Combination of Host Susceptibility and Virulence of Mycobacterium tuberculosis Determines Dual Role of Nitric Oxide in the Protection and Control of Inflammation

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Tuberculosis (TB) remains a global health threat. Although it is generally accepted that TB results from intensive cross-talk between the host and the pathogen Mycobacterium tuberculosis, underlying mechanisms remain elusive. The first evidence of human polymorphisms related to susceptibilities to distinct M. tuberculosis lineages has been gathered. Confrontation of limited host resistance with heightened bacterial virulence forms a most hazardous combination. We investigated extreme combinations, confronting inducible nitric oxide synthase–deficient (iNOS−/−) and wild-type (WT) mice with 2 related M. tuberculosis strains that differ markedly in virulence, namely, the M. tuberculosis laboratory strains H37Rv and H37Ra. We provide evidence that deregulated chemokine signaling and excessive neutrophil necrosis contribute to disproportionate neutrophil influx and exacerbated TB in iNOS−/− mice infected with virulent M. tuberculosis (strain H37Rv), whereas resistant and susceptible mice controlled attenuated H37Ra equally well. Thus, a combination of host susceptibility and M. tuberculosis virulence determines the role of iNOS in the protection and control of inflammation.

Tuberculosis (TB) remains a global health threat, with 9 million new cases and up to 2 million deaths annually [1]; however, 90% of infected individuals stay healthy but harbor the pathogen throughout their lives [2]. Accumulating data reveal a phylogenetic tree of Mycobacterium tuberculosis with 6 major lineages of different global distribution [3]. Moreover, evidence for lineage-and strain-specific virulence is increasing [4, 5]. Although genetic aspects of susceptibility to M. tuberculosis in humans are far from understood, genes involved in innate immunity have been identified as decisive factors. These include polymorphisms in the Toll-like receptor 2 signaling machinery [6] and in genes that encode interferon (IFN)–γ and inducible nitric oxide synthase (iNOS) [7]. In consequence, confrontation of gene polymorphisms responsible for reduced resistance of the host by highly virulent M. tuberculosis strains will affect the course, severity, and clinical manifestations of disease [6, 8].

Experimental infection of mice with M. tuberculosis is widely exploited to investigate the pathogenesis of TB [2, 9]. Mutant mouse strains lacking genes encoding IFN–γ [10] or iNOS [11] suffer from exacerbated TB. These mutants fail to control the growth of virulent M. tuberculosis strains in lungs, resulting in death within 2–4 weeks. These in vivo findings are consistent with those of in vitro studies that suggest a central role of iNOS in M. tuberculosis control [12]. Induction of iNOS results in large quantities of reactive nitrogen intermediates (RNIs), which are bactericidal [13]. Although the role of RNIs in the control of human TB was originally questioned, compelling evidence for a critical role of iNOS and RNIs has been gathered more recently. Thus, iNOS
inhibition in human alveolar macrophages causes reduced antimycobacterial activity [14], patients with active TB exhale abundant nitric oxide [15], and iNOS-producing cells are detected in biopsy specimens of granulomas [16].

The virulent M. tuberculosis laboratory strain H37Rv and the attenuated strain H37Ra were isolated in 1934 from the H37 strain, which had been derived from a clinical isolate in 1905 [17]. Recently, mutation of transcriptional regulator PhoP in H37Ra has been linked to loss of virulence [18]. In addition, H37Ra exhibits reduced secretion of virulence factors and impaired synthesis of virulence-associated lipids [19]. The role of these factors is incompletely understood. However, iNOS−/− mice control H37Ra growth to the same extent as wild-type (WT) mice, whereas H37Rv infection is detrimental to them [20]. Moreover, H37Rv, but not H37Ra, inhibits apoptosis in vitro [21] and causes exacerbated necrosis in macrophages in vitro [22].

To better understand the cross-talk between M. tuberculosis virulence and host resistance in TB, we characterized H37Ra and H37Rv infections in WT and iNOS−/− mice as an extreme combination between these 2 parameters. Our data reveal a role of RNIs in the control of inflammation during TB, which extends direct antimycobacterial effects and influences the balance of host resistance and M. tuberculosis virulence.

MATERIALS AND METHODS

Mice and M. tuberculosis infection. C57BL/6 (WT) mice were purchased from Charles River Laboratories, and NOS2tm1/lau (iNOS−/−) mice were purchased from Jackson Laboratories. Mice were bred in our facilities and kept under specific pathogen-free conditions with food and water ad libidum. All animal experiments were conducted according to German animal protection law. M. tuberculosis H37Ra (ATCC 25177) and M. tuberculosis H37Rv (obtained from William Jacobs, Albert Einstein College of Medicine, New York) infection stocks were grown to mid-log phase in Middlebrook 7H9 medium (albumin-dextrose-catalase [ADC] supplement, 0.05% Tween 80), and 1-mL aliquots were frozen at −80°C until use.

Mice were infected with ~200 cfu H37Rv and ~2000 cfu H37Ra with use of an aerosol chamber (Glas-Col). Bacterial counts of inocula and in homogenized lungs, spleens, and livers, measured in colony-forming units, were determined at various time points after infection by plating serial dilutions in PBS/0.05% Tween 80 on Middlebrook 7H11–oleic acid–ADC–ampicillin plates and incubating cultures at 37°C for 3–4 weeks.

Immunohistochemistry. Lungs were fixed with paraformaldehyde (PFA), dehydrated, and embedded in paraffin. Cut tissues (4 μm) were mounted on slides, deparaffinized, and subjected to epitope retrieval by boiling (0.1 mol/L sodium citrate, pH 6.0) for 2 min [23]. Slides were rinsed in cool water, washed (Tris-buffered saline; pH, 7.4), and incubated for 30 min with polyclonal antibodies against CD3 (N1580, Dako; 1:10) or myeloperoxidase (MPO-7, A0398, Dako; 1:10,000) or monoclonal antibodies (MABs) against Ki-67 (TEC-3, Dako; 1:500) or F4/80 (BM8, eBioscience; 1:50). For detection, biotinylated rabbit anti-rat (Dako) or donkey anti-rabbit (Dianova) antibodies were used, followed by the streptavidin–alkaline phosphocyanin kit (K5005, Dako). Fast Red (Dako) conversion for 10 min revealed alkaline phosphatase.

Flow cytometry. Lung cells were isolated as described elsewhere [24]. Typically, right lung lobes were cut in small pieces, digested in collagenase-containing medium (60 min, 37°C, 7% CO2), and pressed through cell strainers (70 μm). Cells of 2 lungs were pooled, erythrocytes were lysed, and cells were filtered (50 μm) and enumerated (with a hemocytometer). Routinely, 2 × 10⁶ cells per sample were used.

Major histocompatibility complex (MHC) class I tetramer staining was done as described elsewhere [24]. In short, cells were incubated with rat serum, anti-CD16/CD32 MAB, and streptavidin and were stained with phycoerythrin (PE)–labeled peptide A (PepA) H-2Dd MHC-I tetramers loaded with peptide GAPINSATAM (PepA, derived from M. tuberculosis protein Mtb32), anti-CD8–Pacific Blue (53.67, BD Pharmingen), and anti-CD62L–fluorescein isothiocyanate (FITC; MEL-14, ATCC) MAb. Staining with alloparycin-labeled annexin V (APC annexin V, BD Pharmingen) and 7-amino-actinomycin D (7-AAD, BD Pharmingen) was performed according to the manufacturer’s specifications after staining with anti-Gr-1–Pacific Blue (RB6–8CS, ATCC) and anti–MHC-II-PE (TIB120, ATCC) MAb. Regulatory T (Treg) cells were detected using the Mouse Regulatory T Cell Staining Kit (eBioscience), according to the manufacturer’s recommendations.

Intracellular cytokine staining was done as described elsewhere [25]. In brief, cells were cultured for 6 h in 200 μL of RPMI 10 complete medium containing 10 μg/mL brefeldin A. In parallel, cells were supplemented with M. tuberculosis–derived peptides at 10−4 mol/L concentrations: Ag85A141–160 (QDAYNAGGGHNGVDFDPDSG), Ag85B240–260 (FQDAYNAGGGHNGVDFDPDSG), Ag85B240–260 (FQDAYNAGGGHNAVFNPPNG), and ESAT44–46 (MTEQQWNFAGIEAAASAIQG) for stimulating CD4+ T cells and PepA for stimulating CD8+ T cells. Alternatively, 5 μg/mL anti-CD3 MAB and 5 μg/mL anti-CD28 MAB were supplemented for polyclonal stimulation. Cells were stained with anti-CD4–Pacific Blue (IM7, eBioscience) and anti-CD8–perindopril chlorophyll protein (53–67, BD Pharmingen). Fixed cells (PBS/2% PFA) were permeabilized (saponin buffer) and stained with anti–IFN-γ–PE-cyanine 7 (XMG1.2, BD Pharmingen), anti-tumor necrosis factor (TNF)–α–FITC (XT-22, ATCC), and anti–interleukin (IL)-17–PE (TC11–18H10.1, BD Pharmingen) MABs. The absolute numbers of cytokine-producing cells without stimulation were subtracted from the numbers after M. tuberculosis–specific or polyclonal stimulation.
tion. Routinely, 30,000–100,000 cells were acquired (FACS-Canto II) and analyzed (Diva software, version 6.1.1; BD Bioscience).

Quantification of cytokines, chemokines, and myeloperoxidase. One-quarter of the total lung was homogenized in 500 µL of PBS/2 × protease inhibitor cocktail (EDTA free, Roche), centrifuged, and sterile filtered. Cytokine content was quantified with Milliplex MAP Mouse Cytokine/Chemokine Multiplex assay (Millipore), according to the manufacturer’s specifications. The myeloperoxidase content of pooled homogenates was quantified by ELISA (Hycult Biotechnology), according to the manufacturer’s specifications.

Statistical analyses. Bacterial burdens were analyzed by the Mann-Whitney U test, and Student’s t test was performed for other analyses (GraphPad Prism software, version 4). Statistical significance was categorized as *P < .05, **P < .01, ***P < .001, or not significant.

RESULTS

Bacterial burdens in WT and iNOS−/− lungs after infection with H37Rv and H37Ra. We first determined the bacterial burden in lungs (in colony-forming units) after M. tuberculosis infection. Initial growth of H37Rv and H37Ra organisms in iNOS−/− and WT lungs peaked at approximately day 30 after infection (figure 1A and 1B). Bacterial counts of H37Ra increased to the same extent in iNOS−/− and WT lungs and steadily decreased beginning at day 30 after infection (figure 1A). H37Ra titers in iNOS−/− lungs were lower than those in WT lungs throughout the later infection phase. In contrast, H37Rv counts in iNOS−/− lungs exceeded those in WT lungs by 1–2 orders of magnitude by day 30 after infection (figure 1B). Soon thereafter, iNOS−/− mice succumbed to infection, whereas WT mice entered a chronic infection phase, with stable but elevated H37Rv counts (figure 1B). H37Rv organisms rapidly disseminated from lungs into spleens and livers in both mouse strains, whereas H37Ra organisms did not disseminate significantly (figure 1C and 1D). H37Rv counts increased in peripheral organs until day 30 after infection and remained elevated. Thus, our observations on bacterial burden confirm and extend previous findings [20].

CD8+ and CD4+ T cell responses to infections with H37Rv. To investigate the interplay of host resistance and M. tuberculosis virulence, we first investigated adaptive immune responses of iNOS−/− and WT mice to infection with H37Rv or H37Ra. Lung infiltrating leukocytes were detected early after infection (day 14). Their numbers increased to similar extents in WT and iNOS−/− mice infected with either M. tuberculosis strain (figure 2A). Leukocyte infiltration after infection with lower doses of H37Ra (∼200 cfu) tended to decrease, but absolute numbers were not significantly lower than those observed in H37Rv-infected lungs (data not shown). In all experiments, lymphocyte counts increased over time, with profound proportional increases in CD4+ T cell counts, which exceeded absolute CD8+ T cell counts by day 30 after infection (figure 2B). Although pathologic features differed profoundly between WT and iNOS−/− mice (figure 3), absolute leukocyte numbers and T cell accumulations in lungs were comparable. Moreover, both M. tuberculosis strains in-
duced similar increases in the absolute numbers of CD8+ and CD4+ T cells.

More detailed investigations showed that *M. tuberculosis*-specific CD8+ T cells (stained with PepA-loaded MHC-I tetramers) were hardly detectable at day 14 after infection (data not shown) but increased in number thereafter (day 30 after infection) (figure 2C). These *M. tuberculosis*-specific CD8+ T cells expressed an activated phenotype, indicated by CD62L down-regulation on the cell surface (figure 2C). Intracellular cytokine staining after ex vivo stimulation with an *M. tuberculosis*-specific peptide mix to stimulate CD8+ and CD4+ T cells substantiated our findings (figure 2D and 2E). Accordingly, hardly any *M. tuberculosis*-specific T cell responses were detected at day 14 after infection (data not shown), but absolute numbers of cytokine-producing CD8+ and CD4+ T cells increased profoundly by day 30 after infection, after *M. tuberculosis*-specific (figure 2D and 2E) or polyclonal stimulation (data not shown). Both CD4+ and CD8+ T cells predominantly produced T helper type 1 (Th1) cytokines IFN-γ and TNF-α, whereas almost no cells produced IL-17 (figure 2D and 2E). We conclude that highly susceptible iNOS−/− mice can mount adaptive immune responses comparable to those in resistant WT animals, independent of *M. tuberculosis* virulence.

Treg cells (defined as CD4+ Foxp3+ CD25+ T cells) could impair protective T cell responses [25]. However, compared with H37Ra infection, H37Rv infection did not increase the absolute numbers of Treg cells by day 30 after infection (figure 2F), and the absolute num-

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**Figure 2.** Comparable adaptive immune responses in lungs after infection with H37Rv (Rv) or H37Ra (Ra) *Mycobacterium tuberculosis* in wild-type (WT) and inducible nitric oxide synthase–deficient (iNOS−/−) mice. A, Leukocytes from lungs of WT and iNOS−/− mice infected with H37Rv or H37Ra, counted early (day 14 [d14p.i]) and late (day 30 [d30p.i]) during infection. B, Absolute numbers of lymphocytes, determined with anti-CD4 and anti-CD8 monoclonal antibodies (MAbs). C, CD8+ T cells, stained with peptide A–loaded major histocompatibility complex class I tetramers (tet+) and anti-CD8 and anti-CD62L MAbs. D, Cytokine-producing CD8+ T cells in lungs after *M. tuberculosis*-specific stimulation. E, Cytokine-producing CD4+ T cells in lungs after *M. tuberculosis* stimulation. F, Regulatory CD4+ T cells in lungs, detected by staining with anti-CD4 and anti-CD25 MAbs and intracellularly with anti-Foxp3 MAb. Data are means ± standard error of means, representative of 2 experiments performed with 6 mice per group. No significant differences were detected between different combinations by Student’s t test. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.
bers of Treg cells in lungs of WT and iNOS−/− mice were comparable. Finally, the absolute numbers of CD4+ Foxp3+ CD25+ T cells, constituting a peripheral reservoir of committed Treg cells [26], were also comparable in all combinations. Therefore, despite differences in TB disease outcome, robust and comparable T cell responses were induced independently of M. tuberculosis virulence and host susceptibility.

Severe lung pathology and profound neutrophil influx during H37Rv infection. Infection with H37Ra resulted in comparable cell infiltrations in lungs of WT and iNOS−/− mice (figure 4). Compared with H37Rv-infected mice, granulomatous infiltrates were generally smaller and limited to peribronchial and perivascular areas. Whereas CD3+ T cells and macrophages were evenly distributed throughout infiltrates, neutrophils were hardly detectable in H37Ra-infected lungs (figure 4). However, pathologic features in H37Rv-infected iNOS−/− and WT lungs differed distinctively (figure 3). Comparable lung portions were affected by day 14 after infection with H37Rv in WT and iNOS−/− mice.

Figure 3. Neutrophils, T cells, and proliferating cells in H37Rv-infected lungs of wild-type (WT) and inducible nitric oxide synthase−deficient (iNOS−/−) mice. Lungs of WT mice (A, C, E, G) or iNOS−/− mice (B, D, F, H) infected with H37Rv were fixed at day 14 (d14p.i) (E, F) or day 30 (d30p.i; A–D, G, H) after infection. Cross-sections were stained with biotinylated polyclonal antibodies against CD3 (T cells; A, B) or myeloperoxidase (MPO; neutrophils; E–H) or monoclonal antibodies against Ki-67 (proliferating cells; C, D) and visualized by Fast Red (Dako) conversion with streptavidin-allophycocyanin (visible in red). Representative samples are shown from lung sections of ≥5 mice per group.
but pathologic alterations differed profoundly by day 30 after infection (figures 3 and 5).

Neutrophil infiltrates in H37Rv-infected mice differed most markedly between resistant and susceptible hosts. Although similar neutrophil clusters were visible in early infiltrates of iNOS−/− and WT lungs (at day 14 after infection), neutrophil proportions within inflammatory aggregates increased markedly in iNOS−/− mice but decreased in WT mice (figure 3E–3H). Neutrophils clustered in central areas of infiltrates in H37Rv-infected iNOS−/− lungs and spread to other areas. Extensive lung areas were affected, and infiltrate sizes in iNOS−/− mice exceeded those in WT mice. They extended beyond perivascular areas and

Figure 4. Neutrophils, T cells, and proliferating cells in H37Ra-infected lungs of wild-type (WT) and inducible nitric oxide synthase–deficient (iNOS−/−) mice. Lungs of WT (A, C, E, G) or iNOS−/− mice (B, D, F, H) infected with H37Ra were fixed at day 14 (d14p.i.; E, F) or day 30 (d30p.i.; A–D, G, H) after infection. Cross-sections were stained with biotinylated polyclonal antibodies against CD3 (T cells; A, B) or myeloperoxidase (MPO; neutrophils; E–H) or monoclonal antibodies against Ki-67 (proliferating cells; C, D) and visualized by Fast Red (Dako) conversion with streptavidin-allophycocyanin (visible in red). Representative samples are shown from lung sections of ≥5 mice per group.
frequently merged into confluent infiltrates affecting large portions of lung tissue. Widespread lung structure disruption and edema were visible in H37Rv-infected inOS−/− lungs, and central areas of large granulomatous structures often became necrotic. In contrast, infiltrates in H37Rv-infected WT mice remained localized in the vicinity of airways and blood vessels and did not necrotize. In H37Rv-infected inOS−/− lungs, diffuse infiltrates of CD3+ T cells and proliferating cells were found surrounding central necrotic areas, within which they were undetectable (figure 3B and 3D). In contrast, CD3+ T cells and proliferating cells were focally distributed throughout smaller infiltrates in WT lungs (figure 3A and 3C). Macrophages were visible throughout infiltrates but were strongly concentrated in the vascular vicinity in both mouse groups (data not shown).

Therefore, neutrophil accumulation and formation of necrotic areas were distinguishing hallmarks of the combination of virulent M. tuberculosis and susceptible host.

**Increased neutrophil infiltration and cell death as hallmarks of H37Rv infection in inOS−/− mice.** We then analyzed the impacts of mycobacterial virulence and host resistance on innate immune responses. We therefore determined absolute numbers of neutrophils (Gr-1<sup>high</sup>/MHC-II<sup>low</sup>) and macrophages (Gr-1<sup>low</sup>/MHC-II<sup>med-high</sup>/FITC<sup>low/d0</sup>) in lungs (figure 6). In addition, annexin V (AnnV) and 7-AAD staining were applied to detect apoptotic (AnnV<sup>+/−7-AAD−</sup>) and necrotic (AnnV<sup>+/7-AAD+</sup>) cells (figure 6). The absolute macrophage numbers in H37Rv-infected lungs exceeded those in H37Ra-infected lungs in both susceptible and resistant mice (figure 6A). H37Rv caused significantly more macrophage necrosis than did H37Ra (figure 6B). The absolute numbers of apoptotic macrophages were comparable in most combinations (figure 6B). Neutrophil accumulation was the discriminating hallmark of H37Rv-infected inOS−/− mice (figure 6C). The absolute numbers of neutrophils increased only in H37Rv-infected inOS−/− mice, but they remained at similar, lower levels in all other lungs. These findings were reflected in levels of myeloperoxidase in lungs (figure 6E). Marked increases in absolute numbers of both necrotic and apoptotic neutrophils were unique to H37Rv-infected inOS−/− mice (figure 6D). In conclusion, the degree of cell death depended on M. tuberculosis virulence only, whereas neutrophil accumulation and necrosis were distinctive consequences of combined mycobacterial virulence and host susceptibility.

**Deregulated chemokine signaling as a probable cause of detrimental neutrophil influx into lungs of H37Rv-infected inOS−/− mice.** To identify mechanisms that cause pulmonary neutrophil influx resulting from combined M. tuberculosis virulence and host susceptibility, we investigated cytokines involved in neutrophil attraction. Combined virulence and host susceptibility caused the greatest production of all cytokines that were measured (figure 7). Macrophage inflammatory protein (MIP)−2 and granulocyte colony-stimulating factor (G-CSF) production in H37Rv-infected inOS−/− lungs increased most markedly. MIP-2 production was detected almost exclusively in inOS−/− mice, with markedly increased levels in H37Rv-infected inOS−/− mice (figure 7A). Production of CXCL1 (KC), IL-1α, and G-CSF was elevated in all mice (figure 7B–7D) but increased markedly in H37Rv-infected inOS−/− mice. Thus, production of proinflammatory cytokines directly depended on combined M. tuberculosis virulence and host susceptibility, thereby providing a functional explanation for increased neutrophil influx with this combination.

**DISCUSSION**

The course, severity, and clinical manifestation of TB are dictated by combined M. tuberculosis virulence factors and host resistance. Evidence suggests that combined genetic susceptibility polymorphisms in the human Toll-like receptor 2 signaling pathway with the Beijing genotype family of the East-Asian lineage of M. tuberculosis results in increased risk of meningeal TB [8]. In the same context, the Euro-American lineage is more prone to cause pulmonary TB rather than severe, disseminated disease, including meningeal TB [8].

To analyze the impact of this cross-talk between pathogen and host, we chose highly susceptible inOS−/− mice and their resistant WT cognate as the host pair and M. tuberculosis H37Rv and
its attenuated cognate H37Ra as the pathogen pair under defined conditions. As expected, H37Rv infection of highly susceptible iNOS−/− mice resulted in the most severe outcome of TB [20]. More importantly, RNI deficiency caused exacerbated inflammatory syndrome, which contributed to accelerated death of mice. This inflammatory syndrome was characterized by elevated secretion of proinflammatory cytokines and chemokines in lungs, notably MIP-2, KC, and IL-1α, as well as neutrophil differentiation factor G-CSF. Accordingly, H37Rv-infected iNOS−/− mice experienced massive pulmonary neutrophil influx, heightened necrotic cell death, and consequently, acute inflammatory lung injury. This inflammatory syndrome was not observed in any other combination. We conclude that inflammation during M. tuberculosis infection is tightly controlled by host resistance, including RNI production, as well as by M. tuberculosis virulence factors that are absent in H37Ra but present in H37Rv. The most likely candidates for these virulence factors are genes under control of the transcriptional regulator PhoP. The DNA binding domain of PhoP is mutated in H37Ra [18], and PhoP deletion mutants are highly attenuated [19]. As a transcriptional regulator, PhoP can affect many genes that affect virulence, ranging from lipid production to proteinaceous factors [19]. Control of H37Ra depends on TNF-α production [27], IFN-γ, and T cells [20], implying involvement of Th1-type acquired immune responses.
Without doubt, acquired T cell immunity is critical for long-term control of *M. tuberculosis*. Although the role of CD4+ T cells is unquestioned [28], increasing evidence suggests additional participation of CD8+ T cells [29], particularly at later stages of disease [30]. Although robust TH1 responses are essential for control of infection, exacerbated TH1 responses can be detrimental [31]. Immune restoration inflammatory syndrome is a severe inflammatory syndrome, prominent in patients coinfected with *M. tuberculosis* and HIV who are receiving antiretroviral therapy [1]. Thus, exuberant T cell responses can cause a shift from beneficial to detrimental T\(_{H1}\) responses in immune restoration inflammatory syndrome, thereby contributing to TB severity. Our data revealed that all combinations of H37Rv and H37Ra with iNOS\(^{-/-}\) and WT mice induced comparable CD4+ T cell– and CD8+ T cell–mediated T\(_{H1}\) responses. Moreover, no differences in T\(_{reg}\) cell induction were observed in the various combinations. Therefore, acquired immunity was unaffected by differential host resistance or susceptibility and pathogen virulence, at least in the initial phase of acquired immunity that was analyzed here. Even the most hazardous combination of H37Rv-infected iNOS-deficient hosts resulted in robust T cell responses that did not develop into exuberant and potentially dangerous T cell immunity.

In contrast, marked differences were observed in initial innate immune responses. Excessive pulmonary neutrophil influx was a critical hallmark of the inflammatory syndrome in H37Rv-infected iNOS\(^{-/-}\) mice. The role of neutrophils in TB remains controversial. On the one hand, neutrophils can contribute to protection against TB [32]. On the other hand, excessive neutrophil responses can lead to tissue damage and severe inflammation [33]. Earlier studies showed a minimal protective role of neutrophils in murine TB [34]. However, granulocyte infiltration and necrosis in lungs of *M. tuberculosis*–susceptible mouse strains were suggested to correlate with bacterial burden [35]. Our data confirm that excessive neutrophil influx and death in susceptible iNOS\(^{-/-}\) mice in-

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**Figure 7.** Chemokine and cytokine production in *Mycobacterium tuberculosis*–infected lungs. Lung samples of wild-type (WT) or inducible nitric oxide synthase–deficient (iNOS\(^{-/-}\)) mice infected with H37Rv (Rv) or H37Ra (Ra) were homogenized. Cytokine and chemokine abundance were determined by multiplex assay on days 14 (d14) and 30 (d30) after infection. **A,** Macrophage inflammatory protein 2 levels in *M. tuberculosis*–infected lungs. **B,** KC levels in *M. tuberculosis*–infected lungs. **C,** Interleukin 1 levels in *M. tuberculosis*–infected lungs. **D,** Granulocyte colony-stimulating factor levels in *M. tuberculosis*–infected lungs. Median values with interquartile ranges are displayed for groups of 8–10 mice. *P < .05, **P < .01, ***P < .001; n.d., not detectable (by Student’s *t* test).
fected with virulent H37Rv had a profound effect on the pathogenesis of TB.

We observed marked increases of late apoptotic and necrotic neutrophils exclusively in lungs of H37Rv-infected iNOS−/− mice (figure 6). It has been shown in vitro that H37Rv and H37Ra induce apoptosis with equal efficiency in human neutrophils [36]. Because no detrimental neutrophil influx occurred in H37Ra-infected iNOS−/− lungs, apoptosis induction in neutrophils by phagocytosed M. tuberculosis is probably insufficient to cause such detrimental neutrophil influx. However, virulent M. tuberculosis affect macrophage functions [28, 37, 38]. Furthermore, RNIs interfere with M. tuberculosis–mediated inhibition of phagosome maturation [39], further impairing infected iNOS−/− macrophages. Therefore, M. tuberculosis–infected macrophages probably fail to clear apoptotic cells efficiently. Pro- or anti-inflammatory sequelae of apoptosis are mostly governed by the ability of local phagocytes to remove apoptotic cells before they enter secondary necrosis [40], which provides strong proinflammatory stimuli [41]. Our observations of enhanced necrosis of macrophages in H37Rv-infected lungs substantiate in vitro findings of enhanced induction of necrosis by H37Rv relative to H37Ra [22].

Quantification of selected cytokines revealed underlying mechanisms of exacerbated pulmonary influx of neutrophils in H37Rv-infected iNOS−/− mice. We detected highly elevated MIP-2, KC, G-CSF, and IL-1 production in H37Rv-infected iNOS−/− mice (figure 7). IL-1α is a potent stimulus for neutrophil responses [42]. G-CSF signals are involved in neutrophil generation [33] and inhibition of neutrophil apoptosis [43], which can cause neutrophil accumulation in lungs. KC and MIP-2 signal through CXCR2 and are potent neutrophil attractants [44]. Whereas KC levels increased in all mice, high MIP-2 levels were seen only in H37Rv-infected iNOS−/− lungs (figure 7). Nitric oxide suppresses MIP-2 production in macrophages [45], and it is essential for CXCR2 down-regulation in activated neutrophils [46]. Induced apoptosis in iNOS−/− mice up-regulates MIP-2 transcription and exacerbates neutrophil influx [47]. In addition, injection of apoptotic cells causes MIP-2–mediated neutrophil recruitment [48]. Together, these reports link neutrophil recruitment to sites of cell death with MIP-2 and show RNI-mediated regulation of this process.

We conclude that, during TB, profound RNI induction by M. tuberculosis also dampens neutrophil recruitment. At first glance, efficient neutrophil recruitment to CXCR2−/− lungs after Mycobacterium avium infection [49] appears to contrast with our conclusions, but pathogen and host differences can explain why CXCR2 deficiency is overcome. The authors caution that CXCR2−/− mice exhibited an enhanced state of neutrophil response readiness through increased numbers of circulating neutrophils and note an impact of CXCR2 blockade on other infections [49]. Moreover, BALB/c mice, used as controls for M. avium infection, show stronger neutrophil lung recruitment than do C57BL/6 mice [50], which were used as controls in our analysis of M. tuberculosis infection.

We propose that induction of cell death by virulent H37Rv triggers MIP-2–mediated pulmonary inflammation in the absence of RNIs. Therefore, exacerbated inflammation in iNOS−/− mice infected with virulent M. tuberculosis results from (1) inflammatory stimuli provided by insufficiently controlled replication of M. tuberculosis, (2) necrosis caused by virulent M. tuberculosis, and (3) subsequent failure to control inflammation owing to the lack of RNI production.

Our findings underline the importance of host-pathogen cross-talk in defining the course, severity and clinical manifestation in TB. More specifically, our data have revealed novel functions of RNIs during TB. In addition to direct antimycobacterial effects, RNIs also control inflammatory responses during TB by inhibiting detrimental pulmonary neutrophil influx. Therefore, our experiments may form the basis for novel immune intervention strategies against the global health threat caused by M. tuberculosis.

Acknowledgments

We thank M. L. Grossman for critical reading of the manuscript.

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