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Role of IFN-γ in Regulating T2 Immunity and the Development of Alternatively Activated Macrophages during Allergic Bronchopulmonary Mycosis[1]

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Pulmonary *Cryptococcus neoformans* infection of C57BL/6 mice is an established model of a chronic pulmonary fungal infection accompanied by an "allergic" response (T2) to the infection, i.e., a model of an allergic bronchopulmonary mycosis. Our objective was to determine whether IFN-γ plays a role in regulating the pulmonary T2 immune response in *C. neoformans*-infected C57BL/6 mice. Long-term pulmonary fungistasis was lost in IFN-γ knockout (KO) mice, resulting in an increased pulmonary burden of fungi at wk 3. IFN-γ was required for the early influx of leukocytes into the lungs but was not required later in the infection. By wk 3, eosinophil and macrophage numbers were elevated in the absence of IFN-γ. The inducible NO synthase to arginase ratio was lower in the lungs of IFN-γ KO mice and the macrophages had increased numbers of intracellular cryptococci and YM1 crystals, indicative of alternatively activated macrophages in these mice. There was evidence of pulmonary fibrosis in both wild-type and IFN-γ KO mice by 5 wk postinfection. IFN-γ production was not required for the development of T2 cytokine (IL-4, IL-5, IL-13) producing cells in the lungs and lung-associated lymph nodes or induction of an IgE response. At a number of time points, T2 cytokine production was enhanced in IFN-γ KO mice. Thus, in the absence of IFN-γ, C57BL/6 mice develop an augmented allergic response to *C. neoformans*, including enhanced generation of alternatively activated macrophages, which is accompanied by a switch from a chronic to a progressive pulmonary cryptococcal infection. ***The Journal of Immunology,*** **2005, 174: 6346–6356.**

Pulmonary *Cryptococcus neoformans* infection of C57BL/6 mice is an established model of an allergic bronchopulmonary mycosis, i.e., a chronic pulmonary fungal infection that is accompanied by an "allergic" response (T2) to the active infection. This model has been used to address the role of immunomodulatory agents such as OX40, *Mycobacterium* bacillus Calmette-Guérin, α-galactosylceramide (a CD1 ligand), IL-5 antagonists, and anti-capsular Abs in addition to antifungal drugs in modulating immunity and promoting protective host responses (1–8). Compared with "resistant" mouse strains such as CBA/J, BALB/c, and C.B-17, lung leukocytes from *C. neoformans*-infected "susceptible" C57BL/6 mice produce less IFN-γ and more IL-5 following intratracheal infection (9–11). The mice develop a chronic pulmonary *C. neoformans* infection that is accompanied by a pulmonary eosinophil infiltrate (10, 11). Between wk 5 and 7 postinfection, significant amounts of the eosinophilic crystalline protein YM1 begin to accumulate in the lungs, and the macrophages harbor large numbers of cryptococci (11, 12). C57BL/6 mice can survive >12 wk while harboring a stable *C. neoformans* burden in the lungs of 10^6–10^7 CFU (10, 11).

So, what is the role of IFN-γ in regulating the chronicity of the pulmonary infection in this murine model of allergic bronchopulmonary mycosis (ABPM)?[3] Although C57BL/6 mice make less IFN-γ than C.B-17 and BALB/c mice in response to *C. neoformans* infection, they still make significant amounts of IFN-γ (10). One possibility is that the amount of IFN-γ produced is insufficient to drive protective T1 immunity. Another possibility is that IFN-γ production does not regulate the T2 response in C57BL/6 mice. Can T1 and T2 responses coexist in the lungs without cross-regulating each other? In one set of studies, Th1 and Th2 cells from mice expressing a TCR that is specific for an influenza PR8 hemagglutinin peptide (SFE) were adoptively transferred into naive recipients (13). Following intratracheal challenge of mice with the peptide, the SFE-specific Th1 cells did not inhibit SFE (Ag) or infection (influenza)-induced lung eosinophilia demonstrating that Th2-mediated lung inflammation can coexist with a Th1-mediated response (neutrophilia) stimulated by the same Ag/infection (13). In OVA-immunized IFN-γR-deficient mice, there is no difference in pulmonary eosinophilia or production of IL-4 and IL-5 from lung T cells following repeated airway OVA challenge compared with that observed in wild-type (WT) mice (14), again demonstrating that airway T2 responses can exist that are not cross-regulated by IFN-γ.

Another possibility is that IFN-γ may augment the airway T2 response to *C. neoformans*. T2-mediated allergic lung inflammation is often associated with a vigorous T1-mediated response (13),

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[3] Abbreviations used in this paper: ABPM, allergic bronchopulmonary mycosis; aaMac, alternatively activated macrophage; caMac, classically activated macrophage; LALN, lung-associated lymph nodes; KO, knockout; WT, wild type; ABPA, allergic bronchopulmonary aspergillosis; HKC, heat-killed cryptococci; SFE, influenza PR8 hemagglutinin peptide; iNOS, inducible NO synthase.

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probably owing to the fact that IFN-γ can prime alveolar macrophages to release proinflammatory cytokines during allergic reactions (15). Whereas the effects of IFN-γ and IL-4 are often viewed as antagonistic, IL-18-dependent IFN-γ production is IL-4 dependent in *C. neoformans*-infected C57BL/6 mice (5). Finally, passive transfer of "detrimental" IgG3 anticapsular mAb requires IFN-γ for the negative regulatory effect on host defense (16). Thus, IFN-γ production can also play a role in augmenting T2 responses and/or destructive pathology.

Finally, it has recently been recognized during tumor growth and parasitic infections that both IL-4/IL-13 and IFN-γ can activate macrophages but the phenotype of these macrophages are strikingly different (17–19). Classically activated macrophages (caMac) are generated in high IFN-γ to IL-4/13 environments, and these macrophages produce high levels of NO and proinflammatory cytokines such as TNF-α (17–19). Alternatively activated macrophages (aaMac) are generated in high IL-4/13 to IFN-γ environments. aaMac produce arginase (which decreases NO levels), express the chitinase-related protein YM1, have increased fungal phagocytosis (due to increased mannose receptor expression), have decreased intracellular killing, promote fibrosis, and produce less TNF-α (17–19). Of all these properties, production of YM1 protein is a distinctive histological feature unique to aaMac activation (20–23). The objective of our studies was to determine whether IFN-γ plays a role in either up-regulating or down-regulating the pulmonary T2 immune response in *C. neoformans*-infected C57BL/6 mice and to determine whether the production of IFN-γ plays a critical role in regulating macrophage activation (alternative vs classical) in this T2 environment.

Materials and Methods

Mice

Female WT and IFN-γ KO mice on a C57BL/6 genetic background (16 ± 2 g) were obtained from The Jackson Laboratory. Mice were 6–8 wk of age at the time of infection. Mice were housed in sterilized cages covered with a filter top. Sterile food and water were given ad libitum. The Unit for Laboratory Animal Medicine at University of Michigan (Ann Arbor, MI) maintained the mice, in accordance with regulations approved by the University of Michigan Committee on the Use and Care of Animals.

C. neoformans

C. neoformans strain 52D was obtained from the American Type Culture Collection (24067-E). For injection, yeast were grown to stationary phase (48–72 h) at 37°C in Sabouraud dextrose broth (1% neopeptone and 2% dextrose; Difco) on a shaker. The cultures were then washed in nonpyrogenic saline, counted on a hemocytometer, and diluted to 3.3×10^5 CFU/ml in sterile nonpyrogenic saline.

Surgical intratracheal inoculation

Mice were anesthetized by i.p. injection of pentobarbital (0.074 mg/g weight of mouse) and 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 diluted *C. neoformans* culture. The needle was inserted into the trachea, and 30 μl of inoculum (10^4 CFU) was dispensed into the lungs. The needle was removed, and the skin closed with cyanoacrylate adhesive. The mice recovered with minimal visible trauma.

CFU assay

For determination of lung and lung-associated lymph nodes (LALN) CFU, small aliquots were collected from lung digests or lymph node suspensions, respectively (described below). Ten-microliter aliquots of the lungs and lymph nodes were plated out on Sabouraud dextrose agar plates in duplicate 10-fold dilutions and incubated at room temperature. *C. neoformans* colonies were counted 2–3 days later, and the number of CFU was calculated.

Lung leukocyte isolation

Individual lungs were excised, minced, and enzymatically digested for 30 min in 15 ml of digestion buffer (RPMI 1640, 5% FCS, antibiotics, 1 mg/ml collagenase, and 30 μg/ml DNase). The cell suspension and undigested fragments were further dispersed by drawing 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 by resuspending them in ice-cold NH_4Cl buffer (0.83% NH_4Cl, 0.1% $KHCO_3$, and 0.037% Na_2EDTA, pH 7.4). A 10-fold excess of medium was added to return the solution to isotonicity. The isolated leukocytes were repelleted and resuspended in complete medium. Total lung leukocyte numbers were counted in the presence of trypan blue using a hemocytometer.

Lung leukocyte subsets

Macrophages, neutrophils, and eosinophils were visually counted in Wright-Giemsa-stained samples of lung cell suspensions cytospun onto glass slides (Shandon Cytospin). For Wright-Giemsa staining, the slides were fixed for 2 min with a one-step methanol-based Wright-Giemsa stain (Harleco; EM Diagnostics) followed by steps two and three of the Diff-Quik whole blood stain kit (Diff-Quik, Baxter Scientific). A total of 200–300 cells was counted from randomly chosen high power microscope fields for each sample. The percentage of a leukocyte subset was multiplied by the total number of leukocytes to give the absolute number of that type of leukocyte in the sample.

Numbers of B, CD4, and CD8 T cells were determined by flow cytometry. Lung leukocytes (5×10^5) were incubated for 30 min on ice in a total volume of 120 μl of staining buffer (FA buffer; Difco), 0.1% NaN_3, and 1% FCS. Each sample was incubated with 1 μg of the respective FITC- or PE-labeled mAb (BD Pharmingen), or isotype-matched rat IgG. The samples were washed in staining buffer and fixed in 1% paraformaldehyde (Sigma-Aldrich) in buffered saline. Stained samples were stored in the dark at 4°C until analyzed on a flow cytometer (C; Beckman Coulter). The percentage of a lymphocyte subset was multiplied by the total number of leukocytes to give the absolute number of that type of lymphocyte in the sample.

Induction of T cell deficiency

Mice were treated with 300 μg of anti-CD4 plus 300 μg of anti-CD8 mAb (GK1.5 and YTS 169.4, respectively) on day 0 of the infection and boosted with 100 μg of each mAb at days 7 and 14. T cell depletion was analyzed by flow cytometry of spleen cells. Depletion was >99% for $CD4^+$ T cells and >95% for $CD8^+$ T cells (data not shown).

Hydroxyproline assay

Total lung collagen levels were determined using a previously described assay (24). Briefly, a 1-ml sample of lung homogenate was added to 1 ml of 12 N HCl for a minimum of 8 h at 120°C. To a 5-μl sample of the digested lung, 5 μl of citrate/acetate buffer (5% citric acid, 7.2% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid, pH 6.0) and 100 μl of chloramine-T solution (282 mg of chloramine-T, 2 ml of *n*-propanol, 2 ml of distilled water, and 16 ml of citrate/acetate buffer) were added. The resulting samples were then incubated at room temperature for 20 min before 100 μl of Ehrlich's solution (Aldrich Chemical) was added. These samples were incubated for 20 min at 65°C, and cooled samples were read at 550 nm in a Beckman DU 640 spectrophotometer. Hydroxyproline concentrations were calculated from a standard curve of hydroxyproline. The results are expressed as hydroxyproline levels per gram weight of mouse to control for the increasing collagen content of the lung with increasing whole body weight.

Immunohistochemical analysis of lung tissue

Formalin-fixed paraffin-embedded histological sections were used for immunohistochemical analysis of macrophage cells. Sections were deparaffinized and rehydrated through a xylene/alcohol series to a final wash in PBS. The slides were microwaved in the presence of 10 mM citric acid (pH 6.0) for 15 min for Ag retrieval. To quench endogenous peroxidase activity, samples were incubated with 3% H_2O_2 for 5 min. After blocking the tissue with normal rabbit serum for 20 min, the sections were incubated with either rat isotype control Ab or anti-YM1 mAb (1:500) for 30 min at room temperature. Subsequently, slides were washed in buffer and stained with biotinylated rabbit anti-rat Ig for another 30 min. Finally, slides were incubated with VECTASTAIN ABC *Elite* reagent (Vector Laboratories) and developed using peroxidase substrate solution for 3 min, dehydrated, and mounted. Specimens were examined using light microscope.

RT-PCR

Total RNA was prepared from whole lung samples removed from C57BL/6 (WT) and IFN-γ KO mice 5 wk following challenge with *C. neoformans*. RNA was isolated using TRIzol reagent (Invitrogen Life

Technologies) according to the manufacturer's directions. The purified RNA was subsequently reverse transcribed and DNA was amplified using Access-RT PCR kit (Promega). The following murine oligonucleotide primers (5′-3′ sequences) were used for RT-PCR analysis: arginase-1 sense, CAGAAGAATGGAAGAGTCAG; arginase-1 antisense, CAG ATATGCAGGGAGTCACC; inducible NO synthase (iNOS) sense, TTT GCTTCCATGCTAATGCGAAAG; iNOS antisense, GCTCTGTTGAG GTCTAAAGGCTCCG; β-actin sense, TGGAATCCTGTGGCATCC ATGAAAC; β-actin antisense, TAAAACGCAGCTCAGTAACAGT CCG. Arginase-1 and β-actin were cycled 50 times (denatured at 95°C for 30 s, annealed at 55°C for 5 s and elongated at 72°C for 12 s); iNOS, cycled 35 times (denatured at 95°C for 30 s, annealed at 58°C for 60 s and elongated at 68°C for 90 s). Final amplification step was done for arginase-1 and β-actin at 72°C for 7 min and for iNOS at 68°C for 7 min. After amplification, the samples were separated on a 2% agarose gel containing 0.3 μg/ml ethidium bromide, and bands were visualized and photographed using UV transillumination.

Histology

Lungs were fixed by inflation with 10% neutral buffered formalin. After paraffin embedding, 5-μm sections were cut and stained with H&E, periodic acid Schiff (to stain mucus and mucus-secreting goblet cells), or Masson's trichrome (collagen deposition stains blue).

Preparation of lymph nodes

LALN from two to three mice were pooled and processed into a cell suspension by gently passing tissues through nylon mesh. Cells were then washed and resuspended in complete RPMI 1640 medium. Total viable cell numbers were assessed by trypan blue exclusion and counting on a hemocytometer.

Lung leukocyte and lymph node cultures

Isolated lung leukocytes or lymph node cells (5×10^6/ml) were cultured for 24 h in 24-well plates with 2 ml of complete RPMI 1640 medium at 37°C and 5% CO_2 with or without additional stimulus. Cultures were supplied with heat-killed *C. neoformans* at 1×10^7/ml when indicated.

Cytokine production

Culture supernatants were harvested at 24 h and assayed for IFN-γ, IL-4, IL-5, IL-13, TNF-α, IL-12, and IL-10 production by sandwich ELISA using the manufacturer's instructions supplied with the cytokine-specific kits (BD Pharmingen and R&D Systems).

Total serum IgE

Serum was obtained by tail vein bleed of the mice and spinning the blood to obtain the serum. Serum samples were then assayed using an IgE-specific sandwich ELISA (BD Pharmingen).

Statistics

Analysis of data was conducted using Microsoft Excel. Data are expressed as means ± SE (SEM) for each group of combined data derived from two experiments. Statistical analysis between groups was performed using *t* test, with significance being $p < 0.05$ for comparison between WT and the IFN-γ KO mice.

Results

Effect of IFN-γ deficiency on the pulmonary growth of C. neoformans

We first investigated whether the IFN-γ produced by C57BL/6 mice plays a role in controlling the growth of *C. neoformans* in the lungs. IFN-γ KO and WT C57BL/6 mice were inoculated intratracheally with *C. neoformans*-strained 52D. Lung CFU increased >100-fold in WT mice from the time of infection through wk 1 but then remained relatively level from wk 1–3, with less than a 10-fold difference between wk 1, 2, and 3 (Fig. 1*A*). In contrast, lung CFU in IFN-γ KO mice progressively increased between each time point throughout the study. By wk 3, lung CFU was >30-fold higher in the absence of IFN-γ (Fig. 1*A*). Thus, production of IFN-γ by C57BL/6 mice limits the growth of *C. neoformans* in the lungs; however, the amount of IFN-γ produced is insufficient to clear the infection. The end result is a chronic level of infection.

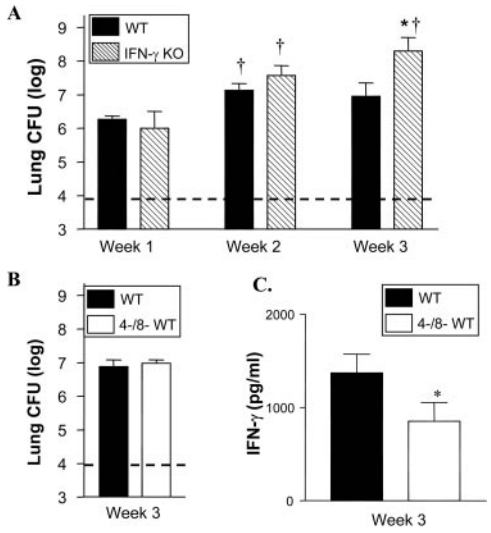

FIGURE 1. Pulmonary fungal burden in WT vs IFN-γ KO (*A*) and CD4/CD8-deficient C57BL/6 mice (*B*) after intratracheal inoculation of *C. neoformans*. Data shown are mean CFU/whole lungs ± SEM. Dashed line represents initial inoculum. To generate a CD4/CD8 T cell deficiency, mice were treated with 300 μg of anti-CD4 plus 300 μg of anti-CD8 mAb (GK1.5 and YTS 169.4, respectively) on day 0 of the infection and boosted with 100 μg of each mAb at days 7 and 14. T cell depletion analyzed by flow cytometry was >99% for $CD4^+$ T cells and >95% for $CD8^+$ T cells (data not shown). ∗, $p < 0.05$ compared with WT mice at the same time point. †, $p < 0.05$ compared with CFU at wk 1 within the same group (WT or IFN-γ KO). $n = 6–8$ mice/group/time point from two independent experiments. *C*, IFN-γ levels in CD4/CD8 T cell-deficient B6 mice as measured by ELISA.

To determine the role of T cells in controlling the infection in WT C57BL/6 mice, WT mice were treated with anti-CD4 and anti-CD8 mAb to render the mice T cell deficient. This resulted in >99% depletion of $CD4^+$ T cells and >95% depletion of $CD8^+$ T cells (data not shown). Removal of T cells from WT mice did not increase lung CFU to the levels seen in IFN-γ KO mice (Fig. 1*B*). We next measured IFN-γ levels in cultures of lung leukocytes from T cell-depleted B6 mice. Depletion of T cells in B6 mice did not eliminate IFN-γ production by lung leukocytes. There was approximately a 50% reduction in IFN-γ levels in cultures from CD4/CD8-deficient mice compared with CD4/CD8-replete B6 mice (Fig. 1*C*). Overall, these results demonstrate the following: 1) elimination of CD4 and CD8 T cells does not eliminate the fungistatic ability in the lungs at wk 3 postinfection; 2) however, in the absence of IFN-γ, this fungistatic ability is lost and the pulmonary burden of *C. neoformans* increases. Thus, there is a significant non-CD4/CD8 T cell source of IFN-γ production in the lungs of *C. neoformans*-infected C57BL/6 mice that is required for fungistasis at wk 3 postinfection.

Effect of IFN-γ deficiency on the pulmonary inflammatory response to C. neoformans

To determine the role of IFN-γ in the development of the pulmonary inflammatory response in C57BL/6 mice, lung leukocyte numbers were enumerated at wk 1, 2, and 3 postinfection. Total lung leukocytes were isolated and quantified following enzymatic digestion of the lungs (described in *Materials and Methods*). The initiation of the inflammatory response in IFN-γ KO mice was delayed by 1 wk compared with the inflammatory response in the WT mice (Fig. 2*A*). However, by wk 2, both IFN-γ KO and WT mice developed significant inflammatory responses in the lungs.

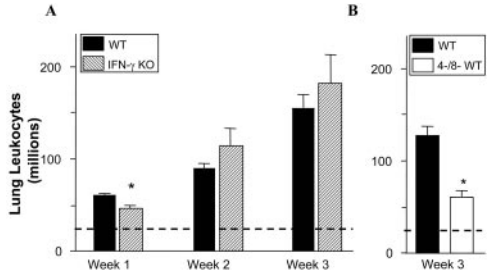

FIGURE 2. Leukocyte recruitment into the lungs of WT, IFN-γ KO, and CD4/CD8-deficient C57BL/6 mice after intratracheal inoculation of *C. neoformans* as described in Fig. 1. Total lung leukocytes were isolated from whole lungs of individual mice following enzymatic digestion. Dashed line represents total leukocytes in the lungs of uninfected mice. Values are means ± SEM. *, $p < 0.05$ compared with WT at the same time point. $n = 6–8$ mice/group/time point from two experiments.

Between wk 2 and 3, the number of inflammatory cells nearly doubled in both groups of mice. Thus, IFN-γ is required for the early influx of leukocyte into the lungs but is not required for leukocyte recruitment later in the infection (after wk 1).

Next, we assessed whether leukocyte recruitment into the lungs of C57BL/6 mice has a T cell-dependent component. WT mice were treated with anti-CD4 and anti-CD8 mAb to render the mice T cell deficient (as described above) before infection. Pulmonary leukocyte recruitment at wk 3 was reduced by 60% in T cell-deficient C57BL/6 mice (Fig. 2*B*). Altogether, these studies demonstrate that pulmonary inflammation at wk 3 can occur in the absence of IFN-γ. However, CD4 and CD8 T cells are required for maximal inflammatory cell recruitment.

Effect of IFN-γ deficiency on the cellular composition of the pulmonary inflammatory response during C. neoformans *infection*

Our next objective was to determine whether the cellular make-up of the inflammatory response changed in the absence of IFN-γ. Leukocytes isolated from whole lung enzymatic digests were identified by Wright-Giemsa stain or flow cytometry. At wk 1, there were only small differences in the types of lung leukocytes between IFN-γ KO and WT mice (Fig. 3). There were no differences in neutrophil numbers between the two groups at any of the time points examined. At wk 2, there was no difference in lymphocyte numbers in the lungs, but at wk 3, T cell recruitment into the lungs of IFN-γ KO mice was significantly less than that in WT mice. In contrast, macrophage recruitment was significantly greater in IFN-γ KO mice at wk 3 compared with WT mice (Fig. 3). Both IFN-γ KO and WT mice developed a prominent pulmonary eosinophilia. However, the numbers of eosinophils were significantly greater in IFN-γ KO compared with WT mice. There were >2-fold more eosinophils in the lungs of IFN-γ KO mice at wk 2 and 3 than in WT mice (Fig. 3). Thus, IFN-γ plays a role in modulating the cellular composition of the inflammatory response such that by wk 3, eosinophil and macrophage numbers were markedly elevated in IFN-γ KO mice.

We further analyzed the pathology of the response in WT and IFN-γ KO mice. Both IFN-γ KO and WT infected mice had extensive areas of consolidated inflammation in the lungs (Fig. 4, *B* and *C*) and fibrosis at wk 3 (Fig. 4, *E* and *F*) compared with the uninfected controls (Fig. 4, *A* and *D*). To quantitate fibrosis, hydroxyproline levels (collagen content) were measured in whole lung samples from uninfected and infected-WT and IFN-γ KO mice. There was an approximately 4-fold increase in the levels of hydroxyproline in infected animals compared with uninfected B6

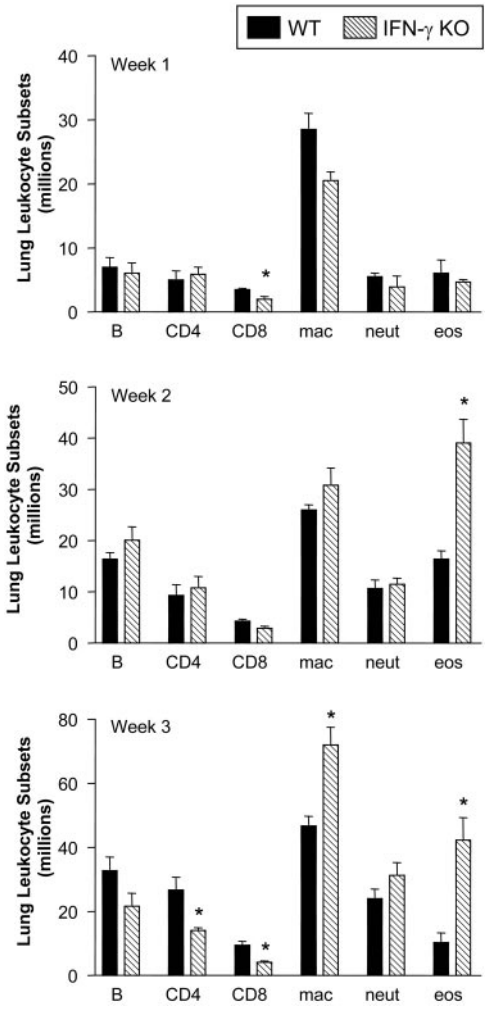

FIGURE 3. Total lung leukocyte differentials in *C. neoformans*-infected WT and IFN-γ KO C57BL/6 mice at wk 1, 2, and 3 postinfection. Total lung leukocytes were isolated from whole lungs of individual mice following enzymatic digestion. Leukocyte suspensions were spun onto slides using cytocentrifuge and stained with Wright-Giemsa for visual quantification of macrophages ¯, neutrophils (neut), and eosinophils (eos), whereas B cells, CD4, and CD8 T cells were analyzed by flow cytometry. Values are means ± SEM. *, $p < 0.05$ compared with WT mice at the same time point. $n = 6–8$ mice/group/time point.

mice. However, there was no significant difference between infected WT and IFN-γ KO mice (Fig. 5*A*). Thus, a pulmonary *C. neoformans* infection in C57BL/6 mice induces an inflammatory response that includes the development of IFN-γ-independent pulmonary fibrosis.

Production of YM1-containing macrophages in IFN-γ KO mice in response to C. neoformans *infection*

In addition to an increase in the number of macrophages (Fig. 3), the macrophages were also morphologically different in IFN-γ KO mice (Fig. 4). As illustrated in Fig. 4*H*, macrophages in IFN-γ KO mice were larger and contained numerous intracellular cryptococci and eosinophilic crystals. Eosinophilic crystals in *C. neoformans*-infected macrophages in vitro have been previously described and were identified as YM1, a chitinase-like protein that is produced by aaMac (12, 18, 21). To confirm that the crystals in the lungs *C. neoformans*-infected IFN-γ KO mice were YM1, we performed immunohistochemical staining using an anti-YM1 mAb on paraffin-embedded lung sections from IFN-γ KO mice at wk 3 postinfection. In contrast to the isotype control mAb (Fig. 4*I*), anti-YM1

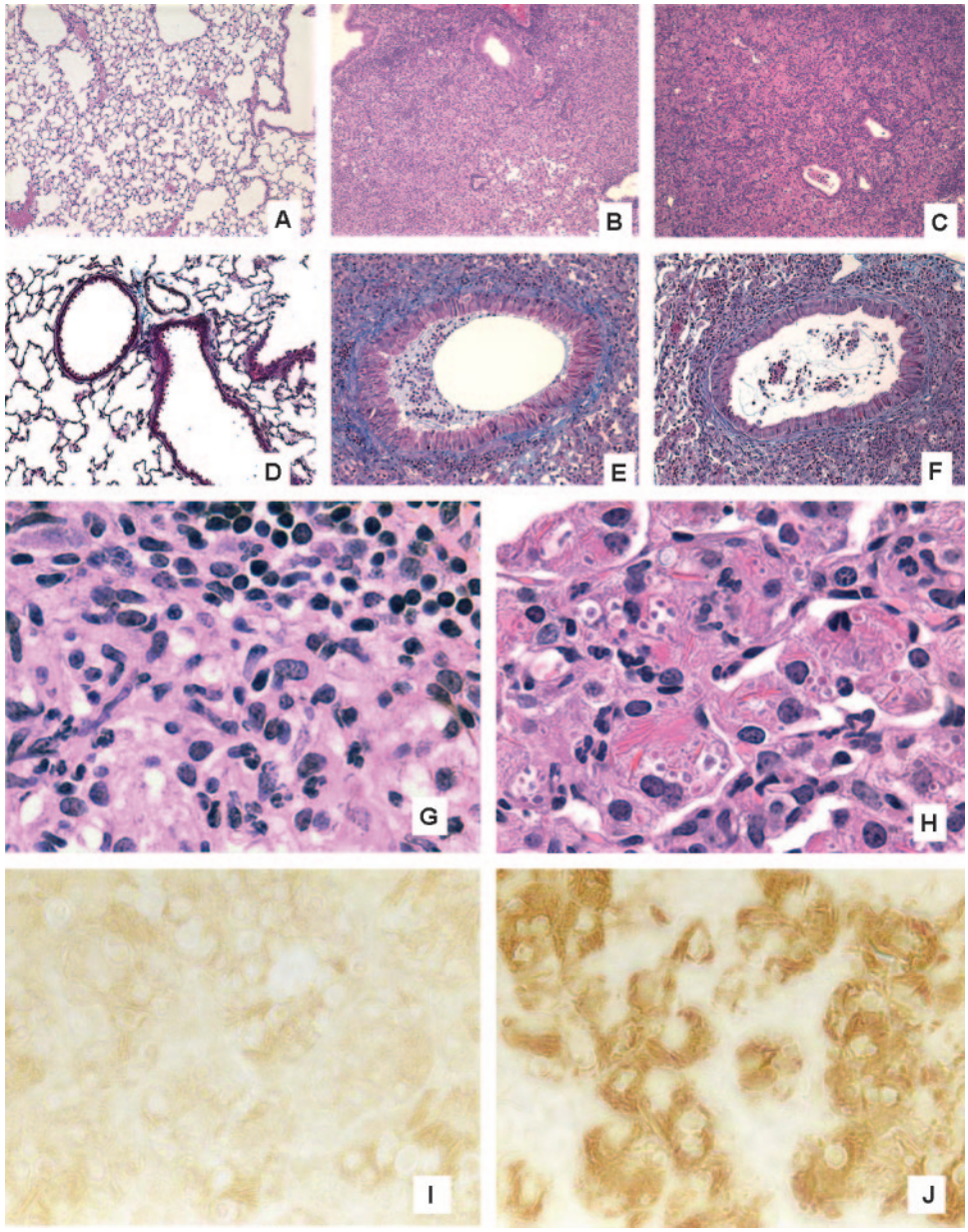

FIGURE 4. Photomicrographs of *C. neoformans*-infected lungs from WT (*B*, *E*, *G*) and IFN-γ KO (*C*, *F*, *H*) C57BL/6 mice at wk 3 postinfection compared with an uninfected lung (*A*, *D*). *A*, *B*, *C*, ×100, H&E; *D*, *E*, *F*, ×200, Masson's trichrome (collagen stains blue); and *G*, *H*, ×1000, H&E demonstrating enhanced crystal deposition and large macrophages containing numerous intracellular cryptococci in IFN-γ KO mice (*H*) compared with WT mice (*G*). Immunohistochemical staining of infected lungs from IFN-γ KO mice at wk 3 postinfection with either isotype-matched Ab (*I*) or anti-YM1 mAb (*J*). Crystals seen in the macrophages from the lungs of IFN-γ KO mice stain positive for YM1.

mAb stained the crystals inside the macrophages (Fig 4*J*). Similar crystals were not observed in *C. neoformans*-infected WT mice at this time point (data not shown); however, they have previously been seen much later in the infection (7 wk) (11). Thus, the magnitude and type of macrophage response in IFN-γ KO mice was markedly different from WT mice at wk 3, including the presence of YM1 crystals in the macrophages from IFN-γ KO mice.

iNOS-arginase balance in WT vs IFN-γ KO C. neoformans*-infected mice*

One of the hallmarks of classical activation of macrophages in mice is the generation of NO by iNOS (18). In contrast, generation of aaMac is consistent with induction of arginase (an enzyme that catalyzes conversion of L-arginine to L-ornithine and urea). We analyzed the mRNA expression of iNOS and arginase in whole lungs from WT vs IFN-γ KO mice at wk 3 following *C. neoformans* infection. There was a strong induction of arginase in both WT and IFN-γ KO mice compared with uninfected mice and the levels appeared to be slightly higher in IFN-γ KO mice. In contrast, the levels of iNOS expression were markedly lower in IFN-γ KO mice compared with WT mice (Fig. 5*B*). Thus, the ratio of iNOS/arginase expression was significantly lower in the lungs of IFN-γ KO mice compared with WT mice.

Role of IFN-γ in regulating pulmonary T2 and inflammatory cytokine production during C. neoformans *infection*

The next objective was to determine whether the production of IL-4, IL-5, and IL-13 was up-regulated in the absence of IFN-γ. Whole lung leukocytes were isolated following enzymatic digestion and placed into culture overnight with or without heat-killed cryptococci (HKC). Supernatants from these cultures were assayed by ELISA for IFN-γ, IL-4, IL-5, and IL-13. Since these leukocytes are prepared from actively infected lungs, there is a significant amount of live cryptococci already in the preparation (as both intracellular and intercellular yeast). These organisms serve as an endogenous source of Ag for stimulating these cultures. However, we also included cultures set up with heat-killed organisms as a source of exogenous Ag. Addition of exogenous Ag did not alter the relative cytokine expression pattern in the lungs except that the levels of IL-4 generally increased (Fig. 6).

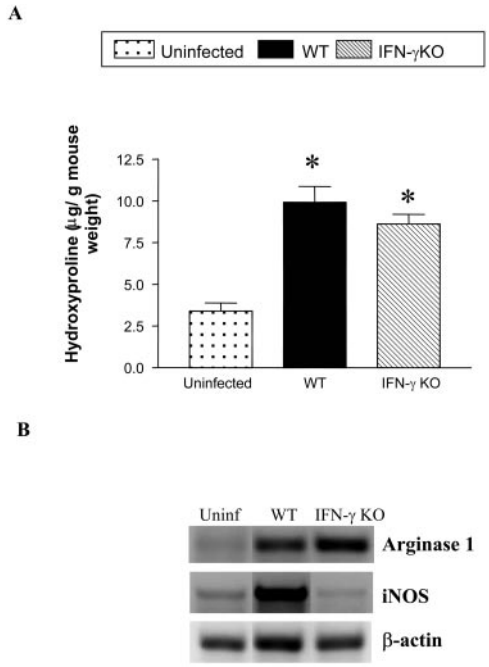

FIGURE 5. *A*, Hydroxyproline levels in whole lung homogenates from uninfected and infected WT and IFN-γ KO mice at wk 3 postinfection. *, $p < 0.05$ compared with uninfected group. Values are expressed as mean ± SEM, $n = 3$ mouse/group. *B*, RT-PCR analysis from the whole lung for expression of iNOS, arginase-1, and β-actin from uninfected and infected WT and IFN-γ KO mice at wk 3 postinfection. Each lane represents one of four animals per group.

Lung leukocyte cultures (with or without exogenous Ag) from infected mice produce significantly higher levels of all cytokines assayed compared with lung leukocyte cultures from uninfected mice (data not shown). Lung leukocytes from infected WT mice produced significant levels of IFN-γ (Fig. 6). In vitro depletion of CD4 and CD8 T cells before culture decreased IFN-γ levels by ~40% (data not shown), indicating that there is both a T cell and non-T cell source of IFN-γ in the lungs of *C. neoformans*-infected C57BL/6 mice. This is consistent with the T cell deficiency studies in Figs. 1 and 2. In IFN-γ KO mice, IL-4, IL-5, and IL-13 were all produced at levels equivalent or significantly higher than that seen in lung leukocyte cultures from WT mice (Fig. 6). IL-5 levels were significantly higher in cultures at wk 1 and 3, IL-13 at wk 1 and 2, and IL-4 at wk 2. Thus, IFN-γ production was not required for the development of T2 cytokine-producing cells in the lungs, and at a number of time points, T2 cytokine production was higher in the absence of IFN-γ.

We next analyzed whether production of TNF-α and IL-10 was modulated in the absence of IFN-γ. Using the culture system described above, TNF-α production by lung leukocytes was significantly diminished in IFN-γ KO mice (Fig. 7). IL-10 production was not significantly different between the two groups of mice at wk 1 or 2 in the presence or absence of exogenous heat-killed organisms (Fig. 7). However, IL-10 production by lung leukocytes in the presence of added HKC was significantly higher in WT mice compared with IFN-γ KO mice at wk 3 (Fig. 7). Overall, the presence of T2 cytokines (IL-4, IL-5, IL-13), absence of IFN-γ, and decreased TNF-α levels in lung leukocyte cultures from IFN-γ KO mice was consistent with the increased eosinophilia, YM1 production, intracellular cryptococci and fibrosis, and decreased fungistasis observed in the lungs at wk 3.

Effect of IFN-γ deficiency on the growth of C. neoformans *in LALN and their cytokine production*

To determine whether IFN-γ had an affect on the growth of *C. neoformans* at extrapulmonary sites, LALN were isolated from *C. neoformans*-infected WT and IFN-γ KO mice at wk 1, 2, and 3. LALN CFU was assayed as described in *Materials and Methods*. As early as wk 1, LALN CFU could be detected in both WT and IFN-γ KO mice. Following wk 2 and 3, LALN CFU in WT mice remained similar. In contrast, fungal burden in the LALN of IFN-γ KO mice continued to increase progressively (Fig. 8), similar to the increase observed in the lungs between wk 2 and 3 (Fig. 1).

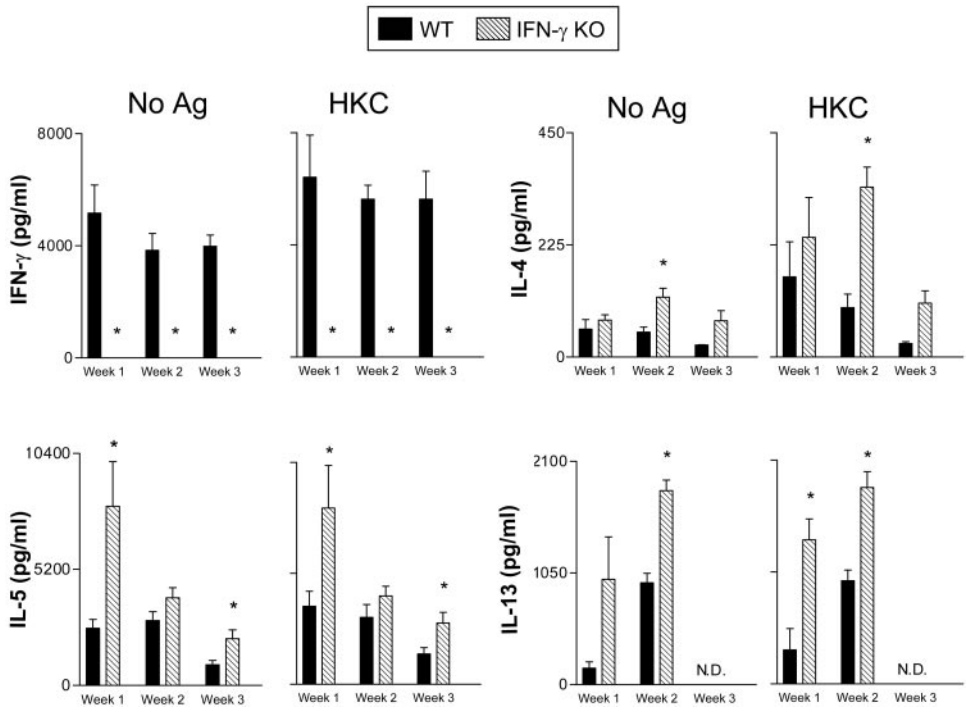

FIGURE 6. Cytokine (IFN-γ, IL-4, IL-5, and IL-13) production by lung leukocytes isolated from *C. neoformans*-infected WT or IFN-γ KO C57BL/6 mice. Total lung leukocytes were isolated following enzymatic digestion of the lungs at wk 1, 2, and 3 postinfection and cultured for 24 h at 5×10^6 cells/ml in the absence of exogenous Ag (No Ag) or in the presence of heat-killed *C. neoformans* (HKC) at 1×10^7/ml. Supernatants were harvested, and cytokine levels were measured by ELISA. *, $p < 0.05$ compared with WT. $n = 6–8$ mice/group/time point from two experiments. Values are means ± SEM. N.D., Not done.

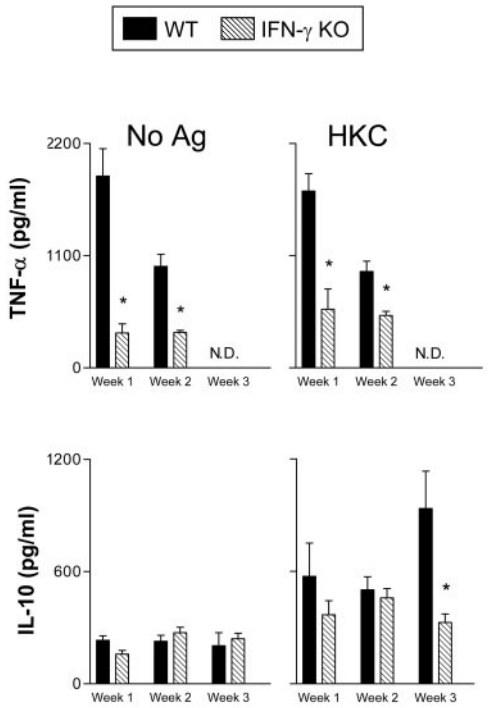

FIGURE 7. TNF-α and IL-10 production by lung leukocytes isolated from *C. neoformans*-infected WT or IFN-γ KO C57BL/6 mice. Leukocytes were isolated at wk 1, 2, and 3 postinfection as described in Figs. 6 and 7 and cultured for 24 h at 5×10^6 cells/ml in the absence of exogenous Ag (No Ag) or in the presence of heat-killed *C. neoformans* (HKC) at 1×10^7/ml. Supernatants were harvested, and cytokine levels were measured by ELISA. *, $p < 0.05$ compared with WT. $n = 6–8$ mice/group/time point from two experiments. Values are means ± SEM. N.D. = Not done.

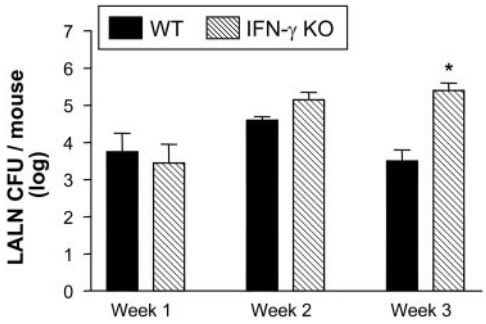

FIGURE 8. *C. neoformans* burden in the LALN. Data shown are mean CFU/total LALN ± SEM. *, $p < 0.05$, comparing WT with IFN-γ KO mice at the same time point. $n = 6–8$ mice/group from two experiments.

The lymphocyte composition of the LALN was also analyzed by flow cytometry. The percentage of CD8 T cells in the LALN from IFN-γ KO mice was less than that observed in the LALN from WT mice throughout the course of the infection. For WT mice, CD8 T cells in the LALN ranged from 21% (wk 1) to 16% (wk 2 and 3). For IFN-γ KO mice, CD8 T cells range from 11 to 13% (wk 1–3); however, these differences never reached statistical significance. The LALN from IFN-γ KO mice contained a higher percentage of CD4 T cells at wk 1 (18.5 vs 29.9%) but not at wk 2 or 3. No differences in the B cell composition of the LALN between these two groups were observed (data not shown). Thus, there were only slight differences in the lymphocyte composition of the LALN between WT and IFN-γ KO mice during a *C. neoformans* infection even though the cellular response in the lungs of these two groups of mice was significantly different at wk 2 and 3.

Overnight cultures were set up with LALN cells to determine whether the production of IL-4, IL-5, and IL-13 by LALN cells was augmented in the absence of IFN-γ. Cells were cocultured with or without exogenous HKC. Generally, cytokine production by LALN cells in the absence of exogenous Ag is very low. Addition of exogenous Ag augmented the production of IFN-γ, IL-5, and IL-13 but not IL-4 (Fig. 9). In the absence of IFN-γ, IL-5, and IL-13 were all produced at levels equivalent or significantly higher than that seen in LALN cultures from WT mice (Fig. 9). IL-4 was not detectable in either culture system for either group (Fig. 9). It is interesting to note that, in the absence of exogenous Ag, WT mice produce significant levels of IFN-γ, and IFN-γ KO mice produce significant levels of IL-5 and IL-13 at wk 2 (Fig. 9). The source of Ag in these cultures is most likely the endogenous cryptococci in the lymph nodes. Overall, the production of IL-5 and IL-13 and absence of IFN-γ in LALN cultures from IFN-γ KO mice was consistent with the general T2 cytokine profile in the lungs of IFN-γ KO mice.

Production of serum IgE by WT and IFN-γ KO mice during ABPM

We also investigated whether IFN-γ plays a regulatory role in the production of IgE during *C. neoformans* infection in C57BL/6 mice. Blood from IFN-γ KO and WT mice was collected, and the serum was assayed for total IgE levels by ELISA. Between wk 1 and 2, serum IgE levels increased in WT C57BL/6 mice and remained elevated at wk 3 (Fig. 10). Serum IgE levels in IFN-γ KO mice were similar to WT mice at wk 1, 2, and 3 (Fig. 10). Thus, the production of IgE in response to a pulmonary *C. neoformans* infection in C57BL/6 mice is independent of IFN-γ.

Discussion

This is the first study to analyze the development of aaMac during fungal infection. During parasitic infections, aaMac develop when macrophages are activated by IL-4/13 with minimal or no IFN-γ signaling. IL-4/13 and IFN-γ have antagonistic activation properties for macrophages in vitro, whereas IL-10 simply prevents activation (18). aaMac produce arginase (which decreases NO levels), express the chitinase-related protein YM1, have increased fungal phagocytosis (due to increased mannose receptor expression), have decreased intracellular killing due to lack of NO, promote fibrosis, and produce less TNF-α (17–19). *C. neoformans*-infected IFN-γ KO mice produced high amounts of IL-4 and IL-13, and the inflammatory response was consistent with that mediated by aaMac. Compared with *C. neoformans* infection of "resistant" mouse strains such as CB-17 and CBA, infected C57BL/6 mice probably have more aaMac, and the number of aaMac is significantly increased in IFN-γ KO C57BL/6 mice. *C. neoformans*-infected C57BL/6 mice will eventually develop all the histological features of aaMac-mediated pathology (fibrosis, YM1 deposition, large numbers of intracellular cryptococci, lack of NO).

The balance of iNOS vs arginase is an important feature in the polarization of macrophages during infection. Several groups have demonstrated that IL-4/IL-13 and IFN-γ show reciprocal inhibition of activities of iNOS and arginase, respectively (25, 26). Both iNOS and arginase compete for the same substrate L-arginine to catalyze its conversion to NO/L-citrulline or urea/L-ornithine, respectively. IFN-γ can induce iNOS in macrophages both in vitro and in vivo (27), whereas IL-4 is known to suppress the activation of iNOS in murine macrophages (28). Lower iNOS/arginase ratio in the lungs of IFN-γ KO mice (Fig. 5*B*) is consistent with these mice having decreased intracellular yeast killing and overall higher

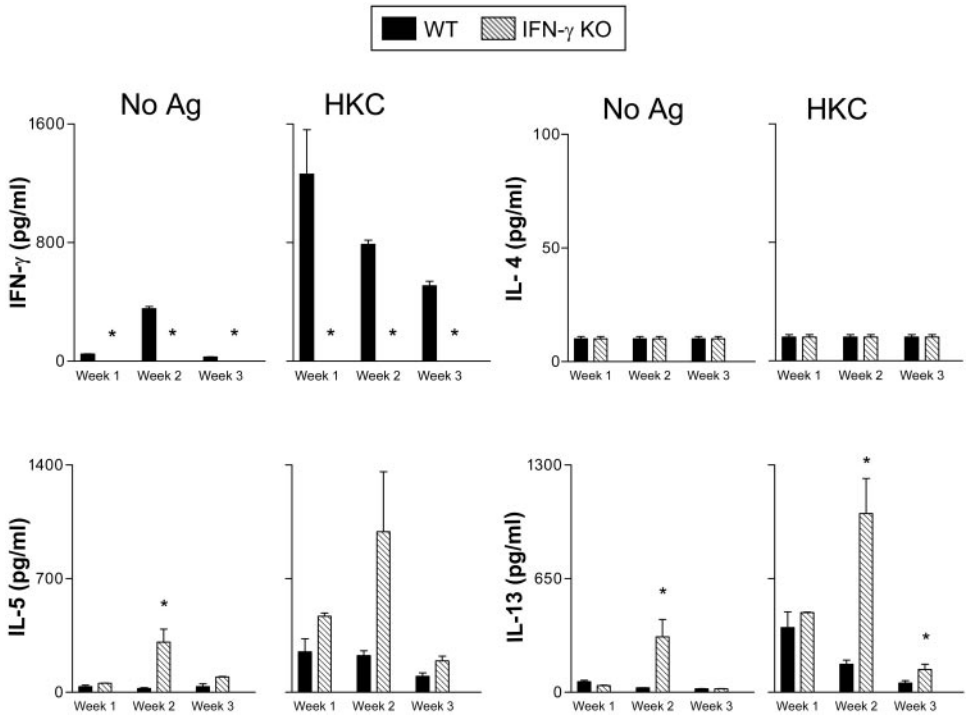

FIGURE 9. Cytokine (IFN-γ, IL-4, IL-5, and IL-13) production by LALN leukocyte isolated from *C. neoformans*-infected WT or IFN-γ KO C57BL/6 mice. LALN cells were isolated at wk 1, 2, and 3 postinfection and cultured for 24 h at 5×10^6 cells/ml in the absence of exogenous Ag (No Ag) or in the presence of heat-killed *C. neoformans* (HKC) at 1×10^7/ml. Supernatants were harvested, and cytokine levels were measured by ELISA. *, $p < 0.05$ compared with WT. $n = 6–8$ mice/group/time point from two experiments. Values are means ± SEM.

fungal burden. Our observation that WT B6 mice can induce arginase similar to IFN-γ KO mice is consistent with a previously unexplained observation that C57BL/6 mice express iNOS after pulmonary *C. neoformans* infection but produce almost no NO (29). The production of arginase would decrease substrate availability for NO production despite induction of the enzyme NO synthase (18).

We have previously reported that neutralization of IL-5 also decreases YM1 crystal formation (11). Eosinophils could be a significant source of IL-4 in the lungs during *C. neoformans* infection, as has been reported for models of allergic diseases (30). Since anti-IL-5 decreases the number of eosinophils, this would indirectly decrease YM1 production by macrophages. If eosinophils are a significant source of IL-4, this could also explain why IL-4 was readily detectable in lung but not lymph node cultures in our studies. Overall, IFN-γ plays a significant role in antagonizing the development of aaMac and aaMac-associated pathology during pulmonary cryptococcosis in C57BL/6 mice, a pulmonary immune response where high levels of IL-4 and IL-13 are produced in the lungs.

The studies in this manuscript, together with those of other investigators, demonstrate that the pathogenesis of pulmonary cryptococcosis in C57BL/6 mice shares many features with murine models of allergic bronchopulmonary aspergillosis (ABPA). These include high IgE, elevated peripheral blood and lung eosinophils, pulmonary inflammation, elevated levels of IL-4, IL-5, and IL-13, production of IFN-γ, pulmonary fibrosis and chronic fungal colonization/persistence (10, 11, 31–34). In murine models of ABPA, the T2 cytokines IL-4, IL-5, and IL-13 are required for these pathologic features of the host response (31–34). In murine ABPA models, there is also an inverse correlation between IFN-γ and IL-4 production in the lungs (34–39). Other studies examining the role of CCR2, NK T cells, IL-12, IL-18, and IL-4 during pulmonary cryptococcosis have also noted this inverse correlation between IFN-γ and IL-4 levels in the lungs (40–43). In "resistant" C.B-17 mice, anti-IFN-γ Abs can up-regulate IL-4 and IL-5 production by LALN and lung leukocytes (43). *C. neoformans*-infected CCR2 KO mice also display defects in IFN-γ production while expressing high levels of IL-4, resulting in the pathologic features of ABPM including YM1 crystal deposition (41). Thus, cytokine imbalances during *C. neoformans* infection can induce aaMac development that will lead to an ABPM, implicating aaMac as major cellular mediators of the disease.

IFN-γ is a T1 cytokine that can be produced by all lymphoid cells (44, 45); however, the role of IFN-γ as an inducer of T1 immunity is influenced by other signals. IL-12 is the primary cytokine driving Th1 differentiation (46) and IFN-γ KO and IFN-γ receptor KO mice can develop normal Th1 responses (47–49). IFN-γ plays an important role in the protective Th1 response to infection with intracellular pathogens such as *Leishmania*, *Toxoplasma*, and *Listeria*. However, mice from a genetically resistant background lacking the IFN-γ receptor are susceptible to infection with *Leishmania* major but still develop a polarized Th1 response (47). Our studies suggest that a non-T cell source of IFN-γ is responsible for the low-level protection of C57BL/6 during pulmonary cryptococcosis. NK cells and NK T cells have been identified as sources of IFN-γ during *C. neoformans* infection (50–52). Lung CFU were higher in IFN-γ KO mice at wk 3 than in WT or T cell-deficient WT mice (Fig. 1, *A* and *B*) and removal of CD4 and CD8 T cells in WT mice before culturing only partially decreased the IFN-γ levels (Fig. 1*C*). We have also shown that IFN-γ produced in *C. neoformans*-infected C57BL/6 mice is not cross-regulated by IL-4 and IL-10 (53), which would not be consistent with strictly T cell sources of IL-4 and IFN-γ. Altogether, these three observations support the concept that the main cellular source of IFN-γ in the lungs of C57BL/6 mice is not a CD4 or CD8 T cell.

Pulmonary fibrosis is a histologic feature of the bronchopulmonary response to *C. neoformans* in C57BL/6 mice. The most likely mechanism underlying this fibrotic response is the elevated production of IL-4, IL-5, and IL-13, similar to the mechanisms proposed for bleomycin, *Schistosome*, and ABPA-driven fibrosis (34–37, 54–62). Although IL-13 clearly promotes fibrosis, the roles of IL-4, IL-5, and IFN-γ in pulmonary fibrosis depend on the

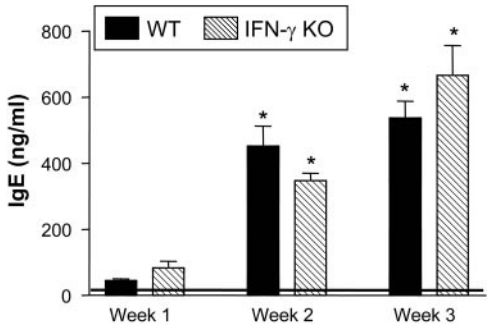

FIGURE 10. Total serum IgE levels in *C. neoformans*-infected WT and IFN-γ KO C57BL/6 mice. Total serum IgE levels were determined by ELISA. Line represents serum IgE levels in uninfected mice (both WT and IFN-γ KO). Values are means ± SEM. *, $p < 0.05$ compared with uninfected WT or IFN-γ KO mice. $n = 6–8$ mice/group/time point.

profibrotic stimulus with differences noted between bleomycin, *Schistosome*, *Cryptococcus*, and ABPA-driven fibrosis (34–37, 54–62). Since TGF-β is one of the major driving factors in fibrosis (63), we also predict that TGF-β will be up-regulated in the lungs of *C. neoformans*-infected IFN-γ KO and WT C57BL/6 mice. Pulmonary infections with *Paracoccidioides, Histoplasma*, and *Aspergillu*s have all been reported to promote fibrotic responses either as a response to the infection or as a hypersensitivity response (34, 64–67). Fibrosis is not a feature of pulmonary cryptococcosis during a protective response, i.e., when the ratio of IFN-γ to Th2 cytokine production is high (9, 68). Overall, the fibrotic response to *C. neoformans* infection in the lungs has been largely ignored in the literature, but our studies clearly indicate pulmonary fibrosis is a significant feature of *C. neoformans*-induced ABPM (Figs. 4, *D–F*, and 5*A*).

Pulmonary eosinophilia was enhanced in *C. neoformans*-infected IFN-γ KO compared with WT C57BL/6 mice, indicating a down-regulatory role for IFN-γ. Similar observations have been made for murine ABPA (38). In allergic airway models, adenoviral gene transfer of IFN-γ into the airways inhibits airway eosinophilia (69). Adenoviral gene transfer of IL-12, IL-18, IL-10, or IFN-inducible protein-10 also inhibits airway eosinophilia in murine models of allergic airway responses but the effect of all these modalities is dependent on IFN-γ (70–73). The mechanism underlying the augmented recruitment of eosinophils is probably due to the increase in IL-5 production in IFN-γ KO mice because IL-5 is a critical mediator of pulmonary eosinophilia during *C. neoformans* infection (11). In addition, IFN-γ may enhance eosinophil apoptosis (74). The continued production of IL-12 in the absence of IFN-γ also probably plays a role in augmenting pulmonary eosinophilia, as has been reported for pulmonary T2 responses during *Schistosome* infection (75). Increased IL-5 levels may also play a role in the enhanced fibrotic response in *C. neoformans*-infected IFN-γ KO mice (62).

Antibody responses can also promote the development of protective T cell responses against *C. neoformans* (76, 77). Recently, it was demonstrated that passively administered Ab in *C. neoformans*-infected C57BL/6 mice could down-modulate the inflammatory responses to the infection (78). Thus, an alternative explanation for "fungal chronicity" in C57BL/6 mice unlike other strains of mice is that B6 mice fail to produce a protective Ab response that might be required to generate an effective cell-mediated immune response. In this scenario chronicity could result from chronic host damage caused by poorly regulated host responses.

For all chronic fungal infections, one of the major questions is "what are the host factors responsible for chronicity?" Immunologically, chronic fungal infections likely involve an inappropriate cytokine balance. Thus, driving the cytokine balance toward T1 and inflammatory cytokines should enhance clearance. In the C57BL/6 model of chronic allergic bronchopulmonary cryptococcosis, administration of *Mycobacterium* bacillus Calmette-Guérin, OX40, or α-galactosylceramide enhances clearance (1–5). The mechanism of this enhancement is through the augmentation of multiple T1/inflammatory cytokines leading to a stronger T1 response with down-regulation of the T2 response (1–5). In murine models of cryptococcosis, production of IFN-γ correlates strongly with protective immunity and neutralization/deficiency of IFN-γ renders mice more susceptible to infection (79). IFN-γ-neutralized C.B-17 mice have increased numbers of eosinophils and higher levels of IL-5 compared with control mice although production of IL-4 and IL-10 is largely unaffected (43). In contrast, IFN-γ-deficient/neutralized CBA/J and BALB/c mice are more susceptible to *C. neoformans* infection but do not demonstrate the same increase in eosinophils and IL-5 (G. Huffnagle, unpublished observations). Altogether, studies in genetically disparate "resistant" mouse strains indicate that deficient IFN-γ production does not necessarily lead to a T1 to T2 switch in polarization of cell-mediated immunity. Our current studies demonstrate that deficient IFN-γ production in "susceptible" C57BL/6 mice results in continued high level production of T2 cytokines (IL-4, IL-5, and IL-13) in the lungs and the alternative activation of macrophages, leading to a loss in fungistasis and a switch from a chronic to a progressive pulmonary cryptococcal infection.

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Disclosures

The authors have no financial conflict of interest.

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