Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with *Mycobacterium tuberculosis*

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Infection usually leads to the development of acquired immune responses associated with clearance or control of the infecting organism. However, if not adequately regulated, immune-mediated pathology can result. Tuberculosis is a worldwide threat, and development of an effective vaccine requires that the protective immune response to *Mycobacterium tuberculosis* (Mtb) be dissected from the pathological immune response. This distinction is particularly important if new vaccines are to be delivered to Mtb-exposed individuals, as repeated antigenic exposure can lead to pathological complications. Using a model wherein mice are vaccinated with bacille Calmette-Guérin after Mtb infection, we show that repeated vaccination results in increased IL-17, tumor necrosis factor, IL-6, and MIP-2 expression, influx of granulocytes/neutrophils, and lung tissue damage. This pathological response is abrogated in mice deficient in the gene encoding IL-23p19 or in the presence of IL-17–blocking antibody. This finding that repeated exposure to mycobacterial antigen promotes enhanced IL-17–dependent pathological consequences has important implications for the design of effective vaccines against Mtb.

Abbreviations used: BCG, bacille Calmette-Guérin; DLN, draining LN; Mtb, *Mycobacterium tuberculosis*; TB, tuberculosis.

Tuberculosis (TB) remains one of the main threats to mankind. Despite the efforts of the scientific community toward understanding this disease, ~9 million active TB cases and 2 million deaths occur every year (Dye et al., 1999; Dye, 2006). Although immune cell activation is required to limit the growth of *Mycobacterium tuberculosis* (Mtb), if uncontrolled it can damage host tissue. The balance between protection and pathological consequences is the crux of TB pathogenesis. It is therefore crucial to understand and discriminate the components of the immune response that are protective from those that are damaging to effectively intervene in TB. Indeed, the hope for improved vaccination relies on the possibility that these responses can be independently manipulated.

The discrimination between protective and pathological components of the immune response during TB is particularly important because *M. bovis* bacille Calmette-Guérin (BCG), currently the only available vaccine against TB, has variable efficacy (ranging from 0 to 80%; Colditz et al., 1994) and its protection may last for only 10 yr (Sterne et al., 1998; Weir et al., 2008). As a result of this apparent loss of activity, revaccination with BCG, DNA vaccines, or subunit vaccines is considered as a potential control strategy; however, the safety of this strategy in a highly exposed population is not yet fully defined. The issue of safety arises because of the fact that repeated exposure of Mtb-infected animals to mycobacterial antigens can initiate immune-mediated pathology. Specifically, the exposure of Mtb-infected guinea pigs to either live mycobacteria or mycobacterial antigens results in necrotic inflammation at the site of injection.

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challenge, a response that has been termed the Koch phenomenon (Koch, 1891). Although the cellular and molecular determinants of this response are undefined, it appears that the severity of the Koch phenomenon depends on the dose of antigen, as lower doses of antigen are used to induce a delayed-type hypersensitivity response; this is the basis for the current skin test for detecting latently infected individuals (Rich, 1944). A damaging focal response at the site of initial infection can also be triggered by repeated vaccine (BCG or DNA) challenge of Mtb-infected animals. This leads to the development of severe pathology, including necrosis and increased granulocyte influx in preexisting lesions in the lung (Turner et al., 2000; Moreira et al., 2002; Taylor et al., 2003). The perceived risk of increased pathological consequences as a result of vaccination in previously exposed humans has lead to initial safety screens being performed on novel vaccine candidates (Sander et al., 2009). However, results from these types of studies should be interpreted cautiously, as the extent of repeated antigen exposure will differ greatly depending on the level of disease in the community. Therefore, there is a concern that adult postexposure vaccination to prevent the reactivation of TB could lead to pathology, particularly in highly exposed populations.

The importance of the cytokine IFN-γ in the protective response to Mtb is well established (North and Young, 2004); however, the mediator of pathological responses has not been identified. The Koch-like pathologies described in the previous paragraph were consistently associated with neutrophil influx and could therefore be mediated by IL-17 (Miyamoto et al., 2003; Kolls and Linden, 2004). Further, although IL-17 is induced during mycobacterial infection, it does not play a significant role during the early period of infection (up to 100 d; Khader and Cooper, 2008). In addition, IFN-γ is able to regulate the IL-17 response during BCG infection (Cruz et al., 2006), and in the absence of IFN-γ signaling in the stroma, an increase in neutrophil involvement in the TB granuloma is seen (DesVignes and Ernst, 2009); these data reflect an important regulatory activity for IFN-γ in the control of pathology in TB. Therefore, we hypothesized that repeated antigen exposure would allow the IL-17 response to overcome the IFN-γ-mediated regulation and thereby mediate immunopathological consequences, and that the Koch phenomenon may result from an unregulated IL-17 response.

To test our hypothesis and investigate the mechanisms underlying the pathogenic response to repeated antigen exposure, we examined the pathological cellular response in an established mouse model of repeated antigen exposure that leads to increased pathology (Turner et al., 2000; Moreira et al., 2002; Taylor et al., 2003). Specifically, Mtb-infected mice were repeatedly vaccinated subcutaneously with BCG to promote an immunopathologic response. We found that enhanced pathology was associated with a dramatic increase in the number of antigen-specific IL-17–producing cells in the lungs of infected and revaccinated animals. Importantly, in the absence of IL-23 or in the presence of anti–IL-17 antibody, the enhanced pathological response was ablated, along with the granulocyte/neutrophil infiltrate. These results support a pathogenic role of IL-17–producing antigen-specific cells during TB. Thus, the development of Th17 responses after vaccination and their impact on chronic infection should be considered.

RESULTS AND DISCUSSION
Repeated exposure to BCG after Mtb infection results in a significant increase in pathological inflammation in the lungs of Mtb-infected mice
To dissect protective from pathological responses to Mtb we chose to use a previously described model of accelerated host damage wherein Mtb-infected mice receive repeated BCG vaccination (Turner et al., 2000). C57BL/6 (B6) mice were infected with Mtb and repeatedly injected subcutaneously with either saline or BCG. Control mice that were not Mtb-infected underwent the repeated BCG vaccination protocol alone. Macroscopic examination of the lungs at 90 d after Mtb infection demonstrated that repeated BCG exposure resulted in a larger number of detectable lesions (Fig. 1 a; Turner et al., 2000). Consistent with this observation, microscopic analysis of the lesions indicated that although Mtb-infected mice had small yet defined lesions, mice repeatedly vaccinated with BCG exhibited lesions of increased size (Fig. 1 b). Mice that were not Mtb infected but were vaccinated with BCG three times did not show any signs of inflammation or pathological consequences in the lungs (unpublished data). When the histological sections were scored in a blinded manner according to an inflammatory index, there was a clear and reproducible increase in inflammation between the mice receiving three postinfection vaccinations and those receiving either no vaccination or just one vaccination (Fig. 1 c). As previously described, this difference in inflammation was not associated with differences in bacterial burden in the lungs or spleens over the interval tested (Table S1; Turner et al., 2000), suggesting that although lung lesions were increased in size, the hosts’ ability to contain the bacterial burden was not affected.

To determine whether increased pathology was associated with altered expression of effector molecules within the lung, we compared the relative mRNA expression between the unvaccinated and vaccinated groups. The repeatedly vaccinated infected mice exhibited an increase in MIP-2 (Fig. 1 d), a chemokine associated with granulocyte influx, as well as TNF (Fig. 1 e) and IL-6 (Fig. 1 f), both active inflammatory mediators.

Repeated exposure to BCG after Mtb infection results in a significant increase in the number of antigen-specific IL-17–producing cells
To determine whether the pathological inflammation resulted from a change in the nature of the immune response, we next quantified the expression of T cell effector cytokines within the lungs of infected and vaccinated mice. We found that although expression of the protective cytokine IFN-γ mRNA was not significantly altered (Fig. 2 a), for the potentially damaging
cytokine IL-17 it was significantly increased in the mice vaccinated three times after infection compared with those mice receiving only one or no vaccination (Fig. 2 b). To determine if this IL-17 response was associated with the acquired antigen-specific response to infection, we measured the number of antigen-specific cells making the protective cytokine IFN-γ and the potentially damaging cytokine IL-17 using a peptide-driven ELISPOT. Strikingly, repeated vaccination of Mtb-infected mice with BCG induced a significant increase in the number of antigen-specific IL-17–producing cells as compared with nonvaccinated or singly vaccinated mice (Fig. 2 c). In contrast, the number of IFN-γ–producing antigen-specific cells did not change under the conditions tested (Fig. 2 c).

To determine whether the strong IL-17 response at day 90 in the mice vaccinated three times was simply a result of sampling time relative to the most recent BCG vaccination, we analyzed the antigen-specific IFN-γ and IL-17 responses at 45 and 60 d after both a second (Fig. 2 d) and a third (Fig. 2 e) vaccination. We found that in both cases there was an increase in the number of antigen-specific IL-17–producing cells at day 90 in the mice vaccinated three times after infection compared with those mice receiving only one or no vaccination (Fig. 2 b). To determine if this IL-17 response was associated with the acquired antigen-specific response to infection, we measured the number of antigen-specific cells making the protective cytokine IFN-γ and the potentially damaging cytokine IL-17 using a peptide-driven ELISPOT. Strikingly, repeated vaccination of Mtb-infected mice with BCG induced a significant increase in the number of antigen-specific IL-17–producing cells as compared with nonvaccinated or singly vaccinated mice (Fig. 2 c). In contrast, the number of IFN-γ–producing antigen-specific cells did not change under the conditions tested (Fig. 2 c).

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day 45 after vaccination; however, the increased IL-17 response was only sustained to day 60 after vaccination, when animals were challenged three times with BCG (Fig. 2 e). Consistent with the sustained antigen–specific IL-17 response, we observed an increased expression of iL23a in the lungs of the infected mice receiving three BCG vaccinations (Fig. 2 f).

We hypothesize that the repeated antigenic challenge results in conditions in the lung that are permissive for the persistence of IL-17–producing cells.

**Granulocyte accumulation within the granuloma depends on IL-23**

We previously demonstrated that the absence of IL-23 modulates the IL-17 and inflammatory response to primary Mtb infection in the lung (Khader et al., 2005). This, together with data showing the role of IL-23 and IL-17 in neutrophil accumulation and function (Stark et al., 2005; Zelante et al., 2007), prompted us to examine the role of IL-23 in the enhanced pathological response to repeated BCG exposure in the Mtb-infected lung using mice lacking the IL-23a subunit (B6.il23a−/−). Although Mtb-infected B6 mice repeatedly exposed to BCG showed a significantly increased granuloma size compared with the unexposed B6 mice, the repeated BCG exposure did not significantly increase the granuloma size in B6.il23a−/− mice relative to the unvaccinated B6.il23a−/− mice (Fig. 3 a).

Because IL-17 and IL-23 have been implicated in neutrophil recruitment and function and we detected an increase in MIP-2 in the B6 mice (Fig. 1 e), we reasoned that neutrophils may be involved in the pathology induced by BCG reexposure. To investigate this, we assessed the number of GR1+ cells (likely neutrophils) by immunohistochemistry present in the lung lesions. There is a modest level of neutrophil accumulation in the nonvaccinated infected B6 mice (Fig. 3, b and c, green cells), but as we have shown previously this was not dependent on IL-23 (Khader et al., 2005). Importantly, we found that the number of GR1+ cells within the granuloma of BCG-vaccinated Mtb-infected B6 mice was increased when mice were repeatedly vaccinated (Fig. 3, b and c). In contrast to the unvaccinated mice, however, it was clear that this increase in GR1+ cell accumulation and pathology was dependent on the presence of IL-23 (Fig. 3, b and c). In fact, in the absence of IL-23, the number of GR1+ cells actually dropped after vaccination (Fig. 3 b). As we have previously shown that IL-17 responses are regulated by IFN-γ in BCG infections (Cruz et al., 2006), we wanted to determine the potential for the relative levels of IFN-γ– and IL-17–producing cells to be linked to the accumulation of GR1+ cells and pathology; to do this, we calculated the ratios of IFN-γ– to IL-17–producing antigen-specific cells in the lungs by ELISPOT. We found that IL-17–producing cells specific for Ag85 were increased upon vaccination (not depicted for this experiment but similar to that depicted in Fig. 2 c), and that the ratio of IFN-γ– to IL-17–producing cells was significantly different between B6 and B6.il23a−/− mice but not significantly different between vaccinated and unvaccinated mice day 45 after vaccination; however, the increased IL-17 response was only sustained to day 60 after vaccination, when animals were challenged three times with BCG (Fig. 2 e).

Figure 3. **IL-23 is required for the increased immunopathologic response after repeated BCG exposure in Mtb-infected mice.** B6 and B6.il23a−/− mice were infected with Mtb and either left unvaccinated (Mtb; black bars) or vaccinated two (Mtb+2BCG; white bars) or three times (Mtb+3BCG; gray bars) after infection with BCG. (a) Morphometric analysis of the size of inflammatory lesions in the lungs of treated mice. (b) Number of GR1+ cells within lung lesions of B6 or B6.il23a−/− Mtb-infected mice. (c) Immunohistology of GR1+ cells (green signal indicates GR1 stain, red signal indicates activated macrophages, and blue signal indicates cell nuclei) in B6 or il23a−/− Mtb-infected mice either left unvaccinated (top) or vaccinated three times (middle, B6; bottom, il23a−/−) after infection with BCG. Bars, 200 µm. (d) Cells from the lungs of treated mice were restimulated in vitro with Ag85 peptide, IFN-γ– or IL-17–producing cells were measured by ELISPOT, and the ratio of IFN-γ– to IL-17–producing cells was calculated. (e and f) Cells were isolated from the DLNs of B6 or B6.il23a−/− mice at the site of vaccination after two (white bars) or three (gray bars) vaccinations, and the number of IFN-γ– (e) or IL-17–producing cells (f) was determined by ELISPOT. In a and b and d–f, data points represent mean values (n = 4 mice per group). *, P < 0.05; **, P < 0.01; and ***, P < 0.001, determined as described in Materials and methods. For all panels, one representative experiment out of two total is shown.
Brief Definitive Report

In this report we reveal the immune mechanisms leading to pathology caused by reexposure of Mtb-infected animals to BCG vaccination. We show that enhanced pathological consequences are associated with an IL-17–dominated response, with increased production of inflammatory cytokines, recruitment of granulocytes/neutrophils, and increased tissue involvement. This enhanced pathology and inflammatory infiltrate is completely dependent on the cytokines IL-23 and IL-17. This is a critical and novel observation, as the mediators of the pathogenic cellular response in TB have not been previously described. This observation is also critical for rational vaccine development and delivery of immune-mediated intervention vaccines to control this disease. Indeed, this study has important implications that should be considered in the design of new vaccines or vaccination protocols.

Based on our results, one possible suggestion would be to limit the induction of memory T cells capable of generating an IL-17 response, as this would limit immunopathogenesis. However, we have previously demonstrated that IL-17 production is required during the recall response to Mtb challenge in the mouse model for an accelerated expression of protection (Fig. 3 d). In a preliminary assessment of the impact of repeated vaccination and the presence of IL-23 on the induction of T cells, we compared the number of cells producing IFN-γ (Fig. 3 e) or IL-17 (Fig. 3 f) in the draining LNs (DLNs) of the vaccine sites. We found that there was not a significant difference in the number of cytokine-producing cells between mice vaccinated two or three times, and much as we have seen previously (Khader et al., 2007), the absence of IL-23 does not significantly impact early induction of IL-17 responses (Fig. 3 f). Collectively, our data show that vaccine-induced increased immunopathology is associated with IL-17 (Fig. 2, b and c) and dependent on il23a (Fig. 3). We also show that an exacerbated recruitment of GR1+ cells (likely neutrophils) is associated with the pathology caused by BCG reexposure of Mtb-infected mice (Fig. 3, b and c). Further, although an increased presence of IL-17–producing T cells is associated with increased pathology, it does not appear to be associated with an altered ratio of IFN-γ to IL-17–producing cells (Fig. 3 d). Finally, the observation that expression of il23a is increased in the lungs of mice vaccinated three times (Fig. 2 f) suggests that availability of IL-23 in the lung may be responsible for the persistent IL-17 response in these mice.

Neutralization of IL-17 improves the pathology associated with BCG revaccination

Because IL-23 is associated with the development and maintenance of IL-17 responses in Mtb infection (Khader et al., 2005), we tested whether the response to BCG vaccination was dependent on the excessive IL-17 production in the triple-vaccinated Mtb-infected mice. To do this, we treated these mice with anti–IL-17 antibody starting on day 43 after Mtb-infection (2 d before the third BCG vaccination) until day 90. Importantly, neutralization of IL-17 reduced the inflammation induced by revaccination (Fig. 4 a), as well as the granuloma size (Fig. 4 b) and the number of GR1+ cells in the granuloma (Fig. 4 c) and within the lung (Fig. 4 d). The anti–IL-17 treatment also significantly reduced the level of the neutrophil-recruiting cytokine MIP-2 in the lungs of the triple-vaccinated mice (Fig. 4 e). Therefore, it appears that the increase in granuloma size and neutrophil influx resulting from repeated antigen exposure are dependent both on IL-23 and IL-17.

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Figure 4. IL-17 is required for the increased immunopathologic response after repeated BCG exposure in Mtb-infected mice. B6 mice were infected with Mtb and then either left unvaccinated (Mtb) or vaccinated with BCG three times and either treated with isotype control antibody (Mtb+3BCG+Iso) or anti–IL-17 antibody (Mtb+3BCG+antiIL17). (a) Histological images of lung lesions in Mtb-infected mice either left unvaccinated (left) or vaccinated with BCG three times and either treated with isotype control antibody (middle) or anti–IL-17 antibody (right). Bars, 50 µm. (b–d) Morphometric quantification of lesion size (b), number of GR1+ cells within lung lesions (c), and number of GR1+ cells in total lung measured by flow cytometry (d) in Mtb-infected mice either left unvaccinated (black bars) or vaccinated with BCG three times and either treated with isotype control antibody (gray bars) or anti–IL-17 antibody (white bars). (e) Total mRNA was extracted from the lungs of mice treated as in panel a, and the expression of MIP-2 mRNA was assessed by real-time PCR normalized to HPRT. In b–e, data points represent mean values (n = 4 animals per group), and for all panels one representative experiment out of two total is shown. *, P < 0.05; **, P < 0.01; and ***, P < 0.001, determined as described in Materials and methods.
IL-17 is altered in favor of IL-17. In addition to a direct role in (Khader et al., 2005). IL-17 response caused by repeated exposure depends on IL-23 (McGeachy and Cua, 2008). The functional impact of these induced T cells also depends on the expression of stimulatory and inhibitory cytokines at the site of infection/inflammation. Our data suggest that it is not altered induction of Th1 and Th17 cells in the DLNs but rather increased presence of IL-23 at the site of inflammation that supports the persistence of Th17 cells after three vaccinations. It is possible that increased apoptosis at the site of inflammation results in conditions suitable for induction of IL-17–producing cells (Torchinsky et al., 2009), and that the triple vaccination induces cells capable of inducing this apoptosis. That the absence of il23a results in the loss of the immunopathologic consequences of repeated vaccination suggests that, as for the nonpathogenic IL-17 response to mycobacterial infection, the enhanced IL-17 response caused by repeated exposure depends on IL-23 (Khader et al., 2005).

Our data correlating the presence of IL-17 and MIP-2 during the enhanced pathological response suggest that, as in the LPS-induced lung inflammation model (Miyamoto et al., 2003), IL-17 drives MIP-2 that recruits neutrophils to the tissue. The association between IL-17 and the frequency of neutrophils within the lung lesions of the repeatedly vaccinated mice suggests that granulocyte accumulation is a consequence of enhanced IL-17 activity in the Mtb-infected lung. Interestingly the absence of IL-17 during pulmonary mycobacterial infection only modestly alters the early inflammatory response (Khader et al., 2005; Umemura et al., 2007). This suggests that IL-17 plays a minor role early in the response, and that the pathogenic role occurs as the balance between IFN-γ and IL-17 is altered in favor of IL-17. In addition to a direct role in recruiting neutrophils, the current data may reflect differences in the nature of neutrophil activation in the presence and absence of IL-17 and IL-23. Specifically, neutrophils can be protective during fungal infection, but when exposed to excess IL-23 or IL-17, their function is altered and they become more able to mediate tissue damage (Zelante et al., 2007).

The role of neutrophils in TB is currently an area of intense investigation. Careful analysis reveals that neutrophils are a dominant site for bacterial presence in the sputum and bronchoalveolar lavage of patients with active TB, suggesting a role for these cells as permissive hosts (Eum et al., 2010). In addition, rapid accumulation of neutrophils that are permissive for bacterial growth is a dominant feature in genetically susceptible mice (Eruslanov et al., 2005; Keller et al., 2006). Further, recent work has shown that restriction of neutrophil accumulation is dependent on the IFN-γ receptor–dependent activity of indoleamine-2,3-dioxygenase by radio-resistant cells within the lung, which results in increased tryptophan catabolic products that likely act to inhibit IL-17–producing cells in situ (DesVignes and Ernst, 2009). Further, blockade of IL-17 in Mtb-infected mice can limit neutrophil recruitment (Redford et al., 2010). Collectively, these data support a largely negative role for neutrophils in TB pathogenesis, and our data expand these observations by demonstrating that increased pathology is directly dependent on IL-23– and IL-17–mediated neutrophil recruitment.

To generate a host response by vaccination that eliminates the infection, we must continue to define both the protective and pathogenic immune responses to Mtb infection. Otherwise, vaccination may promote detrimental consequences, as we show in this report. In particular, in locations where the disease is highly prevalent, the delivery of a new vaccine to an individual who has been BCG vaccinated and/or has subclinical Mtb infection may result in unexpected tissue damage. The more we know of the natural and vaccine-induced response to Mtb infection, the better placed we will be to improve immunonintervention. In addition, it should be determined whether a high frequency of antigen-specific IL-17–producing cells in patients is indicative of increased lesion size or disruption of granuloma structure.

MATERIALS AND METHODS

Bacteria. The H37Rv strain of Mtb and M. bovis BCG Pasteur were grown in Proskauer-Beck medium containing 0.05% Tween 80 to mid-log phase and frozen at −70°C.

Animals and experimental infection and antibody treatment. 8-wk-old female C57BL/6 (B6) mice were obtained from Charles River or the Jackson Laboratory. IL-23p19–deficient mice (B6.129S2 (Il23a−/−)) were bred at the Trudeau Institute from stock provided by N. Ghilardi and F.J. deSauvage (Genentech, South San Francisco, CA). Mice were either anesthetized with ketamine/medicotamine and injected intranasally with 4 × 10^6 CFU Mtb H37Rv, resulting in a lung dose of ∼10^5 CFU (Figs. 1, 2, and 4), or were infected via the aerosol route (final dose of ∼10^5 CFU) as previously described (Fig. 3; Roberts et al., 2002). Mice were divided into four groups. The first group was only infected with Mtb. The second group was vaccinated subcutaneously with 10^6 BCG 15 d after Mtb infection. The third group received a BCG inoculation at days 15 and 30 after Mtb infection. The fourth group received three doses of BCG at days 15, 30, and 45 after Mtb infection. Control groups received saline instead of Mtb.
and were submitted according to the same protocol as the other groups. In some experiments, mice infected with Mb and vaccinated three times with BCG were treated intraperitoneally with either 100 µg anti–IL-17 mAb (clone S0104; R&D Systems) or isotype control (clone 54447; R&D Systems), starting at day 43 after Mb infection (2 d before the third BCG vaccination) and every 3 d until day 90. Infected mice were killed at 90 d after Mb infection, and the bacterial counts were determined as previously described (Roberts et al., 2002). All animal experiments were approved either by the Trudeau Institute Institutional Animal Care and Use Committee or were performed according to the European Union Directive 86/609/EEC and were previously approved by the National Authority “Direcção Geral de Veterinária.”

Cell preparation and culture. A single-cell suspension was generated from the lungs of experimental mice, and ELISPOTs were performed using an I-Ab–restricted epitope of Ag85A, as previously described (Huygen et al., 1994; Cruz et al., 2006).

Quantitative real-time-PCR analysis. Total RNA from whole lungs was extracted with TRIzol reagent (Invitrogen). Reverse transcription was performed as previously described (Torrado et al., 2010). The cDNA underwent real-time PCR for HPRT, IFN-γ, IL-17, IL-6, TNF, and MIP-2 using a LightCycler (Roche). The specific probes for each cytokine were designed and synthesized by TIB MolBiol and are listed in Table S2. Single acqisition was performed at the end of each annealing step.

Histology and morphometric analysis. Caudal lobes from four mice per group underwent morphometric analysis in a blinded manner using a morphometric tool on a microscope (Axioplan 2; Carl Zeiss, Inc.) that determines the area defined by the squared pixel value for each granuloma. Immunofluorescence was performed on formalin-fixed lung sections as described previously (Khader et al., 2007). In brief, antigens were unmasked and blocked with donkey serum and Fc Block, and endogenous biotin was neutralized. Sections were probed with purified goat anti–iNOS (clone M-19; Santa Cruz Biotechnology, Inc.) and biotinylated rat anti-GR1 (clone RB6-8C5; BD). Binding of the iNOS-specific antibody was detected with a donkey anti–goat antibody conjugated to Alexa Fluor 594 (Invitrogen), and GR1 was visualized by adding streptavidin–Alexa Fluor 488 (Invitrogen). SlowFade Gold antifade with DAPI (Invitrogen) was used to counterstain tissues and to detect nuclei. Images were obtained with an AxioPlan 2 microscope and were recorded with a digital camera (AxioCam; Carl Zeiss, Inc.).

Statistical analysis. The data points represent means ± SD. Statistical significance was calculated by first determining whether the data were normally distributed, ANOVA was performed followed by Tukey’s significance was calculated by first determining whether the data were normally distributed, ANOVA was performed followed by Tukey’s

Online supplemental material. The bacterial burden in the lungs of mice treated as in Fig. 1 is shown in Table S1. The sequences of the PCR primers used in the RT-PCR analysis are shown in Table S2. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100265/DC1.

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## Table S1. Bacterial counts (CFUs) in the lung and spleen 90 d after Mtb infection

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<th>Lung</th>
<th>Spleen</th>
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<tr>
<td></td>
<td>CFU ± SD</td>
<td>CFU ± SD</td>
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<tr>
<td>Mtb</td>
<td>4.2 ± 0.12</td>
<td>3.83 ± 0.77</td>
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<tr>
<td>Mtb + M. bovis BCG (1x)</td>
<td>4.38 ± 0.14</td>
<td>3.44 ± 0.64</td>
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<tr>
<td>Mtb + M. bovis BCG (2x)</td>
<td>4.5 ± 1.13</td>
<td>3.82 ± 0.6</td>
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<td>Mtb + M. bovis BCG (3x)</td>
<td>4.79 ± 0.79</td>
<td>3.86 ± 0.26</td>
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Mice were infected in the lung and either left unvaccinated or subcutaneously vaccinated with BCG one (1x), two (2x), or three (3x) times. Organs from infected mice were harvested and homogenized, diluted, and plated to determine the number of mycobacterial CFUs per organ (n = 4 mice per group from one experiment representative of two). Means were determined to be not statistically different as described in Materials and methods.

## Table S2. Sequences of primers and probes

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<thead>
<tr>
<th>Primer/probe</th>
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<tr>
<td>Sense primer</td>
<td>5’-GCTGGTGA AAAGGACCTCT-3’</td>
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<tr>
<td>Antisense primer</td>
<td>5’-CACAGACTAGAACACCTC-3’</td>
</tr>
<tr>
<td>Probes</td>
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