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Protection against Cryptococcosis by Using a Murine Gamma Interferon-Producing *Cryptococcus neoformans* Strain[▽]

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We evaluated cell-mediated immune (CMI) responses in mice given a pulmonary infection with a *Cryptococcus neoformans* strain engineered to produce the Th1-type cytokine gamma interferon (IFN-γ). Mice given a pulmonary infection with an IFN-γ-producing *C. neoformans* strain were able to resolve the primary infection and demonstrated complete (100%) protection against a second pulmonary challenge with a pathogenic *C. neoformans* strain. Pulmonary cytokine analyses showed that Th1-type/proinflammatory cytokine and chemokine expression were significantly higher and Th2-type cytokine expression was significantly lower in mice infected with the IFN-γ-producing *C. neoformans* strain compared to wild-type-infected mice. This increased pulmonary Th1-type cytokine expression was also associated with significantly lower pulmonary fungal burden and significantly higher pulmonary leukocyte and T-lymphocyte recruitment in mice infected with the IFN-γ-producing *C. neoformans* strain compared to wild-type-infected mice. Our results demonstrate that pulmonary infection of mice with a *C. neoformans* strain expressing IFN-γ results in the stimulation of local Th1-type anti-cryptococcal CMI responses and the development of protective host immunity against future pulmonary cryptococcal infections. The use of fungi engineered to produce host cytokines is a novel method to study immune responses to infection and may be useful in developing vaccine strategies in humans.

Cryptococcus neoformans, the etiological agent of cryptococcosis, is an opportunistic fungal pathogen that has a predilection to invade the central nervous system. Exposure to *C. neoformans* is very common in the general population (31), but almost all cases of clinically recognized infection are thought to be due to reactivation from latency in persons with severe defects in cell-mediated immunity (CMI). Despite advances in antifungal therapy, the acute mortality rate remains between 10 and 25% in medically advanced countries (38), and at least one-third of patients with cryptococcal meningitis who receive appropriate therapy will still experience mycologic and/or clinical failure (41, 43). CMI by T-helper 1 (Th1)-type $CD4^+$ T cells is the predominant host defense mechanism against *C. neoformans* infections, as evidenced by the high incidence of cryptococcosis in individuals with reduced CMI (4, 7, 10, 11, 13, 14, 23, 25, 28). $CD4^+$ T cells mediate protective anti-cryptococcal host immunity through the generation of Th1-type cytokine responses via production of interleukin-2 (IL-2), tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ). These cytokines induce lymphocyte and phagocyte recruitment and activation of anti-cryptococcal delayed-type hypersensitivity responses, resulting in increased cryptococcal uptake and killing by effector phagocytes (1, 6, 12, 18, 26, 27, 32). Studies in mice and humans have shown some efficacy in using systemically administered recombinant Th1-type cytokines to stimulate anti-cryptococcal host responses and to enhance antifungal chemotherapy (5, 15, 19, 20, 22). Specifically, experimental studies with the Th1-type cytokine IFN-γ have yielded some promising results as an adjunctive therapy to antifungal agents (19, 29) and to significantly enhance the anti-phagocytic activity of macrophages against *C. neoformans* in vitro (6, 12, 26, 34, 40). Mucci et al. have engineered a murine macrophage cell line to express IFN-γ in an inducible manner, and this cell line was shown to enhance the anti-cryptococcal activity of microglial cells in a coculture system (33). Administration of recombinant IFN-γ to *C. neoformans*-infected mice results in increased survival times and reduced fungal burden (21), and cytokine treatment enhances the effectiveness of the antifungal drug amphotericin B (19, 29). A randomized, double-blinded, placebo-controlled clinical trial in patients with AIDS-associated cryptococcal meningitis demonstrated a trend towards more rapid sterilization of cerebrospinal fluid cultures in those treated with IFN-γ as an adjunct to antifungals (37). Taken together, the evidence suggests that the administration of IFN-γ is an attractive strategy for the augmentation of host immune responses to invasive cryptococcal infection. However, experimental studies using recombinant IFN-γ therapy alone have failed to induce complete clearance of *C. neoformans* from infected tissues or to help induce protection against subsequent cryptococcal infections. These observations suggest that alternative strategies for modulating host immune responses against cryptococcal infections at the site of infection should be investigated. Therefore, the present study was designed to investigate the efficacy of using a *C. neoformans* strain engineered to produce IFN-γ at the site of the infection to modulate local immunity against experimental pulmonary cryptococcosis. The present study represents the first instance in which a fungal pathogen has been genetically

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altered to express a host immune-modulatory cytokine in vivo to aid in the resolution of the acute infection and confer complete protection against future yeast challenges.

MATERIALS AND METHODS

Mice. Female A/Jcr (H-2[a]) and BALB/c (H-2[d]) mice, 4 to 6 weeks of age (National Cancer Institute/Charles River Laboratories), were used throughout these studies. Mice were housed at the Duke University Medical Center Vivarium and handled according to guidelines approved by the Institutional Animal Care and Use Committee.

Strains. *C. neoformans* H99 strains (serotype A, mating type α) were recovered from 15% glycerol stocks stored at −80°C prior to use in the experiments described herein. The strains were maintained on yeast-extract-peptone-dextrose (YPD) media (1% yeast extract, 2% peptone, and 2% dextrose). Transformants were selected on YPD media supplemented with 100 μg/ml of nourseothricin (clonNAT; Werner Bioagents, Jena, Germany) as previously described (30). The yeast strains were grown for 18 to 20 h at 30°C with shaking in YPD broth (Becton, Dickinson and Company, Sparks, MD), harvested, and washed three times with sterile phosphate-buffered saline (PBS), and viable yeast were quantified using trypan blue dye exclusion in a hemacytometer.

Transformation of *C. neoformans* with a murine IFN-γ construct. Whole spleens were aseptically removed from BALB/c mice, and total RNA was isolated using TRIzol reagent (Invitrogen) and was DNase (Invitrogen) treated to remove possible traces of contaminating DNA according to the manufacturer's instructions. A mouse cDNA library was synthesized using the oligo(dT) primer and reagents supplied in the SuperScrpt III RT kit (Invitrogen) according to the manufacturer's instructions. Murine IFN-γ cDNA was subsequently amplified from the mouse spleen cDNA library by PCR using Ex *Taq* polymerase (Pan Vera Corporation, Madison, WI) using sense primers (5′-TCT-*GGA-TCC*-ATG-AAC-GCT-ACA-CAC-TG-3′) and antisense primers (5′-CAC-*CTC-GAG*-GCA-GCG-ACT-CCT-3′) containing a BamHI and an XhoI site (italics), respectively. The amplicon was digested with BamHI and XhoI (New England Biolabs, Ipswich, MA) restriction enzymes and ligated to the mammalian expression vector pcDNA6/V5-His B (Invitrogen). The promoter region and first exon including the signal sequence of the phospholipase B (*PLB*) gene was amplified from genomic DNA derived from *C. neoformans* strain H99 using sense primers (5′-*AAG-CTT*-AAG-TGC-ACT-GTC-AA-3′) and antisense primers (5′-*GGA-TCC*-AGT-GGC-ATT-TCT-AA-3′) containing a HindIII and a BamHI site (italics), respectively. The amplicon was digested with HindIII and BamHI (New England Biolabs) and ligated to the pcDNA6/V5-His B plasmid upstream of IFN-γ. A plasmid containing the nourseothricin cassette was digested with HindIII and ligated to the HindIII site upstream of the PLB fragment. The plasmid was subsequently used as template in a PCR to obtain a fragment containing the nourseothricin, PLB, and IFN-γ sequences using sense primers (5′-CGA-CGC-TCC-TAC-ACT-CGA-CC-3′) and antisense primers (5′-GCG-ATG-CAA-TTT-CCT-CAT-TT-3′). The PCR fragment was used to transform *C. neoformans* strain H99 using biolistic delivery as previously described (8, 9). Transformants were selected on YPD media supplemented with 100 μg/ml of nourseothricin (clonNAT; Werner Bioagents, Jena, Germany) as previously described (30). Integration of the IFN-γ expression construct into the genome of *C. neoformans* strain H99 was confirmed by colony PCR and Southern blot hybridization of genomic DNA digested with BamHI (New England Biolabs) and probed with a [^{32}P]dCTP-labeled IFN-γ PCR fragment. IFN-γ transcript levels were analyzed by Northern blot hybridization of DNase-treated total RNA probed with a [^{32}P]dCTP-labeled IFN-γ PCR fragment. Culture supernatant of IFN-γ-producing and wild-type *C. neoformans* H99 strains were evaluated for IFN-γ protein production by enzyme-linked immunosorbent assay (ELISA) as described below.

Phenotypic assays. Prior to testing, *C. neoformans* strains H99 and H99-γ were grown for 16 to 20 h at 30°C with shaking in YPD media, harvested, and washed three times in sterile phosphate-buffered saline (PBS). Temperature sensitivity of each strain was analyzed by growth on YPD agar at 30°C and 37°C. Melanin production was assayed by growth on L-dopamine and Niger seed agar at 30°C. Sensitivity to tert-butyl hydroperoxide (t-BOOH) (Sigma) was assayed by culture of yeast (1×10^4) in the wells of a 96-well plate in 200 μl of YPD media alone or in media containing 0.5 mM, 0.025 mM, 0.0125 mM, and 0.00625 mM of t-BOOH for 48 h. Aliquots (5 μl) of the cultures were then spotted onto YPD agar and incubated at 30°C for 72 h. *C. neoformans* capsules were observed by microscopic examination of India ink preparations of yeasts following growth overnight in Dulbecco's modified Eagle medium (DMEM) (GIBCO) at 30°C with 5% CO_2 to stimulate capsule production. Strains were tested for urease activity using Christensen's urea agar and broth (Becton Dickinson, Cockeysville, Md.).

Pulmonary infections. Pulmonary *C. neoformans* infections were initiated by nasal inhalation as previously described (8, 9). Briefly, mice were anesthetized by an intraperitoneal injection of pentobarbital (35 mg/kg of body weight) (Abbott Laboratories, North Chicago, IL) and suspended by their incisors on a silk thread to fully extend their necks. A yeast inocula of 1×10^4 or 1×10^5 CFU of *C. neoformans* strain H99 or H99-γ in 50 μl of sterile PBS was slowly pipetted directly into the nares. Mice that were to experience a secondary pulmonary infection received an initial inoculum of 1×10^4 CFU of *C. neoformans* strain H99 or H99-γ or heat-killed *C. neoformans* yeasts in 50 μl of sterile PBS, allowed 70 days to resolve the infection, and subsequently given a second experimental pulmonary infection with 1×10^4 CFU of *C. neoformans* strain H99 in 50 μl of sterile PBS. The mice remained suspended for an additional 10 min and were closely monitored until recovery from anesthesia. Mice were euthanized on the indicated days or if they appeared to be in pain or moribund using CO_2 inhalation.

Pulmonary leukocyte isolation. Lungs were excised on days 3, 7, and 14 postinoculation and digested enzymatically at 37°C for 30 min in 15 ml of digestion buffer (RPMI 1640 and a 1-mg/ml concentration of collagenase type IV [Sigma Chemical Co., St. Louis, MO.]) with intermittent (every 15 min) stomacher homogenizations. The resultant cell suspension was centrifuged ($250 \times g$) for 1 min to remove tissue debris. Supernatants were then successively filtered through sterile nylon filters of various pore sizes (70 and 40 μm) to enrich for leukocytes. Erythrocytes were lysed by incubation in NH_4Cl buffer (0.859% NH_4Cl, 0.1% $KHCO_3$, 0.0372% Na_2EDTA [pH 7.4]; Sigma) for 3 min on ice followed by a 10-fold excess of Hank's balanced salt solution (HBSS). The resulting leukocyte population was then collected by centrifugation ($800 \times g$) for 10 min, washed twice with sterile HBSS, and enumerated in a hemacytometer using trypan blue dye exclusion. Flow cytometric analysis was used to determine the absolute number of total leukocytes ($CD45^+$) within the lung cell suspension for correction of hemacytometer counts.

Antibodies. For flow cytometry experiments, phycoerythrin-, biotin-, or fluorescein isothiocyanate-conjugated antibodies specific for CD3, CD4, CD8α, CD16/CD32 (Fc Block), CD45, CD45R/B220, Gr-1 (neutrophils) (all purchased from BD Pharmingen Corp, San Diego, CA), and F4/80 (macrophages) (Caltag Laoratories, Burlingame, CA) were used. Biotin-conjugated antibodies were identified with allophycocyanin (APC)-Cy7-conjugated streptavidin (Caltag Laboratories). Fluorochrome-conjugated isotype control antibodies included hamster immunoglobulin G1 (IgG1), rat IgG_{2a}, rat IgG_{2b}, and rat IgM (BD Pharmingen).

Flow cytometry. Standard methodology was employed for the direct and indirect immunofluorescence of pulmonary leukocytes. Briefly, in 1.7-ml Eppendorf tubes 1×10^6 leukocyte-enriched lung cells were incubated with Fc Block (BD Pharmingen) in 50 μl of PBS supplemented with 2% heat-inactivated fetal bovine serum (PBS-FBS) for 5 min to block nonspecific binding of antibodies to cellular Fc receptors. Subsequently, an optimal concentration of fluorochrome-conjugated antibodies (between 0.06 to 0.25 μg/1×10^6 cells) was added in various combinations to allow for dual- or triple-staining experiments in a final volume of 50 μl of PBS and incubated for 30 min on ice. Following incubation, the cells were washed three times with PBS-FBS. Biotinylated samples were then resuspended in 100 μl of PBS-FBS containing an optimal concentration of APC-Cy7-conjugated streptavidin (Caltag Laboratories) for 30 min on ice. All other samples were suspended in 100 μl of PBS-FBS and incubated for 30 min on ice. After incubation, the cells were washed with PBS-FBS and fixed in 400 μl of 1% ultrapure formaldehyde (Polysciences, Inc., Warrington, PA). Cells incubated with either PBS-FBS alone or fluorochrome-conjugated isotype control antibodies were used to determine background fluorescence. Flow cytometry was performed in the Duke Human Vaccine Institute Flow Cytometry Core Facility. The samples were analyzed using software on a BD FACSVantage SE flow cytometer (BD Pharmingen). Dead cells were excluded on the basis of forward angle and 90° light scatter. For data analyses, 10,000 events (cells) were evaluated from a predominantly leukocytic population identified by backgating from $CD45^+$-stained cells and using isotype-specific antibody staining as a negative control. Compensation for each fluorochrome was determined by parallel single-color analysis of cells labeled with each fluorochrome-conjugated antibody. The absolute number of leukocyte subsets (neutrophils, macrophages, and $CD4^+$/$CD3^+$ and $CD8^+$/$CD3^+$ lymphocytes) was determined by multiplying the absolute number of $CD45^+$ cells by the percentage of cells stained by specific fluorescein isothiocyanate-, phycoerythrin-, or APC-labeled antibodies.

Cytokine analysis. Cytokine levels in lung tissues were analyzed using the Bio-Plex Protein Array System (Luminex-based technology) (Bio-Rad Laboratories, Hercules, CA). Briefly, lung tissue was excised and homogenized in

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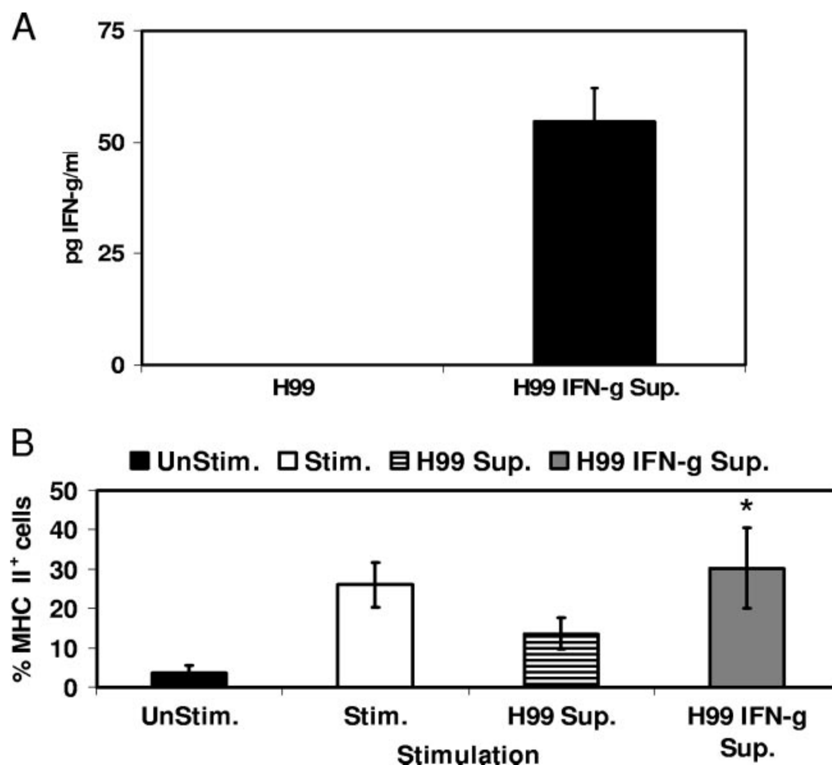

FIG. 1. Confirmation of IFN-γ production in *C. neoformans*. A) Detection of IFN-γ in culture supernatant of the IFN-γ-producing *C. neoformans* strain (H99 IFN-g Sup) compared to the wild-type *C. neoformans* strain H99 (H99). B) Induction of MHC class II expression on J774A.16 cells following culture in DMEM complete media plus LPS (UnStim.), DMEM media plus LPS and recombinant murine IFN-γ (Stim.), or supernatants from *C. neoformans* strain H99 (H99 Sup.) or the IFN-γ-expressing *C. neoformans* strain plus LPS (H99 IFN-g Sup.). (*, $P < 0.05$ compared to supernatants from *C. neoformans* strain H99 plus LPS).

ice-cold sterile PBS (1 ml). An aliquot (50 μl) was taken to quantify the pulmonary fungal burden, and an anti-protease buffer solution (1 ml) containing PBS, protease inhibitors (inhibiting cysteine, serine, and other metalloproteinases), and 0.05% Triton X-100 was added to the homogenate that was then clarified by centrifugation (800 × *g*) for 5 min. Pulmonary homogenates were assayed undiluted for IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-12 p40, IL-12 p70, IL-17, TNF-α, and granulocyte-colony stimulating factor (G-CSF) expression as well as chemokine (macrophage inflammatory protein 1α [MIP-1α] and regulated upon activation, normal T-cell-expressed and secreted [RANTES]) production using the Bio-Plex Protein Array System (Bio-Rad Laboratories).

Murine IFN-γ protein levels in *C. neoformans* strains H99 and H99-γ culture supernatants were analyzed by ELISA. Briefly, supernatants were obtained following incubation of wild-type *C. neoformans* strain H99 or the putative IFN-γ-producing *C. neoformans* strain H99-γ for 16 to 18 h in liquid YPD media at 37°C. Yeast were removed from culture supernatants by centrifugation (12,000 × *g*), and IFN-γ levels were quantified using commercially available capture and biotinylated antibodies supplied in the Mouse IFN-γ OptEIA ELISA kit (BD PharMingen) according to the manufacturer's instructions. The plates were incubated with the substrate *o*-phenylenediamine dihydrochloride (Sigma), and the absorbance values were determined using a Multiskan Ascent microplate reader and Ascent software version 2.4.1 (Labsystems, Helsinki, Finland).

Macrophage assay. The J774A.16 macrophage-like cell line (American Type Culture Collection, Manassas, VA) was maintained at 37°C in 5% CO_2 in culture medium consisting of DMEM supplemented with 10% heat-treated fetal bovine serum, 1× nonessential amino acids, 100 μg/ml penicillin-streptomycin, and 10% NCTC-109 medium. Macrophages were harvested by mechanical dislocation and washed three times in Hank's balanced salt solution (HBSS) (GIBCO), and cell viability and numbers were quantified using trypan blue dye exclusion. Macrophages (1×10^6 cells/ml) were cultured in 24-well tissue culture plates for 48 h in DMEM complete media with 0.3 μg/ml of lipopolysaccharide (LPS) (Sigma) with or without 100 U/ml of murine gamma interferon (Roche Diagnostics GmbH, Mannheim, Germany). In addition, macrophages (1×10^6 cells/ml) were cultured in 24-well tissue culture plates for 48 h in culture supernatants of wild-type *C. neoformans* strain H99 or the IFN-γ-producing strain H99-γ plus 0.3 μg/ml of LPS for 48 h at 37°C in 5% CO_2. Supernatants were obtained from the IFN-γ-producing and wild-type *C. neoformans* H99 strains following incubation for 16 to 18 h at 37°C in 5% CO_2 in DMEM complete media, and supernatants were collected and sterile filtered using a 0.45-μm filter (Millipore, Billerica, MA). The macrophages were subsequently washed with sterile PBS and harvested by mechanical dislocation. Macrophage major histocompatibility complex (MHC) class II expression was analyzed by flow cytometry as described above. Experiments were performed using triplicate wells.

Statistical analysis. The unpaired Student's *t* test (two-tailed) was used to detect significant differences. Significant differences were defined as $P < 0.05$.

RESULTS

Generation and validation of IFN-γ-producing *C. neoformans* strains. We created a heterologous fusion construct where the cryptococcal phospholipase B (*PLB1*) promoter and signal sequence was used to drive expression of the murine IFN-γ coding sequence. The *PLB1* promoter and signal sequence were used to ensure export of the IFN-γ from the yeast cells. The construct was used to transform the wild-type *C. neoformans* strain H99, and stable transformants containing a single insertion into the genome were verified using PCR and Southern blots (data not shown). A single strain, designated H99-γ, was selected for further study, and Northern blot analysis confirmed the production of the IFN-γ transcripts (data not shown). ELISA performed on culture supernatants verified that IFN-γ was exported from the H99-γ strain (Fig. 1A), and the biologic activity of the IFN-γ was demonstrated by quan-

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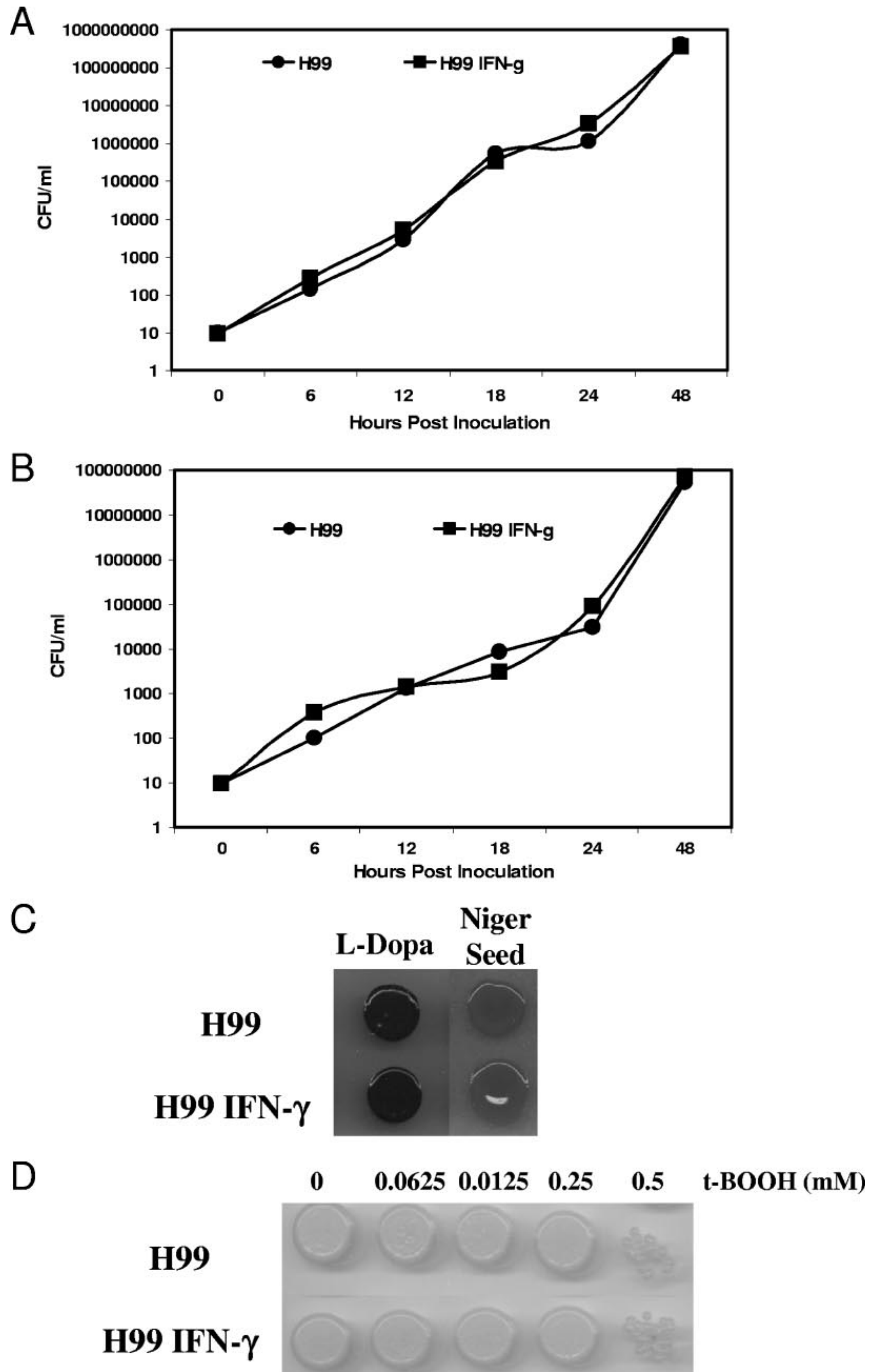

FIG. 2. Analysis of *C. neoformans* in vitro virulence phenotypes. (A) *C. neoformans* strain H99 (H99) and *C. neoformans* strain H99-γ (H99 IFN-g) (10^2 cells in 10 ml of liquid yeast-peptone-dextrose media) was cultured at 30°C and (B) 37°C. (C) An aliquot (5 μl) of an overnight culture

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tifying MHC class II expression in a macrophage-like cell line (Fig. 1B).

Virulence phenotype of IFN-γ-producing *C. neoformans* strain. Evaluation of the in vitro phenotype of the H99-γ strain demonstrated no significant differences in growth rate at 30°C (Fig. 2A) and 37°C (Fig. 2B), melanin production (Fig. 2C), and sensitivity to oxidative stress (Fig. 2D) compared to wild-type *C. neoformans* strain H99. Urease activity and capsule production were also observed to be similar in *C. neoformans* strain H99-γ compared to wild-type *C. neoformans* strain H99 (data not shown). Thus, the H99-γ strain had no defects in the in vitro phenotypes commonly associated with cryptococcal virulence. The virulence of the IFN-γ-expressing *C. neoformans* strain was compared to that of *C. neoformans* strain H99 using the mouse inhalational model. As shown in Fig. 3A, 100% survival of A/Jcr mice infected with 10^4 CFU of the IFN-γ-expressing *C. neoformans* strain H99-γ was observed on day 100, compared to 100% mortality of A/Jcr mice infected with 10^4 CFU of the wild-type *C. neoformans* strain H99 (median survival time of 27 days; $P < 0.0001$). Pulmonary and brain tissue cultures from the surviving mice were all sterile.

Assessment of protective immunity. To evaluate whether prior infection with the IFN-γ-producing strain could confer protection against a subsequent infection with wild-type *C. neoformans*, A/Jcr mice were given a primary intranasal inoculation with sterile PBS, 10^4 CFU of the IFN-γ-producing *C. neoformans* strain H99-γ, or 10^4 heat-killed *C. neoformans* strain H99 yeasts. The mice were allowed 70 days to resolve the infection and then were challenged with 10^4 CFU of *C. neoformans* strain H99. As shown in Fig. 3B, on day 100 postinoculation, all of the A/Jcr mice that received a previous infection with the IFN-γ-producing *C. neoformans* strain survived the second intranasal inoculation with the wild-type *C. neoformans* strain H99. In contrast, 100% mortality was observed in the control mice given a prior inoculation with sterile PBS or heat-killed *C. neoformans* yeast and subsequently challenged with *C. neoformans* strain H99. Brain and pulmonary tissue cultures from surviving mice were sterile, proving that the mice were able to completely eliminate the wild-type strain.

A/Jcr mice are deficient in the C5a component of complement and are oftentimes considered immune deficient. Therefore, we repeated the protection studies using immune competent BALB/c mice. BALB/c mice were given a primary intranasal inoculation with sterile PBS or 10^4 CFU of *C. neoformans* strain H99 or the IFN-γ-producing *C. neoformans* strain H99-γ. We observed 100% mortality of BALB/c mice infected with 10^4 CFU of the wild-type *C. neoformans* strain H99 (median survival time of 25 days; $P < 0.0001$) compared to 100% survival of BALB/c mice infected with 10^4 CFU of the IFN-γ-expressing *C. neoformans* strain H99-γ or PBS on day 100 postinoculation. The surviving BALB/c mice given a prior inoculation with PBS or the IFN-γ-producing *C. neoformans* strain mice were then challenged with 10^4 CFU of *C. neoformans* strain H99. As shown in Fig. 3C, on day 100 postsecondary inoculation, all of the BALB/c mice that received a previous infection with the IFN-γ-producing *C. neoformans* strain survived the second intranasal inoculation with the wild-type *C. neoformans* strain H99. In contrast, 100% mortality was observed in the control mice given a prior inoculation with sterile PBS. Brain and pulmonary tissue cultures from surviving BALB/c mice were sterile, also indicating that the immune competent BALB/c mice were able to completely resolve the second infection with the wild-type strain.

Pulmonary leukocyte recruitment. To investigate the pulmonary inflammatory and lymphocyte response to the IFN-γ-producing *C. neoformans* strain during experimental pulmonary cryptococcosis, BALB/c mice were given an intranasal inoculation with 10^5 CFU of the IFN-γ-producing *C. neoformans* strain or the wild-type *C. neoformans* strain H99, and the pulmonary fungal burden was quantified on days 3, 7, and 14 postinoculation. These time points were chosen so that we could also evaluate and compare local CMI responses to experimental pulmonary cryptococcosis. Time points beyond day 14 postinoculation of 10^5 CFU of *C. neoformans* were not examined due to the high mortality observed in mice infected with the wild-type strain (44). As shown in Fig. 4, mice inoculated with *C. neoformans* strain H99 had a significantly higher pulmonary fungal burden throughout infection compared to mice inoculated with *C. neoformans* strain H99-γ ($P < 0.05$, $P < 0.005$, and $P < 0.0005$ on day 3, 7, and 14 postinoculation, respectively), and the differences between the two strains progressively increased as the infection progressed. Pulmonary leukocyte subpopulations were evaluated using flow cytometry, and we found that the absolute total numbers of pulmonary leukocytes ($CD45^+$ cells) were slightly higher in mice inoculated with the IFN-γ-producing *C. neoformans* strain compared to *C. neoformans* strain H99-infected mice on day 7 postinoculation (Fig. 5A). In addition, we observed a significant increase in the total number of MHC class II^+ cells in mice infected with the IFN-γ-producing *C. neoformans* strain on day 7 postinoculation compared to wild-type-infected mice ($P < 0.05$). Analysis of phagocyte subsets (polymorphonuclear leukocytes [PMNs] and macrophages) during the same time course showed a significantly greater absolute number of PMNs in lung tissues of mice infected with the IFN-γ-producing *C. neoformans* strain compared to wild-type-infected mice on day 7 postinoculation ($P < 0.05$) (Fig. 5B). The absolute number of macrophages that trafficked to the lungs during infection was higher in each group tested; however, no statistically significant differences were observed between the groups (Fig. 5B). Analysis of lymphocyte subpopulations showed that mice infected with the IFN-γ-producing *C. neoformans* strain had a significantly higher absolute number of $CD4^+$ (Fig. 6A) and $CD8^+$ (Fig. 6B) T lymphocytes in lung tissues on day 7 postinoculation compared to wild-type-infected mice. There was no significant difference in the absolute number of B lymphocytes in wild-type compared to IFN-γ-

of *C. neoformans* strains H99 (H99) and H99-γ (H99 IFN-g) were cultured on L-dopamine (L-DOPA) and Niger seed agar for 48 h. (D) *C. neoformans* strains (H99) and H99-γ (H99 IFN-g) were cultured in liquid YPD media containing t-BOOH at the indicated concentration, spotted (5 μl) onto YPD agar, and incubated at 30°C for 72 h.

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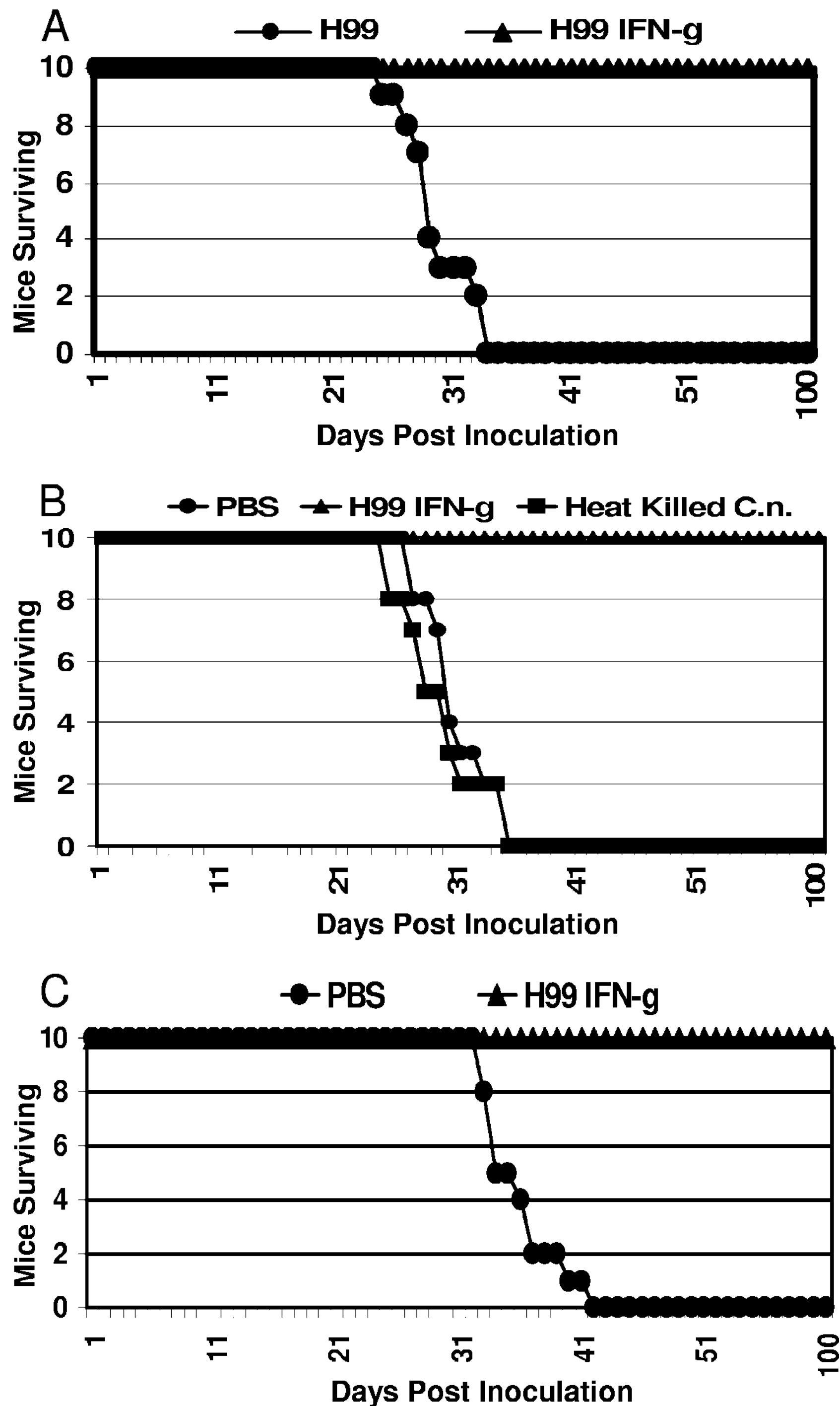

FIG. 3. Pathogenesis of an IFN-γ-expressing *C. neoformans* strain in a murine inhalational model. (A) A/J mice were inoculated with 10^4 CFU of wild-type *C. neoformans* strain H99 (H99) or the IFN-γ-producing *C. neoformans* strain (H99 IFN-g). Data shown are cumulative of one experiment using 10 mice per experimental group. (B) A/J mice received an initial inoculum of sterile PBS or 1×10^4 CFU of IFN-γ-producing *C. neoformans* H99 or heat-killed *C. neoformans* (C.n.) yeasts in 50 μl of sterile PBS, allowed 70 days to resolve the infection, and subsequently given a second challenge with 1×10^4 CFU of *C. neoformans* strain H99 in 50 ml of sterile PBS. Data shown are cumulative of one experiment using 10 mice per experimental group. (C) BALB/c mice (10 mice per inoculation) received an initial inoculum of sterile PBS or 1×10^4 CFU of IFN-γ-producing *C. neoformans* H99 in 50 μl of sterile PBS, allowed 70 days to resolve the infection, and subsequently given a second challenge with 1×10^4 CFU of *C. neoformans* strain H99 in 50 μl of sterile PBS. Survival of primary- and secondary-infected mice was monitored twice daily, and mice that appeared moribund or not maintaining normal habits (grooming) were sacrificed by CO_2 inhalation. Data shown are cumulative of one experiment, each using 10 mice per experimental group.

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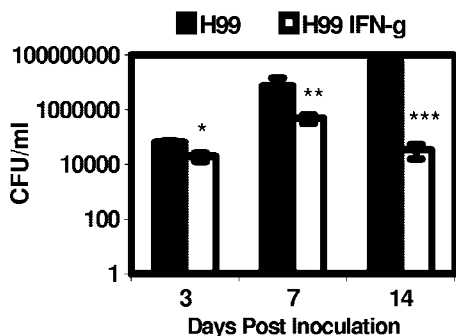

FIG. 4. Effect of IFN-γ-producing *C. neoformans* strain on pulmonary fungal burden. BALB/c mice received an intranasal inoculation of 10^5 CFU of the IFN-γ-producing *C. neoformans* strain (H99 IFN-g) or wild-type *C. neoformans* strain H99 (H99), and the pulmonary fungal burden was quantified. Results are expressed as mean CFU per milliliter ± standard errors of the means. Asterisks indicate where significant decreases were observed compared to mice infected with *C. neoformans* strain H99.

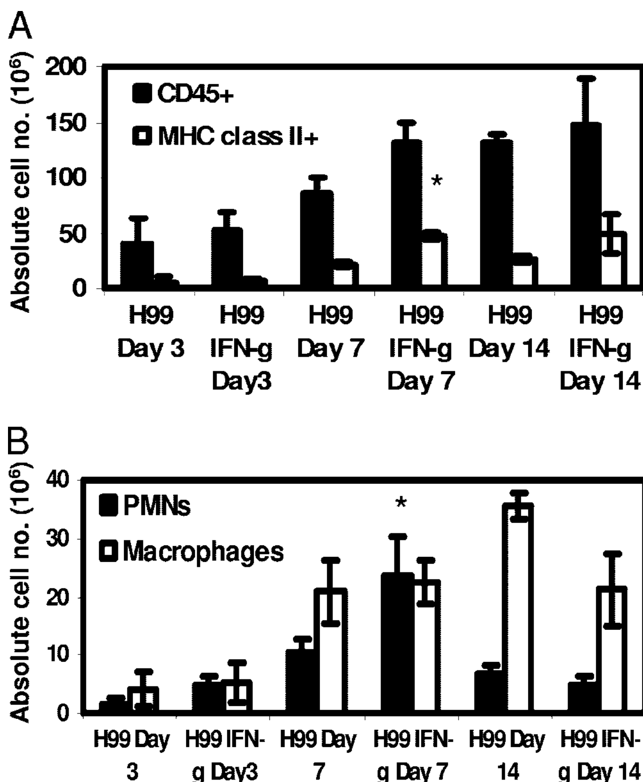

FIG. 5. Pulmonary leukocyte recruitment during experimental pulmonary cryptococcosis. BALB/c mice were given an intranasal inoculation with 10^5 CFU of the IFN-γ-producing (H99 IFN-g) or wild-type (H99) *C. neoformans* strain. Leukocytes were isolated from whole lungs by enzymatic digestion and labeled with anti-CD45 antibodies or anti-IA/IE (MHC class II) (A) or dual labeled with anti-Gr-1 (PMNs) and CD45 or anti-F4/80 (macrophages) and CD45 antibodies (B) and analyzed by flow cytometry. Data shown are cumulative of three experiments using 5 mice per group. Results are expressed as means ± standard errors of the means. Asterisks indicate where significant differences (*, $P < 0.05$) were observed compared to mice inoculated with *C. neoformans* strain H99.

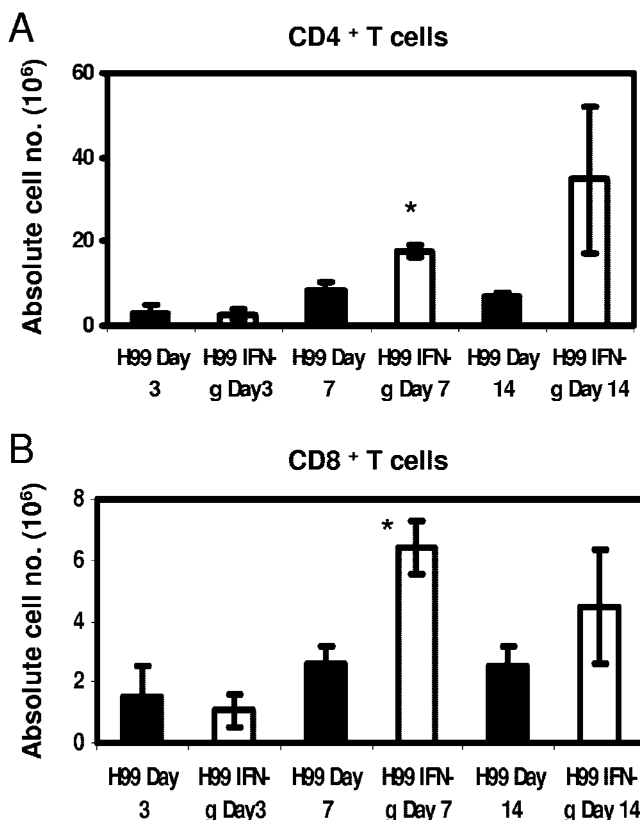

FIG. 6. Pulmonary lymphocyte recruitment during pulmonary cryptococcosis. BALB/c mice were intranasally inoculated with 10^5 CFU of the IFN-γ-producing *C. neoformans* strain (H99 IFN-g) or wild-type *C. neoformans* (H99). Pulmonary leukocytes were isolated from whole lungs by enzymatic digestion. The lung cells were dual labeled with anti-CD4 and anti-CD3 antibodies (A) or anti-CD8 and anti-CD3 antibodies (B) and analyzed by flow cytometry. Data shown are cumulative of three experiments using 5 mice per group. Results are expressed as means ± standard errors of the means. Asterisks indicate where significant differences (*, $P < 0.05$) were observed compared to mice inoculated with *C. neoformans* strain H99.

producing *C. neoformans* strain-infected mice (data not shown).

Pulmonary cytokine expression during experimental cryptococcosis. To evaluate local cytokine responses, lung homogenates were prepared from BALB/c mice infected with the IFN-γ-producing or wild-type *C. neoformans* strains on days 3 and 7 postinoculation and evaluated for Th1-type (IL-2, IL-12 p40, IL-12 p70, and IFN-γ), Th2-type (IL-4 and IL-5), and inflammatory (IL-1α, TNF-α, granulocyte-colony stimulating factor [G-CSF], and IL-17) cytokine expression as well as chemokine (MIP-1α and regulated upon activation, normal T-cell-expressed and secreted [RANTES]) production. As shown in Table 1, Th1-type and inflammatory cytokine and chemokine levels were significantly higher, and conversely Th2-type cytokine expression was significantly lower in lung homogenates derived from mice infected with the IFN-γ-producing *C. neoformans* strain compared to wild-type-infected mice on day 7 postinoculation. The increases in Th1-type cytokines and chemokine expression in mice infected with the IFN-γ-producing strain closely mirrored the increases in pulmonary inflamma-

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TABLE 1. Pulmonary Th1/Th2, inflammatory cytokine, and chemokine levels[a]

Cytokine or chemokine	Level (pg/ml) in pulmonary homogenate tissue at day:			
	3		7	
	H99	H99 IFN-γ	H99	H99 IFN-γ
Th1-type cytokine				
IFN-γ	118.8 ± 27	104.1 ± 27	188.6 ± 27.5	1,426 ± 122.5*
IL-2	143.7 ± 22.5	171.4 ± 17.8	363.5 ± 42.5	582.9 ± 51.9*
IL-12 p40	262.6 ± 17.4	277.3 ± 35.4	427.5 ± 33.8	988.5 ± 118*
IL-12 p70	101.6 ± 11.3	133.6 ± 28.3	222.9 ± 14.3	425.7 ± 48.3*
Th2-type cytokine				
IL-4	9.8 ± 2.2	7 ± 0.6	104.6 ± 11.9*	56.9 ± 4.2
IL-5	312.4 ± 45.0	99.15 ± 26.8	1,031 ± 109.1*	347.9 ± 39.2
Inflammatory cytokine				
IL-1α	195.5 ± 19.3	252.4 ± 38.5	398.1 ± 18.9	665.8 ± 92.2*
IL-17	223 ± 25.5	378.1 ± 70.8	467.9 ± 21.8	1,361 ± 100.4*
TNF-α	132.3 ± 42.1	160.7 ± 47.3	167.1 ± 31.9	541 ± 159.1*
G-CSF	436.4 ± 51.6	590.4 ± 120.1	961.1 ± 78.6	1,324 ± 115.6*
Chemokine				
MIP-1α	648.1 ± 80	719.2 ± 112.3	1,838 ± 179	3,018 ± 428.4*
RANTES	542.6 ± 65.4	546.9 ± 62.37	1,008 ± 75.8	1,652 ± 189.1*

[a] *, $P < 0.05$ compared to the infected counterpart on the same day postinoculation.

tory (Fig. 5A) and lymphocyte cell recruitment (Fig. 6) and subsequent reductions in pulmonary fungal burden observed during infection in this model system.

DISCUSSION

Attenuated microbial pathogens have successfully been used as vectors for targeted cytokine gene therapy against various diseases (16, 24, 35, 36). However, the studies presented herein represent the first instance in which a pathogenic fungus has been genetically altered to express a cytokine with biological effects in vivo towards the resolution of disease. Individuals at high risk for developing invasive *C. neoformans* infections have suppressed Th1-type cell-mediated immune (CMI) responses. Studies have demonstrated that experimental infection with heat-killed or attenuated *C. neoformans* strains do not stimulate Th1-type CMI responses or protect against subsequent cryptococcal infections. Consequently, we reasoned that a *C. neoformans* strain expressing IFN-γ would elicit local protective immunity against acute and subsequent infections. Mice infected with the IFN-γ-expressing *C. neoformans* strain not only resolved the primary infection but also were completely protected against a second challenge with a pathogenic *C. neoformans* strain. The capacity of prior infection with the IFN-γ-producing strain to induce protection in "immune deficient" A/Jcr mice against a second pulmonary *C. neoformans* infection suggests that vaccine strategies designed to stimulate Th1-type anti-cryptococcal host responses can afford some level of protection in immune-compromised individuals. Importantly, infection with the IFN-γ-producing strain resulted in 100% protection against challenge with an extremely virulent wild-type strain, where the organism appears to have been completely eliminated from the animals. Therefore, pulmonary infection of mice with an IFN-γ-producing *C. neoformans* strain resulted in the development of adaptive anti-cryptococcal immune responses that were shown to be protective. This result is dramatically different compared to our previous results using a temperature-sensitive mutant of *C. neoformans* that can survive in the host for approximately 2 weeks, producing some inflammatory responses before elimination (44). However, mice given a previous infection with the temperature-sensitive *C. neoformans* mutant demonstrate no protection against rechallenge with a fully virulent *C. neoformans* strain. These data demonstrate that local secretion of IFN-γ by infecting yeasts can modulate local anti-cryptococcal host immune responses, leading to the development of protective host immunity against subsequent cryptococcal infections.

Resolution of an experimental pulmonary infection with the IFN-γ-expressing *C. neoformans* strain is unlikely to result from attenuation of the strain following integration of the IFN-γ expression construct. Integration of the IFN-γ expression construct and/or IFN-γ production was observed to not affect any of the phenotypes (viability at 30°C and 37°C, urease production, phospholipase activity, melanin production, capsule production, and sensitivity to oxidative stress) in the IFN-γ-producing strain that have been associated with cryptococcal pathogenesis. In addition, we observed an increase in pulmonary fungal burden on day 7 compared to day 3 postinoculation in BALB/c mice infected with the IFN-γ-producing strain (Fig. 4), indicating that integration of the IFN-γ expression construct did not affect growth of the strain in vivo. Lastly, studies demonstrating the inability of immunization with heat-killed or attenuated *C. neoformans* strains to protect mice from a second pulmonary cryptococcal infection (44) indicate that immunization with attenuated cryptococcal strains alone are inadequate at inducing protective anti-cryptococcal immune responses.

To identify an immune correlate for the protective immunity, we analyzed pulmonary cellular responses and cytokine production during infection. Our results indicated that exper-

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imental infection of mice with the IFN-γ-producing *C. neoformans* strain resulted in a significantly greater number of MHC class II$^+$ cells (Fig. 5A), PMNs (Fig. 5B), and CD4$^+$ (Fig. 6A) and CD8$^+$ (Fig. 6B) T lymphocytes in the lungs on day 7 postinoculation compared to wild-type-infected mice. Cytokine analyses of pulmonary homogenates derived from mice infected with the IFN-γ-producing *C. neoformans* strain were demonstrated to have (i) significantly greater levels of the Th-1 type cytokines IFN-γ, IL-2, and IL-12; (ii) significantly greater levels of the inflammatory cytokines/chemokines IL-1α, IL-17, TNF-α, G-CSF, MIP-1α, and RANTES; and, conversely, (iii) significantly lower levels of the Th-2-type cytokines IL-4 and IL-5 compared to wild-type-infected mice on day 7 postinoculation (Table 1). These results strongly suggest that the protection against acute experimental pulmonary cryptococcosis mediated by the IFN-γ-expressing *C. neoformans* strains was due to the selective induction of local Th1-type CMI responses leading to the resolution of the cryptococcal infection.

Although no significant difference in the absolute number of B lymphocytes was observed quantitatively between IFN-γ-producing *C. neoformans* strain- and wild-type-infected mice, we cannot rule out the possibility that certain qualitative differences in the B-lymphocyte response also contributed to the reduction of pulmonary fungal burden in IFN-γ-producing *C. neoformans*-infected mice. Studies suggest that effective anticryptococcal host immune responses are associated with the predominance of "protective" monoclonal antibody isotypes (i.e., IgG1, IgG2a, and IgG2b) against *C. neoformans*. Likewise, although no significant difference in macrophage infiltrate was observed in mice infected with the IFN-γ-producing strain compared to wild-type-infected mice, a recent study has shown that T-helper responses (Th1/Th2) during experimental pulmonary cryptococcosis in mice help determine macrophage activation (classical versus alternative) and their efficacy against cryptococcal infections (2). Specifically, Th2-type polarized responses against pulmonary cryptococcal infection in IFN-γ knockout mice led to the enhanced generation of alternatively activated macrophages and a loss of fungistasis, leading to progressive pulmonary cryptococcal infection. Therefore, a more in-depth study is needed to investigate any role of B lymphocytes and macrophage activation in the host response of mice infected with the IFN-γ-producing strain and should not be ignored as a potential component in host immunity against pulmonary cryptococcosis.

These studies provide a valuable framework from which to advance our understanding in devising strategies for establishing local protective host responses against life-threatening fungal infections. The use of a pathogenic fungus engineered to secrete host cytokines is a novel way to examine the immune response to these pathogens. Further work is under way to evaluate the efficacy of immunization of mice with *C. neoformans* strain H99-γ to confer protection against a second infection with other cryptococcal strains. This study and additional studies using this strain and additional strains engineered to secrete other host cytokines will be helpful in developing immune-based therapies for human mycoses. We are cautious, however, in extrapolating the results of our murine studies to what we may expect in humans. Namely, in vitro studies failed to show an increase in the anti-cryptococcal activity of human macrophages stimulated with IFN-γ (3, 39), perhaps due to differences in macrophage nitric oxide production between mice and humans. However, questions regarding the adequacy of in vitro systems used for evaluating nitric oxide activation in human macrophages remains unresolved (C. Nathan, Letter, Science **312:**1874–1875, 2006). On the other hand, recent clinical studies in patients with cryptococcal meningitis have demonstrated that the administration of IFN-γ augments host anticryptococcal immune responses (37, 42). The necessity of such therapies will continue to gain prominence due to an increasing population of individuals with severe immune dysfunction (i.e., human immunodeficiency virus-infected individuals, individuals receiving corticosteroid therapy, lymphoproliferative disorders, and organ transplant recipients). The viability of therapies using live yeast in immune-compromised individuals is a cause for concern, therefore the proper safeguards such as strains engineered to die in the host need to be investigated and implemented prior to its use. The regulation of essential genes by tetracycline treatment is one possible strategy to control fungal viability (M. Chayakulkeeree and J. R. Perfect, Abstr. 6th Int. Conf. Cryptococcus Cryptococcosis, abstr. 157, 2005). Furthermore, the recent outbreak of cryptococcosis in otherwise healthy individuals on Vancouver Island (17) suggests a necessity of developing strategies to prevent fungal infections in immune-competent and immune-deficient individuals. Since *C. neoformans* infections in immune-competent individuals are usually contained in the lungs, it is reasonable to infer that therapies that enhance local anti-cryptococcal CMI responses could provide additional protection against subsequent or perhaps latent infections in this population. Taken together, the results presented herein support the concept that strategies that induce Th1-type CMI responses against invasive fungal infections such as cryptococcosis can result in protection against subsequent fungal infections and describes a paradigm for future vaccine studies.

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