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Elucidating the Pathogenesis of Spores from the Human Fungal Pathogen *Cryptococcus neoformans*▽

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Cryptococcus neoformans **was first described as a human fungal pathogen more than a century ago. One aspect of the *C. neoformans* infectious life cycle that has been the subject of earnest debate is whether the spores are pathogenic. Despite much speculation, no direct evidence has been presented to resolve this outstanding question. We present evidence that *C. neoformans* spores are pathogenic in a mouse intranasal inhalation model of infection. In addition, we provide mechanistic insights into spore-host interactions. We found that *C. neoformans* spores were phagocytosed by alveolar macrophages via interactions between fungal β-(1,3)-glucan and the host receptors Dectin-1 and CD11b. Moreover, we discovered an important link between spore survival and macrophage activation state: intracellular spores were susceptible to reactive oxygen-nitrogen species. We anticipate these results will serve as the basis for a model to further investigate the pathogenic implications of infections caused by fungal spores.**

Cryptococcus neoformans is an opportunistic fungal pathogen that has emerged as an important cause of morbidity and mortality in people with underlying immune deficiencies. During the last three decades, the incidence of *C. neoformans* cases has dramatically increased, due in large part to the global human immunodeficiency virus pandemic. *C. neoformans* is environmentally ubiquitous and can be found in a variety of soils contaminated with avian guano (11). Cryptococcal infection occurs as a result of environmental exposure and inhalation of aerosolized *C. neoformans* cells. In healthy people, initiation of innate and adaptive cellular immune responses limits the severity of the infection to an asymptomatic and often self-resolving pulmonary infection (11, 35). In contrast, hematogenous dissemination of *C. neoformans* from the lungs to the central nervous system in immunocompromised people can lead to cryptococcal meningoencephalitis, a life-threatening complication requiring aggressive chemotherapeutic intervention (11, 35).

Prior to the discovery in 1975 that *C. neoformans* could produce spores, only the yeast form was considered to be an infectious propagule (29). Numerous studies showed that small, desiccated, encapsulated *C. neoformans* yeast could be recovered from soil, and these cells were readily aerosolized and could cause disease in animal models similar to human disease (10, 11, 15, 32, 36, 39). The discovery that *C. neoformans* had the potential to produce spores led Cohen et al. (12) to hypothesize that spores might also be infectious propagules. This hypothesis was consistent with the infectious life cycles of other human pathogenic fungi; inhalation of spores from *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides* spp., and *Paracoccidioides brasiliensis* can cause pulmonary or disseminated disease in healthy people (38).

For the last three decades, efforts to elucidate the role of spores in the pathogenesis of *C. neoformans* have been severely hampered because of the difficulty of isolating large numbers of pure spores (13, 42, 45). Two previous studies that succeeded in isolating spores from *C. neoformans* var. *neoformans* strains found that spores were infectious in mouse models of cryptococcosis, which suggested that they might contribute to the pathogenesis of *C. neoformans* (42, 45). Although mice inoculated with *C. neoformans* var. *neoformans* spores did develop infections, the animals did not exhibit signs of morbidity or mortality (42, 45), likely because the *C. neoformans* var. *neoformans* strains used in these studies possessed limited virulence potential. Because the spores used in these studies did not kill mice, the specific role of spores in the pathogenesis of *C. neoformans* was not addressed (42, 45).

Our laboratory recently developed a technique to isolate large numbers of pure spores from the *C. neoformans* var. *neoformans* strains, and smaller numbers of pure spores from the virulent *C. neoformans* var. *grubii* strains (4). Our initial biochemical characterization of *C. neoformans* spores revealed that they possessed a cell surface composition different from yeast but physical properties similar to other fungal spores (4). These observations led us to speculate that, like other pathogenic fungi, the infectious life cycle of *C. neoformans* might include an infectious spore form that transitions into a pathogenic form in the lungs. *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides* spp., and *Paracoccidioides brasiliensis* spores undergo a rapid transition to a parasitic form in the lungs, which is essential for establishment of infection and disease progression (30, 33). Pulmonary host defense against these and other pathogens is mediated by the recognition of

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pathogen-associated molecular patterns (PAMPS) by host pattern recognition receptors (PRRs). These interactions facilitate the phagocytosis and killing of fungi and other pathogenic microorganisms by activated alveolar macrophages.

In the present study, we used spores from virulent *C. neoformans* var. *grubii* parental strains to test the hypothesis that spores are infectious propagules. In addition, we used spores from *C. neoformans* var. *neoformans* strains to identify mechanisms that contribute to host-fungal interactions. The rationale for using spores from two different varieties of *C. neoformans* was based on necessity; only a small number of spores could be isolated from the virulent *C. neoformans* var. *grubii* strains, which do not produce abundant spores. Thus, these spores were used for our in vivo animal studies. In contrast, *C. neoformans* var. *neoformans* strains produced abundant spores, which were required in large numbers for our in vitro assays. Overall, the results of the present study (i) provide evidence that spores are infectious propagules, (ii) that spores contribute to the pathogenesis of *C. neoformans*, (iii) provide mechanistic insights into the host-pathogen interactions that contribute to innate pulmonary defense against spores, (iv) and reveal the importance of alveolar macrophage activation state for defense against infection by spores.

MATERIALS AND METHODS

Fungal strains and media. *C. neoformans* var. *grubii* strain H99 (serotype A, mating type α), *C. neoformans* var. *grubii* strain KN99**a** (serotype A, mating type **a**), *C. neoformans* var. *grubii* strain KN99α (serotype A, mating type α), *C. neoformans* var. *neoformans* strain JEC20 (serotype D, mating type **a**), and *C. neoformans* var. *neoformans* strain JEC21 (serotype D, mating type α) were revived from 15% glycerol stocks stored at −80°C and maintained on yeast extract peptone dextrose (YPD; 1% yeast extract, 2% peptone and 2% dextrose) agar plates at 30°C. Prior to use, yeast were grown in YPD liquid medium overnight at 30°C with shaking, collected, washed with sterile phosphate-buffered saline (PBS), and counted with a hemacytometer to determine cell concentrations.

Mouse strains. Three- to six-week-old female AJ/Cr and C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Double-knockout mice lacking inducible nitric oxide synthase (iNOS$^{-/-}$) and NADPH-oxidase (gp47$^{phox-/-}$) were generously provided by Matyas Sandor (University of Wisconsin-Madison). The University of Wisconsin-Madison Institutional Animal Use and Care Committee approved the animal protocol used for the present study.

***C. neoformans* spore isolation.** Crosses with *C. neoformans* var. *neoformans* yeast strains JEC20 and JEC21 were performed on V8 agar plates (15-cm-diameter plates, pH 7). A 1:1 suspension of strains JEC20 and JEC21 in PBS was spotted onto V8 agar plates (10-μl spots). After 5 to 7 days of incubation at room temperature, a rubber cell scraper was used to collect spots, which were suspended in 200 ml of ice-cold PBS containing 60% Percoll Plus (GE Healthcare, Piscataway, NJ) and 0.01% Triton X-100. After the scraped cells from approximately 10 V8 agar plates were suspended in the gradient solution, the cells were homogenized by using a 10-ml pipette with a 1-ml pipette tip fitted on the end. Then, 50-ml tubes were filled to the top with the cell suspension and any remaining gradient solution. Samples were centrifuged in an SS-34 rotor at 12,000 rpm for 30 min at 4°C. Spores were harvested by first aspirating yeast cells from the top of the gradient and then using a 10-ml syringe fitted with a 20-gauge needle (1.5 in.) to remove approximately 10 ml of solution from the bottom of the centrifuge tube. Spores were washed three times at low speed with PBS (1 ml, each wash) to remove Percoll, suspended in 1 ml of PBS, and counted by using a hemacytometer. Using this method, spore preparations with purities of >98% were routinely achieved (data not shown). Crosses with *C. neoformans* var. *grubii* yeast strains KN99**a** and KN99α were performed on V8 agar plates (15-cm diameter plates, pH 5). A 1:1 suspension of strains KN99**a** and KN99α in PBS were spotted onto V8 agar plates (10-μl spots). Plates were incubated in the dark at room temperature until white filamentous growth and spore chains were observed. Filamentous sections of spots were collected with a pipette tip and suspended in 0.5 ml of PBS with 0.01% Triton X-100. 1.2 ml of Percoll Plus (GE Healthcare, Piscataway, NJ) was then added, and tubes were centrifuged at 10,000 × *g* for 30 min. Spores were collected by inserting a needle into the bottom of the tube and removing ~0.1 ml of solution. Spores were washed three times in 1 ml of PBS to remove gradient material, suspended in PBS, and counted by using a hemacytometer.

Phagocytosis assays. Alveolar macrophages were isolated from iNOS$^{-/-}$ gp47$^{phox-/-}$ or wild-type C57BL/6 mice. Bronchoalveolar lavage was performed as previously described (23). Briefly, lungs were lavaged with 1 ml of PBS containing 5 mM EDTA (instilled four times). Recovered cells were washed once with PBS and counted by using a hemacytometer, and 10^5 cells/well were added to a 16-well chamber slide (Nalgene Nunc, Naperville, IL) and allowed to adhere for 2 h. Prior to the addition of calcofluor-labeled *C. neoformans* spores to alveolar macrophages, spores or alveolar macrophages were pretreated with receptor blocking antibodies against CD11b (antibody), CD11c (antibody), Dectin-1 (antibody), polysaccharides (α-mannan and laminarin), or Fc-Dectin. Spores were then added to alveolar macrophages in culture at a multiplicity of infection of 4:1. Spores and alveolar macrophages were coincubated for 4 h at 37°C, and then the wells were washed with sterile PBS to remove extracellular spores. Attachment and/or phagocytosis of spores by alveolar macrophages was visualized and quantified by fluorescence microscopy (Zeiss Axioskop 2 Plus microscope) and flow cytometry. Fluorescent images were captured by using an AxioCam MRm (Carl Zeiss, Thornwood, NY) digital camera and AxioVision 4.5 software (Carl Zeiss). Images were uniformly processed to adjust contrast and image clarity using Adobe Photoshop CS2. Visualization of infected monolayers by confocal microscopy was performed to confirm the intracellular localization of spores. Flow cytometric data was analyzed by using the FlowJo software package (v6.3.3; Tree Star, Inc., Ashland, OR). In preliminary studies we observed that if spores were bound by alveolar macrophages they were phagocytosed (data not shown); thus, quantification of spore-alveolar macrophage interactions refers to the percent inhibition of phagocytosis. The percent inhibition of phagocytosis was determined by comparing the number of spores phagocytosed in the treatment group to the number of spores phagocytosed in the control group. Only experiments in which >19% of alveolar macrophages phagocytosed spores in the untreated control group were used to calculate the percent inhibition of phagocytosis. Statistical analysis was performed by using the JMP statistics package (SAS, Cary, NC). Student *t* test or analysis of variance was used to assess the statistical significance.

In vitro testing. Alveolar macrophages collected from the bronchoalveolar lavage fluid of iNOS$^{-/-}$ gp47$^{phox-/-}$ or wild-type C57BL/6 mice were used to assess intracellular survival of *C. neoformans* spores. Prior to the addition of spores, alveolar macrophages were treated with mIFN-γ (Roche Molecular Biochemicals) and lipopolysaccharide (LPS; Sigma, St. Louis, MO) at concentrations of 10 and 300 ng/ml, respectively. Spores were prepared as previously described, washed three times in PBS, and coincubated for 4 h with alveolar macrophages. After coincubation, wells were washed three times with PBS to remove extracellular spores. Visual examination of the cocultures by microscopy was performed to confirm that extracellular spores were removed and to check the integrity of the macrophage monolayers. The contents of select wells were immediately collected at the zero time point (0 h) to determine the number of *C. neoformans* spores initially present. The remaining wells were incubated for 24 h at 37°C, at which time the total contents of each well were collected by lysis with 0.05% sodium dodecyl sulfate. Wells were washed with 2 volumes of sterile PBS and pooled with the previously collected material. The concentration of SDS used does not affect the growth of *C. neoformans* (21). CFU analysis was then performed to determine *C. neoformans* CFU/ml. A Student *t* test was performed to determine the statistical significance between groups ($P < 0.05$ was considered significant).

In vivo testing. The virulence of *C. neoformans* var. *grubii* spores was assessed by infecting groups consisting of four or eight iNOS$^{-/-}$ gp47$^{phox-/-}$, C57BL/6, or AJ/Cr mice via intranasal inhalation with 10^5 CFU of spores or yeast (in a volume of 50 μl), as described previously (22). The rationale for choosing an inoculum of 10^5 was based on previous susceptibility studies, in which this is a common inoculum size (20–23). For CFU analysis from the lungs, mice were euthanized at days 3 and 6 postinfection, and the lungs were surgically removed and homogenized in sterile PBS. Lung homogenates were serially diluted and plated on YPD agar. CFU were then determined after 2 days of growth at 30°C. In all studies, mice that appeared moribund (i.e., lethargic or exhibiting rapid weight loss) or in pain were euthanized. Mice were monitored twice daily, and the mean time to mortality between the various groups was determined. Statistical analysis was performed by using the JMP statistics package (SAS, Cary, NC). Survival statistics were performed to evaluate mouse survival data for statistical significance. A Kaplan-Meier plot, followed by log-rank or Wilcoxon tests, was prepared. *P* values of < 0.05 were considered statistically significant.

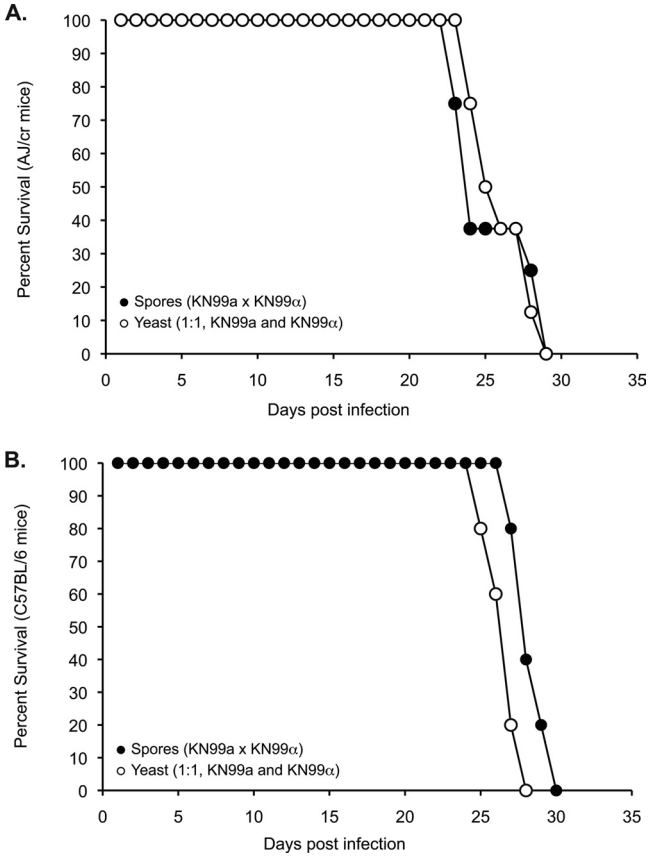


FIG. 1. *C. neoformans* var. *grubii* spores are pathogenic in a murine model of cryptococcosis. Groups of four or eight AJ/Cr (A) or C57BL/6 (B) mice were infected with *C. neoformans* var. *grubii* spores (10^5) or yeast (10^5) via intranasal inhalation. All mice succumbed to infection and died by day 30 postinfection. There was no significant difference in the median time to death of AJ/Cr or C57BL/6 mice infected with *C. neoformans* var. *grubii* spores or yeast ($P > 0.05$). Median times of death for AJ/Cr mice infected with *C. neoformans* var. *grubii* spores or yeast were 24 and 25 days, respectively. Median times of death for C57BL/6 mice infected with *C. neoformans* var. *grubii* spores or yeast were 28 and 27 days, respectively.

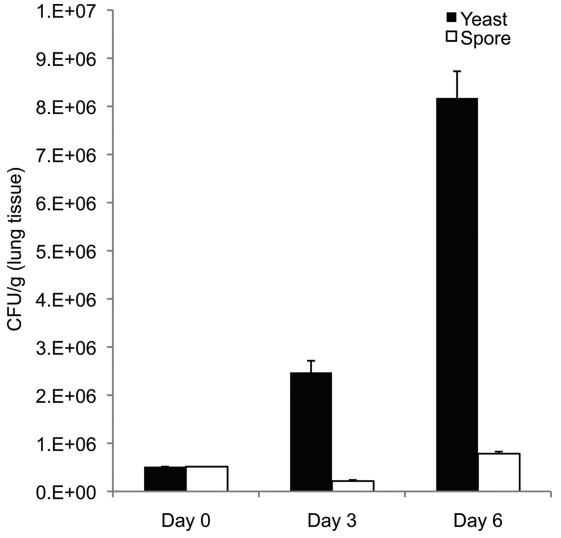


FIG. 2. Mice infected with *C. neoformans* var. *grubii* spores have low lung CFU early in infection. Groups of four C57BL/6 mice were infected with *C. neoformans* var. *grubii* spores (10^5) or yeast (10^5) via intranasal inhalation. Mice were sacrificed 3 and 6 days postinfection. Pulmonary CFU were determined by homogenizing whole lungs in PBS and plating dilutions of homogenates on YPD plates. The CFU/g of lung tissue were calculated. Bars represent mean ± the standard error.

RESULTS

***C. neoformans* spores are pathogenic.** We recently reported the development of a purification method to isolate large numbers of pure spores from *C. neoformans* var. *neoformans* strains (4). In the present study, we adapted this method to isolate spores from *C. neoformans* var. *grubii* strains (KN99**a** and KN99α). Although crosses between these strains do not produce abundant spores, the parental strains do have the advantage of being virulent in a mouse intranasal inhalation infection model and thus are highly relevant for the analysis of pathogenicity. To assess the pathogenicity of *C. neoformans* var. *grubii* spores, groups of four or eight mice from two unique genetic backgrounds (C57BL/6 and AJ/Cr) were infected by intranasal inoculation with 10^5 spores or 10^5 of a 1:1 mixture of parental yeast strains. Spores and yeast were subjected to identical purification processes. By day 30 postinfection, mice in all groups had succumbed to infection and died with no significant difference ($P > 0.05$) in the median time to mortality between the groups (Fig. 1). Necropsy of spore-infected mice indicated that the cause of death was pulmonary failure, a common cause of death in mice infected with *C. neoformans* yeast (11). *C. neoformans* colonies could not be recovered from brain homogenates of spore- or yeast-infected mice, suggesting that dissemination to the central nervous system did not occur in either case (data not shown). These results demonstrated that *C. neoformans* spores from virulent parental strains could cause morbidity and mortality in mice in a manner indistinguishable from parental yeast strains.

The observation that spores were infectious propagules led us to compare the ability of *C. neoformans* var. *grubii* spores and yeast to colonize the lungs. To gain insights into the fate of spores during the early stages of infection, groups of four C57BL/6 mice were infected by intranasal inoculation with spores (10^5) or parental yeast strains (10^5), and CFU analysis was performed at days 3 and 6 postinfection. CFU recovered from spore-infected mice (2.16×10^5 and 7.85×10^5 CFU/g of lung tissue) were significantly lower than yeast-infected mice (2.47×10^6 and 8.17×10^6 CFU/g of lung tissue) ($P < 0.05$) at both time points (Fig. 2). The recovery of 10-fold fewer CFU at days 3 and 6 postinfection from the lungs of spore-infected mice suggested that *C. neoformans* spores might be susceptible to innate pulmonary defense mechanisms.

Alveolar macrophages phagocytose *C. neoformans* spores. Alveolar macrophages provide the first line of innate cellular immunity against inhaled pathogens in the lungs (26, 44). To determine whether alveolar macrophages interacted with *C. neoformans* spores, in vitro phagocytosis assays were performed. Because of the large number of spores required for these assays, it was not feasible to use spores from *C. neoformans* var. *grubii* strains. Thus, we used spores from *C. neoformans* var. *neoformans* strains, which produced spores in abun-

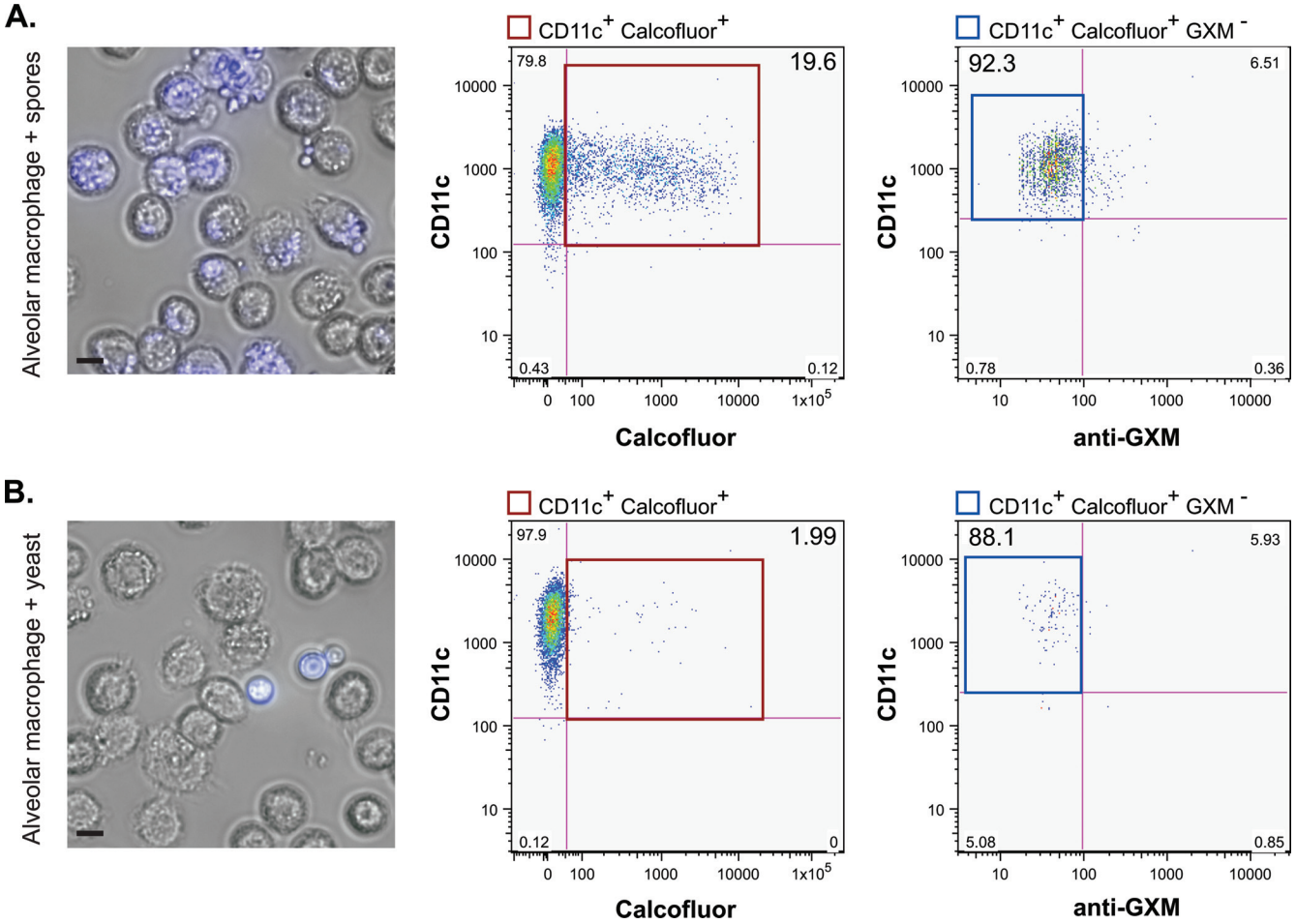


FIG. 3. *C. neoformans* var. *neoformans* spores are phagocytosed by alveolar macrophages. *C. neoformans* var. *neoformans* spores (A) and yeast (B) were coincubated with adherent mouse alveolar macrophages at a multiplicity of infection of 4:1. Fluorescence microscopy images of alveolar macrophages (differential interference contrast) incubated with calcofluor stained (blue) spores and yeast. Only spores are phagocytosed by alveolar macrophages. Flow cytometric analysis of alveolar macrophages incubated with calcofluor-stained spores and yeast confirmed that spores were phagocytosed. An AF488-labeled anti-CD11c antibody was used to detect alveolar macrophages, spores and yeast were labeled with calcofluor white, and an AF594-labeled anti-GXM antibody was used to discern between extracellular and intracellular spores or yeast.

dance (4, 29, 42, 45). For detection purposes, spores and yeast were labeled with calcofluor white (blue color). Both were added to alveolar macrophages at a multiplicity of infection of four spores to one macrophage (4:1). We observed by confocal microscopy that, in contrast to *C. neoformans* yeast, spores were readily phagocytosed by alveolar macrophages (Fig. 3). Z-stack analysis confirmed the intracellular localization of spores.

To further analyze spore-alveolar macrophage interactions, flow cytometric analysis was performed. We took advantage of the fact that the capsular polysaccharide glucuronoxylomannan (GXM) was present on the surface of both spores (4) and yeast (3, 35). An AF488-labeled anti-CD11c antibody was used to detect alveolar macrophages, spores and yeast were labeled with calcofluor white, and an AF594-labeled anti-GXM antibody (5) was used to distinguish between alveolar macrophages with extracellularly attached versus intracellular spores. Based on this staining strategy, alveolar macrophages with attached extracellular spores would be $CD11c^+$ $calcofluor^+$ GXM^+, whereas those with intracellular spores would be $CD11c^+$ $calcofluor^+$ GXM^-. We observed that 19.6% of the alveolar macrophage population interacted with spores, whereas fewer than 2% interacted with yeast (Fig. 3). Interactions between spores and alveolar macrophages resulted in phagocytosis 92% of the time (Fig. 3). The observation that *C. neoformans* yeast were phagocytosed poorly by alveolar macrophages was consistent with previous studies; opsonins such as complement and antibody are required for efficient phagocytosis of *C. neoformans* yeast by macrophages (11). In preliminary experiments, we confirmed that *C. neoformans* var. *grubii* spores were also phagocytosed by alveolar macrophages (data not shown). Because spores from both species were phagocytosed by alveolar macrophages, and *C. neoformans* var. *neoformans* spores were more abundantly available, we chose to use *C. neoformans* var. *neoformans* spores to further investigate spore-alveolar macrophage interactions.

***C. neoformans* spore-alveolar macrophage interactions.** Very little is known about the cell surface composition of *C. neoformans* spores. Thus, we looked to previous studies that investigated macrophage interactions with other pathogenic fungi to

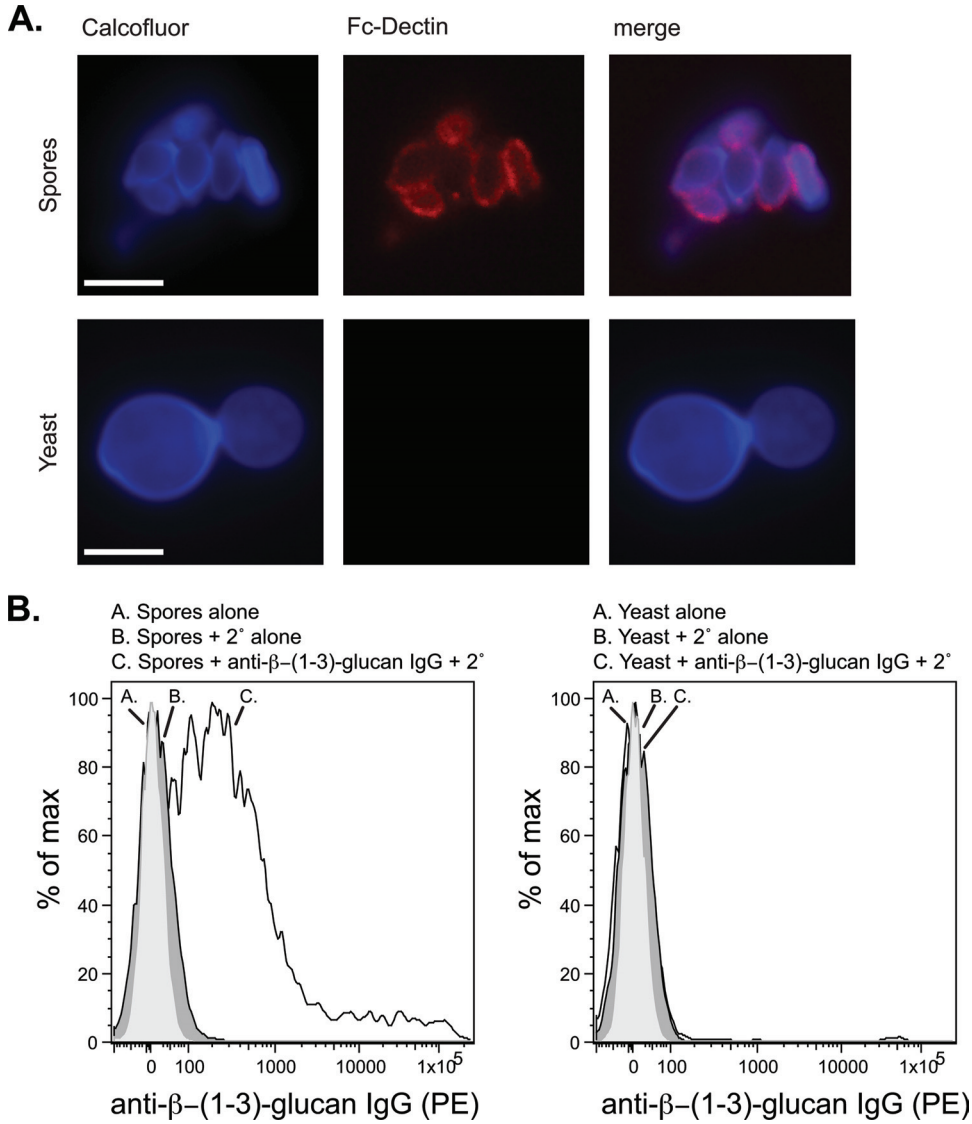


FIG. 4. β-(1,3)-Glucan is accessible on the surface of *C. neoformans* var. *neoformans* spores. (A) Fc-Dectin chimera protein was used to assay for β-(1,3)-glucan on the surface of *C. neoformans* var. *neoformans* spores and yeast. Fluorescence microscopy of Fc-Dectin binding showed that β-(1,3)-glucan was on the surface of spores but not yeast. Bar, 5 μm. (B) Flow cytometric analysis of anti-β-(1,3)-glucan IgG antibody binding to *C. neoformans* var. *neoformans* spores and yeast confirmed that β-(1,3)-glucan was accessible on the surface of spores but not yeast. No binding was observed with the secondary antibody alone control.

identify candidate receptor-ligand interactions for study in our system. These studies identified numerous host PRRs, including Dectin-1, mannose receptor, and CD11b, that were involved in mediating phagocytosis (7). Interactions between the host PRR Dectin-1 and fungal PAMP β-(1,3)-glucan were shown to be particularly important for host defense against a variety of pathogenic fungi (7, 25, 28). We confirmed that β-(1,3)-glucan was accessible on the surface of *C. neoformans* spores (Fig. 4). Two different reagents were used to detect β-(1,3)-glucan: a fluorescently labeled Fc-Dectin chimera protein consisting of the Fc portion of human immunoglobulin G (IgG) and the receptor portion of mouse Dectin-1 (24) was used for confocal microscopy (Fig. 4A), and an anti-β-(1,3)-glucan antibody was used for flow cytometric analysis (Fig. 4B).

The presence of β-(1,3)-glucan on the surface of *C. neoformans* spores led us to investigate whether interactions between β-(1,3)-glucan and Dectin-1 were important for phagocytosis. Competitive inhibition of the Dectin-1 receptor with lamanarin (1 mg/ml), a linear polysaccharide consisting of β-(1,3)- and β-(1,6)-glucan linkages, inhibited phagocytosis by 48% ± 5.21% (Table 1). Likewise, blocking the availability of β-(1,3)-glucan with Fc-Dectin chimera protein or the availability of the Dectin-1 receptor with anti-Dectin-1 blocking antibody inhibited phagocytosis by 66% ± 10.02% and 60% ± 4.56%, respectively (Table 1). Blocking antibodies against CD11b, a component of the leukocyte adhesion receptor CR3 (CD11b/CD18) (8), inhibited phagocytosis by 34% ± 5.80%. A control antibody against CD11c, a component of the leukocyte adhesion receptor CR4 (CD18/CD11c), had no effect on phagocytosis. Surprisingly, the addition of soluble α-mannan (1 mg/

TABLE 1. *C. neoformans*-macrophage receptor ligand interactions[a]

Treatment	Mean % inhibition of phagocytosis ± SE	*P*
Spores treated with Fc-Dectin	66.01 ± 10.02	<0.0001
AM treated with anti-Dectin-1	60.2 ± 4.56	<0.0011
AM treated with lamanarin	48.02 ± 5.21	<0.0001
AM treated with anti-CD11b	33.92 ± 5.80	<0.001
AM treated with anti-CD11c	ND	>0.05
AM treated with α-mannan	ND	>0.05

[a] The percent inhibition uptake is given relative to the uptake of spores by alveolar macrophages (AM) in the absence of treatment. *P* values indicate whether there was a statistically significant difference between treatments and the untreated control. ND, no difference from the untreated control.

ml), which binds mannose receptor (CD206), had no detectable effect on phagocytosis (Table 1). Collectively, these results demonstrate that β-(1,3)-glucan, Dectin-1, and CD11b contribute to the phagocytosis of *C. neoformans* spores.

Alveolar macrophage activation state and spore survival. Previous studies have demonstrated a correlation between the activation state of macrophages and antimicrobial activity (26, 44). Activated alveolar macrophages exhibit an increased ability to phagocytosis and kill pathogenic microorganisms (26). To determine the functional consequences of phagocytosis on the viability of *C. neoformans* spores, we first investigated whether intracellular *C. neoformans* spores could germinate in unstimulated alveolar macrophages. Microscopic examination of alveolar macrophages coincubated with *C. neoformans* spores 24 h postinoculation revealed numerous intracellular yeast (Fig. 5A). The presence of budding daughter cells (white arrow) indicated that yeast cells were viable and replicating intracellularly (Fig. 5A). The halo observed around yeast was suggestive of the production of capsular polysaccharide, a key virulence determinant for *C. neoformans* yeast (35). Confocal microscopy was performed to confirm the intracellular localization of yeast (stained blue with calcofluor) within alveolar macrophages (stained green with phalloidin-fluorescein isothiocyanate) (Fig. 5B). During this analysis, we also observed numerous instances in which yeast appeared to be undergoing phagosome extrusion (Fig. 5B and C), a process that enables intracellular *C. neoformans* yeast to escape into the extracellular space without damaging host cells (1).

Further investigation revealed that intracellular germination of spores and/or replication of yeast were severely restricted when alveolar macrophages were stimulated with LPS and gamma interferon (Fig. 5D). The CFU shown at time point zero represent the number of spores initially phagocytosed by alveolar macrophages, whereas those at the 24-h time point indicate germination and subsequent intracellular replication of yeast over time. To resolve the question of which form of *C. neoformans* was susceptible to killing, spores and yeast were opsonized with anti-GXM IgG and coincubated with stimulated alveolar macrophages. The rationale for doing this was to ensure that phagocytosis proceeded through a common receptor, the Fcγ receptor. We observed an ~10-fold increase in CFU in the yeast group over time but not in the spore group (Fig. 5E). Collectively, these results showed that *C. neoformans* spores were susceptible to killing by stimulated alveolar macrophages.

Reactive oxygen-nitrogen intermediates contribute to host defense against *C. neoformans* spores. One mechanism activated alveolar macrophages use to kill pathogenic microorganisms is the production of reactive oxygen-nitrogen species (6, 17, 27, 31). To determine whether the antimicrobial activity of activated alveolar macrophages was due to the production of reactive oxygen-nitrogen species, we used alveolar macrophages from iNOS$^{-/-}$ gp47$^{phox-/-}$ knockout mice. These mice lack NADPH oxidase and inducible nitric oxide synthase, which are required for the production of reactive oxygen-nitrogen species. In contrast to our earlier results with wild-type alveolar macrophages, stimulation of iNOS$^{-/-}$ gp47$^{phox-/-}$ alveolar macrophages did not dramatically affect spore germination (Fig. 6A). The increase in CFU over time was comparable for both groups, indicating that in the absence of reactive oxygen-nitrogen species spores were able to germinate intracellularly.

To determine the in vivo relevance of this observation, groups of four iNOS$^{-/-}$ gp47$^{phox-/-}$ or C57BL/6 mice were infected by intranasal inoculation with 10^5 *C. neoformans* var. *grubii* spores. The median time to death of iNOS$^{-/-}$ gp47$^{phox-/-}$ mice (25 days) was significantly less ($P < 0.004$) than for wild-type mice (28.4 days) (Fig. 6B), implicating a role for reactive oxygen-nitrogen species in innate immunity against *C. neoformans* spores. Moreover, we also compared the susceptibility of iNOS$^{-/-}$ gp47$^{phox-/-}$ and wild-type mice to infection with *C. neoformans* var. *grubii* strain H99 yeast. Previous studies have shown that *C. neoformans* strain H99 yeast are highly virulent and resistant to the effects of reactive oxygen-nitrogen species (9, 21, 22). Thus, we would anticipate that the absence of reactive oxygen-nitrogen species would not increase the susceptibility of iNOS$^{-/-}$ gp47$^{phox-/-}$ mice to infection by *C. neoformans* strain H99 yeast. To test this hypothesis, groups of four iNOS$^{-/-}$ gp47$^{phox-/-}$ or C57BL/6 mice were infected by intranasal inoculation with 10^5 *C. neoformans* strain H99 yeast. We observed no difference in the median time to death for wild-type and iNOS$^{-/-}$ gp47$^{phox-/-}$ KO mice (Fig. 6C). Taken together, these data indicate that reactive oxygen-nitrogen species contribute to innate immunity against *C. neoformans* spores but not against *C. neoformans* yeast.

DISCUSSION

Spores are the most common form of infectious propagule produced by the human pathogenic fungi. Spores produced by the dermatophytes (*Epidermophyton* spp., *Trichophyton* spp., and *Microsporum* spp.), *Penicillium marneffei*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Aspergillus fumigatus* cause significant morbidity and mortality in humans and other animals (38). The identification of a sexual cycle in *C. neoformans* (29) led Cohen et al. (12) to propose that spores are infectious propagules. Despite a lack of experimental evidence, in the last three decades it has become generally accepted that *C. neoformans* spores are infectious propagules. In the present study, we provide evidence that spores from virulent *C. neoformans* strains are pathogenic in a mouse model of cryptococcosis and provide mechanistic insights into their interactions with alveolar macrophages.

Efforts to elucidate the role of spores in the infectious life cycle of *C. neoformans* have been greatly hindered due to the difficulty of isolating spores from *Cryptococcus* species. There

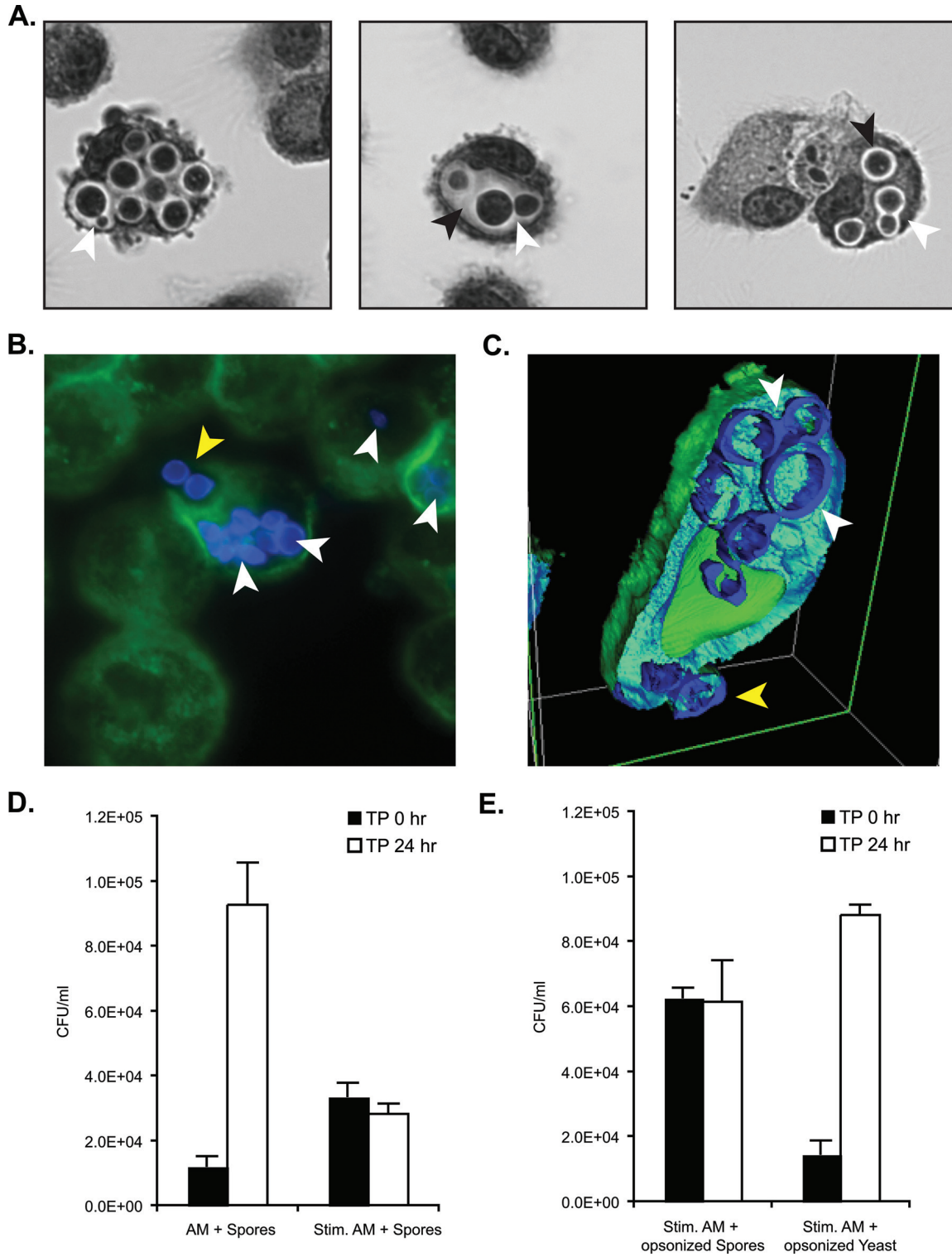
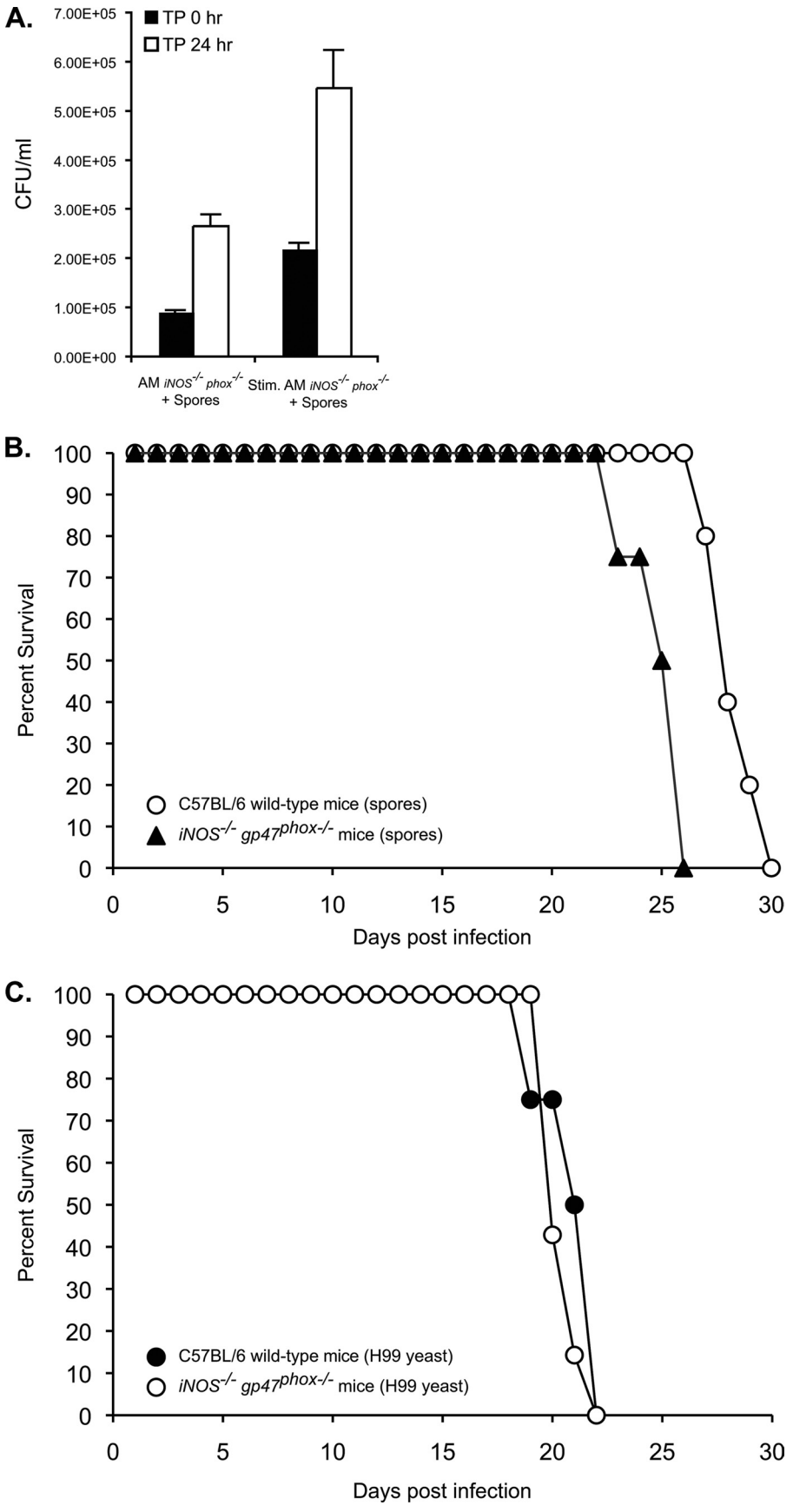


FIG. 5. Intracellular survival of *C. neoformans* var. *neoformans* spores is dependent on alveolar macrophage activation state. (A) *C. neoformans* var. *neoformans* spores were cocultured with untreated alveolar macrophages, and intracellular germination was assessed 24 h postinfection. Numerous budding yeast (white arrowheads) enveloped in capsule (black arrowheads) were observed inside alveolar macrophages. (B) Within 24 h, intracellular *C. neoformans* var. *neoformans* spores germinated into yeast (white arrowheads). Confocal microscopy of calcofluor labeled yeast inside actin stained alveolar macrophages showed yeast exiting intact alveolar macrophages (yellow arrow). (C) Three-dimensional reconstruction of a deconvolved confocal image of calcofluor labeled yeast inside actin-stained alveolar macrophages. The three-dimensional image confirmed the intracellular localization of yeast (white arrowheads) and exit of yeast from the alveolar macrophages (yellow arrowhead). The images are representative of three independent experiments. (D) *C. neoformans* var. *neoformans* spores were cocultured with alveolar macrophages (AM) treated with LPS and IFN-γ (Stim.) or left untreated as a control. After 4 h of coculture the wells were washed to remove extracellular spores and yeast, and cultures were allowed to continue for 24 h. Phagocytosis (time point [TP] 0 h) and inhibitory activity (TP 24 h) were assessed by CFU analysis. The results demonstrate that activation state affects alveolar macrophage (AM) antifungal activity. (E) An identical experiment was performed with spores and yeast opsonized with anti-GXM IgG. Activated macrophages inhibited the growth of spores, but not yeast. The bars represent means plus the standard errors.


A.
TP 0 hr
TP 24 hr
CFU/ml
7.00E+05
6.00E+05
5.00E+05
4.00E+05
3.00E+05
2.00E+05
1.00E+05
0.00E+00
AM iNOS-/- phox-/- + Spores
Stim. AM iNOS-/- phox-/- + Spores
B.
Percent Survival
C57BL/6 wild-type mice (spores)
iNOS-/- gp47phox-/- mice (spores)
Days post infection
C.
Percent Survival
C57BL/6 wild-type mice (H99 yeast)
iNOS-/- gp47phox-/- mice (H99 yeast)
Days post infection

are two previous reports in which *C. neoformans* spores were successfully isolated and tested for virulence (42, 45). In both studies, spores were isolated from *C. neoformans* var. *neoformans* parental strains (42, 45). Although these strains sporulate abundantly, they have the limitation of being minimally virulent. This was reflected in the findings of both studies: *C. neoformans* var. *neoformans* spores were infectious in mice but did not cause morbidity or mortality (42, 45). To extend the findings of these studies, we modified a purification protocol developed for the isolation of *C. neoformans* var. *neoformans* spores (4) and isolated spores from the more virulent *C. neoformans* var. *grubii* strains. We found that spores from virulent *C. neoformans* strains caused morbidity and mortality in mice (from two independent genetic backgrounds) equivalent to that of yeast. These observations demonstrated that *C. neoformans* spores are infectious propagules. Based on these findings, we propose that the infectious life cycle of *C. neoformans* include both yeast and spores as infectious propagules.

Alveolar macrophages provide the first line of innate cellular defense against inhaled pathogens in the lungs (26, 44). The recognition of PAMPs by host PRRs enables alveolar macrophages to phagocytose and kill a wide variety of inhaled pathogens (26, 44). The PRR Dectin-1, which recognizes the fungal PAMP β-(1,3)-glucan, contributes to innate immunity against several pathogenic fungi, including *Aspergillus fumigatus*, *Pneumocystis carinii*, *Histoplasma capsulatum*, and *Candida albicans* (14, 19, 37, 40, 41). We found that Dectin-1 also plays an important role in the recognition of *C. neoformans* spores by alveolar macrophages. The leukocyte adhesion receptor CR3 (CD11b/CD18), a member of the CD18 subfamily of integrins, is another host receptor that binds fungal PAMPs, including β-glucans and mannose. Recognition of these carbohydrate structures is mediated by a lectin-like binding domain in CD11b (7). We found that blocking CD11b inhibited phagocytosis of *C. neoformans* spores by alveolar macrophages, a finding consistent with previous studies in which *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *Candida albicans* were shown to exploit CD11b/CD18 as a means of gaining entry into host cells (2, 18, 34). As a control, we also blocked the CD11c component of leukocyte adhesion receptor CR4 (CD11c/CD18) and observed no inhibition of phagocytosis. Thus, it is unlikely that the effect of blocking CD11b was due to indirect interference with CD18, which is not known to bind carbohydrates. Given that soluble α-mannan did not inhibit phagocytosis, interactions between CD11b and β-(1,3)-glucan may be important for phagocytosis. This also ruled out the possibility that mannose receptor, another host pattern recognition receptor, was involved in phagocytosis of *C. neoformans* spores. The observation that none of our treatments completely inhibited phagocytosis suggests that additional receptor-ligand interactions that remain to be discovered are involved in the phagocytosis of *C. neoformans* spores.

Not surprisingly, the functional consequence of phagocytosis on *C. neoformans* spores was largely dependent on the activation state of alveolar macrophages. However, it was surprising that the antimicrobial activity against spores was mediated by reactive oxygen-nitrogen species. *C. neoformans* yeast possess a highly redundant antioxidant defense system that provides protection against innate immune mechanisms that utilize reactive oxygen-nitrogen species (9, 21, 22). In our in vitro studies, reactive oxygen-nitrogen species accounted for nearly all of the antimicrobial activity of alveolar macrophages against *C. neoformans* spores. We observed equivalent spore germination and subsequent intracellular replication of spore-derived yeast over time in alveolar macrophages from iNOS$^{-/-}$ gp47$^{phox-/-}$ mice, independent of activation state. Whether this is the case in vivo is not clear. It is possible that in vivo, additional components of the pulmonary innate immune response could modulate the response of alveolar macrophages to spores. For example, binding of complement or the surfactant proteins (SP-D and SP-A) to *C. neoformans* spores could have immunomodulatory effects and affect the outcome of phagocytosis (26, 43, 44). The observation that iNOS$^{-/-}$ gp47$^{phox-/-}$ mice exhibited increased susceptibility to infection by *C. neoformans* spores, but not yeast, provided compelling evidence that the production of reactive oxygen-nitrogen species played a role in innate immunity against spores and demonstrated an important relationship between morphotype and pathogenesis. Taken together, our results suggest that the survival of intracellular *C. neoformans* spores is dependent on their ability to germinate into yeast, which are facultative intracellular pathogens (16), before alveolar macrophages become activated.

Based on our in vitro results, intracellular parasitism might explain how mice infected with *C. neoformans* spores or yeast had dramatically different pulmonary fungal burdens but similar median times to death. We observed that spores germinated in alveolar macrophages, and the subsequent yeast replicated intracellularly and escaped into the extracellular space without damaging the host cells. This is consistent with previous studies that have shown that yeast are intracellular pathogens (16). Moreover, it has been shown that intracellular *C. neoformans* yeast replicate faster than extracellular yeast (15), suggesting that alveolar macrophages serve as a protected niche and nutrient source for yeast. Because yeast replication is based on a geometric progression, it is possible that even a very small increase in the replication rate could potentially offset very large differences in the initial population size.

The results of the present study suggest that *C. neoformans* may provide an attractive model for studying host-fungal spore interactions. The robust genetic and immunological tools avail-

FIG. 6. Alveolar macrophage (AM) inhibitory activity is mediated by reactive oxygen-nitrogen species. (A) *C. neoformans* var. *neoformans* spores were cocultured with mouse alveolar macrophages from iNOS$^{-/-}$ gp47$^{phox-/-}$ double-knockout mice for 4 h. Phagocytosis (time point [TP] 0 h) and inhibitory activity (TP 24 h) were assessed by CFU analysis. Stimulated macrophages from knockout mice did not inhibit spore germination. (B) Groups of four iNOS$^{-/-}$ gp47$^{phox-/-}$ or C57BL/6 mice were infected with 10^5 *C. neoformans* var. *grubii* spores via intranasal inhalation. There was a statistically significant difference in the median time to death ($P < 0.004$) of iNOS$^{-/-}$ gp47$^{phox-/-}$ mice infected mice compared to or C57BL/6 wild-type mice. (C) In contrast, there was no significant difference in the median time to death of groups of four iNOS$^{-/-}$ gp47$^{phox-/-}$ or C57BL/6 mice infected with *C. neoformans* var. *grubii* strain H99 yeast (10^5) via intranasal inhalation.

able for the study of *C. neoformans* pathogenesis provide an opportunity to gain insights into the molecular mechanisms that control germination and parasitization of host immune cells and to discover additional fungal PAMPs and host PRRs involved in spore-host immune cell interactions. We anticipate that this model will yield insights into the pathogenic implications of infections caused by spores and facilitate the development of therapeutic interventions for the prevention and treatment of fungal infections.

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REFERENCES

1. **Alvarez, M., and A. Casadevall.** 2006. Phagosome extrusion and host-cell survival after *Cryptococcus neoformans* phagocytosis by macrophages. Curr. Biol. **16:**2161–2165.
2. **Arnaout, M. A.** 1990. Structure and function of the leukocyte adhesion molecules CD11/CD18. Blood **75:**1037–1050.
3. **Bose, I., A. J. Reese, J. J. Ory, G. Janbon, and T. L. Doering.** 2003. A yeast under cover: the capsule of *Cryptococcus neoformans*. Eukaryot. Cell **2:**655–663.
4. **Botts, M. R., S. S. Giles, M. A. Gates, T. R. Kozel, and C. M. Hull.** 2009. Isolation and characterization of *Cryptococcus neoformans* spores reveals a critical role for capsule biosynthesis genes in spore biogenesis. Eukaryot. Cell **8:**595–605.
5. **Brandt, S., P. Thorkildson, and T. R. Kozel.** 2003. Monoclonal antibodies reactive with immunorecessive epitopes of glucuronoxylomannan, the major capsular polysaccharide of *Cryptococcus neoformans*. Clin. Diagn. Lab. Immunol. **10:**903–909.
6. **Brennan, R. E., K. Russell, G. Zhang, and J. E. Samuel.** 2004. Both inducible nitric oxide synthase and NADPH oxidase contribute to the control of virulent phase I *Coxiella burnetii* infections. Infect. Immun. **72:**6666–6675.
7. **Brown, G. D.** 2006. Dectin-1: a signalling non-TLR pattern-recognition receptor. Nat. Rev. Immunol. **6:**33–43.
8. **Brown, G. D., and S. Gordon.** 2003. Fungal beta-glucans and mammalian immunity. Immunity **19:**311–315.
9. **Brown, S. M., L. T. Campbell, and J. K. Lodge.** 2007. *Cryptococcus neoformans*, a fungus under stress. Curr. Opin. Microbiol. **10:**320–325.
10. **Bulmer, G. S., and J. R. Tacker.** 1975. Phagocytosis of *Cryptococcus neoformans* by alveolar macrophages. Infect. Immun. **11:**73–79.
11. **Casadevall, A., and J. R. Perfect.** 1998. *Cryptococcus neoformans.* ASM Press, Washington, DC.
12. **Cohen, J., J. R. Perfect, and D. T. Durack.** 1982. Cryptococcosis and the basidiospore. Lancet **i:**1301.
13. **Cohen, J., J. R. Perfect, and D. T. Durack.** 1982. Method for the purification of *Filobasidiella neoformans* basidiospores by flow cytometry. Sabouraudia **20:**245–249.
14. **Dennehy, K. M., and G. D. Brown.** 2007. The role of the beta-glucan receptor Dectin-1 in control of fungal infection. J. Leukoc. Biol. **82:**253–258.
15. **Diamond, R. D., and J. E. Bennett.** 1973. Growth of *Cryptococcus neoformans* within human macrophages in vitro. Infect. Immun. **7:**231–236.
16. **Feldmesser, M., Y. Kress, P. Novikoff, and A. Casadevall.** 2000. *Cryptococcus neoformans* is a facultative intracellular pathogen in murine pulmonary infection. Infect. Immun. **68:**4225–4237.
17. **Feng, H. M., and D. H. Walker.** 2000. Mechanisms of intracellular killing of *Rickettsia conorii* in infected human endothelial cells, hepatocytes, and macrophages. Infect. Immun. **68:**6729–6736.
18. **Forsyth, C. B., E. F. Plow, and L. Zhang.** 1998. Interaction of the fungal pathogen *Candida albicans* with integrin CD11b/CD18: recognition by the I domain is modulated by the lectin-like domain and the CD18 subunit. J. Immunol. **161:**6198–6205.
19. **Gantner, B. N., R. M. Simmons, and D. M. Underhill.** 2005. Dectin-1 mediates macrophage recognition of *Candida albicans* yeast but not filaments. EMBO J. **24:**1277–1286.
20. **Giles, S. S., I. Batinic-Haberle, J. R. Perfect, and G. M. Cox.** 2005. *Cryptococcus neoformans* mitochondrial superoxide dismutase: an essential link between antioxidant function and high-temperature growth. Eukaryot. Cell **4:**46–54.
21. **Giles, S. S., J. R. Perfect, and G. M. Cox.** 2005. Cytochrome *c* peroxidase contributes to the antioxidant defense of *Cryptococcus neoformans*. Fungal Genet. Biol. **42:**20–29.
22. **Giles, S. S., J. E. Stajich, C. Nichols, Q. D. Gerrald, J. A. Alspaugh, F. Dietrich, and J. R. Perfect.** 2006. The *Cryptococcus neoformans* catalase gene family and its role in antioxidant defense. Eukaryot. Cell **5:**1447–1459.
23. **Giles, S. S., A. K. Zaas, M. F. Reidy, J. R. Perfect, and J. R. Wright.** 2007. *Cryptococcus neoformans* is resistant to surfactant protein A mediated host defense mechanisms. PLoS ONE **2:**e1370.
24. **Graham, L. M., S. V. Tsoni, J. A. Willment, D. L. Williams, P. R. Taylor, S. Gordon, K. Dennehy, and G. D. Brown.** 2006. Soluble Dectin-1 as a tool to detect beta-glucans. J. Immunol. Methods **314:**164–169.
25. **Herre, J., J. A. Willment, S. Gordon, and G. D. Brown.** 2004. The role of Dectin-1 in antifungal immunity. Crit. Rev. Immunol. **24:**193–203.
26. **Holt, P. G., D. H. Strickland, M. E. Wikstrom, and F. L. Jahnsen.** 2008. Regulation of immunological homeostasis in the respiratory tract. Nat. Rev. Immunol. **8:**142–152.
27. **Jackett, P. S., V. R. Aber, and D. B. Lowrie.** 1978. Virulence and resistance to superoxide, low pH, and hydrogen peroxide among strains of *Mycobacterium tuberculosis*. J. Gen. Microbiol. **104:**37–45.
28. **Kimberg, M., and G. D. Brown.** 2008. Dectin-1 and its role in antifungal immunity. Med. Mycol. **46:**631–636.
29. **Kwon-Chung, K. J.** 1975. A new genus, filobasidiella, the perfect state of *Cryptococcus neoformans*. Mycologia **67:**1197–1200.
30. **Medoff, G., M. Sacco, B. Maresca, D. Schlessinger, A. Painter, G. S. Kobayashi, and L. Carratu.** 1986. Irreversible block of the mycelial-to-yeast phase transition of *Histoplasma capsulatum*. Science **231:**476–479.
31. **Murray, H. W., and D. M. Cartelli.** 1983. Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes. Evidence for oxygen-dependent and -independent leishmanicidal activity. J. Clin. Investig. **72:**32–44.
32. **Neilson, J. B., R. A. Fromtling, and G. S. Bulmer.** 1977. *Cryptococcus neoformans*: size range of infectious particles from aerosolized soil. Infect. Immun. **17:**634–638.
33. **Nemecek, J. C., M. Wuthrich, and B. S. Klein.** 2006. Global control of dimorphism and virulence in fungi. Science **312:**583–588.
34. **Newman, S. L., S. Chaturvedi, and B. S. Klein.** 1995. The WI-1 antigen of *Blastomyces dermatitidis* yeasts mediates binding to human macrophage CD11b/CD18 (CR3) and CD14. J. Immunol. **154:**753–761.
35. **Perfect, J. R.** 2005. *Cryptococcus neoformans*: a sugar-coated killer with designer genes. FEMS Immunol. Med. Microbiol. **45:**395–404.
36. **Powell, K. E., B. A. Dahl, R. J. Weeks, and F. E. Tosh.** 1972. Airborne *Cryptococcus neoformans*: particles from pigeon excreta compatible with alveolar deposition. J. Infect. Dis. **125:**412–415.
37. **Rappleye, C. A., L. G. Eissenberg, and W. E. Goldman.** 2007. *Histoplasma capsulatum* α-(1,3)-glucan blocks innate immune recognition by the β-glucan receptor. Proc. Natl. Acad. Sci. USA **104:**1366–1370.
38. **Rippon, J. W.** 1982. Medical mycology: the pathogenic fungi and the pathogenic actinomycetes. W.B. Saunders Company, Philadelphia, PA.
39. **Ruiz, A., and G. S. Bulmer.** 1981. Particle size of airborn *Cryptococcus neoformans* in a tower. Appl. Environ. Microbiol. **41:**1225–1229.
40. **Steele, C., L. Marrero, S. Swain, A. G. Harmsen, M. Zheng, G. D. Brown, S. Gordon, J. E. Shellito, and J. K. Kolls.** 2003. Alveolar macrophage-mediated killing of *Pneumocystis carinii* f. sp. *muris* involves molecular recognition by the Dectin-1 β-glucan receptor. J. Exp. Med. **198:**1677–1688.
41. **Steele, C., R. R. Rapaka, A. Metz, S. M. Pop, D. L. Williams, S. Gordon, J. K. Kolls, and G. D. Brown.** 2005. The beta-glucan receptor dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*. PLoS Pathog. **1:**e42.
42. **Sukroongreung, S., K. Kitiniyom, C. Nilakul, and S. Tantimavanich.** 1998. Pathogenicity of basidiospores of *Filobasidiella neoformans* var. *neoformans.* Med. Mycol. **36:**419–424.
43. **Wright, J. R.** 2005. Immunoregulatory functions of surfactant proteins. Nat. Rev. Immunol. **5:**58–68.
44. **Zhang, P., W. R. Summer, G. J. Bagby, and S. Nelson.** 2000. Innate immunity and pulmonary host defense. Immunol. Rev. **173:**39–51.
45. **Zimmer, B. L., H. O. Hempel, and N. L. Goodman.** 1984. Pathogenicity of the basidiospores of *Filobasidiella neoformans*. Mycopathologia **85:**149–153.

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