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.
challenge, a response that has been termed the Koch phenomen- 
on (Koch, 1891). Although the cellular and molecular de-
terminants of this response are undefined, it appears that 
the severity of the Koch phenomenon depends on the dose of an-
tigen, 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) chal-
lenge of Mtb-infected animals. This leads to the development 
of severe pathology, including necrosis and increased granulo-
cyte 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 vacci-
nation 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 reactiva-
tion 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 previ-
ous paragraph were consistently associated with neutrophil in-
flux 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 regu-
late 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 impor-
tant regulatory activity for IFN-γ in the control of pathology
in TB. Therefore, we hypothesized that repeated antigen ex-
posure 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 ex-
posure, 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 anti-
body, the enhanced pathological response was ablated, along

RESULTS AND DISCUSSION
Repetitive 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, micro-
scopic 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 vacci-
nated with BCG three times did not show any signs of inflam-
mmatory 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 inflam-
mation between the mice receiving three postinfection vac-
cinations and those receiving either no vaccination or just
one vaccination (Fig. 1 c). As previously described, this dif-
ference 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 al-
though lung lesions were increased in size, the hosts’ ability
to contain the bacterial burden was not affected.

To determine whether increased pathology was associ-
ated 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 in-
flammatory 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

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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 after Mtb 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 granulomata 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.

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 and 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.
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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.

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](image.png)

**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–d, 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.
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Importantly, the detrimental role of IL-17 during chronic infection is not associated with control of the bacterial burden but in the level of inflammatory involvement of the interstitium. This is not surprising, as the number of antigen-specific IFN-γ–producing cells is not diminished and it would appear that the enhanced IL-17 response does not inhibit the protective response. Similarly, it appears that although IFN-γ can regulate the IL-17 during mycobacterial infection (Cruz et al., 2006), repeated vaccination overcomes the ability of IFN-γ to do this. How then is repeated vaccination changing the immunopathologic response? We see both higher IL-17 mRNA and a higher frequency and number of antigen-specific IL-17–producing cells in the lungs of repeatedly vaccinated mice. The generation of IL-17–producing T cells as opposed to antigen-specific cells of other functional phenotypes depends on the cytokines available during initial activation of naive T cells (McGeachy and Cua, 2008). The functional impact of these induced T cells also then 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 pathologic 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 immunointervention. 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 (B6.129S7-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 and medetomidine and infected intranasally with 4 × 10^6 CFU (Figs. 1, 2, and 4), or were infected via the aerosol route (final dose of ~10^6 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 50104; 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 infected mice. Submitted: 8 February 2010

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 (Axoplan 2; Carl Zeiss, Inc.) that determines the area defined by the squared pixel value for each granuloma. Immunohistochemistry 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 using the D’Agostino-Pearson omnibus normality test. If the data were not normally distributed, a Kruskal-Wallis test was performed followed by Dunn’s multiple comparison test. Means were considered significantly different if P < 0.05.

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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