Enhanced Innate Immune Responsiveness to Pulmonary Cryptococcus neoformans Infection Is Associated with Resistance to Progressive Infection

Loïc Guillot,1 Scott F. Carroll,1,2 Robert Homer,4 and Salman T. Qureshi1,3*

Centre for the Study of Host Resistance, McGill University, Montreal, Canada1; Department of Human Genetics, McGill University, Montreal, Canada2; Department of Medicine, McGill University, Montreal, Canada3; and Yale University School of Medicine, New Haven, Connecticut4

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Genetically regulated mechanisms of host defense against Cryptococcus neoformans infection are not well understood. In this study, pulmonary infection with the moderately virulent C. neoformans strain 24067 was used to compare the host resistance phenotype of C57BL/6J with that of inbred mouse strain SJL/J. At 7 days or later after infection, C57BL/6J mice exhibited a significantly greater fungal burden in the lungs than SJL/J mice. Characterization of the pulmonary innate immune response at 3 h after cryptococcal infection revealed that resistant SJL/J mice exhibited significantly higher neutrophilia, with elevated levels of inflammatory cytokine tumor necrosis factor alpha (TNF-α) and keratinocyte-derived chemokine (KC)/CXCL1 in the airways, as well as increased whole-lung mRNA expression of chemokines KC/CXCL1, MIP-1α/CCL3, MIP-1β/CCL4, MIP-2/CCL2, and MCP-1/CCL2 and cytokines interleukin 1β (IL-1β) and IL-1Ra. At 7 and 14 days after infection, SJL/J mice maintained significantly higher levels of TNF-α and KC/CXCL1 in the airways and exhibited a Th1 response characterized by elevated levels of lung gamma interferon (IFN-γ) and IL-12/IL-23p40, while C57BL/6J mice exhibited Th2 immunity as defined by eosinophilia and IL-4 production. Alveolar and resident peritoneal macrophages from SJL/J mice also secreted significantly greater amounts of TNF-α and KC/CXCL1 following in vitro stimulation with C. neoformans. Intracellular signaling analysis demonstrated that TNF-α and KC/CXCL1 production was regulated by NF-κB and phosphatidylinositol 3 kinase in both strains; however, SJL/J macrophages exhibited heightened and prolonged activation in response to C. neoformans infection compared to that of C57BL/6J. Taken together, these data demonstrate that an enhanced innate immune response against pulmonary C. neoformans infection in SJL/J mice is associated with natural resistance to progressive infection.

Cryptococcus neoformans infection may cause severe and potentially life-threatening cases of pneumonia, meningitis, and disseminated disease in the immunocompromised host (36, 40). C. neoformans infection disease has been associated with several virulence factors including growth at 37°C (37), synthesis of a polysaccharide capsule (8), melanin (37), and mannitol (10), as well as the expression of urease (13), laccase (41), and extracellular phospholipase (12). Despite the organism’s potential to subvert a broad range of host defenses (36, 52), epidemiologic studies indicate that asymptomatic exposure or mild infection is much more common than severe cryptococcal disease (1, 19). Therefore, the progression and outcome of human cryptococcal infection appears to be determined, to a large extent, by the competence of the host immune response.

Significant insight into the immunopathogenesis of cryptococcal infection has been acquired from experimental animal models. The fundamental importance of T-lymphocyte-mediated immunity in the clearance of experimental pulmonary infection was demonstrated through the depletion of CD4+ and CD8+ T-lymphocyte subsets (34, 47), as well as by infection of congenitally lymphocyte-deficient SCID and athymic mutant mouse strains (7, 24, 35, 58). Additional studies have shown that Th1 polarization of the immune response is protective against C. neoformans infection (28, 49). Macrophages and pulmonary dendritic cells rapidly internalize C. neoformans organisms in murine models of pneumonia (17, 67); however, in vivo depletion of neutrophils unexpectedly enhanced the host defense (45). B-cell-mediated humoral immunity participates in host resistance against cryptococcal infection (2, 57, 65), although its protective efficacy depends on the genetic background of the host (56, 69, 70). A clear role for cytokines such as tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), interleukin 12 (IL-12), and IL-18, as well as chemokines such as monocyte chemoattractant protein-1 (MCP-1/C-C motif ligand 2 [CCL2]) and macrophage inflammatory protein-1-alpha (MIP-1α/CCL3) in leukocyte recruitment and effective host defense (33) has been demonstrated. Conversely, the microbial pattern recognition receptor Toll-like receptor 2 (TLR2) or the cytoplasmic adaptor molecule MyD88 may have a limited role in host resistance to C. neoformans challenge (5, 50, 68). Thus, a complex cellular and molecular network mediates an effective host immune response against C. neoformans infection.

Infection of inbred mouse strains with C. neoformans has revealed significant variations in host susceptibility that are regulated by genetic factors (11, 26, 55, 71). For example,
experimental intratracheal infection with 10^6 CFU of a moderately virulent serotype D isolate of C. neoformans revealed that C57BL/6J mice had natural susceptibility, whereas those of the BALB/c and CBA/J inbred strains did not (26). In this study, C57BL/6J mice developed an allergic bronchopulmonary mycosis (ABPM) characterized by chronic IL-5-dependent eosinophilia and intracellular, as well as extracellular, deposition of Charcot-Leyden-like crystals. Interestingly, C57BL/6J and CBA/J mice were equally susceptible to high-dose (10^6 CFU) intravenous infection with the same cryptococcal isolate, demonstrating that host resistance depends on the dose and route of infection (71). In another report that used an intravenous challenge with 5 x 10^6 CFU of a serotype D C. neoformans isolate, inbred strains lacking the fifth component of complement (C5) had a mean survival time of 4 days, compared to 13 days for C5-sufficient strains (55). Among the 16 inbred strains that were tested in this study, SJL/J mice had the longest mean survival time (mean ± standard error of the mean, 31.3 ± 2.0 days) (55).

Characterizing the genetic basis of host resistance is an important and proven approach to advancing the understanding of microbe-host interactions and disease pathogenesis (6, 64). Despite the importance of host factors in controlling the development and outcome of human cryptococcal disease and the clear evidence for substantial variations in host resistance shown by experimental mouse models of C. neoformans infection, the cellular and molecular bases of these traits remain largely undefined (11). In this study, we hypothesized that genetically controlled variation of the innate response to pulmonary cryptococcal infection is an important mechanism of host resistance. To test this possibility, we compared the early inflammatory response and pathogen burden of the resistant SJL/J mouse with that of susceptible C57BL/6J inbred mouse strains, using a clinically relevant lung infection model (29). Our findings demonstrate that the SJL/J strain generates a significantly stronger lung innate immune response than the innate immune response of C57BL/6J mice, following intratracheal C. neoformans challenge. The heightened innate response of SJL/J mice precedes and instructs the development of Th1 immunity in the lung that is associated with natural resistance to progressive pulmonary and extrapulmonary cryptococcal infection.

MATERIALS AND METHODS

Reagents and media. RPMI 1640 medium, L-glutamine, and penicillin-streptomycin were from Gibco (Grand Island, NY). Heat-inactivated fetal calf serum (FCS) was from HyClone (Logan, UT). Complete RPMI denotes RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS (vol/vol). [γ-32P]UTP was from Perkin-Elmer (Woodbridge, ON, CA). Protease and phosphatase inhibitors (leupeptin, aprotonin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, benzamidine, N-ethylmaleimide, and sodium orthovanadate) were from Sigma (Oakville, ON). The NF-κB inhibitor (BAY11-7082) was obtained from Calbiochem (La Jolla, CA). The phospho- and total-NF-κB p65, phospho- and total-Akt, and α-tubulin antibodies, as well as the phosphatidylinositol 3 kinase (PI3K) inhibitor (LY294002), were obtained from Cell Signaling technology (Beverly, MA).

Animals. Six-week-old male C57BL/6J and SJL/J mice were obtained from Harlan Laboratories (Indianapolis, IN) and maintained in our animal facilities under conditions specified by the Canadian Council on Animal Care. All protocols were reviewed and approved by the McGill University Animal Care Committee.

C. neoformans culture. C. neoformans 24067 (ATCC, Manassas, VA) was grown and maintained on Sabouraud dextrose agar (SDA) (BD, Sparks, MD) (26). For infection, a single colony suspension in Sabouraud dextrose broth (BD, Sparks, MD) was prepared and grown to early stationary phase (48 h) at room temperature with continuous rotation. The stationary culture was then washed with phosphate-buffered saline (PBS), counted on a hemacytometer, and diluted to the desired fungal concentration in sterile PBS. Confirmation of the fungus dose was done before and after experimental infection by plating a diluted aliquot on SDA and counting the CFU after 72 h of incubation at room temperature.

Intratracheal and intranasal administration of C. neoformans. Mice underwent intratracheal infection as described elsewhere (26). Briefly, mice were anesthetized by intraperitoneal injection of ketamine (10 mg/kg of body weight; Ayerst Veterinary Laboratories, Guelph, ON, Canada) and xylazine (125 mg/kg; Bayer Inc., Pittsburgh, PA) in 0.9% sterile saline. A small midline skin incision was made over the trachea, and the underlying tissue was retracted using a two-pronged blunt retractor (Fine Scientific Tools, North Vancouver, BC, Canada). The smooth muscle surrounding the trachea was then dissected to allow for the insertion of a 22-gauge catheter. A dose of 10^6 CFU of C. neoformans 24067 in a 50-µl volume (2 x 10^7 CFU/ml) was then administered through the catheter via a 1-ml tuberculin syringe (BD, Sparks, MD), followed immediately by 50 µl of air. The incision was closed using a 9-mm EZ Clip wound closing kit (Stoelting, Wood Dale, IL). For intranasal infection, mice were lightly anesthetized with 5% isoflurane and placed in a vertical position, and 10^6 CFU of C. neoformans in a 50-µl volume (2 x 10^7 CFU/ml) was administered via the nares.

Lung isolation and CFU assay. After mice were euthanized by anesthesia overdose, their infected lungs were excised and placed in 2 ml of sterile, ice-cold PBS. Individual lungs were then weighed and homogenized using an autoclaved glass tube and pestle attached to a tissue homogenizer (Glas-Col, Terre Haute, IN) at approximately 10 g. Organ homogenates were serially diluted using sterile PBS and plated in duplicate on SDA, and incubated at 37°C for 72 h prior to enumeration of C. neoformans CFU. To detect bacterial contamination or concomitant infection, each homogenate was simultaneously plated on Columbia blood agar and incubated for 72 h at 37°C with 5% CO2.

Collection of BALF. Mice were killed by CO2 exposure at various times, and bronchoalveolar lavage fluid (BALF) was collected from the airways four times with a total volume of 5 ml of ice-cold, sterile PBS. The total cell count for each BALF sample was determined with a hemacytometer, and differential cell counts were determined with 200 cells after cells were subjected to Cytospin centrifugation and staining with Diff-Quick (Dade Behring, Newark, DE). Each BALF supernatant was collected and stored at −20°C for subsequent quantification of cytokines and chemokines after it was centrifuged at 306 x g for 10 min at 4°C.

Histological analyses. Whole lungs were inflated to a fixed pressure of 25 cm of H2O with 10% buffered formalin acetate (Fischer Scientific, Fair Lawn, NJ), embedded in paraffin, and sectioned at 5-µm thicknesses. The sections were stained with periodic acid-Schiff (PAS) to identify mucus-secreting goblet cells. Slide image were captured with a coolSNAP-Pro cf digital capture kit (Media Cybernetics, Bethesda, MD) using an Olympus BX51 light microscope (Olympus Canada Inc., Markham, ON, Canada).

Bronchoalveolar lavage fluid isolation and stimulation. Naïve mice were killed by CO2 exposure, and resident alveolar macrophages were obtained by lung bronchoalveolar lavage with 5 ml (5 x 1 ml) of ice-cold, sterile PBS. Cells were centrifuged at 306 x g for 10 min at 4°C, followed by treatment with red blood cell lysis buffer that was quenched by the addition of 10 volumes of warm complete RPMI and incubated overnight until stimulation was performed. Resident peritoneal macrophages were obtained from individual mice by serial peritoneal lavages with 10 ml of ice-cold RPMI medium that were pooled and centrifuged at 306 x g for 10 min at 4°C. Peritoneal macrophages were subsequently prepared as described for alveolar macrophages. In both cases, adherent cells were stimulated after overnight incubation with various multiplicities of infection (MOI) of C. neoformans 24067. Cell supernatants were collected 24 h later, immediately frozen at −20°C, and subsequently assayed for cytokine and chemokine secretion. For pharmacologic inhibition experiments, macrophages were pretreated for 45 min with individual compounds and then stimulated for 24 h with C. neoformans.

Cytokine and chemokine measurements. Cell culture supernatants were assessed for mouse TNF-α and IL-6, using an OptEIA instrument (detection sensitivity, 15.6 pg/ml; BD Biosciences, San Diego, CA), IL-12/IL-23p40 (detection sensitivity, 62.5 pg/ml), keratinocyte-derived chemokine (KC)/CXCL1 (detection sensitivity, 15.6 pg/ml; IFN-γ (detection sensitivity, 15.6 pg/ml), and IL-4 (detection sensitivity, 15.6 pg/ml) concentrations were determined with a Duoset.
(R&D Systems, Minneapolis, MN) enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions.

**Immunoblotting**. Macrophage cell extracts were prepared from 5 × 10^6 cells and solubilized as described previously (21). An equal amount of protein (10 μg) from each sample was size separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and electrotransferred to a nitrocellulose membrane (Bio-Rad). Immunodetection was performed with antibodies specific for the total and phosphorylated forms of Akt and for the p65 subunit of NF-κB, as well as total α-tubulin. Bound antibodies were detected using an ECL-plus detection system (GE Healthcare/Amersham, Piscataway, NJ) according to the manufacturer’s instructions. Between successive probes, membranes were treated with Western restore blot stripping reagent (Pierce, Rockford, IL). Molecular masses were determined using the biotinylated calibration standards included in each gel (Cell Signaling Technology). Images were recorded with a Gene Genius bioimaging system (Syngene, Frederick, MD).

**RT-PCR**. Reverse transcription (RT) was performed with 1 μg of total RNA that had been extracted using an ABI high-capacity cDNA archive kit (ABI, Foster City, CA). PCR was performed for mouse IFN-γ, using the specific primers sense (5’-CAT TGA AAG CCT AGA AAG TCT G-3’) and antisense (5’-CTC ATG AAT GCA TCC TTT TTC G-3’) (43). Primers for the detection of the internal control house-actin-β-actin were sense (5’-GGT ACC ACC ATG TAC CCA GG-3’) and antisense (5’-ACA TCT GCT TGT GGA TGG TGG AC-3’) (amplon size, 163 bp). PCR amplifications were performed in a Peltier thermal cycler apparatus (MJ Research, Watertown, MA) with AmpliTaq polymerase (ABI, Foster City, CA). The thermocycling protocol performed in a Peltier thermal cycler apparatus (MJ Research, Watertown, MA) with AmpliTaq polymerase (ABI, Foster City, CA). The thermocycling protocol was 94°C for 1 min, followed by 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. Amplification products were resolved on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide and recorded with a Gene Genius bioimaging system.

**RNase protection assay (RPA)**. Total lung RNA was extracted using an RNeasy mini-kit (Qiagen, Mississauga), and the integrity of all samples was confirmed on a denaturing agarose gel. 32P-labeled riboprobes were synthesized with AmpliTaq polymerase (ABI, Foster City, CA). The thermocycling protocol was 94°C for 1 min, followed by 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. Amplification products were resolved on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide and recorded with a Gene Genius bioimaging system.

**RESULTS**

**Restriction of progressive pulmonary infection in SJL/J mice infected with C. neoformans**. To determine whether SJL/J mice exhibit heritable resistance to pulmonary cryptococcal infection, the lung fungal burdens of C57BL/6J and SJL/J mice were determined starting at day 3 after an intratracheal challenge with 10^4 CFU of a serotype D C. neoformans isolate. (A) Fungal burden in the lung was determined at 0, 3, 7, 14, and 28 days after infection. Each point represents the mean ± standard error of the mean of CFU (n = 5; *** P ≤ 0.001). Two of five SJL/J mice had cryptococcal infection of the lung (log CFU = 3.71 ± 0.26), while fungal growth was not observed with any of the five SJL/J mouse brains. C. neoformans-infected SJL/J lungs demonstrate enhanced inflammation without ABPM. The significant difference in pulmonary fungal burden between SJL/J and C57BL/6J mice throughout the duration of experimental infection raised the prospect that these two inbred strains develop distinct lung tissue pathology. To evaluate this possibility, formalin-fixed lung sections from each strain were prepared at days 0, 7, 14, and 28 following intratracheal infection (Fig. 1B). C57BL/6J mice had a significantly higher spleen fungal burden than SJL/J mice (log CFU = 2.82 ± 0.12 versus log CFU = 0.64 ± 0.21, respectively) (P ≤ 0.001). The specificity of this pathology was further demonstrated by PAS staining of SJL/J and C57BL/6J mouse lungs. C. neoformans-infected SJL/J lungs demonstrate enhanced inflammation without ABPM. The significant difference in pulmonary fungal burden between SJL/J and C57BL/6J mice throughout the duration of experimental infection raised the prospect that these two inbred strains develop distinct lung tissue pathology. To evaluate this possibility, formalin-fixed lung sections from each strain were prepared at days 0, 7, 14, and 28 following intratracheal infection (Fig. 1B). C57BL/6J mice had a significantly higher spleen fungal burden than SJL/J mice (log CFU = 2.82 ± 0.12 versus log CFU = 0.64 ± 0.21, respectively) (P ≤ 0.001). Two of five C57BL/6J mice also had cryptococcal infection of the brain (log CFU = 3.71 ± 0.26), while fungal growth was not observed with any of the five SJL/J mouse brains.
FIG. 2. Inflammatory response in the lungs of resistant and susceptible mice following intratracheal C. neoformans infection. Photomicroscopy of PAS-stained lung sections from individual C57BL/6J (A, C, E, and G) or SJL/J (B, D, F, H) inbred mice that were uninfected (A and B) or underwent intratracheal infection with $10^4$ CFU of a serotype D C. neoformans isolate for 7 (C and D), 14 (E and F), and 28 (G and H) days. Goblet cells and mucus stain dark pink (magnification, ×100). Each image is representative of $n = 3$ mice/group/time point.
TABLE 1. Airway cell recruitment following lung infection with C. neoformans

<table>
<thead>
<tr>
<th>Time</th>
<th>BALF cell</th>
<th>% of BALF cells ± SEM (relative to total)</th>
<th>C57BL/6J</th>
<th>SJL/J</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Uninfected</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Macrophage</td>
<td>97.89 ± 1.41</td>
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<td></td>
<td></td>
<td></td>
<td>PMN</td>
<td>2.11 ± 1.41</td>
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<td></td>
<td></td>
<td></td>
<td>Eosinophil</td>
<td>0</td>
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<td>Lymphocyte</td>
<td>0</td>
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<tr>
<td>3 h</td>
<td></td>
<td></td>
<td>Macrophage</td>
<td>92.13 ± 1.77</td>
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<td></td>
<td></td>
<td></td>
<td>PMN</td>
<td>7.53 ± 1.79</td>
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<td></td>
<td></td>
<td></td>
<td>Eosinophil</td>
<td>0.29 ± 0.12</td>
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<td></td>
<td></td>
<td>Lymphocyte</td>
<td>0</td>
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<td>24 h</td>
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<td>Macrophage</td>
<td>44.90 ± 4.25</td>
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<td></td>
<td></td>
<td></td>
<td>PMN</td>
<td>54.00 ± 3.76</td>
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<td></td>
<td></td>
<td></td>
<td>Eosinophil</td>
<td>1.15 ± 0.56</td>
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<td></td>
<td></td>
<td></td>
<td>Lymphocyte</td>
<td>0</td>
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<tr>
<td>7 days</td>
<td></td>
<td></td>
<td>Macrophage</td>
<td>18.06 ± 1.21</td>
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<td></td>
<td></td>
<td></td>
<td>PMN</td>
<td>57.95 ± 3.01</td>
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<td></td>
<td></td>
<td></td>
<td>Eosinophil</td>
<td>21.96 ± 2.84</td>
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<td></td>
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<td></td>
<td>Lymphocyte</td>
<td>2.04 ± 1.35</td>
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<tr>
<td>14 days</td>
<td></td>
<td></td>
<td>Macrophage</td>
<td>15.31 ± 3.17</td>
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<td></td>
<td></td>
<td></td>
<td>PMN</td>
<td>10.64 ± 2.67</td>
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<td></td>
<td></td>
<td></td>
<td>Eosinophil</td>
<td>70.96 ± 6.39</td>
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<td></td>
<td></td>
<td></td>
<td>Lymphocyte</td>
<td>3.09 ± 0.435</td>
</tr>
</tbody>
</table>

a Total and differential cell counts were obtained from the BALF of uninfected C57BL/6J and SJL/J mice at 3 h, 24 h, 7 days, and 14 days after mice were infected. BALF cell counts are reported as the percentage (mean ± standard error of the mean [SEM]) of total cells in the BALF (n = 4 mice/group/time point). PMN, polymorphonuclear leukocyte. **, P ≤ 0.01; ***, P ≤ 0.001.

experimental cryptococcal infection, in contrast to the well-described allergic bronchopulmonary response of C57BL/6J mice.

The resistant phenotype of SJL/J mice is associated with an increased proinflammatory innate host immune response that leads to a Th1-cell-polarized adaptive immune response. To determine whether the marked differences between the fungal burdens observed at 1 week postinfection for C57BL/6J mice and SJL/J mice were associated with heritable variations of the innate immune response, the lung inflammatory profiles of these two inbred strains were examined following an intranasal infection with 10⁶ CFU of a serotype D C. neoformans isolate. Cell counts are reported as the percentage (mean ± standard error of the mean [SEM]) of total cells in the BALF (n = 4 mice/group/time point). PMN, polymorphonuclear leukocyte. ***, P ≤ 0.01; ***, P ≤ 0.001.

C57BL/6J mice are well known to develop a Th2 pattern of lung adaptive immunity to C. neoformans, while relatively resistant strains generate a protective Th1 response. To determine whether the stronger early inflammatory response in SJL/J mice leads to a more robust Th1 pattern of adaptive immune response, we compared the profiles of cellular and soluble mediators in the airways of SJL/J with those of C57BL/6J mice at 7 and 14 days after infection (Table 1).

At day 7 after infection, SJL/J mice had significantly higher airway neutrophilia than C57BL/6J mice (83.91% ± 2.45% versus 57.95% ± 3.01%, respectively) (P ≤ 0.001). By day 14, this difference was even more pronounced: SJL/J mice had 67.77% ± 2.69% BALF neutrophils compared to 10.64% ± 3.67% BALF neutrophils for C57BL/6J mice (P ≤ 0.001). Conversely, at day 7, C57BL/6J mice had significantly higher airway eosinophilia than SJL/J mice (21.96% ± 2.84% versus 0.30% ± 0.30%, respectively) (P ≤ 0.01). At day 14, airway eosinophilia was not detectable in SJL/J mice, while eosinophils represented 70.96% ± 6.39% of the total cells in the airways of C57BL/6J mice. At day 14, SJL/J mice also had a significantly higher percentage of lymphocytes in the BALF than C57BL/6J mice (5.33% versus 3.09% ± 0.44%, respectively) (P ≤ 0.01).

To identify molecular signals that regulate early cell recruitment to the airways, the expression of mediators known to trigger rapid inflammatory responses was then characterized. Cytokine and chemokine analyses of the BALF at 3 h and 24 h postinfection demonstrated significantly higher levels of TNF-α and KC/CXCL1, but not IL-6, in the airways of SJL/J mice than in those of C57BL/6J mice (Fig. 3A, B, and C, respectively). To determine whether the increased response observed in the airways of SJL/J mice also correlated with inflammatory changes in lung tissue, quantitative mRNA expression of a panel of cytokines and chemokines was examined by qRT-PCR at 3 h after infection. Compared to uninfected mice, mice of both the C57BL/6J and SJL/J strains exhibited transcriptional upregulation for the cytokines TNF-α, IL-1α, IL-1β and IL-1Ra (see Fig. S1A in the supplemental material and Table 2) and the chemokines RANTES/CCL5, KC/CXCL1, MIP-1α/CCL3, MIP-1β/CCL4, MIP2/CXCL2, and MCP-1/CCL2 (see Fig. S1B in the supplemental material and Table 2) following the C. neoformans challenge. Relative to the expression levels determined for C57BL/6J mice, infected SJL/J mice exhibited a more than 3-fold increase in expression for all of these mediators except for RANTES/CCL5. Taken together, these results indicate that SJL/J mice naturally mount a stronger early airway and lung tissue inflammatory response to C. neoformans.

Significant differences in the BALF inflammatory cytokine and chemokine levels were also observed between C57BL/6J and SJL/J mice at days 7 and 14 after C. neoformans infection. Indeed, as observed for the early host response, SJL/J mice exhibited significantly higher levels of both TNF-α (Fig. 4A) and KC/CXCL1 (Fig. 4B) at both time points than C57BL/6J. In SJL/J mice, a progressive increase of the TNF-α level was observed from day 7 to day 14, while the highest level of KC/CXCL1 was observed at day 7. Finally, although there were no significant differences between the BALF IL-6 levels begins during the very early stages of the innate immune response in this strain (26).
of C57BL/6J mice and those of SJL/J mice at day 7 after infection, the level of IL-6 was significantly higher in SJL/J mice BALF at day 14 postinfection. ($P < 0.05$) (Fig. 4C).

To compare the adaptive immune response in the airways of SJL/J mice with that in the airways of C57BL/6J mice that had been infected with *C. neoformans*, classical markers of Th1 (IL-12, IFN-γ)- and Th2 (IL-4)-associated immunity were measured in the BALF. SJL/J mice produced increasing amounts of IFN-γ that were significantly higher at day 7 ($P \leq 0.01$) and day 14 ($P \leq 0.01$) than those of C57BL/6J mice (Fig. 4D). At 1 week after infection, IFN-γ mRNA was also detectable in the infected lung tissue of SJL/J mice but not in that of C57BL/6J mice (see Fig. 7G). The level of IL-12/IL23p40 (Fig. 4E) was also significantly higher in SJL/J airways at day 14 ($P < 0.05$). In contrast, IL-4 expression was detectable only in the airways of C57BL/6J mice (Fig. 4F). Collectively, these data demonstrate that C57BL/6J and SJL/J mice develop differential Th1/Th2 polarization of the adaptive immune response following *C. neoformans* infection.

Increased proinflammatory responses of SJL/J macrophages stimulated with *C. neoformans* infection are regulated by NF-κB and PI3K/Akt signaling pathways. To investigate the cellular mediators that underlie the differential innate immune responsiveness of C57BL/6J and SJL/J mice to *C. neoformans*, we studied macrophage responses following in vitro stimulation for 24 h. Differences in macrophage function have been related to susceptibility or resistance to cryptococcal infection (59). Alveolar macrophages from the resistant SJL/J inbred strain secreted significantly higher levels of TNF-α ($P \leq 0.001$) and KC/CXCL1 ($P \leq 0.001$) than that from the C57BL/6J strain (Fig. 5A and C, respectively). Peritoneal macrophages from SJL/J mice also secreted higher levels of TNF-α ($P \leq 0.001$) and KC/CXCL1 ($P \leq 0.001$) under the same conditions (Fig. 5B and D). Comparative kinetic analysis between the TNF-α production of the resistant strain and that of the susceptible inbred strain showed significantly increased production by SJL/J macrophages at 6 h ($P \leq 0.001$) and 24 h ($P \leq 0.01$) after stimulation (Fig. 6A).

To identify potential mechanisms that underlie the strain-dependent variations of macrophage cytokine and chemokine responses following *C. neoformans* stimulation, we analyzed intracellular signaling activity. Induction of the transcription factor NF-κB and activation of PI3K are both central processes during the innate immune response to infection (14, 22). In-

![FIG. 3. Early release of airway cytokines and chemokines following *C. neoformans* lung infection. The secretion of TNF-α (A), KC/CXCL1 (B), and IL-6 (C) in the BALF of C57BL/6J and SJL/J mice was measured at 3 h and 24 h after intranasal administration of $10^6$ CFU of a serotype D *C. neoformans* isolate. Each bar represents the mean ± standard error of the mean (n = 8 mice/group/time point; *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001).](image)

<table>
<thead>
<tr>
<th>Immune response</th>
<th>Mediator</th>
<th>Mean RPA signal intensity ± SD</th>
<th>Fold change SJL/J versus C57BL/6J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines</td>
<td></td>
<td>C57BL/6J plus PBS</td>
<td>C57BL/6J plus C. neoformans</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td>1.4 ± 1.6</td>
<td>4.5 ± 3.3</td>
</tr>
<tr>
<td>IL-1α</td>
<td></td>
<td>2.4 ± 3.2</td>
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</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td>0.1 ± 0.1</td>
<td>1.5 ± 1</td>
</tr>
<tr>
<td>IL-1Ra</td>
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<td>2.4 ± 2.8</td>
<td>3.8 ± 0.5</td>
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<tr>
<td>IL-18</td>
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<td>3.1 ± 3.9</td>
<td>5.2 ± 1.1</td>
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<tr>
<td>MIF</td>
<td></td>
<td>15.7 ± 5.1</td>
<td>18.3 ± 2.8</td>
</tr>
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<td>Chemokines</td>
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<tr>
<td>CCL5</td>
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<td>18.5 ± 5.6</td>
<td>46.4 ± 0.01</td>
</tr>
<tr>
<td>CXCL1</td>
<td></td>
<td>ND</td>
<td>17.0 ± 4</td>
</tr>
<tr>
<td>CCL4</td>
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<tr>
<td>CCL3</td>
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<td>CXCL2</td>
<td></td>
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</tr>
<tr>
<td>CCL2</td>
<td></td>
<td>ND</td>
<td>2.0 ± 2.6</td>
</tr>
</tbody>
</table>

* The intensity of the individual cytokine or chemokine band that was measured by RPA (see Fig. S1 in the supplemental material) was quantified using digital analysis software. Data expressed in the table are the means ± standard deviations (SD) of the signal obtained for each group. ND, not detected. The fold change value is the signal intensity of SJL/J (C. neoformans minus PBS)/C57BL/6J (C. neoformans minus PBS).
Involvement of NF-κB and PI3K in *C. neoformans* FcγR signaling has been shown in human microglial cells (61) and NK cells (66), yet the role of these pathways in *C. neoformans* infection-associated macrophage signaling has not been well studied. Therefore, as a first step in defining the role of these intermediates in the production of TNF-α, we treated resident peritoneal macrophages with pharmacologic inhibitors of NF-κB (BAY11-7082) and PI3K (LY294002) prior to stimulation with *C. neoformans* infection. Pretreatment with either of these compounds significantly reduced the *C. neoformans*-induced production of TNF-α in both C57BL/6J and SJL/J mouse macrophages (Fig. 6B and C).

To confirm the genetic regulation of differential macrophage NF-κB and PI3K signaling between resistant and susceptible mouse strains, we directly determined the activation of these pathways by specific immunoblotting techniques. Treatment of peritoneal macrophages from both strains with *C. neoformans* induced the phosphorylation of the serine/threonine kinase Akt, a downstream target of PI3K (Fig. 7A and B). Kinetic analysis showed that Akt phosphorylation was observable by 15
min after stimulation with *C. neoformans* in both the C57BL/6J and the SJL/J macrophages. Interestingly, the duration of Akt phosphorylation in the C57BL/6J macrophages was relatively brief, lasting for approximately 30 min, while in the SJL/J macrophages, Akt phosphorylation peaked at 1 h and remained visible at 5 h (Fig. 7A and B). For both strains, the total amount of Akt protein remained constant throughout the period of observation (Fig. 7A). At 30 min after *C. neoformans* stimulation, phosphorylation of the p65 subunit of NF-κB was detectable in both strains and appeared to be slightly higher in C57BL/6J macrophages (Fig. 7A and C); subsequent measurements at 1 h and 5 h after stimulation also demonstrated stronger NF-κB p65 phosphorylation in SJL/J macrophages. During this time, the total amount of NF-κB p65 subunit remained constant in both strains (Fig. 7A). Finally, alveolar macrophages were stimulated with *C. neoformans* to determine whether resident cells from the lung also exhibit a distinct strain-dependent signaling profile (Fig. 7D). Stimulation of C57BL/6J and SJL/J alveolar macrophages for 1 h resulted in the phosphorylation of Akt and NF-κB p65 in both strains. Consistent with the results obtained using peritoneal macrophages, alveolar macrophages from SJL/J mice exhibited stronger phosphorylation of both signaling molecules than those obtained from the C57BL/6J inbred strain.

**DISCUSSION**

The host immune response is a major determinant of the outcome of cryptococcal infection; however, the underlying basis for the differences in susceptibility is poorly understood (11, 71). To elucidate the mechanisms of host resistance against *C. neoformans* infection, we have compared the innate and adaptive pulmonary responses using C57BL/6J and SJL/J mice, two strains that have been reported to be susceptible and resistant to experimental pulmonary (26) and systemic (55) cryptococcal infection, respectively. The major findings of our study include the following: (i) SJL/J mice are highly resistant to pulmonary and extrapulmonary *C. neoformans* infection compared to the C57BL/6J inbred strain; (ii) SJL/J mice mount a significantly stronger inflammatory response in the airways,
as well as in lung tissue, than C57BL/6J mice within 3 h of administration and maintain this differential response at days 7 and 14 after infection; and (iii) alveolar and peritoneal macrophages from SJL/J mice exhibit heightened responsiveness to in vitro stimulation with \textit{C. neoformans} infection through differential activation of intracellular signaling cascades. Collectively, these observations clearly demonstrate that genetically regulated innate immune responsiveness in the murine lung is associated with host resistance to progressive cryptococcal infection.

In this report, a moderately virulent clinical isolate of \textit{C. neoformans} was chosen for administration via the mouse respiratory tract to model the development of human cryptococcal infection. Under these conditions, SJL/J mice were naturally resistant to progressive pulmonary infection as well as to dissemination to the spleen and brain compared to C57BL/6J mice. Consistent with previous studies of inbred mouse strains, SJL/J mice developed a Th1 response characterized by significantly higher IL-12 and IFN-\(\gamma\) expression, while susceptible C57BL/6J mice had increased IL-4 expression in the BALF, indicative of Th2 polarization. Previously, SJL/J mice were also shown to be resistant to \textit{C. neoformans} infection, using a very-high-dose (5 \times 10^6 CFU) intravenous challenge model with a mean survival time of 31.3 \pm 2.0 days (55). In that report, resistance and susceptibility were linked to the \(Hc\) locus on chromosome 2 that encodes the wild-type (\(Hc^1\)) and the mutated (\(Hc^0\)) alleles of the fifth component of complement (C5), respectively (54, 55). Although SJL/J male mice have been reported to have a significantly higher serum C5 level than other strains (42), it is important to note that the C57BL/6J mice used in this study also carry the wild-type \(Hc^1\) allele and are C5 sufficient. The highly resistant phenotype of SJL/J mice strongly suggests that genetic factors, in addition to a potential contribution from C5, control host defense against pulmonary \textit{C. neoformans} infection.

It is well established that a Th1 pattern of adaptive immunity is protective against progressive cryptococcal infection (27). Adaptive immunity requires clonal expansion of lymphocytes,
a process that typically develops by 7 days in the naïve host. In the current study, the fungal burden of previously unexposed mice began to diverge at 3 days after infection, implicating innate immune mechanisms in differential host resistance to pulmonary cryptococcal infection. To characterize the initial events that precede and instruct the development of a protective immune response against *C. neoformans* infection, we compared the expression levels of the inflammatory cytokine TNF-α and the neutrophil chemokine KC/CXCL1 in the airways of C57BL/6J mice with those of SJL/J mice at 3 h after pulmonary cryptococcal infection and demonstrated a significant elevation of both proteins in the resistant SJL/J mice. This finding is consistent with a previous study that described an essential role for TNF-α during the afferent phase of the immune response to *C. neoformans* infection (32). More recently, intratracheal delivery of a TNF-α-expressing adenoviral vector switched the natural allergic bronchopulmonary/Th2 response of C57BL/6J mice to *C. neoformans* toward Th1, resulting in the control of infection (46). Therefore, it is likely that the higher TNF-α protein expression during the innate immune response to *C. neoformans* infection contributes to the naturally resistant SJL/J phenotype. On the other hand, the precise role of the neutrophil chemokine KC/CXCL1 in host defense against cryptococcal infection is less well characterized. Recent reports have shown that both *C. neoformans* stimulation of human bronchial epithelial cells in vitro and adenoviral overexpression of TNF-α in the mouse lung induce the expression of KC/CXCL1 (20, 46); however, further studies will be required to determine its contribution to host defense.

IL-1β and IL-Rα were also differentially upregulated in SJL/J mouse lungs 3 h after *C. neoformans* infection. Cryptococcal capsular polysaccharide has been shown to induce IL-1β release by human neutrophils (53), and a recent report described a functional defect of TNF-α, IL-1β, and nitric oxide production by monocytes and neutrophils in an apparently immunocompetent patient with pulmonary cryptococcosis (44). Together, these observations suggest a potential role for the IL-1β-IL-Ra axis in the host innate immune response against *C. neoformans* infection that also warrants further investigation.

Three hours after they received *C. neoformans* infection, differential upregulation of the chemokines MIP-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL2, and MCP-1/CCL2 was demonstrable in the lungs of C57BL/6J and SJL/J mouse strains, while RANTES/CCL5 was upregulated in both strains. Chemokines play an important role during the innate immune response to cryptococcal infection by recruiting and activating cells and influencing subsequent adaptive immunity. Crucial roles for MCP-1/CCL2 and its receptor CCR2 in the development of pulmonary Th1 immunity and cryptococcal clearance have been reported (31, 33, 62, 63). Similarly, depletion of MIP-1α/CCL3 during the efferent phase of pulmonary cell-mediated immunity reduced macrophage/monocyte and neutrophil recruitment and resulted in a threefold higher burden of the moderately virulent *C. neoformans* isolate in the lung (30), while infection of CCL3 knockout mice with a highly virulent cryptococcal strain resulted in a Th2 phenotype with dramatically decreased survival at 12 weeks (51). The specific contributions of MIP-1β/CCL4 and MIP-2/CXCL2 to cryptococcal host defense have not been reported; however, based on existing data, it appears that multiple chemokines are required to orchestrate an effective Th1 response against pulmonary cryptococcal infection. Therefore, it seems very likely that the rapid and heightened innate chemokine response in the SJL/J inbred strain also contributes to a resistant phenotype.

Consistent with differential chemokine expression levels following *C. neoformans* infection, resistant SJL/J mice also exhibited significantly greater airway neutrophilia than C57BL/6J mice at 3 h after challenge. Neutrophils are important in host defense against infection by certain fungi including *Candida* spp. and *Aspergillus* spp.; however, their contribution to host resistance against cryptococci infection is uncertain. Human neutrophils have been shown to kill cryptococci in vitro through oxidative and nonoxidative mechanisms (10, 15, 39), yet, somewhat unexpectedly, transient depletion of neutrophils in BALB/c mice, using a monoclonal antibody, enhanced survival following a high-dose (5 × 10⁶ CFU) cryptococcal challenge without altering the fungal burden, possibly through modulation of the adaptive immune response (45). Due to the important differences between the experimental designs in that study and the current investigation, a potential contribution of neutrophils to the resistant SJL/J phenotype described here cannot be excluded, particularly since differential neutrophilia were also observed at 7 and 14 days after infection in association with a progressive decline in the fungal burden. In contrast, C57BL/6J mice developed a mild eosinophilia during the first 24 h after cryptococcal infection that progressively increased during the phase of adaptive immunity. C57BL/6J mice are known to develop an ABPM associated with chronic cryptococcal infection (4, 23, 26). The rapid development of airway eosinophilia in C57BL/6J mice exposed to *C. neoformans* points to uncharacterized innate mechanisms of eosinophil recruitment to the lung that precede the development of ABPM. It is possible that the rapid recruitment of eosinophils causes significant lung tissue destruction that may contribute to chronic fungal infection in C57BL/6J mice.

To establish a cellular mechanism for the differential innate immune responsiveness between C57BL/6J and SJL/J mice, analysis of macrophage activation was performed. Resident peritoneal and alveolar macrophages from SJL/J mice secreted significantly greater TNF-α and KC/CXCL1 than C57BL/6J mice, following in vitro stimulation with *C. neoformans*. Macrophages play an important role in host defense against cryptococci through various mechanisms including phagocytosis, killing, cytokine and chemokine production, and antigen presentation (38). During chronic pulmonary infection in mice, *C. neoformans* may survive as a facultative intracellular pathogen in alveolar macrophages and may cause cytotoxicity and disruption in association with intracellular polysaccharide production (17, 18). Consistent with the finding that human monocyte-derived macrophages express cellular receptors including TLR4 (60), CD14 (68), and CD18 (16) that are involved in the uptake of GXM (48), no specific opsonin or priming stimulus was required to induce cytokine and chemokine secretion in mouse macrophages. Both NF-κB and PI3K/Akt are well-recognized elements of the innate immune response against infection (14, 22). TLR4 stimulation by cryptococcal GXM has been shown to activate NF-κB (60), and PI3K/Akt has been implicated in the killing of *C. neoformans* organisms by human NK cells (66). Therefore, pharmacologic inhibitors and immu-
n blotting were used to study and confirm the differential activation of NF-xB and PI3K/Akt in SJL/J macrophages following cryptococcal stimulation. As relatively little is known about the signaling pathways that are activated by cryptococci, identification of the receptors and mechanisms of macrophage activation by cryptococci during the innate immune response is an important area for future investigation (9).

In summary, natural variations between the host resistance to cryptococcal infection of the C57BL/6J inbred mouse and that of the SJL/J inbred mouse strain are associated with the differential regulation of immune responsiveness in the airways, lung tissue, and macrophages that is demonstrable within hours of infection. Heightened pulmonary innate immunity precedes and instructs the development of definitive Th1 adaptive responses that lead to a progressive reduction in fungal burden.

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