

MHC Class II Expression Restricted to CD8 α^+ and CD11b $^+$ Dendritic Cells Is Sufficient for Control of *Leishmania major*

Maria P. Lemos,¹ Fatima Esquivel,² Phillip Scott,² and Terri M. Laufer¹

¹Department of Medicine and ²Department of Pathobiology, University of Pennsylvania, Philadelphia, PA 19104

Abstract

Control of the intracellular protozoan, *Leishmania major*, requires major histocompatibility complex class II (MHC II)-dependent antigen presentation and CD4 $^+$ T cell T helper cell 1 (Th1) differentiation. MHC II-positive macrophages are a primary target of infection and a crucial effector cell controlling parasite growth, yet their function as antigen-presenting cells remains controversial. Similarly, infected Langerhans cells (LCs) can prime interferon (IFN) γ -producing Th1 CD4 $^+$ T cells, but whether they are required for Th1 responses is unknown. We explored the antigen-presenting cell requirement during primary *L. major* infection using a mouse model in which MHC II, I-A β^b , expression is restricted to CD11b $^+$ and CD8 α^+ dendritic cells (DCs). Importantly, B cells, macrophages, and LCs are all MHC II-negative in these mice. We demonstrate that antigen presentation by these DC subsets is sufficient to control a subcutaneous *L. major* infection. CD4 $^+$ T cells undergo complete Th1 differentiation with parasite-specific secretion of IFN γ . Macrophages produce inducible nitric oxide synthase, accumulate at infected sites, and control parasite numbers in the absence of MHC II expression. Therefore, CD11b $^+$ and CD8 α^+ DCs are not only key initiators of the primary response but also provide all the necessary cognate interactions for CD4 $^+$ T cell Th1 effectors to control this protozoan infection.

Key words: Langerhans cells • CD4 • macrophages • Th1 • antigen presentation

Introduction

Leishmania major is an intracellular protozoan that infects and multiplies within macrophages, although it can also infect Langerhans cells (LCs), DCs, and neutrophils (1, 2). Clearance of *L. major* in resistant C57Bl/6 mice requires IL-12-driven Th1 CD4 $^+$ T cells and macrophage activation for the killing of intracellular amastigotes (2). This immune regulation is MHC class II (MHC II)-dependent; MHC II-positive B cells, macrophages, and DCs accumulate in draining LNs of resistant mice (3) and class II-deficient (A $\beta^{b-/-}$) mice cannot control the infection (4). However, the class II-positive APCs required for CD4 $^+$ T cell priming, effector differentiation, and parasite control have not been identified.

MHC II $^+$ DCs prime CD4 $^+$ Th1 cells to nominal antigens (5) and could fulfill this role during *L. major* infection (1). CD8 α^+ and CD11b $^+$ DCs can be infected in vitro (6), and T cell priming to an immunodominant *L. major* peptide is mediated by CD11b $^+$ DCs (7). Transfer of *L. major*-infected

LCs can also confer resistance to susceptible strains (8). The DC subsets that initiate Th1 priming in vivo have not been delineated clearly.

The local accumulation of MHC II $^+$ infected macrophages and their role in parasite lysis suggest that these cells are the central APC-mediating parasite control (3, 9). Production of IL-12, TNF α , nitric oxide (NO), and IFN α by macrophages can contribute to Th1 polarization, effector Th1 CD4 $^+$ T cell maintenance, and parasite lysis (9–11). Macrophage secretion of these molecules can be facilitated by MHC II peptide-TCR and CD40-CD40L interactions (12, 13), but noncognate mechanisms such as Toll-like receptor signaling, TNF α , and IFN γ also elicit cytokine secretion and activation (14). Thus, the relative requirement of MHC II expression within different DC subsets and macrophages has not been established.

To study the cognate interactions that mediate control of *L. major*, we took advantage of the CD11c/A β^b transgenic mouse model in which MHC II expression is restricted to CD11b $^+$ and CD8 α^+ DCs (5). We report that CD11c/A β^b mice develop normal immunity to the intracellular parasite, *L. major*, and control this subcutaneous infection, indicating that antigen presentation by CD11b $^+$ and CD8 α^+ DCs is

Address correspondence to Terri M. Laufer, Dept. of Medicine, University of Pennsylvania, 753 BRB II/III, 421 Curie Blvd., Philadelphia, PA 19104. Phone: (215) 573-2975; Fax: (215) 573-7599; email: tlaufer@mail.med.upenn.edu

sufficient for Th1 differentiation and effector functions throughout the primary response.

Materials and Methods

Animals. CD11c/A β^b (5) and A $\beta^{b/-}$ mice were bred in our colony (9 and 22 generations backcrossed to C57Bl/6). TCR $\alpha^{-/-}$, C57Bl/6, and B6.SJL (CD45.1) mice were obtained from Jackson Laboratories. Mice 6–10 wk of age were used in all experiments.

Contact Sensitization and LC Isolation. Mice were anesthetized, shaved, and painted on the back with 30 μ l FITC (Sigma-Aldrich) diluted in 1:1 acetone/dibutyl acetate (Sigma-Aldrich) as described previously (15). 18–24 h after application of FITC, brachial, inguinal, and auricular LNs were digested at room temperature in 0.8 mg/ml collagenase type IV (Worthington Biochemicals) and 0.05% DNase I (Sigma-Aldrich) for 30–45 min. CD11c $^+$ cells were purified using CD11c MACS beads (Miltenyi Biotech) on paramagnetic AUTOMACS columns according to the manufacturer's instructions. CD11c $^+$ cell purity ranged from 85 to 95%.

Flow Cytometry. Single cell suspensions were blocked with anti-FcR2/3 (24G2; American Type Culture Collection). Staining of APCs was performed in PBS with 2% BSA, 2 mM EDTA, 1 μ g/ml mouse IgG (Sigma-Aldrich), and 1 μ g/ml rat IgG (Sigma-Aldrich). All conjugated antibodies used for staining were obtained from BD Biosciences. Intracellular staining was performed on 1% PFA fixed samples, which were permeabilized with 2% BSA and 0.02% saponin in PBS. For intracellular IFN γ staining, LN cells were first stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 2 nM monensin for 5 h at 37°C. Samples were analyzed on a FACSCalibur™ (BD Biosciences) using CELLQuest™ software. All dot plots have a log axis of 10 0 –10 4 .

Polyclonal T Cell Purification. B6.SJL spleen and LN suspensions were incubated with supernatants of 24G2, RA3, M1/70, and M5/114 (American Type Culture Collection) followed by goat anti-rat IgG magnetic beads (Polysciences, Inc.) and cells were removed by attaching to a magnetic stand (Bio-Mag; Polysciences, Inc.).

L. major Infections and Analysis. Purified CD45.1 $^+$ CD4 $^+$ T cell suspensions were transferred i.v. into hosts. 1 d later, the hind footpad was infected with 5 \times 10 5 stationary phase *L. major* promastigotes (MHOM/IL/80/Friedlin) grown in Grace's media as described previously (16). Footpad swelling was measured with a digital caliper and reported as the difference between infected and uninfected hind footpads. Standard deviation represents the difference in measurements within individual mice of the same genotype. Mice with swelling >5 mm were killed.

4, 7, or 9 wk after infection, infected footpads were processed for parasite counts (16) or immunohistochemistry (5). Antibodies GK1.5, M5/114, N418 (American Type Culture Collection), inducible NO synthase (iNOS)–FITC, and IFN γ (BD Biosciences) were used. FITC-stained slides were visualized on an IMT-2 fluorescence microscope (Olympus) and analyzed using SPOT software (HiTech).

Splenic CD4 $^+$ T cells were purified using CD4 MACS beads (Miltenyi Biotech) on AUTOMACS columns. C57Bl/6 T-depleted splenocytes were prepared by low-toxicity complement depletion (Cedarlane) with anti-Thy1 (MMT1) antibodies. 10 6 CD4 $^+$ T cells were cultured with 5 \times 10 6 C57Bl/6 T-depleted splenocytes and 10 μ g/ml soluble *L. major* antigen (16) for 3 d. Supernatants were analyzed by sandwich ELISA for IFN γ production.

Results and Discussion

To test the sufficiency of CD11b $^+$ and CD8 α^+ DC antigen presentation in control of *L. major* infection, we infected CD11c/A β^b mice. In CD11c/A β^b mice, the CD11c promoter reconstitutes A β^b expression in B6 A $\beta^{b/-}$ mice (5). CD8 α^+ and CD11b $^+$ DCs are class II positive, but other DC subtypes, specifically plasmacytoid DCs and LCs lack I-A b expression (5). B cells, macrophages, and parenchymal cells are also MHC II negative (5). We used CD11c/A β^b mice to determine the role of different APCs in the Th1 response to *L. major*.

MHC II Expression in CD11c/A β^b Mice. First, we determined whether APC subsets from CD11c/A β^b mice maintained their MHC II–negative phenotype after exposure to inflammatory signals in vivo. LCs migrating out of skin explants lacked MHC II expression in CD11c/A β^b mice (5). To examine LC expression during inflammation, we painted the ears and dorsal skin of mice with FITC diluted in irritant. In response to the stimulus, FITC-painted epidermal DCs migrated into the draining LNs, and were detected within 16–48 h (reference 15 and unpublished data). FITC-painted cells only migrated into skin-draining LNs, indicating specific painting of LCs (unpublished data). We analyzed FITC-negative and FITC-positive DCs in skin-draining LNs for MHC II expression (Fig. 1 A). FITC-painted LCs from A $\beta^{b+/-}$ mice were MHC II bright; whereas, FITC-painted LCs from CD11c/A β^b mice re-

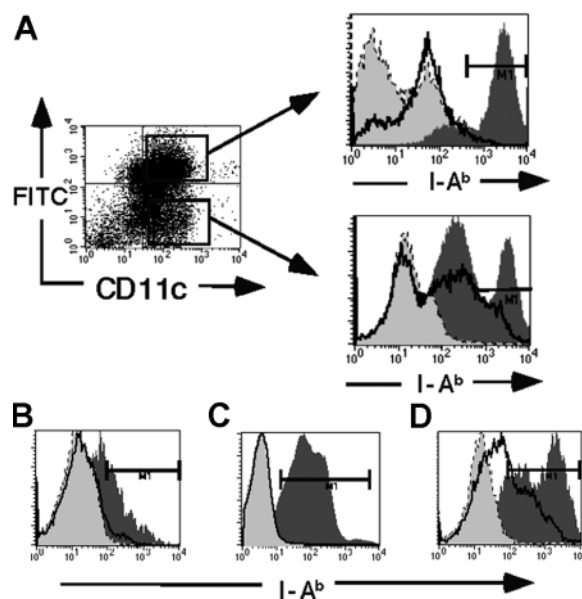


Figure 1. LCs, B cells, and macrophages lack MHC II expression in CD11c/A β^b mice. (A) MHC II expression on LCs (CD11c $^+$ FITC $^+$) or resident LN DCs (CD11c $^+$ FITC $^-$) obtained from the draining LNs of mice after epidermal irritation ($n = 3$ per genotype). (B–D) MHC II levels on APCs harvested from draining LNs of mice infected with *L. major* 4–7 wk earlier. (B) CD11b $^+$ CD11c $^-$ macrophages. (C) CD45R $^+$ B cells. (D) CD11c $^+$ DCs. Histograms correspond to representative A $\beta^{b+/-}$ (light gray with dashed line), CD11c/A β^b (black line), and A $\beta^{b+/-}$ (dark gray) mice.

mained MHC II negative. In contrast, FITC-negative DCs from $A_{\beta}^{b+/-}$ and $CD11c/A_{\beta}^b$ mice (including $CD11b^+$ and $CD8\alpha^+$ cells) stained MHC II positive. Thus, $CD11b^+$ and $CD8\alpha^+$ LN DCs from $CD11c/A_{\beta}^b$ mice, but not LCs, are MHC II positive during inflammation.

To verify that MHC II expression in $CD11c/A_{\beta}^b$ mice remained restricted to $CD8\alpha^+$ and $CD11b^+$ DCs during *L. major* infection, we also examined APCs during infection. 4–7 wk after footpad infection, draining popliteal LNs of $A_{\beta}^{b+/-}$ mice included MHC II^{intermediate} $CD11b^+$ and $CD8\alpha^+$ populations, as well as LCs, identified previously as MHC II^{high} (17). Popliteal LNs of infected $CD11c/A_{\beta}^b$ mice also contained MHC II^{intermediate} populations, but lacked the MHC II^{high} population of LCs (Fig. 1 D). Instead, draining LNs in $CD11c/A_{\beta}^b$ mice contained an MHC II^{negative} population, suggesting that LCs lack MHC II throughout this chronic infection.

Macrophages and B cells in the $CD11c/A_{\beta}^b$ mouse model remain MHC II negative after in vitro exposure to $IFN\gamma$ or LPS (5). Macrophages from *L. major*-infected $A_{\beta}^{b-/-}$ and $CD11c/A_{\beta}^b$ mice also lacked surface MHC II, but $A_{\beta}^{b+/-}$ macrophages were MHC II positive (Fig. 1 B). Similarly, $A_{\beta}^{b+/-}$ B cells had MHC II expression; whereas, $A_{\beta}^{b-/-}$ and $CD11c/A_{\beta}^b$ B cells were MHC II deficient (Fig. 1 C).

Finally, it was important to establish that MHC II expression at the site of infection mirrored that in the LNs. Diffuse I-A^b staining was observed in $A_{\beta}^{b+/-}$ footpads, where MHC II expression was present in macrophages, $CD11c^+$ DCs, and parenchymal cells (Fig. 4 C and not depicted; reference 3). In contrast, $CD11c/A_{\beta}^b$ lesions had MHC II expression limited to isolated $CD11c^+$ foci (Fig. 4 C and not depicted). Thus, antigen presentation in $CD11c/A_{\beta}^b$ mice during *L. major* infection is restricted to $CD8\alpha^+$ and $CD11b^+$ DCs both in the LN and at the primary site of infection.

Control of *L. major*. $CD11c/A_{\beta}^b$ mice lack MHC II expression on cortical thymic epithelium and have no MHC II-restricted $CD4^+$ T cells (5). However, adoptive transfers of naive polyclonal $CD4^+$ T cells into SCID and RAG-deficient mice restore control of *L. major* infections (18, 19). Therefore, we transferred $15\text{--}20 \times 10^6$ polyclonal $CD45.1^+$ T cells into $A_{\beta}^{b-/-}$, $A_{\beta}^{b+/-}$, or $CD11c/A_{\beta}^b$ mice 1 d before infection. $TCR\alpha^{-/-}$ mice were also infected because they have wild-type expression of I-A^b but, like $CD11c/A_{\beta}^b$ mice, would depend on transferred cells for parasite control. Mice were infected in the footpad with 5×10^5 stationary phase promastigotes on day 1, and footpad swelling was monitored weekly (Fig. 2 A). In $A_{\beta}^{b+/-}$ mice, lesion size peaked at week 5 and resolved by week 8. In contrast, $A_{\beta}^{b-/-}$ mice developed large nonhealing lesions requiring euthanasia between weeks 6 and 9. Parasite titers were consistent with lesion size (Fig. 2 B). Thus, control of parasite growth and lesion resolution was MHC II dependent.

The disease courses in $CD11c/A_{\beta}^b$ and MHC II-positive $TCR\alpha^{-/-}$ mice infected with *L. major* were similar. Minimal swelling was detected throughout the infection, suggesting a role for endogenous $CD4^+$ T cells in the development of local inflammation (Fig. 2 A). However, $CD4^+$ T

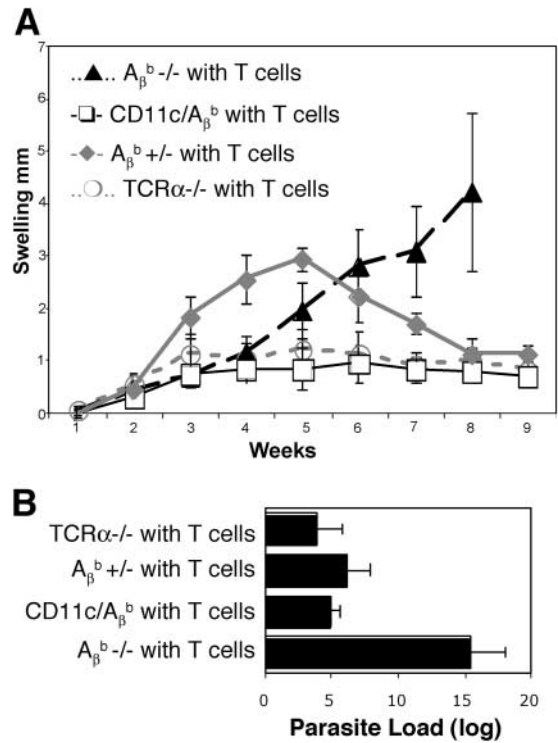


Figure 2. $CD8\alpha^+$ and $CD11b^+$ DC antigen presentation is sufficient for control of *L. major* lesions. (A) Footpad swelling in infected $A_{\beta}^{b-/-}$, $CD11c/A_{\beta}^b$, $A_{\beta}^{b+/-}$, and $A_{\beta}^{b+/-}$ $TCR\alpha^{-/-}$ mice that received $CD45.1^+$ T cells before infection ($n = 5$ mice/genotype). (B) Parasite load at 6–9 wk after infection expressed as logarithm of parasite counts. Standard error of 4–7 mice/genotype is indicated. There was no difference in pathogen clearance amongst $CD11c/A_{\beta}^b$, $A_{\beta}^{b+/-}$, and $TCR\alpha^{-/-}$ mice, but all had significantly lower parasite loads than I- $A_{\beta}^{b-/-}$ mice ($P < 0.002$).

cells transferred into $CD11c/A_{\beta}^b$ and $TCR\alpha^{-/-}$ mice-mediated parasite control efficiently; parasite loads 9 wk after infection were comparable to those of $A_{\beta}^{b+/-}$ mice (Fig. 2 B). No footpad swelling was detected for 15 wk after infection (unpublished data), so $CD4^+$ T cells control *L. major* growth for an extended period rather than simply delay the outgrowth of the parasite. Thus, MHC II-dependent antigen presentation by $CD11b^+$ and $CD8\alpha^+$ DCs mediate all the functions necessary for control of *L. major*.

***L. major*-dependent Th1 Differentiation.** To examine Th1 differentiation in response to *L. major*, $IFN\gamma$ production was assayed. First, $CD4^+$ T cells from draining (popliteal) or distant (cervical) LNs were harvested 4 or 9 wk after infection, and $IFN\gamma$ production was assayed after restimulation with PMA and ionomycin (Fig. 3 A). Distant cervical LNs served as a control for the absence of *L. major*-specific responses. $CD45.1^+$ cells (right quadrants) are transferred MHC II-restricted $CD4^+$ T cells; the $CD45.1^-$ $CD4^+$ T cells are endogenous cells (left quadrants). $A_{\beta}^{b+/-}$ mice have endogenous MHC II-restricted $CD4^+$ T cells, which produced most of the $IFN\gamma$ in the popliteal LNs. However, more $CD4^+$ T cells in the popliteal LNs produced $IFN\gamma$ than at distant sites. In contrast, MHC II-restricted transferred $CD4^+$ T cells did not accumulate in

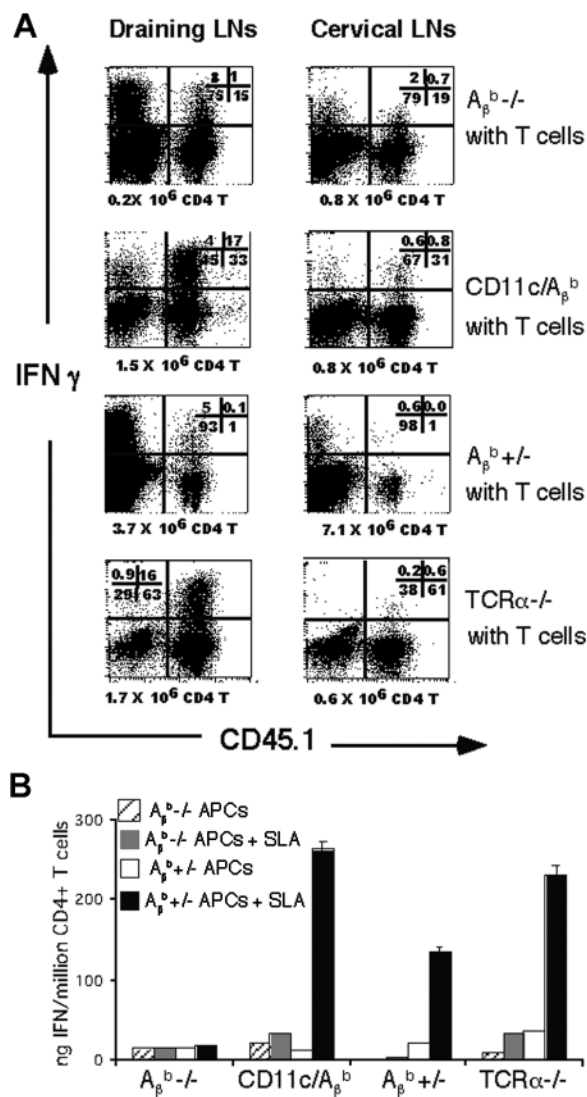


Figure 3. DCs mediate *L. major*-specific Th1 differentiation. (A) Intracellular IFN γ staining from CD4 $^+$ T cells 9 wk after footpad infection. Transferred CD45.1 $^+$ cells and endogenous CD45.1 $^-$ CD4 $^+$ T cells are depicted. ($n = 5-8$ mice/genotype). Under each dot plot, the total number of CD4 $^+$ T cells recovered from the harvested LNs is shown. (B) IFN γ production from purified splenic CD4 $^+$ T cells after 72 h of incubation with the indicated APCs, in the presence or absence of soluble *L. major* antigen (SLA). CD4 $^+$ T cells from three mice of each genotype were combined. This experiment is representative of three individual experiments.

the draining LNs of $A_{\beta}^{b-/-}$ mice (4–6% of the transferred CD45.1 $^+$ cells produced IFN γ at this site, a proportion comparable to the staining in the cervical LNs). Endogenous MHC II-independent CD4 $^+$ cells (probably NKT cells) do accumulate at the site of infection in $A_{\beta}^{b-/-}$ mice, but cannot control the pathogen (4, 20, 21).

CD11c/ A_{β}^b and TCR $\alpha^{-/-}$ mice, which lack endogenous MHC II-restricted CD4 $^+$ T cells, accumulated CD45.1 $^+$ CD4 $^+$ T cells in the popliteal LNs, where 20–35% of the transferred CD4 T cells produced IFN γ (Fig. 3 A). The increased IFN γ production was specific, as <3% of the CD4 $^+$ T cells in the cervical LNs produced this cyto-

kine. To confirm the antigen specificity of this Th1 response, splenic CD4 $^+$ T cells were purified and restimulated in vitro for 3 d. IFN γ in the supernatants was measured by ELISA. Importantly, IFN γ produced by CD4 $^+$ T cells of $A_{\beta}^{b+/-}$, TCR $\alpha^{-/-}$, and CD11c/ A_{β}^b mice was antigen specific and MHC II dependent (Fig. 3 B). Therefore, antigen presentation by DCs is sufficient for the local expansion of *L. major*-responsive MHC II-restricted CD4 $^+$ Th1 cells. We used immunohistochemistry to confirm that *L. major*-specific Th1 cells migrated into the *L. major*-infected footpad. Strikingly, CD4 $^+$ T cells that secreted IFN γ (not depicted) were present in the lesions of I- $A_{\beta}^{b+/-}$, CD11c/ A_{β}^b , and TCR $\alpha^{-/-}$ mice, but not in $A_{\beta}^{b-/-}$ footpads (Fig. 4 C). The result indicated that DC-restricted MHC II expression was sufficient for Th1 CD4 $^+$ T cell differentiation, migration into infected sites, and effector function.

L. major-infected macrophages might not effectively prime CD4 $^+$ T cells, but they have been shown to enhance Th1 polarization in vitro (22). The current data suggest that any macrophage functions required for differentiation of Th1 effectors must be noncognate, perhaps functioning through TNF α , iNOS, or IFN α/β production (9–11). Similarly, LCs are either not necessary for anti-*L. major* responses or they do not need to express MHC II to play a role in Th1 immunity. However, it will be important to assess the requirement for LC MHC II expression during intradermal infection models, where LCs might be essential. However, CD11b $^+$ and CD8 α^+ DC subsets without LC or macrophage involvement are sufficient to mediate antigen presentation and Th1 differentiation during subcutaneous *L. major* infection.

***L. major*-dependent Phagocyte Activation.** Parasite control depends on activation of phagocytes (2). Thus, either macrophage activation in CD11c/ A_{β}^b mice is noncognate, or MHC class II-positive DCs can subsume functions normally attributed to macrophages. To analyze macrophage activation, we examined CD40 up-regulation and iNOS expression in macrophages harvested from popliteal LNs. Macrophages accumulated in the draining LNs of $A_{\beta}^{b+/-}$, CD11c/ A_{β}^b , and TCR $\alpha^{-/-}$ mice, but not I- $A_{\beta}^{b-/-}$ mice (unpublished data). However, surface expression of CD40 staining was equivalent in all genotypes (Fig. 4 A). Thus, CD40 expression is independent of either Th1 effectors or cognate antigen presentation.

iNOS-dependent NO production also contributes to *L. major* control (2). Expression of iNOS in popliteal LN macrophages was only detectable in $A_{\beta}^{b+/-}$, CD11c/ A_{β}^b , and TCR $\alpha^{-/-}$ macrophages (Fig. 4 B). Macrophages in $A_{\beta}^{b+/-}$ and TCR $\alpha^{-/-}$ mice contained more iNOS than those in CD11c/ A_{β}^b mice (Fig. 4 B), suggesting a cognate component to regulation of the enzyme, but this had no effect on parasite loads at weeks 4, 7, and 9 (Fig. 2 B and not depicted). Immunofluorescence of the footpad revealed that iNOS was also present in the infection site of $A_{\beta}^{b+/-}$, CD11c/ A_{β}^b , and TCR $\alpha^{-/-}$, but not $A_{\beta}^{b-/-}$ mice (Fig. 4 E). Thus, cognate macrophage-CD4 T cell interactions are not required for either CD4 $^+$ T cell recruitment to inflamed tissues or macrophage activation and effector functions.

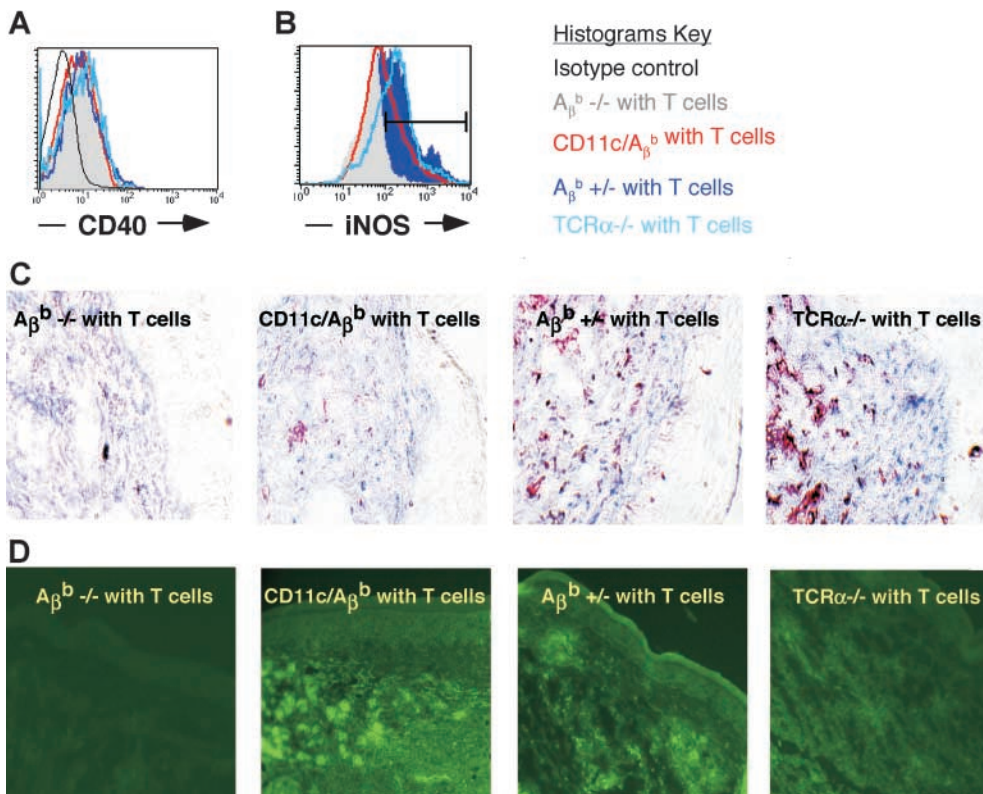


Figure 4. Macrophage effector functions are MHC II-independent. Footpads and popliteal LNs were harvested 4 wk after infection. (A) CD40 levels on draining LN macrophages (CD11b⁺ CD11c⁻ Gr1⁻). The dashed line depicts staining by an isotype control. (B) Intracellular iNOS in CD11b⁺ CD11c⁻ macrophages from popliteal LNs. (C and D) Immunostaining of footpad lesions stained for (C) CD4 and MHC II and iNOS (D).

One interpretation of our results is that IFN γ production by differentiated effector CD4⁺ T cells at the site of infection is antigen dependent, and relies on CD11b⁺ and CD8 α ⁺ DCs. Indeed, we detected recruitment of CD11c⁺ cells to local sites of infection. Therefore, the role of DCs in the primary immune response could extend beyond initiation of CD4⁺ T cell responses to control CD4⁺ T cell functions during the effector phase. Alternatively, IFN γ secretion from activated CD4⁺ T cells could occur in the absence of cognate antigen presentation in vivo. In this scenario, MHC II-TCR interactions would be required for priming, but activated effector CD4⁺ T cells could secrete IFN γ without further TCR triggering. This would imply that the effector cytokine delivery of the CD4⁺ T cell responses might be less specific than previously thought.

Finally, control of *L. major* infection also requires phagocyte activation and the killing of intracellular amastigotes (2). In CD11c/A β^b mice, MHC II-deficient macrophages must respond to IFN γ and/or CD40-CD40L interactions without cognate T cell interactions. Thus, pathogen clearance functions attributed to macrophages, such as NO production, phagocytosis, and lysis of infectious organisms (9), lack the specificity requirements of antigen presentation. Our results imply that the immune system can avoid pathogens' attempts to block antigen presentation within the infected macrophage because antigen presentation by this cell type is not a requirement for activation or parasite lysis.

In conclusion, we have demonstrated the sufficiency of CD11b and CD8 α ⁺ DC antigen presentation in the initiation and effector phases of Th1 immunity to *L. major*. Our

findings suggest that antigen presentation by macrophages, keratinocytes, pDCs, and LCs are not required for either differentiation of Th1 CD4⁺ T cells responding to *L. major* or the effector phases that follow. It will be interesting to determine if other APCs play a more important role in secondary immune responses against *L. major* because memory CD4⁺ T cells survey tissues where resident *L. major*-infected macrophages and LCs might be more important APCs.

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