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The Gamma Interferon Receptor Is Required for the Protective Pulmonary Inflammatory Response to *Cryptococcus neoformans*

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Mice with a null deletion mutation in the gamma interferon (IFN-γ) receptor gene were used to study the role of IFN-γ responsiveness during experimental pulmonary cryptococcosis. *Cryptococcus neoformans* was inoculated intratracheally into mice lacking the IFN-γ receptor gene (IFN-γR$^{-/-}$) and into control mice (IFN-γR$^{+/+}$). The numbers of CFU in lung, spleen, and brain were determined to assess clearance; cytokines produced by lung leukocytes were measured, and survival curves were generated. In the present study, we demonstrate the following points. (i) IFN-γR$^{-/-}$ mice are markedly more susceptible to *C. neoformans* infection than IFN-γR$^{+/+}$ mice. (ii) In the absence of IFN-γ signaling, pulmonary CFU continue to increase over the course of infection, and the infection disseminates to the brain. (iii) In the absence of IFN-γ receptor, recruitment of inflammatory cells in response to pulmonary cryptococcal infection is not impaired. (iv) At week 5 postinfection, IFN-γR$^{-/-}$ mice have recruited greater numbers of leukocytes into their lungs, with neutrophils, eosinophils, and lymphocytes accounting for this cellular increase. (v) IFN-γ signaling is required for the development of a T1 over a T2 immune response in the lung following cryptococcal infection. These results indicate that in the absence of IFN- γ responsiveness, even though the recruitment of pulmonary inflammatory cells is not impaired and the secretion of IFN-γ is not affected, IFN-γR$^{-/-}$ mice do not have the ability to resolve the cryptococcal infection. In conclusion, our data suggest that proper functional IFN-γ signaling, possibly through a mechanism which inhibits the potentially disease-promoting T2 response, is required for mice to confine the cryptococcal infection.

T1-type cell-mediated immunity plays an important role in protection against the fungus *Cryptococcus neoformans* (10, 15, 16, 26, 31). The T1-type response requires CD4^{+} and CD8^{+} T cells in addition to the production of the cytokines tumor necrosis factor alpha, interleukin-12 (IL-12), and gamma interferon (IFN-γ) (9, 14). On the other hand, the development of a chronic eosinophil infiltrate in the lungs is associated with the inability of the host to clear *C. neoformans* from the lungs (11, 12, 42, 43).

IFN-γ is a hallmark T1 cytokine that plays an important role in inducing and modulating an array of immune responses. Previously, we have demonstrated an in vitro effect of IFN-γ on rat alveolar macrophage anticryptococcal activity (32). Levitz and North demonstrated that *C. neoformans* directly stimulates IFN-γ production by T lymphocytes and NK cells (24). A series of studies using anti-IFN-γ monoclonal antibodies (MAbs) demonstrated that this cytokine is a critical molecule to exert host resistance against cryptococcal infection (1, 11, 17, 19–21, 37, 46). The role of NK cells, one of the major producers of IFN-γ, in protection against *C. neoformans* infection has been suggested by using neutralizing anti-IFN-γ MAb (17, 37, 46). Furthermore, this protective activity has been shown to be mediated by stimulating the macrophage production of fungicidal mediators including nitric acid (7, 22, 41, 46). Aguirre et al. demonstrated that both IFN-γ and tumor necrosis factor are important mediators of acquired resistance to cryptococcal meningoencephalitis (1). Finally, the involvement of both IL-12, an important cytokine for the differentiation of T1 cells, and IL-18, an IFN-γ-inducing factor, in the induction of IFN-γ production during the course of *C. neoformans* infection has been profoundly elucidated (11, 17, 19, 20, 46). Mice lacking IFN-γ have been shown to be more susceptible to cryptoccocal infection (17, 45). All of these studies point to the protective role that IFN-γ plays in cryptococcal infection, but a controversial outcome has been demonstrated by Yuan et al. (45). They showed that both cryptococcus-specific immunoglobulin G1 (IgG1)-mediated protection and IgG3-mediated acceleration of infection require IFN-γ. Our objective was to determine the requirement for IFN-γ in pulmonary leukocyte recruitment and cell-mediated immunity during *C. neoformans* infection. Our approach was to utilize an intratracheal infection model in IFN-γR$^{+/+}$ versus IFN-γR$^{-/-}$ mice and measure the parameters of pulmonary T-cell-mediated immunity in these two groups.

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MATERIALS AND METHODS

C. neoformans. *C. neoformans* strain 52D was obtained from the American Type Culture Collection (ATCC 24067, Manassas, Va.). For infection, yeast cells were grown to stationary phase at 35°C for 48 to 72 h in Sabouraud dextrose broth (1% neopeptone, 2% dextrose; Difco, Detroit, Mich.) on a shaker. The yeast cells were then washed in sterile nonpyrogenic saline, counted on a hemocytometer, and diluted to 3.3 × 10^{5} CFU/ml in saline.

Mice. IFN-γR$^{-/-}$ mice (129-*Ifngr^{tm1}*) and control IFN-γR$^{+/+}$ mice (129S3/SvImJ) were purchased from Jackson Laboratory, Bar Harbor, Maine. The mice

were maintained in the animal facility of the University of Michigan Medical Center from 6 weeks of age until they were used.

Intratracheal inoculations. Mice were anesthetized by intraperitoneal injection of pentobarbital (0.074 mg/g of body weight) and were restrained on a small surgical board. A small incision was made through the skin over the trachea, and the underlying tissue was separated. A 30-gauge needle was bent and attached to a tuberculin syringe filled with the *C. neoformans* culture. The needle was inserted into the trachea, and 30 μl of inoculum was dispensed into the lungs (10^4 CFU). The skin was closed with a cyanoacrylate adhesive. The mice recovered with minimal visible trauma.

CFU assay. For lung CFU, small aliquots were collected from lung digests. For spleen and brain CFU, the organs were excised, placed in 2 ml of sterile water, and homogenized. Ten-microliter aliquots of the lungs, spleen, and brain tissue were plated on Sabouraud dextrose agar plates in duplicate serial 10-fold dilutions and incubated at room temperature. *C. neoformans* colonies were counted 3 days later, and the numbers of CFU were determined on a per-organ basis.

Preparation of lung leukocytes. The lungs were excised, minced, and enzymatically digested for 30 min in 15 ml of digest solution (RPMI with 5% fetal calf serum, antibiotics, 1 mg of collagenase [Boehringer Mannheim Biochemical, Chicago, Ill.]/ml, and 30 μg of DNase [Sigma Chemical Co., St. Louis, Mo.]/ml). The cell suspension and undigested fragments were further dispersed by drawing the mixture up and down 20 times through the bore of a 10-ml syringe. The total cell suspension was then pelleted, and the erythrocytes were lysed with ice-cold NH_4Cl buffer (0.83% NH_4Cl, 0.1% $KHCO_3$, 0.037% Na_2EDTA; pH 7.4). Excess Hanks' balanced salt solution (HBSS; Gibco) was added to bring the solution to isotonicity, and the cells were pelleted and resuspended in complete medium. Total lung cells were enumerated by hemocytometer counting in the presence of trypan blue. Subsets of isolated leukocytes (neutrophils, eosinophils, macrophages, and total lymphocytes) were determined by Wright-Giemsa staining of samples cytospun onto slides.

Flow cytometry of lymphocyte subsets. Lung cells (5×10^5/sample) were incubated for 30 min on ice in a total volume of 120 μl of staining buffer (fluorescent antibody [FA] buffer [Difco] with 0.1% sodium azide and 1% fetal calf serum). Each sample was incubated with (i) 0.12 μg of phycoerythrin-labeled anti-CD45 (30-F11) and (ii) either 0.25 μg each of the fluorescein isothiocyanate-labeled MAbs RM4-5 (anti-CD4), 53-6.7 (anti-CD8), and RA3-6B2 (anti-B220) or isotype-matched rat IgG. The samples were washed in staining buffer and fixed with 1% paraformaldehyde (Sigma) in buffered saline. Stained samples were stored in the dark at 4°C until analyzed on a flow cytometer (Coulter Elite ESP, Palo Alto, Calif.). All MAb reagents were purchased from PharMingen, San Diego, Calif. Samples were gated for CD45-positive cells and then analyzed for staining by the specific fluorescein isothiocyanate-labeled antilymphocyte markers. The absolute number of each lymphocyte subset in the sample was obtained by multiplying the percentage of cells in that type of lymphocyte subset by the total number of leukocytes.

Histology. Lungs were fixed by inflation with 10% neutral buffered formalin. After paraffin embedding, 5-μm-thick sections were cut and stained with hematoxylin and eosin stain for routine histology or Masson's trichrome for collagen and other matrix proteins (34) and then examined by light microscopy.

Lung leukocyte culture and cytokine production. Single-cell suspensions of lung leukocytes were cultured at 5×10^6 cells/ml in six-well plates with 3 ml of complete medium with or without heat-killed strain 52D at 37°C and 5% CO_2. Culture supernatants were harvested at 24 h and assayed for cytokines IL-4, IL-5, IL-10, and IFN-γ (OptEIA kits; BD PharMingen), and chemokines macrophage inflammatory protein 2 (MIP-2) and keratinocyte-derived chemokine (KC) (DuoSet kits; R&D Systems) were produced by sandwich enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocols.

Blood cell counts. Blood was collected from the tails of mice into microtainer tubes containing EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, N.J.). Red blood cells were lysed with water for 20 s. Concentrations of leukocytes were counted in a hemocytometer. The concentrations of neutrophils were determined by differential counting on cytospin slides.

Cryptococcal burdens of lung macrophages. At week 4 postinoculation, total lung leukocytes were isolated and cultured in 60-mm-diameter petri dishes at 5×10^6 cells/ml. After 24 h, plates were washed three times with HBSS to remove nonadherent cells. Adherent lung macrophages were isolated with trypsin-EDTA. Lung macrophages were further cultured at 10^5 cells/well in 96-well plates. CFU were determined as described above after 0 and 24 h in culture.

Statistical analysis. Means, standard errors of the means (SEM), and unpaired Student's *t* test results were used to analyze the data. In comparing groups, when *P* values of less than 0.05 were obtained, the groups were considered statistically different. Survival data were analyzed with Kaplan-Meier survival plots followed by the log-rank test.

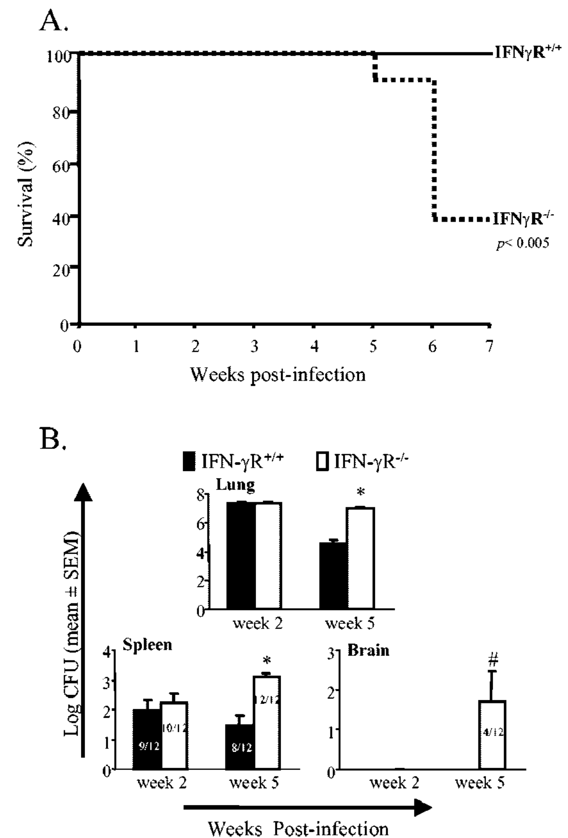

FIG. 1. (A) Effect of IFN-γ receptor deletion on the survival of mice infected with *C. neoformans*. Ten mice from each group of IFN-γ$R^{+/+}$ and IFN-γ$R^{-/-}$ mice were intratracheally inoculated with 10^4 CFU of *C. neoformans* 52D. Survival experiments were carried out to day 49. The difference in survival at day 49 between IFN-γ$R^{+/+}$ and IFN-γ$R^{-/-}$ mice was significant; $P < 0.005$. (B) Effect of IFN-γ receptor deletion on pulmonary growth and dissemination of *C. neoformans* in infected IFN-γ$R^{+/+}$ and IFN-γ$R^{-/-}$ mice. Mice were inoculated intratracheally with 10^4 CFU of *C. neoformans* 52D and assayed at weeks 2 and 5 postinoculation. Total CFU per organ were determined, and numbers in each column indicate the number of mice which had a *C. neoformans* burden in that organ. All mice had detectable CFU in the lungs. #, $P < 0.05$, and *, $P < 0.001$, compared to those for wild-type mice at the same time points; $n = 12$ for each group (two separate experiments of four and eight each). Values are given as means ± SEM.

RESULTS

Effect of IFN-γ receptor deletion on the survival of mice infected intratracheally with *C. neoformans*. We first sought to determine whether the IFN-γ$R^{-/-}$ mice would have increased mortality upon intratracheal infection with *C. neoformans.* IFN-γ$R^{+/+}$ and IFN-γ$R^{-/-}$ mice were intratracheally inoculated in parallel with *C. neoformans* 52D, and survival studies were performed until week 7 after inoculation. There was a rapid die-off of the IFN-γ$R^{-/-}$ mice beginning at day 35, and by day 49, 60% of the knockout mice had died (Fig. 1A). In sharp contrast, none of the wild-type mice died. The difference in survival rates between the IFN-γ$R^{+/+}$ and IFN-γ$R^{-/-}$ mice was highly significant through day 49 ($P < 0.005$). Thus, deletion of the gene for the IFN-γ receptor had an adverse effect on the host immune response, resulting in significantly de-

TABLE 1. Effect of IFN-γ receptor deletion on pulmonary recruitment of leukocytes in IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice infected with *C. neoformans*

Leukocyte type	Leukocyte count[a] (millions/lung) postinfection:					
	Wk 0		Wk 2		Wk 5	
	IFN-γR$^{+/+}$	IFN-γR$^{-/-}$	IFN-γR$^{+/+}$	IFN-γR$^{-/-}$	IFN-γR$^{+/+}$	IFN-γR$^{-/-}$
Total leukocytes	17 ± 0.8	18 ± 2.2	61 ± 9.7	50 ± 4.2	52 ± 5.2	170 ± 19*
Macrophages	12 ± 0.8	13 ± 2.1	23 ± 4.3	23 ± 2.4	20 ± 2.2	28 ± 4.0
Neutrophils	0.3 ± 0.1	0.3 ± 0.3	2.0 ± 1.8	2.5 ± 0.6	5.9 ± 1.7	57 ± 16**
Eosinophils	0.1 ± 0.1	0.2 ± 0.1	11 ± 1.5	13 ± 1.4	0.8 ± 0.3	30 ± 4.2**
Lymphocytes	5.1 ± 0.6	4.6 ± 0.5	20 ± 3.4	13 ± 0.9*	25 ± 2.1	59 ± 6.3*
CD4$^+$ T cells	2.5 ± 0.2	2.4 ± 0.3	8.1 ± 1.4	5.5 ± 0.4	14 ± 1.3	26 ± 2.9*
CD8$^+$ T cells	1.0 ± 0.1	1.0 ± 0.2	3.8 ± 1.2	2.4 ± 0.4	6.8 ± 1.6	17 ± 3.0*
B220$^+$ cells	1.6 ± 0.3	1.1 ± 0.2	8.2 ± 1.6	5.2 ± 0.5	4.5 ± 0.6	13 ± 1.4*

[a] Whole lungs were removed and enzymatically digested to disperse lung leukocytes. Cell counts were then performed, and total lung leukocytes and subsets were identified either by differential counting on cytospun slides or by immunofluorescent staining (total leukocytes and lymphocyte subsets) as outlined in Materials and Methods. Recruited leukocytes in infected mouse = (total number of leukocytes in infected mouse) − (mean number of leukocytes in uninfected mice). *, $P < 0.001$, and **, $P < 0.01$, compared to those for wild-type mice at the same time points; $n = 12$ for each group (two separate experiments of four and eight each). Values are given as means ± SEM.

creased survival following pulmonary infection with *C. neoformans*.

Effect of IFN-γ receptor deletion on pulmonary growth and dissemination of *C. neoformans* in infected IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice. To determine if fungal burden or differences in cryptococcal dissemination could explain why the IFN-γR$^{-/-}$ mice were dying from the infection, mice were infected with 10^4 CFU of *C. neoformans* and cryptococcal burdens in the lungs, spleens, and brains were determined at weeks 2 and 5 postinfection. There was no difference in lung CFU between IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice at week 2 (Fig. 1B). The number of CFU in the lungs of IFN-γR$^{+/+}$ mice diminished from week 2 to week 5, while the number of CFU in the IFN-γR$^{-/-}$ mice remained high in this period. By week 5, IFN-γR$^{-/-}$ mice had significantly higher pulmonary CFU than IFN-γR$^{+/+}$ mice. Thus, IFN-γ receptor is required for long-term control of *C. neoformans* growth in the lungs.

Dissemination of the infection to the spleen and brain was analyzed. The majority of mice from both strains (9 out of 12 in the wild-type mice, 10 out of 12 in the IFN-γR$^{-/-}$ mice) already had cryptococcal dissemination to the spleens at week 2 (Fig. 1B), and there was no significant difference in spleen CFU between the two strains at this time point. By week 5, IFN-γR$^{-/-}$ mice had significantly higher CFU in the spleen compared to IFN-γR$^{+/+}$ mice. No dissemination of cryptococci to the brain in either group occurred by week 2. By week 5, while no brain dissemination occurred in IFN-γR$^{+/+}$ mice, we detected cryptococci in the brains of 4 out of 12 IFN-γR$^{-/-}$ mice. Thus, the organ CFU data correlated with the increased mortality in IFN-γR$^{-/-}$ mice.

Effect of IFN-γ receptor deletion on lung leukocyte recruitment in *C. neoformans*-infected IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice. We next investigated whether pulmonary leukocyte recruitment in response to *C. neoformans* infection was impaired in the absence of IFN-γ receptor, thereby potentially contributing to the decreased clearance of *C. neoformans*. IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice were inoculated intratracheally with *C. neoformans* (10^4 CFU), and the total number of lung leukocytes (CD45$^+$) present in whole lung digests was determined at weeks 0, 2, and 5 postinoculation (Table 1). There was a dramatic increase in lung leukocyte numbers in both IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice at 2 weeks postinfection. In the IFN-γR$^{+/+}$ mice, the number of CD45$^+$ cells did not significantly change between weeks 2 and 5. At week 2, IFN-γR$^{-/-}$ mice showed no significant difference from IFN-γR$^{+/+}$ mice, but by week 5, IFN-γR$^{-/-}$ showed a marked increase in inflammatory cells (175.9 ± 19.2 versus 57.7 ± [5.2 × 10^6] CD45$^+$ cells/lung, respectively). Thus, cellular recruitment in response to pulmonary *C. neoformans* infection was not impaired in the absence of IFN-γ receptor. Rather, the pulmonary inflammatory response was augmented in IFN-γR$^{-/-}$ mice.

Effect of IFN-γ receptor deletion on recruitment of myeloid subsets to the pulmonary parenchyma in response to *C. neoformans* infection. We therefore sought to determine which leukocyte subsets were recruited into the lungs of IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice in response to *C. neoformans* infection. Leukocytes were isolated from whole lungs by enzymatic dispersion, and the leukocyte subsets were analyzed as described in Materials and Methods. At week 2, the numbers of macrophages recruited into the lungs of IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice were similar (Table 1). By week 5, the absolute number of macrophages remained similar, although the percentage of macrophages and monocytes in total leukocytes was higher in IFN-γR$^{+/+}$ mice than in IFN-γR$^{-/-}$ mice (41% versus 16%). There were no significant differences in neutrophil recruitment between IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice at week 2. However, at week 5, there were almost 10 times more neutrophils in the lungs of IFN-γR$^{-/-}$ mice (Table 1). Another considerable difference between IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice was the recruitment of eosinophils into infected lungs. At week 2, eosinophil recruitment was similar in both mouse strains. By week 5, the eosinophil numbers were profoundly decreased in IFN-γR$^{+/+}$ mice while eosinophilia was markedly increased in IFN-γR$^{-/-}$ mice (Table 1). Thus, IFN-γR$^{+/+}$ mice develop a transient pulmonary eosinophilia in the lungs in response to *C. neoformans* infection, while IFN-γR$^{-/-}$ mice develop a chronic pulmonary eosinophilia during the course of infection. The chronic pulmonary eosinophilia in *C. neoformans*-infected IFN-γR$^{-/-}$ mice was evident by histological analysis. At 5

week postinfection, the lungs of infected IFN-$\gamma R^{+/+}$ mice showed only localized areas of leukocyte infiltration, whereas IFN-$\gamma R^{-/-}$ mice exhibited extensive areas of inflammatory infiltration (Fig. 2A and B, respectively). High-power examination of IFN-$\gamma R^{+/+}$ mouse lungs showed the localized infiltrate was predominantly mononuclear cells, whereas affected areas in IFN-$\gamma R^{-/-}$ mouse lungs contained massive eosinophil and neutrophil infiltrates (Fig. 2C and D, respectively). The pronounced pulmonary eosinophilia in the *C. neoformans*-infected IFN-$\gamma R^{-/-}$ mice is suggestive of a T2-type immune response. Previous studies have suggested that eosinophils play a role in the pulmonary fibrosis (6, 36). Masson's trichrome staining for collagen and other matrix proteins from lung sections obtained at day 35 postinfection showed that the lungs of IFN-$\gamma R^{-/-}$ mice (Fig. 2F) have much more intense matrix protein deposition than those of IFN-$\gamma R^{+/+}$ mice (Fig. 2E). Taken together, deficient IFN-γ signaling during pulmonary *C. neoformans* infection results in the development of a chronic granulocytic influx (neutrophils and eosinophils) and pulmonary fibrosis.

Effect of IFN-γ receptor deletion on recruitment of lymphocyte subsets to the pulmonary parenchyma in response to *C. neoformans* infection. Next, we analyzed the recruitment of lymphocytes into infected lungs by flow cytometry. At week 2, the numbers of lymphocytes were significantly lower in the lungs of IFN-$\gamma R^{-/-}$ mice than they were in IFN-$\gamma R^{+/+}$ mice (Table 1). In contrast, at week 5, total lymphocyte recruitment in the IFN-$\gamma R^{-/-}$ mice was markedly higher than that in IFN-$\gamma R^{+/+}$ mice. But the percentage of lymphocytes in the total lung leukocyte homogenate was actually higher in IFN-$\gamma R^{+/+}$ mice (47% versus 34%). The increase in lymphocyte numbers at week 5 in IFN-$\gamma R^{-/-}$ mice occurred in all three subsets of lymphocytes ($CD4^+$, $CD8^+$, and B cells) but was particularly notable for $CD8^+$ T cells and B cells ($B220^+$). Thus, IFN-γR deficiency produced a marked lymphocytic infiltrate in the lungs as the infection progressed.

Production of MIP-2 and KC chemokines by lung leukocytes from *C. neoformans*-infected IFN-$\gamma R^{+/+}$ and IFN-$\gamma R^{-/-}$ mice. As described above, a profound pulmonary neutrophilia was observed in the infected IFN-$\gamma R^{-/-}$ mice after 5 weeks. We suspected that this phenomenon could be caused by the overproduction of chemokines chemotactic for neutrophils. To verify this, we determined the levels of chemokines MIP-2 and KC of lung cell cultures from infected IFN-$\gamma R^{+/+}$ and IFN-$\gamma R^{-/-}$ mice. To our surprise, we found that IFN-$\gamma R^{+/+}$ mice produced significantly higher amounts of MIP-2 and KC than IFN-$\gamma R^{-/-}$ mice did after 3 and 4 weeks of infection (Fig. 3). Thus, the data suggest that chemotactic mediators for neutrophils are not the limiting factors for neutrophil infiltration into the lung, as both strains of mice produced sufficient amounts of MIP-2 and KC chemokines. Next, we hypothesized that IFN-$\gamma R^{-/-}$ mice, with their high blood neutrophil concentration, might account for the pulmonary neutrophilia at the late stage of infection. Blood leukocytes were obtained from mice at weeks 3 and 4 postinfection, and differential counts were made on cytospin slides. As shown in Table 2, the total number of blood leukocytes from IFN-$\gamma R^{-/-}$ mice was higher than that from IFN-$\gamma R^{+/+}$ mice. The neutrophil predominance in the population is the main difference between the two strains. The numbers of blood neutrophils in IFN-$\gamma R^{-/-}$ mice are three to almost six times those in the IFN-$\gamma R^{+/+}$ mice at 3 and 4 weeks postinfection, respectively. Thus, the pulmonary neutrophilia seen in the lungs of infected IFN-$\gamma R^{-/-}$ mice is a result of the accumulation of neutrophils in the circulation.

Lack of IFN-γ signaling impairs the anticryptococcal capacity of recruited lung macrophages. It has been demonstrated that macrophages can acquire anticryptococcal activity in response to IFN-γ (3). In this study, we demonstrated that mice lacking the IFN-γ receptor have a high cryptococcal burden in the lungs at 5 weeks postinoculation. Experiments were designed to test whether the anticryptococcal activity of pulmonary phagocytic cells was impaired. Adherent lung macrophages were obtained from day 28-infected lungs and were cultured at 10^5 cells per well in 96-well plates. At the beginning of the culture, IFN-$\gamma R^{+/+}$ macrophages had a higher cryptococcal burden than IFN-$\gamma R^{-/-}$ macrophages, indicating that the former had ingested more yeasts ($t = 0$ h [Fig. 4A]). After 24 h, the cryptococcal burdens in IFN-$\gamma R^{+/+}$ macrophage cultures were significantly diminished, while IFN-$\gamma R^{-/-}$ macrophage cultures had markedly increased cryptococcal burdens ($t = 24$ h [Fig. 4A]). Indeed, bursting clusters of cryptococci at the surrounding area of macrophages were observed (Fig. 4B). Thus, the data indicate that IFN-$\gamma R^{-/-}$ macrophages have impaired phagocytic activity and are incapable of killing yeast cells in the absence of IFN-γ signaling.

Cytokine production by lung cultures from both *C. neoformans*-infected IFN-$\gamma R^{+/+}$ and IFN-$\gamma R^{-/-}$ mice. Since IFN-$\gamma R^{-/-}$ mice had larger numbers of eosinophils and increased cryptococcal burden in the lungs at week 5 postinfection, we investigated whether IFN-$\gamma R^{-/-}$ mice might produce more T2 cytokines, such as IL-4, IL-5, and IL-10, in their lungs at this time point. We determined the production of various cytokines by lung leukocytes isolated from both IFN-$\gamma R^{-/-}$ and IFN-$\gamma R^{+/+}$ mice infected with *C. neoformans* at week 5. The lung cells from IFN-$\gamma R^{-/-}$ mice produced significantly higher amounts of IL-4, IL-5, and IL-10 than did those from IFN-$\gamma R^{+/+}$ mice (Fig. 5). Interestingly, it was also noted that IFN-$\gamma R^{-/-}$ mice produce more IFN-γ, most likely due to the lack of IFN-γ signaling similar to the increased monocyte chemotactic protein 1 production observed in *C. neoformans*-infected $CCR2^{-/-}$ mice (43). Thus, in the absence of IFN-γ signaling, elevated levels of T2 cytokines were produced by lung leukocytes from *C. neoformans*-infected IFN-$\gamma R^{-/-}$ mice.

DISCUSSION

To verify the role of IFN-γ in the regulation of T1 and T2 immune responses during pulmonary infection with *C. neoformans*, we infected IFN-$\gamma R^{+/+}$ and IFN-$\gamma R^{-/-}$ mice intratracheally with *C. neoformans* and analyzed the resulting immune response. We show that IFN-γ signaling is required for generating a protective host defense against cryptococcal infection. The immune response in IFN-$\gamma R^{-/-}$ mice was characterized by uncontrolled growth of *C. neoformans*, chronic pulmonary neutrophilia and eosinophilia, epithelial disruption of lower respiratory tract (data not shown), and tissue fibrosis.

IFN-γ receptor is required for T1 protective immune responses in lungs. In the absence of IFN-γ receptor, our results suggest that the infected mice eventually die from immunopathological pulmonary damage instead of dying from enceph-

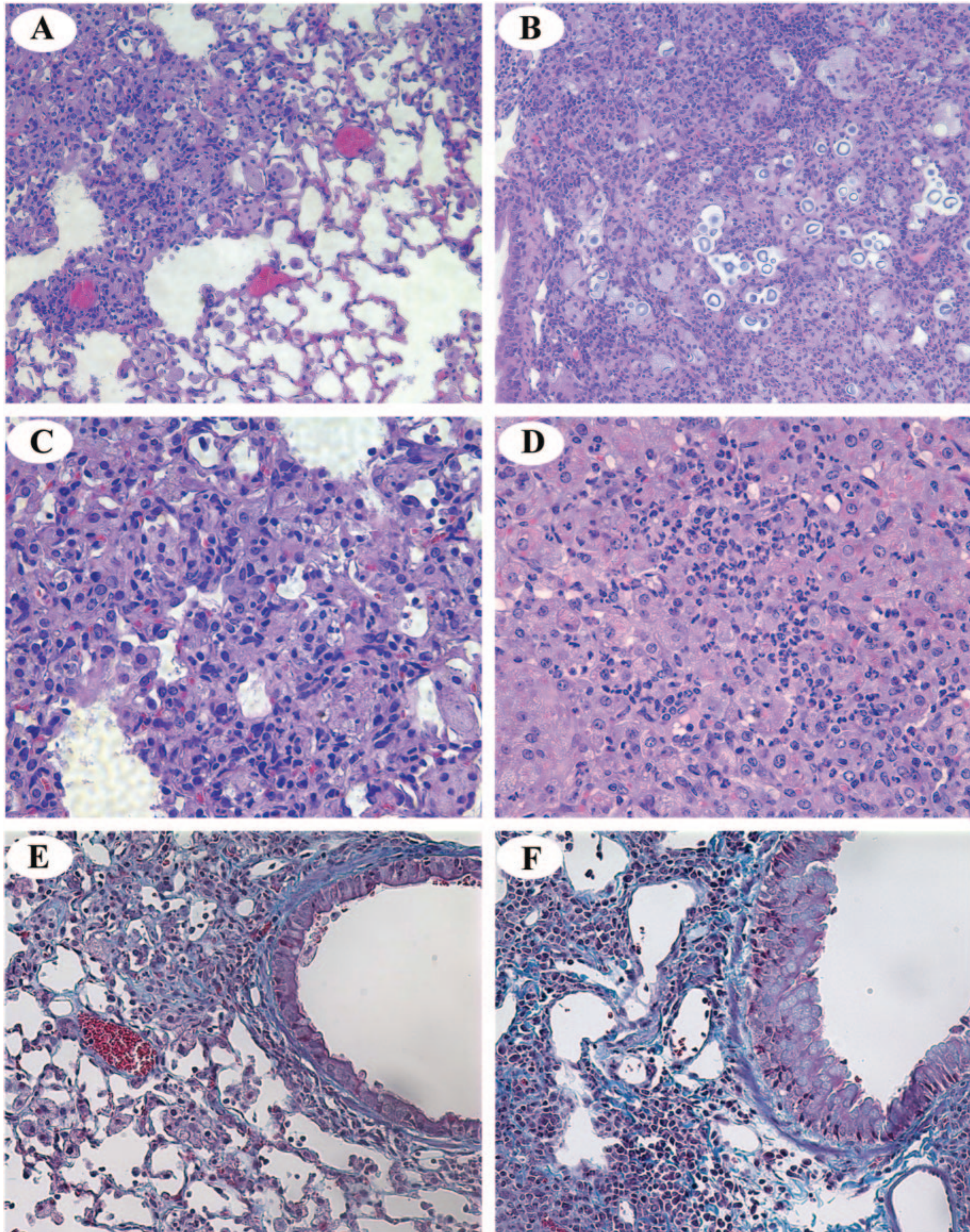

FIG. 2. Lung pathology in IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice infected with *C. neoformans* at 5 weeks postinfection. (A) Photomicrograph of hematoxylin-and-eosin-stained sections from the lung of a *C. neoformans*-infected IFN-γR$^{+/+}$ mouse (magnification, ×66). Note only the localized area of leukocyte infiltrate. (B) Photomicrograph of hematoxylin-and-eosin-stained sections from the lung of a *C. neoformans*-infected IFN-γR$^{-/-}$ mouse (magnification, ×66). Note the widespread leukocyte infiltrates surrounding numerous encapsulated cryptococci. (C) High-powered examination of an inflammatory focus in an IFN-γR$^{+/+}$ mouse lung showing the leukocyte infiltrate with predominantly mononuclear cells and no obvious cryptococci (magnification, ×132). (D) High-powered examination of an inflammatory focus in IFN-γR$^{-/-}$ mouse lung showing massive infiltration of eosinophils (magnification, ×132). (E. and F) Representative lung sections were stained with Masson trichrome for collagen and other matrix proteins showing that the IFN-γR$^{-/-}$ mouse (panel F) had more intense deposition than the IFN-γR$^{+/+}$ mouse (panel E) (magnification, ×66).

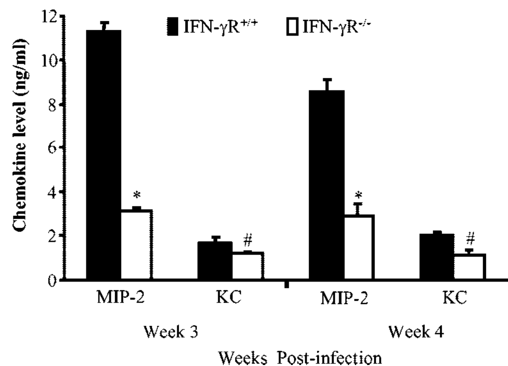

FIG. 3. Production of MIP-2 and KC chemokines by lung leukocytes from *C. neoformans*-infected IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice. At week 3 and week 4 postinoculation, total lung leukocytes were isolated and cultured at 5×10^6 cells/ml with heat-killed strain 52D for 24 h. Culture supernatants were analyzed by ELISA for chemokines as described in Materials and Methods. #, $P < 0.05$, and *, $P < 0.001$, compared to those for wild-type mice at the same time points; $n = 9$ per data point, pooled from two separate experiments. Values are given as means ± SEM.

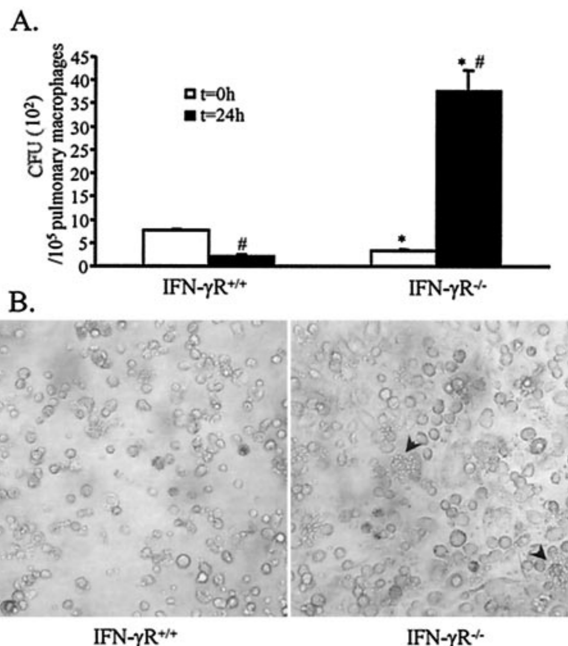

FIG. 4. (A) Cryptococcal burdens of lung macrophages from *C. neoformans*-infected IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice. At week 4 postinoculation, total lung leukocytes were isolated and cultured in 60-mm-diameter petri dishes at 5×10^6 cells/ml. After 24 h, plates were washed three times with HBSS to remove nonadherent cells. Adherent lung macrophages were isolated with trypsin-EDTA and then cultured at 10^5 cells/well in 96-well plates. CFU were determined as described above at 0 and 24 h after culture. *, $P < 0.001$, compared to cultures from wild-type mice at the same time point; #, $P < 0.001$, compared to cultures from the same mice at the different time point; $n = 8$ per data point, pooled from two separate experiments. Values are given as means ± SEM. (B) Photomicrographs of lung macrophages cultured in vitro for 24 h. Cryptococcal foci (shown with arrows) grew in cultures of lung macrophages from IFN-γR$^{-/-}$ mice, but not those from IFN-γR$^{+/+}$ mice.

alitis. That is the reason that we focus on the pulmonary responses in this study. A surprising finding was that even in the absence of IFN-γ receptor, there were more inflammatory cells recruited into the lungs of IFN-γR$^{-/-}$ mice, notably increased numbers of eosinophils, neutrophils and T cells. This strongly indicates that cellular recruitment in response to cryptococcal infection was not impaired in the absence of IFN-γ signaling. However, the numerous extracellular organisms seen in the lung tissue section of day 35 IFN-γR$^{-/-}$ mice (Fig. 2B) suggest that cryptococcal killing by macrophages is impaired.

The ratio of macrophages (2×10^7) to cryptococci (10^5) in the lungs of IFN-γR$^{+/+}$ mice is about 100 times higher than that (2.7×10^7 macrophages to 1.3×10^7 cryptococci) in the IFN-γR$^{-/-}$ mice. Thus, in the absence of IFN-γ signaling, pulmonary macrophages are recruited but not activated. These observations are supported by in vitro experiments showing that isolated IFN-γR$^{-/-}$ macrophages from day 28-infected lung have a diminished ability to phagocytose and kill cryptococci.

Our results indicate that the host response in 129/J mice is similar to that in BALB/c mice. This observation is intriguing because mice with different genetic backgrounds differ in their susceptibility to pulmonary cryptococcal infection, and this correlates with the degree of pulmonary eosinophilia. C57BL/6 mice, which mount a chronic pulmonary eosinophilia during the course of infection, are more susceptible to cryptococcal infection than CBA and C.B-17 mice, which develop no or little eosinophilia and greater resistance to infection (11–13, 16). BALB/c mice develop a transient pulmonary eosinophilia at the early stage of infection and are considered to have intermediate resistance to cryptococcal infection (12). Infiltrated eosinophils could be detected in the lungs of both groups at 2 weeks after infection. In wild-type controls, the number of eosinophils decreased greatly between weeks 2 and 5 postinfection, while in IFN-γR$^{-/-}$ mice, eosinophil numbers continued to increase markedly. IFN-γR$^{-/-}$ mice infected with *Mycobacterium bovis* BCG also developed a nonprotective pulmonary eosinophilia in the lung (23). It has been suggested that IFN-γ may enhance eosinophil apoptosis (29). Thus, in the absence of IFN-γ signaling, mice are unable to control the influx of eosinophils to the site of infection. The eosinophil influx indeed may exacerbate the cryptococcal infection by interfering with macrophage activation and function or by providing an intracellular habitat which promotes cryptococcal growth.

The mechanisms underlying the high neutrophil level in

TABLE 2. Blood leukocytes obtained from infected IFN-γR$^{+/+}$ and IFN-γR$^{-/-}$ mice[a]

Wks post infection	No. of blood leukocytes (10^3)[b]			
	IFN-γR$^{+/+}$		IFN-γR$^{-/-}$	
	Total WBC	Neutrophils	Total WBC	Neutrophils
3	2.8 ± 0.5	1.6 ± 0.4	6.6 ± 1.1*	4.8 ± 0.7**
4	3.7 ± 0.6	2.5 ± 0.4	16 ± 1.2**	14 ± 1.2**

[a] Blood was obtained from the tail and analyzed by cytospin. *, $P < 0.01$; **, $P < 0.001$ compared to those for wild-type mice at the same time points; $n = 9$ for each group (two separate experiments of four and five each). Values are given as means ± SEM.
[b] WBC, white blood cells.

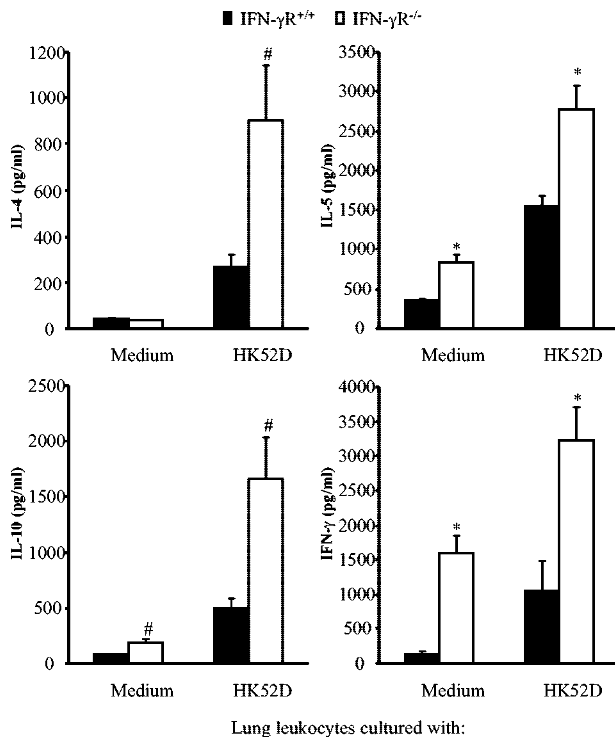

FIG. 5. Production of T1- and T2-type cytokines by lung leukocytes from *C. neoformans*-infected IFN-$\gamma R^{+/+}$ and IFN-$\gamma R^{-/-}$ mice. At week 5 postinoculation, total lung leukocytes were isolated and cultured at 5×10^6 cells/ml for 24 h in vitro with or without heat-killed strain 52D. Culture supernatants were analyzed by ELISA for cytokines as described in Materials and Methods. #, $P < 0.05$, and *, $P < 0.005$, compared to those for wild-type mice at the same time points; $n = 9$ or 12 per data point, pooled from four separate experiments. Values are given as means ± SEM.

IFN-$\gamma R^{-/-}$ mice are still unclear. Our data indicate that production of chemotactic factors for neutrophils at the site of infection is not the cause for the neutrophilia observed in IFN-$\gamma R^{-/-}$ mice, as wild-type mice produce even higher amounts of MIP-2 and KC in the lungs. It is possible that the elevated peripheral neutrophil concentrations in IFN-$\gamma R^{-/-}$ mice could be the result of the accumulation of neutrophils in the circulation because of enhanced neutrophil survival. It has been shown that neutrophils can undergo apoptosis in response to IFN-γ (30).

Alternatively, altered hematopoiesis, mediated by a disrupted regulatory feedback loop, might cause the bone marrow to increase neutrophil production. It will be intriguing to determine the levels of granulocyte colony-stimulating factor and IL-17 in the mice in serum, as they have been described to induce increased granulopoiesis (25, 38). Another possible explanation for increased neutrophil numbers in the presence of lower CXC chemokine levels is that adhesion receptors on the leukocytes may be activated to a high-affinity state in IFN-$\gamma R^{-/-}$ mice, which can modify the migration of leukocytes into tissues (28).

The abundant neutrophil recruitment observed in IFN-$\gamma R^{-/-}$ mice could cause the lung damage. Activated neutrophils have been shown to be capable of killing or inhibiting the growth of *C. neoformans* in vitro and in vivo in the periphery but not in the lungs (27, 33). Studies by Herring et al. have also shown that neutrophils do not play a role in clearing *C. neoformans* from the lung (8). Eosinophils were present in the lungs over the course of infection and were accompanied by eosinophilic crystal deposition and tissue fibrosis in the lungs of IFN-$\gamma R^{-/-}$ mice. Uncontrolled growth of *C. neoformans* at a later stage of infection can also result in destruction of lung architecture. Taken together, the combination of uncontrolled cryptococcal growth, inactivated macrophages, pulmonary neutrophilia and eosinophilia, damaged epithelia, and tissue fibrosis likely produce the lung damage and lethality observed in IFN-$\gamma R^{-/-}$ mice.

IFN-γ has been described as playing a role in determining the development of T1- and T2-type immune responses by blocking T2-cell development (5, 39, 44). Because IL-5, a T2 cytokine, is responsible for the recruitment and development of eosinophils during cryptococcosis (12), it would be reasonable to expect that pulmonary leukocytes isolated from IFN-$\gamma R^{-/-}$ mice would have a tendency toward generating more T2 cytokines than the wild type in response to cryptococcal infection. Analyzing the pattern of cytokine production by lung-infiltrating leukocytes showed that there was no significant difference between two strains at week 2 postinfection (data not shown). In contrast, at week 5, IFN-$\gamma R^{-/-}$ mice produced significantly higher amounts of IL-4, IL-5, and IL-10 than those from control mice. Several findings have clearly shown that IFN-γ is required for initiating a protective T1 response to pulmonary cryptococcosis in resistant mice (1, 2, 11, 17–19, 35). Previous studies using neutralizing anti-IFN-γ antibodies demonstrated that this cytokine was critical for host resistance against cryptococcal infection (1, 11, 37). Its protective activity was mediated by stimulating the macrophage production of fungicidal mediators including nitric oxide (41). Using IFN-γ gene-disrupted mice, Kawakami et al. (17) have recently demonstrated that IFN-γ is required for host resistance to cryptococcal infection. Taken together, in the present study, this cytokine likely exerts its ability to restrict the infection in IFN-$\gamma R^{+/+}$ mice through the aforementioned mechanisms.

An unexpected result was that the elevated production of IL-4, IL-5, and IL-10 in the IFN-$\gamma R^{-/-}$ mice did not correlate with a decrease in IFN-γ production. Indeed, IFN-γ was produced and accumulated in IFN-$\gamma R^{-/-}$ mice. Four very intriguing points were raised from this surprising observation. (i) There is a source of IFN-γ production that appears to be independent of regulation by IL-4, IL-5, and IL-10. This observation is supported by two previous reports showing that IFN-γ-secreting Th1 cells were also identified in the IFN-$\gamma R^{-/-}$ mice infected with *Leishmania major* and *M. bovis* BCG (4, 40). (ii) The presence of IFN-γ is important to induce a protective response to *C. neoformans*. Accumulation of IFN-γ in susceptible IFN-$\gamma R^{-/-}$ mice not only shows that the presence of IFN-γ is not sufficient to induce a protective response but also verifies that IFN-γ–IFN-γR interactions are required for IFN-γ to be fully utilized in order to produce a protective effect. (iii) Our results suggest that IFN-γ signaling is not required for the production of IFN-γ-secreting cells in an immune response induced by *C. neoformans*. (iv) In the absence of IFN-γ signaling, IFN-$\gamma R^{-/-}$ mice did not default toward a T2 response, but rather both T1 and T2 subsets were likely generated during the infection.

In conclusion, we demonstrate that IFN-γ is a potent cytokine for host resistance against infection with *C. neoformans*. Over the first 5 weeks of infection, there are two phases of cell-mediated immune response to *C. neoformans*. An IFN-γ-independent phase (the first 2 weeks postinfection) is characterized by almost exactly the same inflammatory response in both infected IFN-$\gamma R^{+/+}$ and IFN-$\gamma R^{-/-}$ mice, indicating IFN-γ is not an innate effector molecule. The second phase (week 2 to week 5) is IFN-γ dependent, and IFN-$\gamma R^{-/-}$ mice are unable to control the growth of *C. neoformans* and eventually develop a T2 response, allowing disease to progress. Thus, IFN-γ may signal for T1-T2 differentiation during the afferent phase of the response, but the effects of defective IFN-γ signaling do not manifest themselves until the efferent phase of the response.

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