Brief Definitive Report

An Essential Role for Interferon γ in Resistance to *Mycobacterium tuberculosis* Infection

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Summary

Tuberculosis, a major health problem in developing countries, has reemerged in recent years in many industrialized countries. The increased susceptibility of immunocompromised individuals to tuberculosis, and many experimental studies indicate that T cell–mediated immunity plays an important role in resistance. The lymphokine interferon γ (IFN-γ) is thought to be a principal mediator of macrophage activation and resistance to intracellular pathogens. Mice have been developed which fail to produce IFN-γ (gko), because of a targeted disruption of the gene for IFN-γ. Upon infection with *Mycobacterium tuberculosis*, although they develop granulomas, gko mice fail to produce reactive nitrogen intermediates and are unable to restrict the growth of the bacilli. In contrast to control mice, gko mice exhibit heightened tissue necrosis and succumb to a rapid and fatal course of tuberculosis that could be delayed, but not prevented, by treatment with exogenous recombinant IFN-γ.

Tuberculosis remains the largest cause of morbidity and mortality in the world from a single infectious agent (1). Immune mechanisms necessary for protection are poorly understood, although studies involving animal models and human infections have implicated T cells as an essential component of the immune response to *Mycobacterium tuberculosis*. The lymphokine IFN-γ, produced primarily by T cells and NK cells, has been shown to be an important mediator of macrophage activation in controlling a number of intracellular pathogens, including *Leishmania major* (2, 3), *Leishmania donovani* (4, 5), and *Listeria monocytogenes* (6–8). Abundant IFN-γ mRNA has been found in pleural tissues from tuberculous pleuritis patients (9) and in the lesions of the self-healing form of leprosy (10). Injection of recombinant IFN-γ into lesions in lepromatous leprosy patients resulted in the migration of large numbers of Th cells and monocytes to the site of injection (11, 12) and a decrease in acid-fast bacilli (11). T cells which adoptively transferred protection against a virulent *M. tuberculosis* challenge produce IFN-γ when stimulated in vitro (13). We have tested directly the importance of IFN-γ in resistance to *M. tuberculosis* infection using a genetic "knockout" mouse in which the single gene encoding IFN-γ was disrupted (14). Mice homozygous for the targeted IFN-γ gene disruption (gko) produce no IFN-γ, but are healthy in the absence of pathogens and appear not to have obvious alterations in splenic or thymic cell populations. However, in vitro activation of macrophages from gko mice was greatly reduced and the ability of the mice to survive a BCG vaccine strain infection was impaired (14).

Materials and Methods

Mice. Littermates from heterozygote (gko/+) breeding pairs (Charles River Laboratories, Wilmington, MA) were genotyped (14). All mice were maintained in a specific pathogen-free environment, and found to be free of all 12 mouse pathogens tested.

Bacterial Strains and Mouse Infections. Virulent *M. tuberculosis* Erdman strain (Trudeau Institute, Saranac Lake, NY) was passed through mice, and grown in culture once before storing in aliquots at −70°C and titered. Before use, aliquots diluted in PBS containing Tween 80 (0.5%) were briefly sonicated and 10^6 viable *M. tuberculosis* was delivered intravenously.

Organ Harvesting. Organs retrieved from infected mice were homogenized in PBS-Tween 80 (0.5%) in plastic bags using a homogenizer (Stomacher; Tekmar Co., Cincinnati, OH), diluted, and plated on supplemented 7H10 media (Difco Laboratories, Inc., Detroit, MI). Colonies were counted after 21 d incubation at 37°C.

Histology. Sections (5-μm) from paraffin blocks containing lung, liver, and spleen were stained with hematoxylin and eosin, or by the Ziehl-Neelsen method for acid-fast bacilli (AFB).

Cytokine Assays. Spleen cells from *M. tuberculosis*–infected mice were harvested 14 d after infection and stimulated with Con A (5 μg/ml) or tuberculin purified protein derivative (PPD) (10 μg/ml). Supernatants were collected after 72 h, filtered, and IFN-γ or IL-4 production was determined by ELISA (Pharmingen, San Diego, CA).

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Assessment of Reactive Nitrogen Intermediates (RNI) in Sera of Infected Mice. In aqueous phase, nitric oxide (NO) generated by the activation of the macrophage L-arginine–dependent mechanism forms nitrite and nitrate. To assess RNI production, total serum nitrite and nitrate concentrations were quantitated. Serum from infected mice was pooled and filtered (0.45 μm). Nitrate was converted into nitrite using the *Pseudomonas oleovorans* nitrate reductase (15) by incubation of 20 ml serum with live *P. oleovorans* at 37°C for 2 h. Bacteria were removed by centrifugation and proteins precipitated with 30% $ZnSO_4$. Total nitrite content was measured by the Greiss reaction, as previously described (16).

Reconstitution of gko Mice with Exogenous IFN-γ. Reconstitution doses were based on unpublished pharmacokinetic studies of murine IFN-γ performed by Sharon Chen (Genentech, Inc., South San Francisco, CA). Mice were injected intramuscularly with 17,280 U of IFN-γ or PBS every third day for the length of the experiment. For continuous infusion, mice were subcutaneously implanted with osmotic pumps (Alza Corp., Palo Alto, CA) delivering 4 U IFN-γ/min or PBS.

Assessment of In Vivo TNF-α and Nitric Oxide Synthase (NOS) mRNA Production. *M. tuberculosis*–infected mice were killed at 14 d after infection. Spleen tissue was frozen in liquid nitrogen, and stored at −70°C until use. RNA was extracted as previously described (17). Any residual DNA was removed by treatment with DNase. Reverse transcription reactions on 5 μg total RNA were performed using specific 3′ primers or random hexamers, at 45°C, for 1 h. PCR reactions were performed on cDNA using TNF-α–specific primers (Stratagene, La Jolla, CA) or NOS-specific primers (5′ AATACGACTCACTATAGGGCTGTCAGAGCCTCGTGGCTTTGG, 3′ ATTAACCCTCACTAAAGGGCCCTTCCGAAGTTTCTGGCAGCAGC) deduced from the published sequence (18) and the following conditions: at 94°C for 45 s, at 60°C for 45 s, and at 72°C for 2 min for 30 cycles, and then at 72°C for 5 min. The reactions were electrophoresed on 2% agarose gels, transferred to nitrocellulose, and probed with a TNF-α or NOS-specific fragment amplified from RNA from the murine macrophage cell line RAW264.7.

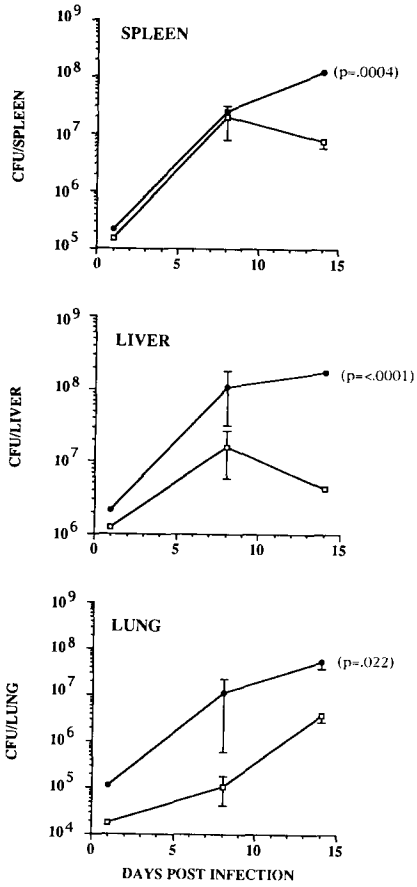

Figure 2. *M. tuberculosis* CFU recovered from organs of infected gko (—●—) and +/+ (—□—) mice. Data shown are combined data from three separate experiments. Error bars, SE.

Results and Discussion

Course of M. tuberculosis Infection in gko Mice. Gko and wild-type (+/+) littermates were infected intravenously with virulent *M. tuberculosis* (10^6, Erdman strain). The mean survival time for *M. tuberculosis*–infected gko mice was 15 ± 1 d, compared with >60 d for +/+ littermate controls (Fig. 1), at which point the experiments were terminated. At 14 d after infection, 10–100-fold more viable bacteria were found in the organs of gko mice compared with +/+ controls (Fig. 2). Histopathological examination of the mice revealed striking differences between gko and +/+ mice. Although both +/+ and gko mice formed granulomas upon infection, by 14 d after infection ~90% of the granulomas in the liver and 20% in the lungs of the gko mice were necrotic and contained apparently extracellular AFB (Fig. 3, *A* and *C*). The spleens

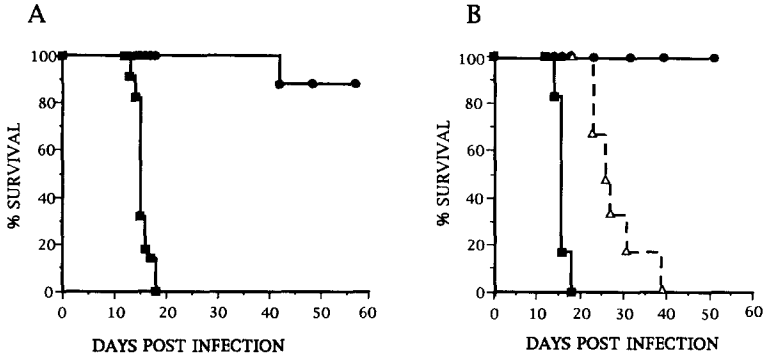

Figure 1. Survival curves of mice infected with *M. tuberculosis*. (*A*) gko (■) and +/+ (●) littermates were infected intravenously with 10^6 *M. tuberculosis* strain Erdman. Data presented are from three separate experiments, and represent 18 mice in each group. $p = < .0001$ in paired Student's *t* test. (*B*) gko mice were implanted subcutaneously with Alzet osmotic pumps loaded with IFN-γ (10^4 U/h) (△) or PBS (■), and infected as above. Infected +/+ littermates (●) were used as controls. Each group represents six mice. $p = .0036$ for PBS and IFN-γ–treated gko mice.

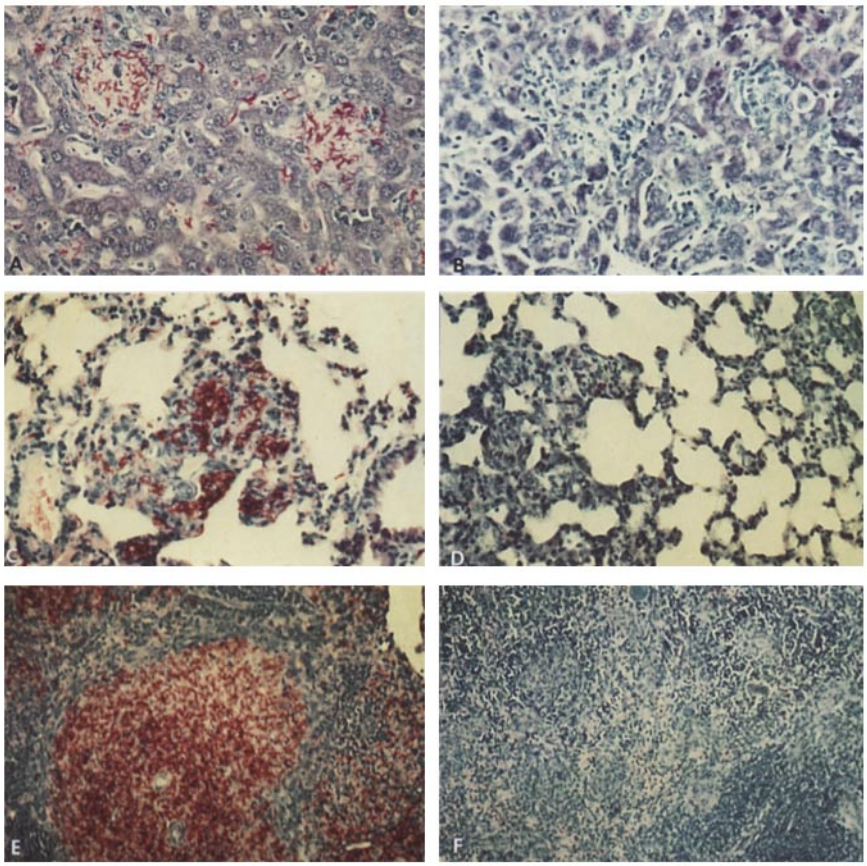

Figure 3. Histology sections. Mice were killed 2 wk after infection and formalin-fixed sections were stained for AFB using the Ziehl-Neelsen method. Mycobacteria stain red, whereas tissues stain blue. (*A*) Liver, gko, and necrotic granulomas (×140); (*B*) liver, +/+ (×140); (*C*) lung, gko (×70); (*D*) lung, +/+ (×70); (*E*) spleen, gko, with extensive necrosis (×70); and (*F*) spleen, +/+ (×70).

from gko mice were consumed by large necrotizing granulomas filled with AFB, with much of the tissue destroyed (Fig. 3 *E*). Necrosis was not seen in any of the organs from either +/+ or +/− mice. The survival curves, together with the CFU and histological data, indicate that IFN-γ is a lymphokine essential to the survival of mice infected with *M. tuberculosis*. Necrosis, which is generally not observed in immunocompetent mice, but is observed in human tuberculous infections, may be the consequence of the products of the increased bacterial load in gko mice, or of the immune response, e.g., possible overproduction of TNF-α in the infected mice, or both.

Reconstitution of gko Mice with Exogenously Added IFN-γ. Since the defect in the gko mice was a genetically defined one, the possibility of reconstituting immune function by exogenous recombinant lymphokine was investigated. It was reported previously that a relatively susceptible strain of mice, BALB/c, when infected with *M. tuberculosis*, exhibited increased survival if infused with IFN-γ, compared with BALB/c mice given heat-inactivated cytokine (19). We attempted reconstitution of gko mice by either of two methods: intramuscular injections of IFN-γ or continuous infusion via surgically implanted osmotic pumps. Mice were infected with *M. tuberculosis* 2 d after initiation of IFN-γ treatment. When IFN-γ was given by injection, the gko mice survived 4 d longer on average than gko mice treated with PBS (19 ± 0.7 vs 15 ± 0.3 d, $p = <.0001$), and there was a 10–100-fold decrease in the number of bacilli recovered from the lungs and livers, respectively, in the IFN-γ–treated mice, compared with the PBS-treated mice at 14 d after infection (lung mean CFU $5.2 \pm 3.5 \times 10^6$ vs $5.1 \pm 3.1 \times 10^7$; liver mean CFU $3.8 \pm 2.7 \times 10^6$ vs $1.9 \pm 0.1 \times 10^8$). All control mice (+/+ and +/− littermates) survived the infection, and administration of IFN-γ had no effect on the numbers of bacilli recovered from the organs of these mice. When reconstitution was attempted using implantable osmotic pumps to deliver IFN-γ or PBS, a 12 d increase in average survival time was seen in gko mice treated with IFN-γ compared with PBS-treated mice (28 ± 2.5 vs 16 ± 0.4 d, $p = .003$) (Fig. 1), again with a significant decrease in numbers of bacilli recovered from the organs of IFN-γ–infused mice at 14 d after infection (lung mean CFU $3.1 \pm 1.6 \times 10^6$ vs $1.6 \pm 0.7 \times 10^7$; liver mean CFU $3.0 \pm 0.1 \times 10^6$ vs $3.3 \pm 0.9 \times 10^8$). Histological samples from this time point showed extensive necrosis with large numbers of AFB in the liver and spleen of PBS-infused gko mice, whereas the IFN-γ–infused gko mice did not exhibit necrosis.

Thus, exogenously added IFN-γ reduced the bacterial load and increased survival of gko mice, demonstrating the importance of IFN-γ in the immune response to *M. tuberculosis*. Nevertheless, treatment with exogenous IFN-γ was unable to reconstitute fully protective immune function and prevent mortality of gko mice using this challenge dose. Although the reconstitution conditions tested were very limited, a

number of possibilities for this outcome require consideration. Since the circulating half-life of administered IFN-γ in mice is ~1 h (Chen, S., personal communication), it is unclear whether the IFN-γ was available in effective concentrations, especially in the granulomas. The increased survival of the mice receiving continuous IFN-γ infusion vs IFN-γ injections supports the view that the inability to fully reconstitute the IFN-γ defect may largely be a problem of delivery and bioavailability. It is also possible that the absence of IFN-γ in gko mice has a regulatory effect on the expression of other cytokines that are necessary to control *M. tuberculosis* infection, although no differences in the expression levels of TNF-α, another cytokine involved in macrophage activation, in gko mice compared with +/+ mice were observed (Fig. 4, and our unpublished results). Finally, since exogenous IFN-γ significantly reduced the number of bacilli recovered from the mice, which probably accounts for the increased time of survival, more dramatic effects might be seen at lower *M. tuberculosis* challenge doses.

To examine the T cell cytokine profiles from gko mice, spleens from *M. tuberculosis*–infected gko and +/+ mice were stimulated in vitro with Con A or tuberculin PPD. Although splenocytes from all mice tested proliferated to the antigens, only +/+ mice produced IFN-γ (>200 ng/ml with both antigens), whereas gko mice failed to produce any detectable IFN-γ when stimulated with either antigen (<25 pg/ml). Of interest, the lack of IFN-γ did not result in a discernible increase in T cells producing IL-4, as IL-4 production was low but indistinguishable between the strains.

In Vivo Production of RNI from M. tuberculosis–infected gko Mice Is Greatly Reduced. Macrophage activation is considered essential to the resolution of *M. tuberculosis* infection. We and others (16, 19, 20) have previously reported that NO, and its related RNI, are responsible for destruction of virulent tubercle bacilli by murine macrophages, a response that requires treatment in vitro with both IFN-γ and TNF-α. Consequently, we examined the in vivo production of RNI in these mice. It was reported previously that peritoneal macrophages recovered from BCG-infected gko mice required the addition of exogenous IFN-γ to generate RNI in vitro (14). At 8 d after infection with *M. tuberculosis*, neither gko nor +/+ mice had measurable levels of RNI in their sera, nor was it possible to detect RNI in the sera of uninfected gko or +/+ mice. However, at 14 d after infection, +/+ mice showed high serum levels of RNI, comparable with those of mice injected with LPS (21), whereas gko mice had very low serum RNI levels (Table 1). Production of RNI in mice infected with mycobacteria sharply increases at ~10 d after infection (15) (Chan, J., and B. R. Bloom, unpublished observation).

Table 1. *RNI in Sera of M. tuberculosis–infected gko and +/+ mice*

Mouse	Days after infection	Treatment	NO_2^-
			μM
gko	–	None	<4.5
+/+	–	None	<4.5
gko plus *M. tb*	8	PBS	<4.5
gko plus *M. tb*	8	IFN-γ	<4.5
+/+ plus *M. tb*	8	None	<4.5
gko plus *M. tb*	14	PBS	22.5
gko plus *M. tb*	14	IFN-γ	<4.5
+/+ plus *M. tb*	14	None	373.0

Sera obtained from mice were treated to reduce NO_3 to NO_2, and NO_2 was measured by the Greiss reagent. 4.5 μM was the lower limit of detection in this assay. Experiment was repeated once, with similar results. *M. tb*, M. tuberculosis.

The key enzyme involved in the production of NO from arginine, NOS, is produced by a number of cell types in the mouse, including an inducible isoform in macrophages, the gene for which has recently been cloned (18, 22, 23). We examined the in vivo levels of NOS mRNA produced by *M. tuberculosis*–infected gko and +/+ mice by RNA-PCR using NOS-specific primers. NOS-specific mRNA was present in the spleens of +/+ mice at 14 d after infection, but at consistently lower or undetectable levels in spleens from gko mice infused with PBS or IFN-γ (Fig. 4). In contrast, mRNA for TNF-α, which is also produced by macrophages, was present in +/+ as well as gko mice. The results from these experiments indicated that in vivo gko mice make far less RNI than wild-type controls, and are consistent with the interpretation that production of RNI, at least in the murine model, may be a necessary mechanism for the control of *M. tuberculosis* infection.

Findings in the gko model may have relevance to tuberculosis in AIDS. As CD4 function declines in HIV infection, IFN-γ production by T cells is reduced (24, 25), and the pattern of *M. tuberculosis* infection is frequently more diffuse than that seen in classical tuberculosis, but characterized by extensive tissue necrosis (26, 27). There are some striking similarities in the pattern of infection in the gko mouse: there is greater bacterial growth and dissemination, and necrosis particularly of liver and spleen, relative to infection in +/+ control mice. Initial attempts to compensate for diminished T cell function in these mice indicated that continuous infu-

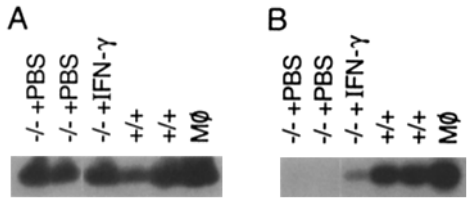

Figure 4. In vivo expression of TNF-α (*A*) and NOS (*B*) mRNA in *M. tuberculosis*–infected mice using RNA-PCR. Gko mice (−/−) were treated with PBS or IFN-γ via osmotic pump. +/+ are littermate control mice. IFN-γ stimulated RAW264.7 cells were used as a source of control RNA (Mϕ). Control PCR reactions with β2-microglobulin and β-actin primers were performed (data not shown).

sion of exogenous IFN-γ reduced bacillary load and prolonged survival of infected gko mice, but ultimately failed to protect mice infected with a high challenge dose from death. The implications are that effective lymphokine therapy, used as a single agent, may be difficult to achieve in diseases such as tuberculosis which require localization of lymphokines in granulomas. However, IFN-γ given in combination with appropriate chemotherapy could contribute significantly to reduction in bacillary numbers and prevention of dissemination. Clearly, gko mice represent an extreme model in which the host is unable to produce any IFN-γ necessary for activating microbicidal activities. Thus, the therapeutic possibility of IFN-γ therapy in *M. tuberculosis*–infected HIV$^+$ individuals who retain some level of CD4 T cells, particularly those infected with multidrug-resistant strains, should be considered.

In conclusion, the results presented in this and in the accompanying paper (28) establish IFN-γ as a necessary lymphokine for a protective immune response to *M. tuberculosis* infection. Our data further suggest that a key role played by IFN-γ in mediating protection in the mouse is the induction of NOS of macrophages, allowing production of RNI, an important microbicidal mechanism for protection against *M. tuberculosis* infection.

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