Materials and Methods

Mice. GKO mice were generated as described previously (10). Briefly, a normal IFN-γ allele in mouse embryonic stem cells was replaced with a defective gene using a targeted vector which introduced a termination codon after the first 30 amino acids of the mature IFN-γ protein. The altered stem cells were injected into C57BL/6j blastocysts and transmitted via the germline. Heterozygous offspring of the chimeras were intercrossed to generate mice homozygous for the altered (GKO) and wild type (WT) allele. The GKO mice were previously characterized as normal in terms of spleen and thymus cell number and expression of CD3, B220, CD4, and CD8 surface markers, and were shown to be incapable of IFN-γ secretion (10).

Experimental Infections. The Erdman strain of M. tuberculosis was grown in Proskauer Beck medium containing 0.01% Tween 80 to mid-log phase. Mice were infected intravenously via a lateral tail vein with an inoculum of 107 M. tuberculosis suspended in 0.2 ml PBS. For airborne infections, mice were placed in the exposure chamber of an airborne infection apparatus (Glas-col Inc., Terre Haute, IN). The nebulizer compartment was filled with 10 ml of a suspension of M. tuberculosis at a concentration previously calculated to provide an uptake of ~50 viable bacilli within the lungs over a 30-min exposure (1, 2).

The numbers of viable bacteria in target organs was followed against time by plating serial dilutions of whole organ homogenates on nutrient Middlebrook 7H11 agar (GIBCO BRL, Gaithersburg, MD) and counting bacterial colony formation after 21 d incubation at 37°C in humidified air.

Histological Analysis. Tissues were fixed in 10% formal saline, set in paraffin blocks, sectioned, and stained using the Ziehl-Neelsen method to visualize acid-fast bacilli. For electron microscopy, tissues were fixed in 1% glutaraldehyde in HBSS, postfixed in 1% OsO4, dehydrated in acetone, and embedded in Spur’s resin. Sections were cut and stained in 1% uranyl acetate and Reynolds’ lead.
Results

In this study, we first wished to ensure that T cells in the GKO mice were in a similar physiological state as the WT controls. Flow cytometric staining of T cells for the CD44 marker, which characteristically changes on activated cells (11), was found to be expressed similarly in both groups of mice. In addition, in assurance of the effectiveness of the gene disruption, abundant mRNA message encoding IFN-γ was detected in the tissues of infected WT controls, but was completely absent in GKO mice (data not shown).

Course of Infections. The course of tuberculosis infection in the GKO and WT mice was followed against time after delivery by two separate routes. In the first, animals were infected intravenously with a normally sublethal dose (10⁸ bacilli) of the virulent Erdman strain of M. tuberculosis. As shown in Fig. 1a, evidence of control and containment of the infection in the primary target organs (liver, spleen, and lungs) of WT mice was clearly evident from day 10 onwards. In contrast, however, the infection grew progressively in the GKO mice, reaching very high numbers. The experiment was humanely curtailed on day 28, when remaining GKO mice showed visible signs of severe illness.

A notable observation in the GKO mice was the presence of large numbers of bacteria in the kidney and bone marrow, as well as an obvious bacteremia, indicating severe dissemination of the infection. In contrast, no bacteria were detected in these tissues in the control animals.

In a second experiment, mice were infected aerogenically with a low dose (~5 × 10⁸) of bacteria using an aerosol generator (Fig. 1b). In the WT control animals, containment of the pulmonary infection was evident after 30–40 d, consistent with our previous observations (12). In addition, some degree of hematogenous seeding to the spleen and liver was observed; again, this is consistent with earlier reports (1). In the GKO mice, however, there was no containment of the infection, which grew progressively. When the experiment was curtailed on day 50 of the experiment, bacterial numbers in the lungs had reached over 10⁹, an extraordinary tissue load. Again, bacteria could be harvested from the kidney, bone marrow, and blood of the GKO mice, but not from these tissues in control animals.

Histology of Infection. Previous studies have demonstrated that emergence of the protective immune response to intravenous infection peaks ~2 wk after inoculation (1). At this point both WT and GKO mice showed evidence of some initiation of the granulomatous response, with small perivascular accumulations of mononuclear cells, associated with a few acid-fast bacilli, visible in target organs such as the liver. By week 4, however, the GKO mice exhibited spectacular histopathology, with multifocal necrotic areas containing vast numbers of intralysosomal acid-fast bacteria evident throughout tissue sections of the spleen, liver, lungs, and kidneys. Fig. 2 shows such an area in the liver, in which a very large halo of bacteria surrounded a large area of necrosis and nuclear debris. Beyond this halo, a few neutrophils, eosinophils, and histiocytes could be seen, but any evidence of mononuclear cell accumulation was completely absent. Similar widespread damage was seen in the lungs, in which there was substantial interstitial hemorrhaging, with only a sparse inflammatory cell infiltrate comprised primarily of neutrophils and eosinophils. In addition, there were bacterial clumps evident throughout the alveoli and in the perivascular areas.

Similar pathology was observed by electron microscopy (Fig. 3). In WT control mice infected 4 wk previously by the aerosol route, discrete, well-formed accumulations of mononuclear cells delineated the main sites of infection. Most of the bacteria within the cells of the granulomas were degraded. In contrast, the lungs of GKO mice were almost totally devoid of mononuclear cells. Instead, the tissue had been extensively infiltrated by neutrophils and eosinophils. The lung tissue had lost its cohesive structure, and exhibited advanced caseous necrosis and multiple foci of bacterial growth. A few bacterial clumps were extracellular, after apparent degradation of their host cell, but the majority had clearly been engulfed by eosinophils. These bacteria appeared to be situated within membrane-bound vacuoles, many of which had fused with the lysosomal granule contents of the cell, although with no apparent damage to the bacilli. Recently, Mycobacterium has been shown to enter eosinophils (13), and a role for granulocytes in host defense has been proposed (14). The present results clearly indicate, however, that eosinophils alone are not capable of limiting the growth of M. tuberculosis.
Figure 2. Representative tissue histology obtained from GKO mice 4 wk after infection. Similar patterns of widespread tissue destruction and florid bacterial dissemination were observed after either infection route. (Top) A massive halo of bacteria surrounding a large area of complete tissue destruction in the liver; Ziehl-Neelsen stain. ×40. (Center) Edge of halo showing presence of a few granulocytes and histiocytes, but complete absence of any mononuclear cell response. ×160. (Bottom) Perivascular/parenchymal tissue in lungs containing numerous large clumps of mycobacteria. ×160.

Figure 3. Electron microscopic examination of lung tissues 4 wk after aerogenic infection. (a) Discrete granulomatous formation in WT control mice. Some monocytes possess obvious vacuoles, a few of which may contain bacterial remnants. The size bar at the bottom of this photograph corresponds to 5 μm. (b) An area of extensive lung tissue necrosis and bleeding in GKO mice. Scattered granulocytes, and large rafts of extracellular bacteria are evident. Size bar = 5 μm. (c) Phagocytosis of several mycobacteria by an eosinophil in the lung of a GKO mouse. Size bar = 2.5 μm.
Discussion

This study shows that mice in which the gene for the molecule IFN-γ has been disrupted, a normally sublethal inoculum of *M. tuberculosis*, delivered by either the intravenous or aero- genic routes, grew progressively to lethal levels. Histological examination of these mice revealed widespread caseous necrosis throughout the major target organs such as the lungs and liver, with no evidence of a surviving mononuclear cell response at a time when bacterial loads reached very high levels. These data convincingly demonstrate, therefore, that mice lacking a functional gene for IFN-γ are totally unable to contain and control a virulent *M. tuberculosis* infection. Since we were able to observe the beginnings of a mononuclear cell granulomatous response occurring early during the course of the infection in the GKO mice, it seems reasonable at this time to hypothesize that the central deficiency in these animals lies in their inability to adequately activate both infected macrophages and arriving monocytes to halt the progressive growth of the infection. These data suggest in turn, therefore, that other cytokines can mediate the initiation of the granuloma, but that infiltrating phagocytes, in the absence of IFN-γ-mediated activation, become heavily infected and destroyed leading to the observed widespread tissue necrosis.

In this regard, the cytokine TNF has also been implicated in having a key role in such mechanisms. Kindler et al. (15) found that neutralizing Ab to TNF disrupted the architecture of granulomas and increased bacterial numbers in mice infected with the avirulent BCG strain of *M. bovis*. Similarly, Amiri et al. (16) observed that TNF could reconstitute the granulomatous response to *Schistosoma* eggs in *scid* mice. In this latter model, however, such mice are known to retain NK cells that can also be a source of IFN-γ. Collectively, therefore, it seems reasonable to hypothesize that, while TNF may clearly contribute in some way to the formation of the granuloma, IFN-γ appears to be essential for the successful retention of its integrity, including the expression of bactericidal mechanisms.

The expression of cutaneous sensitivity to skin test reagents containing mycobacterial antigens (DTH reactions) is also believed to represent a type of granulomatous response in sensitized individuals, involving as it does the local accumulation of monocytes (17). Although this reaction remains a mainstay in the clinical diagnosis of tuberculosis, the basis of the reaction, including the role of cytokines such as IFN-γ, still lacks clear definition. In this regard, both WT and GKO mice mounted significant swelling responses to skin test antigens after footpad inoculation on day 12 of the intravenous infection (0.43 ± 0.10 mm in WT, 0.67 ± 0.37 mm in GKO). Whereas these reactions followed the kinetics of characteristic 24-h DTH responses, we are unable at this time to provide hard histological evidence that these reactions represented true DTH. Should this subsequently be proven to be the case however, then these data would indicate that whereas the loss of the ability to secrete IFN-γ has a devastating effect on protective immunity against *M. tuberculosis*, it may not be essential to the DTH response in which other cytokines such as TNF, migrating inhibitory factor, and IL-8 (15, 18–20) released by macrophages or T cells may be more important. This in turn provides evidence for a possible dissociation between protection and DTH, although it does not necessarily mean that separate T cell populations are involved.

Evidence for a low state of activation of macrophages harvested from GKO mice infected with *M. bovis* BCG has been reported previously (10). Such cells had low levels of reactive oxygen and nitrogen radical production, and were poor expressers of class II MHC molecules. It is of no surprise, therefore, that such cells were incapable of controlling the proliferation of *M. tuberculosis*, and hence were destroyed. Furthermore, many bacteria were present (especially in lung tissues) in areas of extensive caseous necrosis, a pathology not previously believed (17) to exist in the mouse. This model, and another recently published report using β2-microglobulin gene-disrupted mice (3), refutes this earlier contention.

In conclusion, the model described both here and in a parallel publication (21) may provide useful new information in the evaluation of several important areas. In particular, from the perspective of infectious disease, it provides an excellent new model whereby the precise role of IFN-γ in the expression of protective immunity to a variety of microorganisms can be thoroughly investigated. In addition, as a model of the severely immunocompromised host, the GKO mouse may prove very useful for the evaluation and testing of new strategies of immunotherapy and chemotherapy of tuberculosis, and other intracellular infections.

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References

1. Orme, I.M. 1987. The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with Mycobacterium tuberculosis. J. Immunol. 138:293.

2. Orme, I.M., E.S. Miller, A.D. Roberts, S.K. Furney, J.P. Griffin, K.M. Dobos, D. Chi, B. Rivoire, and P.J. Brennan. 1992. T lymphocytes mediating protection and cellular cytolysis during the course of Mycobacterium tuberculosis infection. Evidence for different kinetics and recognition of a wide spectrum of protein antigens. J. Immunol. 148:189.

3. Flynn, J.L., M.M. Goldstein, K.J. Triebold, B. Koller, and B.R. Bloom. 1992. Major histocompatibility class I restricted T cells are required for resistance to Mycobacterium tuberculosis infection. Proc Natl. Acad. Sci. USA. 89:12013.

4. Orme, I.M., P. Andersen, and W.H. Bloom. 1993. T cell response to Mycobacterium tuberculosis. J. Infect. Dis. 167:1481.

5. Nathan, C.F., H.W. Murray, M.E. Wiebe, and B.Y. Rubin. 1983. Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J. Exp. Med. 158:670.

6. Nathan, C.F. 1986. Interferon gamma and macrophage activation in cell-mediated immunity. In Mechanisms of host resistance to infectious agents, tumors and allografts. R.M. Steinman, and R.J. North, editors. The Rockefeller University Press, New York. 165–184.

7. Flesch, I.E.A., and S.H.E. Kaufman. 1990. Activation of tuberculous macrophage functions by gamma interferon, IL-4, and tumor necrosis factor. Infect. Immun. 58:2675.

8. Denis, M. 1991. Interferon-gamma–treated murine macrophages inhibit growth of tubercule bacilli via the generation of reactive nitrogen intermediates. Cell. Immunol. 132:150.

9. Appelberg, R., I.M. Orme, M.I. Pinto de Sousa, and M.T. Silva. 1992. In vitro effects of interleukin-4 on interferon-γ–induced macrophage activation. Immunology. 76:5533.

10. Dalton, D.K., S. Pitts-Meek, S. Keshav, I.S. Figari, A. Bradley, and T.A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon-γ genes. Science (Wash. DC). 259:1739.

11. Lee, W.T., and E.S. Vitetta. 1991. The differential expression of homing and adhesion molecules on virgin and memory T cells in the mouse. Cell. Immunol. 132:215.

12. Orme, I.M. 1988. A mouse model of the recrudescence of latent tuberculosis in the elderly. Am. Rev. Respir. Dis. 137:716.

13. Gil Castro, G., N. Esaguy, P.M. Macedo, A.P. Aguas, and M.T. Silva. 1991. Live but not heat-killed mycobacteria cause rapid chemotaxis of large numbers of eosinophils in vivo and are ingested by the attracted granulocytes. Infect. Immun. 59:3009.

14. Brown, A.E., T.J. Holzer, and B.R. Andersen. 1987. Capacity of human neutrophils to kill Mycobacterium tuberculosis. J. Infect. Dis. 156:985.

15. Kindler, V., A.-P. Sappino, G.E. Grau, P.-F. Piguet, and P. Vassalli. 1989. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell. 56:731.

16. Amiri, P., R.M. Locksley, T.G. Parslow, M. Sadick, E. Rector, D. Ritter, and J. McKerrow. 1992. Tumor necrosis factor α restores granulomas and induces parasite egg-laying in schistosome-infected SCID mice. Nature (Lond.) 356:604.

17. Dansenberg, A.M. 1991. Delayed-type hypersensitivity and cell-mediated immunity in the pathogenesis of tuberculosis. Immunol. Today. 12:228.

18. David, J.R. 1966. Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell–antigen interaction. Proc. Natl. Acad. Sci. USA. 65:72.

19. Bloom, B.R., and B. Bennett. 1966. Mechanism of a reaction in vitro associated with delayed type hypersensitivity. Science (Wash. DC). 153:80.

20. Freidland, J.S., D.G. Remick, R. Shattuck, and G.E. Griffin. 1992. Secretion of interleukin-8 following phagocytosis of Mycobacterium tuberculosis by human monocye cell lines. Eur. J. Immunol. 22:1373.

21. Flynn, J.A., J. Chan, K.J. Triebold, D.K. Dalton, T.A. Stewart, and B.R. Bloom. 1993. An essential role for IFN-γ in resistance to M. tuberculosis infection. J. Exp. Med. 178:2249.