IRF4 in Dendritic Cells Inhibits IL-12 Production and Controls Th1 Immune Responses against *Leishmania major*

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Abstract

IRF4 is a transcription factor from the IRF factor family that plays pivotal roles in the differentiation and function of T and B lymphocytes. Although IRF4 is also expressed in dendritic cells (DCs) and macrophages, its roles in these cells in vivo are not clearly understood. In this study, conditional knockout mice that lack IRF4 in DCs or macrophages were generated and infected with *Leishmania major*. Mice lacking DC expression of IRF4 showed reduced footpad swelling compared with C57BL/6 mice, whereas those lacking IRF4 in macrophages did not. Mice with IRF4-deficient DCs also showed reduced parasite burden, and their $CD4^+$ T cells produced higher levels of IFN-γ in response to *L. major* Ag. In the draining lymph nodes, the proportion of activated $CD4^+$ T cells in these mice was similar to that in the control, but the proportion of IFN-γ–producing cells was increased, suggesting a Th1 bias in the immune response. Moreover, the numbers of migrating Langerhans cells and other migratory DCs in the draining lymph nodes were reduced both before and postinfection in mice with IRF4 defects in DCs, but higher levels of IL-12 were observed in IRF4-deficient DCs. These results imply that IRF4 expression in DCs inhibits their ability to produce IL-12 while promoting their migratory behavior, thus regulating $CD4^+$T cell responses against local infection with *L. major*.

Introduction

$CD4^+$ T cell immune responses are polarized to distinct Th cell types, such as Th1, Th2, Th17, and induced regulatory T cells (Tregs), which produce different cytokines (**1**). The infection model of *Leishmania major* has been used for the study of Th1/Th2 differentiation of $CD4^+$ T cells (**2**). In susceptible mice, such as BALB/c, *L. major*–specific immune responses are shifted toward the Th2 type, which is unable to control parasite infection. In resistant mice, such as C57BL/6, immune responses are shifted to a Th1 type, which clears the infection. Whether $CD4^+$ T cells differentiate toward a Th1 type versus a Th2 type during infection with *L. major* is controlled, in part, by macrophages and dendritic cells (DCs). Macrophages are primary host cells for the parasite infection and are also responsible for the elimination of the parasites. Infection with *Leishmania* parasites modulates the protective immune response induced by macrophages by inhibiting their ability to produce IL-12 (**3**). In contrast, DCs are critical for mounting protective T cell responses against *Leishmania* infections. In cutaneous *Leishmania* infection, DCs in the skin migrate to draining lymph nodes (LNs), where they prime Ag-specific T cells (**4**). Studies revealed the involvement of different DC subsets in the induction of host T cell responses against infection with *L. major*, depending on the stage of infection. During the early phase of infection, DCs that initiate parasite-specific immune response in the draining LNs are not directly infected with *L. major*. $CD11c^{hi}CD11b^{hi}Langerin^-$ resident DCs in the LNs acquire soluble *Leishmania* Ags released by parasites and present these Ags to specific $CD4^+$ T cells, while, at the same time, producing IL-12 (**5**, **6**). Several days postinfection, $CD8^-Langerin^-$ DCs within draining LNs present *L. major* Ag to $CD4^+$ T cells (**7**), whereas $Langerin^+$ DCs present *Leishmania* Ag to $CD8^+$ T cells (**8**). During the late phase of *L. major* infections, dermal monocyte-derived DCs ($CD11c^{int}Ly6C^{lo}MHC\ II^{hi}DEC\text{-}205^{int}$) are the major APCs that activate specific $CD4^+$ T cells and are the main source of IL-12 (**9**). Within the migrating dermal DC types, Langerhans cells were thought to be responsible for the priming of *Leishmania*-specific T cells during infection, but recent studies suggest that they drive expansion of Tregs and are inhibitory for the protective immune responses when small doses of *L. major* are used to inoculate C57BL/6 mice (**10**).

IRF4 is a transcription factor in the IRF family whose expression is limited to immune cells, such as lymphocytes, macrophages, and DCs (**11**–**14**). We and other investigators showed that, within the T cell compartment, IRF4 is essential for the development of Th2, Th17, and follicular Th cells (**12**, **15**–**18**) and is critical for the functions of Tregs (**19**). We previously demonstrated that, in macrophages, IRF4 negatively regulates production of proinflammatory cytokines in response to TLR ligands (**20**, **21**). IRF4 interacts with MyD88 and acts as negative regulator of TLR signaling by competing with IRF5 (**21**). IRF4 is also expressed in different DC subsets and is essential for the development of $CD8^-CD11b^+$splenic DCs (**13**, **22**). Bajaña et al. (**23**) evaluated the roles of IRF4 expression in DCs using $Irf4^{-/-}$ mice. They showed that development and residency of tissue DCs were not disrupted by the lack of IRF4, but Langerhans cells and dermal DCs did not express the

chemokine receptor CCR7, and their migration to LNs was impaired. However, they were unable to evaluate the ability of *Irf4*$^{-/-}$ DCs to prime T cells in *Irf4*$^{-/-}$ mice, because T cell function is also IRF4 dependent and is impaired in *Irf4*$^{-/-}$ mice.

We previously reported that, after s.c. infection with *L. major* into the footpad, *Irf4*$^{-/-}$ mice show significantly reduced footpad swelling 2–6 wk postinfection but show worsening of footpad swelling and a greater extent of infection later (**16**). The cell type responsible for the reduction of the lesion was not clear. In this study, we examined the possibility that IRF4 deficiency in macrophages or DCs causes enhanced immunity against *L. major*infection by using conditional gene knockout mice that lack IRF4 in macrophages or DCs. The study showed that IRF4 deficiency in DCs induces early and enhanced Th1-biased anti-*Leishmania* CD4^{+} T cell responses and causes a lesser degree of footpad swelling and reduced parasite burden. Recruitment of migratory DCs (mDCs) to the draining LNs also was reduced, but IRF4-deficient DCs produced increased levels of IL-12, suggesting that a higher level of IL-12 production from DCs induced enhanced Th1 immune responses against *L. major*.

Materials and Methods

Animals

Mice containing loxP-flanked *Irf4* alleles (*Irf4*$^{fl/fl}$) were kindly provided by Dr. U. Klein (Columbia University, New York, NY) (**24**). CD11c-Cre mice (**25**) were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129P2-Lyzs < tm1(cre)Ifo > (LysM-Cre) mice (**26**) were provided by the Riken BioResource Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.*Irf4*$^{fl/fl}$ mice were crossed with CD11c-Cre or LysM-Cre mice to generate *Irf4*$^{fl/fl}$CD11c-Cre^{+}or *Irf4*$^{fl/fl}$ LysM-Cre^{+} mice, respectively. *Irf4*$^{-/-}$ mice were described previously (**11**). OT-II–and OT-I–transgenic mice expressing TCR specific for $OVA_{323–339}$/IA^{b} and $OVA_{257–264}$/K^{b}, respectively, were provided by Dr. H. Kosaka (Osaka University, Osaka, Japan) (**27**, **28**). B6.SJL and OT-II mice were interbred, and offspring were intercrossed to obtain CD45.1^{+}OT-II mice. C57BL/6 and BALB/c mice were purchased from SLC (Shizuoka, Japan). These mice were maintained in the Laboratory Animal Center for Animal Research at Nagasaki University and were used at the age of 8–14 wk. The animal experiments were approved by the Institutional Animal Care and Use Committee of Nagasaki University and were conducted according to the guidelines for Animal Experimentation at Nagasaki University.

Parasite infection

L. major (MHOM/SU/73-5-ASKH strain) was provided by Dr. K. Himeno (Kyushu University, Fukuoka, Japan). *L. major* Friedlin clone Vl expressing OVA (PHOC *L. major*) (**29**) was kindly provided by Dr. D.F. Smith (University of York, York, U.K.). Cells from the popliteal LNs of

infected mice were cultured in 199 medium supplemented with 10% FCS and penicillin/streptomycin. After 5–6 d, *L. major* promastigotes (5×10^6) were collected and injected s.c. in the left hind footpad or in the ears of mice. The thickness of the infected (left) and the contralateral uninfected (right) footpad was measured once per week by using a vernier caliper, as described (**16**). The increase in footpad thickness was calculated as follows: ([thickness of infected footpad] − [thickness of uninfected footpad]/[thickness of uninfected footpad]) × 100. Parasite burden was determined by real-time PCR analysis of*L. major* DNA, as described previously (**30**). Briefly, footpads were dissected, dropped in Isogen (1 ml; Nippon Gene, Tokyo, Japan), and homogenized using a mechanical homogenizer. DNA was extracted from aliquots of homogenates, and real-time PCR on each DNA sample was run in quintuplicate on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Carlsbad, CA). To quantify DNA standards, 120-bp PCR products were amplified from *L. major* cDNA using the primer pairs described previously (**30**) and cloned into a pGEM-T Easy vector (Promega, Madison, WI). A serial dilution of this plasmid was used as standard to determine the parasite copy numbers, and all samples were normalized using *G3PDH* or *18S* as a housekeeping gene.

To block IL-12 in vivo, an anti–IL-12 mAb (C17.8), prepared from the ascites of hybridoma cells, was purified using HiTrap-protein G (GE Healthcare). Mice were inoculated i.p. with anti–IL-12 mAb (800 µg/mouse) on days 0 and 7 postinfection with *L. major*. Three weeks postinfection, parasite burden in the mice footpads was determined by real-time RT-PCR.

Flow cytometry

LNs and spleens were incubated in HBSS (5 ml) containing collagenase (1 mg/ml) at 37°C for 30 min and then washed before RBCs were lysed using Gey's solution. The cells were then blocked with anti-FcR mAb (2.4G2) and stained for CD103 (2E7), CD3 (145-2C11), CD4 (GK1.5), CD8a (53-6.7), Ly-6G/Gr-1 (RB6-8C5), F4/80 (BM8), CCR7/CD197 (4B12), CD11a (M17/4), CD11b (M1/70), CD11c (N418), CD49d (R1-2), MHC class II (M5/114.15.2), CD45.1 (A20), or isotype controls. All mAbs were purchased fromeBioscience (San Diego, CA), except where specifically indicated. For intracellular staining, cells were stained for surface markers, washed, fixed, permeabilized, and stained using a Fixation/Permeabilization kit (BD Biosciences, San Jose, CA) and stained with Abs for Langerin (929F3.01; Dendritics, San Diego, CA), IL12- p40/p70 (C15.6; BD Bioscience), IFN-γ (XMG1.2), IRF4 (3E4), or isotype control. Surface staining of CCR7 was performed at 37°C, following the manufacturer's recommendations (eBioscience). Data from the stained cells were collected on a FACSCanto II (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR).

Cell culture

$CD4^+$ T cells were enriched from LN single-cell suspensions using anti-CD4 IMag (BD Biosciences), whereas DCs from the spleens of naive C57BL/6 mice were separated using anti-CD11c magnetic beads and an AutoMACS magnetic cell separator, according to the

manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, glutamine, penicillin/streptomycin, 2-ME, nonessential amino acids, and sodium pyruvate. Crude *L. major* Ag was prepared by freezing and thawing of promastigotes five times. $CD4^+$ T cells (2×10^5) and DCs (2×10^4) were cultured in the presence or absence of *L. major* Ag (6×10^5 parasite equivalent) in 96-well flat culture plates for 72 h. Levels of cytokines in the supernatants were determined by sandwich ELISA, as described previously (**16**). To examine the $CD4^+$ T cell subpopulation responses, $CD4^+CD11a^{hi}CD49d^+$ and $CD4^+CD11a^{lo}CD49d^-$ cells were purified, using a FACSAria II (BD Biosciences), from popliteal LN cells harvested from C57BL/6 mice 2 wk postinfection with *L. major* in the footpad. Purified $CD4^+$ T cells (1×10^5) and DCs (1×10^4) were cultured in the presence of *L. major* Ag, and IFN-γ production was determined as described above.

For intracellular staining of IFN-γ, cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) in the presence of monensin for 4 h, following the manufacturer's recommendation (BD Biosciences). For the staining of IL-12, cells were cultured in the presence of monensin alone for 4 h.

Preparation of skin cells

Epidermal Langerhans cells and dermal cells were prepared as previously described with modifications (**31**). Briefly, mouse ears were rinsed in PBS and split into dorsal and ventral halves. After incubation in trypsin-EDTA medium (Sigma) with the epidermal side down, at 37°C for 1 h, the epidermis was separated from the dermis. Dermal tissue was minced into small pieces and digested in RPMI containing collagenase (1 mg/ml) at 37°C for 1 h. Epidermal sheets and digested dermal tissue were smashed gently with a plunger, and cell clumps and debris were removed by passing the cell suspension through a 70-μm nylon mesh.

CFSE labeling and T cell adoptive-transfer experiments

$CD4^+$ and $CD8^+$ T cells were purified from OT-II and OT-I mice using anti-CD4 and anti-CD8 IMag (BD Biosciences), respectively; labeled with 15 μM CFSE, according to the manufacturer's protocols (Molecular Probes, Eugene, OR); and adoptively transferred into recipient mice, as described previously (**32**). Mice were infected with PHOC *L. major* in the left hind footpad 24 h after transfer of OT-II or OT-I cells. Popliteal LNs were dissected 72 h postinfection, and cell suspensions were analyzed for diminution of CFSE using a FACSCanto II.

Statistical analysis

Results are shown as mean ± SD. The statistical significance of the differences between two groups was determined using unpaired two-tailed Student *t* tests. A p value < 0.05 was considered significant.

Results

Reduced footpad swelling postinfection with *L. major* due to IRF4 deficiency in DCs

To examine the role of IRF4 in DCs and macrophages, *Irf4*$^{fl/fl}$ mice with a C57BL/6 background were crossed to CD11c-Cre or LysM-Cre mice to generate mice lacking IRF4 in DCs (*Irf4*$^{fl/fl}$CD11c-Cre mice) or in macrophages (*Irf4*$^{fl/fl}$LysM-Cre mice), respectively. Swelling of the footpad peaked at 3–4 wk postinfection with *L. major* and then gradually decreased and healed in control C57BL/6 mice, whereas swelling continued to increase in BALB/c mice, as described previously (**Fig. 1A**, **1B**) (**4**). In *Irf4*$^{-/-}$ mice with a C57BL/6 background, the degree of footpad swelling was lower than that in C57BL/6 mice during the 2–6 wk of infection, but it continued to increase afterward without healing, as described previously (**16**). The continued footpad swelling in the late stages of infection was likely due to the loss of cellularity in the draining LNs and enhanced apoptosis of T cells that do not express IRF4 (**16**, **33**). *Irf4*$^{fl/fl}$CD11c-Cre mice showed a lower degree of footpad swelling, similar to *Irf4*$^{-/-}$ mice 2–5 wk postinfection, but the swelling of the footpad decreased after 6 wk of infection and eventually dissolved, unlike in *Irf4*$^{-/-}$ mice, which showed worsening of the lesion during the latter period of infection (**Fig. 1A**). In contrast, *Irf4*$^{fl/fl}$LysM-Cre mice showed footpad swelling similar to C57BL/6 mice, suggesting that expression of IRF4 in macrophages does not play a significant role in the protection against *L. major* infection (**Fig. 1B**). These results suggest that the reduced footpad swelling in *Irf4*$^{-/-}$ mice 2–6 wk postinfection with *L. major* was mainly due to the lack of IRF4 in DCs and not macrophages.

Because the gene construct in *Irf4*$^{fl/fl}$ mice allowed GFP expression in cells that were depleted of IRF4 (**24**), we determined the expression of GFP and IRF4 in *Irf4*$^{fl/fl}$CD11c-Cre and *Irf4*$^{fl/fl}$LysM-Cre mice. In *Irf4*$^{fl/fl}$CD11c-Cre mice, the majority of DCs in popliteal LNs express GFP, indicating that these cells showed depletion of IRF4, whereas only a small proportion of T cells, B cells, and macrophages expressed GFP (**Fig. 1C**). IRF4 expression was detectable in the DCs of naive *Irf4*$^{fl/fl}$ mice, but not *Irf4*$^{fl/fl}$CD11c-Cre mice, by intracellular staining with a specific mAb, whereas macrophages in both mice expressed IRF4 at similar levels (**Fig. 1D**). The majority of DCs, as well as T and B cells, remained GFP^{-} in *Irf4*$^{fl/fl}$LysM-Cre mice (**Fig. 1E**).

Th1 responses against *L. major* are enhanced in *Irf4*$^{fl/fl}$CD11c-Cre mice

We examined the mechanism underlying the reduction of footpad swelling in *Irf4*$^{fl/fl}$CD11c-Cre mice. Production of IFN-γ by CD4^{+} T cells is critical for the control of infection with *L. major* (**34**, **35**). However, a previous study (**36**) showed that CD4^{+} T cells from resistant C57BL/6 mice produce IL-4 transiently during the first week of *L. major* infection. Thus, we determined whether IFN-γ and IL-4 were produced by CD4^{+} T cells during *L. major*infection. Therefore, we obtained CD4^{+} T cells from the draining LNs at different time points postinfection and stimulated them with *L. major* Ag in the presence of wild-type DCs in vitro

(**Fig. 2A**). The level of IFN-γ production was significantly higher in $CD4^+$ T cells from*Irf4*$^{fl/fl}$CD11c-Cre mice than in control *Irf4*$^{fl/fl}$ mice until 3 wk postinfection. Six weeks postinfection, we did not observe any significant differences in specific IFN-γ production by $CD4^+$ T cells. The production of IL-4 was detected in *Irf4*$^{fl/fl}$ mice at early time points postinfection, as previously described (**35**), but this response was barely detectable in $CD4^+$ T cells from *Irf4*$^{fl/fl}$CD11c-Cre mice, suggesting that their Th1 bias began early in the infection. We next used cell surface expression of CD11a and CD49d as markers of activated Ag-specific $CD4^+$ T cells, as reported in virus-infected mice (**37**). All $CD4^+$ T cells producing IFN-γ in response to *L. major* Ag were enriched in the $CD11a^{hi}CD49d^+$population obtained from the LNs of infected mice (**Fig. 2B**). $CD4^+$ T cells from the draining LNs were stained for surface markers and intracellular IFN-γ (**Fig. 2C–F**). Two weeks postinfection with *L. major*, the proportion of $CD11a^{hi}CD49d^+$ $CD4^+$ T cells increased by ∼10% in both *Irf4*$^{fl/fl}$CD11c-Cre and *Irf4*$^{fl/fl}$ mice, suggesting that clonal expansion of specific $CD4^+$ T cells was not significantly affected by the absence of IRF4 in DCs. However, the proportion of IFN-γ–producing $CD4^+$ T cells was significantly higher in*Irf4*$^{fl/fl}$CD11c-Cre mice, suggesting the Th1 bias of the response. We also determined parasite burden in the infected footpads. As expected, it was significantly reduced in*Irf4*$^{fl/fl}$CD11c-Cre mice compared with control *Irf4*$^{fl/fl}$ mice (**Fig. 2G**). Our results indicate that the Th1 immune response against *L. major* is enhanced in mice lacking IRF4 in DCs, beginning as early as 4 d after the infection, leading to the effective clearance of parasites.

To confirm our results of a Th1-biased immune response in *Irf4*$^{fl/fl}$CD11c-Cre mice, we performed a second, independent experiment using PHOC *L. major* expressing OVA (**29**).*Irf4*$^{fl/fl}$CD11c-Cre and *Irf4*$^{fl/fl}$ mice were transferred with CFSE-labeled OT-II $CD4^+$ T cells and infected with PHOC *L. major* (**Fig. 3A**). Three days later, the proportion of OT-II cells within the $CD4^+$ T cell population in the draining LNs of *Irf4*$^{fl/fl}$CD11c-Cre mice was lower than that in *Irf4*$^{fl/fl}$ mice (**Fig. 3B**, **3C**). However, the proliferation of OT-II cells, as determined by the diminution of CFSE, was similar in both groups (**Fig. 3B**, **3D**). Production of IFN-γ in OT-II cells, as well as in the recipient $CD4^+$ T cell compartment, was higher in*Irf4*$^{fl/fl}$CD11c-Cre mice (**Fig. 3B**, **3E**). These results confirmed that the enhanced Th1 response in *Irf4*$^{fl/fl}$CD11c-Cre mice is due to the lack of IRF4 expression in DCs and not in T cells. In the $CD8^+$ T cell compartment, postinfection with PHOC *L. major*, OVA-specific transgenic $CD8^+$ T cells from *Irf4*$^{fl/fl}$CD11c-Cre mice were more abundant and showed higher proliferation and a higher proportion of IFN-γ production compared with those from*Irf4*$^{fl/fl}$ mice (Supplemental Fig. 1).

IRF4 is necessary for migration of DCs to draining LNs postinfection

We next examined the composition of DC subsets in the spleen and LNs. The proportion of $CD4^+$ DC subsets was severely reduced in the spleens of *Irf4*$^{fl/fl}$CD11c-Cre mice compared with *Irf4*$^{fl/fl}$ mice, as we previously reported (**Fig. 4**) (**13**). In the LNs, DCs can be divided into two subpopulations according to the expression levels of MHC class II and CD11c: resident

conventional DCs, which are MHC II^{+}CD11c^{hi}, and mDCs, which are MHC II^{hi}CD11c^{int} (**Fig. 4**) (**23**, **38**). Interestingly, the proportion of CD4^{+} DCs within conventional DC subpopulations in the LNs of *Irf4*$^{fl/fl}$CD11c-Cre mice was not significantly different from that in their controls. Thus, IRF4 is critical for the development of CD4^{+} DCs in the spleen but does not appear to be required for the development of CD4^{+} conventional DCs in the LNs. However, the proportion of mDCs in LNs was reduced in *Irf4*$^{fl/fl}$CD11c-Cre mice compared with that in *Irf4*$^{fl/fl}$ mice, whereas the proportion of conventional DCs was similar to that in their controls; this finding is consistent with a previous result showing defective migration of skin mDCs in *Irf4*$^{-/-}$ mice (**Figs. 4**, **5A–C**) (**23**).

We next examined DC subpopulations in the draining LNs before and 3 d postinfection with*L. major*. The number of conventional DCs in the LNs of uninfected *Irf4*$^{fl/fl}$CD11c-Cre mice was not significantly different from that in *Irf4*$^{fl/fl}$ mice, and the number increased similarly postinfection (**Fig. 5A**, **5B**). MHC II^{hi}CD11c^{int} mDCs (mDCs) can be divided into Langerin^{-}mDCs, which include CD11b^{lo} and CD11b^{hi} subsets, and Langerin^{+} mDCs, which include epidermal Langerhans cells and CD103^{+} dermal DCs (**Fig. 5A**) (**23**, **38**). The number of mDCs in LNs of naive *Irf4*$^{fl/fl}$CD11c-Cre mice was significantly lower than that in *Irf4*$^{fl/fl}$mice. Postinfection, the number of mDCs in LNs increased by ∼2-fold in control *Irf4*$^{fl/fl}$mice, but the increase in mDCs in *Irf4*$^{fl/fl}$CD11c-Cre mice was modest (**Fig. 5C**). A similar pattern of reduction in DC numbers was observed in LNs before and postinfection in all subsets of mDCs, with the exception of CD103^{+} DCs, which had a very small absolute number (**Fig. 5D**). To determine the expression of IRF4 in each DC subset, we stained LN cells from naive and infected mice with subset-specific cell surface markers and intracellular IRF4. All conventional DC and mDC subsets in *Irf4*$^{fl/fl}$ mice expressed IRF4, and the level of expression did not change 1–3 d postinfection (**Fig. 5E**, data not shown). Because IRF4 promotes CCR7 expression, we measured the expression of CCR7 in mDC subsets (**Fig. 5F**) (**23**). As expected, the majority of mDC subsets in *Irf4*$^{fl/fl}$CD11c-Cre mice, in particular Langerin^{-} DCs, expressed CCR7 at levels lower than those in *Irf4*$^{fl/fl}$ mice.

We also examined skin DC populations during homeostasis and postinfection. In the epidermis, the proportions of MHC II^{+} cells and Langerhans cells were similar between*Irf4*$^{fl/fl}$ and *Irf4*$^{fl/fl}$CD11c-Cre mice both before and postinfection (**Fig. 6A–C**). In the dermis, the proportions of MHC II^{+} cells and Langerhans cells also were not significantly different between *Irf4*$^{fl/fl}$CD11c-Cre and *Irf4*$^{fl/fl}$ mice. However, the proportions of CD11b^{+} and CD103^{+} dermal DCs in *Irf4*$^{fl/fl}$CD11c-Cre were significantly higher than those in *Irf4*$^{fl/fl}$ mice both before and postinfection (**Fig. 6D–F**). These data suggest that the development of epidermal and dermal DCs is not impaired in *Irf4*$^{fl/fl}$CD11c-Cre mice and that these cells stay in the skin postinfection as a result of defects in their ability to migrate. Taken together, our model using conditional knockout mice that lack IRF4 in DCs showed that IRF4 plays an important role in the migration of mDCs in the steady-state and postinfection, consistent with the observation in a previous study using *Irf4*$^{-/-}$ mice (**23**).

IRF4 inhibits IL-12 production by mDCs

IL-12 is a key cytokine for the induction of Th1 immune responses and IFN-γ production postinfection with *L. major*. We hypothesized that IL-12 production by IRF4-deficient DCs is responsible for this higher Th1 induction in *Irf4*$^{fl/fl}$CD11c-Cre mice. Draining LN cells from*Irf4*$^{fl/fl}$CD11c-Cre and *Irf4*$^{fl/fl}$ mice were collected 3 d after the infection and measured for IL-12 production by intracellular cytokine staining. All subsets of IRF4-deficient mDCs, with the exception of minor CD103^{+} DCs, produced higher amounts of IL-12 compared with*Irf4*$^{fl/fl}$ mice (**Fig. 7A**, **7B**). To confirm the role of IL-12 in the enhanced protective immunity observed in *Irf4*$^{fl/fl}$CD11c-Cre mice, IL-12 was neutralized in vivo using an anti–IL-12 mAb (**Fig. 7C**). Although parasite burden in *Irf4*$^{fl/fl}$CD11c-Cre mice was lower than that in *Irf4*$^{fl/fl}$mice without IL-12 blockade, both groups showed similarly high levels of parasite burden when IL-12 activity was neutralized in vivo. We also examined the expression of costimulatory molecules in IRF4-deficient and control DC subsets. All of these DC subsets expressed CD80, CD86, and CD40 at high levels in both *Irf4*$^{fl/fl}$CD11c-Cre and *Irf4*$^{fl/fl}$ mice (data not shown). These results suggest that DCs produce higher levels of IL-12 early postinfection in *Irf4*$^{fl/fl}$CD11c-Cre mice, leading to Th1-biased immune responses.

Discussion

In this study, we evaluated the role of IRF4 expressed in macrophages and DCs during infection with *L. major.* Macrophages are definitive host cells for survival and replication of*Leishmania* parasites and they are also one of the major effector cells. We reported previously that IRF4$^{-/-}$ macrophages produce higher levels of cytokines, such as TNF-α, IL-12, and IL-6, in response to TLR signaling (**20**, **21**). However, in this study, IRF4-deficient macrophages did not affect the course of the infection with *L. major*. The production of IL-12 by infected macrophages was reported to be inhibited by resident *L. major* parasites (**3**); thus, the effect of IRF4 deficiency in macrophages may not have been apparent following *L. major* infection. DCs are the main producers of IL-12, which is critical for Th1 differentiation and IFN-γ production by CD4^{+} T cells during infection with *L. major*(**39**). In our study, IRF4 deficiency in DCs enhanced the protective Th1 immunity against *L. major* infection, leading to better clearance of parasites, thus suggesting that IRF4 expressed in DCs may play a regulatory role in the production of IL-12 in vivo. One caveat of this study is the leakiness of *Irf4*$^{fl/fl}$CD11c-Cre mice. We observed small, but significant, proportions of lymphocytes and macrophages expressing GFP, indicating that these cells lack IRF4 (**Fig. 1C**). Therefore, a possibility that IRF4 expressed in a subpopulation of macrophages is involved in the protection cannot be completely excluded, although we think that it is less likely.

The proportion of Ag-specific CD4^{+} T cells in the draining LNs of *Irf4*$^{fl/fl}$CD11c-Cre and*Irf4*$^{fl/fl}$ mice, as judged by the proportion of CD11a^{hi}CD49d^{+} CD4^{+} T cells, was not significantly different, suggesting that the levels of activation/proliferation of Ag-specific CD4^{+} T cells were equivalent in both groups. However, CD4^{+} T cells in *Irf4*$^{fl/fl}$CD11c-Cre mice showed a strong Th1 bias as early as 4 d postinfection and during the peak response

against *L. major*. These results were confirmed in experiments using mice transferred with OT-II cells and infected with OVA-expressing *L. major*. The expansion of OT-II cells, as evaluated by the diminution of CFSE, was not significantly different between *Irf4*$^{fl/fl}$CD11c-Cre and control mice, whereas the proportion of OT-II cells in the draining LNs was lower in*Irf4*$^{fl/fl}$CD11c-Cre mice. We speculated that this reduction was due to the reduced recirculation of OT-II cells to the LNs of *Irf4*$^{fl/fl}$CD11c-Cre mice, in which the number of mDCs was reduced, because it was reported that lymphocyte recirculation is controlled by DCs expressing lymphotoxin (**40**). Functionally, the IFN-γ response in OT-II cells was higher than in *Irf4*$^{fl/fl}$CD11c-Cre mice. These results collectively suggest that Ag-specific CD4$^+$ T cells primed by IRF4-deficient DCs expand at levels similar to control, but their responses are more biased toward a Th1 type. Thus, IRF4 expression in DCs plays a pivotal role in priming Th1 cells.

We previously reported that the proportion of the CD11b^{high}CD4$^+$ DC subset is severely reduced in the spleen of *Irf4*$^{-/-}$ mice (**13**). We confirmed that this population was also reduced in the spleens of *Irf4*$^{fl/fl}$CD11c-Cre mice. However, the CD4$^+$ DC subset was not reduced in the LNs of *Irf4*$^{-/-}$ or *Irf4*$^{fl/fl}$CD11c-Cre mice compared with controls, suggesting that IRF4 is not essential for the development of CD4$^+$ DCs in LNs. The differences in IRF4 dependency for the presence of splenic and LN CD4$^+$ DCs suggest that these two DC subsets might be derived from different developmental pathways. Alternatively, the LN environment might compensate for the IRF4 dependency on the development, survival, or migration of CD4$^+$ DCs. Recently, studies showed that the proportions of CD103$^+$CD11b$^+$DCs in the lamina propria of the small intestine and mesenteric LNs, as well as CD24$^+$CD11b$^+$ DCs in the lung, are reduced in mice with IRF4-deficient DCs. These mice failed to support the development of Th17 cells after immunization or infection (**41**, **42**). In these studies, IRF4 was shown to be required for the survival of a CD11b$^+$ DC subpopulation in the intestine and the lung, rather than their development. Thus, the IRF4 dependency of DCs appears to differ depending on DC subtype and localization. Further study is required to clarify the role of IRF4 and other transcription factors in the development and homeostasis of different DC subtypes in different tissues.

Prior to infection, the numbers of resident DCs in the LNs of control and *Irf4*$^{fl/fl}$CD11c-Cre mice were similar, and they increased similarly postinfection. However, the number of mDCs in *Irf4*$^{fl/fl}$CD11c-Cre mice was lower than that in the control prior to infection, and it did not increase significantly postinfection. In the epidermis and dermis of *Irf4*$^{fl/fl}$CD11c-Cre mice, the proportion of Langerhans cells was not reduced postinfection with *L. major*, but CD11b$^+$ and CD103$^+$ DCs were increased, suggesting that migration of these DC subsets from the skin to the draining LNs was impaired in the absence of IRF4. CCR7 expression was reduced in mDCs lacking IRF4, consistent with the reduced ability of these DCs to migrate to the LNs. While this study was in progress, Bajaña et al. (**23**) reported that migration of CD11b$^+$ DCs, as well as Langerhans cells, from the dermis to LNs was impaired in *Irf4*$^{-/-}$ mice. Our study using *Irf4*$^{fl/fl}$CD11c-Cre mice is consistent with their study and further shows that

the defective migration of DCs occurs independently of the IRF4 defect in the lymphocyte compartment. Interestingly, priming of *L. major*–specific $CD4^+$ T cells was not reduced in *Irf4*$^{fl/fl}$CD11c-Cre mice, despite reduced migration of skin DCs to draining LNs. We speculate that the activation of $CD4^+$ T cells during the early period after *L. major* infection depends on the resident DCs in the draining LNs, which receive soluble *Leishmania* Ag through the lymph conduit network, as shown previously (**6**), and, therefore, was not severely affected by the reduced migration of DCs from footpads.

IL-12 released by DCs plays a pivotal role in Th1 development and IFN-γ production in $CD4^+$ cells (**43**). In *Irf4*$^{fl/fl}$CD11c-Cre mice, DC subsets produced IL-12 at levels higher than that in the control during infection with *L. major*, which explains, at least in part, why $CD4^+$T cells are more prone to Th1 skewing in *Irf4*$^{fl/fl}$CD11c-Cre mice. TLR delivers critical signals that induce IL-12 production in DCs during immune responses against infection with*L. major*. It was shown that MyD88$^{-/-}$ C57BL/6 mice produce reduced levels of IL-12 and exhibit lesions similar to Th2-prone susceptible BALB/c mice during infection with *L. major*(**44**). TLR2, TLR4, and TLR9 are suggested to be involved in the recognition of *Leishmania*molecules, and TLR9 appears to be the most important TLR required for the development of Th1 responses (**45**). We showed previously that IRF4 negatively regulates TLR signaling and production of proinflammatory cytokines, including IL-12 in macrophages (**20**, **21**). Thus, it is likely that, similar to what is observed in macrophages, production of IL-12 in response to *L. major* molecules is enhanced in IRF4-deficient DCs during infection, leading to accelerated Th1-type $CD4^+$ T cell responses. We examined the expression of IRF4 and IL-12 production in DC subsets in LNs. In contrast to splenic DCs that showed heterogeneous expression of IRF4 (**13**), all DC subsets in LNs showed IRF4 expression. IL-12 production was detected in all DC subsets, including resident and mDCs, in our intracellular cytokine-staining assay. The proportions of DCs producing IL-12 were significantly higher in the majority of IRF4-deficient mDC subsets compared with controls, consistent with the inhibitory role of IRF4 in TLR signaling. IL-12 production by resident DCs from *Irf4*$^{fl/fl}$CD11c-Cre mice was not significantly different from that in *Irf4*$^{fl/fl}$ mice. However, $CD4^+$ T cells showed strong Th1-biased protective immunity against *L. major*. Perhaps, enhanced production of IL-12 by mDCs established a Th1-biased environment.

In this study, we showed that IRF4 expressed in DCs is inhibitory for their IL-12 production in vivo during *L. major* infection and that it plays a pivotal role in regulating Th1 differentiation of $CD4^+$ T cells. Bajaña et al. (**23**) reported that IRF4 is critical for the CCR7-mediated migration of $CD11b^+$ DCs from the dermis to LNs. Recent studies (**41**, **42**) suggest that IRF4 in DCs is critical for the survival of a subset of $CD11b^+$ DCs in the lamina propria of the intestine and lung, as well as mesenteric LNs, and support Th17 differentiation after immunization or infection. Taken together, these studies highlight critical roles for IRF4 in controlling DC homeostasis and function and, thus, regulating functional differentiation of $CD4^+$ T cells. IRF4 in DCs is required for Th17 development but is inhibitory for Th1 development of $CD4^+$ T cells. It would be interesting to examine whether IRF4 expressed in DCs also affects

differentiation of $CD4^+$ T cells to other lineages, including follicular Th and induced Tregs, in different models. Furthermore, previous studies (**46**, **47**) showed that the expression and function of IRF4 can be modulated by PGE_2 and immunophilin FKBP52, respectively. Thus, IRF4 expressed in DCs could be a target of drug-mediated immune modulation. Further study is required to fully elucidate the role of IRF4 in regulating DC subtypes and the immune responses that they regulate. This will be of particular importance when developing vaccines or novel strategies that modulate immune responses.

Disclosures

The authors have no financial conflicts of interest.

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Abbreviations used in this article:

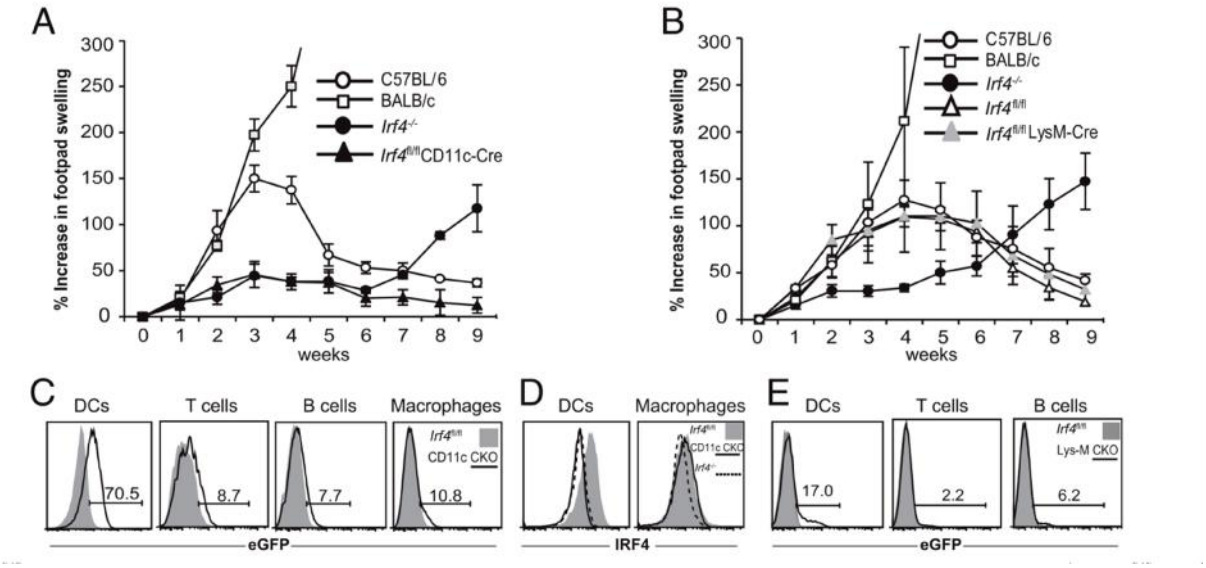
DC dendritic cell

LN lymph node

mDC migratory DC

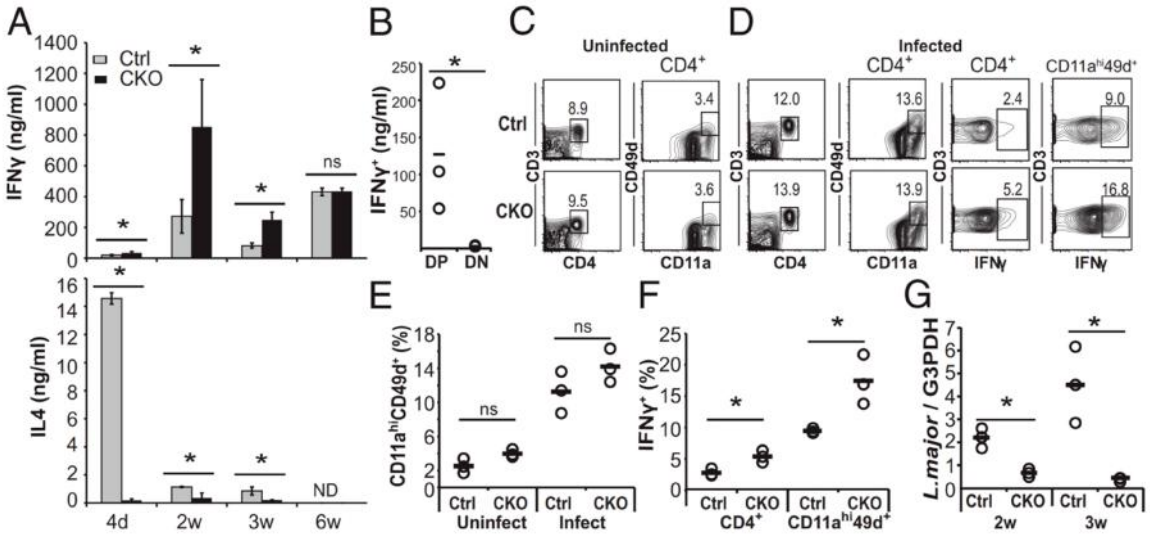
Treg regulatory T cell.

FIGURE 1.



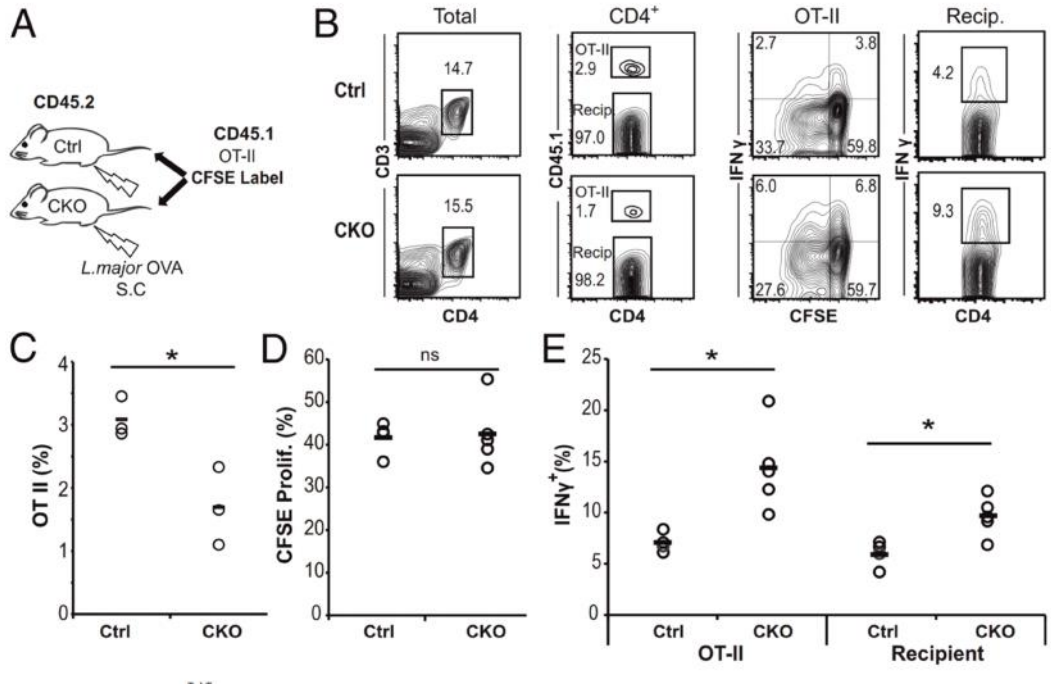
Irf4$^{fl/fl}$CD11c-Cre mice show reduced footpad swelling postinfection with *L. major*. (**A** and **B**) C57BL/6, BALB/c, *Irf4*$^{-/-}$, *Irf4*$^{fl/fl}$, *Irf4*$^{fl/fl}$CD11c-Cre, and *Irf4*$^{fl/fl}$LysM-Cre mice were infected in the left hind footpad with *L. major* promastigotes (5×10^6), and footpad swelling was measured every week until 9 wk postinfection. Graphs show mean ± SD. Data represent three independent experiments (4–5 mice/group) with similar results. (**C** and **E**) Expression of GFP in DCs (MHC II$^+$CD11c$^+$ cells), T cells (CD3$^+$ cells), B cells (CD19$^+$ cells), and macrophages (Gr-1$^-$CD11b$^+$CD11c$^-$F4/80$^+$ cells) in the popliteal LN cells from *Irf4*$^{fl/fl}$ (filled graphs), *Irf4*$^{fl/fl}$CD11c-Cre (C, line), and *Irf4*$^{fl/fl}$LysM-Cre (E, line) mice. The numbers indicate the proportions (%) of GFP$^+$ cells in each cell type. (**D**) IRF4 staining in DCs (CD11c$^+$MHC II$^+$cells) and macrophages (Gr-1$^-$CD11b$^+$ CD11c$^-$F4/80$^+$ cells) from LNs of *Irf4*$^{fl/fl}$(filled graph), *Irf4*$^{fl/fl}$CD11c-Cre (black line), and *Irf4*$^{-/-}$ (dashed line) mice.

FIGURE 2.



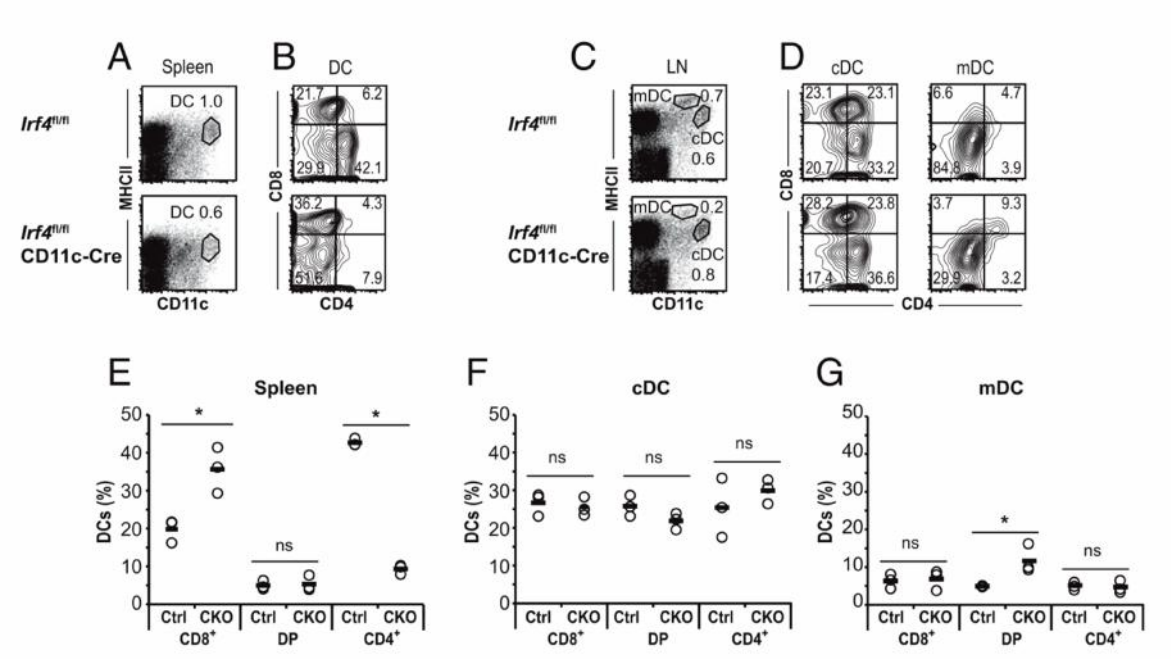
Irf4$^{fl/fl}$CD11c-Cre mice show Th1-biased immune responses against infection with*L. major*. (**A**) At different time points postinfection with *L. major* (4 d and 2, 3, and 6 wk), CD4$^+$ T cells from the draining LNs were cultured in the presence of splenic DCs from C57BL/6 mice and *L. major* Ag for 72 h. The amounts of IFN-γ and IL-4 in the supernatants were determined by ELISA. Graphs show mean ± SD. Representative data of three similar results are shown. (**B**) Two weeks postinfection with *L. major*, popliteal LN cells were prepared from C57BL/6 mice and stained for CD4, CD3, CD49a, and CD11a. CD11a^{hi}CD49d$^+$CD4$^+$ T cells (DP) and CD11a^{lo}CD49d$^-$ CD4$^+$ T cells (DN) were FACS sorted from the popliteal LNs and cultured in the presence of DCs from uninfected C57BL/6 mice and crude *L. major* Ag for 3 d. The levels of IFN-γ in the supernatant were determined by ELISA. (**C**–**F**) Postinfection with *L. major*, right (uninfected) and left (infected) popliteal LN cells were prepared from *Irf4*$^{fl/fl}$ (Ctrl) and *Irf4*$^{fl/fl}$CD11c-Cre (CKO) mice and stained as in (B). (C and D) Flow cytometry profiles of CD4$^+$ T cell gating, as well as CD49d versus CD11a expression of CD4$^+$ T cells, are shown. After culture for 4 h with ionomycin/PMA, the left LN cells were stained for cell surface markers, fixed, permeabilized, and stained for IFN-γ. (D) CD3 and IFN-γ profiles of total CD4$^+$ and CD11a^{hi}CD49d$^+$CD4$^+$ cells (*right panels*). Summary of the proportions (%) of CD11a^{hi}CD49d$^+$ cells in total CD4$^+$ T cells (E) and the proportions (%) of IFN-γ$^+$ cells within total CD4$^+$ T cells and in CD11a^{hi}CD49d$^+$CD4$^+$ T cells (F). The horizontal line represents the mean value in each group. (**G**) Two and three weeks postinfection, DNA from the left footpads (*n*= 3 mice/group) were subjected to real-time PCR, and the relative ratio of *L. major*to the *G3PDH* housekeeping gene was calculated. The horizontal line represents the mean value in each group. *$p < 0.05$. ND, not detectable.

FIGURE 3.



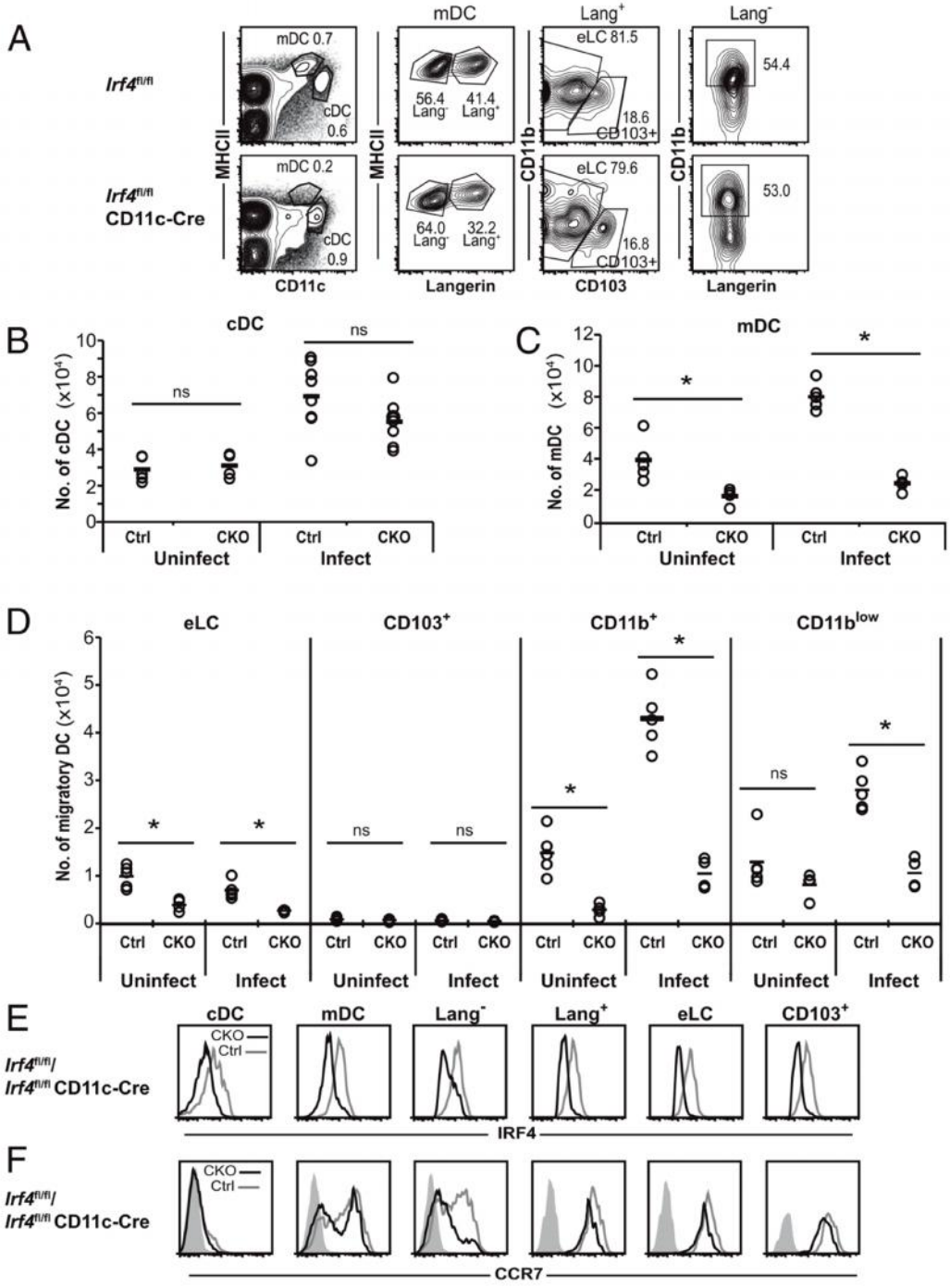
OT-II cells transferred to *Irf4*$^{fl/fl}$CD11c-Cre mice show Th1-biased responses to the infection with *L. major*-OVA. (**A**) *Irf4*^{flfl} (Ctrl) and *Irf4*$^{fl/fl}$CD11c-Cre (CKO) mice were adoptively transferred with CFSE-labeled CD4$^+$ T cells from OT-II mice (CD45.1) and were infected with PHOC *L. major* (*L. major* OVA) (5 × 10^6) in the left hind footpad. Three days postinfection, LN cells were stimulated with ionomycin/PMA for 4 h and stained for CD3, CD4, CD45.1, and IFN-γ. OT-II cells were gated as CD4$^+$CD3$^+$CD45.1$^+$ cells. (**B**) CFSE versus IFN-γ profiles of OT-II cells and IFN-γ staining of recipient CD4$^+$ cells. The numbers in the flow cytometry data indicate the proportions (%) of each cell population. The proportion of OT-II cells within the CD4$^+$ T cell population (**C**) and the proportion of IFN-γ$^+$ cells in the OT-II and recipient CD4$^+$ T cell populations (**E**) are shown for *Irf4*^{flfl} (Ctrl) and*Irf4*$^{fl/fl}$CD11c-Cre (CKO) mice. (**D**) The proportions of OT-II cells that divided more than once were determined by CFSE dilution. In (D) and (E), data from two independent experiments with similar results (n = 2–3 mice/group) were pooled. *p< 0.05.

FIGURE 4.



The numbers of $CD4^+$ conventional DCs are reduced in the spleen but not in LNs of $Irf4^{fl/fl}$CD11c-Cre mice. Cells were prepared from spleen (**A**, **B**, **E**) and popliteal LNs (**C**, **D**, **F**, **G**) of $Irf4^{flfl}$ (Ctrl) and $Irf4^{fl/fl}$CD11c-Cre mice (CKO) and were stained for MHC class II, CD11c, CD4, and CD8. CD4 versus CD8 expression of splenic DCs ($CD11c^+MHC\ II^+$), migratory LN DCs (mDCs; $MHC\ II^{hi}CD11c^+$), and conventional LN DCs (cDCs; $MHC\ II^+CD11c^{hi}$) is shown. The proportion (%) of each subpopulation is indicated. *$p < 0.05$.

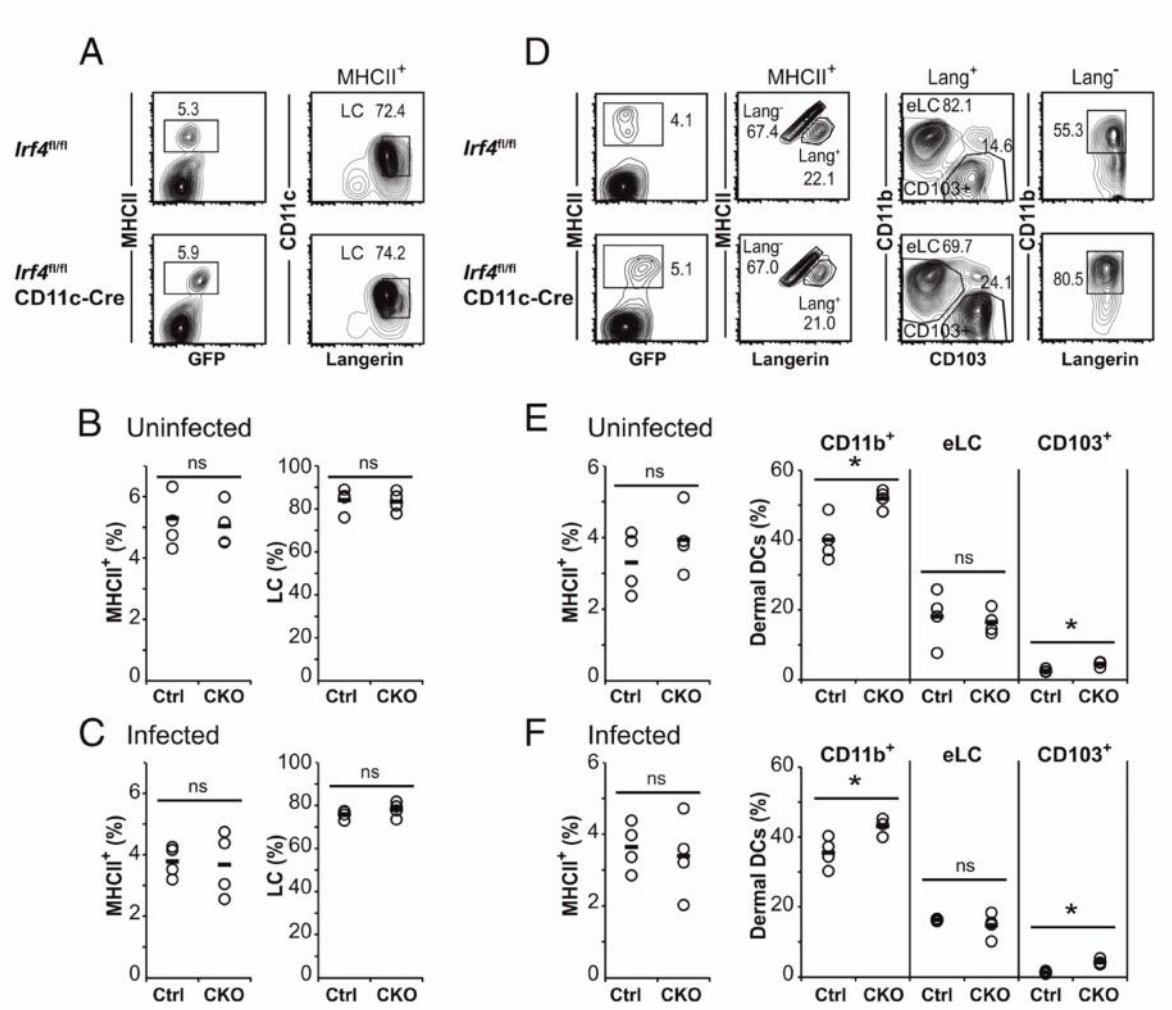
FIGURE 5.



IRF4 is important for the migration of mDCs. *Irf4*^{flfl} and *Irf4*$^{fl/fl}$CD11c-Cre mice were uninfected or infected for 3 d with *L. major* in the left footpad. After staining for CD11c, MHC II, CD11b, and CD103, LN cells were permeabilized and intracellularly stained for Langerin. (**A**) Staining profiles of LNs of uninfected mice and the gating strategies used for

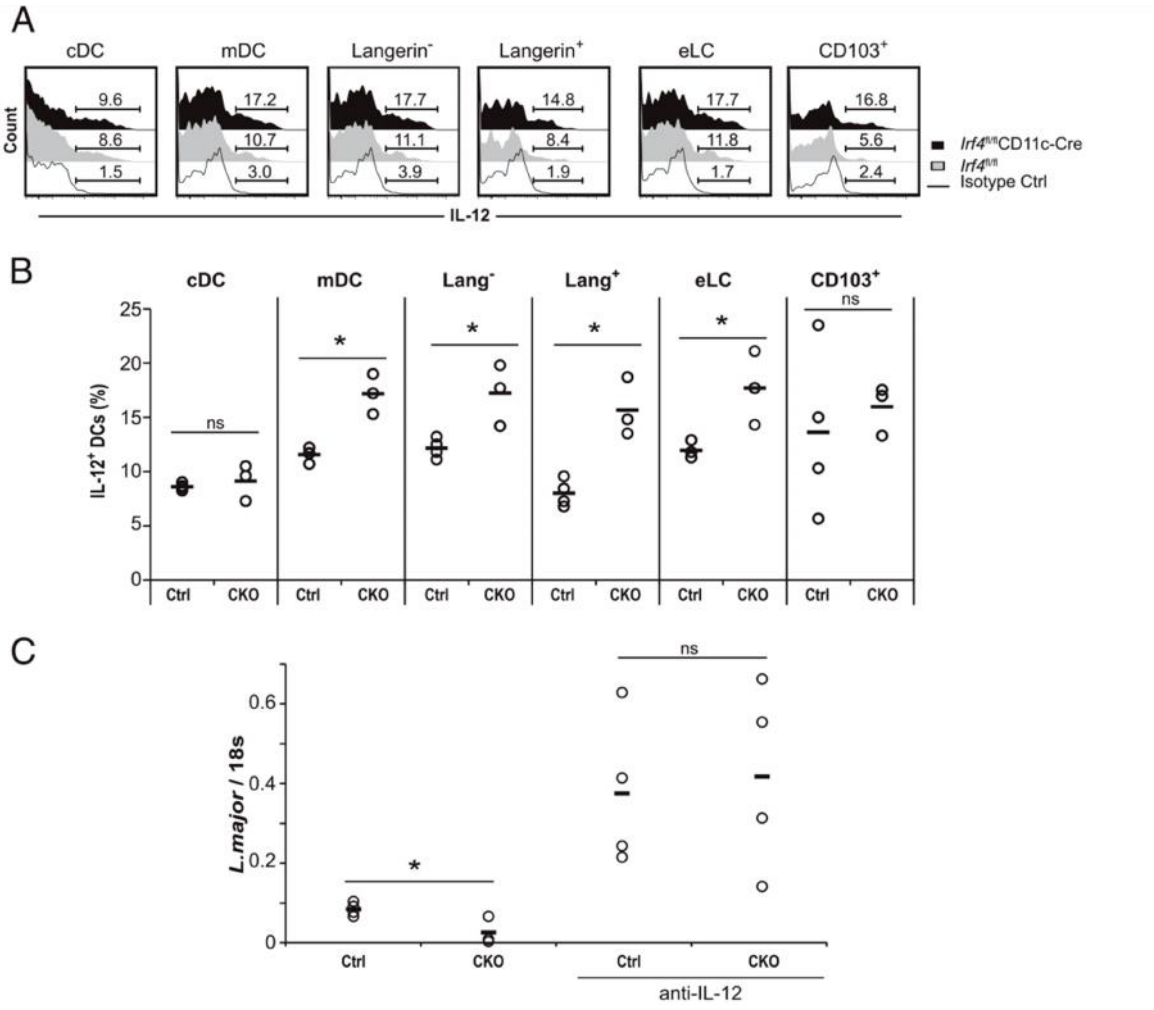
the identification of different Three days postinfection of *Irf4*^{flfl} (Ctrl) and *Irf4*$^{fl/fl}$CD11c-Cre (CKO) mice with *L. major* in the left footpad, the numbers of conventional DCs (cDC) (**B**), mDCs (**C**), and subpopulations of mDCs (**D**) in the left popliteal LNs (infected) and right popliteal LNs (uninfected) were determined. LN cells from *Irf4*$^{fl/fl}$ (gray line) and*Irf4*$^{fl/fl}$CD11c-Cre (black line) mice were stained for cell surface markers to distinguish DC subsets and were fixed, permeabilized, and stained for intracellular IRF4 (**E**) or were costained with anti-CCR7 mAb (**F**). LN cells from *Irf4*$^{fl/fl}$ mice were stained with an isotype control (F, shaded graphs). The expression of IRF4 (E) or CCR7 (F) is shown after gating for each subpopulation, as shown in (A). Data represent two independent experiments with similar results. The proportion (%) of each population is indicated. $*p < 0.05$. CD103^{+}, CD103^{+} DCs; cDC, conventional DCs; eLC, epidermal Langerhans cells; Lang^{+}, Langerin^{+} DCs; Lang^{-}, Langerin^{-} DCs.

FIGURE 6.



Dermal DCs increased in *Irf4*$^{fl/fl}$CD11c-Cre mice both in homeostasis and postinfection. (**A**) Epidermal Langerhans cells (MHC II$^+$CD11c$^+$Langerin$^+$) were identified within cell suspensions of epidermal sheets from *Irf4*^{flfl} and *Irf4*$^{fl/fl}$CD11c-Cre mice. (**B** and **C**) The proportion of MHC II$^+$ cells within epidermal cells (*left panel*) and the proportion of Langerhans cells within the MHC II$^+$ fraction (*right panel*) were determined before (B) and postinfection (C). (**D**) Dermal DC subpopulations were identified after staining dermal cells for MHC II, CD11b, CD103, and Langerin. The proportions of MHC II$^+$ cells in the dermis and the proportion of each DC subset within the MHC II$^+$ cells were determined prior to (**E**) and 3 d postinfection (**F**). Data represent two independent experiments (n = 4 mice/group) with similar results. *$p < 0.05$.

FIGURE 7.



Enhanced IL-12 production by mDCs from *Irf4*$^{fl/fl}$CD11c-Cre mice. (**A** and **B**) Draining LN cells from *Irf4*$^{fl/fl}$ and *Irf4*$^{fl/fl}$CD11c-Cre mice were prepared 3 d postinfection with *L. major*. Cells were cultured for 4 h in the presence of monensin; stained for CD11c, MHC II, CD11b, and CD103; permeabilized; and stained with allophycocyanin–anti-Langerin and PE–anti-IL-12 mAb. Subsets of DCs were gated as shown in **Fig. 5A**, and the profiles of IL-12 staining of DC subsets in *Irf4*^{flfl} (shaded graphs) and *Irf4*$^{fl/fl}$CD11c-Cre (filled graphs) mice are shown (A). The isotype control (open graph) contained cells from *Irf4*$^{fl/fl}$ mice. (B) Proportions of IL-12^{+} cells in subsets of mDCs and conventional DCs (cDCs). Experiments were performed three times (*n* = 4 mice/group) with similar results. Representative results of one experiment are shown. (**C**) *Irf4*$^{fl/fl}$ and *Irf4*$^{fl/fl}$CD11c-Cre mice were inoculated i.p. with anti–IL-12 mAb on day 0 and day 6 postinfection with *L. major*. Parasite burden in the infected footpads was determined 3 wk postinfection by real-time RT-PCR, and the relative ratio of *L. major* to *18S* gene was

calculated. The horizontal line represents the mean value. *$p < 0.05$. $CD103^+$, $CD103^+$ DCs; eLC, epidermal Langerhans cells; $Lang^+$, $Langerin^+$ DCs; $Lang^-$, $Langerin^-$ DCs

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