Interleukin 12p40 is required for dendritic cell migration and T cell priming after *Mycobacterium tuberculosis* infection

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Migration of dendritic cells (DCs) to the draining lymph node (DLN) is required for the activation of naive T cells. We show here that migration of DCs from the lung to the DLN after *Mycobacterium tuberculosis* (Mtb) exposure is defective in mice lacking interleukin (IL)–12p40. This defect compromises the ability of IL–12p40–deficient DCs to activate naive T cells in vivo; however, DCs that express IL–12p40 alone can activate naive T cells. Treatment of IL–12p40–deficient DCs with IL–12p40 homodimer (IL–12(p40)$_2$) restores Mtb–induced DC migration and the ability of IL–12p40–deficient DCs to activate naive T cells. These data define a novel and fundamental role for IL–12p40 in the pathogen-induced activation of pulmonary DCs.

The importance of IL-12p70 in the CD4 T cell response to *Mycobacterium tuberculosis* (Mtb) has been demonstrated both experimentally (1–3) and by the increased susceptibility of humans deficient in the IL-12 pathway to tuberculosis (4, 5) and other mycobacterial infections (6–8). IL-12p70 is made up of IL-12p35 and IL-12p40 (9). In murine models of tuberculosis, IL-12p35 deficiency is less detrimental to host response than IL-12p40 deficiency (2, 3), suggesting that IL-12p40 has IL-12p35–independent bioactivity.

Three cytokines use the IL-12p40 subunit: IL-12p70, IL-23 (p40 and p19) (10), and IL-12(p40)$_2$ (homodimeric IL-12p40; see Table I) (9). The majority of the protective response to tuberculosis in the absence of IL-12p70 is dependent on IL-23 (11); however, exogenous IL-12(p40)$_2$ partially restores protection in mycobacteria-infected IL-12p40–deficient mice (2). How IL-12(p40)$_2$ affects the cellular response to Mtb is unknown. IL-12(p40)$_2$ cannot induce IFN-γ production in T cells and blocks IL-12p70 activation of T cells (9). Myeloid cells express the IL-12R (12–14), the ligation of which initiates NF-κB migration (12, 15, 16).

IL-12(p40)$_2$ also acts as a chemoattractant for macrophages (14) and induces TNF-α (17) and nitric oxide synthase (18). Thus, IL-12p40 has the potential to directly modify myeloid cell activity.

The DC population lining the lung is key to the initiation of T cell responses after pulmonary challenge (19). These cells remain quiescent until activated to migrate to the draining LN (DLN) (20), present antigen, and initiate T cell activation (21). Effector cell development depends upon whether DCs are stimulated to produce IL-12p70 (22, 23). Although this role of DC-derived IL-12p70 is accepted, the early secretion of only IL-12p40 during lung inflammation (24–26) supports an independent function for IL-12p40.

Here we investigate the role of IL-12p40 in DC migration from the lung to the DLN and in activation of CD4 T cells. We show that IL-12p40 deficiency results in reduced migration of DCs and reduced activation of naive T cells. This deficiency is not seen in mice that express only IL-12p40 and can be overcome with exogenous IL-12(p40)$_2$. These data support a novel and important role for IL-12p40 in initiation of the adaptive immune response to bacterial challenge in the lung.

RESULTS

Mice lacking IL–12p40 fail to generate activated CD4 T cells after Mtb infection

Induction of antigen-specific CD4^{+} T cells in response to respiratory pathogens such as Mtb is required to mediate protection. In previous studies we showed that in the absence of IL-12p70, both antigen-specific IFN-γ and delayed-type hypersensitivity responses were induced after Mtb infection but that in the absence of IL-12p40, no such responses could be detected (3). To confirm that Mtb-specific IFN-γ–producing CD4 T cells depend upon IL-12p40 for their induction, we used an IA[b]-restricted peptide of an immunodominant Mtb antigen (ESAT-6$_{1-20}$) to analyze the CD4^{+} T cell response in gene-deficient mice. We show that in the absence of IL-12p40, very few ESAT-specific IFN-γ–producing CD4 T cells accumulate in the lung by day 21 (Fig. 1 a). In contrast, both IL-23p19–deficient and IL-12p35–deficient mice accumulate a significant number of ESAT-specific IFN-γ–producing CD4 T cells, although this response is reduced in the IL-12p35–deficient compared with the B6 and IL-23p19–deficient mice (Fig. 1 a). We have recently shown that the residual IFN-γ–producing CD4^{+} T cell effector response observed in the IL-12p35–deficient mice is due to IL-23 as this response is lost in the IL-12p35–/IL-23p19–deficient mice (11). However, we have not determined the relative roles of IL-23 and IL-12p40 on activation of naive T cells after Mtb infection. Therefore, to determine whether IL-12p40 alone can initiate CD4 T cell activation after Mtb infection, we measured the total number of activated (i.e., CD44^{high}) CD4 T cells accumulating in the lung at day 20 after infection in B6 and gene-deficient mice. As shown in Fig. 1 b, the number of activated CD4 T cells in the lung of
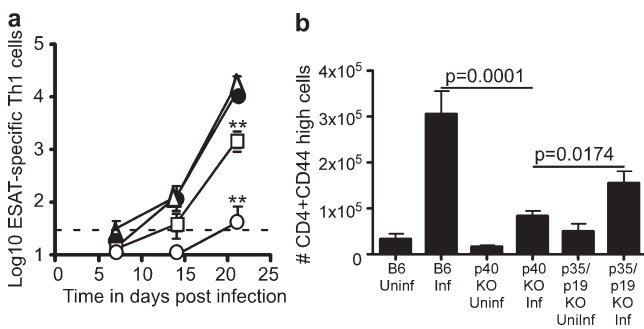
normal mice increases significantly upon infection with Mtb, but this response is reduced in the absence of IL-12p40. In contrast, mice that express IL-12p40 alone accumulate a significantly higher number of activated CD4^{+} T cells upon infection than in the absence of IL-12p40. These data demonstrate that the absence of IL-12p40 is detrimental to the induction of activated CD4 T cells within the lung and that although generation of IFN-γ–producing effector cells is dependent on IL-12p70 and IL-23, the accumulation of activated CD4 T cells within the lung can be initiated by IL-12p40 alone.

IL–12p40–deficient DCs can activate naive CD4 T cells when not required to migrate

The ability of IL-12(p40)$_2$ to mediate protection against mycobacterial disease (2), as well as our data indicating a unique role for IL-12p40 in T cell activation (Fig. 1 b), makes understanding the function of this cytokine subunit in T cell responses to Mtb an important goal. As DCs are key to the activation of naive cells and also express IL-12R subunits, we began by analyzing the effect of IL-12p40 deficiency on DC function. Initially, we investigated the ability of
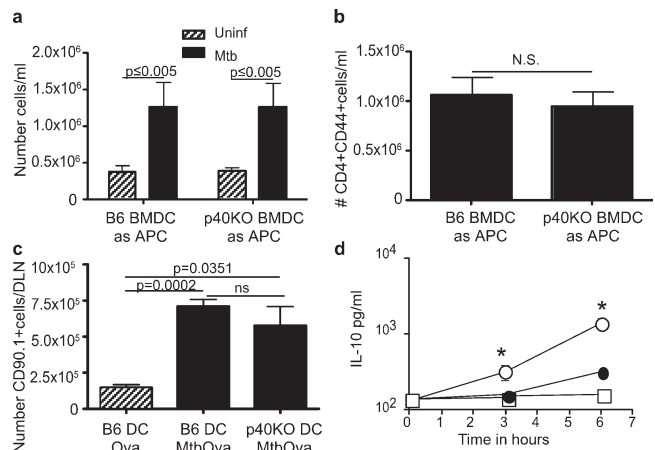

Figure 1. T cell activation after Mtb infection requires IL–12p40.
(a) B6 (filled circle), IL-23p19–deficient (open triangle), IL-12p35–deficient (open square), and IL-12p40–deficient (open circle) mice were infected with ~75 CFU Mtb via the aerosol route, and the number of antigen-specific IFN-γ–producing CD4 T cells in the lung on day 21 was determined by ELISPOT. Data points represent the mean number of Th1 cells/time point ($n = 4$). One experiment representative of three total experiments is shown. The dashed line represents the limit of detection for this assay. **, $P \leq 0.001$ relative to B6 value determined by Student's t test. (b) The number of activated CD4 T cells in the lungs of mice infected for 21 d was determined by flow cytometry. Data points represent the mean number of T cells/time point ($n = 8-16$). p-values were determined by the Student's t test.

Figure 2. IL–12p40–deficient BMDCs can stimulate naive T cells if not required to migrate. BMDCs were generated from B6 and IL-12p40–deficient (p40KO) mice and either treated with Mtb (filled bars) or left uninfected (striped bars) and pulsed with OVA$_{323-339}$. Naive OVA-specific TCRTg CD4 T cells were cultured with the BMDCs for 3 d. The number (a) and phenotype (b) of cells present on day 3 were determined. Data points represent the mean of each group ($n = 3$). One experiment representative of two total experiments is shown. p-values were determined by the Student's t test. (c) BMDCs were generated as described for (a) and delivered intravenously into mice that had previously received naive TCRTg OVA-specific T cells. The number of T cells was determined at day 4. Data points represent the mean number of T cells ($n = 3-4$). One experiment is shown. (d) BMDCs from B6 (filled circles), IL-12p40–deficient (open circles), and IL-12p35–deficient (open squares) mice were cultured with Mtb, and the amount of IL-10 released over time was determined by ELISA. Data points represent the mean for each group ($n = 3$). One experiment representative of two total experiments is shown. *, $P \leq 0.01$ for the difference between B6 and IL-12p40–deficient BMDCs as determined by the Student's t test.
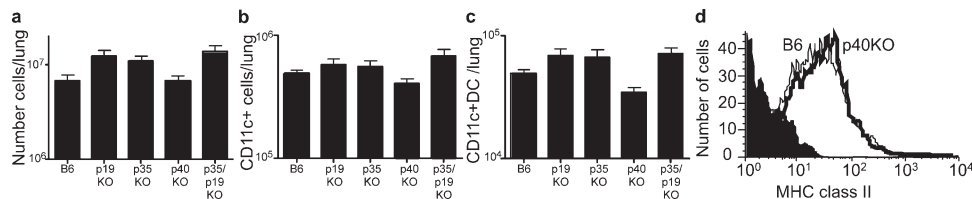
Figure 3. IL–12p40–deficient steady–state lung DCs are not compromised. The number of total live cells (a), the total number of CD11c^{+} cells (b), and the total number of low autofluorescence CD11c^{+} cells was determined (c). Data points represent the mean for each group ($n = 5$). One experiment representative of two total experiments is shown. The

expression MHC class II was analyzed on low-autofluorescence CD11c^{+} cells (d). Black histogram represents the isotype control, gray histogram represents B6 cells, and the open line shows expression of class II on IL–12p40–deficient (p40KO) cells. One mouse representative for each group is shown.

IL–12p40–deficient bone marrow–derived DCs (BMDCs) activated by Mtb to present cognate antigen (OVA$_{323-339}$ peptide) to naive TCR transgenic (Tg; OT-II) CD4 T cells both in vitro and in vivo. The IL–12p40–deficient BMDCs were not compromised in their ability to initiate T cell activation in response to antigen in vitro, as T cell expansion was equivalent in cultures stimulated by B6 or IL–12p40–deficient BMDCs (Fig. 2 a). CD44 was also up-regulated on CD4 T cells after activation by IL–12p40–deficient BMDCs as shown by the equivalent numbers of CD4^{+}CD44^{high} cells in the wells stimulated by B6 and IL–12p40–deficient BMDCs (Fig. 2 b). When IL–12p40–deficient BMDCs were exposed to Mtb, loaded with OVA$_{323-339}$, and delivered intravenously into mice that had received a bolus of naive OT-II TCRTg CD4 T cells, they were able to stimulate T cell expansion (Fig. 2 c) as effectively as B6 BMDCs. These data indicate that IL–12p40–deficient DCs are capable of inducing expansion and activation of naive CD4 T cells when the DCs are in close proximity (either in vitro or in vivo) with naive T cells.

To determine whether IL–12p40 modulates the DC response to Mtb, we measured cytokine mRNA and protein and found that IL–12p40–deficient DCs generate more IL-10 both at the mRNA (not depicted) and protein level than B6 DCs (Fig. 2 d). These data indicate that although Mtb-exposed IL–12p40–deficient DCs are able to activate naive T cells, IL–12p40 acts to reduce the IL-10 response to Mtb in DCs.

IL–12p40–deficient mice have an equivalent population of DCs in the lung compared with intact mice

We show that activated T cells fail to accumulate in the lungs of Mtb-infected IL–12p40–deficient mice despite the fact that IL–12p40–deficient DCs can activate naive T cells. Thus, it was possible that DCs were not developing appropriately in the lungs of IL–12p40–deficient mice and were therefore unable to prime T cells in vivo. To determine whether DCs in the lungs of naive normal mice differ substantially from the DCs in the lungs of IL–12p40–deficient mice, we used a simple set of criteria to identify and characterize lung DCs. We stained for the integrin CD11c, a marker expressed by most DCs (27), and gated on CD11c^{+} cells that have low autofluorescence, as these cells have been characterized as lung DCs (28). Using this designation, we determined that there are similar numbers of total lung cells (Fig. 3 a), CD11c^{+} cells (Fig. 3 b), and DCs (Fig. 3 c) in B6, IL–12p35–deficient, IL–23p19–deficient, and IL–12p40–deficient mice. To further characterize these cells we also stained for MHC class II (Fig. 3 d), CD80, CD86, and CD40 (not depicted) expression and found that the apparent activation state of these cells in naive mice is equivalent regardless of the availability of the IL–12p40–dependent cytokines. These data indicate that in the steady-state lung, DCs are not substantially altered in terms of number or activation state in the absence of IL–12p40. There are no significant detectable changes in the numbers or phenotype of myeloid cells in the lung during the first 20 d of low-dose aerosol infection (not depicted).
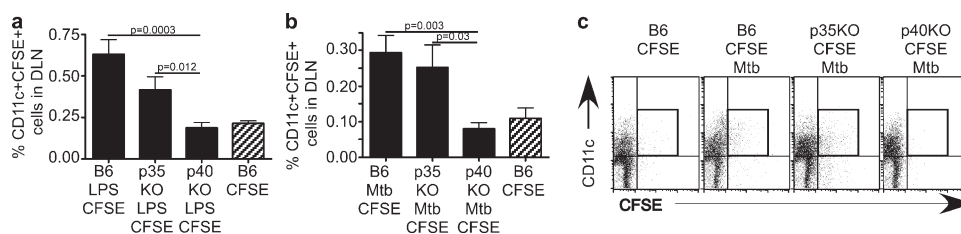

Figure 4. Pathogen-activated DCs fail to migrate from the lungs of IL–12p40–deficient mice. CFSE and either LPS (a) or irradiated Mtb (b) were delivered intratracheally to B6 and gene-deficient mice, and the frequency of CFSE-labeled CD11c^{+} cells in the DLN and lung was determined by flow cytometry after 18 h. Data points represent the mean for

each group ($n = 3-4$). One experiment representative of two total experiments is shown. p-values were determined by the Student's t test. (c) Representative histogram illustrating the population of CFSE^{+}CD11c^{+} cells within the DLNs of B6 and gene-deficient mice at 18 h after delivery of Mtb to the lungs of CFSE-treated mice.
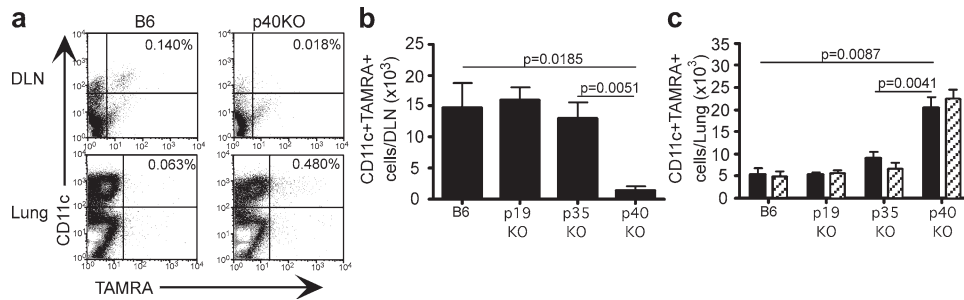
Figure 5. BMDCs from IL–12p40–deficient mice fail to migrate from the lung when activated by Mtb. BMDCs from B6 and gene-deficient mice were exposed to irradiated Mtb for 3 h, washed, labeled with TAMRA orange, and delivered intratracheally to B6 mice. The frequency (a) and number (b) of CD11c^{+}/TAMRA-labeled cells within the DLN was then determined over time using flow cytometry. The frequency and number of CD11c^{+}/TAMRA-labeled cells within the lung were also determined on day 2 (filled bars) and day 4 (striped bars) (c). Data points represent the mean for each group (n = 3–4). One experiment representative of two total experiments is shown. p-values were determined by the Student's t test.

DCs fail to migrate from the lungs of IL–12p40–deficient mice after pathogen exposure

Because the DCs in the lungs of the IL-12p40–deficient mice are present in normal numbers, we next considered whether these DCs were able to respond to Mtb challenge and migrate from the lung to the DLN (21). Therefore, we labeled cells in the airways of B6 and gene-deficient mice with CFSE and activated the DCs by administering LPS or irradiated Mtb intratracheally. We then determined the frequency of CFSE^{+}/CD11c^{+} DCs within the DLN 18 h after delivery (Fig. 4). We found that the frequency of CD11c^{+}/CFSE^{+} DCs in the DLNs of B6 mice exposed to either LPS (Fig. 4 a) or Mtb (Fig. 4 b) was significantly increased compared with mice that received CFSE alone (Fig. 4, a and b; there was no difference in frequency or number of CFSE^{+}/CD11c^{+} cells in the DLNs of B6, IL-12p35–deficient, or IL-12p40–deficient mice treated with CFSE alone). However, there was a significantly reduced frequency of CD11c^{+}/CFSE^{+} DCs in the DLNs of IL-12p40–deficient mice challenged with either agent (Fig. 4, a and b). Although the IL-12p35–deficient mice had a slightly lower frequency of CD11c^{+}CFSE^{+} cells in the DLNs than the B6 mice, this reduction did not reach significance and these mice had a significantly higher response to both agents compared with IL-12p40–deficient mice (Fig. 4, a and b). These data demonstrate that in the absence of IL-12p40, CD11c^{+} cells are less able to migrate from the lung to the DLN.

The experiment described above does not distinguish an intrinsic defect in the IL-12p40–deficient DCs from defective signaling within the gene-deficient lung interstitium. To address this issue, we generated BMDCs from B6 and gene-deficient mice, activated these cells with Mtb for 3 h, and labeled them with TAMRA orange. These cells were then delivered intratracheally into the lungs of B6 mice, and the frequency of TAMRA-labeled CD11c^{+} cells in the DLN was determined by flow cytometry (Fig. 5 a). We found that the frequency and total number of Mtb-activated, B6, IL-23p19–deficient, and IL-12p35–deficient BMDCs within the DLNs of the host mice was equivalent (Fig. 5 b). In con-

trast, there were many fewer BMDCs from IL-12p40–deficient mice in the DLNs (Fig. 5 b). The fact that the transferred IL-12p40–deficient BMDCs remained in the lung at a higher frequency and total number than the BMDCs from the B6, IL-23p19–deficient, and IL-12p35–deficient mice supports the hypothesis that these cells were not able to move from the lung to the DLN (Fig. 5 c). We conclude that DCs activated by Mtb need to be able to produce IL-12p40, but not IL-23 or IL-12p70, to be able to migrate from the lung to the DLN.

BMDCs from IL–12p40–deficient mice fail to become responsive to chemokines after exposure to Mtb but can be rescued by IL–12(p40)$_2$

The inability of BMDCs from IL-12p40–deficient mice to migrate to the DLN after transfer suggested that Mtb-exposed DCs might not be capable of trafficking to the LN due to an inability to respond to specific chemokine signals. One facet of DC activation in response to microbial stimuli is the increased ability of these cells to respond to homeostatic chemokines such as CCL19 and CCL21 that facilitate migration to the LN. To determine whether the IL-12p40–deficient BMDCs are less able to respond to homeostatic chemokines after Mtb exposure, we performed in vitro transwell migration assays on Mtb-activated BMDCs from B6 and gene-deficient mice. We found that BMDCs not exposed to Mtb exhibited limited migration in response to the CCR7 ligands CCL19 (Fig. 6 a) or CCL21 (not depicted), but after a 3-h exposure to Mtb, B6 and IL-23p19–deficient BMDCs migrated efficiently in response to both chemokines. In contrast, the IL-12p40–deficient BMDCs were unable to up-regulate their response to either chemokine even after exposure to Mtb (Fig. 6 a). IL-12p35–deficient BMDCs responded to chemokines significantly better than the IL-12p40–deficient BMDCs after Mtb exposure, but this response was reduced compared with the B6 BMDCs (Fig. 6 a). We also compared induction of the *ccr7* gene, which encodes the principal CCL19 and CCL21 receptor CCR7, between B6 and IL-12p40–deficient BMDCs over the 3-h exposure to Mtb.
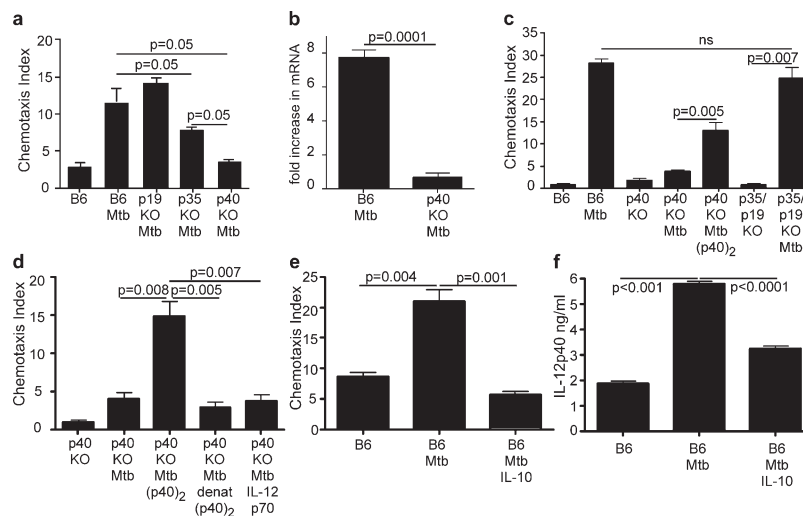
Figure 6. IL–12p40–deficient DCs fail to become responsive to homeostatic chemokines when stimulated by Mtb. BMDCs from B6 and gene-deficient mice were either left uninfected or infected with Mtb for 3 h. BMDCs were then placed in the upper chamber of a transwell plate and CCL19 was added to the lower chamber. The cells that migrated from the upper to the lower chamber within 90 min were then counted. The data are shown as chemotaxis index, which is the fold increase in induced migration over spontaneous migration. (a) The chemotaxis index was determined for B6 and gene-deficient mice. (b) The mRNA from untreated B6 and IL–12p40–deficient (p40KO) BMDCs treated with Mtb for 3 h was analyzed for the relative induction of mRNA for CCR7 by real-time PCR. The level of *ccr7* gene expression in unstimulated B6 and IL–12p40– deficient (p40KO) BMDCs was not significantly different (not depicted). (c) The chemotaxis index for Mtb-activated IL–12p40–deficient (p40KO) BMDCs treated with $IL-12(p40)_2$ or Mtb-activated BMDCs producing IL–12p40 alone was determined. Data points represent the mean for each group ($n = 3$–4). (d) The chemotaxis index for Mtb-activated p40-deficient BMDCs was determined when $IL-12(p40)_2$, denatured $IL-12(p40)_2$, or IL–12p70 was added to p40-deficient BMDCs. (e) The chemotaxis index for Mtb-activated BMDCs was determined in the presence and absence of exogenous IL–10. (f) The production of IL–12p40 from B6 BMDCs activated by Mtb for 3 h in the presence or absence of exogenous IL–10 was determined by ELISA. One experiment representative of two total experiments for each of panels a–f is shown. p-values were determined by the Student's *t* test.

We found that this gene is poorly induced in the Mtb-treated IL–12p40–deficient BMDCs compared with the Mtb-treated B6 BMDCs (Fig. 6 b). These data demonstrate that IL–12p40–deficient DCs have a compromised response to Mtb in that they fail to up-regulate the mRNA for the chemokine receptor associated with migration.

Because IL–12p40–deficient mice lack both IL–12p40 and IL–12p70, we next asked whether IL–12p40 alone was sufficient to induce the differentiation of DCs into cells capable of migrating in response to CCL19. Therefore, we tested the ability of BMDCs containing only IL–12p40 (i.e., IL–23p19–/IL–12p35–deficient) to become responsive to CCL19 when exposed to Mtb. These BMDCs were as able as B6 BMDCs to migrate to CCL19 after Mtb exposure (Fig. 6 c), indicating that IL–12p40 was sufficient for the development of this phenotype. Similarly, the addition of exogenous $IL-12(p40)_2$ for the 3-h period of Mtb exposure dramatically increased the ability of IL–12p40–deficient BMDCs to migrate in response to CCL19 (Fig. 6, c and d), albeit not to the level seen in B6 BMDCs. To confirm the specificity of the $IL-12(p40)_2$ reagent, it was denatured by boiling. This ablated its ability to restore migration in the IL–12p40–deficient BMDCs (Fig. 6 d). The addition of IL–12p70 was also unable to restore migration in the IL–12p40–deficient BMDCs (Fig. 6 d). As IL–12p40–deficient BMDCs produce more IL–10 upon exposure to Mtb than B6 BMDCs (Fig. 2 d), we deter-

mined whether exogenous IL–10 could recapitulate the IL–12p40–deficient BMDC phenotype. Indeed, the addition of IL–10 to Mtb-activated B6 BMDCs reduced the responsiveness of these cells to CCL19 to background level (Fig. 6 e). The addition of IL–10 also significantly reduced the Mtb-induced IL–12p40 produced by the Mtb-activated B6 BMDCs (Fig. 6 f). Collectively, these data confirm that IL–12p40, probably in the form of $IL-12(p40)_2$, is sufficient to mediate the Mtb-induced migratory phenotype in DCs and that a balance between IL–10 and IL–12p40 may dictate the ability of BMDCs to respond to CCL19.

Failure of BMDCs to migrate from the lung to the DLN dramatically compromises the ability of the cells to activate antigen–specific cells in vivo

To determine whether reduced migration of DCs from the lung to the DLN seen in the IL–12p40–deficient mice results in reduced activation of naive antigen-specific CD4^{+} T cells, we pulsed Mtb-activated BMDCs with OVA[323–339] and transferred these cells into the lungs of B6 mice that had received a bolus of naive OVA-specific TCRTg (OT-II) CD4 T cells 48 h previously. The ability of the transferred DCs to initiate activation of naive T cells was measured by the frequency of activated (CD44^{high}/CD69^{high}) TCRTg T cells within the LN (Fig. 7 a). The data demonstrate that intratracheal delivery of Mtb-activated OVA[323–339]-pulsed

B6, IL–23p19–deficient, or IL–12p35–deficient BMDCs results in the increased accumulation (Fig. 7 b) of activated antigen-specific cells (Fig. 7 c; also true for CD44^{high} cells; not depicted) within the DLN compared with nonactivated BMDCs. Further, although the delivery of BMDCs with OVA$_{323–339}$ alone initiated the expansion of TCRTg cells, the expansion was significantly reduced compared with Mtb-activated BMDCs (5.4^3 ± 1^3 CD44^{high} TCRTg cells in the LN after OVA$_{323–339}$ alone compared with 1.3^5 ± 2.7^4 after OVA$_{323–339}$ and Mtb-activated BMDCs; n = 3, P = 0.012 by Student's t test). In contrast, the transfer of OVA$_{323–339}$-pulsed Mtb-activated IL–12p40–deficient DCs resulted in a reduced frequency of activated antigen-specific cells (Fig. 7 a) and a dramatically reduced accumulation of antigen-specific T cells in the DLN (Fig. 7, b and c). To confirm that

these reduced numbers reflected reduced activation of T cells, we compared the dilution of CFSE in naive cells after the transfer of OVA$_{323–339}$-pulsed Mtb-activated B6 and IL–12p40–deficient DCs. It is clear that although there is some CFSE dilution in those mice receiving IL–12p40–deficient DCs, this is significantly reduced compared with those receiving B6 DCs (Fig. 7 e). These data demonstrate that lung DCs can initiate proliferation and activation of naive T cells within the DLN and that this ability requires IL–12p40, but not IL–12p70 or IL–23, expression by the DC. In light of data demonstrating IL–12p40 production from both human and murine airway epithelial cells (14, 25, 29), we wanted to determine whether host-derived IL–12p40 was required for T cell activation. To do this we examined the ability of B6 Mtb-activated OVA$_{323–339}$-pulsed BMDCs to stimulate
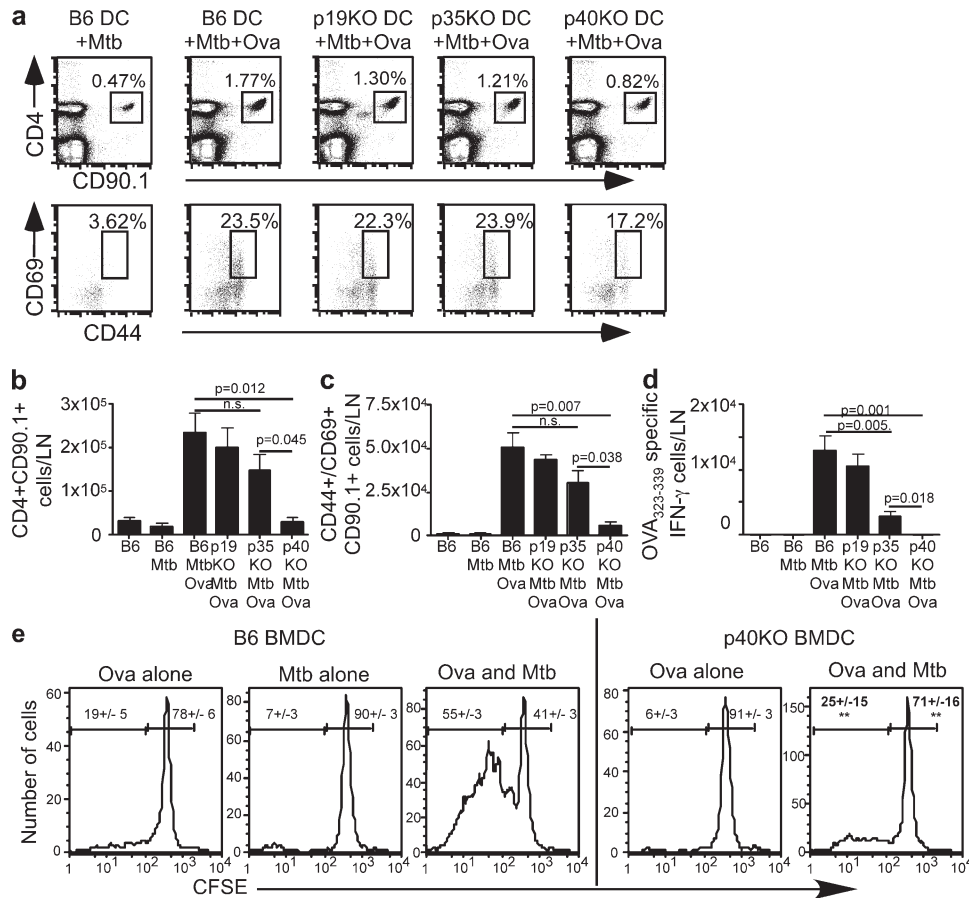

Figure 7. DCs from IL–12p40–deficient mice are unable to activate naive T cells when delivered into the lung. BMDCs generated from B6 and gene-deficient mice were exposed to irradiated Mtb for 3 h, pulsed with OVA$_{323–339}$, and delivered intratracheally into mice that had received intravenous delivery of naive TCRTg CD4 T cells 48 h earlier. The frequency of CD4$^+$CD90.1$^+$ OVA-specific cells in the DLN cells was determined by flow cytometry for mice receiving BMDCs from each of the groups (a, top). The frequency of activated CD4$^+$CD90.1$^+$ cells in the DLN was also determined by flow cytometry (a, bottom). The total number of CD4$^+$CD90.1$^+$ T cells (b) and the total number of activated CD4$^+$CD90.1$^+$ T cells (c) were calculated. ELISPOT was used to determine the number of IFN-γ–producing OVA-specific T cells within the DLN (d). Data points represent the mean for each group (n = 3–4). One experiment representative of two total experiments is shown. p-values were determined by the Student's t test. CFSE-labeled naive TCRTg CD4 T cells were delivered to mice, and the loss of CFSE 4 d after intratracheal delivery of B6 or IL–12p40-deficient (p40KO) BMDCs was determined by flow cytometry (e). Graphs are representative of three to seven mice and of two experiments Numbers indicate the mean frequency of cells within the undivided and divided gates for each group. **, P ≤ 0.005 compared with the B6 values by Student's t test.

TCRTg T cell expansion in IL-12p40–deficient hosts. We found that T cell expansion was as efficient as for B6 BMDCs within B6 hosts ($1.5^5 \pm 1.4^4$ CD44^{high} TCRTg cells in the LNs of IL-12p40–deficient hosts compared with $1.3^5 \pm 2.7^4$ in B6 hosts; $n = 3$, $P = 0.4563$ by Student's t test). These data demonstrate that IL-12p40 from the interstitium is not required for the DC-induced expansion of T cells.

DCs not only drive the proliferation and activation of naive T cells, they also facilitate cytokine production by the newly activated T cells. Therefore, we measured the frequency of cytokine-producing T cells in the DLNs of the mice described above. B6 DCs were able to initiate a Th1 response within the TCRTg T cells, as were DCs derived from IL-23p19–deficient mice (Fig. 7 d). In contrast, DCs from mice lacking IL-12p70 were substantially compromised in their ability to induce a Th1 response, although they were significantly better than DCs from IL-12p40–deficient mice in this respect (Fig. 7 d). These data demonstrate that the formation of IFN-γ–producing T cells in the LN is dependent on IL-12p40 expressed by the DC, and this is due to a large degree on IL-12p70.

Availability of IL-12(p40)$_2$ during Mtb activation results in the ability of IL-12p40–deficient BMDCs to induce activation of antigen-specific T cells

We have demonstrated that the ability of Mtb-activated DCs to respond to homeostatic chemokines is dependent on IL-12p40 and that this activity can be restored in IL-12p40–deficient DCs by the addition of IL-12(p40)$_2$ (Fig. 6 c). We next asked whether treatment of Mtb-exposed IL-12p40–deficient DCs with IL-12(p40)$_2$ could rescue T cell activation and priming in the LN. To do this we incubated IL-12p40–deficient BMDCs with Mtb, OVA$_{323-339}$, and IL-12(p40)$_2$ for 3 h, transferred these cells to the lungs of mice that had received a bolus of naive TCRTg T cells 48 h previously, and monitored T cell activation in the DLNs. We show here that

antigen-specific CD4$^+$ T cells accumulate in significantly higher numbers in the DLNs of mice receiving the IL-12(p40)$_2$–treated, Mtb-exposed IL-12p40–deficient DCs than in mice receiving untreated, Mtb-exposed IL-12p40–deficient DCs (Fig. 8 a). These antigen-specific T cells expressed high levels of CD44 and CD69, indicative of an activated phenotype (Fig. 8 b). In fact, the ability of the IL-12(p40)$_2$–treated IL-12p40–deficient BMDCs to induce activation and accumulation of antigen-specific cells was equivalent to that of the B6 BMDCs. In further support of this finding, Mtb-exposed antigen-pulsed BMDCs capable of expressing only IL-12p40 are also able to initiate T cell expansion and activation after intratracheal delivery (Fig. 8, c and d), indicating that expression of IL-12p40 alone by DCs is sufficient to drive T cell activation and expansion. In contrast, the availability of IL-12p40 either in dimeric form (Fig. 8 e) or from p19/p35-deficient BMDCs (not depicted) was unable to initiate the generation of IFN-γ–producing cells. We conclude from these data that IL-12p40 provides sufficient stimulus to reconstitute the T cell–activating phenotype of IL-12p40–deficient DCs but that it is insufficient to restore a Th1 phenotype within the newly activated T cells. These data fit with our previous observation that the development of Th1 cells in the absence of IL-12p70 is entirely dependent on IL-23 (11).

DISCUSSION

We demonstrate a novel and important function for IL-12p40 in the initiation of T cell responses. This observation improves our limited understanding of the factors required for T cell activation upon microbial challenge to the lung. We demonstrate that a key component of Mtb-initiated T cell activation is the induction of chemokine responsiveness in DCs and that this induction is dependent on IL-12p40 production by DCs. This requirement for IL-12p40 does not reflect the need for IL-23 or IL-12p70, as
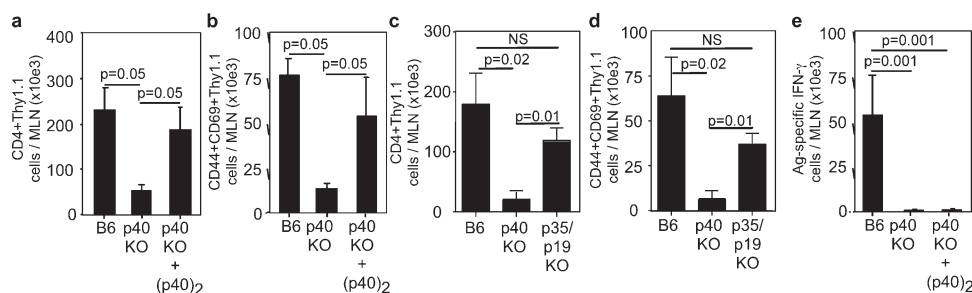

Figure 8. DCs expressing IL-12p40 alone or IL-12p40–deficient DCs treated with IL-12(p40)$_2$ can activate naive T cells when delivered to the lung. BMDCs from B6 or IL-12p40–deficient (p40KO) mice were treated with irradiated Mtb alone or Mtb in the presence of IL-12(p40)$_2$ for 3 h. The cells were pulsed with OVA$_{323-339}$ and transferred intratracheally into B6 mice that had received a bolus of naive CD4 TCRTg T cells. The number (a) and activation phenotype (b) of CD4$^+$CD90.1$^+$ T cells within the DLN at 4 d were determined by flow cytometry. BMDCs from B6, IL-12p40–deficient (p40KO), or IL-12p35/IL-23p19–deficient (p35/p19KO) mice were treated as described above and delivered to mice, and the number (c) and activation phenotype (d) of transferred naive T cells were determined at day 4 by flow cytometry. ELISPOT was used to determine the total number of antigen-specific IFN-γ–producing cells in the DLN for IL-12(p40)$_2$–treated IL-12p40–deficient (p40KO) DCs (e). Data points represent the mean for each group ($n = 3-4$). One experiment representative of three total experiments for each of panels a–e is shown. p-values were determined by the Student's t test.

the migratory phenotype is induced in mice lacking these cytokines and can be reconstituted by providing IL-12(p40)$_2$ to IL-12p40–deficient DCs. The data also support the importance of pathogen-induced DC migration in the initiation of T cell responses after aerosol delivery of antigen.

Pathogen-mediated activation facilitates migration of DCs from the periphery to the secondary lymphoid organs, and the failure of Mtb-exposed IL-12p40–deficient DCs to become migratory implicates this cytokine as a key mediator of this process. This novel role for IL-12p40 provides a rationale for the fact that IL-12p40 production is one of the earliest events after DC activation (24, 30–32) and that IL-12p40 is produced more rapidly and in excess of IL-12p70 during pulmonary inflammation (24–26).

Although migration of DCs to the LN is the first step in initiating T cell responses, DCs must also be able to activate naive T cells. Our data showing that DCs making only IL-12p40 as well as IL-12p40–deficient DCs treated with IL-12(p40)$_2$ can initiate activation of naive T cells confirm that IL-12p40, probably as the IL-12(p40)$_2$, is sufficient to mediate the Mtb-induced maturation of DCs to a T cell–activating phenotype. The failure of DCs making only IL-12p40 and of IL-12p40–deficient DCs treated with IL-12(p40)$_2$ to promote effector cell development indicates that polarization of the T cell during activation requires, as expected, IL-12p70 and/or IL-23 (as discussed in reference 11). In a preliminary analysis of Mtb-infected B6, IL-12p40–deficient, and IL-12p35/IL-23p19–deficient mice, we have not detected differences in the frequency or number of IL-4– or IL-10–producing CD4 T cells in the lungs (not depicted), indicating that activation in the presence of IL-12p40 alone does not drive a Th2 type of CD4 T cell response.

Our understanding of this novel and important activity of IL-12p40 is in its infancy and the mechanisms involved are not yet clear. However, the data suggest that events progress rapidly. Specifically, changes in chemokine responsiveness occur within 3 h, with exogenous IL-12(p40)$_2$ also able to restore responsiveness within this timeframe. This rapidity means that up-regulation of mRNA for the chemokine receptor CCR7 is unlikely to be the key IL-12(p40)$_2$–mediated event. Rather, a change in the ability of the receptor to initiate migration may be more likely. Thus, IL-12(p40)$_2$ may act to overcome negative signals limiting pulmonary DC responsiveness. Interestingly, we see increased IL-10 mRNA and protein production in IL-12p40–deficient DCs, and the addition of IL-10 to B6 DCs reduced chemokine responsiveness to the level seen in IL-12p40–deficient DCs. Further, IL-10 specifically inhibits migration of DCs to CCL19 by uncoupling the CCR7 receptor from protein kinase B phosphorylation (33). Thus, it is plausible that rapid, early induction of IL-12(p40)$_2$ in response to a pathogen allows lung DCs to overcome an IL-10–induced inhibition of migration. This pathway is further supported by the fact that autocrine IL-10 reduces trafficking of pulmonary DCs to the DLN after mycobacterial infection (34). This pathway may also explain the apparently contradictory data showing that

although DCs in IL-12p35–deficient mice exhibit a moderately compromised migratory response to Mtb, IL-12p35–deficient DCs generated in vitro do not show a defect. The relative importance of IL-12p70 on Mtb-induced migration may thus reflect the levels of IL-10 within the in vivo and in vitro environments.

We have not determined the receptor(s) by which IL-12(p40)$_2$ stimulates DCs to become migratory; however, as IL-12(p40)$_2$ can induce NF-κB mobilization in myeloid cells (18), this cytokine may act directly on DCs to initiate chemokine responsiveness. IL-12(p40)$_2$ likely binds the IL-12Rβ1 (35); however, as the IL-12Rβ2 chain is the primary signal-transducing element of the IL-12R (9) and ligation of the IL-12Rβ2 chain requires the presence of IL-12p70 (36, 37), it is not clear how IL-12(p40)$_2$ induces responses in myeloid cells. However, there are differences in the affinity of the IL-12R on myeloid and lymphoid cells that may allow IL-12(p40)$_2$ signaling in myeloid cells (12, 15, 16). We are currently investigating the requirement for each IL-12R chain in the Mtb-induced migration of pulmonary DCs.

Whether IL-12(p40)$_2$–induced DC migration is specific for the lung is not yet known. Increased sensitivity of IL-12p40–deficient mice over IL-12p35–deficient mice has been seen in *Francisella tularensis* (38), *Cryptococcus neoformans* (39), *Salmonella enteriditis* (40), and *Mycobacterium bovis* BCG (2). In none of the above studies was the initiation of cellular response studied; therefore, it is unclear whether these models reflect a role for IL-12p40 in either pulmonary or other DC function. We are currently extending our studies to determine whether IL-12p40 is required for migration of DCs from the skin. This is particularly interesting in light of the recent publication showing that human skin migratory DCs produce IL-12p40 and can stimulate allogeneic T cells in the absence of IL-12p70 (41). In contrast, we think that if antigen is systemic, IL-12p40–mediated DC migration is not required. This is supported by the fact that IL-12p40–deficient DCs can initiate T cell activation as efficiently as normal DCs in culture and when injected intravenously (shown here and in reference 42). Similarly, after *Toxoplasma gondii* infection, the activation of CD4 T cells is equivalent in B6, IL-12p35–deficient, and IL-12p40–deficient mice (43), suggesting that when a potent and systemic infection occurs, the role of IL-12p40 in DC migration is not required.

The data presented here impact our understanding of the natural history of Mtb infection and the initiation of the immune response in the lung after microbial challenge. The data also have ramifications for the generation of vaccines that act via the pulmonary route, in that IL-12p40 may prove to be an effective adjuvant for the initiation of T cell responses in the lung.

MATERIALS AND METHODS

Animals. C57BL/6J (B6), IL-12p35–deficient (B6.*Il12a*$^{-/-}$, IL-12p35–deficient) and IL-12p40–deficient (B6.*Il12b*$^{-/-}$, IL-12p40–deficient) mice were purchased from The Jackson Laboratory. IL-23p19 gene-disrupted mice (B6.*Il23a*$^{-/-}$, IL-23p19–deficient) were generated at Genentech Inc. by F. deSauvage and N. Ghilardi as described previously (44). Mice lacking

both IL-12p35 and IL-23p19 (B6.$Il12a^{-/-}$x$Il23a^{-/-}$, IL-12p35–/IL-23p19–deficient) were generated at the Trudeau Institute as described previously (11). The genotype and phenotype of the gene-deficient mice are presented in Table I. OT-II αβTCRTg male mice (OT-II), which are MHC class II I-A^b restricted and specific for OVA$_{323–339}$ (45) were obtained from the Trudeau Institute Inc.'s Animal Breeding Facility. Experimental mice were age and sex matched and used between the ages of 8 and 12 wk. Mice were used in accordance with the Institutional Animal Care and Use Committee guidelines of the National Research Council and the Trudeau Institute.

Experimental infection. The H37Rv strain of Mtb was grown in Proskauer Beck medium containing 0.05% Tween 80 to mid-log phase and frozen in 1-ml aliquots at −70°C. For aerosol infections, subject animals were infected with ∼75 bacteria using a Glas-Col airborne infection system as described previously (46).

Lung and DLN cell preparation. The lung tissue was prepared as described previously (46). Single cell suspensions were prepared from either digested lung tissue or DLN by dispersing the tissue through a 70-μm nylon tissue strainer (BD Falcon). The resultant suspension was treated with Geys solution to remove any residual red blood cells, washed twice, and counted (46).

Detection of IFN-γ–producing cells by ELISPOT assay. Antigen-specific IFN-γ–producing CD4 T cells from infected lungs or DLNs were counted using peptide-driven ELISPOT as described previously (11). In brief, wells were coated with monoclonal anti–mouse IFN-γ and blocked. Cells from lungs and DLNs were seeded at an initial concentration of 10^5 cells/well and diluted. Irradiated B6 splenocytes were used as APC at a concentration of 10^6 cells/well along with 10 μg/ml ESAT-6$_{1–20}$ (47, 48) or 5 μM OVA$_{323–339}$ peptide and IL-2. After 24 h, plates were washed and probed with biotinylated anti–mouse IFN-γ. Spots were visualized and enumerated visually under a dissection microscope. Neither cells cultured in the absence of antigen nor cells from uninfected mice produced detectable spots.

Generation of BMDCs. DCs were generated from the bone marrow of B6 and gene-deficient mice as described previously (49). In brief, cells were extracted from the femurs of mice and cultured in supplemented DMEM (sDMEM) containing 10% FBS and 20 ng/ml of recombinant murine GM-CSF (rmGM-CSF; PeproTech). On days 3, 6, and 8, cultures were supplemented with additional sDMEM. On day 10 of culture, nonadherent cells were collected by centrifugation and used as BMDCs. ELISA was used to measure the production of IL-10 and IL-12p40 by BMDCs exposed to Mtb. Some Mtb-activated BMDCs were cultured with 500 ng/ml IL-12(p40)$_2$, 500 ng/ml IL-12(p40)$_2$ denatured by boiling for 20 min, 500 ng/ml IL-12p70, or 10 ng/ml IL-10 (all from R&D Systems).

In vivo tracking of BMDCs. Day 10 in vitro–generated BMDCs were used for in vivo tracking as described previously (50, 51). In brief, BMDCs were activated in vitro with 500 μg/ml of irradiated Mtb H37Rv whole cells (obtained under National Institutes of Health [NIH] contract AI-75320) and 5 μM OVA$_{323–339}$ peptide for 3 h. Cells were washed once and stained with 6 μg/ml TAMRA orange (Invitrogen) for 3 min at 37°C, whereupon they were washed and resuspended in PBS. 10^6 Mtb-exposed OVA$_{323–339}$-

pulsed BMDCs were then instilled intratracheally into B6 mice, and at various times after instillation, lungs and DLNs were harvested and processed to produce single cell suspensions.

In vivo tracking of lung CD11c$^+$ DCs. Some mice received a suspension of 5 μg of irradiated Mtb in a 5-mM CFSE (Invitrogen) solution delivered intratracheally, whereas other mice received 5 μg/ml LPS in 5 mM CFSE. Control mice received CFSE alone. 18 h after instillation, both the lung and the DLN were harvested, and single cell suspensions were prepared as described above. Flow cytometry was used to determine the frequency and total number of CFSE-labeled CD11c$^+$ cells within the lung and DLN.

Naive CD4$^+$ T cell isolation and in vivo effector priming. Naive CD4$^+$ T cells were positively sorted from single cell suspensions of the spleens and LNs of OT-II αβTCRTg (TCRTg) mice using magnetic beads labeled with anti-CD4 antibody (clone GK1.5; Miltenyi Biotec) and a MACS column according to the manufacturer's instructions (Miltenyi Biotec). 10^7 T cells (>95% CD4$^+$) were resuspended in 100 μl of physiological saline and injected intravenously into B6 mice. 2 d later, host mice were intratracheally instilled with 10^6 BMDCs activated in vitro with 500 μg/ml of irradiated Mtb H37Rv whole cells in the presence of 5 μM of the OVA$_{323–339}$ peptide for 3 h. Some BMDCs were also exposed to 500 ng/ml IL-12(p40)$_2$ during in vitro stimulation. The IL-12(p40)$_2$ was obtained from R&D Systems (499-ML/CF). It is 97% homodimer as determined by SDS-PAGE and has <1 EU/μg of endotoxin by the Limulus amoebocyte assay method. Control mice received untreated BMDCs, Mtb-treated BMDCs, or OVA-treated BMDCs alone. On day 4, after intratracheal instillation, the DLNs were harvested, single cell suspensions were generated, and the frequency of specific cell types was determined by ELISPOT and flow cytometry. Some mice received the same BMDCs via the intravenous route, and T cell responses were monitored by flow cytometry on day 4.

Flow cytometry. Single cell suspensions were stained with fluorochrome-labeled antibodies specific for CD4 (clone GK1.5), CD90.1 (Thy1.1; clone HIS51), CD69 (clone HI2F3), CD44 (clone IM7), CD11c (clone HL3), MHC class II I-A^b (clone AF6-120.1), CD80 (B7-1; clone 16-10A1), CD86 (B7-2; clone GL1), CD40 (clone 145-2C11), and CD11b (clone M1/70). Cells were collected using CELLQuest software on a Becton Dickinson FACSCalibur flow cytometer. Lymphocytes were gated based on their forward and side scatter characteristics, and adoptively transferred CD4$^+$ T cells (CD90.1$^+$) were tracked in CD90.2$^+$ congenic hosts. DCs were designated as CD11c$^+$ cells that exhibit low autofluorescence in the FL1 channel as defined previously (28). Lung cells were sorted based on CD11c expression and autofluorescence using a three-laser FACSVantage SE with DIVA option.

Chemotaxis assay. DCs were activated with 500 μg/ml of irradiated Mtb for 3 h, and their ability to respond to the chemokines CCL-19 (ELC, or EB1 ligand chemokine) or CCL-21 (SLC, or secondary lymphoid organ chemokine; both from R&D Systems) was determined using a previously described chemotaxis assay (52). In brief, 10^5 activated BMDCs were added to the upper chambers of 24-well transwell plates (Costar) containing a polycarbonate filter with a 5-μm pore size. Either CCL-19 or CCL-21 was added to the lower chamber and the plates were incubated at 37°C for

Table I. Genotype and phenotype of gene-deficient mice

Common name[a] (figure name)	IL-12p35–deficient (p35KO)	IL-23p19–deficient (p19KO)	IL-12p35–/IL-23p19–deficient (p35/p19KO)	IL-12p40–deficient (p40KO)
Missing gene	Il12a	Il23a	Il12a, Il23a	Il12b
Missing protein	p35, IL-12p70	p19, IL-23	p19, p35, IL-12p70, IL-23	p40, IL-12p70, IL-23, IL-12(p40)$_2$
Protein available	IL-23, IL-12(p40)$_2$	IL-12p70, IL-12(p40)$_2$	IL-12(p40)$_2$	None

[a]Used in text. The names in brackets are used in the figures.

90 min. The transmigrated cells were collected from the lower chamber, fixed, and counted on a flow cytometer. To determine the absolute number of cells in each sample, a standard number of 20-μm-size fluorescent microspheres (Polysciences, Inc.) was added to each tube and counted with the cells. The results are expressed as a chemotaxis index, which is calculated as the fold increase in the number of migrated cells in response to chemoattractant over spontaneous migration to control medium.

Real–time PCR. RNA was extracted from BMDCs as described previously (11). RNA samples ($n = 3$) were treated with DNase and reverse transcribed, cDNA was amplified using Taqman reagents on the ABI Prism 7700 sequence detection system, and fold increase in signal over that derived from uninfected BMDCs was determined using the ΔΔct calculation. The primer and probe sequence for murine GAPDH was as described previously (11). The sequences for the CCR7 reaction were designed and validated by the Trudeau Institute Inc.'s Molecular Biology Core Facility and were as follows: forward, 5′-ACCATGGACCCAGGG-AAAC; reverse, 5′-GGTATTCTCGCCGATGTAGTCAT; and probe, 5′-CTTGTCATTTTCCAGGTGTGCTTC-TGCCAA.

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