Infection with the dimorphic fungus *Histoplasma capsulatum* is endemic to the midwestern and southeastern regions of the United States and causes a severe, highly fatal disease in most individuals exposed to the pathogen (6). However, if the immune system is impaired, individuals often exhibit a progressive infection with a potentially fatal outcome (12). Moreover, reinfection is particularly severe in patients who manifest preexisting cellular immunodeficiency (6).

Both CD4+ and CD8+ cells play an important role in host resistance to the fungus. Depletion of CD4+ T cells results in death of mice infected with a sublethal inoculum. In addition, adoptive transfer of monoclonal or polyclonal CD4+ T cells confers protection (2, 11). Although CD8+ T cells are not crucial for survival, they are required for optimal clearance of the organism from infected organs. Thus, mice given anti-CD8 monoclonal antibody (MAb) and β2-microglobulin-deficient mice that lack CD8+ T cells and class I major histocompatibility complex demonstrate impaired clearance of *H. capsulatum* (5).

One principal mechanism by which T cells contribute to clearance of *H. capsulatum* is the release of cytokines that activate macrophages, the principal effector cells, to restrict intracellular growth of the organism. A critical cytokine involved in the protective immune response in mice against *H. capsulatum* is gamma interferon (IFN-γ). In vitro studies have demonstrated that IFN-γ activates murine peritoneal macrophages to inhibit intracellular growth of the organism. An additional activation signal such as lipopolysaccharide is required for splenic macrophages to express anti-*H. capsulatum* activity (13). Recently, Zhou et al. (22) reported that treatment with MAb to IFN-γ resulted in increased mortality of mice infected intravenously (i.v.) with a sublethal inoculum of *H. capsulatum*. An earlier study examined the effect of neutralizing anti-IFN-γ MAb on *H. capsulatum* infection. Although no data regarding survival or quantitative cultures of organs were provided, the authors did state that anti-IFN-γ was inimical to host resistance in mice infected i.v. (21). However, studies to determine the role of IFN-γ in pulmonary histoplasmosis have not been performed, although *H. capsulatum* is acquired via inhalation and the lung is the primary initial target organ. In this study, we analyzed in an experimental model of pulmonary histoplasmosis the contribution of IFN-γ in controlling intrapulmonary infection in naive mice as well as in mice which had been previously exposed to *H. capsulatum*. The reexposure studies were performed because reinfection is particularly germane to the human condition in areas of endemicity. Moreover, we examined the intrapulmonary levels of cytokines that are known to be involved in host resistance to *H. capsulatum* but not interleukin-12 (IL-12) or IL-4. To determine if IFN-γ is required for clearance of pulmonary histoplasmosis in naive and reexposed mice.

**Materials and Methods**

*Animals.* Male BALB/c mice, 8 to 10 weeks old, with disruption of the IFN-γ gene (IFN-γ knockout [GKO]) (−/−) and wild-type (WT) (+/+) littermates were kindly provided by F. D. Finkelman, Division of Rheumatology and Immunology, College of Medicine, University of Cincinnati. All animal experiments were done in accordance with the Animal Welfare Act guidelines of the National Institutes of Health.

*Preparation of *H. capsulatum* and infection of mice.* *H. capsulatum* yeasts were grown at 37°C in Ham’s F-12 medium supplemented with glucose (18.2 g/liter), glutamic acid (1 g/liter), HEPES (6 g/liter), and cysteine (8.4 mg/liter) for 36 h. Yeast cells were washed three times, suspended in balanced salt solution, and enumerated. To produce a sublethal infection in naive mice, animals were infected intranasally with 2.5 × 10^6* H. capsulatum* yeasts in a 50-µl volume. For reexposure histoplasmosis, mice were initially inoculated with 10^6 yeast cells intranasally. Nineteen weeks later previously exposed animals were rechallenged with 2.5 × 10^6 yeasts.

*Organ culture for *H. capsulatum*.* Lungs, livers, and spleens were homogenized in balanced salt solution and serially diluted. One hundred microliters was dispensed onto plates containing brain heart infusion agar (2% agar [wt/vol]) supplemented with 5% (vol/vol) fibrillated sheep erythrocytes, 1% glucose, and 0.01% (wt/vol) cysteine hydrochloride. Plates were incubated at 30°C, and CFU were enumerated 7 to 10 days later. Data are expressed as numbers of CFU per organ.
Induction and measurement of DTH responses to CW/M. Six to eight WT and six to eight GKO mice inoculated with 10⁶ viable H. capsulatum organisms were challenged intradermally with 1 μg of cell wall-cell membrane extract (CW/M) in a volume of 0.05 ml (5). Footpad swelling was measured 24 h later. Delayed-type hypersensitivity (DTH) was expressed as percent increase in footpad thickness.

Cytokine measurement. Lungs from mice were removed on days 3, 5, and 7 postinfection, and the tissue was homogenized in 2 ml of RPMI 1640, centrifuged at 1,500 × g, filter sterilized, and stored at −70°C until assayed. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to measure tumor necrosis factor alpha (TNF-α), interleukin-4 (IL-4), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Endogen, Cambridge, Mass.). IL-12 was assayed by sandwich ELISA (Pharmingen, San Diego, Calif.) specific for mouse IL-12 p70 protein. The sensitivity was >100 pg/ml.

Histology. Lungs were removed, and tissues were fixed in 10% formalin and embedded in paraffin blocks. Sections (5 μm thick) were stained with hematoxylin and eosin or with silver (Grocott) for fungal elements. To quantify inflammatory cells within lung sections, 3 high-power fields per lung (n = three mice) were examined in a blinded fashion. Data are expressed as mean percents ± standard deviations.

Statistical analyses. The log rank test was used to analyze differences in survival; the Wilcoxon rank sum test was employed to analyze differences in cytokine production and fungal burden of organs.

RESULTS

Treatment with neutralizing MAb to IFN-γ increases host susceptibility to i.v. infection with H. capsulatum (22). However, the impact of IFN-γ during pulmonary histoplasmosis has not been studied. First, we sought to investigate the course of primary H. capsulatum infection in mice congenitally deficient in IFN-γ. GKO mice were challenged intranasally with a sublethal inoculum of 2.5 × 10⁶ yeast cells. All GKO mice died by day 9 postinfection, whereas 100% of WT mice survived the infection throughout the observation period of 45 days (Fig. 1A).

FIG. 1. Survival of naive (A) and reexposed (B) GKO and WT mice after intranasal infection with 2.5 × 10⁶ H. capsulatum yeast cells. Survival was monitored for 45 days. Five to seven mice per group were used in each experiment. Similar results were obtained in a second experiment.

Subsequently, we determined whether the increased mortality in GKO mice resulted from an increased fungal burden in affected organs. Therefore, lungs, livers, and spleens from infected animals were cultured for H. capsulatum on day 7 postinfection. The fungal loads of GKO mice (mean log₁₀ CFU ± standard deviation) were 8.8 ± 0.3 in lungs, 7.7 ± 0.2 in livers, and 6.9 ± 0.2 in spleens, whereas WT mice contained 6.5 ± 0.4 in lungs, 6.2 ± 0.3 in livers, and 5.0 ± 0.2 in spleens. Compared to WT mice, GKO animals manifested a 1.5- to 2-log increase in each organ examined (P < 0.01). These findings underline the pivotal role of IFN-γ in controlling primary infection with a sublethal intranasal challenge of H. capsulatum.

To characterize the histopathological changes in lungs of infected GKO and WT mice, organs were removed on day 7 postinfection and examined. The lungs of WT mice contained a moderate multifocal peribronchiolar granulomatous pneumonia with few organisms present in silver stains (Fig. 2A). The inflammatory response was quantified by enumerating the cell populations in the lungs (three high-power fields per lung; n = 3 mice). The principal cell population was lymphocytes, which constituted (mean ± standard deviation) 71 ± 9% of total inflammatory cells. Macrophages and neutrophils comprised 22 ± 6% and 7 ± 4% of the cells, respectively. In sharp contrast, analysis of the lungs of GKO mice revealed a severe multifocal peribronchiolar granulomatous pneumonia with extension into the alveolar parenchyma. The infiltrates were comprised predominantly of polymorphonuclear cells (70 ± 7%), while lymphocytes were almost absent (4 ± 2%) within the area of inflammation. Macrophages constituted 26 ± 7% of the inflammatory cells. Moreover, extensive debris was present in the bronchiolar lumens (Fig. 2B), and numerous yeasts were scattered throughout the pulmonary parenchyma. Thus, the changes in cell populations, namely, the extensive increase in neutrophils in GKO mice, represent an alteration in the character of the inflammatory response.

Although we established that the protective immune response was strikingly altered in GKO mice, we investigated the impact of this perturbation on the generation of cytokines known to modulate host defenses to H. capsulatum. Therefore, we chose to measure IFN-γ, TNF-α, GM-CSF, IL-12, and IL-4 in lung tissue, since these cytokines have been implicated in affecting host susceptibility to H. capsulatum (15, 19, 22). We assayed for possible differences on days 3, 5, and 7 postinfection because GKO mice did not survive a sublethal challenge with the organism beyond day 9. No IFN-γ levels were measurable in lungs of GKO mice (n = 5), whereas lungs of WT mice (n = 5) demonstrated a dramatic increase in IFN-γ by day 5 (from 2,636 ± 771 pg/ml on day 3 to 15,734 ± 1,266 pg/ml on day 5) and a subsequent decrease by day 7 (10,403 ± 1,394 pg/ml).

As shown in Fig. 3A, steady levels of TNF-α were observed at all time points measured. In contrast, TNF-α levels in GKO mice demonstrated a sharp increase by day 5 of infection, reaching a 10-fold difference compared to levels in WT mice. By day 7 the levels were declining; however, they remained 4-fold elevated compared to lungs of wt mice (P < 0.01 at days 5 and 7).

GM-CSF has been implicated in the growth restriction of H. capsulatum (15). We hypothesized that GM-CSF levels could be elevated in GKO mice as a compensatory effort to restrict fungal growth. GM-CSF levels in lungs of WT mice demonstrated a small increase by day 5 postinfection and plateaued by day 7 (Fig. 3B). In contrast, lungs from GKO mice showed a vigorous increase by day 5, and levels remained elevated on day 7 postinfection (P < 0.05). The pattern of production of
IL-12 in GKO mice was comparable to that in WT mice (Fig. 3C) \((P > 0.05)\). Thus, the results indicate that endogenous IFN-\(\gamma\) is not required as a priming agent for IL-12 production, a requirement that has been demonstrated in infection with \textit{Mycobacterium bovis} (8). We did not detect a significant difference in levels of IL-4 between GKO and WT mice \((P > 0.05)\).

It is likely that reinfection with \textit{H. capsulatum} commonly occurs within areas of endemicity (6). Therefore, we sought to investigate the role of IFN-\(\gamma\) in infection in mice previously exposed to \textit{H. capsulatum}. For reinfection histoplasmosis, we challenged mice with \(10^4\) viable \textit{H. capsulatum} yeasts intranasally. Exploratory experiments revealed that GKO mice were unable to restrict growth of this number of yeasts, and death ensued by day 10 postchallenge. Therefore, we instituted treatment with amphotericin B \((3 \text{ mg/kg intraperitoneally [i.p.] on alternate days})\) on day 5 postinfection. WT and GKO mice were treated for 3 weeks, and animals were observed for 6

\begin{figure}[h]
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\caption{Lung histopathology of naive and reexposed WT (A and C) and GKO (B and D) mice infected intranasally with \(2.5 \times 10^6\) \textit{H. capsulatum} yeasts. Hematoxylin and eosin stain was used. Magnification, \(\times 400\).}
\end{figure}
weeks after antifungal therapy was stopped in order to ensure that GKO mice would not die from uncontrolled endogenous infection. Two weeks after the last dose of amphotericin B, the DTH response to the antigen extract, CW/M, was measured. GKO mice demonstrated (mean ± standard deviation) a 22.2% ± 6.3% increase in footpad thickness, compared to 19.3% ± 3.4% for WT mice. Thus, the groups were able to mount equivalent DTH responses. In contrast, the DTH responses in GKO mice (1.5% ± 1.7%) and WT mice (1.6% ± 1.3%) not previously exposed to *H. capsulatum* were significantly less (P < 0.01) than those in mice infected with *H. capsulatum* and treated with amphotericin B.

Nine weeks after the exposure to 10⁴ *H. capsulatum* yeasts, GKO and WT animals were rechallenged with 2.5 × 10⁶ yeast cells intranasally and survival was observed. All GKO mice died within 6 days of challenge, whereas all WT mice survived throughout the observation period (Fig. 1B). Next, we determined whether the decrease in survival correlated with fungal burden in affected organs. In WT mice, fungal burdens (mean log₁₀ CFU ± standard deviation) were 5.3 ± 0.4 (lungs), 3.4 ± 0.4 (livers), and 4.1 ± 0.2 (spleens), compared to 8.1 ± 0.2 in lungs, 6.5 ± 0.1 in livers, and 5.5 ± 0.2 in spleens of GKO mice at day 6. Thus, GKO mice contained significantly more CFU in each organs examined than WT mice (P < 0.001). In contrast to WT mice, GKO mice failed to control *H. capsulatum*. Hence, IFN-γ is directly involved in growth restriction of the organism in reexposure disease.

The lungs of WT mice demonstrated a moderate multifocal peribronchial granulomatous inflammation (Fig. 2C), compared to a severe multifocal peribronchial granulomatous pneumonia in GKO mice (Fig. 2D) which extended into the alveolar parenchyma. The inflammatory cell infiltrate of WT lungs was comprised primarily of lymphocytes (85% ± 4%), whereas macrophages (12% ± 3%) and neutrophils (3% ± 1%) were substantially less abundant. Lungs from GKO mice contained 35% ± 15% lymphocytes, 25% ± 8% macrophages, and 40% ± 10% neutrophils. In addition, neutrophils and debris were found in the bronchial lumens of GKO mice, and very little functional alveolar space remained.

The short life span of GKO reexposed mice limited us to assaying lung cytokines on days 3 and 5 postinfection. The lungs of WT mice exhibited dramatically elevated IFN-γ levels on day 3 (32,454 ± 2,803 pg/ml) with a decline by day 5 (13,316 ± 1,125 pg/ml); no IFN-γ levels were detected in lungs of infected GKO mice. TNF-α levels in lungs of WT mice were similar on days 3 and 5 (Fig. 4A). In contrast, levels of TNF-α in GKO mice rose substantially by day 5 postinfection and were significantly higher (P < 0.01) than those in WT animals. A similar pattern was observed with GM-CSF levels (Fig. 4B). In WT mice, GM-CSF levels remained constant, whereas these levels in lungs of GKO mice were elevated by day 5 postinfection (P < 0.05). IL-12 and IL-4 levels did not differ between groups (Fig. 4C and D) (P > 0.05).

**DISCUSSION**

The data presented herein demonstrate the pivotal influence of IFN-γ in both primary and reinfection histoplasmosis. This cytokine also is critically important in controlling primary and secondary infection with *Listeria monocytogenes* (20). Although these experiments are performed in an established model of listeriosis, in which the organism is delivered i.p., they do not necessarily reflect the human disease condition, since listeriosis is acquired via the intragastric route. In a similar vein, IFN-γ is necessary for control of acute and chronic infection of mice with *Toxoplasma gondii* (10). Although the results of the two
studies stress the significance of IFN-γ in either rechallenge or chronic infection, these studies do not address the influence of this cytokine in models that mimic the natural route of infection, nor do they systematically examine expression of cytokines also involved in protective immunity in IFN-γ-deficient mice.

The lungs of GKO mice contained significantly more TNF-α and GM-CSF than those of WT animals. On the other hand, IL-12 and IL-4 levels were similar in the two groups. The stimulus for the elevated TNF-α and GM-CSF levels in lungs of GKO mice may be attributed to the increased fungal burden, although no direct correlation between CFU and cytokine levels could be discerned. However, the massive number of CFU in lungs of GKO mice did not alter generation of measurable IL-12 and IL-4, both of which are known to affect host resistance to *H. capsulatum* (1, 22). Thus, increased numbers of CFU may not necessarily cause universal changes in release of cytokines.

Alternatively, the raised levels of TNF-α and GM-CSF may be a compensatory response to the absence of IFN-γ. However, the mechanism(s) that prompts increases in levels of these particular cytokines remains to be determined. It is possible that IL-12 may, in the absence of IFN-γ, enhance production of TNF-α since IL-12 can augment TNF-α production in experimental viral infections (16). Nevertheless, it is clear that the increases in TNF-α and GM-CSF levels are insufficient to overcome the adverse effects of IFN-γ deficiency. In fact, the elevations of TNF-α levels may promote the severity of illness because this cytokine does alter the integrity of vascular homeostasis (7).

The failure to detect differences in IL-4 production between GKO and WT mice does not exclude the possibility that IL-4 could exert detrimental effects in GKO mice. Previous studies in our laboratory revealed that administration of anti-IL-4 MAb to mice made IFN-γ deficient as a consequence of treatment with anti-IL-12 MAb restored the protective immune response (1). One possible explanation for the present findings is that the bioactivity of IL-4 in the absence of IFN-γ may be unopposed and therefore lead to disease exacerbation. However, the exact mechanism by which IL-4 might influence the outcome of infection requires further study.

Another important finding of this study was that IFN-γ was absolutely required for resistance in reexposure histoplasmosis. This is reflected in 100% mortality of GKO mice and the enormous increase in numbers of CFU compared to WT mice. In contrast to WT mice, which demonstrated an accelerated clearance of *H. capsulatum*, GKO mice failed to exhibit any clearance of *H. capsulatum*, suggesting that IFN-γ is directly involved in growth restriction of the organism as demonstrated by previous in vitro studies (13).

![Graphs showing cytokine production in lung tissue of reexposed GKO and WT mice.](image-url)

**FIG. 4.** Cytokine production in lung tissue of reexposed GKO and WT mice. Animals were infected intranasally with $10^4$ viable *H. capsulatum* yeasts and treated beginning on day 5 of infection with amphotericin B (3 mg/kg i.p.) on alternate days for 3 weeks. Six weeks after the last dose mice were inoculated with $2.5 \times 10^5$ *H. capsulatum* yeasts. Lungs were removed on days 3 and 5 of infection and homogenized in RPMI 1640, and cytokine levels were determined by ELISA. Five to six lungs were analyzed per group. Results of one representative experiment of two are shown.
The kinetics of cytokine production in lungs and the survival curves of naive GKO mice and those exposed to *H. capsulatum* were quite similar. On these bases, there appear to be no differences between naive and previously exposed GKO mice. However, we were able to distinguish these two groups of mice by the nature of the inflammatory response in lungs and DTH reactivity. The lungs of GKO mice exposed to 10^7 yeast cells contained 35% lymphocytes, compared to 4% in naive GKO mice infected with *H. capsulatum* (P < 0.01). Moreover, lungs of naive GKO mice contained 70% neutrophils, compared to 40% (P < 0.01) in GKO mice with prior exposure to yeast cells.

GKO mice exposed to *H. capsulatum* and treated with amphotericin B mounted a DTH response to CW/M that was similar in magnitude to that measured in WT mice treated identically. DTH reactivity in both groups of animals was significantly greater than that observed in uninfected GKO and WT mice. This finding was surprising since IFN-γ is presumably critical for expression of DTH reactivity (9). Others have shown, however, that in mice deficient in IFN-γ, DTH responses can be elicited with antigen (4, 17). Thus, our data extend these observations and provide additional evidence that exposure to antigen in mice depleted of IFN-γ can be detected by measurement of cutaneous reactivity.

In a recent commentary, Seder et al. (18) indicated that IFN-γ was not necessary for control of secondary exposure to *H. capsulatum*. They reported that survival of normal mice immunized i.v. with *H. capsulatum*, when rechallenged i.v. with a lethal inoculum of *H. capsulatum*, were unaffected by neutralizing antibody to IFN-γ, IL-12, and IFN-γ receptor (18). Their results contrast sharply with our own. The discordant findings may in part be explained by the model system. That is, the same stimulus produces contrasting results in mice congenitally deficient in a cytokine and in immunocompetent mice with an acquired deficiency. Alternatively, the disparities may be attributed to the route by which the fungus is delivered. Thus, the protective immune response induced by i.v. inoculation may differ considerably from that generated by the natural route of infection.

In order to perform studies of reinfection in GKO mice, it was necessary to treat them with amphotericin B for 3 weeks. There is ample evidence that amphotericin B exerts immunomodulatory effects. Among them are the induction of macrophage activation and the increased production of TNF-α (3, 14). There should have been little, if any, persistence of these effects, since we had discontinued treatment with amphotericin B 2 weeks before DTH response measurements and 6 weeks prior to rechallenge with *H. capsulatum.*

As with the primary infection, we observed an increase in TNF-α and GM-CSF protein levels in the lungs of GKO mice reexposed to *H. capsulatum*. In contrast, IL-12 and IL-4 levels did not differ between rechallenged GKO and WT animals. The dramatic increase in IFN-γ levels by day 3, which was approximately 10-fold greater in reexposed than naive WT mice, indicate that peak production of this cytokine in secondary challenge with *H. capsulatum* is generated earlier than that of primary infection. Thus, the evolution of the protective response appears to develop earlier in previously exposed mice.

In summary, congenital deficiency of IFN-γ results in a pronounced loss of protective immunity in mice exposed via the pulmonary route to *H. capsulatum*. In association with this perturbation of immunity, there are changes in the generation of particular cytokines that contribute to the protective immune response. Manipulation of TNF-α and/or GM-CSF production in GKO mice may ameliorate the course of disease. Studies are under way to address this issue.

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