The Journal of Immunology

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J Immunol 2002; 168:3419-3427; ;
doi: 10.4049/jimmunol.168.7.3419
http://www.jimmunol.org/content/168/7/3419

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Antibody Efficacy in Murine Pulmonary *Cryptococcus neoformans* Infection: A Role for Nitric Oxide[1]

Johanna Rivera,* Jean Mukherjee,† Louis M. Weiss,‡§ and Arturo Casadevall[2]*§

We investigated the pathogenesis of pulmonary *Cryptococcus neoformans* infection and passive Ab efficacy in mice deficient in inducible NO synthase (NOS2$^{-/-}$) and the parental strain. Parental mice lived significantly longer than NOS2$^{-/-}$ mice after intratracheal infection, despite having a higher lung fungal burden. Administration of Ab reduced lung CFU in both NOS2$^{-/-}$ and parental mice, but prolonged survival and increased the inflammatory response only in parental mice. Ab administration was associated with increased serum nitrite and reduced polysaccharide levels in parental mice. Eosinophils were present in greater numbers in the lung of infected NOS2$^{-/-}$ mice than parental mice, irrespective of Ab administration. *C. neoformans* infection in NOS2$^{-/-}$ mice resulted in significantly higher levels of IFN-γ, monocyte chemoattractant protein-1, and macrophage-inflammatory protein-1α than parental mice. Ab administration had different effects on infected NOS2$^{-/-}$ and parental mice with respect to IFN-γ, monocyte chemoattractant protein-1, and macrophage-inflammatory protein-1α levels. Ab administration increased lung levels of IFN-γ in parental mice and reduced levels in NOS2$^{-/-}$ mice. The results indicate that NO is involved in the regulation of cytokine expression in response to cryptococcal pneumonia and is necessary for Ab efficacy against *C. neoformans* in mice. Our findings indicate a complex relationship between Ab efficacy against *C. neoformans* and cytokine expression, underscoring the interdependency of cellular and humoral defense mechanisms. *The Journal of Immunology*, **2002, 168: 3419–3427.**

Cryptococcus neoformans is a facultative intracellular pathogen (1) that causes life-threatening infections, particularly in patients with AIDS. Administration of Ab specific for the capsular polysaccharide modifies the course of *C. neoformans* infection in mice (2). Currently, Ab therapy is being evaluated for treatment of human cryptococcosis (3), and vaccines are being developed that mediate protection by eliciting protective Ab responses (4). In recent years, there has been a renaissance in studies of humoral immunity using mAb to demonstrate Ab-mediated protection against a variety of intracellular pathogens including *Mycobacterium tuberculosis* (5), *Listeria monocytogenes* (6), and *Ehrlichia chaffeensis* (7). However, the mechanisms by which certain Abs can mediate protection against intracellular pathogens are not well understood. Furthermore, the variables that affect Ab-mediated efficacy against intracellular pathogens remain to be defined.

For *C. neoformans*, several potential mechanisms of Ab-mediated protection have been described, including enhancement of phagocytosis by macrophages (8), complement activation (9), and clearance of polysaccharide Ag, which causes a variety of deleterious effects on the host immune response (10–12). Mice infected with *C. neoformans* and given specific Ab mount a more intense pulmonary granulomatous response than control mice, suggesting that Ab-mediated protection involves enhancement of cellular immunity (13). Hence, the current consensus is that humoral immunity protects against *C. neoformans* by enhancing the efficacy of the cellular immune response (14). In support of this, in vitro studies have shown that Ab binding to the capsule can enhance Ag presentation and induce changes in cytokine and costimulatory molecule expression (15, 16). However, the mechanism by which Ab alters the inflammatory response to *C. neoformans* in vivo and the variables that affect the outcome of humoral-cellular collaboration are unknown.

The generation of NO by inducible NO synthase (NOS2)[3] has been implicated in the antimicrobial activity of activated macrophages against a variety of intracellular pathogens, including *M. tuberculosis* (17), *Leishmania major* (18), and *L. moncytogenes* (19). Macrophages play a central role in host defense against *C. neoformans* through both oxygen- and nitrogen-derived molecular mechanisms (20, 21). Depending on the concentration, chemically generated NO is fungistatic or fungicidal for *C. neoformans* (22). Furthermore, NO derived from cultured human astrocytes is fungistatic against *C. neoformans* (23), and the appearance of NOS expression in tissue is associated with the clearance of *C. neoformans* in immunocompetent mice and rats (24, 25). Hence, there is considerable evidence that NO is important for host defense against *C. neoformans*.

Because NO is important for host defense against *C. neoformans* and because Ab appears to depend on an effective cellular response for activity, we examined the efficacy of Ab administration against cryptococcal infection in mice with a targeted deletion of NOS2.

*Department of Microbiology and Immunology and †University of Massachusetts Medical School, Worcester, MA 01605; and Departments of ‡Pathology and §Medicine, Division of Infectious Diseases, Albert Einstein College of Medicine, Bronx, NY 10461

Received for publication August 6, 2001. Accepted for publication January 25, 2002.

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[1] J.R. is supported by the Minority Access to Research Careers predoctoral fellowship 5-F31-GM18951. L.M.W. is supported by National Institutes of Health Awards AI39454 and AI31788. A.C. is supported by National Institutes of Health Awards AI33774, AI3142, and HL-59842, and a Burroughs Wellcome Development Therapeutics Award. The data in this paper are from a thesis to be submitted by J.R. in partial fulfillment of the requirements of the degree of doctor of philosophy in the Sue Golding Graduate Division of Medical Science, Albert Einstein College of Medicine, Yeshiva University (Bronx, NY).

[2] Address correspondence and reprint requests to Dr. Arturo Casadevall, Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. E-mail address: casadeva@aecom.yu.edu

[3] Abbreviations used in this paper: NOS2, inducible NO synthase; GXM, glucuronoxylomannan; IT, intratracheal(ly); MCP, monocyte chemoattractant protein; MIP, macrophage-inflammatory protein.

0022-1767/02/$02.00

NOS2$^{-/-}$ mice were found to be more susceptible to *C. neoformans* infection, and Ab administration was ineffective in prolonging survival. Our results provide additional support for the importance of NO in defense against *C. neoformans* and demonstrate, for the first time, a dependence of Ab function on NO.

Materials and Methods

C. neoformans *and GXM Ag*

Strain ATCC 24067 (serotype D; American Type Culture Collection (ATCC), Manassas, VA) was grown from frozen stock in Sabouraud's dextrose broth (Difco, Detroit, MI) at 30°C for 48 h. This strain was selected because it has been extensively used in Ab studies and is well characterized (26). Inoculum was confirmed by counting CFUs on Sabouraud dextrose agar (Difco). Glucuronoxylomannan (GXM) was isolated from culture supernatant of strain 24067, as described (27).

Antibodies

mAb 2H1 (IgG1) binds the capsule polysaccharide GXM of *C. neoformans* (28). This mAb protects mice against *C. neoformans* infection in a variety of infection models (13, 28–30). Murine ascites containing mAb 2H1 protein was prepared by injecting 2H1 hybridoma cells into the peritoneum of pristine-primed BALB/c mice. NSO (control) is the nonproducing mouse myeloma fusion partner of the hybridoma 2H1, and ascites produced using this cell line was used in some experiments. For some experiments, mAb 2H1 was purified from ascites fluid using protein G affinity chromatography (Pierce, Rockford, IL), as instructed by the manufacturer, and PBS was used as the control.

Mice

Breeding pairs of C57BL/6J × 129/SvEv (parental) and NOS2$^{-/-}$ mice (gift of C. Nathan, Weill Medical College of Cornell University, New York, NY) were maintained at the Animal Institute of Albert Einstein College of Medicine. Additional 6- to 8-wk-old female C57BL/6J × 129/SvEv (parental-F_1), C57BL/6J, and NOS2$^{-/-}$ were obtained from The Jackson Laboratory (Bar Harbor, ME). These mice were kept in a specific pathogen-free barrier facility in microisolator cages, fed irradiated rodent food, provided with autoclaved bedding, and routinely monitored for serologic evidence of exposure to common murine pathogens. All serological testing was negative.

Macrophage assays

Alveolar and peritoneal macrophages were isolated from parental and NOS2$^{-/-}$ mice. For alveolar macrophage isolation, the tracheas of euthanized mice were exposed by a skin incision, and a 20-gauge angiocath (BD Biosciences, Sandy, UT) was advanced into the trachea 3 mm and sutured in place. The lungs were then lavaged 10 times through the catheter with sterile calcium and magnesium-free HBSS without Phenol Red (Life Technologies, Grand Island, NY) with 1 mM EGTA (Sigma-Aldrich, St. Louis, MO) using 0.8 ml per wash (31). The lavage fluids were pooled, and cells were collected by centrifugation. Peritoneal macrophages were collected from the same mice by washing the abdominal cavity five times with HBSS using a Pasteur pipette. The total cell suspension was collected by centrifugation, and erythrocytes were lysed by resuspending in ice-cold 0.17 M NH_4Cl and incubating on ice for 10 min. A 10-fold excess of RPMI 1640 solution was then added to make the solution isotonic, the cells were collected by centrifugation and suspended in staining solution (PBS, 1% FBS), and live cells (trypan blue exclusion) were counted in a hemocytometer chamber. The cells were then suspended in DMEM (Life Technologies), 10% NCTC-109 medium (Life Technologies), and 1% nonessential amino acids (Cellgro; Mediatech, Washington, D.C.) containing penicillin and streptomycin (Life Technologies). The cells were plated at a density of 8×10^4/well in a 96-well tissue culture plates, and incubated overnight at 37°C.

The protocols for macrophage phagocytosis and killing assays have been described (13). Briefly, macrophages were stimulated overnight with 100 U/ml murine rIFN-γ (Boehringer Mannheim, Indianapolis, IN). The media were replaced with fresh media that contained 100 U/ml IFN-γ and 1 μg/ml LPS (Sigma-Aldrich). Phagocytosis was measured in media with or without 10 μg/ml mAb 2H1. *C. neoformans* cells were added at a macrophage to yeast ratio of 5:1, and the suspensions were incubated at 37°C for 2 or 4 h. The macrophage monolayer was then washed several times with sterile PBS, fixed with cold absolute methanol, and stained with 1:20 solution of Giemsa. The phagocytic index was determined by microscopic examination at a magnification of ×600. The phagocytic index is the number of attached and ingested cryptococci divided by the number of macrophages per field. Four fields were counted in four wells for each measurement. For killing assays, activated macrophages and *C. neoformans* were coincubated for 18 or 24 h at 37°C. Cell supernatants were collected and cells were lysed by addition of 0.1 of sterile distilled water to each well and incubating for 30 min at room temperature, followed by aspirating and ejecting the lysate with a pipette several times to complete cell disruption. PBS (0.1 ml) was used to rinse each well, and the cell supernatant, lysate, and rinse from each well were pooled, vortexed, diluted, vortexed again, and spread on Sabouraud's dextrose agar (32). Nitrite in cell supernatant was measured using Griess reagent (1% sulfanilamide, 0.1% *N*-1-napthylethylenediamine, 2.5% phosphoric acid) after reducing nitrate to nitrite with *Pseudomonas oleovorans* nitrate reductase, as described (33).

In vivo studies

Survival analysis. Parental and NOS2$^{-/-}$ (6- to 8-wk-old) mice were infected intratracheally (IT) with 10^6 *C. neoformans*, as previously described (13). Ab was administered i.p. 24 h before infection. Mice were monitored daily for mortality and morbidity. On day 107, surviving mice were killed, their lungs were removed, and the right upper lobes were fixed in 10% buffered formalin (Fisher, Pittsburgh, PA) for histology.

CFU experiments. Parental and NOS2$^{-/-}$ mice were given mAb and infected, as described above. At day 14 postinfection, mice were killed by cervical dislocation and organ CFUs were determined by homogenizing the tissue and plating on Sabouraud's agar, as described (13). Blood was obtained from the orbital sinus, and serum was isolated by centrifugation of blood. Paraffin-embedded lung tissue sections were stained with H&E or mucicarmine for histological examination.

GXM levels and clearance. GXM clearance experiments were performed as described (34). Briefly, parental and NOS2$^{-/-}$ mice were treated with mAb i.p. 24 h before i.v. administration of GXM (50 μg). Blood was obtained from the orbital sinus immediately before mAb administration and 2, 6, 23, 35, 59, and 93 h after i.v. GXM administration. Serum was isolated by centrifugation of blood. Mice were sacrificed by cervical dislocation, and organs were removed immediately. Organs were homogenized (Ultra Turrax T25 homogenizer; Janke and Kunkel, Staufen, Germany), and organ homogenate and serum were treated with proteinase K (1 mg/ml, 1 h, 37°C). GXM-capture ELISA was used to detect GXM in the serum, lung, liver, spleen, and kidney of mice, as previously described, except that mAb 2D10 was used for capture and mAb 2H1 was used for detection (35).

Serum nitrite levels. Parental-F_1 mice were fed a chemically defined amino acid diet (L-arginine, nitrate and nitrite free; Ziegler Brothers, Gardner, PA). mAb 2H1 and control were administered 24 h before infection IT with 10^6 *C. neoformans*. Mice were bled at various intervals from the retroorbital sinus, and serum nitrite was measured using Griess reagent (1% sulfanilamide, 0.1% *N*-1-napthylethylenediamine, 2.5% phosphoric acid) after reducing nitrate to nitrite with *P. oleovorans* nitrate reductase, as described (33). The concentration of serum nitrite was calculated from the absorbance at 540 nm using a standard linear curve (0–250 μM).

Cytokine and chemokine studies. Parental and NOS2$^{-/-}$ mice (6–8 wk old) were infected, as described above. Experimental groups were given purified mAb 2H1 or PBS 24 h before infection with yeast. Sham-infected groups were given mAb 2H1 or PBS 24 h before IT administration of PBS. Mice were sacrificed at day 14 postinfection, and the right lungs were homogenized in 2 ml PBS in the presence of protease inhibitors (Complete Mini; Boehringer Mannheim). The homogenates were centrifuged at $6000 \times g$ for 10 min to remove cell debris, and the supernatant was frozen at −80°C until tested. The supernatants were assayed for concentrations of IL-2, IL-4, IL-6, IL-10, IL-12p70, monocyte chemoattractant protein (MCP)-1, and macrophage-inflammatory protein (MIP)-1α using ELISA kits (BD PharMingen (San Diego, CA) and R&D Systems (Minneapolis, MN)). The detection limits of cytokine assays are 3.1 pg/ml for IL-2, 7.8 pg/ml for IL-4, 15.6 pg/ml for IL-6 and TNF-α, 31.3 pg/ml for IL-10 and IFN-γ, and 62.5 pg/ml for IL-12 p40, as stated by the manufacturer. The detection limits of the chemokine assays are 4.7 pg/ml for MIP-1α and 15.6 pg/ml for MCP-1, as determined by the manufacturer.

Endotoxin precautions. For the cytokine and cellular response experiments, great care was taken to avoid the contamination of endotoxin. One person, in a laminar flow hood, did all work involving purification of mAb reagents and handling of reagents. Solutions were made with endotoxin-free water or PBS. Extensive use was made of disposable pyrogen-free plastic ware, pipettes, pipet tips, microcentrifuge tubes, etc. Endotoxin concentration in mAb solutions measured by *Limulus* amebocyte assay (BioWhittaker, Walkersville, MD) was below the limit of detection of the assay.

Preparation of lung leukocytes. Parental and NOS2$^{-/-}$ mice were given mAb and infected, as described above. At day 14 postinfection, lungs were excised, minced, homogenized using a sterile 70-μm nylon mesh (BD Biosciences), and digested for 60 min using 10 ml/lung digestion buffer: RPMI 1640, 10% FCS, 1 mg/ml collagenase (Boehringer Mannheim), and 30 μg/ml DNase I (Sigma-Aldrich). The total cell suspension was collected by centrifugation; erythrocytes were lysed, as described above.

Cell staining and analysis. Neutrophils, eosinophils, lymphocytes, and monocytes/macrophages were visually counted from Giemsa-stained samples of lung cell suspensions centrifuged onto glass slides (Cytospin; Thermo Shandon, Pittsburgh, PA). A total of 200–400 cells was counted from randomly chosen high-power microscope fields for each sample. For FACS analysis, lung leukocytes (10^6) were stained for 30 min on ice with 100 μl of one of the following Abs diluted in staining buffer: 2 μg/ml R-PE-labeled anti-CD45, 5 μg/ml FITC-labeled anti-mouse CD4, 2 μg/ml CyChrome-labeled anti-mouse CD8, 5 μg/ml FITC-labeled anti-mouse CD19, and 5 μg/ml FITC-labeled anti-mouse MAC-3 (all mAbs from BD PharMingen). The samples were washed twice in staining buffer and fixed in 1% paraformaldehyde. Stained samples were stored in the dark at 4°C until analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest (BD Biosciences) software. Live cells were gated as judged from forward and side laser scatter and CD45$^+$ cells. Controls consisted of isotype-matched irrelevant Abs.

Statistics

All data were analyzed by the Student *t* test, Kruskal-Wallis test (Primer; McGraw-Hill, New York, NY), and log rank analysis (Sigmastat, Chicago, IL).

Results

In vitro phagocytosis and killing assay

We investigated the ability of alveolar macrophages isolated from parental and NOS2$^{-/-}$ mice to phagocytose and kill *C. neoformans*. There were no differences in phagocytosis of *C. neoformans* by parental and NOS2$^{-/-}$ alveolar macrophages ($p = 0.82$). The phagocytic indices were significantly higher in the mAb 2H1-treated groups for parental and NOS2$^{-/-}$ alveolar macrophages in both 2- (data not shown) and 4-h assays (Fig. 1*A*). Incubation of parental and NOS2$^{-/-}$ alveolar macrophages with *C. neoformans* 24067 resulted in a significant reduction in CFU after 18 (data not shown) and 24 h (Fig. 1*B*). Alveolar macrophages from parental mice were more effective at inhibiting *C. neoformans* than NOS2$^{-/-}$ alveolar macrophages. To demonstrate that parental alveolar macrophages produced NO during the killing assay, nitrite levels were measured. Administration of mAb 2H1 enhanced production of NO correlating with reduction of CFU (Fig. 1*C*). As anticipated, NO was not detected from NOS2$^{-/-}$ alveolar macrophages during the killing assay (data not shown).

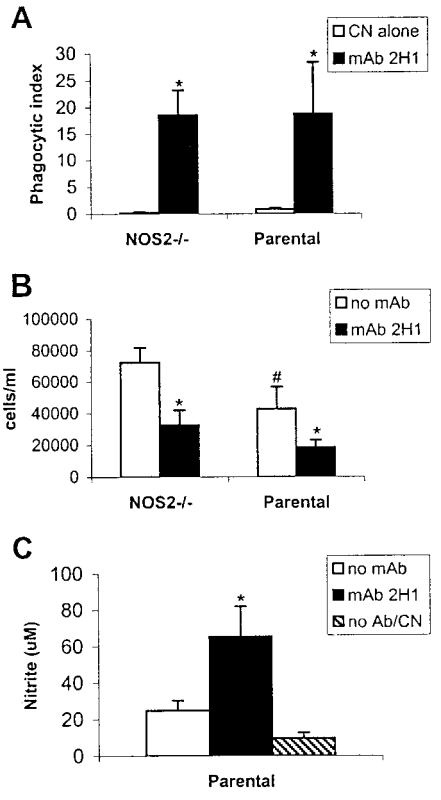

FIGURE 1. Analysis of murine alveolar macrophages from NOS2$^{-/-}$ and parental mice. *A*, Phagocytosis (4 h) of *C. neoformans* in vitro in the presence of mAb 2H1 or the addition of comparable volume of media. Bars denote the average of measurements from four wells. This experiment was done twice with similar results. ∗, Values with mAb 2H1 are significantly different relative to conditions with no mAb 2H1 for NOS2$^{-/-}$ and parental macrophages. *B*, Killing of *C. neoformans* in the presence of mAb 2H1. ∗, Values with mAb 2H1 are significantly different relative to conditions with no mAb 2H1 for NOS2$^{-/-}$ and parental macrophages. #, Values are significantly different relative to NOS2$^{-/-}$ macrophages in the absence of mAb. *C*, Nitrite levels in the supernatant of macrophages from parental during killing assay. ∗, Values are significantly different relative to conditions with no mAb 2H1 and/or *C. neoformans*. Nitrite levels were detected using Griess assay. A value of $p < 0.05$ is considered significant. Error bars denote SDs.

In vivo experiments

Survival. Parental mice infected with *C. neoformans* survived longer than NOS2$^{-/-}$ mice (median survival of 31 and 16 days, respectively; $p < 0.001$) (Fig. 2). Administration of mAb 2H1 (IgG1) prolonged survival of *C. neoformans*-infected parental mice (median survival of 85 days; $p < 0.05$) relative to parental control mice, whereas administration of mAb 2H1 did not prolong survival of NOS2$^{-/-}$ mice (median survival of 17 days; $p = 0.938$) (Fig. 2). The experiment was terminated on day 107, at which time all surviving mice (three parental control, six parental mAb 2H1-treated, and one mAb 2H1-treated NOS2$^{-/-}$ mice) had detectable lung CFU, indicating chronic infection. In summary, mAb administration before infection prolonged survival in parental mice given lethal *C. neoformans* infection, but not in NOS2$^{-/-}$ mice.

Fungal burden. To further understand the difference in survival and the failure to protect with mAb in NOS2$^{-/-}$ mice, we examined fungal organ burden and circulating polysaccharide. Two independent CFU experiments are summarized in Table I. At day 14, the lung fungal burden in parental mice was significantly higher than in NOS2$^{-/-}$ mice ($p < 0.05$), but there were no differences in brain fungal burden (Table I). Administration of mAb 2H1 significantly reduced lung CFU in parental mice compared with parental control mice ($p = 0.014$). To a lesser extent, administration of mAb 2H1 reduced lung CFU in NOS2$^{-/-}$ mice ($p = 0.223$).

Histological analysis. At day 14, the lungs of parental control mice exhibited minimal inflammation with large extracellular collections of *C. neoformans* yeast cells in the alveolar spaces (Fig. 3*A*). In contrast, the lungs of parental mice treated with mAb 2H1 exhibited an intense granulomatous inflammation composed of macrophages and epithelioid cells (Fig. 3*B*). Perivascular cuffs composed of lymphocytes and polymorphonuclear leukocytes were also present. Histological examination revealed a different inflammatory response in the lungs of parental and NOS2$^{-/-}$ mice (Fig. 3, *C* and *D*). A mixed inflammatory infiltrate composed of polymorphonuclear leukocytes (neutrophils and eosinophils), lymphocytes, macrophages/monocytes, and epithelioid cells

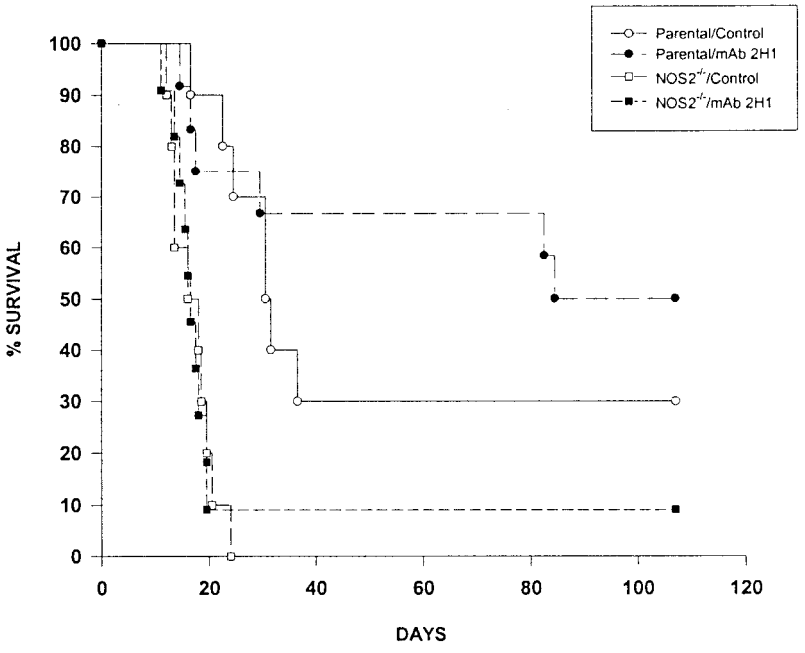

FIGURE 2. Survival of 6- to 8-wk-old parental mice ($n = 10$) and NOS2$^{-/-}$ mice ($n = 10$) following IT infection with 10^6 *C. neoformans* ATCC strain 24067. This experiment was done twice with similar results. Parental vs NOS2$^{-/-}$: $p < 0.001$. Control/Parental vs mAb 2H1/parental: $p < 0.05$.

was present in the perivascular spaces and parenchyma. Mucin staining demonstrated both extracellular and intracellular *C. neoformans* (Fig. 3, *insets*). There were no differences in the type and the extent of inflammation in mAb 2H1-treated NOS2$^{-/-}$ mice compared with NOS2$^{-/-}$ control mice.

Analysis of cellular composition of lung infiltrate. Lung weights, which reflect the magnitude of the inflammatory response, GXM deposition, and organ edema, were similar in parental and NOS2$^{-/-}$ mice (Fig. 4*A*). Accordingly, we found no differences in the total number of cells recruited to the lung of parental and NOS2$^{-/-}$ mice after infection (Fig. 4*B*). On day 14, FACS analysis of lung cells showed similar percentage of lymphocytes and macrophages in the inflammatory infiltrate of parental and NOS2$^{-/-}$ mice (Fig. 4*C*). However, analysis of granulocyte composition revealed significant differences in the proportion of eosinophils and neutrophils in the inflammatory response of parental and NOS2$^{-/-}$ mice. There were higher percentages of eosinophils, irrespective of mAb treatment, in NOS2$^{-/-}$ mice compared with parental mice ($p = 0.001$) (Fig. 4*D*). In addition, NOS2$^{-/-}$ mice exhibited higher percentage of macrophages/monocytes compared with parental mice ($p = 0.025$), but no significant differences were seen between mAb 2H1-treated parental and NOS2$^{-/-}$ mice ($p = 0.473$). In contrast, parental mice contained higher percentage of lymphocytes and neutrophils compared with NOS2$^{-/-}$ mice ($p < 0.001$, for both). There were no differences in the percentage of lymphocytes and neutrophils between mAb 2H1-treated parental and NOS2$^{-/-}$ mice ($p = 0.262$ and $p = 0.098$) (Fig. 4*D*).

GXM serum levels, clearance, and distribution. There was a trend toward lower serum GXM levels in parental mice treated with mAb 2H1 relative to the parental control mice, but this difference was not significant ($p = 0.122$) (Table I). In contrast, infected NOS2$^{-/-}$ mice exhibited significantly higher serum GXM levels compared with parental mice, irrespective of Abtreatment. Given the significantly higher serum GXM levels in NOS2$^{-/-}$, we evaluated the clearance and distribution of GXM Ag in the presence and absence of mAb 2H1 in NOS2$^{-/-}$ and parental mice. The overall pattern of GXM clearance and distribution was similar in NOS2$^{-/-}$ and parental mice, and comparable with that reported previously (34). Serum GXM was rapidly cleared in both NOS2$^{-/-}$ and parental mice given mAb 2H1 and deposited in liver and spleen (Fig. 5).

Nitrite levels in serum

C. neoformans-infected parental mice were bled at various times postinfection, and nitrite concentration in the serum was measured as described (33). Production of nitrate/nitrite correlates with in vivo generation of reactive nitrogen intermediates (33). In the first 8 days postinfection, nitrite levels were significantly higher in

Table I. *Organ CFU and serum GXM in C57BL/6 × 129/SvEv (parental) and NOS2$^{-/-}$ mice 14 days after infection with* C. neoformans[a]

		Parental CFU ($\log_{10}$)			NOS2$^{-/-}$ CFU ($\log_{10}$)		
Expt.	Group	Brain	Lung	GXM (μg/ml)	Brain	Lung	GXM (μg/ml)
I	No treatment	2.99 ± 1.28	7.74 ± 0.15	1.64 ± 1.2	2.74 ± 0.60	7.31 ± 0.53[b]	12.3 ± 19.0
II	Control	2.15 ± 0.29	7.15 ± 0.31	26.5 ± 24.6	2.09 ± 0.15	6.38 ± 0.86	302.1 ± 385.0[c]
	MAb 2H1	2.60 ± 0.88	5.94 ± 1.36[d]	9.8 ± 9.4	2.10 ± 0.21	5.52 ± 1.26[e]	280.1 ± 612.4[f]

[a] NOS2$^{-/-}$ and parental mice received 1 mg of mAb 2H1 or control 24 h prior to infection with 10^6 *C. neoformans*. Mice were killed 14 days later. Statistical analysis was performed using the Kruskal-Wallis test. $n = 10$ for each experimental group.
[b] Comparison with parental mice ($p = 0.031$).
[c] Comparison with parental control mice ($p = 0.013$).
[d] Comparison with parental control mice ($p = 0.014$).
[e] Comparison with NOS2$^{-/-}$ control mice ($p = 0.223$).
[f] Comparison with parental mAb 2H1-treated mice ($p = 0.012$).

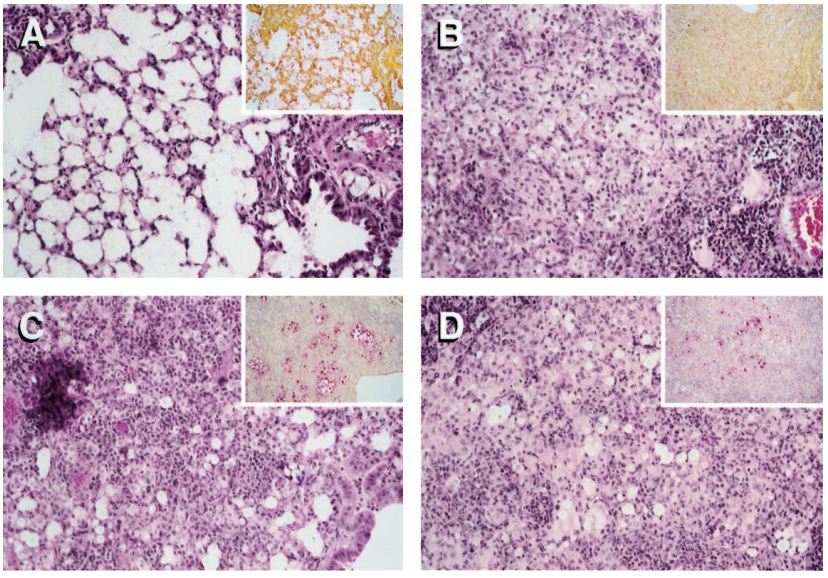

FIGURE 3. Histopathology of lung following IT infection with *C. neoformans* stained with H&E or mucicarmine (*insets*), which stains capsular polysaccharide. Mice were treated with PBS or purified mAb 2H1 24 h before infection. *A*, Control-treated parental mice, day 14. Large collections of extracellular organisms are seen in airspaces, with little inflammation. *B*, mAb 2H1-treated parental mice, day 14. Granulomatous inflammation is seen with fewer cryptococci in airspaces. The inflammatory cells include polymorphonuclear leukocytes, lymphocytes, epithelioid cells, and macrophages. *C*, Control-treated NOS2$^{-/-}$ mice, day 14. There are collections of yeast cells with granulomatous inflammation. *D*, mAb 2H1-treated NOS2$^{-/-}$ mice, day 14. Extensive granulomatous inflammation is associated with small collections of yeast cells. Original magnification, ×50.

mAb 2H1-treated parental mice compared with parental control mice (Fig. 6). From day 14 to day 28 postinfection, serum nitrite levels continued to increase but were not affected by Ab treatment (data not shown).

Cytokine and chemokine studies

To better understand the immune response in NOS2$^{-/-}$ and parental mice, in the presence and absence of Ab, cytokine and

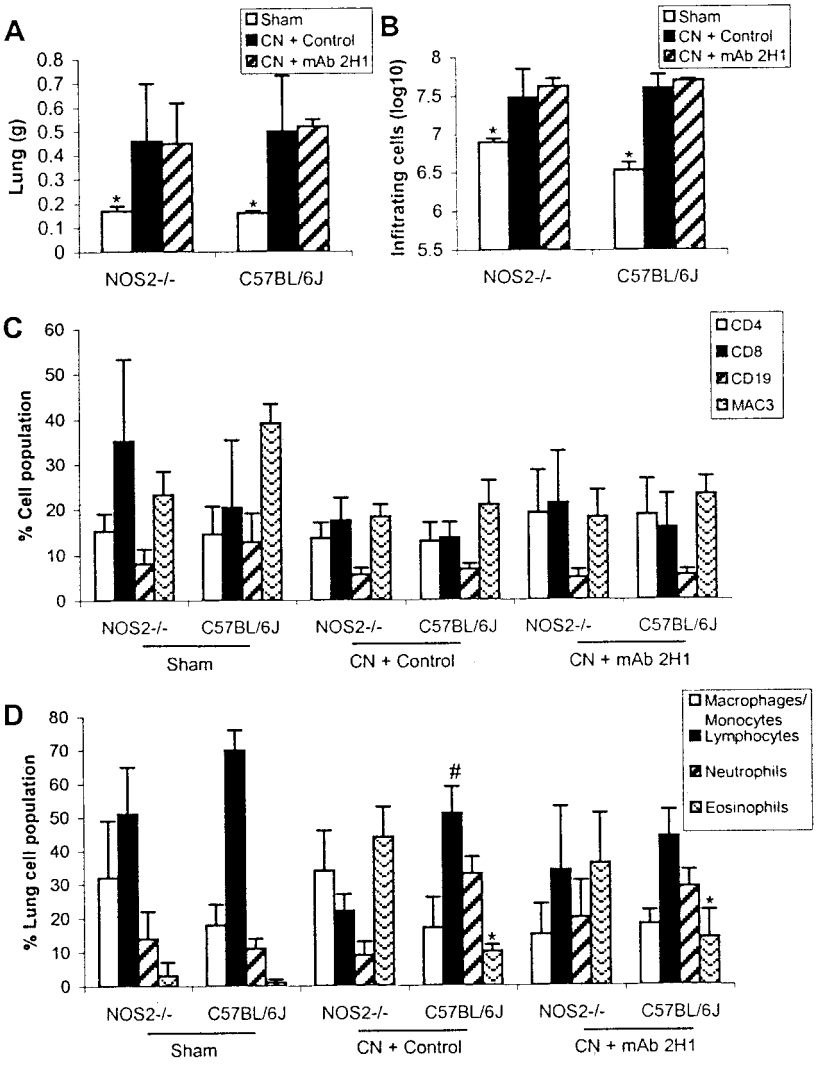

FIGURE 4. Characterization of inflammatory response of *C. neoformans*-infected NOS2$^{-/-}$ and parental mice 14 days postinfection. *A*, Lung weights of infected NOS2$^{-/-}$ and parental mice. Bars denote mean organ weight. ∗, Values are significantly different from infected NOS2$^{-/-}$ and parental mice ($p < 0.05$). *B*, Total number of cells that infiltrated the lungs of infected NOS2$^{-/-}$ and parental mice. ∗, Values are significantly different from infected NOS2$^{-/-}$ and parental mice ($p < 0.05$). Bars denote mean lung cells. *C*, FACS analysis of infiltrating pulmonary B cells, T cells, and macrophages. Bars denote mean percentage. *D*, Morphological analysis of pulmonary macrophages/monocytes, lymphocytes, neutrophils, and eosinophils from infected NOS2$^{-/-}$ and parental mice. ∗, Values are significantly different compared with parental mice ($p = 0.001$). #, Values are significantly different compared with NOS2$^{-/-}$ mice ($p = 0.034$). Mouse groups: sham, $n = 3$; control and mAb 2H1, $n = 6$. Error bars denote SDs.

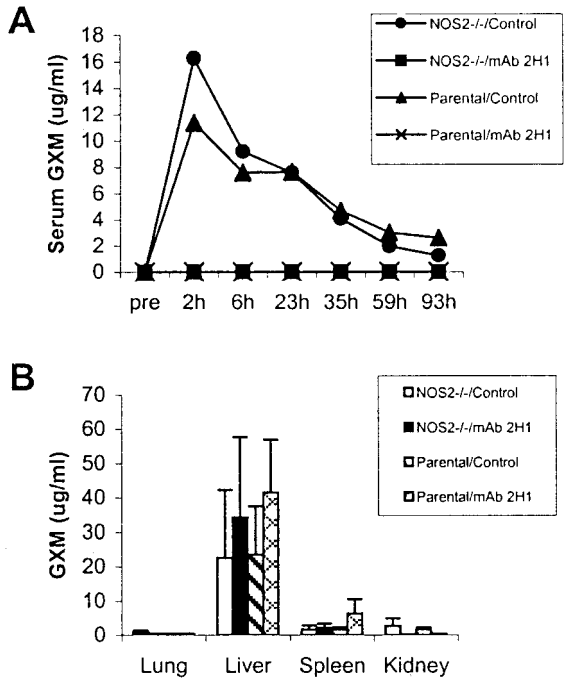

FIGURE 5. Serum polysaccharide clearance and sequestration of polysaccharide in the liver of infected NOS2$^{-/-}$ and parental mice. *A*, Mice were given 50 μg polysaccharide IV with control or mAb 2H1 i.p. and measured at times shown. Each point is average serum GXM concentration at time indicated ($n = 6$). *B*, Polysaccharide levels in organs were measured at the 93 h. Bars denote mean organ polysaccharide concentration ($n = 6$). Error bars denote SDs.

chemokine expression were examined. Preliminary studies using the RNase protection assay revealed differences in cytokine/chemokine expression at day 14, but not at days 1 and 7 postinfection (data not shown). Hence, we proceeded to measure cytokine protein levels at day 14 of infection. At day 14 postinfection, NOS2$^{-/-}$ mice produced significantly higher pulmonary concentrations of IFN-γ than parental mice, irrespective of mAb 2H1 treatment (Fig. 7, *upper panel*). MCP-1 and MIP-1α are induced in the lungs of NOS2$^{-/-}$ and parental mice infected with *C. neoformans* (Fig. 7, *middle* and *lower panels*). NOS2$^{-/-}$ mice produced significantly higher concentrations of MCP-1 and MIP-1α than parental mice, which is consistent with the intense inflammatory response seen in these mice. Notably, mAb significantly decreased IFN-γ, MCP-1, and MIP-1α ($p = 0.02$) in the lungs of NOS2$^{-/-}$ mice. Additional cytokines were measured (IL-2, IL-4, IL-6, IL-10, IL-12, and TNF-α), but no differences were noted between parental and NOS2$^{-/-}$ mice (Table II). However, for some cytokines, the levels measured after infection were lower than in sham-infected mice (Table II). IL-2 and TNF-α were lower in lungs of infected NOS2$^{-/-}$ than in sham-infected NOS2$^{-/-}$ mice. Similarly, IL-4 and IL-10 were lower in the lungs of infected parental mice than in sham-infected mice. Ab administration had little or no effect on the levels of these cytokines during infection, except for IL-4, which was increased in parental mice.

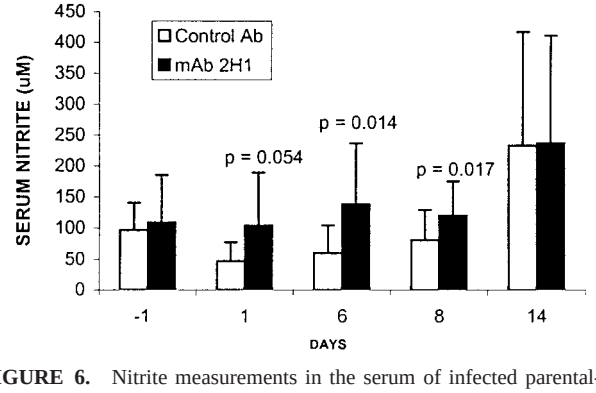

FIGURE 6. Nitrite measurements in the serum of infected parental-F_1 mice ($n = 10$) treated with control or mAb 2H1. Mice were bled before administration of Ab or control (day -1). Serum nitrite levels were detected using Griess assay. A value of $p < 0.05$ (control vs mAb 2H1) is considered significant. Error bars denote SDs.

Discussion

NOS2$^{-/-}$ mice were more susceptible to *C. neoformans* infection, consistent with previous studies using NOS inhibitors (24, 36) and NOS2$^{-/-}$ mice (37). We found no significant differences in either in vitro phagocytic or antifungal activity of pulmonary macrophages from NOS2$^{-/-}$ or parental mice. Macrophages from NOS2$^{-/-}$ mice presumably retain fungicidal activity as a result of their ability to produce microbicidal reactive oxygen intermediates and proteins (38) that can be fungicidal for *C. neoformans* (39, 40).

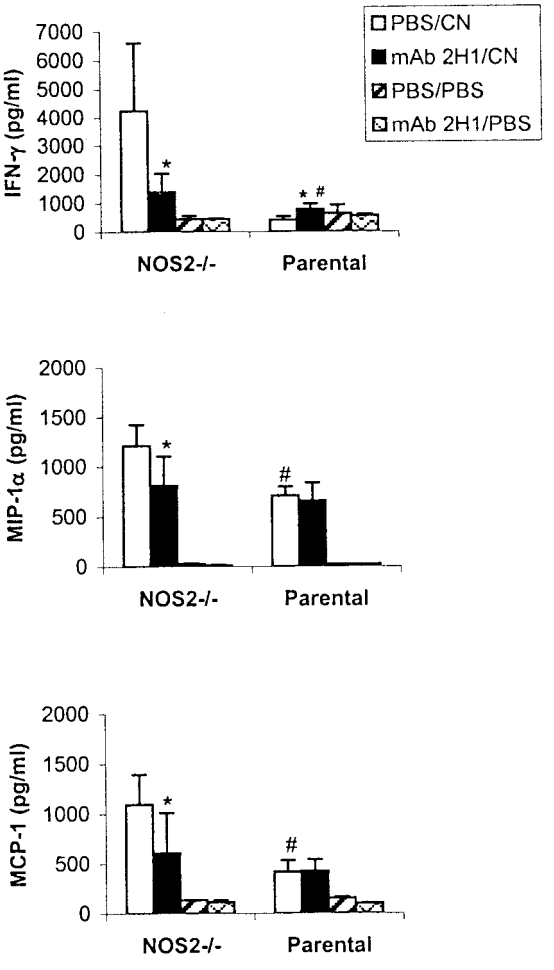

FIGURE 7. Differences in cytokine and chemokine expression in lung of infected NOS2$^{-/-}$ and parental mice. Cytokine and chemokine levels were measured by ELISA at day 14 postinfection. Bars denote mean protein concentration. Mouse groups: PBS/CN and mAb 2H1/CN, $n = 6$; PBS/PBS and mAb 2H1/PBS, $n = 3$. Error bars denote SDs. $*$, Values are significantly different relative to conditions with no mAb 2H1. #, Values are significantly different from protein level measured for NOS2$^{-/-}$ mice. CN, *C. neoformans*.

Table II. *Protein cytokine levels in NOS2$^{-/-}$ and parental mice 14 days after infection with* C. neoformans[a]

Cytokine[b]	Mice	PBS/CN (pg/ml)	mAb 2H1/CN (pg/ml)	PBS/PBS[c] (pg/ml)	mAb 2H1/PBS[c] (pg/ml)
IL-2	NOS2$^{-/-}$	653.5 ± 53.9[d]	826.0 ± 283.9	1078 ± 7.2[d]	1160 ± 18.2
	Parental	1083.5 ± 168.8	1419.6 ± 316.1	1391.6 ± 122.5	1241.3 ± 44.5
IL-4	NOS2$^{-/-}$	674.0 ± 75.6	849.9 ± 296.1	663.0 ± 2.8	722.5 ± 133.6
	Parental	672.3 ± 92.9[e]	1032.3 ± 215.9[f]	852.5 ± 65.8[e]	527.5 ± 77.1[f]
IL-6	NOS2$^{-/-}$	856 ± 238	558 ± 56	635 ± 17	663 ± 134
	Parental	676 ± 122	861 ± 193	692 ± 116	582 ± 35
IL-10	NOS2$^{-/-}$	618.2 ± 93.2[g]	613.1 ± 227.3	1141.3 ± 38.1[g]	1077.1 ± 237.6
	Parental	564.4 ± 103.5[h]	720.8 ± 251.2	1111.9 ± 277.2[h]	980.5 ± 28.3
IL-12	NOS2$^{-/-}$	288.5 ± 27.3	276.6 ± 50.2	287.5 ± 49.5	291.3 ± 12.4
	Parental	229.6 ± 37.2	308.0 ± 125.9	341.0 ± 119.7	263.7 ± 16.5
TNF-α	NOS2$^{-/-}$	404.4 ± 31.2[i]	394.5 ± 126.6[i]	798.17 ± 21.2[i]	771.5 ± 176.8[i]
	Parental	523.4 ± 155.7	697.8 ± 231.1	722.6 ± 175.9	782.0 ± 134.4

[a] NOS2$^{-/-}$ and parental mice received 1 mg of mAb 2H1 or PBS 24 h prior to infection with 10^6 *C. neoformans.* Mice were killed 14 days later. Statistical analysis was performed using the Student's *t* test. $n = 6$ for each group.
[b] No statistical difference was seen with the above cytokines between NOS2$^{-/-}$ and parental mice.
[c] Sham-infected mice were given PBS at the time of infection; $n = 3$ for each group.
[d] Comparison with NOS2$^{-/-}$ control mice ($p = 0.003$).
[e] Comparison with parental mAb 2H1-treated and control mice ($p = 0.003$).
[f] Comparison with parental control mice ($p < 0.05$).
[g] Comparison with NOS2$^{-/-}$ control mice ($p < 0.001$).
[h] Comparison with parental control mice ($p = 0.004$).
[i] Comparison with NOS2$^{-/-}$ control mice ($p < 0.05$).

In this regard, phagocyte oxidase and NOS2 can compensate for each other in providing resistance to commensal bacteria (41). Hence, the enhanced susceptibility of NOS2$^{-/-}$ mice to *C. neoformans* does not appear to be a consequence of impaired macrophage antifungal activity. Nonetheless, NOS2$^{-/-}$ mice died more rapidly than parental mice after pulmonary *C. neoformans* infection, despite a lower pulmonary fungal burden. This observation provides yet another example of dissociation between survival and microbial burden, a phenomenon that has been noted during murine infection with *C. neoformans* (13), *Candida albicans* (42), and *M. tuberculosis* (5). Dissociation between survival time and microbial burden suggests that mortality is caused by factors other than the total number of organisms.

NO is a critical microbicidal molecule (17) that is also a mediator of immunopathology (17, 43, 44), possibly through regulatory effects on Th1 cells (45). Histological studies revealed that the appearance of an inflammatory response in the lungs of NOS2$^{-/-}$ mice was different from the inflammatory response found in the lungs of parental mice. However, there was no difference in lung weights or total inflammatory cells in the lungs of NOS2$^{-/-}$ and parental mice. Similarly, lungs from parental mice treated with Ab had similar lung weights and total inflammatory cells compared with parental mice not receiving Ab. The finding of differences in histological appearance for lungs that contained the same number of inflammatory cells suggests that similar numbers of cells migrate into the lung in response to infection, but that these achieve different tissue distribution. Qualitative analysis of the lung infiltrate revealed similar numbers of CD4, CD8, and B lymphocytes. However, we noted that the number of eosinophils in lungs from NOS2$^{-/-}$ mice was significantly higher than parental mice, irrespective of Ab administration. Eosinophils are recruited to inflammatory sites to a variety of infections (46–49). Eosinophilic granules contain polycationic proteins that are highly toxic to several pathogens including *C. neoformans* (49), as well as mammalian cells including respiratory epithelial cells (50, 51). Eosinophils are potentially toxic to host tissues because of their toxic granule contents and their ability to generate superoxide radicals and leukotrienes (reviewed in Ref. 52). In fact, eosinophils can damage lung tissue by a novel mechanism that involves brominating proteins (53). The marked eosinophilia in the inflammatory response of NOS2$^{-/-}$ mice may account for the seemingly paradoxical effects of shorter survival as a result of increased lung damage and lower CFU through the powerful antifungal action of eosinophil granules.

The differences in inflammatory response in NOS2$^{-/-}$ and parental mice suggest the involvement of NO in the regulation of the inflammatory response to *C. neoformans*. In this regard, we found greater production of IFN-γ, MIP-1α, and MCP-1 in the lungs of *C. neoformans*-infected NOS2$^{-/-}$ compared with parental mice, suggesting that NO may function as an important regulator of the inflammatory response through its effect on the expression of certain cytokines. Recently, Aguirre et al. (37) reported no differences in IFN-γ production in bronchoalveolar lavage fluid between NOS2$^{-/-}$ and parental mice during IT *C. neoformans* infection. The discrepancy between our observation and that of Aguirre et al. (37) is probably due to methodological differences, because we measured total lung cytokine level, while they studied the concentration in alveolar lavage. In addition, we measured lower levels of IL-2 and TNF-α in NOS2$^{-/-}$-infected mice than in sham-infected mice, a phenomenon that was not observed for parental mice. These observations may reflect NO-mediated effects on cytokine expression and inflammation, and are consistent with in vitro studies demonstrating that NO can inhibit both mitogen and Ag-induced proliferation of lymphocytes (54, 55).

Ab administration prolonged survival in parental mice, but not in NOS2$^{-/-}$ mice. This result is consistent with our prior studies that showed that passive administration of IgG1 mAb prolonged survival of *C. neoformans*-infected mice (13, 28–30). Serum nitrite levels were significantly higher in Ab-treated parental mice in the days immediately following cryptococcal infection, whereas parental control mice manifested a reduction in serum nitrite levels after infection. Because *C. neoformans* reduces macrophage production of NO in vitro (56), the reduced levels of serum nitrite in parental control mice may be an in vivo correlate of this phenomenon. The higher levels of serum nitrite in Ab-treated mice could also reflect enhanced NO synthesis due to FcR cross-linking (57). The observation that Ab administration prevents a decrease in serum nitrite suggests a new mechanism of action for specific Ab against *C. neoformans* that could have profound effects on host defense given the protean effects of nitrogen-related radicals on

immune function (17). We also noted that Ab-treated parental mice had higher IL-4 levels. Although we did not measure a cellular correlate for higher IL-4 levels in parental mice, it is conceivable that higher levels of this inflammation-inhibiting cytokine contributed to prolonged survival as a result of reduced lung damage.

The most striking result of our study was the observation that Ab prolonged survival in parental, but not NOS2$^{-/-}$ mice, despite producing a modest reduction in NOS2$^{-/-}$ lung fungal burden. To our knowledge, Ab efficacy has not been studied in NOS2$^{-/-}$ mice against any pathogen. The cytokine response in NOS2$^{-/-}$ and parental mice was different such that infection increased the pulmonary level of IFN-γ in NOS2$^{-/-}$ mice, but reduced it in parental mice. Furthermore, Ab administration had different effects on cytokine production in NOS2$^{-/-}$ and parental mice, reducing IFN-γ in NOS2$^{-/-}$ mice and increasing it in parental mice. These observations suggest that when the stimulus for increased lung IFN-γ is Ab, passive Ab administration is effective in prolonging survival. However, when the stimulus for high levels of lung IFN-γ is infection, in the setting of genetic deficiency of NOS2, passive Ab is not effective in prolonging survival. Thus, NO may exert its effect on Ab-mediated efficacy through effects on the regulation of IFN-γ. This result is consistent with reports that NO has protective effects against T cell-dependent shock induced by *Toxoplasma gondii* (58), staphylococcal enterotoxin B (59, 60), and *L. major* (61) by down-regulating IFN-γ and TNF-α production. In the absence of NO, Ab may deliver an inhibitory signal, which reduces IFN-γ production in NOS2$^{-/-}$ mice. This is consistent with previous studies demonstrating antiinflammatory activity of Ig through FcRs (62).

Ab-treated NOS2$^{-/-}$ mice had very high serum levels of GXM relative to Ab-treated parental mice, despite lower organ fungal burden and Ab-mediated clearance of immune complexes with deposition of polysaccharide Ag in liver, as reported in prior studies of normal animals (34). Because Ab-mediated clearance of GXM in NOS2$^{-/-}$ mice appears to be intact, the mechanism responsible for the much higher levels of GXM in Ab-treated NOS2$^{-/-}$ mice is unknown. Potential mechanisms include the existence of an unidentified Ag clearance defect in NOS2$^{-/-}$ mice, or enhanced polysaccharide production by *C. neoformans* in inflammatory tissues that are devoid of NO. Remarkably, NOS2$^{-/-}$ mice with *C. neoformans* infection had intense inflammation despite high levels of serum GXM. Higher cytokine/chemokine levels in NOS2$^{-/-}$ mice could have mitigated any antiinflammatory properties or other effects that GXM may have, and it is conceivable that, in certain hosts, GXM itself is proinflammatory (63, 64). Thus, irrespective of its effects on CFU reduction and/or the modulation of inflammatory responses, Ab-mediated clearance of GXM may be pivotal and required for Ab efficacy against *C. neoformans*.

Our study explored the mechanisms responsible for Ab efficacy against *C. neoformans*, confirmed an important role for NOS in host defense against experimental murine cryptococcal infection, and established that Ab efficacy was dependent on NO production. Furthermore, our results strongly suggest that NO is an important regulator of immune responses that can affect Ab efficacy. IFN-γ appears to be beneficial or harmful depending on its tissue level and is likely to have a critical role in the mechanisms that determine either early death or survival in NOS2$^{-/-}$ and parental mice, respectively. The emerging picture indicates that Ab exerts profound effects on the inflammatory response by mechanisms that include changes in IFN-γ, serum polysaccharide levels, and nitrite production, in addition to the more classical role of Ab as an opsonin. The requirement for NO in host defense and Ab function was illustrated by the dramatic differences observed in Ab efficacy, inflammatory response, and cytokine production in NOS2$^{-/-}$ and parental mice infected with *C. neoformans* in the presence and absence of specific Ab. Our results provide additional evidence for the interdependency of the humoral and cellular arms of the immune system, and suggest a need to carefully dissect mechanisms of Ab function based on its effect on the expression of immune mediators. The requirement for NO in Ab-mediated protection against *C. neoformans* identifies a new variable in Ab efficacy that may also be applicable to other pathogens.

Acknowledgments

We thank Marta Feldmesser, Bettina Fries, David Goldman, Josh Nosanchuk, and Liise-anne Pirofski for critical reading of the manuscript. We thank Jorge Bermudas for excellent histology. We thank John MacMicking (Cornell University), Carl Nathan (Cornell University), and John Mudgett (Merck Research Laboratories, Rahway, NJ) for providing us with the NOS2$^{-/-}$ and parental mice. We thank Carlos P. Taborda and Marcelas I. Torres for their invaluable help with experiments.

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