Inheritance of Immune Polarization Patterns Is Linked to Resistance versus Susceptibility to Cryptococcus neoformans in a Mouse Model

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Genetic background variation between inbred strains accounts for different levels of susceptibility to Cryptococcus neoformans in the mouse infection model. To elucidate the inheritance of immunophenotypic traits and their associations with clearance outcomes during cryptococcal infection, we compared C57BL/6, BALB/c, and their first-generation hybrid, CB6F1 (F1), mice. Mice from each group were infected with C. neoformans (10^4 CFU) and analyzed at weekly intervals over a 6-week period. BALB/c mice progressively cleared the cryptococcal infection in the lungs and showed a Th1-skewed immune response: a Th1-shifted cytokine profile, modest lung pathology, and no significant elevation in the systemic immunoglobulin E (IgE) level. In contrast, C57BL/6 mice developed a chronic infection with a Th2-skewed immune response: a Th2-shifted cytokine profile, pulmonary eosinophilia, severe lung pathology, elevated serum IgE, fungemia, and cryptococcal dissemination in the central nervous system. F1 mice demonstrated intermediate resistance to C. neoformans, with a stronger resemblance to the immunophenotype of the resistant (BALB/c) mice. F1 mice also demonstrated enhanced pulmonary recruitment of lymphocytes, especially CD8^+ T cells, in comparison to both parental strains, suggesting positive heterosis. We conclude that the inheritance of traits responsible for early cytokine induction in the infected lungs and dendritic-cell maturation/activation status in draining nodes is responsible for the intermediate immune response polarization and clearance outcome observed initially in the lungs of F1 mice. The enhanced pulmonary lymphocyte recruitment could be responsible for a gradual shutdown of the undesirable Th2 arm of the immune response and subsequently improved anticryptococcal resistance in F1 mice.

Cryptococcus neoformans is one of the major opportunistic fungal pathogens worldwide and is a leading cause of fatal mycosis in immunocompromised individuals (3). Clearance of C. neoformans infection requires the development of a protective T-cell-mediated immune response. The lack of this T-cell-mediated response, as is seen in patients with human immunodeficiency virus (HIV)-positive patients or patients undergoing aggressive immunosuppressive therapies, is a major risk factor for clinical C. neoformans infections (7, 24, 36). Invasive cryptococcosis has also been found in noncompromised patients, indicating that C. neoformans can evade the host’s immune responses even in the absence of apparent defects in the immune system (14, 15, 40). Recently Marroni et al. (31) suggested that specific genetic defects in the natural immune system could be responsible for pulmonary cryptococcosis in an apparently immunocompetent patient. Therefore, genetic predisposition is one of the possible factors that could account for the increased susceptibility to C. neoformans infection in noncompromised patients.

In mice, the host’s genetic background has significant effects on the pulmonary immune responses to cryptococcal infections. The inbred mouse strains CBA/J, BALB/c, and C.B-17 are able to clear C. neoformans infection, while C57BL/6 mice develop chronic pulmonary infections (13, 16–19). In addition, host factors such as gender and age have an influence on susceptibility to cryptococcosis (28). Experimental C. neoformans infections in knockout mice lacking specific cytokine or immunological receptor genes have shown a wide spectrum of effects, including a complete lack of clearance (2, 5), diminished clearance (33), and in some instances enhanced clearance rates (11) compared to results for their wild-type counterparts. These studies indicate that a single defect in the immune response can greatly alter the clearance phenotype from resistant to susceptible and vice versa.

The different resistance/susceptibility patterns of cryptococcal infection in various strains of mice have been linked to differential phenotypes of the inflammatory responses (13). Successful clearance of C. neoformans relies on the development of a T1 immune response, while a T2-polarized immune response elicits enhanced susceptibility in a number of mouse models (12, 20, 22, 34, 38). Many of the observed differences strongly suggest that susceptibility/resistance are linked with differential polarization of the immune responses in specific strains of mice (13, 16). However, very little is known about the inheritance of the resistance/susceptibility patterns and how these inherited elements may be important in the resistant phenotype. Investigating the patterns of inheritance of a number of immunological factors required for clearance in resistant versus susceptible strains could reveal how genetic differences affect clearance.

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Apart from the well-established role of T cells in host defenses against *C. neoformans*, the role of pulmonary macrophages and their activation profile has been increasingly appreciated. Alveolar macrophages are involved in the initial recognition of *C. neoformans* and in secretion of cytokines/chemokines which aid the development of the adaptive immune response (2, 39). When properly activated, macrophages play an important role in cryptococcal clearance as effector cells that destroy *C. neoformans* following phagocytosis. The secretion of cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 12 (IL-12) by macrophages supports the development of protective immunity and is crucial to the host’s ability to clear fungal infections (12, 17, 21, 22). Alternatively, macrophages can harbor live *C. neoformans* without induction of proper proinflammatory cytokine signals. In this case, protective immunity does not develop and macrophages become a reservoir of *C. neoformans* in the infected host (2, 39). It is likely that the ability of alveolar macrophages to rapidly recognize *C. neoformans* and deliver robust danger signals, such as TNF-α, is an important component of inherited resistance to this pathogen. Furthermore, the critical role of alveolar macrophages as the central effector cells of the host innate immune system has been attributed to the differences in susceptibility to pulmonary cryptococcal infection in rats and mice (23, 35).

Although work analyzing susceptibility and resistance patterns in wild-type mouse strains has been performed, the patterns of inheritance of host factors that affect the clearance process remain largely unknown. Our studies focus on the inheritance of a wide variety of immune factors that are expressed during cryptococcal infection in mice. In order to elucidate the immunological phenotypic components caused by differential host genetic backgrounds, parallel analysis of *C. neoformans* infection in the widely used susceptible C57BL/6 and resistant BALB/c mice and the first-generation hybrid of these two mouse strains (CB6F1/J, denoted as F1 mice) was performed.

**MATERIALS AND METHODS**

**Mice.** These studies were approved by both the VA and University of Michigan committees for animal use and care. Female wild-type C57BL/6 and BALB/c mice and their first-generation hybrid CB6F1/J (F1) used in these studies were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were raised in specific-pathogen-free conditions at the Ann Arbor VA Medical Center using sterilized cages covered with a filter top and were fed sterile food/water ad libitum. Mice were aged to 6 to 8 weeks at the time of infection.

**Cultures of *C. neoformans*.** *C. neoformans* strain 52D (ATCC 24067) was obtained from the American Type Culture Collection (Manassas, VA). For infection, yeast were grown to stationary phase after an incubation period of 48 to 72 h at 37°C in Sabouraud dextrose broth (1% neopepton and 2% dextrose; Difco, Detroit, MI) on a shaker. Cultures were washed twice with saline, counted on a hemocytometer, and diluted to 3.3 x 10^5 CFU/ml in nonpyrogenic saline.

**Intratracheal inoculation of *C. neoformans*.** Mice were anesthetized by an inhalation of a one-step methanol-based Wright-Giemsa stain followed by steps two and three of the Diff-Quik whole blood stain kit. Total lung leukocytes from each of the 30 mice were enumerated in a hemocytometer using a trypan blue exclusion assay. The percentage of each lymphocyte subset by the total number of leukocytes from cell debris and epithelial cells. Total lung leukocyte numbers were enumerated in a hemocytometer using a trypan blue exclusion assay.

**Leukocyte subset analysis.** Macrophages, neutrophils, eosinophils, monocytes, and lymphocytes were visually counted in Wright-Giemsa-stained samples of lung cell suspensions cytospun onto glass slides. Slides were stained by fixing for 2 min with a one-step methanol-based Wright-Giemsa stain followed by steps two through three of the Diff-Quik whole blood stain kit. Total lung leukocytes from each sample were then counted from a randomly chosen field using a high-powered microscope. The percentage of a leukocyte subset was multiplied by the total number of leukocytes to give the absolute number of the specific leukocyte subset in the sample.

**Lung leukocyte culture.** Isolated lung leukocytes were diluted to 5 x 10^4 cells/ml and were cultured in 24-well plates with 2 ml of complete RPMI medium at 37°C and 5% CO₂ for 24 h. Plates were centrifuged to remove leukocytes, and supernatants were collected for subsequent enzyme-linked immunosorbent assay (ELISA) tests.

**Antibody staining and flow cytometric analysis.** All staining reactions were performed on ice. Cells were incubated with 2.4G2 Fc receptor antibody to minimize nonspecific binding. Data were collected on a FACS LSRII flow cytometer using DIVA software and analyzed using FlowJo software (Tree Star Inc., San Carlos, CA). A minimum of 50,000 cells were analyzed per sample. Initial gates were set based on light scatter characteristics to exclude debris, red cells, and cell clusters. To detect dendritic cells (DC) in the lung-associated lymph node (LALN), cells were isolated and stained with allophycocyanin-labeled anti-CD11c (HL3), phycoerythrin-labeled anti-high-molecular-weight complex II (MHCII) (Iaβ3) antibodies. The phenotype of CD11c⁺ MHCII⁺ cells was determined by using either PerCP-Cy5.5-labeled anti-CD80 or fluorescein isothiocyanate-labeled anti-CD80 and CD40 antibodies. To detect lung lymphocyte subsets, single cells were stained with PerCP-Cy5.5 single cells were stained with PerCP-Cy5.5 and CD45. The lymphocyte subsets of CD45⁺ cells were determined by using allophycocyanin-labeled anti-CD8, phycoerythrin-labeled anti-CD19 (for B cells), and fluorescein isothiocyanate-labeled anti-CD4 antibodies. All monoclonal antibody reagents were purchased from PharMingen (San Diego, CA). The absolute number of each lymphocyte subset in the sample was obtained by multiplying the percentage of that type of lymphocyte subset by the total number of leukocytes.

**Bronchoalveolar lavage (BAL).** Euthanized mice were lavaged after cannulation of the trachea with polyethylene tubing (PE15), which was attached to a 30-gauge needle and inserted in the trachea. The delivery of 30 μl of inoculum (10⁶ CFU) into the trachea was visibly confirmed. The needle was removed and the skin closed with a cyanoacrylate adhesive. Mice recovered with minimal visible trauma.

**Organ CFU assay.** For determination of CFU in lung tissue, small aliquots were collected from lung digests. For determination of CFU in spleen and brain tissue, the respective organs were excised, placed in 2 ml of sterile water, and homogenized. Series of 10-fold dilutions of the lung, spleen, and brain samples were plated on Sabouraud dextrose agar plates in duplicates of 10-μl aliquots and incubated at room temperature. *C. neoformans* colonies were counted 3 days later, and the number of CFU was determined on a per-organ basis.

**Preparation and enumeration of lung leukocytes.** Mice were sacrificed by CO₂ asphyxiation. To isolate pulmonary leukocytes, the abdominal vena cava of the mice was severed to remove red blood cells from the lungs prior to excision. Individual lungs were excised from sacrificed mice, minced with scissors, and enzymatically digested for 30 min in 15 ml of digestion buffer (RPMI, 5% fetal calf serum, penicillin, and streptomycin, 1 mg/ml collagenase, 0.25 ml/mouse of DNase) at 37°C. The resulting cell suspension was further dispersed through the bore of a 10-ml syringe 20 times and centrifuged. Following enzymatic digestion, the erythrocyte pellets were lysed by the addition of 3 ml of NH₄Cl buffer for 3 min. Ten milliliters excess medium was added to return the solution to isotonicity. Resuspended cells were filtered through a 70-μm sterile nylon screen and centrifuged for 25 min at 3,000 rpm in the presence of 20% Percoll to separate leukocytes from cell debris and epithelial cells. Total lung leukocyte numbers were enumerated in a hemocytometer using a trypan blue exclusion assay.

**Histological analysis.** Lungs were fixed by inflation with 1 ml of 10% neutral buffered formalin. After paraffin embedding, 5-μm sections were cut and stained with hematoxylin and cosin. Sections were analyzed using light microscopy.

**Preparation of lung tissue for electron microscopy.** Lungs were serially sectioned (4 μm) onto glass and stained with periodic acid-Schiff for identification of alveolar macrophages and other nonspecific material. Post-stained sections were visualized with a high-powered microscope. The percentage of a leukocyte subset was multiplied by the total number of leukocytes to give the absolute number of the specific leukocyte subset in the sample.

**Lung leukocyte culture.** Isolated lung leukocytes were diluted to 5 x 10⁴ cells/ml and were cultured in 24-well plates with 2 ml of complete RPMI medium at 37°C and 5% CO₂ for 24 h. Plates were centrifuged to remove leukocytes, and supernatants were collected for subsequent enzyme-linked immunosorbent assay (ELISA) tests.
Thus, F1 mice did not demonstrate the clearance of either

**C. neoformans** in lungs postinfection. The F1 mice and the parental strains were infected with 10^4 CFU of **C. neoformans** 52D, and the pulmonary **C. neoformans** burden was evaluated at weeks 1 to 6 postinfection. Values represent means ± SEM (n = 6 mice/group/time point). *, significant difference from results for F1 mice; **, significant difference from results for BALB/c mice. Data were pooled from three parallel experiments.

Calculations and statistics. Statistical significance was calculated using one-way analysis of variance (ANOVA) with the Student-Newman-Keuls test post hoc, and the Kruskal-Wallis test was used whenever data did not follow a normal distribution. A two-way ANOVA (with mouse strain and time as between-subject factors) was used to analyze the overall strain effect when multiple time points were compared. Means with P values of <0.05 were considered significantly different. All values are reported as means ± standard errors (SEM).

**RESULTS**

**F1 mice inherit the ability to clear** **C. neoformans** infection from their lungs. Our first objective was to assess the clearance phenotype in the first-generation hybrid (F1) cross between resistant (BALB/c) and susceptible (C57BL/6) mouse strains. The F1 mice and the parental strains were infected with **C. neoformans** and evaluated at weeks 1 through 6 postinfection (Fig. 1). Rapid pulmonary growth of **C. neoformans** (~1,000-fold) was observed at week 1 postinfection in all three strains of mice. The cryptococcal load reached a plateau at week 2 postinfection and subsequently followed differential clearance patterns between the three strains of mice (weeks 3 to 6). C57BL/6 mice developed a chronic infection, i.e., a constant fungal burden averaging above 10^7 CFU, for the duration of the study. In contrast, BALB/c mice demonstrated gradual clearance of **C. neoformans** beginning at week 2, marked by a 1,000-fold decrease in the pulmonary fungal load between weeks 2 and 6 postinfection. F1 mice maintained a constant level of infection throughout week 3; however, at the later time points (weeks 4 to 6 postinfection), F1 mice exhibited a progressive decrease in the pulmonary load. The pulmonary microbial burden in F1 mice was lower than that in C57BL/6 mice from week 3 on, throughout the remaining course of infection, but it was also significantly higher than that in BALB/c mice at weeks 3 and 6 postinfection (Fig. 1). Although the difference in clearance between C57BL/6 and F1 mice was more pronounced, the factorial analysis showed a significant overall CFU difference between F1 and BALB/c strains (P = 0.001). Thus, F1 mice did not demonstrate the clearance of either parental strain; F1 mice could clear **C. neoformans** from their lungs, in contrast with C57BL/6 mice, but with less efficiency than the resistant BALB/c mice.

**F1 mice show intermediate susceptibility to fungemia and central nervous system dissemination.** Dissemination from the local site of infection (lung) into the blood and into the brain is another measure of susceptibility to **C. neoformans** infection. Therefore, spleen and brain cryptococcal loads were compared at week 3 postinfection. All of the susceptible C57BL/6 mice developed fungemia, illustrated by 100%-positive spleen CFU (Fig. 2A). In contrast, only 67% of resistant BALB/c mice and 83% of F1 mice demonstrated detectable dissemination into spleen tissue. The logarithmic spleen CFU values were significantly different between the three strains (n = 6 animals per group). Consistent with the observed propensity for fungemia, the highest frequency of dissemination into brain tissue was observed in C57BL/6 mice (50%), while none of the BALB/c mice had detectable CFU in the brain (Fig. 2B). F1 mice demonstrated a modest degree of dissemination into the brain, with 33% of positive brain cultures. While CFU loads in the brain were not significantly different between the groups because of low numbers of positive animals (3/6 mice in the C57BL/6 group and 2/6 in the F1 group), the distribution indicates that F1 mice were not entirely protected from cryptococcal dissemination into the brain. Thus, F1 mice show an intermediate pattern of susceptibility/resistance to systemic **C. neoformans** dissemination compared to parental C57BL/6 and BALB/c mice.

**Increased cellular inflammatory response in** **C. neoformans-infected** lungs of F1 mice does not correlate with pulmonary clearance. To determine if the fungal clearance correlated with the magnitude and/or timing of the development of inflammatory responses in the lungs of infected F1, C57BL6, and BALB/c mice, pulmonary leukocytes were enumerated. At week 1 postinfection, all three strains of mice demonstrated a similar, mild increase in pulmonary leukocyte numbers, consistent with the development of an inflammatory immune response (Fig. 3). Between weeks 1 and 2 postinfection, the leukocyte numbers increased significantly and maintained elevation through week 4 in all three strains. Interestingly, both
the F1 and C57BL/6 strains demonstrated greater inflammation than BALB/c mice (Fig. 3). At week 6, all three strains exhibited significant differences in the number of pulmonary leukocytes; C57BL/6 mice maintained high numbers of leukocytes, while BALB/c and F1 mice showed a gradual resolution of the inflammatory response. Thus, F1 mice initially exhibit the highest accumulation of pulmonary leukocytes; however, the magnitude of the inflammatory response does not correlate directly with the rate of *C. neoformans* clearance.

**F1 mice exhibit a “first-generation hybrid effect” on pulmonary lymphocyte recruitment in response to *C. neoformans* infection.** Clearance of *C. neoformans* is T cell dependent. Our next objective was to compare lymphocyte populations in the lungs of infected F1, C57BL6, and BALB/c mice. No differences in relative frequencies of pulmonary lymphocytes were observed between the parental strains at any given time points postinfection. However, F1 mice showed higher percentages of pulmonary lymphocytes compared to both parental strains at week 1 and to C57BL6 mice at weeks 2 and 3 (Table 1). These increases in lymphocyte percentages resulted from significantly greater numbers of lymphocytes that F1 mice recruited into the lungs from week 1 on in comparison to both parental strains (20.0 million ± 2.0 million/lung versus 12.8 million ± 1.8 million/lung and 16.2 million ± 2.8 million/lung in C57BL6 and BALB/c mice, respectively). These differences prompted a subsequent flow cytometry study to enumerate specific lymphocyte subsets at weeks 0, 1, and 3 postinfection for all three strains of mice (Fig. 4). All three strains exhibited significant differences in the frequency of CD4+ cells, with BALB/c mice showing the highest, F1 intermediate, and C57BL/6 the lowest CD4+ cell frequencies prior to the infection. However, the absolute numbers of pulmonary CD4+ cells in uninfected mice were not significantly different between all three strains. BALB/c mice continued to show significantly higher CD4+ cell frequencies at weeks 1 and 3 postinfection than did F1 and C57BL/6 mice. However, C57BL/6 mice showed lower absolute numbers of CD4+ cells in the lungs at weeks 1 and 3 than both BALB/c and F1 mice (Fig. 4). In terms of CD8+ and B220+ cells, there was no difference in their frequencies or absolute numbers between any of the uninfected mouse strains. There was also no difference in the recruitment of these cells into the lungs in the infected parental C57BL/6 and BALB/c mouse strains. However, F1 mice recruited higher numbers of CD8+ cells into their lungs (week 1 to 3) than either parental strain. Additionally, strong trends of elevated B220+ cells were found in the lungs of F1 mice, with a significant increase over levels for C57BL/6 mice at week 1. Taken together, these results demonstrate that F1 mice had increased recruitment of lymphocytes (enhanced CD8+ and to some degree B220+ cell numbers) compared to parental strains and increased the total CD4+ cell number (but not the frequency) compared to C57BL/6 mice.

**Chronic pulmonary eosinophilia observed in *C. neoformans*-infected C57BL/6 mice was not inherited by F1 mice.** Since the differences in pulmonary lymphocyte numbers could not explain the intermediate *C. neoformans* clearance pattern in F1 mice, we analyzed eosinophil populations (a hallmark of a nonprotective Th2 response in the lungs) as well as the remain-

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**TABLE 1. Relative leukocyte subset frequencies for *C. neoformans*-infected BALB/c, F1, and C57BL/6 mice**

<table>
<thead>
<tr>
<th>Infection status or time postinfection</th>
<th>Leukocyte subset</th>
<th>BALB/c (%)</th>
<th>F1 (%)</th>
<th>C57BL/6 (%)</th>
</tr>
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<tr>
<td><strong>Uninfected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocyte</td>
<td>36.3 ± 3.9</td>
<td>39.5 ± 6.7</td>
<td>39.0 ± 6.7</td>
<td></td>
</tr>
<tr>
<td>Macrophage</td>
<td>28.9 ± 6.7</td>
<td>30.9 ± 6.9</td>
<td>29.0 ± 7.9</td>
<td></td>
</tr>
<tr>
<td>Eosinophil</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.2</td>
<td>0.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Neutrophil</td>
<td>2.6 ± 0.4</td>
<td>1.9 ± 0.6</td>
<td>3.4 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>31.2 ± 4.5</td>
<td>26.8 ± 5.2</td>
<td>28.0 ± 3.9</td>
<td></td>
</tr>
<tr>
<td><strong>Wk 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Monocyte</td>
<td>32.5 ± 2.8</td>
<td>33.8 ± 1.6</td>
<td>28.9 ± 1.6*</td>
<td></td>
</tr>
<tr>
<td>Macrophage</td>
<td>22.8 ± 2.4</td>
<td>18.7 ± 1.2</td>
<td>23.1 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Eosinophil</td>
<td>5.0 ± 1.1</td>
<td>6.4 ± 1.3</td>
<td>13.3 ± 2.0**</td>
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<tr>
<td>Neutrophil</td>
<td>18.1 ± 1.8*</td>
<td>13.2 ± 1.6</td>
<td>12.6 ± 1.7**</td>
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<tr>
<td>Lymphocyte</td>
<td>21.7 ± 2.1*</td>
<td>27.0 ± 1.7</td>
<td>22.3 ± 1.7*</td>
<td></td>
</tr>
<tr>
<td><strong>Wk 2</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Monocyte</td>
<td>35.2 ± 1.6*</td>
<td>28.3 ± 1.5</td>
<td>29.4 ± 1.8**</td>
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<td>Macrophage</td>
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<tr>
<td>Eosinophil</td>
<td>8.4 ± 1.1*</td>
<td>13.1 ± 2.3</td>
<td>23.1 ± 3.7***</td>
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<td>Neutrophil</td>
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<td>Lymphocyte</td>
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<td>22.9 ± 2.1</td>
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<td><strong>Wk 3</strong></td>
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<tr>
<td>Monocyte</td>
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<td>31.6 ± 1.5</td>
<td>32.3 ± 2.1**</td>
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<tr>
<td>Macrophage</td>
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<td>22.3 ± 1.9</td>
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<td>Eosinophil</td>
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<td>6.4 ± 1.2</td>
<td>19.5 ± 2.5**</td>
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<tr>
<td>Neutrophil</td>
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<td>Lymphocyte</td>
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<td>17.4 ± 1.5*</td>
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<td><strong>Wk 4</strong></td>
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<tr>
<td>Monocyte</td>
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<td>32.5 ± 4.2</td>
<td>36.9 ± 2.4**</td>
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<tr>
<td>Macrophage</td>
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<tr>
<td>Eosinophil</td>
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<td>1.5 ± 0.4</td>
<td>8.5 ± 3.5*</td>
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<tr>
<td>Neutrophil</td>
<td>7.8 ± 1.2</td>
<td>13.9 ± 4.5</td>
<td>11.6 ± 2.3</td>
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<tr>
<td>Lymphocyte</td>
<td>20.0 ± 2.1</td>
<td>20.1 ± 2.4</td>
<td>17.6 ± 2.5</td>
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*Leukocyte frequencies were evaluated using high-powered-microscope counts of Wright-Giemsa-stained lung cell suspensions on cytospun slides. Frequencies of leukocyte subsets are expressed as percentages of the total leukocytes present in the sample to illustrate alterations in the cellular composition in the infected lungs. Values represent means ± SEM (n = 6 to 20 mice/group/time point). *, significantly different from result for F1 mice; **, significant difference from result for BALB/c mice. Data were pooled from five parallel experiments.
ing subsets of pulmonary leukocytes. Table 1 shows percentages of each leukocyte subset evaluated microscopically in enzymatically digested lungs. No differences in relative frequencies of pulmonary leukocyte subsets were observed between the strains prior to the infection. From week one, C57BL/6 mice demonstrated elevated eosinophil percentages compared with both F1 and BALB/c mice. This elevated frequency and number of pulmonary eosinophils in C57BL/6 mice was observed consistently throughout the entire course of infection (Fig. 5A). At week 2 postinfection, F1 mice had significantly increased recruitment of eosinophils compared to BALB/c mice and showed total numbers similar to those for

FIG. 4. Lymphocyte subset recruitment following pulmonary C. neoformans 52D infection. Samples of leukocyte suspensions from infected mice were stained with fluorochrome-labeled antibodies specific for CD4\(^\text{+}\), CD8\(^\text{+}\), and B220\(^\text{+}\) lymphocytes and analyzed by flow cytometry as described in Materials and Methods. Results are illustrated as percentages or absolute numbers of each lymphocyte subset in the total lung leukocyte sample. Values represent means ± SEM (n = 4 to 6 mice/group/time point). *, significant difference from results for F1 mice; **, significant difference from results for BALB/c mice. Data were pooled from two parallel experiments.

FIG. 5. T2 hallmarks: pulmonary eosinophilia and elevated levels of serum IgE. (A) Pulmonary eosinophils following C. neoformans 52D infection. Aliquots of leukocyte suspension were cytospun onto slides and cell subsets enumerated at weeks 0, 1, 2, 3, and 5 postinfection. Results are illustrated as numbers of eosinophils in the total lung leukocyte isolate. Values represent means ± SEM (n = 6 to 20 mice/group/time point). (B) Total serum IgE levels were evaluated using serum samples from BALB/c, F1, and C57BL/6 mice. Samples were evaluated for the concentration of total IgE using the ELISA. Values represent means ± SEM (n = 6 to 10 mice/group/time point). *, significant difference from results for F1 mice; **, significant difference from results for BALB/c mice. Data were pooled from three parallel experiments.
C57BL/6 mice (Fig. 5A) and an intermediate frequency of eosinophils (Table 1). However, except for week 2, both F1 and BALB/c mice demonstrated similar frequencies and total numbers of eosinophils throughout the course of the infection (Table 1; Fig. 5A). Thus, only C57BL/6 mice developed persistent pulmonary eosinophilia, while F1 mice exhibited a transient elevation in lung eosinophil numbers at week 2 postinfection comparable to that found in C57BL/6 mice.

F1 mice show no evidence of systemic Th2 polarization during infection with C. neoformans. Since we observed transient but significant pulmonary eosinophilia in F1 mice, we suspected the possibility of a temporal shift to the Th2 immune response in these mice. To address this concern, we compared serum IgE levels (a systemic hallmark of a Th2 response) between C. neoformans-infected F1, C57BL/6, and BALB/c mice. All three mouse strains exhibited only minimal IgE production during the initial 2 weeks of infection. As expected, C57BL/6 mice demonstrated a significant increase in serum IgE over time (Fig. 5B) (ANOVA; \( P = 0.001 \)). Furthermore, C57BL/6 mice showed significant increases in serum IgE antibody levels compared to F1 mice and BALB/c mice at week 5 postinfection. Surprisingly, compared to results for C57BL/6 and F1 mice, BALB/c mice demonstrated a small elevation of serum IgE that reached statistical significance at week 2. However, BALB/c IgE levels remained constant throughout the subsequent time points. Although F1 mice showed some modest increase in serum IgE at week 5 compared with the other time points (\( P < 0.05 \)), this was not significantly higher than the IgE level for BALB/c mice (Fig. 5B). Thus, unlike C57BL/6 mice, the F1 mice did not appear to develop the systemic Th2 response.

Host genetics of F1 mice affects the development of lung pathology in C. neoformans infection. To determine if C. neoformans infection in F1 mice resulted in the development of lung pathology that resembled that of either of the paternal strains, infected lungs of F1, C57BL/6, and BALB/c mice were examined histologically 6 weeks postinfection.

As previously reported, C57BL6 mice developed significant lung pathology with overwhelming features of lesions driven by type 2 immunity (2, 5). Microscopic examination revealed consolidation of large portions of lung and loss of functional lung tissue in C57BL/6 mice (Fig. 6A). The mixed inflammatory infiltrate filling the air space was marked by numerous eosinophils and multinucleated giant macrophages, many of which contained intracellular C. neoformans (Fig. 7A). Moreover, infected lungs contained both intracellular and extracellular eosinophilic YM1/YM2 crystals (2), consistent with the presence of alternatively activated macrophages (AAMs).

In BALB/c mice, infected lungs had fewer areas of inflammatory infiltrates and fewer intra- and extracellular cryptococci than the lungs of C57BL/6 and F1 mice (Fig. 6). The air spaces of BALB/c lungs were largely free of microbes and inflammation. Very few eosinophils and no evidence of AAMs (YM1/YM2 crystals) were observed in C. neoformans-infected lungs of BALB/c mice (Fig. 7C). The tight mononuclear infiltrates of predominantly small mononuclear cells surrounding infected lung areas were consistent with the effective cryptococcal clearance and the absence/resolution of pathological lesions.

In F1 mice, large areas of lung tissue were replaced by dense inflammatory infiltrates, much as in C57BL/6 mice, but F1 mice also exhibited areas of tight mononuclear infiltrates as seen in BALB/c mice (Fig. 6B). Some granulocytes were observed in F1 mice; however, they were much fewer in number than in C57BL/6 mice. Although F1 mice exhibited areas of lung pathology filled with multinucleated giant macrophages, fewer macrophages with a morphological AAM phenotype were found in C57BL/6 mice and no areas with YM1/YM2 crystal deposition were observed (Fig. 7B). Thus, F1 mice inherited an immune response that resulted in a mixed phenotype of pathological findings, with aspects of Th2-driven pathology as in parental C57BL/6 mice and Th1-driven clearance as in BALB/c mice.

F1 mice generate an intermediate Th1/Th2 cytokine profile during early infection that evolves toward a weaker Th1 response. To determine if F1 mice inherited a preference for either Th1 or Th2 immune polarization of the response, the levels of TNF-\( \alpha \), IL-12, IFN-\( \gamma \), IL-10, and IL-4 were analyzed at day 7 postinfection in BAL fluid. Compared with C57BL/6 and F1 mice, BALB/c mice produced significantly more of the pro-Th1 cytokines TNF-\( \alpha \) and IL-12 (Fig. 8A). BALB/c mice had significantly greater levels of early IFN-\( \gamma \) than C57BL/6 mice and showed a strong trend toward increases in the IFN-\( \gamma \) levels compared with F1 mice. Although IFN-\( \gamma \) levels at week 1 were significantly different between C57BL/6 and F1 mice but not F1 and BALB/c mice, IL-12 levels for F1 mice were similar to those for C57BL/6 mice but different from those for BALB/c mice. F1 mice showed an intermediate level of IL-4 in BAL fluid that was significantly lower than that for C57BL/6 mice but greater than that for BALB/c mice (Fig. 8A), and no difference between the parental strains and the F1 mice was found in the IL-10 BAL level (not shown). To assess the overall Th1/Th2 cytokine balance at the time of immune response generation, the ratios between driving Th2 (IL-4) versus driving Th1 (IFN-\( \gamma \)) cytokines were calculated. F1 mice demonstrated an intermediate IL-4/IFN-\( \gamma \) ratio compared to their parental strains by week 1 of infection, while cytokine production was strongly Th1 polarized in BALB/c mice and Th2 polarized in C57BL/6 mice (Fig. 8B). No differences were found between the F1 mice and the parental strains when uninfected lung leukocyte cytokine ratios were compared (data not shown). Thus, at an early stage of infection, the signals...
leading to Th1/Th2 cytokine polarization in F1 mice showed intermediate trends.

From week 2 on, we monitored changes in the cytokine profile by analysis of cytokine production from isolated lung leukocytes in cultures. As at week 1, BALB/c mice demonstrated the overall highest level of IFN-γ compared to C57BL/6 and F1 mice (Fig. 9); levels of IFN-γ production were consistent with the observed differential rates of cryptococcal

FIG. 7. High-power photomicrographs of lung pathologies from C57BL/6 mice (A), F1 mice (B), or BALB/c mice (C) at week 6 postinfection. Note the multinucleated giant macrophages containing both intracellular YM1/YM2 crystals (YM) and cryptococcal organisms (*) consistent with the presence of AAMs, as well as small red granulocytes consistent with eosinophils (Eo) for C57BL/6 mice; the tight mononuclear cell infiltrate and the absence of AAMs and eosinophils in BALB/c mice, consistent with Th1 granulomatous inflammation; and intermediate lung pathology shown in the lungs of F1 mice. Pictures show hematoxylin-and-eosin-stained lung sections at an objective power of ×40.
clearance. In contrast, only leukocytes from C57BL/6 mice produced a considerable amount of IL-4, while both BALB/c and F1 mice showed similar near-baseline levels of IL-4 production at weeks 2 and 3 (Fig. 9). No differences in IL-10 cytokine production were observed between parental strains and F1 mice. Similar to what was observed at week 1, BALB/c mice produced higher levels of TNF-α than C57BL/6 mice throughout the remaining time of infection. However, TNF-α levels of F1 mice fluctuated, reaching levels similar to those for BALB/c mice at week 2 and decreasing again at week 3 to the level observed for C57BL/6 mice. These cytokine profiles are consistent with F1 mice developing a weaker Th1 response, in contrast with the robust Th1 response in BALB/c mice and the Th2-biased response in C57BL/6 mice.

**F1 mice exhibit an intermediate DC maturation phenotype in lung draining lymph nodes.** The pulmonary cytokine milieu strongly affects the maturation status of DC that migrate from the lungs into the LALN to subsequently induce T-cell responses. To determine the maturation status of DC in LALN in each of the infected mouse strains, flow cytometry analysis of isolated LALN cells was performed. CD11c⁺ MHCII⁺ double-positive DC populations (consistent with conventional DCs) were gated to analyze the surface expression of costimulatory molecules (Fig. 10A). Consistent with DC migration from the infected lungs into the nodes, the LALN from infected mice yielded significantly higher numbers and percentages of CD11c⁺ MHCII⁺ DC than the LALN from uninfected mice (Fig. 10A). No differences in frequencies or total numbers of DC were observed in the nodes from the infected F1, BALB/c, and C57BL/6 mice (data not shown). Up-regulation of the costimulatory molecules CD40, CD80, and CD86 was used as a surrogate of DC maturity, and these cells were

FIG. 8. Cytokine production in BAL fluid from *C. neoformans*-infected BALB/c, F1, and C57BL/6 mice. (A) BAL samples were collected at 7 days postinfection and analyzed for cytokines by ELISA. (B) The IFN-γ/IL-4 ratio was shown to illustrate a general trend in immune response polarization. Data were pooled from two parallel experiments (n = 4 to 6). Values represent means ± SEM in pg/ml supernatant. *, significant difference from results for F1 mice; **, significant difference from results for BALB/c mice.

FIG. 9. Cytokine production in pulmonary leukocyte culture supernatants from *C. neoformans*-infected BALB/c, F1, and C57BL/6 mice at weeks 0, 2, and 3 postinfection. Isolated pulmonary leukocytes (5 × 10⁶/ml) were cultured for 24 h postharvest, and cytokines were evaluated by ELISA. Values represent means ± SEM in pg/ml supernatant (n = 6 and above for each of the analyzed time points). *, significant difference from results for F1 mice; **, significant difference from results for BALB/c mice.
designated CD40hi, CD80hi, and CD86hi, respectively. Figure 10 illustrates representative peak fluorescence intensity plots and summarizes average frequencies of CD40hi, CD80hi, and CD86hi cells in the gated CD11c+ MHCII+ population. Consistent with the robust Th1 response, BALB/c mice had the highest levels of all three maturation markers and significantly higher percentages of DC with strong costimulatory molecule expression compared to results for C57BL/6 mice. However, F1 mice exhibited an intermediate level of fluorescence intensity and percentage of all three markers, showing no significant difference from either parental strain. Thus, the level of co-stimulatory molecule expression by DC in LALN correlated with differences in Th1 and Th2 immune polarization exhibited in all three strains of mice, with F1 mice expressing an intermediate DC phenotype.

**DISCUSSION**

This study analyzed the inheritance pattern of susceptibility versus resistance to cryptococcal infection by the first-generation hybrid between resistant (BALB/c) and susceptible (C57BL/6) mice. Our data show the following: (i) F1 mice inherit intermediate resistance to C. neoformans but show a stronger resemblance to the immunophenotype of the resistant (BALB/c) mice; (ii) some of the Th1/Th2 polarization traits are inherited independently from each other in the F1 generation, but the clearance and dissemination patterns shows good correlation with the overall Th1/Th2 balance; (iii) early cytokine production in the lungs and the DC maturation/activation status in LALN play significant roles in predicting the Th1/Th2 bias of the immune response and the subsequent cryptococcal infections.
clearance; (iv) apart from DC in LALN, other cell types in the lungs, such as CD8 cells, may influence the immune bias and contribute to the inherited immunophenotype.

Differential patterns of resistance and susceptibility to \( C. \) neoformans infection in different mouse strains have been described previously (16, 41). Our parallel experiments carefully outlined the differences between C57BL/6 and BALB/c immunophenotypes over time and analyzed their inheritance by their first-generation progeny, F1 mice. C57BL/6 mice exhibited a susceptible phenotype marked by ineffective pulmonary clearance and systemic dissemination. Furthermore, they expressed numerous hallmarks of a Th2 response and severe Th2-driven lung pathology. In contrast, BALB/c mice progressively cleared \( C. \) neoformans 52D infection, did not develop severe lung pathology, demonstrated numerous hallmarks of a protective Th1 response, and were protected from central nervous system dissemination. The first-generation hybrid, F1 mice, unlike the C57BL/6 mice, maintained the ability to clear the infection from the lungs, but their clearance was slower than that of BALB/c mice. F1 mice also developed intermediate lung pathology, an intermediate phenotype in regard to early expression of Th1/Th2 immunity hallmarks in the lungs, and an intermediate systemic dissemination of \( C. \) neoformans. However, some of the observed traits in the immune response of F1 mice were not uniform in terms of similarities and differences from the parental strains during the course of the infection. For example, in terms of \( C. \) neoformans clearance from the lungs, F1 mice separated from C57BL/6 mice and BALB/c mice at the same time (week 3) and showed a significantly different cryptococcal lung burden from both parental strains at week 6. However, at weeks 4 to 5, pulmonary clearance was not significantly different from that for BALB/c mice. These outcomes suggest that clearance of the infection is not a linear process and is most likely driven by multiple factors over time.

\( C. \) neoformans affects mostly immunocompromised hosts, and differential recruitment of leukocytes to the lungs could explain a differential clearance phenotype. Interestingly, there was no positive correlation between the total numbers of pulmonary leukocytes and the rates of clearance exhibited by the three strains of mice. The F1 mice recruited on average the highest numbers of leukocytes at weeks 2 to 4 postinfection but exhibited an intermediate rate of clearance. On the other hand, BALB/c mice demonstrated the highest clearance rate while having the lowest number of inflammatory cells in their lungs throughout the study. Finally, C57BL/6 mice did not clear \( C. \) neoformans while recruiting intermediate numbers of leukocytes into their lungs (Fig. 1 and 3).

Subsequent analysis of the leukocyte subsets demonstrated two major differences between C57BL/6 mice and BALB/c mice. First, BALB/c mice recruited greater numbers of CD4\(^+\) cells into the lungs postinfection. Second, the cellular composition (pulmonary eosinophilia and macrophage phenotype in the infected lungs) reflected a differential Th1/Th2 immune bias in the parental strains. With respect to CD4\(^+\) cell numbers, F1 mice recruited numbers of CD4\(^+\) cells similar to those for BALB/c mice. However, CD4\(^+\) cell frequencies were similar between F1 and C57BL/6 mice and differed from those for BALB/c mice. Although, we cannot exclude that the lower CD4\(^+\) cell recruitment in C57BL/6 mice could contribute to the higher sensitivity of these mice to \( C. \) neoformans infection, immune polarization plays a critical role in the outcome of clearance in this otherwise immunocompetent mouse strain. This notion is supported by our previous report that IL-4 gene deletion from C57BL/6 mice results in the absence of a Th2 response and the improved clearance of cryptococcal infection (2).

Eosinophilia in the lungs is a marker of a Th2 response and is associated with the lack of \( C. \) neoformans clearance/pathology (4, 16, 33). We demonstrate that pulmonary eosinophilia in C57BL/6 mice is persistent throughout the course of infection (Table 1; Fig. 5A). In contrast, BALB/c mice had the fewest eosinophils and demonstrated the most effective cryptococcal clearance in the lungs. F1 mice did not show persistent eosinophilia, but they showed initial eosinophil recruitment at week 2 similar to that found in C57BL/6 mice. From week 3 on, eosinophilia in F1 mice subsided, concurrent with the beginning of the clearance process.

Another clue regarding the inheritance of anticycnotococcal resistance and its linkage with the immune bias comes from the morphological appearance of macrophages and the deposition of YM1/YM2 crystals (Fig. 6 and 7). Our group and others previously reported these distinct phenotypes of classically and alternatively activated macrophage (CAM and AAM) in Th1- and Th2-biased immune responses in \( C. \) neoformans-infected lungs (2, 29, 30). Consistent with these reports, an AAM phenotype predominated in C57BL/6 mice, along with the development of Th2-driven lung pathology (Fig. 7). In contrast, there was no morphological evidence of AAM in the lungs of infected BALB/c mice, which showed tight mononuclear cell infiltrates, characteristic for Th1 granulomas with classically activated macrophages. In the infected lungs of F1 mice, both types of macrophage morphologies were present (Fig. 7). This mixed macrophage phenotype in F1 mice corroborated with the development of an immune response that was protective but not as effective as the response in BALB/c mice.

The major hallmark of the polarized systemic Th2 response is an antibody class switch resulting in enhanced production of IgE (2, 6). As expected, C57BL/6 mice showed the most pronounced elevation in serum IgE (Fig. 5B). The IgE levels in infected F1 mice were also significantly elevated by week 5 postinfection compared with the uninfected and week 1 to 3 IgE levels. However, they were not significantly higher in F1 mice than in BALB/c mice, and they were significantly lower than the IgE levels in C57BL/6 mouse sera. This suggests that unlike C57BL/6 mice, F1 mice did not exhibit a significant shift to a systemic Th2 response.

Inheritance of the propensity for a Th1 versus Th2 bias of the immune responses was further demonstrated by the cytokine profile elicited by lung leukocytes in the parental strains and in the F1 mice. At week 1 postinfection, the pulmonary cytokine profiles in the parental strains were skewed in the directions of polarization subsequently seen in these mice. BALB/c mice had higher levels of Th1-driving cytokines (IFN-\(\gamma\), IL-12, and TNF-\(\alpha\)) than C57BL5 mice and a lower IL-4 level (Fig. 8). F1 mice demonstrated an intermediate Th1 cytokine profile, with an IL-12 level lower than that for BALB/c mice, an intermediate TNF-\(\alpha\) level, and an IFN-\(\gamma\) level that was higher for F1 mice than for C57BL/6 mice with a strong decreasing trend compared to results for BALB/c
mice. The IL-4 level was also intermediate for F1 mice, different from those for both C57BL/6 and BALB/c mice. The ratio between driving IL-4 and IFN-γ shows that the balance between the Th1/Th2 responses was significantly different and strongly in line with the resistance levels demonstrated by all three strains of mice (Fig. 1. 2. and 8). Interestingly, the proportions in the Th1/Th2 polarizing cytokine levels did not stay the same during the course of infection for F1 mice. IL-4 levels for F1 and BALB/c mice at weeks 2 and 3 were no longer different, which suggests that F1 mice did not become Th2 biased (Fig. 9). The decline in IL-4 production in F1 mice from week 2 postinfection explains the subsequent resolution of pulmonary eosinophilia, the lack of obvious hallmarks of AAM, and the absence of a significant elevation in serum IgE. On the other hand, the substantially lower IFN-γ levels and fluctuations in TNF-α levels can explain the lower and variable clearance rate in F1 mice. These data combined with previous reports further support the notion that the ability to induce early cytokines in response to C. neoformans is engendered by genetic factors (12, 17). Second, the expression of these early cytokine genes results in differential outcomes of the immune response polarization and anticyclococcal resistance in our model.

It remains unknown which underlying genetic differences are responsible for differential expression of early cytokines between BALB/c and C57BL/6 mice and their F1 progeny. We speculate that the cells of the innate immune system in C57BL/6 mice do not efficiently recognize some of the molecular patterns of C. neoformans and therefore induce an insufficient amount of early proinflammatory cytokines. These quantitative traits are most likely inherited by F1 mice in such way that their early response to C. neoformans is intermediate.

Dendritic cells are the main antigen-sensing and antigen-presenting cells of the immune system. Our previous studies have shown that the early cytokine signals, especially TNF-α priming, are required for optimal DC maturation and activation (12, 20, 22, 34, 38). These studies demonstrated that injection of immature DC pulsed with cryptococcal mannosprotein inhibits development of Th1 immunity and C. neoformans clearance in resistant mice (12, 20, 22, 34, 38) and that the overexpression of adenoviral TNF can prevent the development of Th2 and promote clearance in C57BL/6 mice (32). The present study further illustrates this relationship by showing that the maturation status of DC in draining lymph nodes correlates with the early cytokine profile and the effectiveness of cryptococcal clearance in all three strains of mice. F1 mice demonstrate an intermediate DC maturation phenotype in the regional nodes, which is consistent with the early cytokine profile—in particular, intermediate TNF-α production in F1 mice (Fig. 9 and 10). These data suggest a scenario of intermediate inheritance of early signals leading to a polarization in F1 mice and intermediate activation of DC that migrated into LALN. It is likely that this intermediate DC activation phenotype still present at week 3 postinfection in F1 mice was responsible for a longer period of uncommitted Th1/Th2 bias in these mice. The decrease in IL-4 production observed at week 3 postinfection occurs concurrently with the decline in eosinophil numbers in the lungs of F1 mice (Fig. 5A and 9). This drift in the F1 mouse immune polarization toward a weak Th1 response rather than the development of an intermediate Th0-like response could be a result of local interactions between the T cells recruited into the lungs from LALN within the local lung microenvironment. These effects were reported to play a role in the final development of T-cell effector functions during a pulmonary C. neoformans infection (26). Interestingly, we found a twofold increase in pulmonary CD8+ cells in F1 mice compared to results for either parental strain (Fig. 4), attributed to a first-generation hybrid effect (heterosis) (8–10). CD8+ cells can play a supportive role in generation of a Th1 milieu in the lungs and benefit cryptocooccal clearance (1, 25, 27, 37). Thus, the increased numbers of CD8+ cells could contribute to the gradual shift toward a Th1 phenotype in F1 mice and/or contribute to the improved clearance after week 3 postinfection.

In summary, we found that the susceptibility versus resistance to C. neoformans infection was inherited in a complex fashion and that it was linked to immune response polarization. Although the data presented in this study apply specifically to BALB/c and C57BL/6 parental mouse strains and their first-generation progeny, the mechanisms that contribute to these outcomes may be universally important for the inheritance of traits important for anticyclococcal immunity.

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