An Innate Immune System Cell Is a Major Determinant of Species-Related Susceptibility Differences to Fungal Pneumonia[1]

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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 anticryptococcal 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)[3] 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 anticryptococcal 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 anticryptococcal 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 -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.

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Received for publication March 4, 2005. Accepted for publication June 15, 2005.

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[1] This work was supported by National Institutes of Health Grants AI033142-11, AIO33774-11, AIO52733-02, HL059842-08, and GM071421-01 (to A.C.) and HL064547-05 (to D.L.G.).

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[3] Abbreviations used in this paper: AM, alveolar macrophage; BN, Brown Norway; CL_2MBP, dichloromethylene bisphosphonate; IL-1RA, IL-1R antagonist; TEM, transmission electron microscopy; GXM, glucuronoxylomannan.

0022-1767/05/$02.00

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 dextrose 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 experimentation, two strains of *C. neoformans* were studied, H99 (a serotype A strain) and 24067.

AM depletion

Dichloromethylene bisphosphonate (CL_2MBP) or clodronate was a gift from Roche and was encapsulated in liposomes as described previously (14). CL_2MBP selectively depletes AM after intratracheal instillation (14). Of note, intratracheal administration of liposomal clodronate does not appear 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 CL_2MBP liposomes intratracheally. Three days later, lungs were removed and frozen. Lung sections were stained for the presence of AM using an FITC-conjugated Ab (ED1; Serotec) that specifically recognizes macrophages (20). For mice, the adequacy of AM depletion was confirmed after administration of 0.1 ml of PBS liposomes or CL_2MBP liposomes by either the intranasal or intratracheal route. Three days later, lung tissue was removed and frozen. Sections were stained using biotin-labeled Ab specific for MAC-3 (BD Pharmingen). Biotinylated Ab was then detected with avidin conjugated to HRP, and color was detected with diaminobenzidine. Fischer rats and mice (A/J, BALB/C) treated with liposome clodronate had no detectable macrophage staining (not shown). Intranasal liposome clodronate (A/J) and intratracheal liposome clodronate (BALB/c) treatments gave similar results with regard to AM depletion. For depletion studies, animals were given clodronate liposome or PBS liposome by intratracheal or intranasal administration at the doses described above and then infected 3 days later. To insure ongoing depletion of AM, repeat intratracheal/intranasal injections of liposome clodronate were given weekly (14).

Infection

Rats were anesthetized by exposure to isoflurane and were infected with 1×10^7 *C. neoformans* in 0.3 ml of PBS. *C. neoformans* administration was performed intratracheally, as described previously (2). In other experiments, rats were intratracheally infected with 1×10^5 *C. neoformans*. Mice were anesthetized by i.p. injection with ketamine (10 mg/kg) and xylazine (125 mg/kg) in PBS. The trachea was surgically exposed, and mice were inoculated intratracheally with 1×10^4 *C. neoformans* cells in 0.05 ml of PBS. The smaller inoculum was used for murine studies, because of the enhanced susceptibility of mice to cryptococcal infection. To determine the effects of liposome-clodronate treatment without infection, rats or mice were given sham infection (i.e., PBS alone).

Fungal burden

Groups of animals were killed at various times after infection, and lungs and spleens were removed. Left lungs were finely minced in 5 ml (for rats) and 2 ml (for mice) of PBS with a protease inhibitor mixture (Complete Mini; Roche) and further homogenized by treatment with a Tissue Terrator (Biospec Products). Serial dilutions were plated on Sabouraud's dextrose agar. To ensure that liposome clodronate had no direct effect on C. *neoformans* growth, organisms were incubated in Sabouraud's dextrose broth containing 10% liposome clodronate. At various times (2, 4, 6, 9, and 24 h), an aliquot of culture was removed and tested for *C. neoformans* growth. No differences in growth were observed.

Histology and immunohistochemistry

For rats, right lungs were immersed in O.C.T. compound (Sakura Finetec), snap-frozen, cut into 5-μm-thick sections, and fixed in methanol. Sections were stained with H&E. For glucuronoxylomannan (GXM) immunohistochemistry, sections were treated with 0.3% H_2O_2 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 peroxidase-conjugated, goat anti-mouse isotype-specific IgG (Southern Biotechnology Associates), and color was developed with diaminobenzidine. For mice, lungs were processed in a similar manner.

Cytokine/chemokine measurements

Lung homogenates were centrifuged, and supernatants were frozen at −20°C. Levels of IL-4, IL-10, IL-1 β, IL-1R antagonist (IL-1RA), TNF-α, IFN-γ, TGF-β1, MIP-2, and MCP-1 were measured in rat lung homogenates using commercially available ELISA kits (BD Pharmingen and BioSource International). Levels of IL-10, TNF-α, IFN-γ, IL-1β, TGF-β1, and MCP-1 were measured in murine lung homogenates using commercially available ELISA kits (BD Pharmingen).

Bronchoalveolar lavage

Rats and mice were killed by asphyxiation with CO_2, 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-Aldrich). Lavage fluids were pooled, and cells were collected by centrifugation. RBC were lysed by incubation in 0.17 M NH_4Cl at 4°C for 10 min. Cells were washed with HBSS and resuspended in DMEM (Invitrogen Life Technologies) with 10% heat-inactivated FCS (Bioproducts for Science). Approximately 1×10^6 AM were obtained per rat and 1×10^5 AM per mouse.

Cell size

Cells were lavaged from Fischer rats and BALB/c mice, suspended at 3×10^4/ml, and subjected to centrifugation using a cytospin (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 QI 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. Pictures 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 2.5×10^4/well in 0.2 ml of DMEM-10% FCS and allowed to attach at 37°C. Medium was replaced with fresh medium containing 2.5×10^4 *C. neoformans* (E:T cell ratio, 1:1) alone or with 10% serum (rat or mouse) or mAb 18B7 (10 μg/ml). Cells were incubated together for 4 h and washed twice with fresh medium. The total number of internalized *C. neoformans* per total number of macrophages was calculated. Approximately 200–300 AM/well were counted.

Transmission electron microscopy (TEM)

AM were harvested from Fischer rats and BALB/c mice. Approximately 1×10^6 cells/well were placed in a six-well plate. Cells were incubated with *C. neoformans* (1×10^6 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 with 0.1 M cacodylate, then placed in buffer containing 0.1 M cacodylate. Cells were visualized with a JEOL 1200 EX transmission electron microscope.

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 assay). At 4 h, cultures were washed and returned to the incubator. At various times (4, 8, 16, and 24 h) after inoculation of *C. neoformans*, cells were fixed with cold methanol and stained with Giemsa. The total number of internalized *C. neoformans* per total number of phagocytic AM was determined. Approximately 200–300 AM/well were counted.

The results of these studies were confirmed by measuring changes in fungal burden. For these studies, AM from Fischer rats and BALB/c mice were incubated with *C. neoformans* at a T:E cell ratio of 1:5 in the presence of mAb 18B7 (10 μg/ml) or medium alone. Four hours after coincubation, supernatants were removed, and cells were lysed with water for 1 h at room temperature. Both fractions (i.e., supernatant and lysate) were plated on Sabouraud's dextrose agar. For some cultures, supernatants were removed

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. 0.50 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% CO_2 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 (Scanalytics) 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, luminescence studies were performed. Briefly, cells were washed with HBSS and resuspended at a density of 5×10^5/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 within minutes of adding opsonized organisms (time zero) and every 10 min for 1 h. Luminescence 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 4×10^4 AM from Fischer and BALB/C mice were placed in DMEM and then incubated overnight at 37°C. The following day, mAb 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.

To determine the role of free oxygen free radical production in the enhanced ability of rat AM to limit *C. neoformans* growth, intracellular growth experiments were performed as described above, but catalase (200 μg/ml) or superoxide dismutase (40 μg/ml; both from Sigma-Aldrich) were added to rat and mouse AM cultures at the same time that mAb and *C. neoformans* were added. Fungal burdens in supernatants and lysates were determined at 24 h.

Lysozyme production

Approximately 1×10^5 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 lysodeikticus* as previously described (23). Briefly, 40 μl of a 0.03% 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 was 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. 1*A*) 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 $255 \pm 93 \times 10^4$ vs $23 \pm 9 \times 10^4$, respectively ($p < 0.01$). For A/J mice, lung fungal burden was similar for AM-depleted and control animals on day 3 ($1.36 \pm 0.32 \times 10^6$ vs $1.49 \pm 0.74 \times 10^6$; Fig. 1*D*), but was ~2-fold lower in AM-depleted animals on day 14 ($7.9 \pm 0.37 \times 10^6$ vs $17.0 \pm 0.57 \times 10^6$; $p < 0.001$). 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. 1*E*). 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. 1*F*). 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 Fisher rats on day 3 of infection.

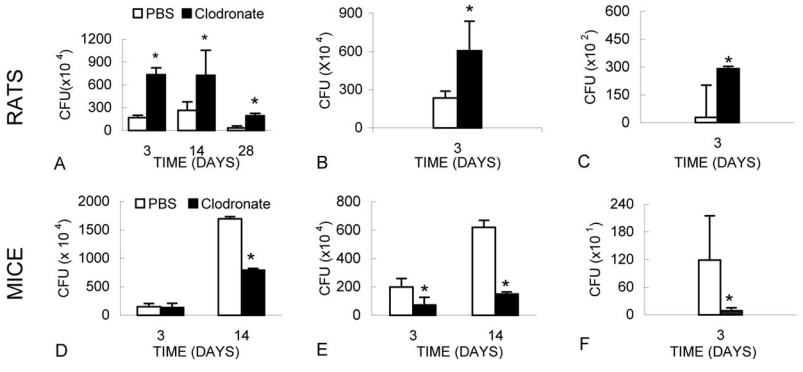

FIGURE 1. Organ fungal burden in two rat strains (*top row*) and three mouse strains (*bottom row*) after depletion of AM with liposome clodronate. *A*, Lung fungal burden after AM depletion in Fischer rats. Lung fungal burden for Sprague-Dawley (*B*) and BN (*C*) rats after AM depletion on day 3. For rats, there were five animals per group. Lung fungal burden for A/J (*D*), BALB/c (*E*), and C57 (*F*) mice ($n = 6$–7/group) after AM depletion is shown. Lung fungal burden was consistently increased in rats and decreased in mice after AM depletion. *, $p < 0.05$ vs liposome PBS. Bars represent 1 SD.

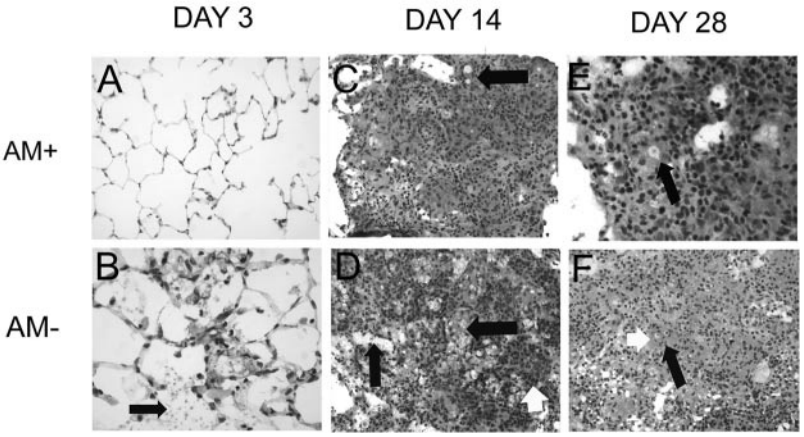

FIGURE 2. Histological appearance of infected lungs from Fischer rats treated with liposome PBS (AM+) or liposome clodronate (AM−). At all times of infection, lungs of rats given clodronate had more inflammation than lungs of PBS-treated rats. Many infiltrating lymphocytes (white arrow) were seen on 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. 4*B*) 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. 4*D*).

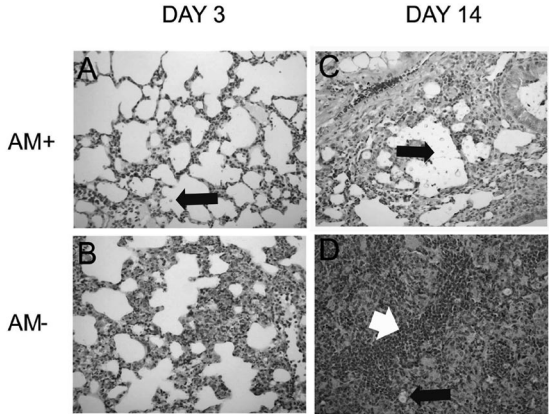

FIGURE 3. Histological appearance of lungs from A/JCr mice after treatment with liposome PBS (AM^+) or liposome clodronate (AM^-). Increased inflammation is present in AM-depleted animals (*B* and *D*). Many infiltrating lymphocytes are present on day 14 in AM^- mice (*D*, white arrow). More *C. neoformans* (black arrows) are present in lungs of AM^+ animals (*A* and *C*). Black arrows point to *C. neoformans*.

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, Fischer 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

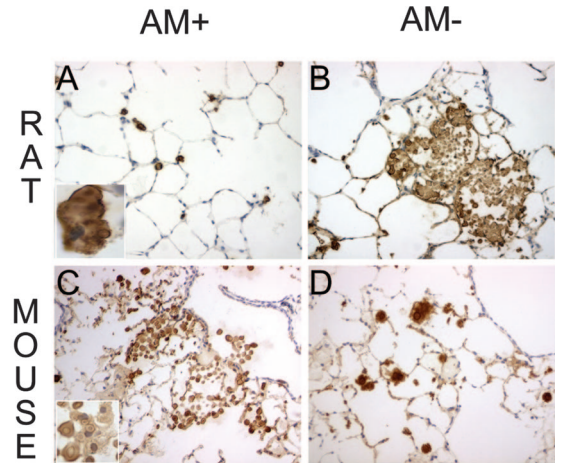

FIGURE 4. Staining for GXM (brown) in lungs of Fischer rats (*A* and *B*) and BALB/c mice (*C* and *D*) on day 3 of infection. *A*, Scattered organisms (brown) are seen throughout the lungs of an infected Fischer rat, treated with liposome PBS (AM^+). The *inset* shows a high magnification view of a 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.

Table I. *Rat and mouse cytokine and chemokine levels*[a]

	Day 3		Day 14		Day 28	
	AM^+	AM^-	AM^+	AM^-	AM^+	AM^-
Fischer rat cytokine and chemokine levels						
IL-1β	1,798 ± 231	1,641 ± 407	2,254 ± 812	2,059 ± 703	3,815 ± 372	3,084 ± 257* ↓
IL-IRA	7 ± 3	18 ± 8* ↑	78 ± 5	111 ± 53	391 ± 12	463 ± 4* ↑
IL-10	59 ± 6	23 ± 11* ↓	57 ± 9	95 ± 24* ↑	50 ± 11	57 ± 6
IFN-γ	nd	nd	nd	nd	65 ± 15	58 ± 10
TNF-α	nd	nd	nd	nd	54 ± 15	48 ± 8
TGF-β	128 ± 33	127 ± 14	92 ± 22	115 ± 14	96 ± 10	114 ± 26
MCP-1	14 ± 3	36 ± 7* ↑	21 ± 6	58 ± 8* ↑	48 ± 9	59 ± 9
MIP-2	216 ± 34	369 ± 53* ↑	200 ± 49	420 ± 55* ↑	367 ± 29	333 ± 38
A/J mouse cytokine and chemokine levels						
IL-1β	1,544 ± 480	1,779 ± 399	1,807 ± 547	3,015 ± 1,028* ↑		
IL-10	695 ± 132	664 ± 249	916 ± 223	517 ± 143* ↓		
TNF-α	255 ± 46	334 ± 129	602 ± 171	353 ± 86* ↓		
TGF-β	9,642 ± 1,869	4,362 ± 3,017* ↓	16,938 ± 7,929	13,542 ± 3,704		
MCP-1	172 ± 47	3,249 ± 2,620* ↑	173 ± 35	350 ± 124* ↑		

[a] Cytokine/chemokine levels in nondepleted (AM^+) and AM-depleted animals (AM^-). All cytokine and chemokine concentrations are given in picograms per milliliter, except rat IL-1RA and rat MCP-1 which are given in nanograms per milliliter. *, $p < 0.05$ compared with control animals at the same time of infection. Arrows represent direction of change in value in association with AM depletion (n = 5–7 animals per group). nd refers to not detected, limit of detection for this assay was 32 pg/ml.

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 MIP-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 anticryptococcal 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 *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 *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 *C. neoformans* were detected for mouse AM that had been cocultured with serum-opsonized *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 *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 *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 *C. neoformans* inside murine macrophages had increased by 3-fold (Fig. 7*A*). Because extracellular organisms

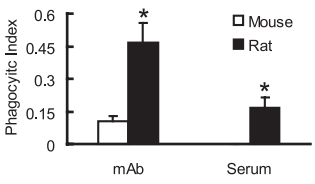

FIGURE 5. Phagocytic indices for AM from Fischer and BALB/c mice. mAb (18B7) and serum were used as opsonins. Bars represent 1 SD. *, $p < 0.05$ for comparison between rat and mouse. This experiment was repeated several times with similar results.

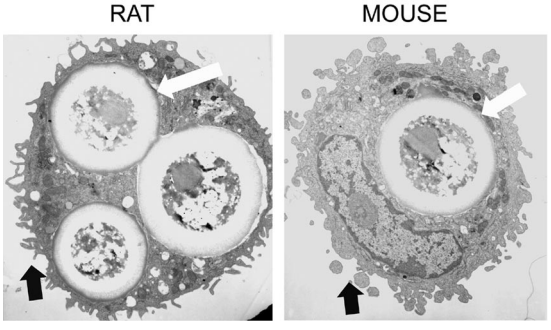

FIGURE 6. TEM micrographs of AM from Fischer rats and BALB/c mice. These studies confirmed our light microscopy findings that Fischer rat AM were larger and more phagocytic than AM from BALB/c mice. White arrows point to *C. neoformans*. Filopodia of rat AM were thinner and more elongate (black arrows) than those of mouse AM.

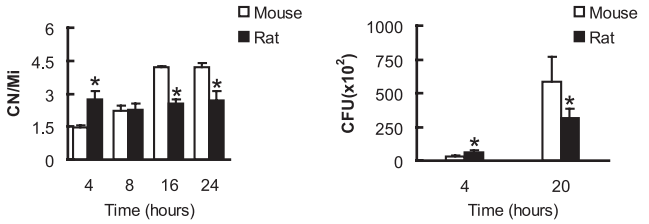

FIGURE 7. Intracellular growth assays. *A*, Average number of intracellular *C. neoformans* per infected macrophage (CN/Mi) at various times after mAb-mediated phagocytosis. *B*, Lysate fungal burden of rat and mouse AM at various times after *C. neoformans* infection. These experiments were performed twice with similar results. □, AM from BALB/c mice; ■, AM from Fischer rats; 1 SD is shown. ∗, $p < 0.05$, rat vs mouse.

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. 7*A*). 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. 7*B*). 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).[4] 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. 8*A*). 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 cells (Fig. 8*B*). In contrast, for mouse AM, the addition of neither catalase nor superoxide dismutase resulted in an increase in lysate fungal burden (Fig. 8*C*). 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

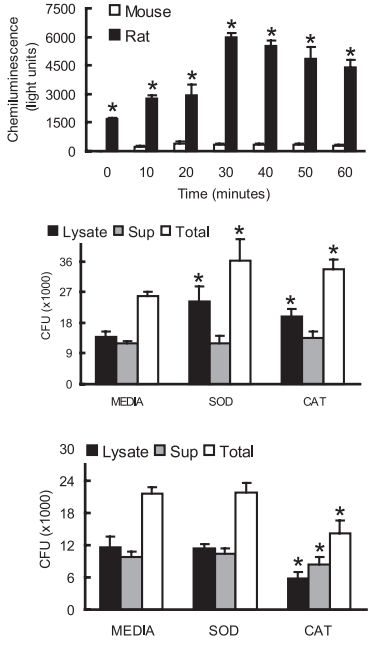

FIGURE 8. The oxidative burst in the context of Ab-mediated phagocytosis of *C. neoformans* was much greater for rat AM than for mouse AM. A luminescence reading represents the average of three separate wells. Errors bars represent 1 SD. ∗, $p < 0.01$. *B*, Fungal burden in lysate, supernatant, and lysate plus supernatant (total) for rat (*B*) and mouse (*C*) AM infected with *C. neoformans* at 24 h. To some cultures, superoxide dismutase (SOD) and catalase (CAT) were added. ∗, $p < 0.05$ compared with infected cells in medium alone.

[4] The online version of this article contains supplemental material.

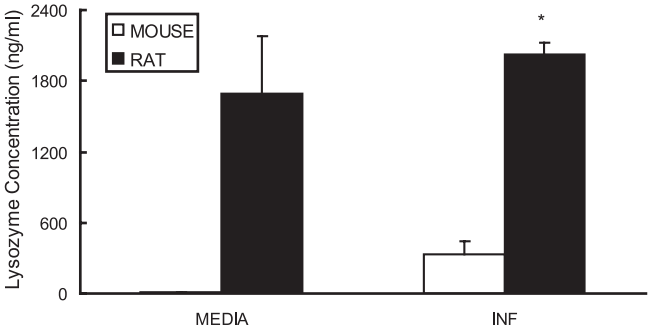

FIGURE 9. Lysozyme levels were higher in the supernatant of rat AM cultures compared with mouse AM cultures. Lysozyme was not detected in the supernatant of mouse AM cultures in the absence of infection (limit of detection, 10 ng/ml). The error bar represents 1 SD. *, $p < 0.05$ compared with mouse AM cultures. INF, Infection.

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 *C. neoformans* infection.

Our findings suggest that differences in anticryptococcal activity among AM from rats and mice correlate with species susceptibility. We note that our studies used three strains of mouse and three strains of rat; consequently, the results are more likely to reflect species differences than variation in intraspecies strain susceptibility. AM appear to play a protective role for rats against cryptococcal infection, but a disease-enhancing role in mice. In support of this idea, we found that rat AM demonstrated enhanced anticryptococcal activity compared with mouse AM, as manifested by increased phagocytosis, enhanced control of intracellular growth, and increased resistance to pathogen-induced cell lysis. The basis for increased effector activity of rat AM compared with that in mice appears to involve both oxidative and nonoxidative mechanisms. These findings are consistent with previous observations noting differences in rat and mouse AM biology (26–29).

Feldmesser et al. (1) noted a 5-fold higher budding index for intracellular *C. neoformans* compared with extracellular *C. neoformans* during pulmonary infection in mice. This observation and our finding of rapid intracellular replication of *C. neoformans* in mouse AM resulting in lysis of the host cells suggested that *C. neoformans* may be able to use the murine AM as a site for enhanced replication. Hence, we hypothesized that macrophage depletion could enhance host defense in the mouse by removing a favorable replication site. Consistent with this hypothesis, depletion of AM in mice resulted in reduced fungal burden. In contrast, the rat AM is intrinsically more active against *C. neoformans*, and depletion of these sites results in increased *C. neoformans* growth.

The ability of rat AM to limit intracellular *C. neoformans* growth and resist pathogen-mediated lysis correlated with enhanced oxidative and nonoxidative activities. These studies were performed with AM from normal noninfected rats in the absence of exogenous cytokine stimulation and would appear to highlight intrinsic differences between rat and mouse AM. Oxidative killing of *C. neoformans* is an important mechanism of action for immune effector cells (30, 31). Rat AM incubated with catalase and superoxide dismutase showed an increase in cryptococcal lysate fungal burden, suggesting the importance of H_2O_2 and reactive oxygen intermediates in limiting intracellular growth. In contrast, mouse AM failed to show an increase in lysate fungal burden after a similar treatment. Compared with murine AM, rat AM also demonstrated higher levels of lysozyme in culture supernatants. Lysozyme has been shown to have potent anticryptococcal activity (32).

Depletion of AM in both rats and mice (all strains tested) resulted in increased inflammation compared with nondepleted animals. Increased inflammation was present in the context of increased (rats) and decreased (mice) *C. 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 anticryptococcal 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 anticryptococcal 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 anticryptococcal 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.

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