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Improved Clearance of *Mycobacterium avium* Upon Disruption of the Inducible Nitric Oxide Synthase Gene

M. Salomé Gomes, Manuela Flórido, Teresa F. Pais, and Rui Appelberg

Mice genetically deficient in the inducible NO synthase gene (iNOS−/−) were used to study the role played by NO during infection by *Mycobacterium avium*. iNOS−/− macrophages were equally able to restrict *M. avium* growth in vitro following stimulation by IFN-γ and TNF-α as macrophages from wild-type mice. In vivo, the infection progressed at similar rates in wild-type and NO-deficient mice during the first 2 mo of infection, but the latter mice were subsequently more efficient in clearing the mycobacteria than the former. The increased resistance of iNOS−/− mice was associated with higher IFN-γ levels in the serum and following in vitro restimulation of spleen cells with specific Ag, increased formation of granulomas and increased survival of CD4+ T cells. We show that NO is not involved in the antmycobacterial mechanisms of *M. avium*-infected macrophages and, furthermore, that it exacerbates the infection by causing the suppression of the immune response to the pathogen. The Journal of Immunology, 1999, 162: 6734–6739.

*Mycobacterium avium* is an opportunistic pathogen of immunocompromised human patients, such as those in advanced stages of AIDS, and occasionally infects immunocompetent individuals such as chronic obstructive pulmonary disease sufferers and children, the latter developing lymphadenitis (1). This pathogen is a facultative intracellular parasite that resides inside the macrophages of the infected host in membrane-bound vacuoles interacting poorly with the lysosomal compartment (2).

In vitro studies in our laboratory and others have shown that although *M. avium* can grow exponentially inside nonactivated macrophages, this growth is restricted if macrophages are stimulated with cytokines, namely IFN-γ and TNF-α (3, 4). The antimicrobial mechanisms used by the activated macrophages to control *M. avium* growth are not clear. The oxygen-reactive intermediates produced by activated macrophages do not seem to play a major role in the control of bacterial proliferation, with the exception of a few strains with limited virulence (5). Recently, it has been argued that the acidification of *M. avium*-containing phagosomes following stimulation of these phagocytes with IFN-γ and LPS was underlying the killing of the infecting mycobacterium (6).

Following the observations by Stuehr and Marletta (7, 8) that macrophages can secrete large amounts of nitrite and nitrate in response to bacterial products and cytokines, there have been countless reports implicating NO in the antimicrobial activity of macrophages against a wide variety of microorganisms (reviewed in Refs. 9 and 10). In the case of mycobacteria, NO is claimed to play a crucial role in the control of *M. tuberculosis* and *M. bovis* bacillus Calmette-Guérin (BCG). Chan et al. (11) have shown that the restriction of *M. tuberculosis* growth induced in macrophages by IFN-γ plus LPS or TNF-α was partially reverted by the addition of an inducible NO synthase (iNOS) inhibitor. The same authors used two NO synthase inhibitors in vivo to treat mice after infection with *M. tuberculosis*. Mice rendered incapable of producing NO during infection showed increased mortality along with increased numbers of mycobacteria in their organs, as compared with control untreated mice (12). These results were more recently confirmed by MacMicking et al. (13) using iNOS gene-disrupted mice. Studying the mechanisms of microbial growth inhibition in IFN-γ-activated macrophages, Flesch and Kaufmann (14) have also found a role for NO in the inhibition of growth of *M. bovis* BCG.

In the case of *M. avium*, the role of NO is not so clearly established. When studying the interaction of *M. avium* with bone marrow-derived macrophages (BMMφ) in vitro, we saw that most strains are not eliminated inside the macrophages, but rather persist and grow. The growth of a wide panel of *M. avium* strains was shown to be inhibited if the macrophages were treated with IFN-γ and/or TNF-α (3, 4). However, this restriction of mycobacterial growth was not reversed in the presence of the NO synthase inhibitor Nω-monomethyl-l-arginine (3). Similar observations were also reported by Bermudez (15). Observations by other groups on the resistance of *M. avium* to nitrite or NO generated in vitro (16, 17) further suggested that the production of nitrogen-reactive species was not a key factor in the control of *M. avium* infection in the mouse. More recently, Doherty and Sher (18), using iNOS gene-disrupted mice, showed that *M. avium* growth was not increased in the absence of iNOS and suggested a negative role for NO in the suppression of T cell function.

We used iNOS-deficient mice to assess the role of NO in *M. avium* infection and found that NO strongly exacerbated *M. avium* infection at late time points, probably by inhibiting the production of IFN-γ by Ag-specific T cells. While this manuscript was being prepared, Karupiah et al. (19) reported that iNOS gene-disrupted mice showed increased resistance to infection by influenza A virus. These authors showed that this increased resistance was associated...
with and dependent on an increased production of IFN-γ by lung cells and that iNOS gene-disrupted mice had less inflammation-related lung pathology.

Materials and Methods

Bacteria

*M. avium* strain 25291, smooth transparent (SmT) variant, was obtained from the American Type Culture Collection (Manassas, VA). Strains 2447, SmT and 2-151 SmD were isolated from AIDS patients and given to us by F. Portaels (Institute of Tropical Medicine, Antwerp, Belgium) and J. Belisle (Colorado State University, Fort Collins, CO), respectively. All mycobacteria were grown in Middlebrook 7H9 broth (Difco, Detroit, MI) with 0.04% Tween 80 (Sigma, St. Louis, MO). Cultures were harvested during log phase, centrifuged, washed in saline with Tween 80, briefly sonicated, and stored in aliquots at −70°C until used.

**Animals**

iNOS-deficient mice were bred in our facilities from a breeding pair kindly provided by Drs. J. D. MacMicking and C. Nathan (Cornell University, New York, NY) and J. Mudgett (Merck Research Laboratories, Rahway, NJ) (20). These mice were kept in HEPA-filter-bearing cages and fed sterilized chow and water. From the initial breeders, males were chosen to start the backcrossing with C57BL/6 females. Backcrossing was monitored by performing PCR of the markers for the disrupted gene on DNA samples from the progeny as described below. In one experiment, the progeny of the seventh backcross were used in an in vivo infection experiment. C57BL/6 mice were purchased from the�

**PCR screening for natural resistance-assorted macrophage protein 1 (Nrampl) allele**

Genomic DNA samples were obtained from each mouse by treating a portion of the ear with proteinase K (Sigma). The amplification of the *Nrampl* gene was performed using Tag DNA polymerase (Ampligène-Oncor, Gaithersburg, MD) and primers specific for the *Nrampl* gene, one oligonucleotide being common to both alleles and the other being specific for either R or S allele, as described elsewhere (21). The amplification was done in a Gene Amp PCR System 9600 (Perkin-Elmer-Roche, Branchburg, NJ). Results for the iNOS−/− mice, as well as 129Sv, C57BL/6, and (129Sv × C57BL/6)F1 mice, are shown in Fig. 1. According to the results of the screening, 129Sv mice were used in all experiments as iNOS−/− mice.

**Infection of BMMφ**

Macrophages were derived from the bone marrow as follows. Each femur was flushed with 5 ml of HBSS. The resulting cell suspension was centrifuged and the cells resuspended in DMEM (Life Technologies, Paisley, U.K.) containing 10% FBS (Life Technologies) and 10% L929 cell-conditioned medium (LCCM) as a source of M-CSF. The cells were distributed in 24-well plates and incubated at 37°C in 7% CO₂ atmosphere. Three days after seeding, another 0.1 ml of LCCM was added. On the 7th day, the medium was renewed.

On the 10th day of culture, when cells were completely differentiated into macrophages, they were infected with *M. avium*. To each well, about 10⁶ CFU of *M. avium* were added, in 0.2 ml of DMEM. Cells were incubated for 4 h at 37°C in a CO₂ atmosphere and then washed with warm HBSS to remove noninternalized bacteria and reincubated in DMEM, with 10% FBS and 10% LCCM. In some of the wells, the macrophages were immediately lysed and the number of viable intracellular bacteria counted as described below (time zero). The other cells were incubated during 7 days to measure the intracellular growth of the bacteria.

The measurement of mycobacterial growth was done by counting CFU. Briefly, 7 days after infection, the cells were lysed by adding 0.1% saponin to each well. The resulting bacterial suspension was serially diluted 1:10 in water containing 0.04% Tween 80. The dilutions were plated on Middlebrook 7H10 agar (Difco) and the number of colonies counted 8–10 days later. For each condition tested, three culture wells were used. The results presented correspond to the mean and SD of these three wells.

In some of the experiments, macrophages were treated from day 0 to day 4 of infection with recombinant murine IFN-γ (100 U/well/day), alone, or in combination with recombinant murine TNF-α (50 U/well/day) (both cytokines from Genzyme, Cambridge, MA).

**In vivo infection**

Each mouse was infected i.v. with 10⁶ CFU of *M. avium*, either strain 2447 SmT or strain 25291 SmT (two independent experiments). At different time points, animals were sacrificed and their livers, spleens, and lungs collected. These organs were homogenized, and serial dilutions of the resulting suspensions were plated in Middlebrook 7H10 agar medium, as described above for BMMφ cultures, and the bacterial colonies counted after culture for 10 days at 37°C.

In one of the experiments, endogenous IFN-γ activity was blocked by treating mice with anti-IFN-γ αG1, obtained from the hybridoma XM1G21 (DNAX, Palo Alto, CA). Control mice were treated with normal rat IgG. Abs were given i.p., 2 mg per animal every 2 wk.

**Characterization of splenic populations by flow cytometry**

A single cell suspension was prepared from half of each spleen and the total cell number determined by counting in a hemocytometer. To assess the proportions of CD3⁺, CD4⁺, and CD8⁺ cells present, cells were stained with FITC-conjugated anti-CD3 (17A2) and PE-conjugated anti-CD4 (L3T4) or FITC-anti-CD4 and PE-anti-CD8 (Ly-2) Abs (all from PharMingen, San Diego, CA) and analyzed in a FACSsort apparatus (Becton Dickinson, Mountain View, CA).

**In vitro stimulation of splenic cells**

Cells obtained from the spleens of each mouse were washed with HBSS, and the erythrocytes were lysed using a hemolytic solution (155 mM NH₄Cl, 10 mM KHCO₃ (pH 7.2)). Cells were then distributed in 96-well plates and incubated in triplicate in DMEM/10% FCS either with no further stimulus or in the presence of mycobacterial Ag (4 μg/ml) or Con A (4 μg/ml; Sigma). Supernatants from the cultures were collected after 3 days in culture, and quantification of the IFN-γ production was done by ELISA. Mycobacterial Ag was prepared as described elsewhere (22). Briefly, the culture supernatant of *M. avium* 25291 SmT grown in Sauton medium was concentrated by ultrafiltration, precipitated with ammonium sulfate, and extensively dialysed against PBS.

**Detection of IFN-γ in the serum and in culture supernatants**

The measurement of IFN-γ was done using an ELISA method. R4-6A2 and biotinylated AN18 anti-IFN-γ mAbs were used as the capture and detection Abs, respectively. Recombinant IFN-γ from Genzyme was used as standard.

**Histology**

Portions of the livers of the infected mice were fixed in buffered formaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin.

**Results**

**Growth of different strains of M. avium inside murine BMMφ**

Most strains of *M. avium* grow exponentially inside unstimulated murine BMMφ, the growth rate being dependent on the particular strain of the bacterium. We have previously shown that when BMMφ were treated with IFN-γ and/or TNF-α, the intracellular growth of *M. avium* was restricted (3). However, we have not identified the mechanism underlying this cytokine-induced bacteriostasis. Since NO is a well-known antimicrobial mechanism of macrophages, we addressed the question of whether the IFN-γ and TNF-α-induced mycobacteriostasis seen in BMMφ was mediated by NO. We took BMMφ from mice that do not express the
In vivo growth of M. avium in mice lacking iNOS; role of IFN-γ

Since our in vitro results indicated that production of NO was not a key factor for the control of M. avium growth inside murine macrophages, we decided to investigate the capacity of iNOS-de-
different capacity to produce IFN-γ in vivo. To assess that, we measured the amount of IFN-γ in the sera of infected mice throughout the course of infection. In mice infected with *M. avium* 25291 SmT, a clear difference in the amounts of IFN-γ present in the serum was seen, with iNOS−/− mice showing at least 10 times more cytokine in the serum than their controls throughout the experiment (Fig. 4B). Uninfected mice of either strain had no detectable IFN-γ in their sera (data not shown).

To relate the differences in IFN-γ production with the number and function of the producing cells, we initially studied the cell populations in the spleen during the course of infection with the 25291 SmT strain of *M. avium*. As shown in Table I, the total cell numbers in the spleen were significantly higher in iNOS−/− mice than in iNOS+/+ mice during the first 2 mo of infection. This difference was also found both among the CD4+ and CD8+ T cells, as assessed by FACS analysis, although the results were not statistically significant. By month 4 of infection, however, the numbers of cells decreased markedly in the two strains of mice. At this late time point, the iNOS-deficient mice had significantly lower numbers of CD4+ and CD8+ T cells as compared to the wild-type controls. Throughout the time course of infection, there was no difference in the relative proportions of CD3+, CD4+, and CD8+ cells in the spleens between the two strains of mice. To assess the state of differentiation of the T cells present in the spleens of infected mice, cell suspensions were prepared that were stimulated in vitro either with *M. avium*-secreted Ag or Con A, as described in Materials and Methods. After 3 days in culture, the supernatants were collected and assayed for IFN-γ. Splenic cells from iNOS−/− mice produced two to four times more IFN-γ than cells from iNOS+/+ mice (Fig. 4C), a difference which could not be accounted for by alterations in the percentage of CD4+ T cells in the cultures (Table I). Ag stimulation of splenic cells from infected mice of either strain led to no detectable secretion of IFN-γ (data not shown).

Histological analysis of granuloma formation was done in liver sections at 2 and 4 mo of infection with *M. avium* 25291 SmT. As shown in Fig. 5, both the number of granulomas and their size were considerably higher in iNOS−/− as compared with their controls. Granulomas of the iNOS-deficient mice consisted of a well-structured core of epithelioid macrophages surrounded by an extensive mantle of lymphoid cells, whereas the organization of granulomas in wild-type mice was very incipient at both time

| Table I. Cell populations ×10^6 in the spleens of mice infected with *M. avium* 25291 SmT |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|        | 1 mo.             | 2 mo.             | 4 mo.             |                 |                 |                 |
| iNOS+/+          | 77.0 ± 8.3        | 135.6 ± 9.0       | 61.0 ± 7.8        |                 |                 |
| CD4+            | 17.5 ± 3.1        | 31.2 ± 4.1        | 8.1 ± 1.4         |                 |                 |
| (22.7%)         | (23.0%)           | (20.0%)           | (13.3%)           |                 |
| CD8+            | 8.7 ± 2.0         | 14.2 ± 2.2        | 2.9 ± 0.5         |                 |                 |
| (11.3%)         | (10.5%)           | (7.8%)            | (4.8%)            |                 |
| iNOS−/−          | 122.5 ± 22.4      | 259.6 ± 46.7*     | 44.2 ± 10.2       |                 |                 |
| CD4+            | 26.6 ± 7.6        | 52.0 ± 20.7       | 4.7 ± 1.8*        |                 |                 |
| (21.7%)         | (20.0%)           | (10.6%)           |                 |                 |
| CD8+            | 11.0 ± 5.9        | 20.3 ± 7.3        | 1.2 ± 0.4**       |                 |                 |
| (9.0%)          | (7.8%)            | (2.7%)            |                 |                 |

* Statistical analysis was performed using the Student’s t test. Statistically significant differences are labeled * for p < 0.05 and ** for p < 0.001.
points. No granulomatous inflammation or signs of any infectious disease were found in uninfected mice of either strain.

Discussion

Although NO has been identified as an important effector of microbial killing inside macrophages, we were previously unable to find such a role in the bacteriostasis of *M. avium* induced by cytokines acting on murine macrophages (3). We extended here those observations by showing that, after cytokine activation, macrophages obtained from iNOS-deficient animals exerted a degree of bacteriostasis of three strains of *M. avium* similar to that of macrophages from wild-type mice. Furthermore, not only were iNOS−/− as resistant as wild-type animals to in vivo infection by *M. avium* during the initial 2 mo of infection, but also they became able to clear the mycobacteria at late time points of infection contrasting with the bacteriostasis found in the wild-type animals. It was already reported that iNOS−/− mice were not more susceptible to infection by a virulent strain of *M. avium* (18). These authors, however, ended their study around the second month of infection, failing to observe the improved clearance of *M. avium* that could have taken place beyond that period of time.

One could argue that the differences in mycobacterial growth between the two groups of animals in our experiments could be related to differences in genetic background other than the iNOS gene. We find this very unlikely for the following reasons. First, the *Nrpm1* gene is the only gene identified so far to have a proven role in determining resistance to *M. avium* in murine models of infection. We screened our breeders of iNOS−/− mice and found them to have the wild-type (resistant) allele of *Nrpm1* gene in homozygosity. Therefore, our in vivo data were obtained by comparing infections of iNOS−/− mice with the 129Sv strain, also harboring the wild-type allele of *Nrpm1*. Second, 129Sv mice are naturally resistant to infection, and, therefore, the iNOS-deficient mice would be expected to be equally resistant or more susceptible to infection rather than more resistant as observed here. Third, the kinetics of the infection showed that the increased resistance of the knock-out mice was a late event, and there have been no such differences in resolution of infection reported for *M. avium* infections when comparing different strains of mice. However, this evidence cannot exclude the possibility that unidentified genes playing a role in determining resistance to *M. avium*, and present in distinct allelic forms in the strains used to obtain the iNOS-deficient animals, could underlie the different susceptibilities to infection observed here. Therefore, we started to backcross the iNOS−/− strain to the C57BL/6 background, and, from a limited cohort of animals, we were able to confirm the data previously generated. It should, however, be stressed that great care should be taken when using recently generated gene-disrupted animals, as there is always a risk that data generated with those animals may depend as much on the genetically engineered deficiency as on unrelated genetic differences between the strains used in the studies.

Our data are consistent with the interpretation that NO produced during infection hampers the development or maintenance of the protective immune response. The numbers of lymphocytes present in the spleen were higher in iNOS−/− mice than in the wild-type controls during the first mo of infection, including the CD4+ T cells, which are the cells required for the development of protective immunity against *M. avium* (4). When the latter cells were stimulated in vitro with the specific Ag, a higher production of IFN-γ was found among cells from infected iNOS−/− mice than from the controls suggesting that there was a loss or reduction of differentiation of Ag-specific Th1 cells when mice had the ability to produce NO in response to infection. Previous work done with T cell clones had suggested that NO has an inhibitory effect on IFN-γ production by Th1 cells (24). The fact that IFN-γ responses to a mitogen were not affected in the same way as the Ag-specific responses in our experiments means that the results with the mycobacterial Ag are not due to in vitro inhibitory effects of NO and suggests that the in vivo effects are most prominent on cells that are responding specifically to Ag rather than a general effect on the whole CD4+ T cell population. Furthermore, there was an in vivo correlate for these results, namely the amounts of serum IFN-γ detected throughout the infection, which were higher in the iNOS-deficient mice as compared with the controls. These results are consistent with those obtained by MacMicking et al. (13), who found higher levels of IFN-γ in the plasma of iNOS−/− vs iNOS+/+ mice infected with *M. tuberculosis* at early time points of infection, when there were no significant differences in bacterial burdens in the organs of infected mice. The negative effect on protective immunity mediated by NO may be particularly evident in our model of infection due to the high resistance of *M. avium* to NO in contrast to other microbes. Data similar to ours have been recently reported for an influenza model of infection (19). In that study, both increased resistance and increased IFN-γ responses were found in iNOS−/− mice as compared with control animals, showing that this scenario may be more than an odd occurrence.

At 4 mo of infection, we found a drastic reduction in the number of T cells in both strains of mice, showing that there are other mechanisms affecting the survival of these cells during an *M. avium* infection. However, despite the marked decrease in total CD4+ cells, the amount of IFN-γ induced by Ag stimulation remained high, suggesting that Ag-specific CD4+ T cells could survive this latter mechanism of T cell depletion or, alternatively, that these cells further differentiated into very high producers of the cytokine, allowing for a certain loss among them.

Work by other groups has already implicated NO in immunosuppression. Secondary immunosuppression following infection with attenuated *Salmonella typhimurium* was shown to be mediated by NO-dependent mechanisms associated with IFN-γ-activated macrophages (25). Likewise, activated macrophages from *Corynebacterium parvum*-treated rats were shown to inhibit lymphyocyte proliferation driven by a mitogen through the production of NO (26). Similar NO-mediated inhibitory mechanisms were described for mouse resident peritoneal macrophages responsible for reducing mitogen-stimulated T cell proliferation (27). On the other hand, NO may regulate macrophage function in an autocrine/paracrine way, as illustrated by the down-modulation of IL-6 secretion in IFN-γ-treated, *Legionella pneumophila*-infected macrophages (28). Gregory et al. (29) showed, in addition, that NO could have a deleterious effect on the development of protective immune responses to *Listeria monocytogenes*, although there is still no consensus as to whether NO may be required for the control of experimental listeriosis (20, 31–33). A tight regulation of NO production appears to be fundamental for the host response. With *M. avium*, however, having no direct role in the killing of the mycobacteria, NO’s role is just a deleterious one. It is still not clear whether its effects are due to a direct toxicity on T cells or to a more subtle regulatory role, such as the determination of the balance between the type of immune response (24). We have already described the state of unresponsiveness to unrelated Ags that develops during *M. avium* infection and the role played by the activated macrophages in this mechanism (33). We speculate that NO may be involved in such nonspecific immunosuppression.

Two of the immunological outcomes of the lack of NO synthesis were the increased production of IFN-γ and the increase in size and number of granulomas. The latter effect of NO depletion has
been described in other models, such as following pulmonary embolization with latex beads coupled to purified protein derivative in sensitized animals (34). It is unclear at the moment if the increased resistance to \textit{M. avium} in iNOS−/− mice is due to either of these effects or whether additional T cell-associated mechanisms may be the culprits of the late killing of \textit{M. avium} seen in the iNOS-deficient mice. The organization of granulomata in mice, namely during mycobacterial infections, has been found different from that in humans. Our results suggest that NO may be related to this. In fact, NO production in human macrophages has been shown to be of a much lesser magnitude and, maybe for that reason, granulomas in man are better organized. We should also stress that we were able to detect differences in granuloma formation in iNOS−/− mice as compared with the controls because they were matched for the allele of \textit{Nramp1} they express. Otherwise, when comparing granuloma formation in the iNOS-deficient mouse strain (naturally susceptible) as compared with the resistant ones, leading to bacterial loads able to trigger a more vigorous granuloma response.

In summary, we report here a counterprotective role for NO in an infectious model, the basis of which probably being related to its immunosuppressive effects. These data illustrate the double-edged sword nature of NO and the delicate equilibrium that should underlie its production. Also, the opposite effects of NO on the resistance of mice to two different mycobacterial species, namely \textit{M. avium} and \textit{M. tuberculosis}, emphasize the distinct nature of the strategies adopted by these pathogens to survive the host’s anti-microbial machinery. Of relevance to human disease is the fact that \textit{M. avium} infection is especially frequent in HIV-infected individuals, in whom it could cause, through NO production, a further role for nitric oxide synthases in human macrophages. J. Exp. Med. 175:1111.


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