown as arithmetic mean + SEM of ten samples and are pooled from three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ unpaired two-tailed Student's *t* test.

an average 1000-fold higher *L. major* titer at 3 and 7 weeks. This lack of local containment resulted in systemic expansion of the parasite [18, 19], leading to higher titers in the dLNs and spleen of *Batf3*$^{-/-}$ mice at 3 and 7 weeks p.i. (Fig. 1B, middle and right).

Lesions in *Batf3*$^{-/-}$ mice persisted at time points at which WT mice were healing or had already completely resolved the wound (Fig. 1A and Supporting Information Fig. 1A and B). At 3 and 7 weeks p.i., the myeloid-cell infiltrate in the ears of infected *Batf3*$^{-/-}$ mice was much greater than in WT mice (Fig. 1C). Accordingly, absolute numbers of infiltrating neutrophils in the ear were significantly higher than in WT mice, reaching an eight-fold difference by the 3rd week p.i. that was maintained throughout the infection (Fig. 1C). These data show that Batf3 is essential for resistance to *L. major* infection.

T-cell response priming to *L. major* is mainly driven by Batf3-independent DCs

Uncontrolled parasite load suggested a major role for Batf3 in the adaptive response to *L. major* infection, prompting us to examine whether antigen presentation was affected in the absence of Batf3. To test the priming of the antigen-specific response, we took advantage of *L. major* expressing OVA (*L. major*-OVA) [20]. WT and *Batf3*$^{-/-}$ mice were transferred with fluorescently labeled (CellTrace^{TM} Violet) OVA-specific CD4$^+$ (OTII) or CD8$^+$ (OTI) T cells from CD45.1 donor mice and subsequently infected i.d. in the ear. Analysis of OTII cell proliferation did not reveal any difference in early priming of CD4$^+$ T-cell responses in the dLNs (Fig. 2A). OTI proliferation to *L. major* OVA infection was also unaffected (Fig. 2B).

To assess the relative contribution of different DC subsets to antigen presentation once the infection is established, CD8α$^-$ cDCs, CD8α$^+$ cDCs, and CD103$^+$ migratory (mDCs) were isolated from the skin dLNs 2 weeks p.i. and exposed ex vivo to polyclonal T cells from *L. major* infected and healed mice. CD8α$^-$ cDC from skin-dLNs of infected mice induced polyclonal IFN-γ production by both CD4$^+$ and CD8$^+$ T cells (Fig. 2C and D). In contrast, CD8α$^+$ cDCs and CD103$^+$ mDCs induced a poor response. Altogether, these results show that antigen presentation during *L. major* i.d. infection is not significantly affected in the absence of Batf3 and suggest that Batf3-independent CD8α$^-$ cDCs are the main DC subset involved in antigen presentation in the dLNs for induction of T-cell immunity against *L. major*.

Batf3 deficiency impairs local Th1 immunity and skews adaptive response to *L. major*

To study how Batf3 deficiency could be affecting immunity against *L. major*, we analyzed T-cell responses in the dLNs of infected mice following restimulation with freeze-thawed *Leishmania* ex vivo. Batf3 deficiency did not significantly affect IFN-γ production by restimulated T cells, whereas IL-10 and IL-4 production were significantly increased at the 2nd and 3rd week p.i. (Fig. 3A). Increased Th2 response in *Batf3*$^{-/-}$ mice was however moderate when compared with BALB/c mice examined in parallel (Supporting Information Fig. 2A).

Taking advantage of the i.d. ear model, restimulation of effector T cells isolated from the infection site revealed a very significant and consistent reduction in the frequency of IFN-γ-producing CD4$^+$ T cells in *Batf3*$^{-/-}$ mice at the time-points explored (Fig. 3B). Ear infiltrates from *Batf3*$^{-/-}$ mice contained significantly higher numbers of FoxP3$^+$ CD4$^+$ CD25$^+$ T cells (Fig. 3C). Batf3-deficiency impaired local Th1-cell responses to a similar extent as BALB/c mice, whereas skewing toward Th2- or Treg-cell differentiation was not as pronounced as in BALB/c mice (Supporting Information Fig. 2B and C). These results suggest that the most significant effect of Batf3 deficiency in the adaptive response to the parasite is the local impairment of Th1 immunity, which is accompanied by skewed CD4$^+$ T-cell immunity.

L. major-infected Batf3$^{-/-}$ mice have defects in monocyte-derived DC and macrophage differentiation

Monocyte-derived DCs are crucial for the generation of Th1 immunity against *Leishmania* and, together with macrophages, mediate nitric oxide production [5, 21]. We therefore explored the possible impact of Batf3-dependent DCs on the differentiation of monocytes in the dermis. CD11b^{hi} CD64$^+$ myeloid cells were analyzed for CCR2, Ly6C, and MHC class II expression, as reported [22] (Fig 4A). CCR2$^+$ Ly6C^{hi} MHC class II$^-$ subset, corresponding to monocytes (P1, Fig. 4A) was increased in *Batf3*$^{-/-}$ mice, both in frequency in the CD11b^{hi} CD64$^+$ CCR2$^+$ subset (Fig. 4B) and number of infiltrating cells with P1 phenotype per ear (Fig. 4C). Monocyte-derived DCs expressing MHC class II (P2 and P3) were reduced in frequency but not in numbers (Fig. 4). CCR2$^-$ MHC class II$^-$ macrophages (P4) were increased in frequency at the expense of a decrease in frequency of the CCR2$^-$ MHC class II$^+$ macrophages (P5), which were reduced in numbers after 3 weeks p.i. (Fig. 4). Thus, Batf3 deficiency partially affects the differentiation of monocytes to monocyte-derived DCs and macrophages, but the impact is limited to overall numbers in the lesion.

We also determined IL-12 production by monocyte-derived DCs in skin dLNs. Only the MHC class II^{hi} subset of monocyte-derived DCs (P3) induced IL-12 production in response to *L. major* 2 weeks p.i. (Supporting Information Fig. 3A). The frequency of IL-12 producers in this subset was reduced in *Batf3*$^{-/-}$ mice, but was compensated in numbers by an increase in infiltrates of this subset in the lesion. We also found reduced iNOS staining in dermal CD11c$^+$ and CD11c$^-$ subsets of Ly6C$^+$ MHC class II$^+$ myeloid cells from *Batf3*$^{-/-}$ mice at 3 weeks p.i. (Supporting Information Fig. 3B). Collectively, these results show that there is a partial impairment of differentiation and function of dermal monocyte-derived DCs and macrophages that could be a consequence rather than the cause of the distorted immunity.

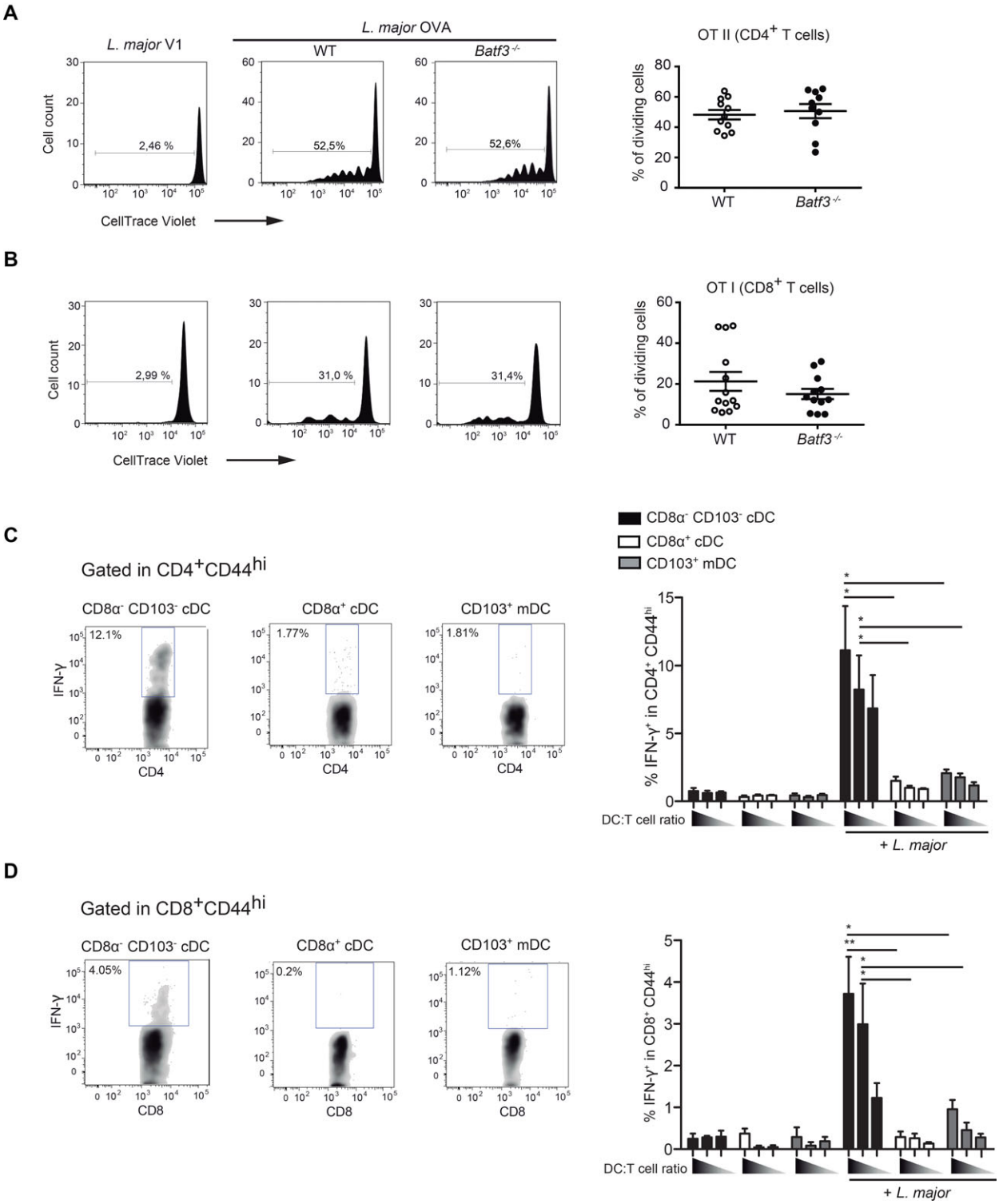

Figure 2. Priming of T-cell responses to *L. major* is mainly driven by Batf3-independent DCs. (A and B) WT and *Batf3*$^{-/-}$ mice were transferred with (A) OTII (CD4$^+$) or (B) OTI (CD8$^+$) OVA-specific T cells labeled with cell violet and infected i.d. in the ear with 2×10^5 *L. major*-OVA parasites. Cell violet dilution was analyzed in the transferred cells present in the dLNs (A) 4 days or (B) 3 days p.i. Left: Representative plots of three independent experiments performed. Right: Data are shown as arithmetic mean ± SEM of individual data ($n = 11$–14 samples) and are data pooled from three independent experiments. (C and D) CD8α$^-$ cDCs, CD8α$^+$ cDCs, and CD103$^+$ mDCs were purified from WT dLNs 2 weeks p.i. and cocultured with polyclonal T cells from *L. major* infected and healed WT mice in different DC: T-cell ratios (2:1; 1:1; 0.5:1). IFN-γ production by (C) CD4$^+$ and (D) CD8$^+$ T cells was analyzed 4 h later by intracellular staining. Left: Representative plots from three independent experiments performed. DCs were pooled from the dLN of ten mice. Right: Data are shown as arithmetic mean + SEM. * $p < 0.05$; ** $p < 0.01$; unpaired ANOVA with Tukey post-hoc test.

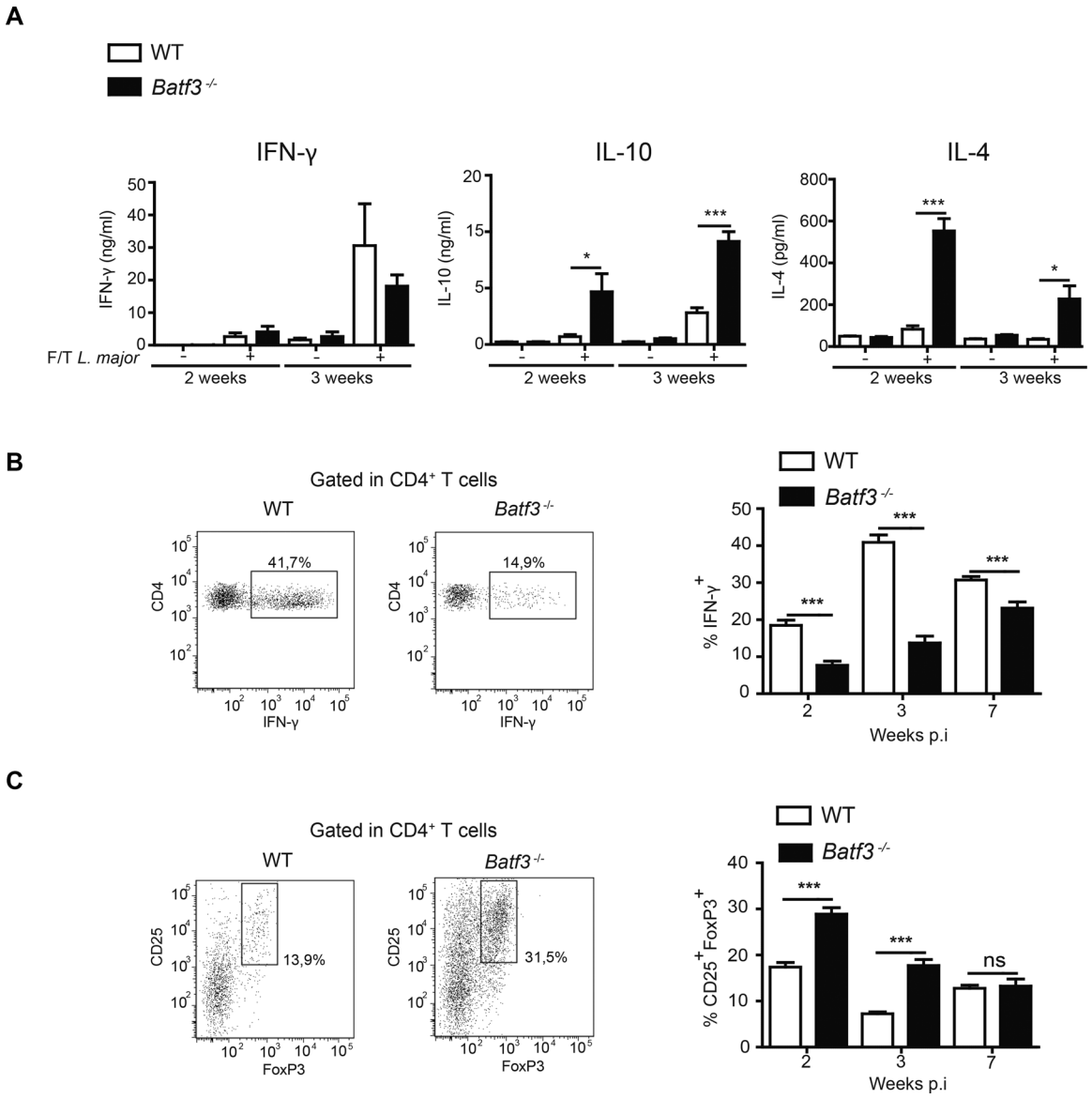

Figure 3. Batf3 deficiency impairs local Th1 immunity and skews the adaptive response to *L. major*. (A) WT and *Batf3*$^{-/-}$ mice were infected i.d. in the ear with 5×10^4 *L. major* parasites. dLN cells (2×10^6) obtained two and three weeks p.i. were restimulated with freeze-thawed (F/T) *L. major*, and IFN-γ, IL-10, and IL-4 were measured in the supernatant. (B, C) Ear cell suspensions obtained as in (A) 2, 3, and 7 weeks p.i. were restimulated with anti-CD3 and anti-CD28 and analyzed for (B) IFN-γ staining or (C) analyzed in steady state for FoxP3 expression. Representative plots of three independent experiments performed. (A–C) Data are shown as arithmetic mean + SEM ($n = 5$ mice (A) or 10 (B and C)) and are from a representative experiment of three performed. * $p < 0.05$; *** $p < 0.001$ unpaired two-tailed Student's *t* test.

Batf-3 dependent CD103$^+$ DCs are major producers of IL-12 during *L. major* infection

We hypothesized that the local impairment in Th1 immunity could result from the loss of a T-cell differentiation signal from Batf3-dependent DCs. The analysis of skin dLNs from *Batf3*$^{-/-}$ mice in the C57BL/6 background revealed normal development of CD8α$^+$ cDCs and a partial but significant reduction in the CD103$^+$ mDCs (Supporting Information Fig. 4A and B), compatible with previous results [11–13]. Through analysis of the ear skin, we further found a significant deficiency in CD103$^+$ dDC in the *Batf3*$^{-/-}$ C57BL/6 mice (Supporting Information Fig. 4C). Similar results were obtained in an analysis 2 weeks after infection with *L. major* (Supporting Information Fig. 5A–C).

Batf3-dependent DCs may provide IL-12 for Th1 CD4$^+$ T-cell differentiation [14, 16]. Examination of IL-12p40 and p35 expression in CD11c$^+$ cells purified from LNs 2 weeks following infection with *L. major* revealed impaired expression of both genes in *Batf3*$^{-/-}$ mice (Fig. 5A). This impaired IL-12 expression was also found in CD45$^+$ cells locally infiltrating the ear 2 weeks after i.d. *L. major* infection (Fig. 5B). These results demonstrate that

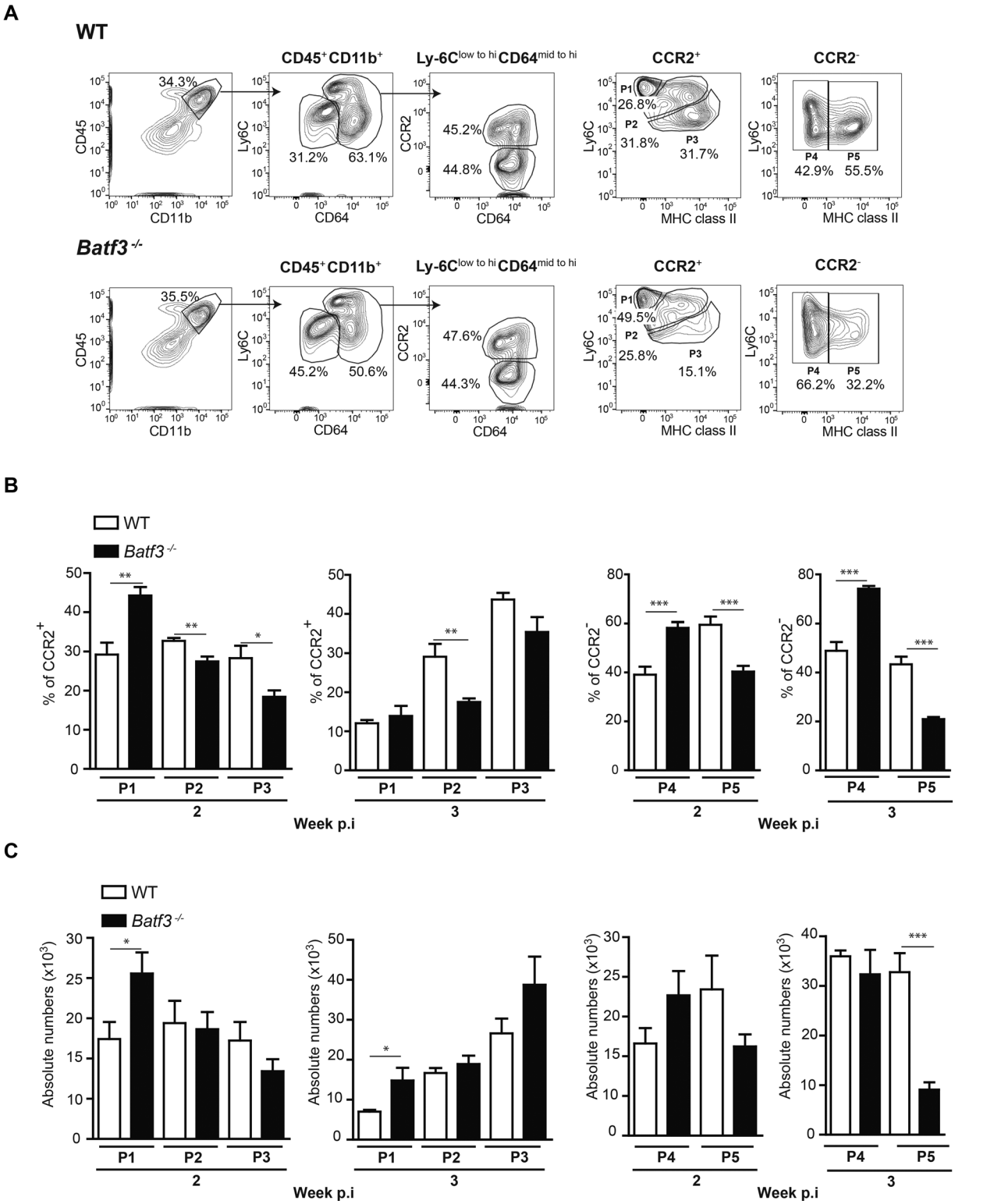

Figure 4. Batf3 deficiency partially affects differentiation of dermal monocyte-derived DCs and macrophages during *L. major* infection. (A) Monocyte differentiation to P1 dermal monocytes (CD11b$^+$ CD64^{mid} CCR2$^+$ Ly6C^{hi}MHC-II$^-$), monocyte-derived DCs (P2: CD11b$^+$ CD64^{hi} CCR2$^+$ Ly6C^{mid} MHC-II^{lo} and P3: CD11b$^+$ CD64^{hi} CCR2$^+$ Ly6C^{lo} MHC-II$^+$) and dermal macrophages (P4: CD11b$^+$ CD64^{hi} CCR2^{lo} MHC-II$^-$ and P5: CD11b$^+$ CD64^{hi} CCR2^{lo} Ly6C^{lo} MHC-II$^+$) was tracked in ears of WT and *Batf3*$^{-/-}$ mice 2 and 3 weeks p.i. with 5×10^4 *L. major* parasites. (A) Representative plots and gating strategy are shown. (B) Right panels: Frequency of P1, P2, and P3 in the CD11b$^+$ Ly-6C$^{lo\text{-}to\text{-}hi}$ CD64$^{lo\text{-}to\text{-}hi}$ CCR2 $^+$ subset; Left panel: P4, and P5 frequency in the CD11b$^+$ Ly-6C$^{lo\text{-}to\text{-}hi}$ CD64$^{lo\text{-}to\text{-}hi}$ CCR2 $^-$ subset. (C) Absolute numbers of the populations in (B) per ear. (B, C) Data are shown as arithmetic mean + SEM ($n = 10$ samples) and are from one experiment representative of three performed. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ unpaired two-tailed Student's *t* test.

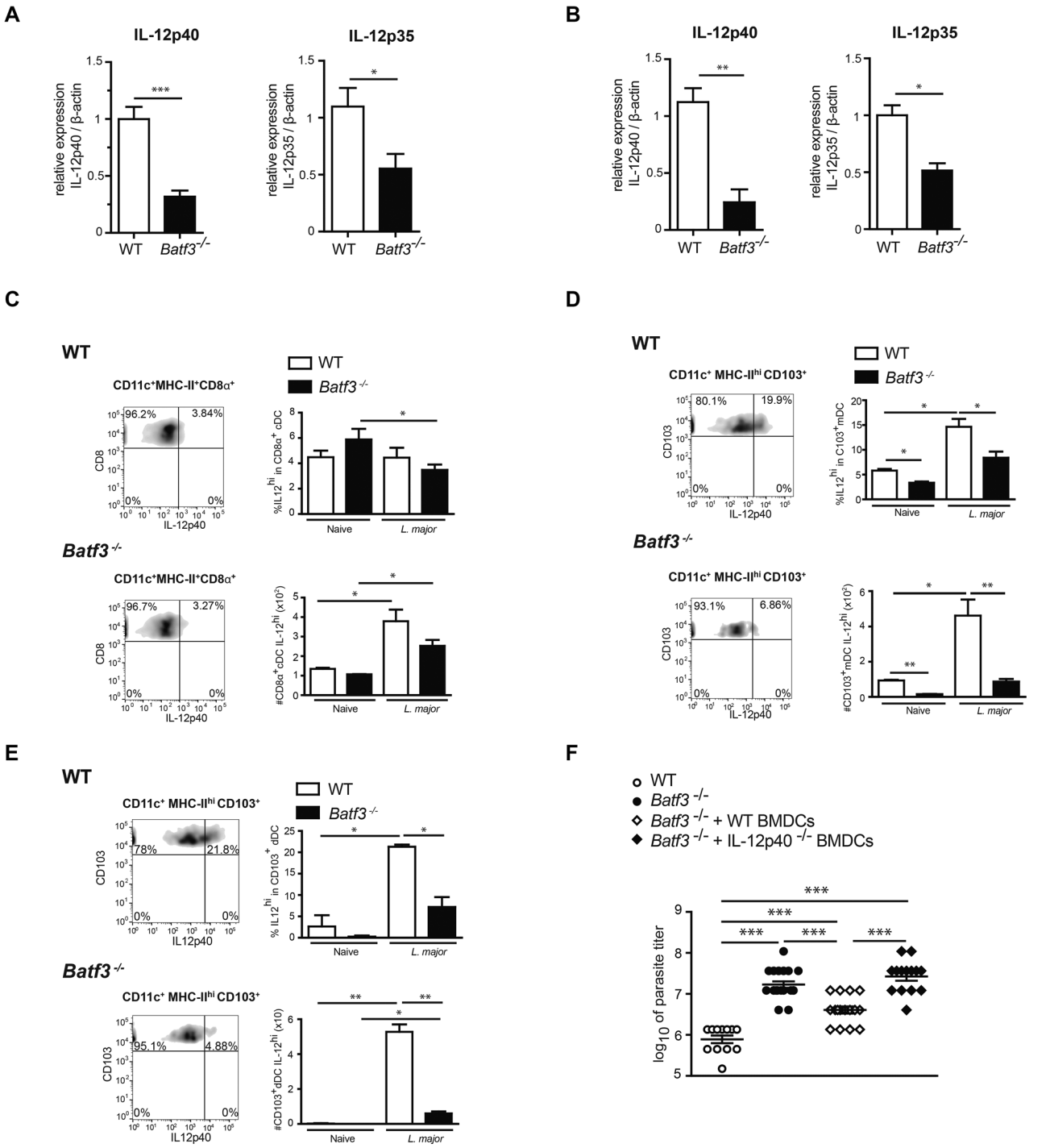

Figure 5. Batf-3-dependent CD103$^+$ DCs are major IL-12 producers. (A–E) WT and *Batf3*$^{-/-}$ mice were infected with 5×10^4 *L. major* parasites i.d. in the ear and analyzed 2 weeks p.i. for IL-12p40 and IL-12p35 expression in (A) purified CD11c$^+$ cells from the dLNs (B) CD45$^+$ cells purified from the infected ears of WT and *Batf3*$^{-/-}$ mice. RNA expression is standardized to the internal β-actin control and shown as fold induction to the WT average. (A and B) Data are shown as mean + SEM ($n = 6$ pooled samples analyzed in triplicate) from three independent experiments. (C–E) Five hours before sacrifice, mice were injected with Brefeldin A (250 μg i.p.). (C and D) dLN cells were stained for CD11c, MHC-class-II, CD8α, CD103, and intracellular IL-12p40. Plots show IL-12p40 staining in (C) CD8α$^+$ CD11c$^+$ MHC class II mid cDCs and (D) CD103$^+$ CD11c$^+$ MHC class II^{hi} DCs. (E) Ear dermal cells were extracted and stained for CD45, CD11c, MHC-class-II, CD103, and intracellular IL-12p40. Left: Plots showing IL-12p40 staining in CD11c$^+$ MHC class II^{hi} CD103$^+$ dermal DCs. Data in C–E are shown as arithmetic mean + SEM of frequency (upper panels) and absolute numbers (lower panels) in naive or infected mice ($n = 5$) and are from a representative experiment of three performed. (F) WT and *Batf3*$^{-/-}$ mice were infected with 5×10^4 *L. major* parasites i.d. in the ear and 10^5 CD24^{hi} cells from Flt3L BMDC cultures were transferred locally every 3 days starting at day 4 p.i. Ears and dLNs were analyzed for parasite load 3 weeks p.i. Individual data and arithmetic mean ± SEM are shown for a representative experiment of two performed. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ unpaired ANOVA with Tukey's post-hoc test.

Batf3-dependent DCs are a key contributor for IL-12 production during *L. major* infection.

The specific Batf3-dependent DC subset that provides IL-12 may vary depending on the infection model [14, 16]. To document the Batf3-dependent DC subset contributing to IL-12 production, we performed intracellular staining of IL-12p40 in naive mice or 2 weeks following *L. major* infection (Fig. 5C–E). In the dLNs, *L. major* infection significantly induced IL-12p40 expression in $CD103^+$ mDCs, but not in $CD8\alpha^+$ cDCs (Fig. 5C and D). In the absence of Batf3, not only were $CD103^+$ mDCs significantly reduced (Supporting Information Figs. 4 and 5), but also their capacity to produce IL-12 in the remaining $CD103^+$ mDCs in response to *L. major* infection was diminished (Fig. 5D). Dermal $CD103^+$ DCs were also major Batf3-dependent IL-12 producers following *L. major* infection (Fig. 5E).

To test whether complementation with Batf3-dependent DCs could rescue the defective elimination of the parasite in *Batf3*$^{-/-}$ mice, we generated Flt3L-BMDC cultures containing $CD24^{hi}$ Batf3-dependent DCs [10, 23, 24]. Transfer of $CD24^{hi}$ DCs could significantly revert the increased parasitemia observed in *Batf3*$^{-/-}$ mice 3 weeks p.i. (Fig. 5F). Notably, transfer of IL-12p40$^{-/-}$ $CD24^{hi}$ DCs did not modify the *Batf3*$^{-/-}$ phenotype (Fig. 5F). These results show that IL-12 produced by Batf3-dependent DCs is crucial for immunity against *L. major*.

Discussion

In this report, we have used the i.d. ear *L. major* infection model to explore the role of Batf3-dependent DCs in the generation of immunity against the parasite. We find that Batf3 deficiency results in exaggerated and unresolved pathology, with skewed T-cell differentiation in the dLNs and an impaired Th1-cell effector response at the infection site, leading to defective control of the parasite. During the preparation of this manuscript, Ashok et al. reported complementary findings, showing that *Batf3*$^{-/-}$ mice exhibit enhanced susceptibility to *L. major* infection [17]. Taken together, the results of the two studies strongly suggest an important role for Batf3-dependent DCs in the generation of immunity against *L. major*. Our study further shows that Batf3 deficiency does not affect antigen presentation to T cells. Our results reveal impaired Th1 immunity at the infection site, with increased parasitemia and neutrophilia. We establish Batf3-dependent $CD103^+$ DCs as key providers of IL-12 for maintenance of local Th1 immunity.

It has been suggested that the $CD8\alpha^+$ DC subset is likely to be the main mediator of the response, since they present *L. major* antigen more efficiently than $CD103^+$ DCs [17]. We find that *L. major* antigen presentation to both $CD4^+$ and $CD8^+$ T cells is largely mediated by $CD8\alpha^-$ cDCs in the dLNs and is Batf3-independent, in agreement with previous results [6, 7]. We detect poor presentation of *L. major* antigens ex vivo by both the $CD8\alpha^+$ DC and the $CD103^+$ DC subsets. Depletion of Langerin$^+$ dermal DCs results in reduced priming of *L. major*-specific $CD8^+$ T cells [9]. Depletion of $CD103^+$ langerin$^+$ dDCs is significant but incomplete in *Batf3*$^{-/-}$ C57BL/6 mice. It is conceivable that remaining $CD103^+$ dDCs could be sufficient for mediating $CD8^+$ T-cell priming in *Batf3*$^{-/-}$ mice. It is also feasible that infected $CD103^+$ dDCs could transport the pathogen to the dLNs [6] and transfer *L. major* antigens to an additional DC subset for presentation. In fact, cross-presentation is mostly TAP-dependent [25], whereas presentation of *Leishmania* antigens on MHC class I molecules is mainly TAP-independent [26]. Moreover, *Leishmania* inhibits cross-presentation [27]. Our data support the notion that susceptibility of *Batf3*$^{-/-}$ mice to *L. major* infection is not caused by deficient antigen presentation.

$CD8\alpha^+$ cDCs are critical for producing IL-12 that drives the Th1 response to *Toxoplasma gondii* [14]. In the setting of *L. major* i.d. infection, we find a low and Batf3-independent contribution of $CD8\alpha^+$ cDC to IL-12 production. In addition to the different pathogen used, the distinct route of immunization could explain this apparent discrepancy: i.d. *L. major* versus i.p. *T. gondii*. In this regard, Th1 immunity in a cutaneous candidiasis model relies on $CD103^+$ dDCs [16]. Notably, we find that $CD103^+$ DCs both in the dLNs and the dermis are the major IL-12 producer after *L. major* i.d. infection. IL-12 impairment does not affect Th1 priming in the dLNs but may cause skewed polarization. This is consistent with IL-12 contributing to the clonal expansion, amplification, and phenotypic stabilisation of already-committed Th1 cells, while negatively regulating the Treg-cell pool [28, 29].

IL-12 is thus important not only for priming but also for the maintenance of Th1 immunity against *L. major* during infection [17, 30]. Local production of IL-12 by $CD103^+$ dDCs may be critical for T-cell homing, migration, and local effector activity, especially when considering the concomitant accumulation of parasite-primed Treg cells [29, 31, 32]. Our proposed role of Batf3-dependent $CD103^+$ DCs in maintenance rather than priming of local Th1 immunity would explain why depletion of cross-presenting DCs between days 17 and 19 p.i. transiently enhances susceptibility to infection [17]. We found that complementation by transfer of WT but not IL-12p40$^{-/-}$ Batf3-dependent DCs significantly improved resistance to the infection. Batf3-dependent $CD103^+$ DCs are thus essential mediators of IL-12 cytokine production that may contribute to maintenance of local Th1 $CD4^+$ T-cell adaptive immunity against the parasite.

L. major infection recruits monocytes to the dermis that generate Th1-promoting dermal monocyte-derived DCs [5]. Our results show that Batf3-deficiency partially affects differentiation of monocytes to DCs or macrophages in the dermis following *L. major* infection, although this is compensated by increased infiltration of myeloid cells as a consequence of higher parasitemia. Rather than a direct effect of Batf3 deficiency, decreased IL-12 and a Th2 environment could affect monocyte differentiation and activation [21]. Reduced iNOS expression in monocyte-derived DCs and macrophages could also be a consequence of decreased IFN-γ production [33].

Our findings suggest that $CD103^+$ Batf3-dependent DCs play a decisive role in providing IL-12 for generation of immunity against *L. major*. Batf3 deficiency is redundant for antigen presentation or even Th1-cell differentiation in the dLNs. IL-12 could however be essential for inhibition of Th2- and Treg-cell responses and for

generation and maintenance of local Th1 immunity against the parasite. This long-term role in maintenance, rather than priming, suggests that CD103$^+$ DC could be potentially targeted throughout the infection for therapy.

Materials and methods

Mice

Mice were bred at the CNIC in specific pathogen-free conditions. *Batf3*$^{-/-}$ mice backcrossed more than ten times to the C57BL/6 background (kindly provided by Dr. Kenneth M. Murphy, Washington University, St. Louis, MO, USA) were further backcrossed with C57BL/6 mice at the CNIC to establish WT and *Batf3*$^{-/-}$ colonies from the heterozygotes. Animal studies were approved by the local ethics committee. All animal procedures conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

Leishmania parasites, preparation, inoculation, and quantification

In vivo experiments were carried out using different *L. major* lines. The *L. major* Friedlin strain FV1 (MHOM/IL/80/Friedlin) was generously provided by Dr. D. Sacks (NIH) [34]. *L. major* FV1 (MHOM/IL/80/Friedlin) parasites expressing ovalbumin (*Leishmania*-OVA) were kindly provided by Prof. Deborah Smith and Prof. Paul Kaye (University of York) [20]. For *Leishmania* challenge, parasites of the different lines were kept in a virulent state by passage in mice. Culture and differentiation of parasites was performed as described [35]. Mice were infected by i.d. injection of 1000 or 5×10^4 metacyclic *L. major* promastigotes into the dermis of both ears. Lesion size in the ear was determined with a digital calliper (Duratool) [34]. The limiting dilution assay was used to determine the number of parasites [35]. The parasite load was expressed as the number of parasites in the whole organ.

Generation and inoculation of mouse BMDCs

WT or IL-12p40$^{-/-}$ BM cells (generously provided by Dr. Salomé LeibundGut-Landmann, Institute of Microbiology, ETH Zürich, Switzerland) were cultured in the presence of 150 ng/mL Flt3L (R&D Systems) and fresh medium was added supplemented with 20 ng/mL murine GM-CSF (Peprotech) as reported [23, 24]. Cells (1×10^5) were i.d. injected every 3 days into the ear, beginning 4 days after *L. major* i.d. infection with 5×10^4 parasites until 3 weeks p.i.

Cell purification, restimulation, ELISA, and RT-qPCR

Single-cell suspensions of LNs and ears were prepared by Liberase/DNAse digestion. At the indicated times after *L. major* infection, ears were recovered from naive or infected mice as described [35]. When further purification of CD4$^+$ or CD8$^+$ T cells was required, cell suspensions were negatively selected using a cocktail of biotin-conjugated antibodies (anti-CD11c, CD11b, B220, MHC-II, NK1.1) followed by separation with streptavidin-microbeads (Miltenyi Biotec). T cells were restimulated to induce cytokine production by incubation over plated anti-CD3 2C11 (10 μg/mL) and anti-CD28 (5 μg/mL, Bio × Cell). dLNs were restimulated with freeze-thawed *Leishmania*. ELISA kits for cytokines (IL-4, IFN-γ, and IL-10) were from BD Biosciences. DCs from LNs and ears were purified with anti-CD11c-microbeads (Miltenyi Biotec) or biotin-conjugated CD45 and streptavidin-microbeads (Miltenyi Biotec). For purification of DC subsets from dLNs, CD11c$^+$ cells enriched using anti-CD11c microbeads were further sorted into CD8α$^+$CD103$^-$, CD8α$^-$ CD103$^+$, and CD8α$^-$ CD103$^-$ DCs in a FACSAria Sorter. RT-qPCR for *Gapdh*, β-*actin*, *Il12p40*, and *Il12p35* was performed using primers from Sigma Aldrich. Total RNA from cells was extracted with the RNeasy Plus Micro Kit (Qiagen, #74034). RNA was reverse transcribed to cDNA using random hexamers and High Capacity cDNA Reverse Transcription Kit (Applied Byosystem, #4368814). Reverse transcription PCR was conducted in a C1000 Thermal Cycler (Bio-Rad). cDNA products were used for quantitative PCR, using the GoTaq® qPCR Master Mix (Promega, #A6001). PCR amplification was performed in a 7900HT Fast Real-Time PCR System (Applied Byosystem, #4329001). All reactions were done in a 20 μL reaction in triplicate, following the manufacturer's protocol. Reverse transcription and PCR amplification of the housekeeping genes Gapdh and β-actin were performed to verify equal loading of RNA and cDNA. PCR primers used for SYBR Green assays were as follows: β-actin sense, (5′) –GGCTGTATTCCCCTCCATCG – (3′), and β-actin antisense, (5′) – CCAGTTGGTAACAATGCCATGT – (3′); GADPH sense, (5′) – TGAAGCAGGCATCTGAGGG – (3′), and GADPH antisense, (5′) –CAGGAAGTAGGTGAGGGCTTG – (3′) IL-12p40 sense, (5′) – GGAAGCACGGCAGCAGAATA– (3′); IL-12p40 antisense, (5′) – AACTTGAGGGAGAAGTAGGAATGG – (3′), IL-12p35 sense, (5′) – TACTAGAGAGACTTCTTCCACAACAAGAG– (3′); IL-12p35 antisense, (5′) – TCTGGTACATCTTCAAGTCCTCATAGA – (3′). Relative expression is defined as the arithmetic mean of triplicate $2^{-\Delta Ct}$ values relative to β-actin RNA. To compare data from different experiments, the biological replicate in each experiment was normalised as fold-induction compared to the average of the WT biological replicates.

Antibodies and flow cytometry

Samples for flow cytometry were stained in ice-cold PBS supplemented with 2 mM EDTA, 1% FCS and 0.2% sodium azide, with the appropriate antibody cocktails. Anti-mouse CD45, CD4, CD8α, CD11b, CD11c, CD103, CD24, Ly-6C, FoxP3, IL-4, and

I-A^b (MHC-II), antibodies conjugated to FITC, PE, PerCP-Cy5.5, V450, or Allophycocyanin were obtained from eBioscience. PE-conjugated CCR2 (R&D), Allophycocyanin-Cy7 CD45, and Allophycocyanin CD25 were from Tonbo Biosciences. PE-conjugated-anti-mouse Ly-6G, Alexa Fluor 647-conjugated anti-mouse CD64-FITC-conjugated anti-mouse iNOS were from BD Biosciences. Allophycocyanin-anti-IFN-γ was from Miltenyi Biotec. Purified anti-FcγRIII/II (2.4G2) was used to block nonspecific antibody binding. Noncell-permeant Hoechst 33258 (0.1 μM) was used as a counterstain to detect necrotic cells. Events were acquired using a FACSCanto or FACSDiva flow cytometer (Becton Dickinson), and data were analyzed with FlowJo software (Tree Star). For the detection of intracellular iNOS, cells were fixed in 2% paraformaldehyde for 15 min and permeabilized in 0.3% saponin and 0.5% BSA in PBS for 20 min before staining, as described [36]. For intracellular IL-12p40 staining *in vivo*, mice were intraperitoneally inoculated with brefeldin A (250 μg/mouse) [37] 2 weeks after *L. major* infection. Ear and ear-dLNs were recovered 5 h after brefeldin A injection. The cells obtained were fixed and permeabilized and were stained with PE-conjugated anti-IL-12p40 antibody (eBioscience). The cells were analyzed using a FACSCanto II flow cytometer (BD Biosciences).

Statistical analysis

Statistical differences were analyzed with Prism software (GraphPad Software, Inc.). Comparisons of samples with a normal distribution (Shapiro–Wilk test for normality) were made using the unpaired two-tailed Student's *t* test for comparison of two groups or ANOVA with Tukey's Post-hoc test for comparison of more than two groups. Differences were considered significant at $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

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Abbreviations: **Batf3**: basic leucine zipper transcription factor, ATF-like 3 · **cDC**: conventional DC · **dDC**: dermal DC · **mDC**: migratory DC · **dLN**: draining lymph node · **i.d.**: intradermal · **p.i.**: postinfection

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