Brief Definitive Report

Mice That Lack the Interferon-γ Receptor Have Profoundly Altered Responses to Infection with Bacillus Calmette-Guérin and Subsequent Challenge with Lipopolysaccharide

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Summary

Mice with a targeted disruption of the interferon γ receptor gene (IFN-γR$^{0/0}$ mice) and control wild-type mice were inoculated with the Bacillus Calmette-Guérin (BCG) strain of *Mycobacterium bovis*. BCG infection was not lethal for wild-type mice whereas all IFN-γR$^{0/0}$ mice died ~7–9 wk after inoculation. Histological examination at 2 and 6 wk after BCG inoculation showed that livers of IFN-γR$^{0/0}$ mice had higher numbers of acid-fast bacteria than wild-type mice, especially at 6 wk. In parallel, the livers of IFN-γR$^{0/0}$ mice showed a reduction in the formation of characteristic granulomas at 2 wk after inoculation. Injection of lipopolysaccharide (LPS) 2 wk after BCG inoculation was significantly less lethal for IFN-γR$^{0/0}$ mice than for wild-type mice. Reduced lethality of LPS correlated with a drastically reduced production of tumor necrosis factor α (TNF-α) in the IFN-γR$^{0/0}$ mice. Interleukin 1α (IL-1α) and IL-6 levels in the serum were also significantly reduced in the IFN-γR$^{0/0}$ mice after BCG infection and LPS challenge. The greatly reduced capacity of BCG-infected IFN-γR$^{0/0}$ mice to produce TNF-α may be an important factor in their inability to resist BCG infection. These results show that the presence of a functional IFN-γ receptor is essential for the recovery of mice from BCG infection, and that IFN-γ is a key element in the complex process whereby BCG infection leads to the sensitization to endotoxin.

Experimental infection of mice with the avirulent Bacillus Calmette-Guérin (BCG) vaccine strain of *Mycobacterium bovis* represents a well-established model for the study of cellular immune responses. Among the alterations in immune responses seen in mice with BCG infection are increased resistance to homologous and heterologous intracellular infectious agents (1, 2), enhanced resistance to transplantable tumors (3), increased production of a number of cytokines including TNF-α (4), IFN-α/β (5), and IFN-γ (6), and greatly increased sensitivity to the lethal action of LPS (7). Although mouse strains differ in the degree of resistance to BCG infection (8, 9), even genetically susceptible mice that develop extensive granulomatous inflammatory lesions in their livers, spleens, and lungs survive infection with high doses of BCG. The numbers of viable bacteria recovered from the livers and spleens of BCG-infected mice reach a peak at 2–3 wk after inoculation and then gradually decline (1, 10).

Kindler et al. (11) demonstrated the importance of TNF in the recovery of genetically susceptible mice from BCG infection. Administration of Abs against murine TNF-α to BCG-infected mice led to a marked increase in the number of mycobacteria present in their livers. The altered course of infection correlated with a defect in the formation of bactericidal granulomas within the livers. The authors concluded that local production of TNF-α within the granulomas was essential for the elimination of mycobacteria and recovery of mice from BCG infection. A beneficial role for TNF was also demonstrated in experimental infections of mice with *M. avium* and *M. tuberculosis* (12, 13). These studies have established a clear role for TNF-α in host resistance to mycobacterial infections.

The role of IFN-γ in host resistance to BCG and related mycobacterial infections is less clearly established. It was reported that the administration of IFN-γ decreased the number of mycobacteria recovered from the spleens of BCG-infected euthymic mice, whereas it apparently exacerbated

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BCG infection in athymic nude mice (14). Some investigators reported that IFN-γ was unable to stimulate bactericidal activity against *M. avium* or *M. tuberculosis* in human monocytes or murine macrophages (15–17), whereas others were successful in demonstrating a decreased mycobacterial growth (18) or increased mycobactericidal activity (19). The administration of IFN-γ was ineffective in the experimental murine infection with *M. avium* (17), whereas a beneficial role for IFN-γ was found in the experimental infection of mice with *M. tuberculosis* (13). The generation of mice with a homozygous targeted disruption of the IFN-γ receptor gene (20) has made it possible to critically determine the role of the IFN-γ receptor in the resistance of mice to BCG infection and in some manifestations of immune responses characteristic for BCG infection.

Materials and Methods

Animals and Reagents. Mice with a deletion in the gene coding for the IFN-γ receptor have been generated as previously reported (20). Homozygous IFN-γR$^{0/0}$ or wild-type (129/SV/EV × C57BL/6)F_2 or (129/SV/EV × 129/SV/EV)F_2 mice aged between 2 and 10 mo, were used in the experiments. ELISA kits for the determination of murine TNF-α and murine IL-6 were purchased from Endogen Inc. (Boston, MA) and ELISA kits for murine IL-1α were from Genzyme Corp. (Cambridge, MA).

BCG and LPS Inoculation. BCG was from Connaught Laboratories Inc. (Swiftwater, PA). Freeze-dried live bacteria were reconstituted with PBS supplemented with 0.025% Tween 80. Mice were inoculated in the tail vein with 0.2 ml of a bacterial suspension containing 2×10^7 viable cells. At 2 wk after inoculation with BCG, some of the BCG-infected as well as uninoculated mice were injected in the tail vein with 25 μg of LPS from *Escherichia coli* 0127:B8 (Sigma Chemical Co., St. Louis, MO). For determination of serum cytokine levels, BCG- and/or LPS-injected mice were bled at 2 h after LPS injection, blood was allowed to clot at room temperature, and serum was collected after centrifugation and stored at −70°C until cytokine assay. Serum levels of TNF, IL-1α, and IL-6 were measured by ELISA.

Histology. Mice were killed 2 and 6 wk after BCG inoculation. Livers, lungs, and spleens were harvested and fixed with 10% formalin. Sections (5 μm) from paraffin blocks containing these organs were cut and stained with hematoxylin and eosin. Randomly selected (n = 20) high-power fields (×400) of liver sections from each animal were examined for the presence of granulomas with the aid of a computerized digitizing tablet. In addition, 5-μm thick paraffin sections of the liver were stained with the Ziehl-Neelsen acid fast stain, and counterstained with Light green. Acid-fast rods were quantitated by microscopic examination in randomly selected (n = 20) high-power fields (×400) of liver sections from each animal.

Results

Infection with BCG is Lethal for IFN-γR$^{0/0}$ Mice. Inoculation of normal mice with the attenuated BCG vaccine strain of *M. bovis* leads to a nonlethal, self-limiting infection (1, 8, 11). To determine the effects of BCG infection in mice lacking the IFN-γ receptor, five IFN-γR$^{0/0}$ mice and five wild-type mice were inoculated intravenously with BCG (2×10^7 live bacteria per mouse). All IFN-γR$^{0/0}$ mice died between 49 and 65 d after BCG inoculation, whereas all wild-type mice survived for at least 180 d (data not shown). Histological examination of the IFN-γR$^{0/0}$ mice showed massive granulomatous pulmonary lesions that were the probable cause of death. The observation that BCG infection is uniformly lethal in mice lacking IFN-γ receptors extends the recently reported finding that mice with a targeted deletion of the structural gene for IFN-γ fail to recover from BCG infection (21).

Altered Granuloma Formation and Increased Number of Acid-fast Bacteria in the Livers of IFN-γR$^{0/0}$ Mice after BCG Infection. Wild-type and IFN-γR$^{0/0}$ mice infected with BCG had granulomatous inflammatory lesions in the liver, spleen, and lungs at 2 and 6 wk after infection, which occurred in a morphological pattern similar to that of miliary tuberculosis. The liver lesions were more well-demarcated from normal tissue than the lesions in spleen and lungs, and the livers were therefore used for qualitative and quantitative assessment of the granulomatous reaction to BCG infection and evaluation of the presence of mycobacteria. The area occupied by granulomatous lesions at 2 wk after infection was 62% less in liver sections from IFN-γR$^{0/0}$ mice than in wild-type mice (Table 1). A strong reduction in the area occupied by lesions occurred in wild-type mice between 2 and 6 wk after infection. In contrast, IFN-γR$^{0/0}$ mice showed, if anything, a slight increase in the percent area occupied by lesions over this time period. The granulomas contained acid-fast bacteria, often inside macrophages (Fig. 1 *e*). The number of acid-fast bacteria present in the liver sections was greater in IFN-γR$^{0/0}$ mice than in wild-type mice. This difference was significant at 2 wk after infection and highly significant at 6 wk (Table 1). In wild-type mice the number of acid-fast bacteria in the liver was considerably lower at 6 than at 2 wk. The decrease in the number of acid-fast bacteria over this time period was much less pronounced in IFN-γR$^{0/0}$ mice.

At 2 wk after BCG inoculation the livers of wild-type mice contained numerous randomly distributed small granulomatous inflammatory areas that were poorly demarcated. These granulomas consisted of macrophages and epithelioid cells, as well as some fibroblasts and occasional lymphocytes, central necrosis was infrequent, and there were no multinucleated (Langerhans) cells (Fig. 1 *a*). In contrast to wild-type mice, IFN-γR$^{0/0}$ mice had well-demarcated lesions that were distinctly more cellular, in part because there appeared to be fewer epithelioid cells, and in part because there were, unlike in the wild-type mice, variable numbers of neutrophils and occasional eosinophils (Fig. 1 *b*).

At 6 wk after BCG inoculation, wild-type mice had distinctly smaller, fewer, and more well-demarcated granulomatous lesions in their livers than at 2 wk. In addition to macrophages, epithelioid cells, and some fibroblasts, the lesions contained more lymphocytes at 6 than at 2 wk, and frequently had some central necrosis, and occasionally a Langerhans cell (Fig. 1 *c*). There were also small and large granulomas consisting of extensive centrally located necrotic, calcified material and a fibrous capsule containing macrophages and lymphocytes. These lesions were mostly located in the walls of large veins, and their morphology was similar to fibrocalcific tuberculosis (Fig. 1 *f*). The granulomatous liver lesions in

Table 1. *Granulomatous Lesions and Acid-fast Bacteria in Liver Sections from BCG-infected Mice*

Time after inoculation	Group of mice	Area occupied by granulomas	No. of acid-fast rods/microscopic field		
			0	1–10	>10
wk		*% ± SEM*		*No. of fields*	
2	Wild-type	24.9 ± 2.1*	1‡	29	30
	IFN-$\gamma R^{0/0}$	8.8 ± 1.7	0	13	47
6	Wild-type	9.3 ± 3.8	12	40	8
	IFN-$\gamma R^{0/0}$	11.5 ± 1.1	1	24	35

* Percent area occupied by granulomatous lesions was determined by examining randomly selected microscopic fields of 310,000–330,000 μm^2 each in liver sections of three mice/group with the aid of a computerized digitizing tablet and Bioquant IV software (R&M Biometrics, Nashville, TN). 20 fields/animal were evaluated. The difference between wild-type and IFN-$\gamma R^{0/0}$ mice was statistically significant at 2 ($p = 0.0238$) but not at 6 wk ($p = 0.7356$), as determined by two-sided *t*-test.

‡ Sections of livers from three mice were examined in each group. Acid-fast rods were counted in 20 randomly selected microscopic fields/mouse. The total number of fields containing 0, 1–10, or >10 acid-fast rods per field is shown for each group of mice. The difference between wild-type and IFN-$\gamma R^{0/0}$ mice was statistically significant at 2 ($p = 0.0049$) and at 6 wk ($p < 0.0001$), as determined by two-sided 2 × 3 X^2 test.

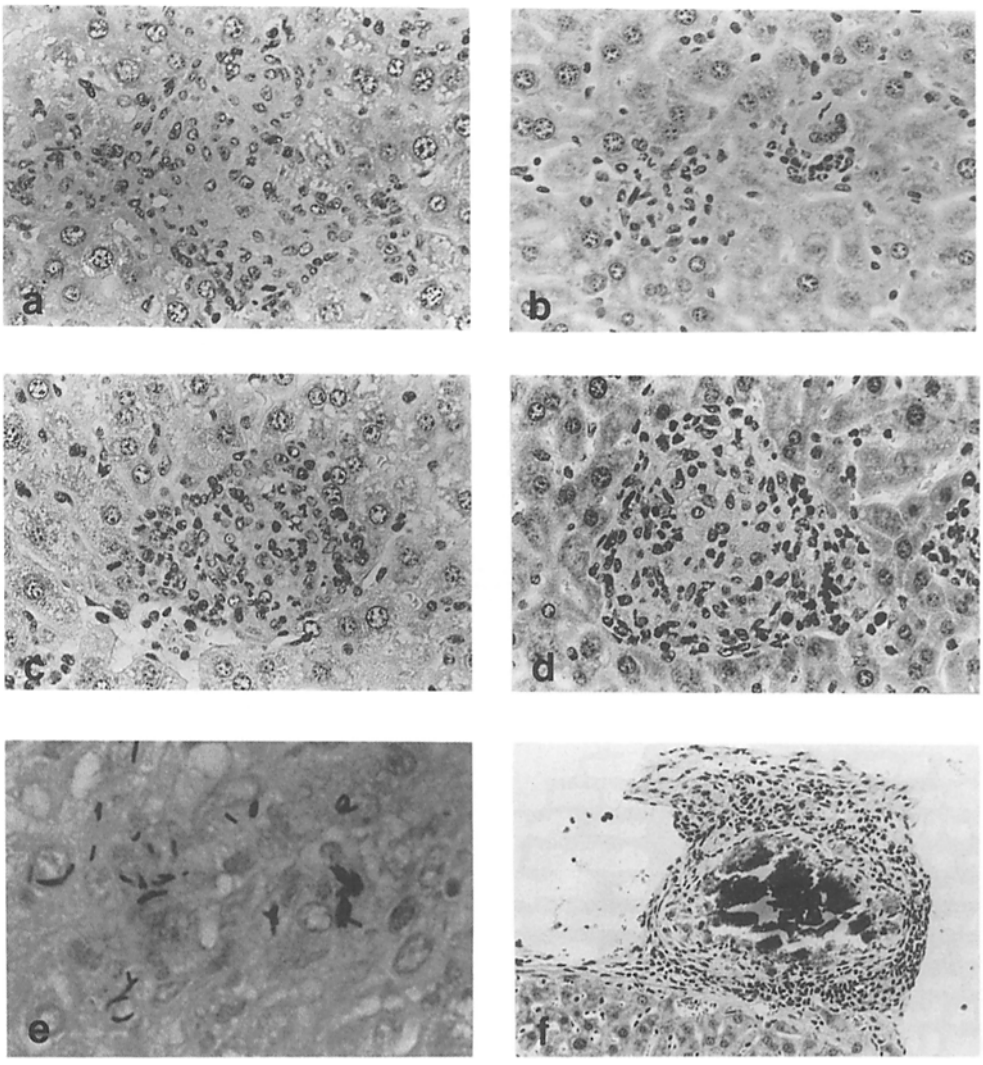

Figure 1. BCG-induced granulomas in wild-type (*a* and *b*) and IFN-$\gamma R^{0/0}$ mice (*c* and *d*) at 2 wk (*a* and *c*) and 6 wk (*b* and *d*) after infection (H&E, ×240). The granulomas shown here are typical examples of the lesions found in the two types of mice at the two time points (see Results). (*e*) Characteristic rod-shaped *Mycobacteria* in a granuloma from an IFN-$\gamma R^{0/0}$ mouse at 2 wk after inoculation, mostly localized within macrophages (Ziehl-Neelsen stain with Light green counterstain, ×950). (*f*) A fibrocalcific granuloma found in a wild-type mouse 6 wk after infection (H&E, ×120).

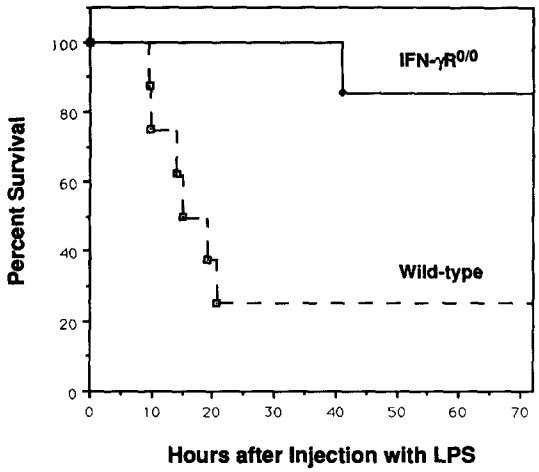

Figure 2. Survival curve of BCG-infected mice after LPS challenge. Wild-type (n = 8) and IFN-$\gamma R^{0/0}$ (n = 7) mice were inoculated in the tail vein with BCG (2 × 10^7 bacteria/mouse). At 2 wk after inoculation, LPS (25 μg/mouse) was injected intravenously. Survival was checked at regular intervals for 72 h.

IFN-$\gamma R^{0/0}$ mice infected with BCG 6 wk earlier consisted of a central zone with variable necrosis, epithelioid cells, and some polymorphonuclear cells, and a more cellular periphery containing macrophages, lymphocytes, neutrophils, eosinophils, and some fibroblasts; there were no Langerhans cells or fibrocalcified granulomas (Fig. 1 *d*).

Reduced Lethality of Endotoxin in BCG-infected IFN-$\gamma R^{0/0}$ Mice. Infection of mice or other laboratory animals with BCG leads to a greatly increased sensitivity to the lethal action of LPS. Mice infected with BCG are killed by an intravenous injection of ~100 times less LPS than is required to kill normal mice (7). To determine whether IFN-γ is required for the development of hyperreactivity to LPS, wild-type and IFN-$\gamma R^{0/0}$ mice were injected intravenously with 25 μg LPS 2 wk after inoculation with BCG. By 24 h, six out of eight wild-type mice had died, whereas none of the IFN-$\gamma R^{0/0}$ mice died within this time period (Fig. 2). One out of seven IFN-$\gamma R^{0/0}$ mice died at 41 h after LPS injection. The difference in the overall survival rate between the two groups of mice is statistically significant (p = 0.0317, as determined by one-sided Fisher's exact test).

Large Increase in the Production of TNF-α and other Cytokines, Seen in BCG-infected Wild-type Mice after Challenge with LPS, Is Abrogated in IFN-$\gamma R^{0/0}$ Mice. Injection of LPS into BCG-infected mice leads to a rapid production of high levels of TNF-α and other cytokines (4–6). In view of the known role of TNF-α as a mediator of LPS toxicity (22), it seemed likely that a decreased production of TNF-α might underlie the markedly reduced lethal effect of LPS in BCG-infected IFN-$\gamma R^{0/0}$ mice. To analyze cytokine production, we injected LPS intravenously into both wild-type and IFN-$\gamma R^{0/0}$ mice, 2 wk after their inoculation with BCG. The serum levels of TNF-α, IL-1α, and IL-6 were determined in these mice 2 h after LPS injection (Fig. 3). In parallel, serum levels of these cytokines were determined 2 h after LPS injection

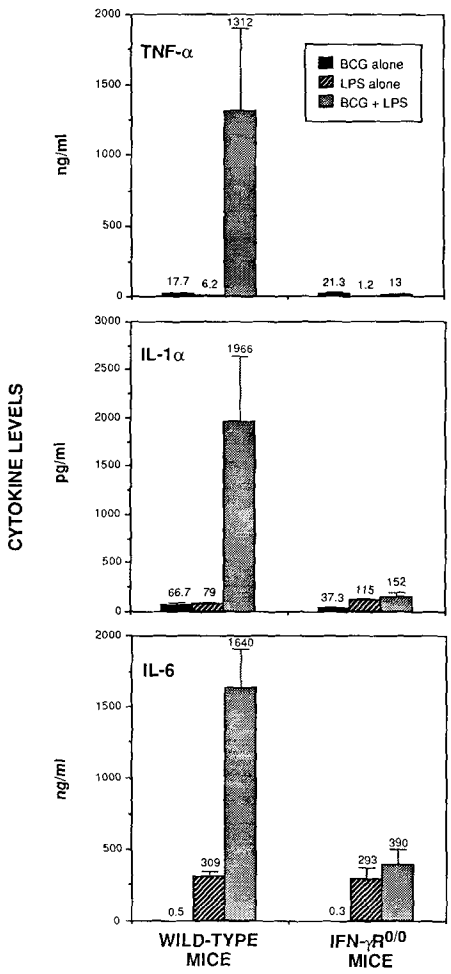

Figure 3. Cytokine levels in the serum of BCG-infected and uninfected mice after LPS challenge. Wild-type and IFN-$\gamma R^{0/0}$ mice (n = 7/group) were inoculated in the tail vein with BCG (2 × 10^7 bacteria/mouse). At 2 wk after inoculation, LPS (25 μg/mouse) was injected intravenously (n = 4/group). The rest of the BCG-infected mice (n = 3/group) were not injected with LPS. Uninfected mice (n = 3/group) were also injected with same dose of LPS. Mice were bled 2 h after LPS injection, and serum cytokine levels were measured by ELISA. (*Error bars*) SD; (numbers, *top*) exact cytokine level, in ng/ml (*TNF-α* and *IL6*) or pg/ml (*IL1α*).

in mice not infected with BCG. Cytokine levels were also measured in the serum of mice that were inoculated with BCG 2 wk earlier, but not challenged with LPS. In wild-type mice, injection of LPS in BCG-infected animals produced a dramatic increase in the serum levels of TNF-α, with the concentration of this cytokine reaching levels that were about 75-fold higher than in mice injected with BCG alone and over 200 times higher than in uninfected LPS-injected mice. This large increase in TNF-α production seen in LPS-challenged, BCG-infected mice was completely abrogated in the IFN-$\gamma R^{0/0}$ mice. Although the increase in IL-1α production elicited by LPS in BCG-infected wild-type mice was somewhat less dramatic (about 25-fold) than the corresponding increase in TNF-α levels, this stimulation too was not seen in the IFN-$\gamma R^{0/0}$ mice. The increase in LPS-induced IL-6 production in BCG-inoculated mice, compared

with uninfected animals, was only about fivefold in the wild-type mice, but this stimulation also was largely absent in the IFN-$\gamma R^{0/0}$ mice.

Discussion

Our results have clearly established the critical role of the IFN-γ receptor in the ability of normal mice to contain infection with the BCG strain of *M. bovis*. IFN-$\gamma R^{0/0}$ mice showed decreased granuloma formation in the livers at 2 wk after inoculation and a reduced ability to eliminate mycobacteria from the liver (Table 1). All IFN-$\gamma R^{0/0}$ mice died between 49 and 65 d after BCG inoculation, whereas all wild-type animals survived the infection. A similar nearly 100% mortality after BCG inoculation was recently reported by Dalton et al. (21) in mice with a targeted deletion of the structural gene for IFN-γ. Earlier, Kindler et al. (11) revealed an essential role for TNF-α in resistance to BCG by demonstrating that the administration of Abs to murine TNF-α converted the normally nonlethal self-limiting BCG infection in mice into a fulminant, progressive infection. The latter authors concluded that TNF-α promotes the formation of bactericidal granulomas that are abundant in the liver and other organs of mice at 2–3 wk after BCG inoculation. Interestingly, our studies revealed a similar role for IFN-γ. Hence, recovery of mice from BCG infection apparently requires both IFN-γ and TNF-α.

It is well known that (*a*) IFN-γ promotes TNF-α synthesis by murine macrophages (23), and (*b*) IFN-γ and TNF-α act synergistically to induce microbicidal activity and nitric oxide release in macrophages (19, 24, 25). Both of these types of interactions between IFN-γ and TNF-α are likely to be important in the murine BCG infection. The serum of BCG-infected IFN-$\gamma R^{0/0}$ mice contained 100 times less TNF-α at 2 h after LPS challenge than the serum of identically treated wild-type mice (Fig. 3). TNF-α levels in the serum at 2 h after LPS challenge are likely to reflect mainly the release of TNF-α from preexisting stores, rather than its de novo synthesis. This conclusion is supported by the high levels of TNF-α mRNA and of immunoreactive TNF-α protein found in the granulomatous liver tissue of mice 2 and 3 wk after BCG infection (11). Yet, without a LPS challenge, TNF-α levels in the serum were low (Fig. 3), which is consistent with the earlier conclusion that TNF-α mRNA and protein accumulate within the disseminated granulomas in the organs, but little or no release of TNF-α protein takes place (11). Hence, in the IFN-$\gamma R^{0/0}$ mice synthesis of TNF-α within the granulomas is likely to be severely impaired. Interestingly, although the area occupied by granulomas in the liver sections was about two-thirds less in IFN-$\gamma R^{0/0}$ mice than in wild-type mice at 2 wk after BCG infection (Table 1), granuloma formation was not completely suppressed. However, the granulomas in IFN-$\gamma R^{0/0}$ mice at 2 wk appeared to contain fewer epithelioid cells, perhaps reflecting an impairment in the process of macrophage differentiation into cells that are capable of killing ingested mycobacteria. Kindler et al. (11) concluded that TNF-α is indispensable for the process of granuloma formation required to contain bacterial dissemination. The likewise indispensable role of IFN-γ then might be explained solely on the basis of the absolute requirement of IFN-γ for the generation of TNF-α in the BCG-infected animals demonstrated in this study. However, it is likely that IFN-γ would also be necessary to act synergistically with TNF-α in promoting the formation of fully functional bactericidal granulomas (19, 24, 25). Our results also demonstrate the essential role of IFN-γ in the production of high serum levels of IL-1α and IL-6 characteristic of BCG-infected mice challenged with LPS (Fig. 3). Failure to produce high levels of IL-1 could contribute to the decreased lethality of LPS in BCG-infected IFN-$\gamma R^{0/0}$ mice.

Our finding that BCG-infected IFN-$\gamma R^{0/0}$ mice fail to produce high cytokine levels in response to LPS (Fig. 3) is related to the recent demonstration that administration of Abs to IFN-γ to mice infected with *Listeria monocytogenes* blocked the production of high levels of TNF and IFN-α/β elicited in these mice by LPS injection (26). The demonstration that IFN-$\gamma R^{0/0}$ mice were more resistant to the lethal effect of LPS at 2 wk after BCG inoculation than wild-type mice (Fig. 2) is reminiscent of two other experimental murine models in which TNF-mediated lethal toxicity could be blocked by antibodies to IFN-γ. One such model is the LPS-induced Shwartzman-like shock in which mAbs to IFN-γ were shown both to reduce mortality and decrease serum TNF levels (27). Another model is experimental murine cerebral malaria, a lethal neurological syndrome that develops in genetically susceptible mice after injection with *Plasmodium berghei*, accompanied by the appearance of high levels of TNF in the serum (28, 29). Mice could be protected from cerebral malaria not only by Abs to TNF-α (28), but also by Abs to IFN-γ (29). Moreover, the protective effect of Abs to IFN-γ was accompanied by a marked reduction in serum TNF levels. The use of IFN-$\gamma R^{0/0}$ mice (20) or of mice with a targeted deletion of the structural gene for IFN-γ (21) should greatly facilitate defining the roles of IFN-γ in cytokine cascades during the pathogenesis of many other types of disorders.

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