An Innate Immune System Cell Is a Major Determinant of Species-Related Susceptibility Differences to Fungal Pneumonia

Xiuping Shao, Aron Mednick, Mauricio Alvarez, Nico van Rooijen, Arturo Casadevall and David L. Goldman

*J Immunol* 2005; 175:3244-3251; doi: 10.4049/jimmunol.175.5.3244
http://www.jimmunol.org/content/175/5/3244

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2005/08/23/175.5.3244.DC1

**References**
This article cites 34 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/175/5/3244.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
An Innate Immune System Cell Is a Major Determinant of Species-Related Susceptibility Differences to Fungal Pneumonia

Xiuping Shao,* Aron Mednick,† Mauricio Alvarez,‡ Nico van Rooijen,§ Arturo Casadevall,†‡ and David L. Goldman2*†§

Rats and mice are considered resistant and susceptible hosts, respectively, for experimental cryptococcosis. For both species, alveolar macrophages (AM) are central components of the host response to pulmonary *Cryptococcus neoformans* infection. We explored the role of AM in three strains of mice and three strains of rats during cryptococcal infection by comparing the outcome of infection after macrophage depletion using liposomal clodronate. AM depletion was associated with enhancement and amelioration of disease in rats and mice, respectively, as measured by lung fungal burden. The apparent protective role for AM in rats correlated with enhanced anticycrococcal activity as measured by phagocytic activity, oxidative burst, lysozyme secretion, and ability to limit intracellular growth of *C. neoformans*. Furthermore, rat AM were more resistant to lysis in association with intracellular infection. In summary, differences in AM function in rats and mice suggest an explanation for the species differences in susceptibility to *C. neoformans* based on the inherent efficacy of a central effector cell of the innate immune system. The Journal of Immunology, 2005, 175: 3244–3251.

Alveolar macrophages (AM) are believed to be of central importance for the initial host response to pulmonary cryptococcal infection by virtue of the fact that they constitute the major resident phagocytic cell in the lung. Consistent with this belief, histological studies reveal an intimate association between AM and yeast cells in mice, rats, and humans (1–3). Potential functions of AM in the host response to *Cryptococcus neoformans* include phagocytosis, killing, polysaccharide sequestration, cytokine production, chemokine production, and Ag presentation (reviewed in Ref. 4). Despite considerable circumstantial evidence of the importance of macrophages in host defense, their contribution has not been experimentally tested. In fact, recent studies suggest that when the role of macrophages is examined experimentally, the results vary with the pathogen and are often unexpected. For example, macrophage depletion enhances resistance of mice to *Mycobacterium tuberculosis* (5), but increases susceptibility to *Streptococcus pyogenes* (6) and has no effect on susceptibility to *Pseudomonas aeruginosa* (7).

AM from mice, rats, and humans are able to ingest *C. neoformans* (8–10). Optimal phagocytosis of encapsulated *C. neoformans* by AM occurs in the presence of opsonin (either serum or Ab) and may be associated with macrophage-mediated killing of *C. neoformans*. However, ingestion may also be accompanied by intracellular fungal replication in vitro and in vivo (11). In vitro experiments suggest that the activation state of macrophages is an important determinant of their anticycrococcal activity. Thus, in the appropriate context, AM may limit the early growth of *C. neoformans* and prevent extrapulmonary dissemination. Alternatively, AM may serve as a site for evasion from the normal host response and promote *C. neoformans* replication.

Both rats and mice have been used to study the pathogenesis of pulmonary cryptococcosis. Nevertheless, these two species differ widely in their susceptibility to pulmonary cryptococcal infection. Rats are significantly more resistant to pulmonary cryptococcosis than mice and typically develop localized subclinical pulmonary infection (2). To better understand these discrepancies in host susceptibility as they relate to macrophage function, we compared the effects of AM depletion on pulmonary cryptococcal infection using liposome clodronate. Additional studies analyzed host species differences in anticycrococcal activity, including phagocytic activity, ability to limit intracellular *C. neoformans* growth, and susceptibility to *C. neoformans*-induced lysis. The results indicate that functional differences between mouse and rat macrophages may have profound consequences on the outcome of cryptococcosis in these animals.

Materials and Methods

Animals

Male Fischer, Sprague-Dawley, and female Brown Norway (BN) rats, weighing 200–250 g, were obtained from Harlan Sprague-Dawley. This weight corresponds to an age of 6 to 11 wk depending on the rat strain. Six- to 8-wk-old female BALB/c and A/J mice were obtained from the National Cancer Institute. C57BL/6 mice were obtained from The Jackson Laboratory. Animals were cared for in accordance with the institutional animal care and use committee of Albert Einstein College of Medicine of Yeshiva University.

### Abbreviations

- CL-1BP, dichloromethylene bisphosphonate
- IL-1RA, IL-1R antagonist
- TEM, transmission electron microscopy
- GXM, glucuronoxylomannan
- BN, Brown Norway
- CL-1BP, dichloromethylene bisphosphonate
- IL-1RA, IL-1R antagonist
- TEM, transmission electron microscopy
- GXM, glucuronoxylomannan

<ref>Received for publication March 4, 2005. Accepted for publication June 15, 2005.</ref>
C. neoformans

For animal studies, American Type Culture Collection strain 24067 was used. This is a serotype D strain that has been studied extensively studied in mouse and rat models of cryptococcosis (2, 12). This strain, also known as 52D, has been used in other laboratories that study the pathogenesis of pulmonary infection (13). The organism was grown in Sabouraud’s dex-
trose broth (BD Biosciences) at 30°C for 3 days, then washed three times with PBS, pH 7.4, and suspended in PBS for infection. For in vitro exper-
imentation, two strains of C. neoformans were studied, H99 (a serotype A strain) and 24067.

AM depletion

Dichloromethylene bisphosphonate (CL₃MBP) or clodronate was a gift from Roche and was encapsulated in liposomes as described previously (14). CL₃MBP selectively depletes AM after intratracheal instillation (14). Of note, intratracheal administration of liposomal clodronate does not ap-
ppear to affect lung dendritic cell density (15, 16). Nevertheless, variable effects (depletion, partial depletion, and nondepletion) on splenic dendritic cells after i.v. administration of liposomal clodronate have been reported (17–19). To confirm the adequacy of AM depletion, rats, five per group, were given 0.8 ml of PBS liposomes or CL₃MBP liposomes intratrache-
ally. Three days later, lungs were removed and frozen. Lung sections were given for the presence of AM using an FITC-conjugated, Ab (ED1; Se-

Histology and immunohistochemistry

For rats, right lungs were immersed in O.C.T. compound (Sakura Fineteck), snap-frozen, cut into 5-μm-thick sections, and fixed in methanol. Sections were stained with H&E. For gluconoxylomannan (GXM) immunohisto-
chemistry, sections were treated with 5% H₂O₂ for 30 min, followed by 10% goat serum in PBS for 1 h. The primary Ab was a murine IgG1, 18B7, at a concentration of 10 μg/ml (21). Primary Ab was detected by peroxi-
dase-conjugated, goat anti-mouse isotype-specific IgG (Southern Biotech-

Cytokine/chemokine measurements

Lung homogenates were centrifuged, and supernatants were frozen at −20°C. Levels of IL-4, IL-10, IL-1β, IL-1R antagonist (IL-1RA), TNF-α, IFN-γ, TGF-β1, MIP-2, and MCP-1 were measured in rat lung homoge-

Bronchoalveolar lavage

Rats and mice were killed by asphyxiation with CO₂, and their tracheas were cannulated with angiocaths (BD Biosciences). Lungs were lavaged (five times for rats and 10 times for mice) with sterile HBSS without phenol red (Invitrogen Life Technologies) with 1 mM EGTA (Sigma-

Cell size

Cells were lavaged from Fischer rats and BALB/c mice, suspended at 3 × 10⁶/ml, and subjected to centrifugation using a cyto spun (Thermo Shandon) at 800 rpm for 6 min. Cells were fixed with ice-cold methanol and stained using a Wright stain. Approximately 150–200 cells/slide were counted under ×1000 magnification. Pictures were taken with a Q Imaging Retiga 1300 digital camera using the Q Capture Suite version 2.46 software (Q Imaging Burnaby). The longest diameter of cells was determined in pixels and then converted into microns using a standard grid of known size. Pic-
tures were processed in Adobe Photoshop 7.0 for Windows.

Opsonins

Both sera and Ab against the cryptococcal polysaccharide were used as opsonins. To obtain sera, animals were bled immediately before lavage. Sera were stored at −4°C and used within 3–4 h of being obtained. For Ab studies, the murine IgG1, 18B7, was used (21).

Phagocytosis assay

AM were obtained from Fischer rats and BALB/c mice. Cells were plated in 96-well tissue culture plates (Costar) at a density of 2.5 × 10⁵/well in 0.2 ml of DMEM-10% FCS and allowed to attach at 37°C. Medium was re-

Transmission electron microscopy (TEM)

AM were harvested from Fischer rats and BALB/c mice. Approximately 1 × 10⁶ cells/well were placed in a six-well plate. Cells were incubated with C. neoformans (1 × 10⁶ organisms/well) in the presence of mAb 18B7 (10 μg/ml) for 2 h. The supernatant was then removed, and cells were fixed with 2.5% glutaraldehyde and stained with Giemsa. The total number of internalized C. neoformans per total number of macrophages was calculated. Approximately 200–300 AM/well were counted.

Intracellular growth

AM from Fischer rats and BALB/c mice were allowed to phagocytize C. neoformans in the presence of mAb 18B7 or serum (see phagocytosis as-

The Journal of Immunology 3245

Downloaded from www.jimmunol.org by guest on April 2, 2017
at 4 h (to remove noninternalized organisms), and cell cultures were returned to the incubator for an additional 20 h. In separate experiments, both H99 and 24067 strains of C. neoformans were studied.

**Imaging of fungal-macrophage interaction**

Visualization of live interactions between C. neoformans (American Type Culture Collection 24067) and primary macrophages was performed with an Olympus IX70 microscope with a ×40 UPlanFL N.A. 0.5 phase 1 objective with an N.A. 0.5 condenser. The halogen lamp was shuttered for each exposure with a Uniblitz shutter (Vincent Associates). The microscope was housed in a Plexiglas box, and temperature was stabilized at 37°C with a forced air heater system. Additionally, 5% CO2 bubbled through water was delivered to a chamber locally at the culture dish. Images were collected with a Cooke Sensicam HQ run by I. P. Lab (Scana-lytics) on a Dell PC with Windows XP. Images for the intracellular replication were collected at 1-min intervals with the ×40 objective. Animations were created using ImageJ software (W. S. Rasband, National Institutes of Health, Bethesda, MD).

**Nitrogen and oxygen free radical production**

AM were obtained by lavage from both Fischer rats and BALB/c mice. To measure oxygen free radical production, luminolucence studies were performed. Briefly, cells were washed with HBSS and resuspended at a density of 5 × 10^6/ml in HBSS containing 50 μM Luminol (sodium salt; Sigma-Aldrich) and C. neoformans (2.5 × 10^6/ml) opsonized with 18B7 (10 μg/ml). Cell suspensions were placed in a luminometer (Monolight 2010; Analytical Luminescence Laboratory). Measurements were obtained with the time of adding opsonized organisms (time zero) and every 10 min for 1 h. Luminolucence readings minus the readings obtained from macrophages in Luminol solution alone were determined. Cultures were performed in triplicate. To assay for nitrogen free radical production, nitrite concentrations in culture supernatants were determined as previously described (22). Approximately 4 × 10^6 AM from Fischer and BALB/c mice were placed in DMEM and then incubated overnight at 37°C. The following day, opsonized C. neoformans (American Type Culture Collection 24067) were added to cultures. At various times (48, 72, and 96 h), supernatants were tested for the presence of nitrite using the Greiss reagent.

**Lysozyme production**

Approximately 1 × 10^6 primary rat and mouse AM were seeded into 96-well plates and allowed to adhere. Medium was then replaced with medium containing mAb 18B7 and C. neoformans (1 × 10^5 cells/well). Supernatants were removed at 5 h and tested for lysozyme using Micrococcus luteus as previously described (23). Briefly, 40 μl of a 0.05% suspension of bacteria in 0.1 M potassium phosphate buffer (pH 6.2) was added to 200 μl of supernatant or lysozyme standard (Roche) and incubated at 37°C for 30 min. Absorbance at 450 nm was then determined.

**Statistics**

For single comparisons between groups, Student’s t test was performed. For multiple comparisons between single groups, a one-way ANOVA was performed. For post-hoc analysis, data were compared using Dunnett’s test if multiple comparisons against a control were made. Otherwise, the Student-Newman-Keuls test was used. A value of p < 0.05 was considered significant. Statistics were calculated using SPSS Base 10.

**Results**

**Effect of macrophage depletion on the outcome of rat and mouse C. neoformans infection**

AM-depleted Fischer rats infected with C. neoformans had 3- to 6-fold higher lung fungal burden at all times tested (i.e., days, 3, 14, and 28; Fig. 1A) compared with normal rats. Spleen fungal burden was also increased on day 3 of infection in AM-depleted animals compared with controls, consistent with increased extrapulmonary dissemination (not shown). For both Sprague-Dawley and BN rats, increases in lung fungal burden (2.5- and 11-fold, respectively) were observed in association with AM depletion on day 3 of infection (the only observation time; Fig. 1, B and C). AM depletion also resulted in an increase in fungal burden for Sprague-Dawley rats infected with a lower inoculum (10^5) of C. neoformans. In these experiments, lung fungal burden for AM-depleted and nondepleted animals were 255 ± 93 × 10^4 vs 23 ± 9 × 10^4, respectively (p < 0.01). For A/J mice, lung fungal burden was similar for AM-depleted and control animals on day 3 (1.36 ± 0.32 × 10^6 vs 1.49 ± 0.74 × 10^6; Fig. 1D), but was ~2-fold lower in AM-depleted animals on day 14 (7.9 ± 0.37 × 10^7 vs 17.0 ± 0.57 × 10^6; p < 0.001). For BALB/c mice, AM depletion was associated with a decrease in lung fungal burden at both 3 days (~2.8-fold; p = 0.002) and 14 days (~4-fold; p < 0.001; Fig. 1E). For C57/BL6 mice, AM depletion was associated with an ~13-fold decrease in lung fungal burden on day 3 (the only observation time; p = 0.003; Fig. 1F). In summary, AM depletion in all three rat strains tested resulted in increased lung fungal burden, whereas AM depletion in all three mouse strains resulted in either no change or a decrease in lung fungal burden.

In the absence of infection, AM depletion was associated with a slight increase in lung inflammation. For Fischer rats, this consisted of scattered inflammatory cells (both multinuclear and mononuclear) in areas surrounding blood vessels (not shown). A similar, although less prominent, pattern was seen for A/J mice. For Fischer rats that were subsequently infected with C. neoformans, AM depletion was associated with significantly more inflammation compared with PBS-treated rats at all times studied (see Fig. 2). Inflammation in AM-depleted Fischer rats was greatest on day 28, at which time relatively few free alveolar air spaces were apparent. At 28 days of infection, lungs from AM-depleted Fischer rats exhibited areas of caseation, with many inflammatory cells exhibiting nuclear condensation and fragmentation consistent with apoptosis. More C. neoformans were present in the lungs of AM-depleted rats. For BN rats, significantly less lung inflammation was present compared with Fischer rats on day 3 of infection.
Increased inflammation is present in AM-depleted animals (AM−). At all times of infection, lungs of rats given clodronate had more inflammation than lungs of PBS-treated rats. Many infiltrating lymphocytes (white arrow) were seen on day 14 (D) in AM-depleted rats. Caseation (white arrow) was also more prominent in AM-depleted rats on day 28 (F) of infection. In addition, increased number of organisms (black arrows) were seen in lungs of AM-depleted animals. Black arrows point to *C. neoformans*. H&E staining; original magnification, ×100, except B and E, where original magnification was ×200.

Nevertheless, the same overall trend was present (i.e., increased number of inflammatory cells in AM-depleted animals; not shown).

For A/J mice that were infected with *C. neoformans*, increased inflammation was observed in association with AM depletion. On day 14, large areas of confluent granulomatous inflammation with infiltrating eosinophils were present in the lungs AM-depleted A/J mice (Fig. 3). Fewer *C. neoformans* were present in the lungs of AM-depleted mice. For both BALB/c and C57 BL/6 mice (not shown), an increase in inflammation was observed in association with AM depletion. In contrast to A/J mice, no eosinophil infiltration was detected in BALB/c and CD57 BL/6 mice regardless of AM depletion.

*C. neoformans* infection is known to be associated with the deposition of GXM in infected tissues (24). GXM staining was performed for both Fischer rats and BALB/c mice. Using this approach, *C. neoformans* were detected inside cells that appeared to be macrophages on day 3 of infection in both rats and mice treated with PBS (Fig. 4, A and C). For Fischer rats, an increase in GXM immunoreactivity was present within lungs in association with AM depletion (Fig. 4B) at all times studied. Some of this reactivity was organism-associated and reflected the increase in lung fungal burden detected by CFU assays (see above). For BALB/c mice, GXM staining revealed a decrease in immunoreactivity in association with AM depletion relative to animals that received PBS. Again, this finding was consistent with CFU assays (Fig. 4D).

**Cytokine response in the presence and the absence of AM**

For Fischer rats, we measured the lung levels of IFN-γ, IL-1β, IL-1RA, IL-4, IL-10, TNF-α, and TGF-β1. We also measured levels of MCP-1 (CCL2) and MIP-2 (CXCL2). IL-1β and IL-RA lung levels increased over the course of infection for both AM-depleted and control rats, with maximal levels on day 28 (see Table I, Fisher rat cytokine and chemokine levels). Compared with controls, AM-depleted animals exhibited lower levels of IL-1β on day 28 and higher levels of IL-1RA on both days 3 and 28 of infection. IL-10 levels for AM-depleted rats were lower on day 3, but higher on day 14 compared with controls. IFN-γ and TNF-α increased over the course of infection for both AM-depleted and control rats, with maximal levels on day 28. Nevertheless, no differences in IFN-γ and TNF-α levels were observed between AM-depleted and control groups throughout the infection.

**FIGURE 4.** Staining for GXM (brown) in lungs of Fischer rats (A and B) and BALB/c mice (C and D) on day 3 of infection. A, Scattered organisms (brown) are seen throughout the lungs of an infected Fischer rat, treated with liposome PBS (AM+). The inset shows a high magnification view of a highly phagocytic cell that appears to be an AM. In contrast, the lungs of rats treated with liposome clodronate (AM−; B) contains a large number of extracellular organisms. Lungs from BALB/c-infected mice treated with liposome PBS (AM+) contain many extracellular *C. neoformans* (C). Inset. A phagocytic cell with intracellular and surrounding extracellular organisms. Fewer organisms were present in the lungs of AM− mice (D). All images were obtained at an original magnification of ×200. Tissues were counterstained with hematoxylin.
control rats. No differences in IL-4 (not shown) and TGF-β1 levels were detected between AM-depleted and control rats. Both MCP-1 and MCP-2 levels were generally higher in AM-depleted animals compared with controls.

For A/J mice, the following cytokines/chemokines were measured: IL-1β, IL-10, TNF-α, TGF-β1, and MCP-1 (see Table I, A/J mouse cytokine and chemokine levels). Compared with control mice, lung levels of IL-1β were higher in AM-depleted mice on day 14 of infection, whereas both IL-10 and TNF-α levels were lower on day 14 of infection for AM-depleted mice. TGF-β1 levels were lower on day 3 of infection for AM-depleted animals compared with controls. MCP-1 levels were higher in AM-depleted mice on both days 3 and 14.

Interaction of AM and yeast cells in vitro

To better understand the basis for the disparate effects of AM depletion in rats and mice, we performed in vitro studies to identify potential differences in anticytotoxic activity between rat and mouse AM. We noted species-associated differences in macrophage size by light microscopy, with AM from Fischer rats being larger than AM from BALB/c mice. The longest diameter of AM from rats was greater than that of AM from mice (23.0 ± 14 vs 16.6 ± 2.3 μm; \( p < 0.001 \)).

Incubation of rat and mouse AM with \( \textit{C. neoformans} \) H99 in the presence of mAb 18B7 resulted in phagocytosis of yeast cells. At 4 h, the phagocytic index was considerably greater for rat AM than for mouse AM (Fig. 5). Serum-mediated phagocytosis of \( \textit{C. neoformans} \) was more efficient for rat AM than mouse AM, although the overall amount of phagocytosis was considerably lower than that observed when capsule-binding mAb was used as the opsonin. No detectable intracellular \( \textit{C. neoformans} \) were detected for mouse AM that had been cocultured with serum-opsonized \( \textit{C. neoformans} \). Similar results were observed with strain 24067 as noted for H99, for both Ab and serum-mediated phagocytosis. The overall efficiency of serum-mediated phagocytosis was lower for strain 24067 for both rat and mouse AM (not shown). All phagocytosis experiments were performed at least twice with similar results.

TEM studies revealed that AM from rats generally ingested more \( \textit{C. neoformans} \) cells than AM from mice. Also, rat AM were larger than mouse AM, and the filopodia of rat AM were generally more elongated and less rounded than the filopodia of mouse AM (Fig. 6.).

Intracellular replication

To study intracellular replication of \( \textit{C. neoformans} \), three types of experiments were performed. In the first study, the number of intracellular organisms was counted at various times after organisms had been ingested. These observations revealed a progressive increase in the number of organisms inside mouse AM, so that by 24 h the numbers of \( \textit{C. neoformans} \) inside murine macrophages had increased by 3-fold (Fig. 7A). Because extracellular organisms

![FIGURE 5](http://www.jimmunol.org/)

Phagocytic indices for AM from Fischer and BALB/c mice. mAb (18B7) and serum were used as opsonins. Bars represent 1 SD. *, \( p < 0.05 \) for comparison between rat and mouse. This experiment was repeated several times with similar results.

![FIGURE 6](http://www.jimmunol.org/)

TEM micrographs of AM from Fischer rats and BALB/c mice. These studies confirmed our light microscopy findings that Fischer rat AM were larger and more phagocytic than AM from BALB/c mice. White arrows point to \( \textit{C. neoformans} \). Filopodia of rat AM were thinner and more elongate (black arrows) than those of mouse AM.
were removed by washing, we conclude that the increase in intracellular numbers reflects intracellular growth. In contrast, the number of *C. neoformans* inside rat macrophages remained relatively constant (Fig. 7A). Similar results were obtained using 10% serum from mouse and rats as opsonin (not shown). These experiments were performed twice with similar results.

In additional experiments, we harvested supernatants and lysates from cell cultures infected with strain 24067 after incubation with Ab-opsonized *C. neoformans* and determined fungal burden. Initially, i.e., at 4 h, the fungal burden of lysates was greater (~2.2-fold) for rat AM cultures than for mouse AM cultures (Fig. 7B). These findings are consistent with our microscopic studies, which indicated enhanced phagocytic activity of rat AM. By 20 h, the fungal burden of lysates for mouse AM cultures had increased greatly and was greater (~1.9-fold) than the fungal burden of lysates from rat AM cultures. Also, at 20 h the fungal burden of supernatants for mouse AM cultures was 2.5-fold greater than that for rat AM cultures (84,750 ± 22,273 vs 38,500 ± 6,000). As with our counting experiments, extracellular organisms were removed at 4 h by washing. As a result, minimal to no organisms were present in the supernatants of cultures at 20 h when opsonin was not used (not shown). Thus, we suspect that organisms found in the supernatants at 20 h originate from an intracellular site. This experiment was repeated with strain H99 with similar results.

To confirm our in vitro studies suggesting that rat AM were more efficient in limiting intracellular growth of *C. neoformans* than mouse AM, we performed live imaging studies in which we focused on a single field and recorded the outcome of macrophage infection for 15 h (see supplemental video). For mouse AM, intracellular budding of *C. neoformans* was noted in five of six macrophages. Furthermore, all infected macrophages lysed by the end of 900 min, with most cells containing many *C. neoformans* at the time of lysis, indicating multiple replications. In contrast, intracellular budding of *C. neoformans* was noted in only two of nine rat AM (*p* = 0.04), with one macrophage containing a *C. neoformans* that underwent a single budding. Lysis was noted in only two of nine rat AM. At the time of lysis, both cells contained only a single organism. Imaging studies were performed twice for both rat and mouse cells with similar results.

**Oxidative burst, and NO and lysozyme production**

To determine the basis of the enhanced anticryptococcal activity of rat AM, we measured oxygen and nitrogen free radical production by rat and mouse AM after Ab-mediated phagocytosis of *C. neoformans*. In the context of mAb-mediated phagocytosis of *C. neoformans*, oxidative burst occurred earlier for rat AM than for mouse AM (see Fig. 8A). Furthermore, the magnitude of this burst was 7- to 17-fold higher for rat AM than for mouse AM. There was no increase in nitrite levels in the supernatant of rat or mouse AM cultures up to 4 days after Ab-mediated phagocytosis of *C. neoformans* (not shown).

To further assess the role of oxygen free radical production in limiting intracellular growth of *C. neoformans* by rat alveolar macrophages, intracellular growth assays were performed in the presence of catalase or superoxide dismutase. For rat AM, the addition of catalase and superoxide dismutase resulted in 45 and 75% increases in lysate fungal burden compared with nontreated controls (Fig. 8B). In contrast, for mouse AM, the addition of neither catalase nor superoxide dismutase resulted in an increase in lysate fungal burden (Fig. 8C). Catalase treatment actually resulted in a decrease in lysate fungal burden. Experiments were performed twice with similar results.

Lysozyme levels were higher in the supernatants of rat macrophage cultures compared with mouse cultures. In the absence of infection, there was no detectable lysozyme (limit of detection, 10 ng/ml) in the supernatant of mouse AM cultures, whereas the average concentration in rat AM cultures was 1694 ± 481 ng/ml. For infected macrophage cultures, the average lysozyme concentrations was ~6-fold greater for rat macrophages compared with mouse AM (Fig. 9). Experiments were performed twice, with similar results.

**Discussion**

Macrophages are considered central to the host defense against cryptococcal infection, but there is a relative dearth of experimental data to support this belief. In this study we examined AM function in experimental pulmonary cryptococcosis by depletion studies and found the effects of macrophage depletion to be host...
species dependent. For these studies we used liposome-clodronate, which is preferentially taken up by alveolar macrophages after intratracheal instillation and is believed to deplete macrophages by inducing apoptosis and cell death (25).

AM depletion of mice resulted in reduced lung fungal burden, whereas AM depletion of rats resulted in increased lung fungal burden and extrapulmonary dissemination. These results correlate with enhanced resistance to pulmonary cryptococcal infection in rats compared with mice, even though rats had to be infected with 100-fold more inocula than mice on a weight basis because these animals are so resistant to Cryptococcus neoformans numbers within the lung. This suggests a conserved role for AM in limiting inflammation in both species. These findings are consistent with the known anti-inflammatory properties of AM, which include production of anti-inflammatory cytokines, phagocytosis of apoptotic cells, and modulation of lymphocyte proliferation. In addition, enhanced function of dendritic cells in the context of AM depletion has been described (16). In keeping with the increase in pulmonary inflammation, we found increased chemokine (MCP-1 and CCL-2) expression in the lungs of both rats and mice depleted of AM. Altered cytokine expression was also present in association with AM depletion, although no specific change in Th-type profile (i.e., Th1 vs Th2) was detected for either mice or rats. A decrease in IL-10 levels was observed in both mouse and rat lungs, and this may reflect a decrease in AM IL-10 production. It may also account for the increased inflammation in association with AM depletion.

Our findings have important implications for understanding the relative importance of innate and adaptive immunities in cryptococcal infection. Previous experiments in mice have highlighted the importance of Th polarization with regard to susceptibility to infection, with enhanced susceptibility to infection linked to Th2 polarization (33). Interestingly, Th2 polarization has not been associated with enhanced susceptibility to cryptococcal infection in rats (34). Based on our studies, we hypothesize that the relative importance of Th polarization to the host response to cryptococcal infection may be dependent on the anticytotoxic activity of the innate immune response, including the ability of AM to limit the initial extent of infection. Thus, for the rat, in which AM are inherently more active against C. neoformans, the relative importance of Th polarization to host defense may be lower. In contrast, for mice, the importance of Th polarization may be considerably greater because of the relatively weak anticytotoxic activity of murine AM.

In summary, our study suggests that the differences in susceptibility to pulmonary cryptococcal infection in rats and mice may result from basic differences in AM–C. neoformans interactions that exist between rats and mice. Enhanced resistance of rats to pulmonary cryptococcal infection correlates with increased anticytotoxic activity of rat AM that involves both oxidative and nonoxidative mechanisms. In contrast, the enhanced susceptibility of mice to cryptococcal infection correlates with a more permissive or disease-enhancing role for AM. Our studies point to important basic differences in the host response to pulmonary fungal infections that occur between animal types and serve as a caution for extrapolating results from one animal system to another.

Disclosures
The authors have no financial conflict of interest.

References


