

Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis

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Mycobacterium tuberculosis (*Mtb*) frequently establishes persistent infections that may be facilitated by mechanisms that dampen immunity. T regulatory (T reg) cells, a subset of CD4⁺ T cells that are essential for preventing autoimmunity, can also suppress antimicrobial immune responses. We use Foxp3-GFP mice to track the activity of T reg cells after aerosol infection with *Mtb*. We report that during tuberculosis, T reg cells proliferate in the pulmonary lymph nodes (pLNs), change their cell surface phenotype, and accumulate in the pLNs and lung at a rate parallel to the accumulation of effector T cells. In the *Mtb*-infected lung, T reg cells accumulate in high numbers in all sites where CD4⁺ T cells are found, including perivascular/peribronchiolar regions and within lymphoid aggregates of granulomas. To determine the role of T reg cells in the immune response to tuberculosis, we generated mixed bone marrow chimeric mice in which all cells capable of expressing Foxp3 expressed Thy1.1. When T reg cells were depleted by administration of anti-Thy1.1 before aerosol infection with *Mtb*, we observed ~1 log less of colony-forming units of *Mtb* in the lungs. Thus, after aerosol infection, T reg cells proliferate and accumulate at sites of infection, and have the capacity to suppress immune responses that contribute to the control of *Mtb*.

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Abbreviations used: ICOS, inducible costimulatory molecule; mLN, mesenteric LN; *Mtb*, *Mycobacterium tuberculosis*; pLN, pulmonary lymph node; RFP, red fluorescent protein.

T cell-mediated immunity is critical for protection against *Mycobacterium tuberculosis* (*Mtb*); mice and humans who lack T cells, are very susceptible to lethal disease (1, 2). Nevertheless, this immunity seems to be subject to limitations. Even the most successful immune response against *Mtb* often manages only to contain, rather than eradicate, the organism. Furthermore, animals and humans persistently infected with *Mtb*, or who have been cured of tuberculosis by chemotherapy, can be superinfected, or reinfected (3–5). In light of these facts, it is perhaps not surprising that the currently used vaccine, BCG, which is an attenuated strain of *Mycobacterium bovis*, is largely ineffective (6). What are the factors that prevent the immune system from eradicating *Mtb* during primary infection, and even prevent an effective memory response from occurring at the time of a subsequent challenge? A better understanding of the reasons for these apparent deficiencies in the immune response

against *Mtb* is needed for the rational design of an effective vaccine.

The structure central to this “limited” immune response evoked by *Mtb* after aerosol infection is the granuloma, which is an organization of immune cells that contains viable bacilli (3, 7–9). The granuloma is composed of an aggregation of macrophages containing intracellular bacilli surrounded by a cuff of lymphocytes, including CD4⁺ T cells and CD8⁺ T cells. Some of these T cells recognize *Mtb*-derived antigens and produce effector cytokines, such as IFN- γ and TNF- α , which promote macrophage activation and control mycobacterial growth. In addition, other T cells express an activated phenotype, but do not produce effector cytokines (10). The granuloma serves the immune system by containing *Mtb* and limiting the extent of inflammation and tissue damage to the host. Conversely, the granuloma also serves the bacillus by providing a niche conducive for its prolonged survival. The pathogen- and host-mediated mechanisms that generate and maintain this balanced immune response are poorly understood.

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Foxp3-expressing regulatory T (T reg) cells are a subset of CD4⁺ T cells, most of which express the cell surface marker CD25. These cells are essential for preventing autoimmunity. Furthermore, T reg cells have recently been shown to also suppress antimicrobial immune responses, especially against pathogens that establish persistent infections (11). In the most well-characterized example, T reg cells have been shown to be essential for establishing and maintaining persistent infection by *Leishmania major* (12–14); elimination of T reg cells in persistently infected mice resulted in the eradication of the parasite from the host (12). In addition, T reg cells protect the host during infections by preventing collateral damage to the host's tissue from excessive inflammation induced by the immune response to the pathogen (11). Although T reg cells have been studied mostly in respect to persistent viral and parasitic infections (11), their involvement has also been implicated in bacterial infections, including infections caused by *Helicobacter pylori* (15–18) and *Listeria monocytogenes* (19). The role of T reg cells in suppressing immunity to *Mtb*, which is the most important bacterial pathogen that establishes a persistent infection in humans in terms of morbidity and mortality, is only beginning to be investigated. In human tuberculosis, three studies have shown an increase in T reg numbers in the blood and at sites of infection during active disease (20–22). In the murine model, there are two studies that have reached different conclusions about the role of T reg cells in tuberculosis. Quinn et al. (23) used anti-CD25 antibodies to reduce the number of T reg cells in mice. Although they observed increased IFN- γ production at early time points after infection, this depletion did not affect the bacterial load. Using a different strategy, Kursar et al. (24) adoptively transferred CD25-negative CD4⁺ T cells with and without CD25⁺ T cells into T cell-deficient (RAG^{-/-}) recipients before infection with *Mtb*; these investigators did observe a lower bacterial load when the number of T reg cells in the recipients was reduced. We seek to further investigate the role of T reg cells in tuberculosis using the murine model.

Monitoring the activity and targeting the depletion of T reg cells during tuberculosis is complicated by the lack of good markers that identify these cells. As in the above studies, expression of CD25 is commonly used to identify CD4⁺ T reg cells, and anti-CD25 antibodies have been used to deplete this population. However, these approaches are problematic during ongoing *Mtb* infection because CD25 is up-regulated on effector T cells, and furthermore, CD25 is not expressed on all T reg cells (25). We describe two approaches to circumvent these problems. First, we have used mice expressing GFP knocked into the locus encoding the forkhead transcription factor, Foxp3 (25). In mice, Foxp3 is uniquely expressed in T reg cells and, in fact, is responsible for promoting the transcriptional program that conveys T regulatory cell development and function (26). Thus, T reg cells could be tracked by their expression of Foxp3-GFP fusion protein independently of CD25 expression. Second, we have reconstituted irradiated recipients with bone marrow from Foxp3^o donors (Thy1.2 expressing) and wild-type B6.PL donors

(Thy1.1-expressing). Using these mixed bone marrow chimeric mice, we were able to eliminate all cells capable of becoming Foxp3-expressing T reg cells in vivo using a depleting anti-Thy1.1 antibody.

RESULTS

During tuberculosis, Foxp3 expression is restricted primarily to CD4⁺ T cells

To characterize the phenotype and function of T reg cells during chronic infection, we infected Foxp3-GFP reporter mice with a low dose of *Mtb* (strain H37Rv) via aerosolization. In uninfected mice, Foxp3 expression is limited to TCR- $\alpha\beta$ ⁺ cells, predominately in the CD4⁺ subset (25). After aerosol infection with *Mtb*, Foxp3 expression remained limited to a subset of TCR- $\alpha\beta$ ⁺ cells (Fig. 1 A), and within this population, >95% were CD4⁺. Foxp3 was not expressed in TCR- $\gamma\delta$ ⁺, NK1.1⁺, CD11b⁺, or CD11c⁺ cells (unpublished data). Thus, even during the highly inflammatory setting

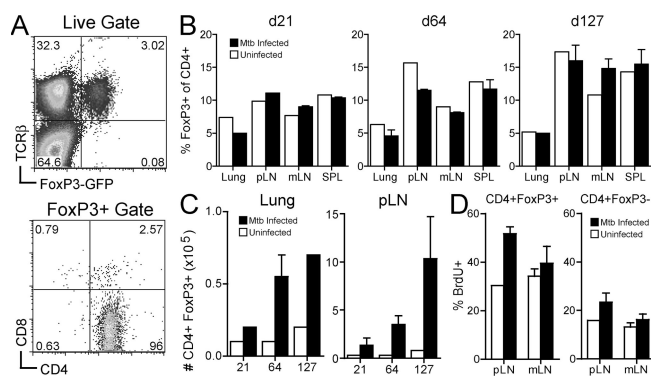


Figure 1. Characterization of Foxp3-expressing cells in *Mtb*-infected mice. (A) PLNs from Foxp3-GFP mice 56 d after aerosol infection with *Mtb* were stained for TCR β , CD4, and CD8. FACS plots are gated on live cells or live FoxP3-GFP⁺ cells. Similar data were obtained gating on cells in the spleen and lung and are representative of two mice in two different experiments. (B) The percentage of CD4⁺ cells that express Foxp3-GFP are shown for lung, pLN, spleen, and mLN 21, 64, and 127 d after infection, as indicated, for uninfected mice (open bars) and infected mice (shaded bars). Numbers represent the mean of three mice per group \pm the SD for infected LNs and the mean of two mice for uninfected LNs. Lung numbers represent a pool of six lobes, two from each of three infected mice, and three each from each of two uninfected mice. Data is representative of four independent experiments. (C) Total numbers of CD4⁺ Foxp3-GFP⁺ cells are shown for lungs and pLNs at 21, 64, and 127 d after infection (black bars). Age-matched controls are shown in white bars. Numbers represent the mean of three mice per group \pm the SD for infected LNs and the mean of two mice for uninfected LNs. Lung numbers represent a pool of six lobes, two from each of three infected mice, and three each from each of two uninfected mice. Data is representative of four independent experiments. (D) Mice infected with *Mtb* (or uninfected controls) were administered BrdU in drinking water beginning 21 d after infection. 14 d later, cells from pLN and mLN from *Mtb*-infected mice (black bars) or uninfected controls (white bars) were stained for CD4, and the percentage of CD4⁺, BrdU⁺ cells were compared between Foxp3-GFP⁺ and Foxp3-GFP⁻ cells in pLNs and mLNs. Data represent the mean of three mice per group \pm the SD.

of tuberculosis, Foxp3 expression is remarkably restricted to a subset of CD4⁺ T cells.

Foxp3⁺ cells accumulate in the lungs and pulmonary lymph nodes (pLNs)

The immune response to *Mtb* after aerosol infection includes a dramatic expansion of lymphocytes in the pLNs and migration of effector cells into the lungs (2). We compared the percentage of Foxp3-expressing CD4⁺ T cells in various tissues after aerosol infection with *Mtb*. Over time, the percentage of CD4⁺ cells expressing Foxp3 increased in both infected and uninfected mice as they aged (Fig. 1 B). However, the percentage at each time point was similar in both infected and uninfected mice in all tissues examined (i.e., lungs, pLNs [draining the lungs], spleen, and mesenteric LNs [mLNs; distal to the infection]). Interestingly, the LNs and spleen contained a higher percentage of Foxp3-expressing CD4⁺ T cells than the lungs, both before and at all time points after infection. Although the T reg population remained a constant percentage of CD4⁺ T cells in each respective tissue, the absolute number of Foxp3-expressing cells increased dramatically at sites of infection as the number of total CD4⁺ T cells increased. Even 21 d after infection, the number of Foxp3-expressing CD4⁺ T cells was elevated at sites of infection, and by later time points had increased ~5- and 20-fold in the lungs and pLNs, respectively (Fig. 1 C). The number of Foxp3-expressing cells did not increase in mLNs distal to the site of infection (unpublished data). Overall, our results indicate that expansion of T reg cells parallels the expansion of CD4⁺ effector T cells in the lung and pLNs after aerosol infection.

Our flow cytometric results suggested that the most profound accumulation of T reg cells after aerosol infection with *Mtb* occurred in the pLNs, but this could reflect either recruitment or increased proliferation. To address this question, we administered BrdU in the drinking water of Foxp3-GFP mice 3 wk after aerosol infection, a time when this accumulation was marked. Proliferation of T cells in vivo in pLNs and mLNs between infected and uninfected animals was compared by incorporation of BrdU. As shown in Fig. 1 D, the percentage of Foxp3⁺ cells that incorporated BrdU increased by 73% in the pLNs of infected animals (52 vs. 30%), compared with uninfected controls. Not surprisingly, BrdU incorporation in Foxp3-negative cells was also increased in the pLNs of infected mice, albeit to a lesser degree (47% increase). In the mLNs, distal to the site of infection, the percentages of Foxp3-expressing and -negative CD4⁺ T cells that incorporated BrdU were similar in infected and uninfected animals. These data indicate that the accumulation of T reg cells in the pLNs after aerosol infection reflects, at least in part, an increased proliferation of these cells.

Foxp3-expressing T reg cells reside in pulmonary granulomas during tuberculosis

To determine the location of T reg cells within the lung during tuberculosis, we stained lung sections from *Mtb*-

infected mice or from uninfected controls with anti-Foxp3 antibodies. Control sections were stained with nonspecific rabbit IgG. In uninfected mice, the few Foxp3-expressing cells observed were seen in perivascular and peribronchiolar regions (Fig. 2 A). In microscopic fields containing vascular and bronchial structures, we counted 1–15 Foxp3-expressing cells at 200× magnification. Almost no T reg cells were observed in the parenchyma or other sites within the lung (unpublished data). In *Mtb*-infected mice (40–120 d after infection), ~70–90% of the lungs were normal in appearance and did not display the inflammatory changes associated with the

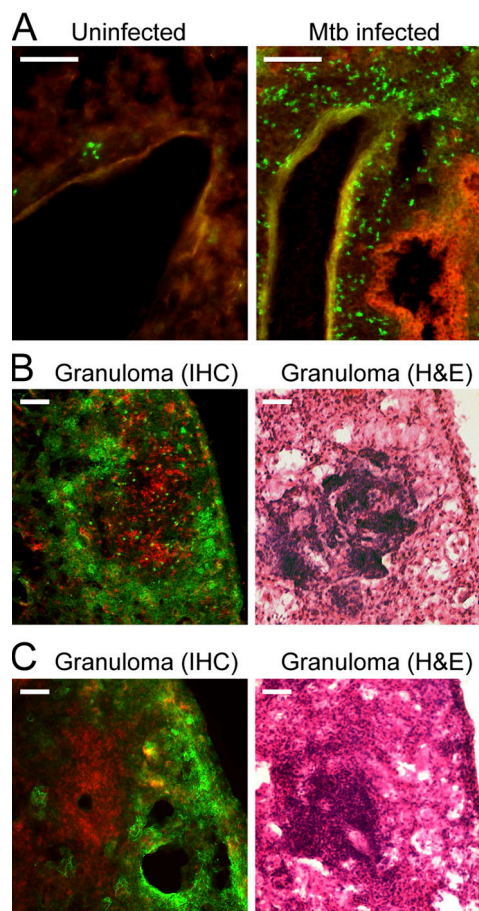


Figure 2. Localization of T reg cells in lungs of infected mice.

(A) Representative sections showing perivascular areas from lungs of uninfected or *Mtb*-infected mice (115 d after infection) stained with anti-Foxp3 (green) and anti-E-cadherin (red) to stain respiratory epithelium. (B) Serial sections showing parenchymal granuloma of an *Mtb*-infected mouse (~60 d after infection) stained with anti-Foxp3 (green) and CD4 (red; left) and hematoxylin/eosin (right). Section is representative of sections of eight mice examined, ranging from 40 to 120 d after infection. (C) Serial sections showing parenchymal granuloma of an *Mtb*-infected mouse stained with nonspecific rabbit IgG (green) and CD4 (red; left), and hematoxylin/eosin (right). Although the nonspecific green fluorescence of macrophages was similar to the section in B, the green pinpoint staining of nuclei within the CD4⁺ region of the granuloma (as seen in B using anti-Foxp3) was not seen using nonspecific polyclonal rabbit IgG as the primary antibodies. Bars, 50 μ m.

infection. In these noninflamed regions of the lung, only a few T reg cells were observed, similar in number and distribution to those seen in uninfected mice (unpublished data). In striking contrast, in regions of the lungs showing inflammatory changes (i.e., ~10–30% of the lung), high numbers of Foxp3-expressing cells were observed in all regions of the lung that contained aggregates of lymphocytes (Figs. 2, A and B). Reflecting pulmonary locations previously described for CD4⁺ T cells in tuberculosis (27), Foxp3-expressing cells were found in abundance in the perivascular and peribronchiolar regions (Fig. 2 A) and within the lymphoid aggregates of granulomas (Fig. 2 B); at these sites, the T reg cells were distributed evenly throughout the aggregates. In the inflamed regions of *Mtb*-infected lungs, most microscopic fields contained >300 Foxp3-expressing cells at 200× magnification. Although many macrophages associated with granulomas displayed nonspecific, cytoplasmic green fluorescence (Fig. 2, B and C), the distinctive staining of T reg cells, represented by the staining of the nuclei of a subset of lymphocytes, was only observed when the anti-Foxp3 primary antibody was used (Fig. 2, A and B). Although a precise quantification of the total number of T reg cells in the lungs is not possible using immunohistochemical techniques, we saw more T reg cells in the lungs of *Mtb*-infected mice relative to uninfected controls than we did by flow cytometry (where we observed a relatively modest ~5× increase in the number of T reg cells in the lung; Fig. 1 C). Thus, we believe it is possible that flow cytometry underestimates the true number of T reg cells that accumulate in the lung in tuberculosis, and that collagenase digestion may not efficiently isolate all the T reg cells from *Mtb*-infected lungs.

To further define the location of T reg cells relative to tuberculous bacilli within the lung, we infected mice with *Mtb* expressing recombinant red fluorescent protein (RFP). Lung sections were examined at various time points after infection to determine the locations of RFP-expressing bacilli and Foxp3-expressing cells. Bacilli were observed primarily within macrophages displaying nonspecific green fluorescence. Some of these infected macrophages were located within the center of granulomas (Fig. S1, B and C, available at <http://www.jem.org/cgi/content/full/jem.20062105/DC1>), and others were distributed around the periphery of the granulomas (Fig. S1 A, top right). Occasionally, bacilli were seen outside of green cells, probably representing either extracellular bacilli or bacilli within fibroblasts or epithelial cells (Fig. S1 A). In contrast, T reg cells were seen throughout the lymphocyte aggregates of granulomas (Fig. 2 and Fig. S1). Only rarely, especially at late time points (~150 d after infection), were T reg cells observed to be directly interacting with clusters of infected cells (Fig. S1 C). Overall, these results show that many of the T reg cells within the lungs of *Mtb*-infected mice are located within granulomas, which is where tuberculous bacilli live, and that they are controlled by the immune system. At a given point in time, however, very few of these T reg cells are interacting directly with the infected cells, but instead are located in lymphocytic

regions of granulomas, which are immediately adjacent to infected macrophages.

Foxp3-expressing T reg cells in tuberculosis do not produce effector cytokines or IL-10

Effector T cells, in response to antigenic stimulation, can develop the ability to make proinflammatory cytokines such as IFN- γ and TNF- α , whereas T reg cells are generally unable to make these cytokines (28). To determine if the cytokine production of Foxp3-expressing cells is altered during chronic infection, cells from the pLNs and lung were stimulated *ex vivo* with anti-CD3 and -CD28 in the presence of monensin. Compared with CD4⁺, Foxp3-GFP-negative cells, the CD4⁺ Foxp3-GFP-expressing cells did not acquire the ability to rapidly make the inflammatory cytokines IFN- γ or TNF- α upon restimulation (Fig. 3). In the lung, 6 and 12.3% of Foxp3-negative CD4⁺ T cells produced IFN- γ or TNF- α , respectively, whereas <1% of Foxp3⁺ CD4s produced these cytokines. Similar results were observed using pLN cells, where the percentage of Foxp3-expressing CD4s capable of producing inflammatory cytokines was 10-fold less than Foxp3-negative CD4s (Fig. 3).

Because IL-10 has been shown to be an important mediator of immunosuppression used by T reg cells in some, but not all, experimental systems (29), we also tested their ability to produce IL-10 using intracellular cytokine staining. We were unable to detect IL-10 production after polyclonal stimulation with both anti-CD3 and -CD28 or after stimulation with PMA and ionomycin at any time point after infection (unpublished data). Thus, we believe it is unlikely that IL-10 produced by T reg cells plays a major role in T reg cell-mediated immunosuppression in tuberculosis, at least in B6 mice. However, other suppressive pathways, such as TGF- β , cell-cell contact mediated by CTLA-4, or other mechanisms may be involved.

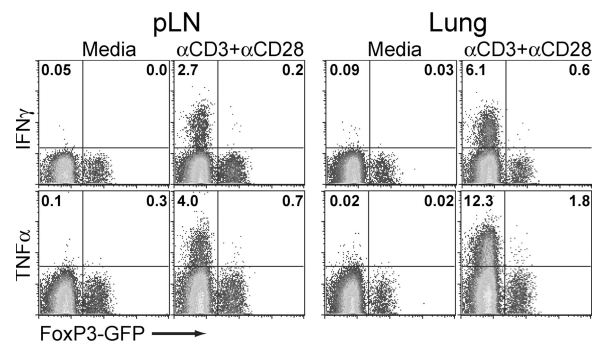


Figure 3. Foxp3⁺ cells do not make inflammatory cytokines after *Mtb* infection. 91 d after infection, cells from the lung and pLNs were stained for IFN- γ and TNF- α after stimulation for 4 h with anti-CD3 and -CD28 or media controls in the presence of monensin. Plots are gated on live CD4⁺ cells. The percentage of Foxp3-GFP-negative or Foxp3-GFP⁺ cells that stain positive for the cytokines are shown. Representative FACS plots are shown for five independent experiments with two to three mice per group.

Foxp3⁺ cells at sites of infection express altered cell surface phenotypes

To determine if the expansion of T reg cells during tuberculosis is associated with a change in their cell surface phenotype, we evaluated the expression of several activation markers. Consistent with previous reports (29), T reg cells in uninfected mice displayed a surface phenotype characteristic of antigen-experienced T cells; most are CD44^{hi}, CD25^{hi}, CD62L⁺, and also express high levels of β 7 integrin and CD103. These markers did not change significantly on the T reg cells that accumulated in the lung and pLN during tuberculosis (unpublished data). Expression of some activation markers and costimulatory molecules, however, was altered after infection. The percentage of Foxp3⁺ CD4⁺ cells expressing inducible costimulatory molecule (ICOS) increased by >50% in the pLNs, but not in the mLNs (Fig. 4, A and B). T reg cells in the lung contained relatively high levels of ICOS, even in uninfected mice, but expression was up-regulated even further during tuberculosis (Figs. 4, A and B). The expression of the inhibitory receptor PD-1 also changed on the regulatory T cell population at late time points during chronic infection. At early time points (<150 d after infection), Foxp3-expressing CD4⁺ T cells did not express significantly higher levels of PD-1 than T reg cells from uninfected age-matched controls (unpublished data). At later time points, however, T reg cells in the pLNs and lung, but not in the T reg cells in the mLNs, expressed higher levels of PD-1 than T reg cells at these same sites in age-matched controls (Fig. 4, A and B). Thus, T reg cells at sites of infection in tuberculosis displayed cell surface phenotypes associated with increased activation.

T reg cell-depleted mice have decreased bacterial burdens in their lungs

The accumulation of T reg cells at primary sites of *Mtb* infection leads to the possibility that they may influence the ability of effector T cells to promote protective immune responses. To determine if Foxp3⁺ cells have the potential to modulate immune responses to *Mtb*, we set up conditions to temporally deplete Foxp3⁺ cells before infection with *Mtb*. As most Foxp3⁺ T reg cells express CD25, depletion with anti-CD25 antibody is a common experimental approach, but depletion is incomplete because not all T reg cells express high levels of CD25⁺, and because some Foxp3-expressing cells survive, but simply down-regulate CD25 after antibody treatment (30, 31). This approach is also problematic because some CD25⁺ effector T cells may also be eliminated by this treatment. These reasons may explain why other groups have not seen a change in the bacterial load in mice that have been depleted of CD25⁺ T cells before (23) (unpublished data; Flynn, J., personal communication) or after (unpublished data; Kornfeld, H., personal communication) infection with *Mtb*.

To facilitate a more complete removal of Foxp3⁺ cells, we generated a mixed bone marrow chimeric system. We reconstituted an irradiated WT C57BL/6-Thy1.1⁺ host with a mixture of WT C57BL/6-Thy1.1⁺ bone marrow and either

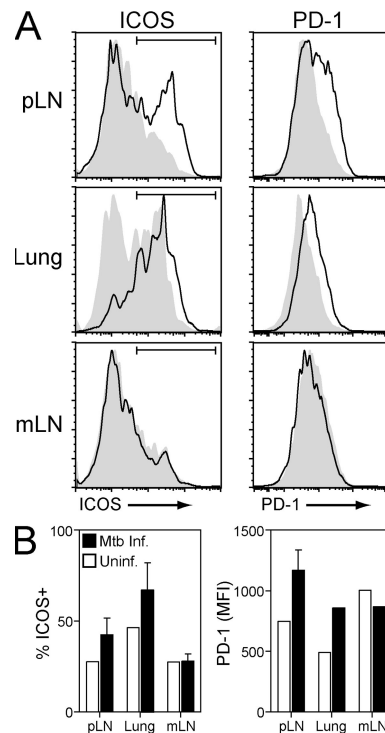


Figure 4. Foxp3⁺ cells up-regulate activation markers at the site of infection. (A) Cells from lung, pLNs, and mLNs of *Mtb*-infected mice (solid line) or uninfected controls (gray area) were stained for CD4 and ICOS (64 d after infection) or PD-1 (242 d after infection). Histogram plots are gated on live CD4⁺FoxP3-GFP⁺ cells. Representative FACS plots are shown for eight independent experiments with two to three mice per group. (B) FACS analysis of ICOS and PD-1 expression among CD4⁺FoxP3⁺ cells from indicated organs of *Mtb*-infected (shaded bars) or uninfected mice (open bars). The percentage of cells expressing ICOS was calculated from the gate indicated on graphs in A. PD-1 mean fluorescence intensity (MFI) was calculated from all CD4⁺ Foxp3⁺ cells. Increased ICOS expression at sites of infection was seen at all time points examined (between 21 and 242 d after infection) in all experiments ($n = 8$). In contrast, increased PD-1 expression was seen in 3 of 3 experiments performed 150 d after infection, but expression was not increased in 2 of 2 experiments at earlier time points.

WT C57BL/6-Thy1.2⁺ (as a control) or Foxp3KO-Thy1.2⁺ bone marrow (Fig. 5 A). After a 10-wk reconstitution, chimeras were bled and the relative ratios of Thy1.1/Thy1.2 expression were compared. As shown in Fig. 5 B, time point 1 (Fig. 5 B, 1), mature T cells expressing either Thy1.1 or Thy1.2 developed at an ~55:45 ratio. 1 wk before infection, mice were treated with anti-Thy1.1 antibody to remove Thy1.1⁺T cells. In both the control (Thy1.1WT/Thy1.2WT) and the experimental (Thy1.1WT/Thy1.2KO) chimeras, the only T cells that remained were Thy1.2⁺ cells. Thus, as shown in Fig. 5 B (2; top), 7 d after depletion, uninfected experimental chimeras were successfully depleted of >95% of the Thy1.1⁺ T cells that could express Foxp3.

Experimental chimeras [KO:WT] and controls [WT:WT] were infected with an aerosol dose of *Mtb* 1 wk after depletion with anti-Thy1.1. After infection, mice were treated

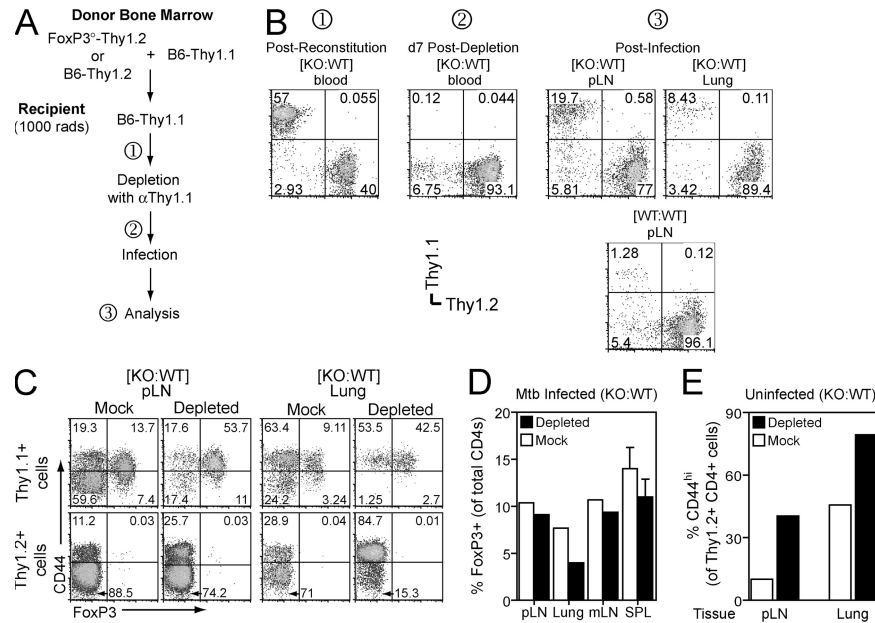


Figure 5. Depletion of T reg cells in bone marrow chimeric mice. (A) Schematic of mixed bone marrow chimeras and depletion of T reg cells. Mixed bone marrow chimeras were generated between Thy1.1⁺ B6 and Thy1.2⁺ Foxp3KO (designated [KO:WT]) or between Thy1.1⁺ B6 and Thy1.2⁺ B6 control (designated [WT:WT]), followed by a 7-d depletion of Thy1.1⁺ T cells, infection with *Mtb* on day 8, and sacrifice 23 d after infection (31 d after depletion). Mice were analyzed at time points indicated by encircled numbers. Chimeras were analyzed in the blood after reconstitution and before depletion with anti-Thy1.1 (time point 1) and 7 d after depletion of Thy1.1⁺ cells (preinfection; time point 2). They were subsequently killed 23 d after infection, and the lungs, pLNs, mLNs, and spleens were analyzed (time point 3). (B) FACS analysis of bone marrow chimeras at various time points, as designated in A. Plots are gated on live, TCRβ⁺ cells from the indicated tissue, and the percentage of total live cells is shown. Data shown are representative plots of three to five mice in each group from two independent experiments. (C) FACS analysis of CD44 and FoxP3 expression from CD4⁺ cells. CD4⁺ cells in lung or pLN from mock or Thy1.1-depleted, *Mtb*-infected, [KO:WT] chimeric mice were compared for CD44 and FoxP3 expression among Thy1.1⁺ and Thy1.2⁺ cells. FACS plots are gated on live CD4⁺ cells and separated into Thy1.1⁺ or Thy1.2⁺ events for analysis. (D) Comparison of frequencies of Foxp3⁺ CD4⁺ T cells in different tissues from *Mtb*-infected mock or Thy1.1-depleted chimeric mice (KO:WT). Data shown are the percentages of Foxp3⁺ cells, as determined by intracellular staining for Foxp3, within the CD4⁺ populations from pooled samples from the lung, pLNs, or mLNs from each group (three to four mice per group), or the mean of three to four spleens per group ± the SD 23 d after infection. (E) Comparison of CD44^{hi}-expressing CD4⁺ cells in pLN and lung from uninfected mock or Thy1.1-depleted chimeric mice (KO:WT). Data shown represent the percentages of CD44^{hi} cells within the Thy1.2⁺ CD4⁺ populations of Thy1.1-depleted (shaded bars) or mock-depleted (open bars) mice.

with weekly injections of anti-Thy1.1 in an attempt to deplete residual or rebounding cells. 23 d after infection, mice were killed and analyzed for T cell function and CFU of *Mtb* in the lungs and spleen. Despite weekly treatment with the depleting antibody, treatment was insufficient to maintain complete depletion in some tissues in the experimental chimeras. In the pLNs of infected mice, ~20% of the T cells expressed Thy1.1 (Fig. 5 B, 3, top left), whereas in the lungs, <10% expressed Thy1.1 (Fig. 5 B, 3, top right). This was true in uninfected experimental chimeras as well (unpublished data). In control [WT:WT] chimeras that were depleted of Thy1.1-expressing cells, the remaining WT Thy1.2⁺ cells could express Foxp3, and the rebound of Thy1.1-expressing cells after depletion was not observed (Fig. 5 B, 3, bottom). A high percentage of the rebounding Thy1.1⁺ CD4⁺ T cells in the [KO:WT] chimeras expressed Foxp3 (45.2% in the lung and 64.7% in the pLNs; Fig. 5 C), suggesting that the homeostatic expansion of Foxp3-expressing cells was one of the factors driving the rebound of Thy1.1-expressing cells in T reg cell-depleted chimeras. In fact, despite the effective elimination of T reg

cells in these mice at the time of infection (Fig. 5 B), the percentage of CD4⁺ cells expressing Foxp3 had almost returned to normal levels at the time of analysis (23 d after infection; Fig. 5 D).

Consistent with the findings in a recent study in which T reg cells were effectively eliminated in genetically manipulated mice (32), we observed signs of robust immune activation in our Thy1.1-depleted chimeras. Even in uninfected mice, LNs in T reg cell-depleted chimeras were 10 times larger than in mock-depleted mice (unpublished data). In addition, fourfold more CD4⁺ T cells in the pLNs, and almost twofold more CD4⁺ T cells in the lungs, expressed high levels of CD44 (Fig. 5 E), suggesting activation of a polyclonal, and possibly autoreactive, population of T cells. These signs of autoimmunity were observed in experimental [KO:WT] chimeras that were depleted of T reg cells, whether or not they were infected with *Mtb*, but not in experimental chimeras that were not depleted, or in control chimeras. Thus, despite the reappearance of Foxp3-expressing cells at the time of analysis (Fig. 5 D), the effective elimination of the

T regulatory population in the experimental chimeras before their infection, and likely for substantial period of time thereafter (even for 2–3 wk), was supported by their development of robust autoimmune activation. We believe that the activated immune response in these mice may explain why some Thy1.1-expressing cells rebounded in the T reg cell-depleted chimeras, but not in the depleted controls, as the elevated immune activation may have facilitated an immune response against the depleting antibody, rendering it ineffective in these animals at later time points.

The bacterial loads were compared between depleted infected experimental chimeras and mock-depleted experimental chimeras. The bacterial load in the spleen showed no statistical difference between the two groups (Fig. 6 A); however, the lungs displayed lower CFU values after depletion of T cells capable of expressing Foxp3 (~ 0.9 log CFU less in experiment 1 and ~ 1.1 log CFU less in experiment 2; Fig. 6 B). The lower CFU in T reg cell-depleted chimeras was correlated with a higher percentage of activated T cells in the lung (Fig. 5 C) with the capacity to rapidly produce IFN- γ in response to polyclonal stimulation ($\sim 17\%$ of CD4 $^{+}$ T cells in the depleted lungs produced IFN- γ after stimulation with PMA and ionomycin, compared with only $\sim 9\%$ in the mock-depleted lungs; Fig. 6 C). However, the number of effector T cells producing IFN- γ in response to the MHC class II-restricted, *Mtb*-specific peptide, ESAT-6 $_{1-20}$ was not increased in either the lung or the pLN (Fig. 6 C and D), probably because the reduced bacterial load resulted in reduced stimulation of T cells specific for this peptide. Overall, these results demonstrate that T reg cells have the capacity to suppress immune responses that control *Mtb*, especially in the lung.

DISCUSSION

It is becoming increasingly clear that Foxp3-expressing T reg cells are not only critical for suppressing immune responses to self-antigens and preventing autoimmunity, but also regulate immunity to foreign antigens, especially derived from pathogens that establish persistent infections, including *L. major*, *H. pylori*, hepatitis C, and HIV (11). We track the activity of Foxp3-expressing T reg cells after aerosol infection of mice with *Mtb*.

The transcription factor Foxp3 is exclusive to TCR- $\alpha\beta^{+}$ in uninfected mice and is expressed primarily by a subset of CD4 $^{+}$ T cells (25); we find this is true even during chronic infection with *Mtb*. We have observed a significant accumulation of T reg cells within the lungs and pLNs that parallels the accumulation of nonregulatory CD4 $^{+}$ T cells at these sites of infection. We have shown that some of this accumulation is caused by increased proliferation, as T reg cells in the pLNs of infected mice show an increased level of BrdU incorporation compared with cells in distal sites. During persistent infection, T reg cells continue to accumulate at these infected sites at a rate that remains similar to the rate of non-T reg cells. Therefore, although the absolute number of T reg cells increases dramatically at these sites, their percentage among the CD4 $^{+}$ T cells does not change significantly. This differs

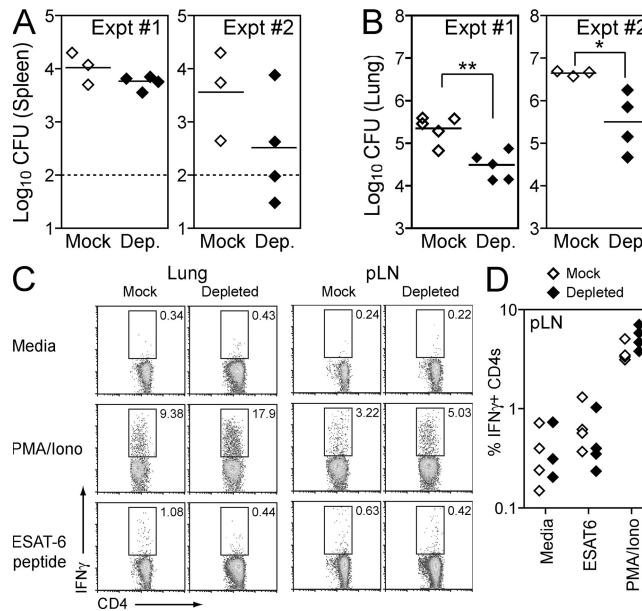


Figure 6. Reduced *Mtb* CFU in lungs of T reg cell-depleted mice.

(A and B) CFU analysis was performed 23 d after infection in Thy1.1-depleted (filled diamonds) or mock-depleted (open diamonds) mice in the spleen (A) or lung (B), as indicated. CFU counts from each organ were determined from 10-fold serial dilutions, and the numbers of viable bacteria are shown for each mouse from two independent experiments (**, $P < 0.01$; *, $P < 0.05$). The limit of detection for these experiments was 100 CFU per organ and is indicated on the graph by the dotted line. (C) Analysis of IFN- γ production by CD4 $^{+}$ cells from lungs or pLNs from mock- or depleted [KO:WT] chimeras. Data represent analysis of pooled lung cells from all mice in each group, or, in the case of pLNs, from individual mice, representative of each group. Percentage of IFN- γ -producing cells (gated on CD4 $^{+}$ cells) is shown after in vitro restimulation with media alone, PMA/ionomycin, or ESAT6 $_{1-20}$ peptide. (D) IFN- γ production by CD4 $^{+}$ cells in the pLN. The percentage of pLN CD4 $^{+}$ T cells producing IFN- γ after in vitro restimulation with media alone, ESAT6 $_{1-20}$ peptide, or PMA/ionomycin. Using the same gates shown in C, data from each individual mouse 23 d after infection from Thy1.1-depleted (filled diamonds) or mock-depleted (open diamonds) mice are represented.

from the situation during persistent infection with *L. major*, where the percentage of T reg cells also increases dramatically in the LNs, draining the site of infection (12). Nevertheless, the accumulation of T reg cells within pulmonary granulomas during tuberculosis raises the possibility that these cells play a role in suppressing immune responses at this site. A recent study shows that signals mediated through DC-SIGN, which is an important receptor for *Mtb* on macrophages and dendritic cells (33, 34), promote the differentiation of dendritic cells into a tolerogenic phenotype and may help explain the expansion of T reg cells during tuberculosis (35).

In addition to displaying increased proliferation, T reg cells at the site of *Mtb* infection (but not distal to the site of infection) change their cell surface phenotype, providing further evidence for the activation of these cells during tuberculosis. The cell surface molecule that is consistently up-regulated at all time points after infection is ICOS, which is a costimulatory

molecule that is highly expressed on activated T reg cells and has been implicated in their suppressive activity (36). A second molecule that is dramatically up-regulated on T reg cells in the lung and pLNs at very late time points (but not earlier time points) after infection is PD-1. PD-1 is an inhibitory molecule that is expressed at high levels on effector T cells that have been “exhausted” by chronic stimulation during persistent infection with lymphocytic choriomeningitis virus. Interestingly, blocking PD-1-mediated signals restores the function of these effector T cells and allows them to clear the viral infection (37). In tuberculosis, PD-1 appears to be most up-regulated on T reg cells at late time points (>150 d after infection), and is less up-regulated on effector T cells. It is unclear whether PD-1 expression on T reg cells would inhibit or augment T reg function, or what effect blockade of PD-1-mediated signals would have on *Mtb*-infected mice during the late chronic stage of persistent infection.

The dramatic accumulation of Foxp3⁺ cells, particularly at infected sites, begs the question of whether these cells have the potential to limit the ability of the immune system to respond effectively and control the infection. Upon depletion of Foxp3⁺ cells, we observed that the bacterial burden in the lungs was reduced by ~10-fold, whereas the bacterial burden in the spleen did not significantly change with depletion. This result is surprising because it is often easier to reduce the bacterial burden in the spleen by immunization than to reduce the bacterial burden in the lung (38, 39). It is possible that part of this difference may be attributable to a slightly more robust rebound of Foxp3-expressing cells in the spleen compared with the lung at the time of analysis (Fig. 5 D). It is tempting to speculate, however, that another contributing factor may be the relative importance of T reg cells in modulating immunity, specifically in the lung. In support of this idea, the lung has been shown to harbor subsets of tolerogenic dendritic cells that may prime T reg cells and suppress pulmonary inflammation (40–46).

The level of reduction of the bacterial burden in T reg cell-depleted, naive mice (~1 log) is similar to the reduction that is achieved in mice that have been immunized by BCG or other comparable vaccines (2). In our system, we achieved near-complete elimination of all cells that could potentially express Foxp3 and observed signs of profound immune activation, including lymphadenopathy and activation of the majority of T cells, mimicking the phenotype seen in Foxp3-null animals (26, 47) or adult animals in another system in which T reg cells were efficiently eliminated (32). Although we observed a rebound of some T reg cells in depleted chimeras when we analyzed these mice 23 d after infection, it is likely that the reappearance of these T reg cells allowed us to perform the experiment by keeping the animals alive, as adult mice that are efficiently and persistently depleted of T reg cells have recently been shown to die of autoimmunity in 2 wk (32). The polyclonal activation of effector T cells at the onset of infection with *Mtb* could have induced a non-specific activation of macrophages, resulting in reduction of bacterial burden. Thus, the specificity of the T reg cell

response during tuberculosis remains an important question that needs to be addressed. Nevertheless, this reduction in the bacterial load after T reg cell depletion suggests that Foxp3⁺ cells have the capacity to suppress immune responses that control *Mtb*.

In our experiments, T reg-depleted mice infected with *Mtb* exhibited a lower bacterial load in the lungs, but had similar, or fewer, numbers of pathogen-specific effector T cells. This differs from reports using other pathogens in which T reg-depleted mice contained more pathogen-specific cells (11). We believe this difference may be explained by the relative abundance of pathogen-derived antigens in different infections. During tuberculosis, compared with other infections, the slow rate of antigen presentation seems to profoundly limit T cell proliferation, and the degree of T cell expansion is highly correlated with the bacterial load. Whereas the secondary T cell response to most infections is higher than the primary response, despite better control of the infection (48, 49), this is not the case for *Mtb*. During tuberculosis, memory mice mount a smaller secondary T cell response than do mice responding to *Mtb* for the first time because they halt bacterial growth sooner and have a lower bacterial load (5). Thus, it is possible that T reg cell-depleted mice infected with *Mtb* would have relatively high numbers of pathogen-specific T effector cells if they were compared with mice with normal numbers of T reg cells and the same bacterial load. In support of this idea, Quinn et al. (23) have shown that anti-CD25-treated mice that have a less effectively eliminated T reg cell population and no change in their bacterial burden display an enhanced IFN- γ response to *Mtb* antigens.

A recent work shows that the vast majority of Foxp3⁺ T reg cells at the site of infection in mice persistently infected with *L. major* are specific for the parasite (14). Furthermore, the study shows that adoptive transfer of these parasite-specific T reg cells confers the recipient with a profound inability to control the pathogen. It will be very important to determine if the T reg cells responding during tuberculosis are similarly pathogen-specific, and furthermore, if pathogen-specific T reg cells are critical for controlling immunity. Although most T reg cells were not observed to be directly interacting with infected macrophages (Fig. S1), it is still possible that they recognize *Mtb*-derived antigens presented by MHC class II molecules on other antigen-presenting cells or that occasional interactions with infected cells in the lung are sufficient for their activity. If most T reg cells that respond during tuberculosis are not pathogen-specific, but simply expand in response to inflammation and tissue damage, then manipulating T reg cells function will probably hold little potential for preventing or treating tuberculosis because suppressing T reg function would probably result in autoimmunity. However, if the T reg cells that are most important for controlling immune responses during tuberculosis are specific for *Mtb*, it may be possible to achieve some beneficial outcomes by circumventing these pathogen-specific T reg cells, especially during immunization.

BCG, which is an attenuated strain of *Mycobacterium bovis*, is closely related to *Mtb*, and is widely used globally to try to prevent tuberculosis. However, its efficacy varies greatly in different studies. Interestingly, although BCG seems to have some efficacy in regions of the world with colder climates, it is largely ineffective in tropical regions that have high levels of exposure to environmental mycobacteria (6). Like *Mtb*, *M. bovis*, including *M. bovis* BCG, has evolved to induce a “limited” immune response in hosts, which facilitates its persistence, and BCG can persist in vaccinated hosts for months or years (50–52). Because BCG is known to share many antigens with *Mtb*, and also with environmental mycobacteria, it will be important to determine the extent to which BCG and environmental mycobacteria may prime and maintain populations of T cells that may cross react with *Mtb*, including T reg cells, and control immunity against tuberculosis. If this is found to be true, devising strategies to overcome or avoid T reg cell-mediated suppression may be critical for the design of an effective vaccine against tuberculosis.

MATERIALS AND METHODS

Mice. Age-matched C57BL/6 or C57BL/6.PL mice were purchased from The Jackson Laboratory. FoxP3^o (backcrossed six generations to C57BL/6) and FoxP3-GFP (maintained by intercrossing C57BL/6xJ129 F1 animals) mice were previously described (25, 26) and maintained under specific pathogen-free conditions at the University of Washington (Seattle, WA). All experiments were performed in compliance with the University of Washington Institutional Animal Care and Use Committee.

Bacteria and aerosol infections. A stock of *Mtb* strain H37Rv was sonicated before use, and mice were infected in an aerosol infection chamber (Glas-Col) with ~50–100 CFU deposited in the lungs of each mouse. In some experiments, a recombinant strain of H37Rv (provided by L. Ramakrishnan, University of Washington, Seattle, WA) that expressed RFP (dsRed2; CLONTECH Laboratories, Inc.) under the control of the constitutive msp12 promoter (53, 54) was used. Mice were killed on day 1 after infection to determine the infectious dose in each experiment.

To determine viable numbers of CFUs, the spleen and one lobe of the lung were homogenized in PBS + 0.05% Tween-80. 10-fold serial dilutions were made in PBS + 0.05% Tween-80 and plated on Mitchison 7H10 plates. Colonies were counted after 21 d of incubation at 37°C, and CFUs per organ were determined.

Cell surface staining. Red blood cell-depleted, single-cell suspensions of intraparenchymal lymphocytes (prepared by collagenase digestion of perfused lungs), and cells from spleens and LNs were prepared as previously described (55). Fc receptors were blocked with anti-CD16/32 (clone 2.4G2). Cells were stained at saturating conditions using antibodies specific for CD4 (clone RM4-5), CD8 (clone 53-6.7), NK1.1 (clone PK-136), TCR β (clone H37-597), TCR $\gamma\delta$ (clone GL3), CD11b (clone M1/70), CD11c (clone HL3), CD25 (clone PC61), CD44 (clone IM7), CD62L (clone MEL-14), β 7 integrin (clone M293), CD103 (clone M280), ICOS (clone 7E.17G9), PD-1 (clone J43), Thy1.1 (clone HIS51), and Thy1.2 (clone 53-2.1) obtained from BD Biosciences. Samples were fixed in a 2% paraformaldehyde solution in PBS for at least 1 h, acquired on a FACSCalibur or FACSCanto (BD Biosciences), and analyzed using FlowJo software (Tree Star, Inc.).

Intracellular staining. 96-well plates were coated with 50 μ g/ml anti-hamster IgG (clone G94-90.5; BD Biosciences) overnight at 4°C, washed three times with PBS, and coated for 1 h at 37°C with 1 μ g/ml anti-CD3 (clone 145-2C11; BD Biosciences). Intracellular IFN- γ staining was performed using a kit as instructed by the manufacturer (BD Biosciences), with

a few modifications. In brief, lung or LN cells were stimulated with plate-bound, anti-CD3 and soluble anti-CD28 (1 μ g/ml; clone 37.51; BD Biosciences) for 4 h in complete RPMI (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 10 mM Hepes, 0.5 μ M 2-ME, 100 U/ml penicillin, and 100 μ g/ml streptomycin) in the presence of monensin. Cells were washed, stained with anti-CD4, and fixed in 2% paraformaldehyde for at least 1 h to kill the *Mtb* before removing the cells from the biohazard level 3 facility. Cells were subsequently permeabilized and stained with anti-IFN- γ -allophycocyanin (clone XMG1.2; BD Biosciences) and anti-TNF- α -PE (clone MP6-XT22; BD Biosciences). In some experiments, cells were permeabilized and stained for intracellular Foxp3 using anti-Foxp3-APC and an intracellular staining kit according to the instructions provided by the manufacturer (eBioscience). Stained cells were acquired on a FACSCanto and analyzed using FlowJo software.

BrdU incorporation. *Mtb*-infected and uninfected Foxp3-GFP mice were given sterile drinking water containing 0.8 mg/ml BrdU (Sigma-Aldrich) that was made fresh and changed daily for 14 d beginning 21 d after aerosol infection with *Mtb*. Single-cell suspensions of lymphocytes from lungs or LNs (pulmonary or mesenteric) were stained for surface markers, followed by intracellular BrdU staining using the BrdU Flow kit (BD Biosciences) according to the manufacturer's instructions. Stained cells were acquired on a FACSCanto and analyzed using FlowJo Software.

Immunohistochemistry. Lung tissue was frozen in Cryo-Gel embedding medium (Instrumedics, Inc.), cut into 8–10- μ m sections, mounted on slides using the CryoJane Tape Transfer System (Instrumedics, Inc.), and fixed by immersion in cold acetone (–20°C) for 20 min. Slides were rinsed with PBS and blocked according to manufacturer's instructions with an avidin/biotin blocking reagent kit (Vector Laboratories) in PBS buffer also containing 1% BSA, 0.05% Tween-80, and 5% normal goat serum. After washing the slides in PBS, primary antibodies (including polyclonal rabbit anti-Foxp3 (56), nonspecific, polyclonal rabbit IgG [Millipore], anti-E-cadherin rat monoclonal antibody, ECCD-2 (57), and biotinylated anti-CD4 [clone RM4-5; BD Biosciences]) were diluted in PBS containing 1% BSA and 0.05% Tween-80 for 1 h. Slides were washed in PBS and a secondary cocktail containing FITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich) and either Alexa Fluor 546-conjugated streptavidin (Invitrogen) or Alexa Fluor 546-conjugated goat anti-rat IgG (Invitrogen) diluted in a solution containing PBS, 1% BSA, 0.05% Tween-80, and 5% normal goat serum was applied for 30 min. Sections were washed with PBS, and slides were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). In some experiments, serial sections were mounted on different slides and one slide was processed for immunohistochemistry as described in this paragraph, and after acetone fixation, the other slide was stained with hematoxylin and eosin. Images were acquired using a camera (Eclipse E600; Nikon) equipped with a 40 \times /NA 0.90 objective lens (Nikon) and Retiga EX charge-coupled device camera (QImaging). Images were analyzed using Photoshop 7.0 software (Adobe).

Generation of bone marrow chimeras. Bone marrow was harvested from C57BL/6, FoxP3^o, and C57BL/6.PL mice. Single-cell suspensions were made and red blood cells were lysed as previously described (55). Bone marrow cells were depleted of T cells using anti-CD3 ϵ -biotin (clone 145-2C11; BD Biosciences), anti-biotin MACS beads (Miltenyi Biotec), and AutoMACS separation (Miltenyi Biotec) according to manufacturer's instructions. Recipient C57BL/6.PL mice were irradiated with 1,000 rads using a cesium-source and reconstituted with 2×10^6 T cell-depleted bone marrow cells at a 1:4 ratio from C57BL/6.PL and C57BL/6 or FoxP3^o. 10 wk after bone marrow reconstitution, the chimerism of the mice was confirmed by bleeding the mice and cell surface staining their peripheral blood cells with anti-CD4, -CD8, -Thy1.1, and -Thy1.2.

Depletion with anti-Thy1.1. Anti-Thy1.1 antibody producing hybridoma (clone 19E12, mouse IgG2b) was grown in serum-free media (HB101;

Irvine Scientific). Antibody was purified via protein A binding (Pierce Biotechnology), exchanged into PBS, and sterile filtered. Chimeric mice were depleted of Thy1.1-expressing cells beginning 10 wk after reconstitution. For experimental groups, mice were injected with 500, 250, and 250 μ g of antibody 7, 4, and 1 d before infection, respectively, followed by infection on day 0. Depletion was maintained with weekly IP injections of 250 μ g of antibody over the course of infection. Control mice were mock-depleted with equivalent doses of mouse IgG2b (The Jackson Laboratory).

Online supplemental material. Fig. S1 shows the relative location of Foxp3-expressing T reg cells to tuberculous bacilli within granulomas in the lung.

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