The Relative Susceptibility of Mouse Strains to Pulmonary *Cryptococcus neoformans* Infection Is Associated with Pleiotropic Differences in the Immune Response[▽]

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CBA/J mice were highly susceptible to intratracheal (i.t.) *Cryptococcus neoformans* infection relative to BALB/c mice, while both strains were equally susceptible to intravenous (i.v.) infection. Increased susceptibility in i.t. infection was associated with higher brain CFU, lower serum immunoglobulin M (IgM) and IgG responses to glucuronoxylomannan (GXM), lack of IgE regulation during infection, and alveolar macrophage permissiveness to intracellular replication in vitro. In contrast, for BALB/c mice, relative resistance was associated with increased interleukin-12 (IL-12) and decreased IL-10 pulmonary levels. In CBA/J mice, relative susceptibility was associated with a decreased proportion of CD4$^+$ and CD8$^+$ T cells and an increase in macrophage percentage in pulmonary infiltrates. In contrast, no significant differences in these cytokines or cell recruitment were observed in the i.v. model, consistent with no differences in the survival rate. Passive antibody (Ab) protection experiments revealed a prozone effect in the BALB/c mice with i.v. infection, such that Ab efficacy decreased at higher doses. In the i.t. model using CBA/J mice, low Ab doses were disease enhancing and protection was observed only at high doses. Our results show (i) that differences in mouse strain susceptibility are a function of the infection model, (ii) that susceptibility to pulmonary infection was associated with macrophage permissiveness for intracellular replication, and (iii) that the efficacy of passive Ab in pulmonary infection is a function of dose and mouse strain. The results highlight significant differences in the pathogenesis of cryptococcal infection among inbred mice and associate their relative susceptibility with differences in numerous components of the innate and adaptive immune responses.

Cryptococcus neoformans is a fungal pathogen for individuals with late-stage human immunodeficiency virus infection. The initial infection is usually controlled and either cleared or contained by immunocompetent hosts into a latent asymptomatic state, and there is evidence that persistent infection might be associated with reactive airway disease (24). However, in immunocompromised individuals dissemination and/or reactivation of latent infection can lead to a life-threatening meningoencephalitis (for a review, see reference 9).

C. neoformans has several well-characterized virulence factors, including a polysaccharide capsule, melanin production, phospholipase, urease, and the alpha mating factor, among others (for reviews, see references 9, 42, and 55). A recent study analyzing the relative contribution of each virulence factor showed that the capsular polysaccharide makes the largest contribution to *C. neoformans* virulence (41). The polysaccharide capsule is composed primarily of glucuronoxylomannan (GXM). The capsule participates in virulence through pleiotropic effects on the immune system (9, 71, 72). Furthermore, *C. neoformans* strains that differ in virulence elicit different immune responses in the host (5, 14).

The susceptibility of a host to cryptococcosis reflects a complex interplay between host and microbial attributes. Both weak and overexuberant immune responses can result in host damage after infection with *C. neoformans* (10). Resistance to infection is associated with a Th1 response, implying that the effective inflammatory response involves granuloma formation and cellular recruitment into the lungs (9, 28, 66).

T cells play a predominant role in the defense against *C. neoformans* (30, 31, 37, 45, 61). Gamma interferon (IFN-γ) is required for the protective effect (4, 12, 21, 38, 46), as is the IFN-γ receptor (17). Th2-polarized responses are associated with enhanced susceptibility to *C. neoformans* in mice (for a review, see reference 9). Neutrophils contribute to host defense against systemic infection but can polarize the immune response toward Th2 such that neutrophil depletion is associated with enhanced resistance to pulmonary infection (44). Despite considerable evidence that Th2-polarized responses are less protective than Th1-polarized responses, there is conclusive evidence from multiple laboratories that certain antibodies (Abs) affect the course of disease during *C. neoformans* infection (19, 39, 49, 51, 62). The Ab response includes both protective and nonprotective Abs, with the functional quality depending on specificity and isotype as well as on the mouse strain (59, 74, 75). Further complicating the assessment of humoral immunity are large dose-dependent effects such as the prozone-like effects that can occur in passive Ab experiments, whereby the protective efficacy of the Ab is abrogated in conditions where large doses are administered (67, 68).

Mice are a particularly useful species to study cryptococcal

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infection because they are susceptible and affordable, many knockout strains are available, and the histology of cryptococcal disease closely approximates that observed in human cryptococcosis. In this regard, several reports have documented differences in the susceptibility of the most commonly used mouse laboratory strains, including BALB/c, C57BL/6J, A/J, CBA/J, and DBA/J, to experimental cryptococcal infection (13, 29). Mouse strains also demonstrate different susceptibility depending on the route of infection (1). However, the host factors that influence susceptibility are not well understood.

In this work, we explore the immunological basis for differences in the susceptibility of BALB/c and CBA/J strains to *C. neoformans* infection. We demonstrate that the CBA/J strain is highly susceptible to *C. neoformans*, whereas the BALB/c strain is relatively resistant. Susceptibility to infection in this system was associated with permissiveness of alveolar macrophages to *C. neoformans* intracellular replication, an imbalance in the Th2-Th1 response, higher dissemination of the pathogen to the brain, and lower Ab production.

MATERIALS AND METHODS

Yeast strain and preparation of inocula for infection. *Cryptococcus neoformans* ATCC 24067 (ATCC, Manassas, VA) was used in all experiments. This strain has been extensively used in studies of pulmonary pathogenesis and was used here to maintain continuity with the prior literature (30). For mouse infection, the cells were grown in Sabouraud medium until late logarithmic phase, washed and suspended in phosphate-buffered saline (PBS), and counted with a hemocytometer. Yeast cells were suspended in PBS at a cell density that would provide a fixed number of cells in the volume that was used to infect the mice (50 μl for intratracheal [i.t.] infections and 100 μl for intravenous [i.v.] infection). Inocula were confirmed by plating on Sabouraud dextrose agar and counting CFU (Difco, Detroit, MI).

Mouse strains, Ab administration, and routes of infection. Female BALB/c and CBA/J mice (6 to 8 weeks old; Jackson Laboratories, Bethesda, MD) were used in this study. We chose female mice because this sex was previously used in immunology studies in our laboratory. For the Ab protection experiments, the protective immunoglobulin G1 (IgG1) monoclonal Ab (MAb) 18B7 (7) was administrated intraperitoneally 24 h prior to infection. MAb 18B7 was purified by protein A affinity chromatography (Pierce, Rockford, IL) following the manufacturer's recommendations. Different amounts of Ab (1, 0.1 or 0.01 mg) were injected in a total volume of 0.5 ml. i.t. infections were performed as described previously (20). For i.v. infections, 100 μl of fungal cell suspension was injected in the caudal vein of the mouse tail.

Organ fungal burden. Organ fungal burden was inferred from the number of CFU per organ on plates. Infected mice were sacrificed, and the lung, brain, and spleen were removed and homogenized in PBS buffer. Various dilutions of the organ homogenate were plated in Sabouraud plates, and after 2 days of incubation at 30°C, the number of colonies was enumerated.

Serum GXM measurement. The amount of GXM in serum was determined by capture enzyme-linked immunosorbent assay (ELISA) as described previously (8). Briefly, serum samples were treated with proteinase K to destroy associated Ab and interfering proteins (1 mg/ml, overnight at 37°C; Roche, IN). Ninety-six-well plates were coated with goat anti-mouse IgM (1 μg/ml; Southern Biotechnologies, Birmingham, AL) followed by capture Ab 2D10 (2 μg/ml) (48). Samples were added, and GXM of known concentration was used as a standard. Bound GXM was detected using IgG1 MAb 18B7 (2 μg/ml) and secondary goat anti-mouse IgG1 Ab conjugated to alkaline phosphatase (1 μg/ml; Southern Biotechnologies, Birmingham, AL). A 1-mg/ml concentration of *p*-nitrophenyl phosphate dissolved in substrate buffer (1 mM MgCl$_2$ · 6H$_2$O, 50 mM Na$_2$CO$_3$) was used for color development, and the absorbance was measured at 405 nm using a microplate reader after approximately 20 min of incubation at room temperature.

Ab measurement. Serum IgM and IgG to GXM were determined by ELISA as described previously (8). Total IgE levels were determined by capture ELISA using specific Abs (Pharmingen, San Diego, CA) according to the manufacturer's instructions.

Ab binding to *C. neoformans* cells in vivo. Mice were infected with *C. neoformans* strain 24067 as described above and sacrificed after 7 days, and cells were
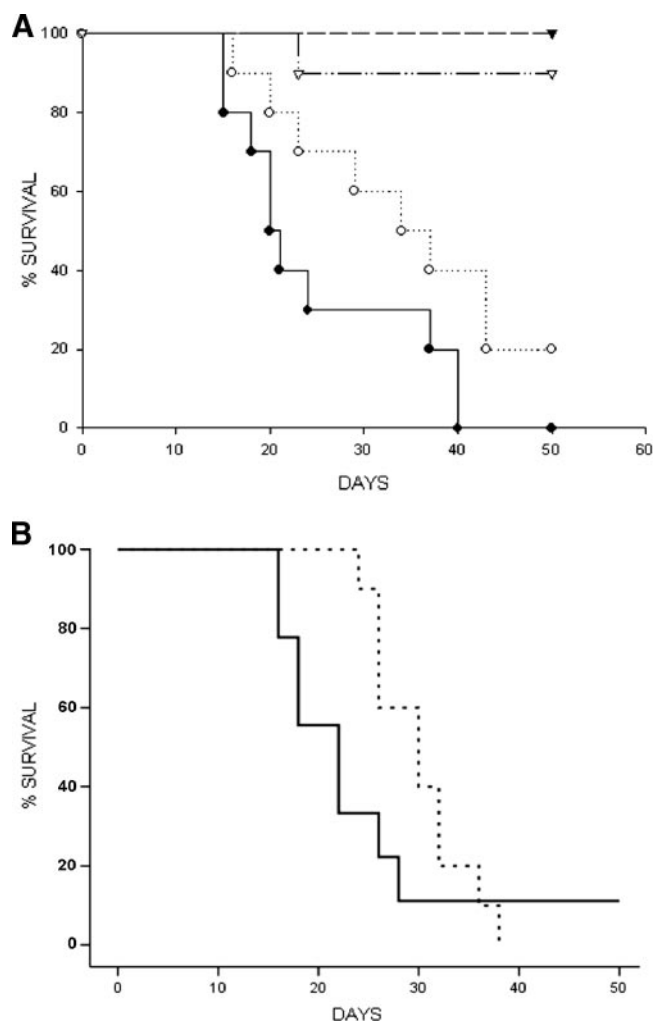

FIG. 1. Survival of BALB/c and CBA/J mice after *C. neoformans* infection. (A) CBA/J (circles) or BALB/c (inverted triangles) mice were infected i.t. with 10^5 (open symbols) or 10^6 (closed symbols) yeast cells, and survival was monitored daily. Each group contained between 8 and 10 mice ($P < 0.05$). (B) CBA/J (dotted line) and BALB/c (solid line) mice were infected i.v. with 10^5 yeast cells, and survival was monitored as for panel A. Each group contained between 8 and 10 mice.

obtained as described previously (76). Abs bound to the capsule were then detected by immunofluorescence using secondary Abs to mouse IgG or IgM conjugated to tetramethyl rhodamine isothiocyanate (Southern Biotechnologies Associates, Birmingham, AL).

Cytokines. Cytokine and chemokine levels were determined in whole organ lysates. Briefly, whole lungs were excised from *C. neoformans*-infected mice and sham-infected controls and homogenized in 2 ml of PBS containing proteases inhibitors (Boehringer Mannheim, Indianapolis, IN). The lysates were centrifuged, and the supernatants were kept at −80°C until they were analyzed. Cytokine and chemokine levels were determined using the Pharmingen (San Diego, CA) ELISA kit according to the manufacturer's instructions.

FACS. Analysis of inflammatory cell content in the lung was performed by fluorescence-activated cell sorting (FACS) as described previously (44). Briefly, mice were infected with 10^6 *C. neoformans* cells i.t. or i.v., and after 7 days of infection, they were sacrificed. The lungs (i.t. infection) or spleens (i.v. infection) were isolated and homogenized. Lysates were then treated with collagenase A (1 mg/ml; Boehringer Mannheim, Chicago, IL) in digestion buffer (RPMI medium, 10% fetal calf serum) and incubated for 1 h at 37°C with occasional shaking. Cells were centrifuged and suspended in 0.17 M NH$_4$Cl to lyse erythrocytes for 10 min at 4°C. A 10-fold excess of RPMI solution was then added to make the
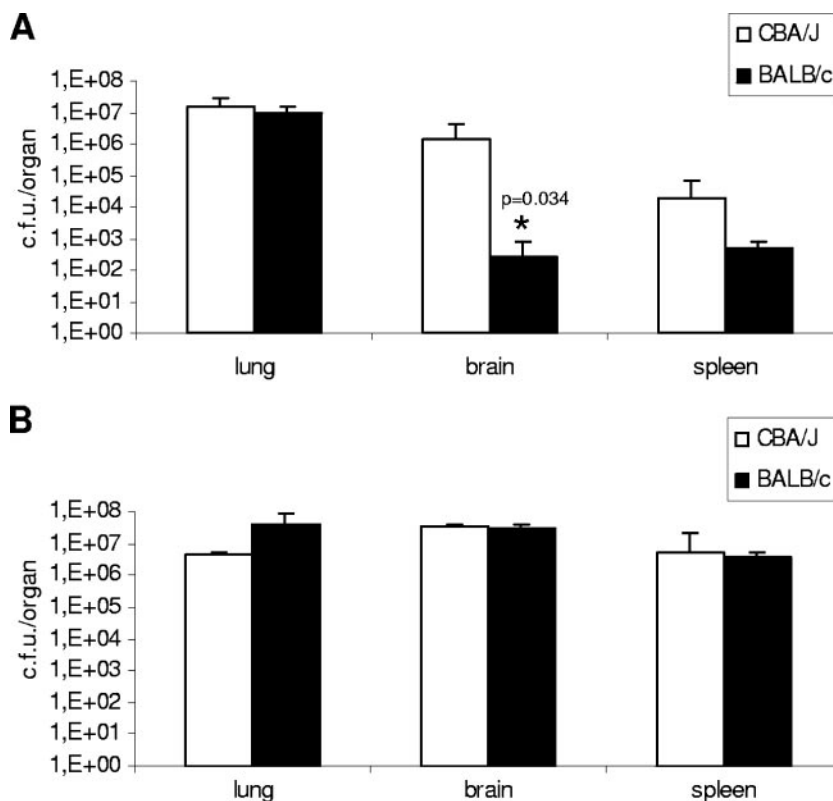
FIG. 2. Organ fungal burden after *C. neoformans* infection. CBA/J (open bars) and BALB/c (closed bars) mice infected with 10^6 yeast cells i.t. (A) or i.v. (B) were sacrificed 14 days after infection, and the organ fungal burden was counted as indicated in Materials and Methods. Mean values and standard deviations from five different mice are plotted. Significant differences determined using a nonparametric test (Kruskal-Wallis test) are highlighted with an asterisk, and the *P* value is shown.

solution isotonic, and the cells were collected by centrifugation and suspended in staining buffer (PBS, 1% fetal bovine serum). The number of cells was determined by counting in a hemocytometer. 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 of R-phycoerythrin-labeled anti-CD45$^+$ per ml, 5 μg of fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4$^+$ per ml, 2 μg of Cy-Chrome-labeled anti-mouse CD8$^+$ per ml, 5 μg of FITC-labeled anti-mouse CD19$^+$ per ml, or 5 μg of FITC-labeled anti-mouse MAC-3 per ml (all MAbs were from Pharmingen, San Diego, CA.). 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 FASCalibur flow cytometer (Becton Dickinson, Mountain View, CA) with CellQuest software (Becton Dickinson). Live cells were gated as judged from forward and side laser scatter and CD45 staining.

In vivo phagocytosis. 10^7 *C. neoformans* ATCC 24067 cells were injected i.t. into both CBA/J and BALB/c mice. After 2 h, the mice were sacrificed, and alveolar lavage was then performed 10 times. The cell suspension was allowed to attach to 96-well plates for 2 h, fixed with methanol, and then stained with Giemsa stain as described previously (77). The phagocytic index (PI) was expressed as the number of internalized *C. neoformans* cells per 100 macrophages counted.

Bronchoalveolar lavage, in vitro phagocytosis, and subsequent imaging. Mice were sacrificed by asphyxiation with CO_2, and their tracheas were cannulated with angiocaths (BD Biosciences, San Jose, CA). Lungs were lavaged 10 times with sterile PBS with 1 mM EGTA (Sigma-Aldrich, St. Louis, MO). Lavage fluids were pooled, and cells were collected by centrifugation. Red blood cells were lysed by incubation in 0.17 M NH_4Cl at 4°C for 10 min. Cells were washed with PBS and suspended in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA) with 10% heat-inactivated fetal calf serum (Gemini Bioproducts, Woodland, CA), 10% NCTC-109 medium (Gibco, Carlsbad, CA), and 1% nonessential amino acids (Mediatech Cellgro, Washington, DC). Cells were then plated on polylysine coverslip-bottom MaTek plates (Ashland, MA) at a density of 1×10^5 per well in feeding medium and allowed to

adhere at 37°C with 10% CO_2 for 1 h prior to incubation with *C. neoformans* for an additional 1 h and subsequent microscopic imaging. The medium was then removed from MaTek plates and replaced with fresh medium containing *C. neoformans* cells (*C. neoformans*-to-macrophage ratio of 5:1) along with 50 μg/ml MAb 18B7. Macrophages were incubated with a solution that contained the yeast cells, 0.3 μg/ml lipopolysaccharide (Sigma, St. Louis, MO), and 50 units/ml of murine IFN-γ for 60 min. After this period of time, plates were washed twice with fresh medium to remove extracellular *C. neoformans* cells. Plates were then replenished with 2 ml feeding medium, and macrophages with ingested yeast cells were imaged under the microscope for 24 h. Images were collected at a magnification of ×40 every 3 min using an Axiovert 200 M inverted microscope and photographed with an AxiocamMR camera controlled by the Axio Vision 4.4 software (Carl Zeiss Micro Imaging, NY). Movies were compiled and analyzed using the ImageJ software (W. S. Rasband, National Institutes of Health, Bethesda, MD) (http://rsb.info.nih.gov/ij/, 1997 to 2006).

Statistics. CFU, cytokine and FACS data were analyzed using the *t* test or the Kruskal-Wallis test after assaying normality by using the Shapiro-Wilk test with the Unistat software for Excel (Unistat, Ltd., London, United Kingdom). Statistical differences in survival rates were assessed using log rank analysis with SPSS software (SPSS, Chicago, IL). Statistical differences in intracellular replication inside alveolar macrophages were determined according to Fisher's exact test following the guidelines described at http://www.exactoid.com/fisher/index .php?a = 11&b = 24&c = 1&d = 26.

RESULTS

Differences in survival between strains at different infection doses. Previous studies (29) and our own laboratory experience have shown great differences in the susceptibility of mouse strains to *C. neoformans* infection. We revisited this question by a preliminary study that compared the outcome of *C. neo-*
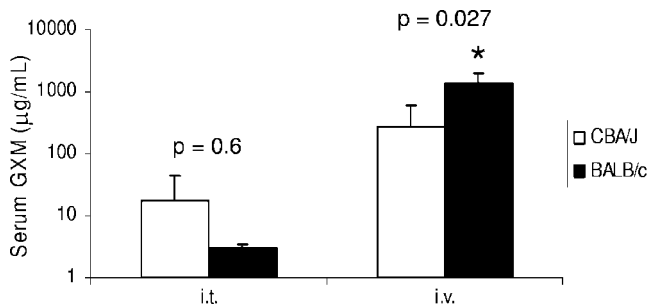
FIG. 3. Serum GXM levels. Sera from mice infected i.t. or i.v. after 7 days of infection were obtained, and GXM levels were measured by capture ELISA as described in Materials and Methods. Significant differences determined using a nonparametric test (Kruskal-Wallis test) are highlighted with an asterisk, and P values are shown. Mean values and standard deviations from five different mice are plotted.

formans pulmonary infection in BALB/c, A/J, C57BL/6J, and CBA/J strains and found that the BALB/c and CBA/J strains were the least and most susceptible, respectively (Fig. 1A). Therefore, we hypothesized that comparison of the immune responses of these two different mouse strains after *C. neoformans* infection could reveal factors involved in the resistance or susceptibility to cryptococcosis.

Measurement of organ fungal burden revealed that the enhanced susceptibility of CBA/J mice to pulmonary infection was associated with greater extrapulmonary dissemination, as evidenced by higher CFU in brain than measured for BALB/c mice (Fig. 2). We considered that the higher brain and spleen CFU in CBA/J mice could reflect greater dissemination from the primary pulmonary infection or a less effective systemic immune response. To investigate these possibilities, we examined the relative susceptibility of these strains to i.v. infection.
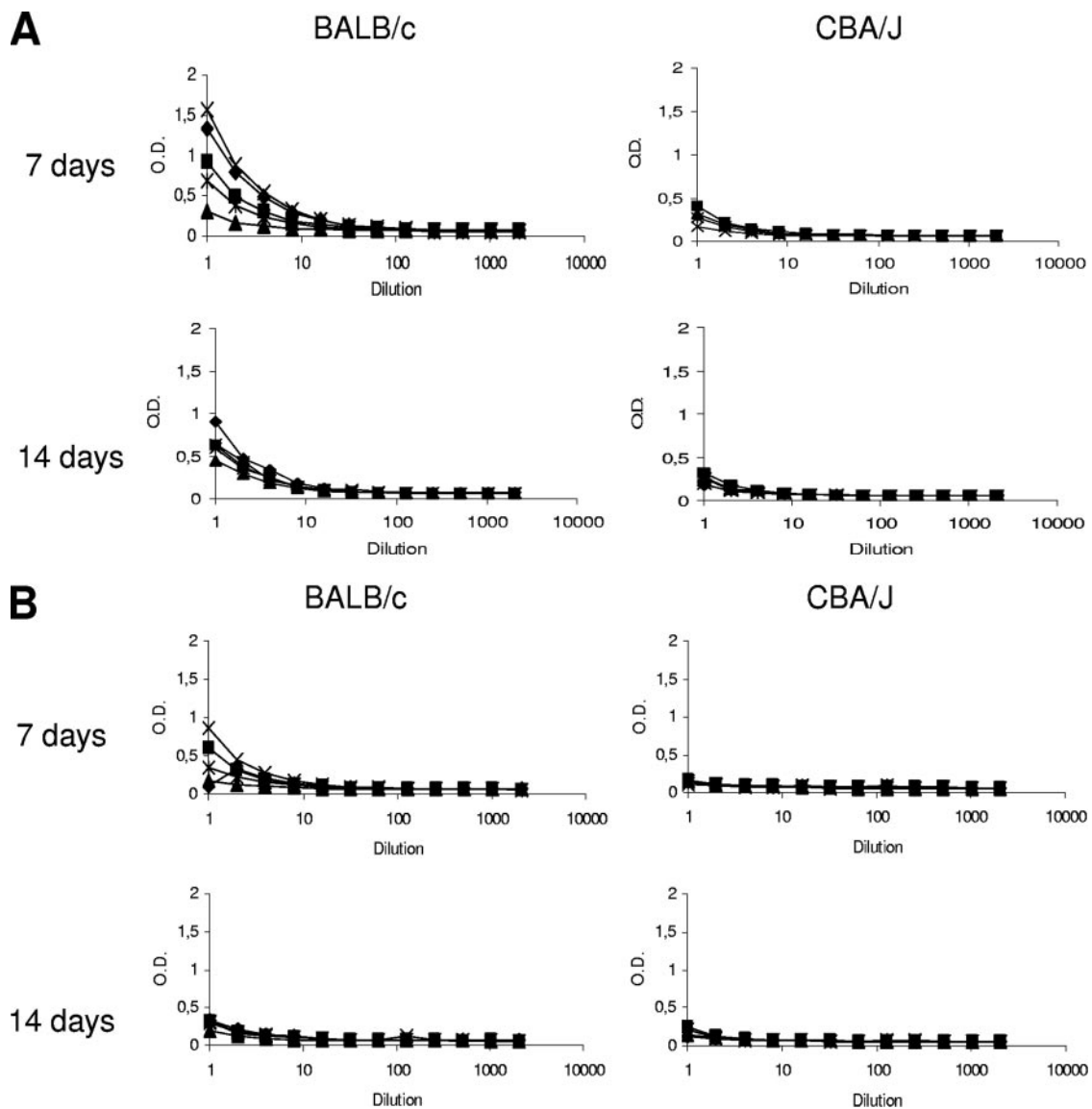

FIG. 4. Measurement of Ab levels in response to GXM. The serum samples described in legend for Fig. 3 (i.t. infected mice) were tested for levels of IgG (A) and IgM (B) to GXM by ELISA as described in Materials and Methods. Each line and symbol refer to the data obtained from each mouse.

We reasoned that if the difference in susceptibility between CBA/J and BALB/c mice was due to a difference in extrapulmonary dissemination, then the strains should have comparable susceptibility to i.v. challenge. Using an i.v. challenge of 10^5, BALB/c mice were equally susceptible as CBA/J mice (Fig. 1B). Analysis of organ fungal burden after i.v. infection revealed, as expected, dissemination of the yeast through all the organs cultured in both mouse strains (Fig. 2B); in some organs, such as the lung, there was a trend in the BALB/c mice for higher fungal burden, but this did not achieve statistical significance. Given comparable BALB/c and CBA/J susceptibility to systemic infection, the higher extrapulmonary CFU observed in CBA/J mice after pulmonary infection probably reflects increased dissemination from the lungs.

GXM levels in serum. Serum GXM levels positively correlated with host fungal burden. Hence, we measured GXM levels in serum after *C. neoformans* infection in both strains. After i.t. infection, there was a trend to find higher GXM levels in the more susceptible CBA/J mice ($P = 0.6$), suggesting a more pronounced disease (Fig. 3). However, when the mice were infected i.v., there were significantly higher GXM levels in the BALB/c strain ($P = 0.027$), consistent with the relatively equivalent susceptibility change of this strain to i.v. challenge.

Ab responses in CBA and BALB/c mice. Ab responses to GXM after i.t. infection were higher in BALB/c mice than in CBA/J mice (Fig. 4). Both IgM and IgG to GXM were present in the sera of infected BALB/c mice (Fig. 4). When the mice were infected i.v., the Ab response to GXM was much lower than in the i.t. model, such that for BALB/c mice, Ab titers were barely above the detection limit of the method used, and no Abs to GXM could be detected in the sera of infected CBA/J mice (results not shown). Endogenously produced mouse Abs to *C. neoformans* have been reported to have immunofluorescence characteristics of nonprotective Abs with a punctate pattern (76). Hence, we studied the pattern of binding of serum Abs in these mouse strains. We found a great heterogeneity in the size of the cryptococcal cells isolated from lungs. *C. neoformans* cells recovered from the lungs of the resistant mouse strain (BALB/c) were coated with Abs that gave a diffuse annular pattern with some fluorescence dots. However, in the susceptible strain, the larger cells were not labeled, and only the small cells gave a punctate pattern (Fig. 5).

Inflammatory response. *C. neoformans* infection is associated with increased IgE levels (3, 59, 60). Given that IgE levels reflect the balance between Th1 and Th2 responses, we measured serum IgE before and after infection in both mouse strains. In BALB/c mice, the IgE levels dropped significantly after i.t. infection (around a 30-fold decrease; $P = 0.01$), while in the susceptible strain, these levels did not respond to the infection (result not shown). When the mice were infected i.v., the response of serum IgE levels differed for the BALB/c strain, such that for this strain total IgE levels in the serum increased after infection (threefold increase; $P = 0.02$), indicating a change in the Th1-Th2 response regulation. As in the i.t. model, CBA/J mice did not regulate IgE levels upon i.v. infection.

After i.t. challenge, interleukin-6 (IL-6), monocyte chemoattractant protein 1 (MCP-1), and IFN-γ increased in response to *C. neoformans* infection in lung extracts, but no difference in
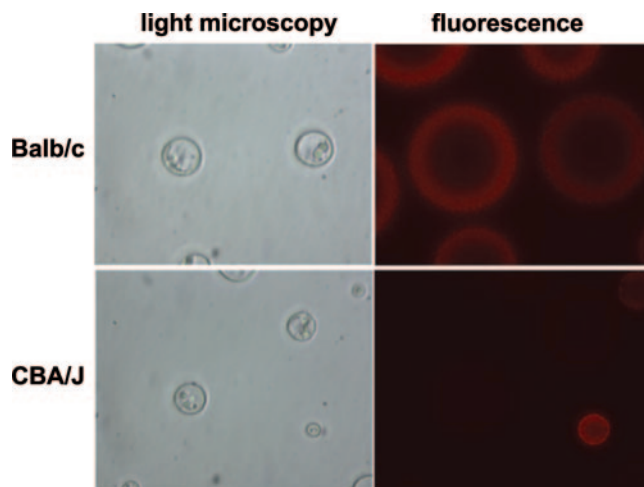

FIG. 5. Fluorescence of Abs bound to *C. neoformans* cells in vivo. *C. neoformans* cells were obtained from the lungs of infected mice as described previously (76), and Ab binding pattern was observed by fluorescence using secondary goat anti IgG plus IgM tetramethyl rhodamine isothiocyanate-labeled Abs. The right panels show the fluorescence patterns of the cells shown in the left panels. The scale bar in left upper panel (10 μm) applies to the all of the pictures.

their regulation during infection was found between the two strains (Fig. 6). However, lung IL-10 and IL-12 levels were differently regulated in the two mouse strains. In the susceptible strain (CBA/J), IL-10 was up-regulated ($P = 0.014$), and IL-12 levels did not change after infection. In the resistant strain (BALB/c), IL-10 levels decreased ($P = 0.05$) and IL-12 increased ($P = 0.02$) with infection. To analyze the cytokine response to infection in the i.v. model, we measured cytokine levels in the spleen, since it was the organ macroscopically most affected as evidenced by its greater inflammation. We found no difference between the strains, except in MCP-1, which was moderately increased in BALB/c mice after infection. The lack of IL-10 and IL-12 regulation after i.v. challenging is consistent with the similar susceptibility shown by the two strains in this model.

Cellular recruitment into the lung was measured by FACS using cell type-specific Abs to enumerate both the absolute number of cells and the relative frequency of the various cell types. For this experiment, we infected the mice, isolated the inflammatory cells from the lung, and detected the proportions of macrophages, CD4$^+$ cells, and CD8$^+$ cells by FACS (Fig. 7). The inflammatory infiltrate in the CBA/J strain manifested a decrease in the proportion of T cells in the lung, both CD4$^+$ and CD8$^+$, and an increase in the proportion of macrophages compared to that in the BALB/c strain (Fig. 7). We performed a similar experiment with mice infected i.v. When the two mouse strains were infected i.v. with *C. neoformans*, there was no difference in cell recruitment in the spleen, a finding consistent with the lack of difference in mortality between the strains in this condition.

In vivo phagocytosis and intracellular replication. We measured phagocytosis in vivo by evaluating the proportion of alveolar macrophages that contain ingested yeast cells. The PIs were similar in the two mouse strains (PI$_{CBA/J}$, 21 ± 12;
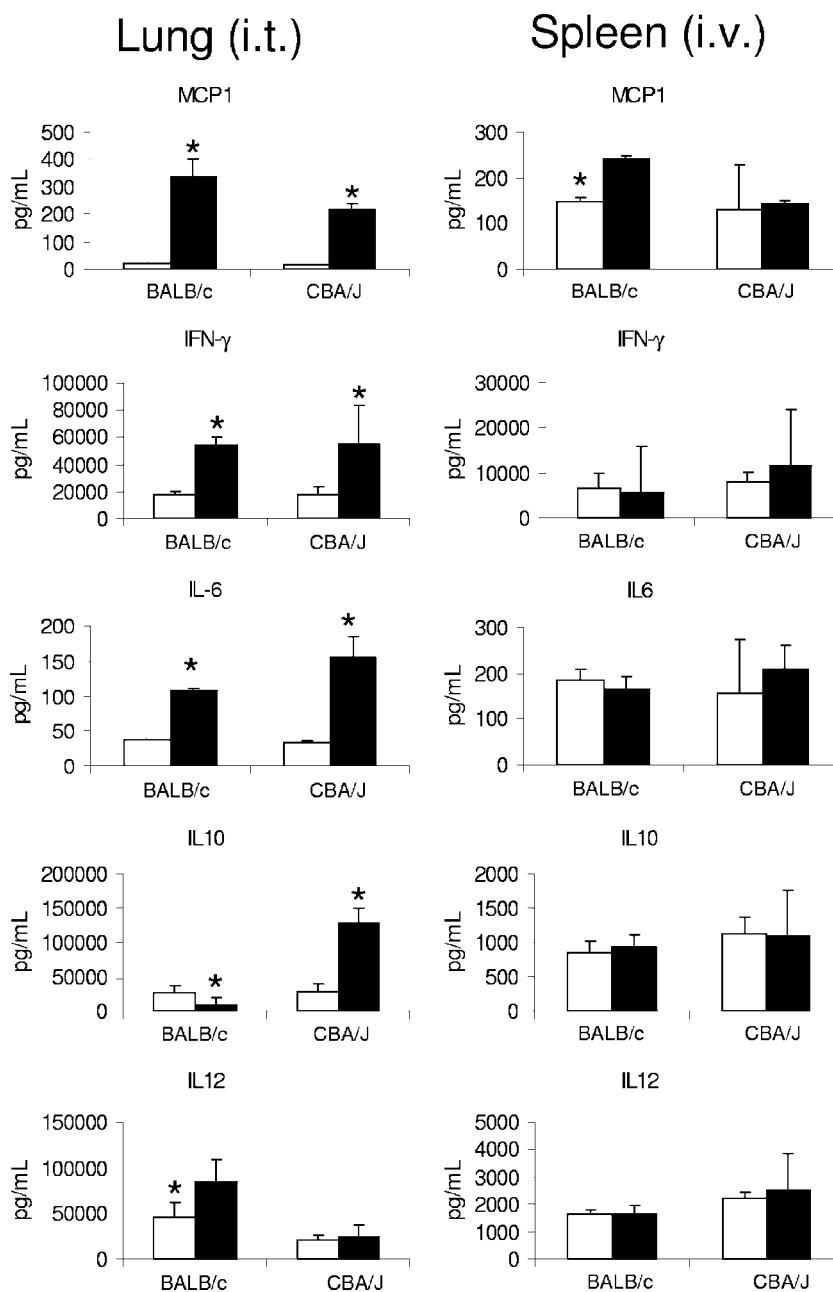
FIG. 6. Cytokine and chemokine levels in target organs after i.t or i.v. infection. BALB/c and CBA/J mice ($n = 5$) were infected i.t. or i.v. with 10^6 *C. neoformans* cells (closed bars) or injected with PBS as a sham control (open bars). The mice were sacrificed after 7 days, and cytokine and chemokine levels in the lung or spleen homogenates were measured by capture ELISA as described in Materials and Methods. Asterisks denote statistical differences ($P < 0.05$) determined using the nonparametric Kruskal-Wallis test. Mean values and standard deviations from five different mice are plotted.

$PI_{BALB/c}$, 20 $\pm$ 4 [$P > 0.05$]), suggesting that susceptibility differences were not associated with phagocytosis efficiency.

Recent studies demonstrate that differences in susceptibility between rats and mice correlate with differences in *C. neoformans* intracellular replication inside macrophages. Consequently, we evaluated intracellular replication of *C. neoformans* in alveolar macrophages isolated from CBA/J and BALB/c mice. For this purpose, we followed the intracellular fate of the yeast cells by live microscopy imaging for 24 h. We

observed that replication occurred more frequently in macrophages from CBA/J mice (Table 1) than in those from BALB/c mice, where intracellular replication was observed in only a small percentage of the macrophages. Interesting, the level of yeast extrusion by the macrophages remained similar between macrophages from BALB/c and CBA/J mice (data not shown).

Efficacy of passive Ab efficacy in i.t. and i.v. models. Passive MAb administration can modify the outcome of cryptococcal infection in mouse models of cryptococcosis. Hence, we stud-

FIG. 7. Cell recruitment after *C. neoformans* infection. Mice were infected as described for Fig. 6, and cell suspensions were obtained from the lungs (i.t. infection) or the spleens (i.v. infection) of infected and sham-infected animals. The proportions of CD4$^+$ cells, CD8$^+$ cells, and macropha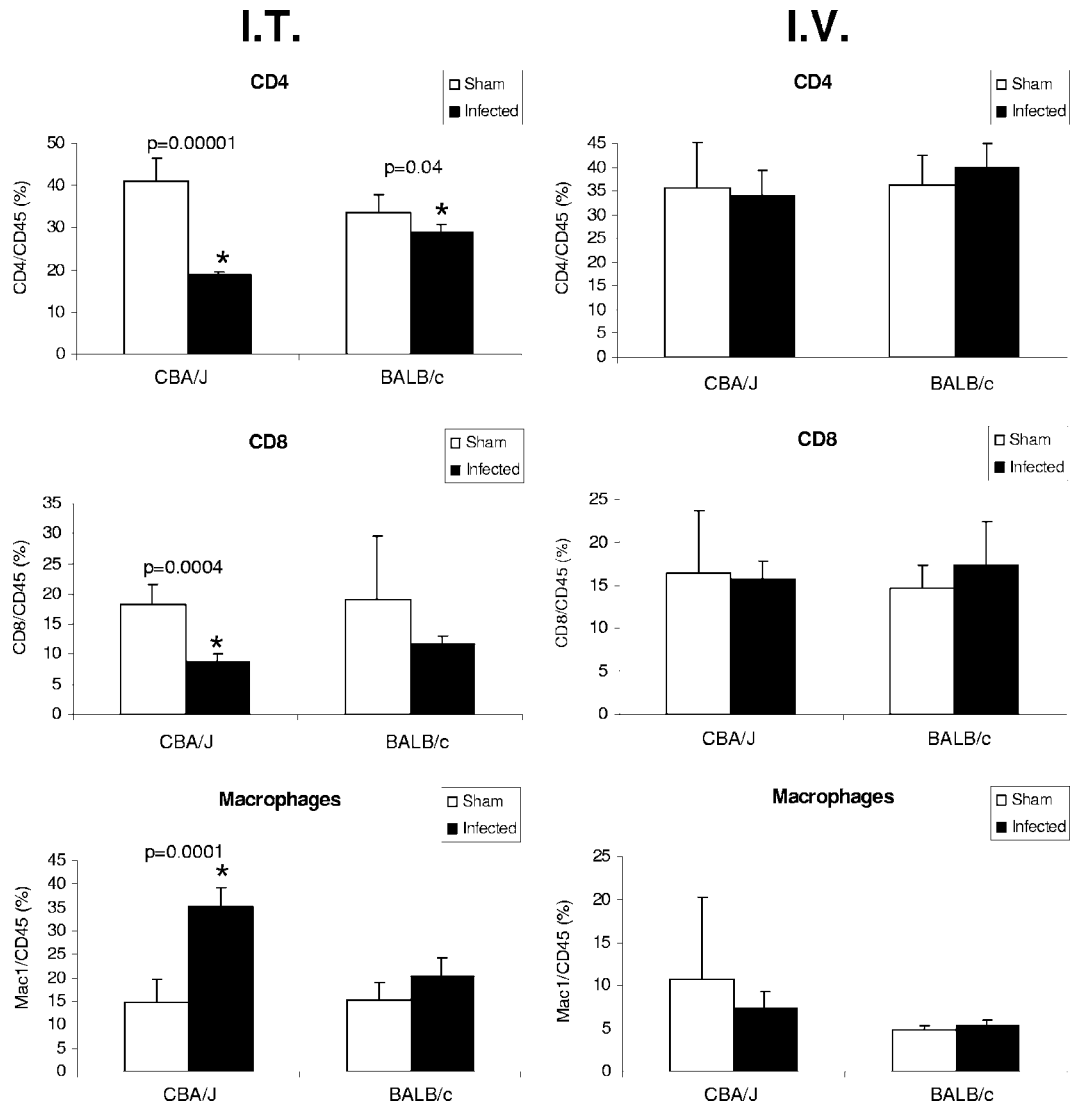ges among the leukocyte population, determined by using the CD45$^+$ marker, were obtained by FACS. Asterisks denote significant differences (obtained using Student's t test), and P values are shown. Mean values and standard deviations from five different mice are plotted.

TABLE 1. Outcome of the interaction between *C. neoformans* and alveolar macrophages isolated from CBA/J and BALB/c mice[a]

Expt	CBA/J macrophages			BALB/c macrophages		
	Total	Replicated	Nonreplicated	Total	Replicated	Nonreplicated
1	7	4	3	8	0	8
2	9	1	8	7	0	7
3	6	3	3	3	0	3
4	13	3	10	3	1	2
5				3	0	3
Total	35	11	24	24	1	23

[a] Alveolar macrophage isolation and phagocytosis experiments were performed as described in Materials and Methods. MAb 18B7 (50 µg/ml) was added as an opsonin. After imaging for 24 h, we focused on various macrophages with ingested *C. neoformans* cells (total) and counted the number of macrophages in which *C. neoformans* divided (replicated) or did not divide (nonreplicated).

ied the effect of MAb 18B7 administration in the two most susceptible infection models observed for CBA/J and BALB/c mice: i.t. infection for CBA/J mice and i.v. infection for BALB/c mice. Administration of small amounts of MAb 18B7 (10 µg) to CBA/J mice after i.t. infection tended to enhance the disease (although the difference was not statistically significant), while large amounts of MAb (1 mg) prolonged survival compared to that of the nontreated mice ($P = 0.044$) (Fig. 8A). To assess the impact of passive Ab administration on the course of i.v. infection, BALB/c mice were infected with 10^4, 10^5, and 10^6 *C. neoformans* cells in the presence and absence of MAb 18B7. MAb administration, irrespective of Ab concentration, did not have any protective effect in BALB/c mice infected with 10^5 and 10^6 yeast cells (Fig. 8C and D). BALB/c mice infected with 10^4 yeast cells and treated with 100 µg of MAb survived longer (median survival of 282 days) than mice treated with PBS or with 1 mg or 10 µg of MAb 18B7 (median

survival of 153, 51, and 153 days, respectively) ($P < 0.05$). In fact, administration of 1 mg of MAb accelerated mortality (although this was not significant [$P = 0.06$]), and 10 μg of MAb did not have any effect on survival (Fig. 8B).

DISCUSSION

We have compared the immune response to *C. neoformans* for two mouse strains that differ in susceptibility. A preliminary experiment evaluated four mouse strains and confirmed significant interstrain differences in susceptibility. To focus our study, we selected the BALB/c and CBA/J mice for additional work, since these represented the most resistant and susceptible strains, respectively. A prior study had reported that CBA/J mice were relatively resistant to infection (29). Both our study and the prior study used the same route of infection and pathogen strain. Although there might appear to be a discrepancy between these studies, they differ in the inoculum used, with the prior study using a significantly lower infecting dose (10^4 cells per mouse). In addition, *C. neoformans* strains can undergo microevolution under laboratory conditions (22), a phenomenon that might also account for interlaboratory variability. *C. neoformans* strains can manifest different virulence in the same model of infection (5, 14), and caution is warranted when generalizing from the experience with one system. In our approach, by comparing two mouse strains and two routes of infection, we identified a system where the relative susceptibility of a strain could vary depending on the infection route.

The relative resistance of a mouse strain to *C. neoformans* was a function of the route of infection. Hence, BALB/c mice were more resistant to i.t. infection than CBA/J mice, but both strains were equally susceptible to i.v. infection, and moreover, BALB/c mice were slightly more susceptible at higher i.v. infection doses. The differences in the relative susceptibility of the BALB/c mice were associated with significant changes in the immune response, such as dissemination and inflammation. The extent of extrapulmonary dissemination after pulmonary infection was different for the two mouse strains, suggesting that immune elements in the lung play a key role in local control of the fungal burden. The mechanisms by which *C. neoformans* disseminates to different organs are not completely known, but dissemination depends on factors from both the host and the pathogen. For example, microbial factors, such as laccase, inositol phosphosphingolipid-phospholipase C, phospholipase D, and capsule structure differences, have each been shown to play an important role during dissemination (16, 23, 56, 65). The mechanism by which *C. neoformans* can cross different barriers remains uncertain. Using an in vitro system, both endocytosis and transcytosis processes have been shown

FIG. 8. Passive Ab administration to CBA/J or BALB/c mice. Mice were treated with different amounts of MAb 18B7 and then infected with *C. neoformans* strain 24067. (A) i.t. infection. CBA/J mice ($n = 8$ to 10) were treated with 10, 100, and 1,000 μg of MAb 18B7 and then infected with 10^6 *C. neoformans* cells. (B to D) Survival of 6- to 8-week-old female BALB/c mice following i.v. infection with 10^4 (B), 10^5 (C), and 10^6 (D) *C. neoformans* strain 24067 organisms, respectively, and treatment with different amounts of MAb 18B7 ($n = 5$ for each experimental group).

to occur at the level of the brain-blood barrier (15). Since we used the same strain in this study yet witnessed major differences in dissemination, one must conclude that this process is also affected by host factors. In this regard, several elements from the immune response have been implicated in avoiding dissemination. CD4$^+$ and CD8$^+$ T lymphocytes are each important in pulmonary containment (26). Furthermore, the expression of some Th1 cytokines, such as IL-12 and IFN-γ, is associated with pulmonary control of cryptococcal infection (34, 35). Interestingly, some elements from the innate immune response, such as some complement receptors or phagocytes, seem to be required for dissemination of the fungal cells to different organs, such as the brain (32, 63). *C. neoformans* is an intracellular pathogen that can survive and replicate inside macrophages (2, 20, 70), and this provides the fungal cell with a mechanism for dissemination, whereby the phagocytic cell serves as a Trojan horse. In our study we noted that in vivo phagocytosis was similar in both the resistant and susceptible strains, yet there was a large difference in intracellular replication after *C. neoformans* was ingested by BALB/c and CBA/J alveolar macrophages. This result suggests that differences in macrophage function between susceptible and resistant mouse strains could be responsible for differences in susceptibility to i.t. infection and extrapulmonary dissemination, as has been suggested to explain differences in species susceptibility (64).

We found that Ab titers to *C. neoformans* capsular polysaccharide were higher after i.t. challenge than after i.v. injection. We do not have a clear explanation for this result, but it might reflect the function of antigen-presenting cells in the lung after i.t. infection. We also found that after i.t. infection, the Ab titers to GXM were higher in the resistant strain, which might be due to the higher proportion of CD4$^+$ cells in the lungs, since these cells are necessary to activate B cells and induce Ab production. Ab titers dropped from 7 to 14 days of infection, a phenomenon that may be related to the Ab unresponsiveness produced by GXM (36, 54). The extent and pattern of Ab binding to fungal cells in tissue differed between the two mouse strains. In susceptible CBA/J mice only a few cryptococcal cells were stained with Ab in a punctate pattern, while in the resistant BALB/c mice most cells were stained with Ab and manifested a mixed annular/punctate immunofluorescence pattern. We believe that this difference is due to both the difference in the Ab titers between the two mouse strains and the different Th1-Th2 polarization response. This could in turn influence B-cells isotype switching to yield Abs with different specificity (43, 69). We previously observed in another susceptible mouse strain (C57BL/6J) that the in vivo binding pattern was punctate (76), a finding that has been associated with lack of protection (11, 57). Although an efficient response seems to be mainly Th1 polarized and T-cell mediated, the role of Abs in natural infection remains uncertain. B cells appear to have an important role in host defense during cryptococcosis, as indicated by the increased susceptibility of B-cell-deficient mice (40, 60). Th2-polarized responses are associated with susceptibility to the disease. Our findings indicate that higher Ab production during the early stage of infection does not necessarily increase susceptibility to infection and is in fact associated with relative resistance. This is in agreement with the fact that MAbs to the capsule can affect the course of infection, and Abs can be protective, nonprotec-

tive, or even disease enhancing depending on their specificity and isotype (6, 7, 19, 39, 49–53, 62). In fact, a given MAb can be protective or disease enhancing depending on a variety of factors, such as Ab dose, a phenomenon known as the prozone effect (25, 67, 68). In our conditions, we observed both prozone and disease-enhancing effects in the i.v. and i.t models, respectively. Although the mechanisms responsible for these effects are not known, the results provide strong, dramatic evidence for the concept that Ab dosing is a critical determinant of Ab efficacy in both i.v. and i.t. infection models.

The increased resistance of BALB/c mice to i.t. infection relative to CBA/J mice was associated with a stronger Th1 response in BALB/c mice, as shown by IgE level regulation and cellular recruitment measured by FACS. The lower accumulation of CD4$^+$ and CD8$^+$ T cells in the lungs of infected CBA/J mice provides another mechanism that explains the difference between the responses of these mouse strains to pulmonary infection. The increased proportion of macrophages in the inflammatory response of the susceptible strain appears paradoxical and might be expected to be associated with resistance given that macrophages are essential host defense cells. However, we believe that in our conditions this increased macrophage influx into the lungs might have deleterious effects for the host by providing permissive cells for fungal reproduction that in turn promote a higher fungal pathogen burden and dissemination through the mechanism explained above. In fact, alveolar macrophage depletion has been associated with reduced susceptibility in mice (64). Interestingly, we noted that in CBA/J mice, some Th1 cytokines, such as IFN-γ and MCP-1, increased in response to *C. neoformans*, suggesting that a simple increase in the level of cytokines associated with protection is not sufficient to mediate resistance to the disease. We found differences in the regulation of IL-10 and IL-12 that could be important in host susceptibility. IL-10 is considered a Th2 cytokine that inhibits Th-1 cytokine production and is a potent inhibitor of macrophage function. During cryptococcal infection, increases in IL-10 are associated with higher susceptibility (33, 47, 73). IL-12 is considered a Th1 cytokine and is required for an effective response to *C. neoformans* (18, 27, 35, 58). In our model, resistance to infection was associated with increased IL-12 production and reduced IL-10 production, which in turn will produce a more active Th1 response. These cytokines may play a crucial role in the dissemination of the fungus to other organs and also by modulating the action of phagocytic cells. In the i.v. system, none of these cytokines were regulated during infection, which is consistent with the higher susceptibility shown by both strains in these conditions.

In summary, we have shown that susceptibility to pulmonary cryptococcal infection in CBA/J mice is associated with numerous differences in the immune response, including alveolar macrophage permissiveness to intracellular replication, lower Ab responses, and fewer Th1-polarized responses. Our results highlight the importance of the dose-response relationship in Ab efficacy in the lung by showing that small amounts of Ab are disease enhancing while large amounts are required for protection. The evolving picture is that both humoral and cellular components of the innate and adoptive immune systems can affect the outcome of cryptococcal pulmonary infection, sug-

gesting that successful outcomes require a layered defense that begins with the alveolar macrophage.

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