Blastomyces dermatitidis Yeast Cells Inhibit Nitric Oxide Production by Alveolar Macrophage Inducible Nitric Oxide Synthase

Nicole M. Rocco, John C. Carmen, and Bruce S. Klein

Microbiology Doctoral Training Program, Departments of Pediatrics, Internal Medicine, and Medical Microbiology and Immunology, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin 53792

Received 24 November 2010/Returned for modification 17 December 2010/Accepted 17 February 2011

The ability of pathogens to evade host antimicrobial mechanisms is crucial to their virulence. The dimorphic fungal pathogen Blastomyces dermatitidis can infect immunocompetent patients, producing a primary pulmonary infection that can later disseminate to other organs. B. dermatitidis possesses a remarkable ability to resist killing by alveolar macrophages. To date, no mechanism to explain this resistance has been described. Here, we focus on macrophage production of the toxic molecule nitric oxide as a potential target of subversion by B. dermatitidis yeast cells. We report that B. dermatitidis yeast cells reduce nitric oxide levels in the supernatants of activated alveolar macrophages. This reduction is not due to detoxification of nitric oxide, but rather to suppression of macrophage nitric oxide production. We show that B. dermatitidis yeast cells do not block upregulation of macrophage inducible nitric oxide synthase (iNOS) expression or limit iNOS access to its arginine substrate. Instead, B. dermatitidis yeast cells appear to inhibit iNOS enzymatic activity. Further investigation into the genetic basis of this potential virulence mechanism could lead to the identification of novel antifungal drug targets.

Without exception, pathogens must evolve strategies for evading the antimicrobial mechanisms of their host’s immune system. For pulmonary pathogens, this means negotiating the microbicidal arsenal of the lung-resident alveolar macrophage. This arsenal includes a highly efficient phagocytic infrastructure and complex enzymatic systems devoted to the production of reactive nitrogen species (ROS) and reactive nitrogen species (RNS) (18). These systems are triggered by the recognition of an invading pathogen, most commonly via pattern recognition receptors (PRRs) that recognize evolutionarily conserved pathogen-associated molecular patterns (PAMPs) (20). Thus, any pathogen invading the lung environment risks activating a network of deadly antimicrobial systems. Complicating matters is the potential for amplification of these systems by proinflammatory cytokines produced by adaptive immune cells, most notably gamma interferon (IFN-γ) produced by CD4+ helper T cells (12). This means that pathogens that survive the initial period of colonization and replication in the lung must withstand an even more potent round of antimicrobial activation once the adaptive immune system is fully engaged.

Understanding the mechanisms by which pathogens evade these antimicrobial systems to infect their hosts is key to the identification of novel drug targets and the generation of safe live attenuated vaccine strains. These goals are of particular importance for fungal infections, since current drug therapies are limited and no vaccines are available (9). The dimorphic fungi are a major cause of systemic fungal infections in both immune-compromised and immunocompetent individuals, accounting for several million infections worldwide each year (8, 19). This group of fungi has the characteristic ability to grow in two different morphotypes: a sporulating hyphal form at ambient temperature in the environment and a pathogenic yeast form at elevated temperatures within the host. Blastomyces dermatitidis is a typical dimorphic fungus with a hyphal form that grows in the soil and generates infectious conidia, which are aerosolized and inhaled into the lungs of a susceptible host. Once in the lung, these conidia undergo their characteristic phase transition and begin replicating as budding yeast (3).

Pathogenic fungi are adept at avoiding killing by innate immune effector cells, including lung-resident alveolar macrophages. For example, B. dermatitidis yeast cells demonstrate remarkable resistance to the antimicrobial mechanisms of the alveolar macrophage. In cocultures, resting alveolar macrophages have no discernible effect on the growth of B. dermatitidis yeast cells. Even activated alveolar macrophages are only weakly effective against B. dermatitidis yeast cells, killing approximately 25% of the inoculum (5). In comparison, activated alveolar macrophages kill more than 90% of the inoculum when incubated with the nonpathogenic fungus Saccharomyces cerevisiae (N. M. Rocco and B. S. Klein, unpublished data). Perhaps more importantly, even this small amount of growth inhibition is transient, and B. dermatitidis yeast cells overcome macrophage defenses to continue replicating during a 72-h coculture (4). Since alveolar macrophages are not only the first immune cells likely to encounter B. dermatitidis in the lung, but also major targets of Th1 (35) and Th17 (36) cells during the adaptive phase of immunity, yeast resistance to killing by these cells is likely to play an important role in the virulence of the pathogen.

Despite extensive investigations into the importance of macrophage generation of the RNS nitric oxide in antifungal immunity and fungal virulence, the role of the molecule remains elusive. Work with the pathogenic fungi Histoplasma capsula-
tum (2, 24), Paracoccidioides brasiliensis (17, 22), Coccidioides immitis (15), and Cryptococcus neoformans (31) has shown that the macrophage nitric oxide-producing enzyme, inducible nitric oxide synthase (iNOS), promotes host clearance of invading fungi. Conversely, each of these fungal pathogens has evolved the means to subvert nitric oxide production or resist killing by nitric oxide (6, 10, 13, 23, 25, 30, 32, 37). Thus, the potentially devastating production of nitric oxide by alveolar macrophages is a selective pressure that has shaped the evolution of anti-RNS virulence factors among pathogenic fungi. Modulation of such a pivotal system, by impairing either the host’s ability to produce RNS or the pathogen’s ability to produce anti-RNS virulence factors, could play a crucial role in tipping the balance toward success of the host or the pathogen.

Consistent with this theory, it is clear that the nitric oxide production apparatus is essential to the host’s ability to eliminate B. dermatitidis yeast cells from the lungs during infection. Wild-type mice that are vaccinated with a live-attenuated strain of B. dermatitidis are more efficient at clearing B. dermatitidis yeast cells than vaccinated iNOS-deficient mice, as demonstrated by significantly lower fungal burdens. In contrast, the ability of the host to produce nitric oxide appears to be dispensable during primary infection, with iNOS-deficient mice succumbing to infection at the same time as wild-type mice (35). This dichotomy suggests the possibility that both host and pathogen survival strategies target the production of nitric oxide. In the case where the host prevails, vaccination gives the host a critical head start against B. dermatitidis, allowing the adaptive immune system to fully activate essential antimicrobial mechanisms and clear yeast from the lungs in a nitric oxide-dependent manner. We hypothesize that in the case where the pathogen prevails, the immune system of the naive host is unable to combat yeast virulence factors that target host nitric oxide production, leading to unchecked growth of the invading pathogen.

Here, we investigated whether B. dermatitidis yeast cells interfere with the macrophage nitric oxide system. We show that B. dermatitidis yeast cells reduce nitric oxide levels in the supernatants of activated macrophages. We further demonstrate that this reduction occurs, not by detoxification of nitric oxide, but by suppression of macrophage nitric oxide production. Our data eliminate two common mechanisms by which other pathogens suppress nitric oxide production: reduction of iNOS expression and limitation of macrophage access to arginine, the iNOS substrate. Our results instead point to interference with iNOS enzymatic activity as a potential mechanism by which B. dermatitidis yeast cells avoid killing by macrophages.

**MATERIALS AND METHODS**

**Reagents.** All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. RAW264.7 cell cultures. RAW 264.7 murine macrophage-like cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and maintained in complete RPMI: RPMI 1640 medium (HyClone, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (FBIS, Atlanta Biologicals, Lawrenceville, GA) and penicillin-streptomycin (Fisher Chemical, Fairlawn, NJ). Yeast cultures. B. dermatitidis strain 26199 was obtained from the ATCC and maintained at 39°C on slants of Middlebrook 7H10 agar. The slants were passaged at 6-day intervals, and fresh stocks were thawed from liquid nitrogen stores after 20 passages. Cryptococcus neoformans var. grubii serotype A was obtained from the ATCC and maintained at 37°C on yeast-peptone-dextrose (YPD) agar.

**Bone marrow-derived macrophages.** Bone marrow-derived macrophages were obtained as previously described (28). Briefly, total bone marrow was obtained from the femurs of 6- to 10-week-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME). The bone marrow cells were cultured overnight in tissue culture plates in complete RPMI. Cells free in suspension were collected and replated onto tissue culture plates in RPMI plus 20% FBS and 20% L929 cell supernatant as a source of macrophage colony-stimulating factor. The cells were cultured for 7 days before they were used in experiments.

**Alveolar macrophages.** Primary alveolar macrophages were obtained as previously described (5). Six- to 10-week-old male C57BL/6 mice (Jackson Laboratory) were anesthetized with isoflurane (Halocarbon Laboratories, River Edge, NJ) and then exsanguinated from the orbital vein and euthanized with CO2. The trachea was exposed for insertion of a catheter (Fisher Scientific, Pittsburgh, PA). The lungs were flushed repeatedly (10 times per animal) with 1 ml of phosphate-buffered saline (PBS) containing 0.6% EDTA. The recovered fluid was kept on ice, and the cells were centrifuged at 1,500 rpm. The cells were resuspended in complete RPMI, counted, and plated at a density of 1 × 107 cells per well in 96-well plates in 200 μl of medium. The cells were allowed to adhere for 3 to 16 h before thorough washing to remove red blood cells. The remaining monolayer of alveolar macrophages was used for subsequent experiments.

**Yeast-macrophage cocultures.** Macrophages were plated in tissue culture plates at a final density of 1 × 105 cells/250 μl in 96-well plates in 20 μl 48-well plates, or 4 × 107 cells/1 ml in 24-well plates. All macrophages were allowed to adhere for 3 to 16 h before subsequent treatments. Resting cells were incubated in complete RPMI. Where indicated, the cells were primed overnight with exposure to 50 U/ml of recombinant murine IFN-γ. The primed cells were subsequently activated with 100 μg/ml of zymosan. Where indicated, macrophages were incubated with the iNOS inhibitor L-NG-monomethyl arginine citrate (L-NMMA) (Cayman Chemical, Ann Arbor, MI) to prevent nitric oxide production or the nitric oxide donor diethylamine NONOate (DETA NONOate) (Cayman Chemical), at a concentration of 100 μM, as an exogenous source of nitric oxide.

In some experiments, macrophages were incubated with B. dermatitidis yeast cells at the indicated multiplicity of infection (MOI). Yeast cells were prepared for coculture following harvest from 2- to 3-day-old slants. Yeast cells were suspended in complete RPMI medium and passed 5 times through a 22-gauge needle to disrupt aggregates. The cells were pelleted at 2,300 rpm and counted using eosin to distinguish live from dead cells. Where indicated, yeast cells were heat killed by incubation for 1 h at 65°C.

**Nitrite determination.** At the indicated times, supernatants were harvested from the macrophage-yeast cocultures and used for nitrite determination via the Griess reaction, as previously described (25). Briefly, samples were centrifuged at 1,500 rpm to pellet cells. One hundred microliters of the remaining yeast-conditioned medium, a freshly passaged culture was incubated for 48 h. Nitrite concentrations of the supernatants were normalized to the calculated number of live cells for each sample.

**Preparation of yeast-conditioned medium.** B. dermatitidis yeast cells were harvested from 2- to 3-day-old slants and resuspended in liquid histoplasma macrophage medium (HMM) supplemented with penicillin-streptomycin. Yeast cultures were grown in flasks at 37°C and 250 rpm. Cells were passaged 2 to 3 times at 3-day intervals before their use in experiments. For generation of yeast-conditioned medium, a freshly passaged culture was incubated for 48 h. Cells were pelleted at 2,300 rpm, and the remaining supernatant was passed through a 0.2-μm filter and used immediately in macrophage cultures.

**Yeast assays.** B. dermatitidis yeast cells were harvested from slants and acclimated to growth in liquid culture as described above. For use in growth curves and detoxification assays, yeast cells were added to HMM plus penicillin-streptomycin at a concentration of 2.5 × 107 cells/ml. C. neoformans yeast cells were acclimated to growth in liquid YPD medium, as described for B. dermatitidis. For use in detoxification assays, yeast cells were added to YPD medium plus penicillin-streptomycin at a concentration of 2.5 × 107 cells/ml. DETA NONOate was added where indicated. The cultures were grown at 37°C and 250 rpm. For growth curves, samples were harvested at the indicated times for measurement of absorbance at 600 nm. The optical density at 600 nm (OD600) for each sample was compared to a standard curve to calculate the number of cells. A fresh standard curve was generated at the time of each experiment. For detoxification
assays, supernatants were analyzed at the indicated times for nitrite concentrations as described above.

**Western blotting.** Macrophage cell lysates were prepared after the monolayers were washed 3 times with PBS, RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, and Roche’s [Indianapolis, IN] Complete Mini EDTA-Free Proteasome Inhibitor Tablets) was added to the monolayer. The cells were scraped from the surface, transferred to Eppendorf tubes, and incubated on ice for 30 min. Debris was pelleted at 13,000 × g for 10 min, and the supernatants were transferred to fresh tubes. Samples were boiled in SDS sample buffer for 5 min, run on 10% SDS gels, and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h in TBS-T (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) plus 5% milk and then incubated with a 1:1,000 dilution of anti-iNOS primary antibody (BD Biosciences, Sparks, MD) and a 1:1,000 dilution of anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primary antibody (Sigma Chemical Co.) in TBS-T plus 5% milk for 1 h. The membranes were washed 3 times in TBS-T and incubated for 1 h with a 1:2,000 dilution of anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) in TBS-T plus 5% milk. The membranes were washed 3 times with TBS-T, and Pierce (Rockford, IL) ECL Western Blotting chemiluminescent substrate was added for 2 min. Bands of interest were visualized by development of X-ray film after exposure to the blot. The bands were quantified using Quantity One software from Bio-Rad Laboratories (Hercules, CA).

**Real-time PCR.** RNA was isolated from macrophage monolayers using a Qiagen (Valencia, CA) RNeasy Kit. cDNA was amplified using Applied Biosciences (Carlsbad, CA) Multiscribe Reverse Transcriptase. Real-Time PCR (RT-PCR) was performed using Bio-Rad’s iQ SYBR Green-containing Supermix. The following protocol was used for real-time PCR: 5 min at 95°C, followed by 40 cycles of 15 s at 95°C and 45 s at 60°C. Transcript levels for targets were normalized to 18S rRNA. Sequences and Integrated DNA Technologies (iDT) reference numbers for the primers are as follows: iNOS (Forward, GCAACCATCAATGCGGACAAC; reference number 388404018; Reverse, CGTACCGCCTCGG; reference number 388411029); arginine (Forward, GCCACCTGCTGCTCTGCAAG; reference number 384537253; Reverse, GTCTGA; reference number 34843943); CAT2B (Forward, GTGTCCTTTCTCCTGAAG; reference number 34843942; Reverse, GGTCTAGCTGTGAA; reference number 38841029); arginase (Forward, GCAACCTGCTGCTCTGCAAG; reference number 348537253; Reverse, GGTCTCGGACCCAGCAATG; reference number 38453943); CAT2B (Forward, GTTCTGGCGTGTGCTGTGATTAC; reference number 37122461; Reverse, TCACAAACCGACGCAAATG; reference number 37122462); 18S rRNA (Forward, GCCCGCTAGAGCTGA; reference number 38347252; Reverse, GGAACCTCCGTTGCTGTT; reference number 38347253).

**Arginine uptake assay.** The macrophage arginine uptake assay was performed as previously described (16). Briefly, yeast-macrophase cocultures were grown as described above. After 24 h, the monolayers were washed, and radiolabeled arginine from Perkin Elmer (Waltham, MA) was added to the cells. The macrophages were incubated with the arginine for 30 min at 37°C and 5% CO₂ and the monolayers were washed with PBS to remove excess arginine. The macrophages were lysed with sterile double-distilled H₂O (ddH₂O), and the resulting lysate was analyzed in the scintillation counter to determine the amount of radiolabeled arginine that was taken up by each sample.

**Statistical analysis.** Differences between treatment groups were analyzed for statistical significance using Student’s t test. A P value of <0.05 was considered statistically significant.

## RESULTS

*B. dermatitidis* yeast cells impair the accumulation of nitric oxide in supernatants of activated macrophages. The production of reactive nitrogen species by macrophages is a crucial part of the immune response to many pathogens, including dimorphic fungi. We used the murine macrophage-like cell line RAW 264.7 to investigate the ability of innate immune cells to produce the RNS nitric oxide during exposure to *B. dermatitidis* yeast cells. We cocultured *B. dermatitidis* yeast cells with resting RAW 264.7 cells and measured nitrate accumulation as an indicator of nitric oxide production after 48 h. There was no detectable nitric oxide production by resting macrophages in response to *B. dermatitidis* yeast cells (Fig. 1A). We then cocultured *B. dermatitidis* yeast with RAW 264.7 cells that had been primed for activation via overnight incubation with 50 U/ml recombinant IFN-γ. To ensure that the macrophages were exposed to a high enough concentration of yeast to trigger nitric oxide production in this experiment, we added *B. dermatitidis* at different MOIs between 0.5 and 10. At all MOIs tested, there was no detectable nitric oxide production by primed macrophages in response to *B. dermatitidis* yeast cells (Fig. 1B).

To determine whether the lack of nitric oxide in the macrophage supernatants was due to a lack of macrophage response or to suppression or detoxification on the part of the yeast, we cocultured *B. dermatitidis* yeast cells with RAW 264.7 cells that were fully activated by overnight exposure to 50 U/ml of recombinant murine IFN-γ and subsequent exposure to the yeast cell wall component zymosan. These cells normally produce large amounts of nitric oxide. However, when the macrophages were exposed simultaneously to both zymosan and *B. dermatitidis* yeast cells, the nitric oxide levels were significantly reduced (Fig. 1C).

To determine whether the reduced amount of nitric oxide in the supernatants of activated macrophages was limited to the RAW 264.7 cell line, we used two types of primary murine macrophages: bone marrow-derived macrophages and alveolar macrophages. As we observed with RAW 264.7 cells, the nitric oxide detected in the supernatants of both of these cell types after activation with IFN-γ and zymosan was significantly reduced upon coculture with *B. dermatitidis* yeast cells (Fig. 1D and E).

To exclude the possibility that macrophage death in the presence of *B. dermatitidis* yeast cells was responsible for the observed reduction of nitric oxide, we enumerated viable cells after coculture of RAW 264.7 cells with *B. dermatitidis* yeast cells. Nitric oxide was determined as a function of the number of viable cells. Upon this analysis, *B. dermatitidis* yeast cells still impaired the accumulation of nitric oxide in the supernatants of activated macrophages (Fig. 1F). Individual wells were also examined microscopically during each experiment. Significant differences in zymosan internalization, yeast internalization, macrophage morphology, and monolayer confluence were not observed between wells (data not shown).

The reduction of nitric oxide by *B. dermatitidis* is dependent on the yeast dose, viability, and contact with macrophages. If *B. dermatitidis* yeast cells are able to specifically reduce nitric oxide levels, either by removal of nitric oxide from supernatants or by suppression of nitric oxide production, exposure of macrophages to increasing numbers of yeast cells should cause a more severe reduction in detectable nitric oxide. To test this prediction, we incubated activated primary murine alveolar macrophages with *B. dermatitidis* yeast cells at MOIs of 0.05, 0.5, and 5. Nitric oxide measurements over a 72-hour time course showed that higher concentrations of yeast caused a greater reduction in detectable nitric oxide (Fig. 2A).

We further hypothesized that *B. dermatitidis* yeast cells at a high enough MOI (such as that found during a progressive pulmonary infection) would be able to completely eliminate detectable nitric oxide from the macrophage supernatants. Nitrite levels were measured in supernatants of activated macrophages incubated for 48 h with *B. dermatitidis* yeast cells at MOIs ranging from 1 to 40. At an MOI of 40, *B. dermatitidis* yeast cells were able to reduce the nitric oxide concentration to that of resting cells (Fig. 2B).

There are many mechanisms by which *B. dermatitidis* yeast cells could reduce nitric oxide levels in cocultures with acti-
vated macrophages. To address this issue, we incubated activated macrophages with either live \textit{B. dermatitidis} yeast cells or \textit{B. dermatitidis} yeast cells that had first been killed by exposure to high temperature. We found that heat-killed yeast cells were unable to reduce the levels of nitric oxide detected in supernatants of activated macrophages (Fig. 2C). These data are compatible with two possibilities: (i) a heat-labile component of the \textit{B. dermatitidis} cell wall could be responsible for the reduction in supernatant nitric oxide levels or (ii) \textit{B. dermatitidis} yeast cells could reduce nitric oxide levels via an active process dependent on yeast viability.

One mechanism by which viable yeast could reduce nitric oxide levels is through the constitutive secretion of a detoxification enzyme or immunosuppressant molecule into the super-

FIG. 1. \textit{B. dermatitidis} yeast cells impair accumulation of nitric oxide in supernatants of activated macrophages. (A) Monolayers of resting RAW 264.7 cells were treated individually with lipopolysaccharide (LPS) or zymosan (as positive controls for nitric oxide production) or with \textit{B. dermatitidis} yeast cells (Bd) at an MOI of 0.5. (B) Monolayers of RAW 264.7 cells were primed by overnight exposure to IFN-\gamma before stimulation with LPS or zymosan (as positive controls for nitric oxide production). \textit{B. dermatitidis} yeast cells were added to the primed monolayers at a range of MOIs. Supernatant nitrite levels were measured 48 h later. In panels A and B, the asterisks indicate a \textit{P} value of <0.05 versus medium alone. (C to E) Monolayers of RAW 264.7 (C), bone marrow-derived (D), or alveolar (E) macrophages were fully activated by overnight incubation with IFN-\gamma and subsequent addition of zymosan as a positive control (Control). \textit{B. dermatitidis} yeast cells at an MOI of 0.5 were added to some wells at the time of zymosan addition. Supernatant nitrite levels were measured after 48 h. (F) Alveolar macrophages were treated as in panel E. After 48 h, supernatant nitrite levels were measured and macrophage viability was assessed by trypan blue exclusion. Nitrite levels are represented relative to the number of viable macrophages observed. In panels C to F, the asterisks indicate a \textit{P} value of <0.05 versus control macrophages. In all panels, the data are represented as the mean ± standard deviations (SD) of 2 or 3 samples per treatment group, representative of 2 experiments.
FIG. 2. The reduction of nitric oxide by *B. dermatitidis* is dependent on the yeast dose, viability, and contact with macrophages. (A) Monolayers of alveolar macrophages were activated with IFN-γ and zymosan (Control). *B. dermatitidis* yeast cells were added to monolayers of activated alveolar macrophages at MOIs of 0.05, 0.5, and 5. Supernatant nitrite levels were measured at distinct time points over a 72-hour time course. The asterisks indicate a $P$ value of $<0.05$ versus control macrophages at the same time point. (B) Monolayers of alveolar macrophages were activated with IFN-γ and zymosan (Control). *B. dermatitidis* yeast cells were added to monolayers of activated macrophages at MOIs between 4 and 40 (Bd 4 to Bd 40). Supernatant nitrite levels were measured after 48 h. The asterisks indicate $P$ values of $<0.05$ versus medium alone. (C) Monolayers of alveolar macrophages were activated with IFN-γ and zymosan (Control). Live *B. dermatitidis* yeast cells (Bd MOI 0.5) or yeast that had been heat killed by a 1-h incubation at 65°C (HK Bd MOI 0.5) were added to the activated macrophages. Nitrite was measured at distinct time points over a 48-h time course. The asterisks indicate $P$ values of $<0.05$ versus control macrophages at the same time point. (D) Yeast-conditioned medium was prepared by filtration of the supernatant from a 48-hour liquid culture of *B. dermatitidis* yeast cells. The yeast-conditioned medium was added at the indicated dilutions (Bd Sup 1:2 to Bd Sup 1:16) to cultures of RAW 264.7 cells that were activated by the addition of IFN-γ and zymosan (Control). Nitrite levels were measured after 24 h. (E) Alveolar macrophage monolayers were grown in 24-well plates and activated by the addition of IFN-γ and zymosan (Control). *B. dermatitidis* yeast cells were added either in direct contact with the macrophages (Bd MOI 0.5 Lower) or separated from the macrophages by a Transwell insert (Bd MOI 0.5 Upper). After 48 h, nitrite levels in the culture supernatants were measured. In panels D and E, the asterisks indicate a $P$ value of $<0.05$ versus control macrophages. In all panels, data are represented as the means ± SD of 2 or 3 samples per treatment group, representative of 2 experiments.
natant. To determine whether the reduction in nitric oxide is mediated by a secreted molecule, we collected HMM in which B. dermatitidis yeast cells had grown for 48 h. The yeast-conditioned medium was added to wells of activated macrophages in increasing concentrations, and nitric oxide levels were measured after 48 h. The yeast-conditioned medium failed to reduce the amount of nitric oxide in the supernatants of activated macrophages at any concentration tested (Fig. 2D).

The above-mentioned results indicate that soluble products from viable yeast do not reduce the nitric oxide levels of macrophages, implying that contact between the yeast and macrophage may be necessary. To address this issue, we grew macrophages in 24-well plates and added B. dermatitidis yeast cells either directly to the wells or to Transwell plate inserts. These inserts allow the diffusion of molecules between the two compartments of the well but prevent the yeast from coming into direct contact with the macrophages. After 48 h of coculture, nitrite levels were measured. When the yeast cells were cocultured in direct contact with the macrophages in the bottom of the well, nitric oxide levels were reduced. However, when the yeast cells were cocultured in the Transwell inserts at the top of the well, no significant reduction in nitric oxide levels was observed (Fig. 2E). Thus, B. dermatitidis yeast cells must be in close proximity to macrophages to suppress nitric oxide levels.

**B. dermatitidis yeast cells are sensitive to chemically generated nitric oxide but fail to remove it from culture supernatants.** The active reduction of nitric oxide levels by B. dermatitidis yeast cells suggests that nitric oxide is a mechanism by which macrophages are able to inhibit the growth of B. dermatitidis yeast cells during infection. This hypothesis predicts that B. dermatitidis yeast cells will be sensitive to nitric oxide even in the absence of macrophages, as when grown in the presence of a nitric oxide-generating compound. To test this prediction, we grew B. dermatitidis yeast cells in the presence of DETA NONOate, a compound that generates nitric oxide with a 24-h half-life, which is similar to the time line over which macrophages produce nitric oxide during coculture with B. dermatitidis yeast cells. Growth of the yeast over a 72-hour time course was assayed by comparing the OD_{600} to a standard curve of B. dermatitidis yeast cells. We observed that B. dermatitidis yeast cells are indeed sensitive to nitric oxide, with significant growth delays at all concentrations tested and complete inhibition of growth at a concentration of 10 mm DETA NONOate (Fig. 3A).

We further hypothesized that if B. dermatitidis yeast cells reduce the amount of detectable nitric oxide by a detoxification mechanism, yeast grown in the presence of DETA NONOate would similarly reduce the amount of detectable nitric oxide in the medium. To test this, we incubated B. dermatitidis yeast cells with increasing concentrations of DETA NONOate for 24 h and measured nitrite in the medium. There was no significant reduction in nitric oxide levels when DETA NONOate was incubated with B. dermatitidis yeast cells compared to incubation with medium alone (Fig. 3B). As a positive control for detoxification, we also grew the pathogenic fungus C. neoformans in the presence of DETA NONOate. As expected, C. neoformans was able to significantly reduce the amount of nitrite in the medium compared to DETA NONOate alone (Fig. 3C). These results indicate that the nitric oxide generated by DETA NONOate can be detoxified by an organism with the capacity for nitric oxide detoxification under our experimental conditions.

Because we showed above (Fig. 2E) that B. dermatitidis yeast cells must be in close proximity to macrophages to reduce nitric oxide levels in coculture supernatants, we wondered whether contact with macrophages might be a trigger for the activation of a yeast nitric oxide detoxification mechanism. To address this question, we set up a system in which B. dermatitidis yeast cells could be incubated with activated macrophages, but the only source of nitric oxide would be exogenously added DETA NONOate. We prevented macrophages from producing their own nitric oxide by the addition of the iNOS inhibitor L-NMMA. We then added DETA NONOate to the cells at a concentration that would generate a level of nitric oxide in the biological range of macrophage nitric oxide production. When B. dermatitidis yeast cells were added to these macrophages, they were no longer able to reduce the levels of nitric oxide (Fig. 3C). We postulate that the failure of B. dermatitidis yeast cells to reduce nitric oxide levels in such a system indicates that nitric oxide reduction by yeast occurs not by detoxification, but instead, by suppression of macrophage nitric oxide production.

**B. dermatitidis yeast cells do not suppress nitric oxide production by reducing expression of iNOS.** One mechanism by which a pathogen could suppress production of nitric oxide by macrophages is by interfering with signaling pathways that trigger upregulation of transcript for iNOS. To determine whether exposure to B. dermatitidis yeast cells causes a reduction in iNOS transcript in activated primary alveolar macrophages, we used RT-PCR to measure the level of iNOS transcript inside the macrophages over a 72-hour time course. Activated macrophages were incubated with B. dermatitidis yeast cells at MOIs of 0.05, 0.5, and 5. The amount of iNOS transcript detected within the macrophage cultures was unaffected by the concentration of B. dermatitidis yeast cells. In fact, macrophages exposed to B. dermatitidis at the highest MOI had iNOS transcript levels that were indistinguishable from those of macrophages that were activated by IFN-γ and zymosan as a positive control (Fig. 4A). Thus, B. dermatitidis does not suppress the production of nitric oxide by reducing the amount of iNOS transcript.

A reduction in iNOS activity could also be achieved through a reduction in the amount of iNOS protein in the macrophage. To determine whether B. dermatitidis yeast cells prevent the normal accumulation of iNOS protein in activated macrophages, we used Western blotting to visualize the levels of iNOS in macrophages that were activated by IFN-γ and zymosan (as a positive control) and macrophages that were cocultured with B. dermatitidis yeast cells. Because we previously observed that reduced levels of nitric oxide upon exposure to B. dermatitidis yeast cells are apparent after just 24 h of coculture (Fig. 2A), we examined iNOS protein levels at 12 h and 24 h of coculture. Our blots showed no significant reduction in iNOS levels between activated macrophages and macrophages cultured with B. dermatitidis yeast cells at an MOI of either 0.5 or 5 (Fig. 4B). In fact, quantification of the bands showed that macrophages cultured with B. dermatitidis yeast cells may contain slightly higher levels of iNOS protein than macrophages that were activated by IFN-γ and zymosan as a control (Fig. 4C). Thus, B. dermatitidis yeast cells do not suppress macrophage production of nitric oxide by reducing iNOS expression.
B. dermatitidis yeast cells do not suppress nitric oxide production by reducing the availability of the arginine substrate. Another potential mechanism for pathogens to suppress nitric oxide production by immune cells is to limit the amount of arginine to which iNOS has access. Because arginine is the substrate from which iNOS produces nitric oxide, this strategy limits the amount of nitric oxide that can be produced by the macrophage. One way to decrease the

FIG. 3. B. dermatitidis yeast cells are sensitive to chemically generated nitric oxide but fail to remove it from culture supernatants. (A) B. dermatitidis yeast cells were grown in liquid medium containing the nitric oxide donor DETA NONOate at the indicated concentrations. Yeast growth was determined by monitoring the OD_{600} at distinct time points over a 72-hour time course. The asterisks indicate a *P* value of <0.05 versus 0 mM DETA NONOate at the same time point. (B) The nitric oxide donor DETA NONOate was added to liquid medium alone (Medium) or in combination with B. dermatitidis (left) or C. neoformans (right) yeast cells (Yeast). After 48 h at 37°C, nitrite levels were measured. The asterisks indicate a *P* value of <0.05 versus medium at the same time point. (C) Monolayers of alveolar macrophages were activated by incubation with IFN-γ and zymosan (Control). B. dermatitidis yeast cells were added to activated macrophages at an MOI of 5. Activated macrophages were also treated with an iNOS inhibitor alone (l-NMMA) to prevent macrophage nitric oxide production or with l-NMMA and the nitric oxide donor DETA NONOate (L-NMMA DETA) to restore nitric oxide levels exogenously. Finally, B. dermatitidis yeast cells were added at an MOI of 5 to activated macrophages that were treated with l-NMMA and DETA NONOate (L-NMMA DETA Bd) to determine whether B. dermatitidis yeast cells, when in contact with activated macrophages, can reduce levels of exogenous nitric oxide. For all treatment groups, nitrite was measured after 24 h. The asterisks indicate a *P* value of <0.05 versus control macrophages. There was no significant difference between the L-NMMA DETA and L-NMMA DETA Bd treatment groups. In panels A and C, data are represented as the mean ± SD of 2 or 3 samples per treatment group, representative of 2 experiments. In panel B, data are represented as the mean ± SD of 3 experiments, each with 2 or 3 samples per treatment group.

B. dermatitidis yeast cells do not suppress nitric oxide production by reducing the availability of the arginine substrate.
availability of arginine is to enzymatically convert it to another metabolite. This could be achieved by hijacking the macrophage's cellular machinery to increase the expression of macrophage arginase, which competes with iNOS for access to arginine.

To determine whether *B. dermatitidis* yeast cells limit macrophage access to arginine in this way, we first looked at transcript levels for arginase by RT-PCR. Upon exposure of activated macrophages to *B. dermatitidis* yeast cells for 12 and 24 h, we observed no significant increase in arginase expression (Fig. 5A). There was a slight increase in arginase expression after 48 h of exposure to *B. dermatitidis*, but since our previous results indicated that nitric oxide reduction occurs by 24 h (Fig. 2A), this increase does not seem to explain the ability of *B. dermatitidis* yeast cells to reduce nitric oxide production.

Nevertheless, to rule out the delayed increase in macrophage arginase expression as a mechanism of nitric oxide suppression, and because pathogens can also produce their own arginine-degrading enzymes (11), we looked at the ability of arginine supplementation to restore maximal nitric oxide production. Exogenous arginine was added to cocultures of activated macrophages and *B. dermatitidis* yeast cells (MOI = 0.5), and nitrite levels were measured after 48 h. Arginine supplementation at concentrations up to 10 mM was unable to increase nitric oxide production to the level of macrophages stimulated with IFN-γ and zymosan as a positive control (Fig. 5B).

To ensure that the macrophages are able to take up the additional arginine in Fig. 5B and supply it to iNOS, we investigated transcript levels of the arginine transporter CAT2B in activated macrophages cocultured with *B. dermatitidis* yeast cells. Because CAT2B is a transporter dedicated to supplying iNOS with arginine (26), it is quickly upregulated upon macrophage exposure to pathogens. RT-PCR indicated an early upregulation of CAT2B transcript in macrophages activated with IFN-γ and zymosan as a positive control. Transcript levels remained activated for 48 to 72 h. There was no significant reduction in CAT2B transcript levels when activated macrophages were exposed to *B. dermatitidis* yeast cells at an MOI of 0.5 or 5 (Fig. 5C).

Because transcript levels do not address transporter function, we also used an arginine uptake assay to determine whether macrophages exposed to *B. dermatitidis* yeast cells were able to adequately take up extracellular arginine. After a short incubation with radiolabeled arginine, macrophage lysates were subjected to scintillation counting to determine the capacity of the cells to take up arginine. When control macrophages, activated by IFN-γ and zymosan, were compared to macrophages incubated with *B. dermatitidis*, there was no significant reduction in arginine uptake due to the yeast (Fig. 5D). These data indicate that *B. dermatitidis* does not suppress nitric oxide production by limiting the ability of macrophage iNOS to access its arginine substrate, and they are compatible with the hypothesis that inhibition of iNOS enzymatic activity is the mechanism underlying *B. dermatitidis* nitric oxide suppression.

---

**FIG. 4.** *B. dermatitidis* yeast cells do not suppress nitric oxide production by reducing expression of iNOS. (A) Monolayers of alveolar macrophages were activated by incubation with IFN-γ and zymosan (Control). *B. dermatitidis* yeast cells were added to the activated macrophages at the indicated MOIs. At distinct time points over a 48-h time course, RNA was harvested from the macrophage monolayers and iNOS transcript levels were measured by real-time PCR. The data were normalized using primers to 18S rRNA. There were no significant differences between treatment groups. The data are represented as the mean ± SD of 2 or 3 wells per treatment group, representative of 2 experiments. (B) Monolayers of alveolar macrophages were activated by incubation with IFN-γ and zymosan. *B. dermatitidis* yeast cells were added to activated macrophages at an MOI of 0.5 (0.5) or an MOI of 5 (5). M indicates resting macrophages incubated with medium alone. Protein was harvested after 12 and 24 h. iNOS levels (visible at 120 kDa [numbers on the right and left]) were analyzed by Western blotting. Protein loading was controlled with an antibody against GAPDH (visible at 38 kDa). (C) iNOS and GAPDH bands from the Western blot shown in panel B were quantified at each time point. iNOS expression data were normalized to GAPDH expression and are represented as the fold change relative to macrophages treated with medium alone. Each lane represents a single sample comprised of pooled protein from 3 individual wells, representative of 2 experiments.
DISCUSSION

The ability of *B. dermatitidis* to avoid clearance by the innate immune system, in both the early and late stages of infection, likely plays a pivotal role in the virulence of the pathogen. Alveolar macrophages are key players in this interaction, since they are both the first immune cells to come into contact with a pathogen invading the lung and also a major target for activation by cytokine-producing T cells. Previous studies have shown that *B. dermatitidis* yeast cells are able to survive in coculture with both resting and activated macrophages (4, 5). Our study has now identified a mechanism by which *B. dermatitidis* interferes with the macrophage antimicrobial arsenal.

We discovered that *B. dermatitidis* yeast cells reduce the levels of nitric oxide in supernatants of activated alveolar macrophages. Further, we showed that *B. dermatitidis* yeast cells do not remove nitric oxide from these supernatants, but rather prevent macrophages from producing nitric oxide, potentially by interfering with the activity of iNOS, the enzyme responsible for generating nitric oxide.

We investigated the interaction of *B. dermatitidis* yeast cells with the macrophage nitric oxide production apparatus because previous data demonstrated that iNOS is essential for fungal clearance in vaccinated mice but dispensable during primary infection. Despite the clear importance of this system in our model, it must be noted that, unlike murine macrophages, human macrophages do not generate high levels of nitric oxide. Our findings remain relevant for several reasons. The role of nitric oxide has been studied extensively in the fungal literature, making this topic important for understanding not only differences between the murine and human im-

FIG. 5. *B. dermatitidis* yeast cells do not suppress nitric oxide production by reducing availability of the arginine substrate. (A and C) Monolayers of alveolar macrophages were activated by incubation with IFN-γ and zymosan (Control). *B. dermatitidis* yeast cells were added to activated macrophages at an MOI of 0.5 or an MOI of 5. At distinct time points over a 72-h time course, RNA was harvested from the monolayers and arginase (A) and CAT2B (C) transcript levels were analyzed by real-time PCR. 18S rRNA levels were used for normalization. In panel A, the asterisks indicate a *P* value of <0.05 versus control macrophages at the same time point. In panel C, no treatment group showed a statistically significant reduction in transcript levels versus control macrophages. (B) Monolayers of alveolar macrophages were activated by incubation with IFN-γ and zymosan (Control). *B. dermatitidis* yeast cells were added to the activated macrophages at an MOI of 0.5. Some treatment groups were supplemented with arginine at the indicated concentrations, and supernatant nitrite levels were measured after 48 h. The asterisks indicate a *P* value of <0.05 versus control macrophages at the same arginine concentration. (D) Monolayers of alveolar macrophages were activated by incubation with IFN-γ and zymosan (Control). The activated macrophages were exposed to *B. dermatitidis* yeast cells at an MOI of 0.5 or an MOI of 5 for 24 h. Macrophage monolayers were then incubated with radiolabeled arginine, washed, and lysed. The macrophage lysates were subjected to scintillation counting to quantify uptake of the radiolabeled arginine. There were no significant differences between treatment groups. In all panels, the data are represented as the mean ± SD of 2 or 3 samples per treatment group, representative of 2 experiments.
mune systems, but also differences between fungal pathogens. This information is critical for the design of effective antifungal therapies and vaccines. Further, the complexity of the mammalian immune system makes it possible that a fungal strategy for evading macrophage nitric oxide production in mice might also be essential for avoiding clearance by the human immune system, for example, by evading nitric oxide production by another cell type or inhibiting the activity of a human enzyme critical for the clearance of infection. Finally, blastomycosis is not only of human medical significance. In regions of endemicity, canine blastomycosis is a common and costly disease with a high fatality rate. Thus, an understanding of the interaction of \textit{B. dermatitidis} with the macrophage nitric oxide production apparatus could also facilitate the development of drugs or vaccines of use to the veterinary industry.

We analyzed the interaction of \textit{B. dermatitidis} yeast cells with macrophages \textit{in vitro} in models that approximate two distinct time points in infection: resting macrophage cocultures represent the interaction of naïve alveolar macrophages with \textit{B. dermatitidis} yeast cells during the first 5 to 7 days of infection, while activated macrophage cocultures represent the interaction of \textit{B. dermatitidis} yeast cells with alveolar macrophages that have been activated by T cell cytokines during the second week of infection. Both systems are relevant, because \textit{B. dermatitidis} yeast cells avoid clearance by both the innate and adaptive immune systems, with mice succumbing to infection in less than 3 weeks (35). We were surprised to discover that resting macrophages produce no detectable nitric oxide in response to \textit{B. dermatitidis} yeast cells. Further, and perhaps more importantly, \textit{B. dermatitidis} yeast cells reduce the amount of nitric oxide detected in supernatants of activated macrophages. These results highlight the potential for \textit{B. dermatitidis} to manipulate the host immune system and thwart the ability of innate effector cells—macrophages—to clear the invading fungus.

Further investigation revealed that \textit{B. dermatitidis} yeast cells reduce supernatant nitric oxide levels in a dose-dependent manner. In fact, supernatant nitric oxide is completely eliminated at an MOI of 40. This number is likely to be biologically relevant in our infection model, in which there are approximately $5 \times 10^5$ alveolar macrophages in the lungs of a mouse (5), and \textit{B. dermatitidis} numbers can approach $1 \times 10^8$ before the mouse succumbs to infection (35). We also showed that the ability of \textit{B. dermatitidis} yeast cells to reduce supernatant nitric oxide is dependent not only on the viability of the yeast, but also on the ability of the yeast to come into close proximity to the macrophages. These results indicate that reduction of nitric oxide levels likely occurs as a result of yeast production of a virulence factor in response to interaction with macrophages.

Importantly, we found that \textit{B. dermatitidis} yeast cells are sensitive to the toxic effects of nitric oxide. In fact, the growth of \textit{B. dermatitidis} is inhibited by the nitric oxide donor DETA NONOate at a concentration between 5 and 10 mM, which corresponds to a nitrite level of between 2.5 and 5 mM. While this level is much higher than the nitrite levels detected in supernatants of activated macrophages, we believe that this level of sensitivity is likely to be biologically relevant for several reasons. First, other fungal pathogens, particularly \textit{C. neoformans} (14) and \textit{H. capsulatum} (27), show similar sensitivity to NONOate compounds. Second, the concentration of nitric oxide produced by macrophages \textit{in vitro} may not reflect the concentration of nitric oxide \textit{in vivo} within an infected alveolus or the phagosome of an activated macrophage. Third, the nitric oxide by-product peroxynitrite, produced when nitrate is combined with reactive oxygen species, is also fungicidal against \textit{B. dermatitidis} (data not shown) under conditions similar to those reported with \textit{Candida albicans} (33). Finally, nitric oxide plays a role \textit{in vivo} in the clearance of \textit{B. dermatitidis} yeast cells from the lungs of vaccinated mice. Thus, our data support the theory that nitric oxide inhibits the growth of \textit{B. dermatitidis}, exerting a selective pressure on the invading pathogen and likely influencing the development of virulence factors that target this system.

The ability to interfere with the alveolar macrophage nitric oxide system is a common characteristic of pathogenic fungi. \textit{C. neoformans} undergoes a massive transcriptional response to nitric oxide and is able to reduce levels of nitric oxide in supernatants of immune cells (7, 21, 25, 37). Although no definitive mechanism has been discovered, \textit{C. neoformans} appears to detoxify nitric oxide to a less toxic molecule (32). \textit{H. capsulatum} also undergoes a transcriptional response after exposure to nitric oxide that includes upregulation of the enzyme nitric oxide reductase, which has been postulated to promote the virulence of the organism (6, 27).

Since many pathogens, including fungi, encode enzymes that convert nitric oxide to less toxic molecules, we explored the role of detoxification in the observed reduction in nitric oxide levels by \textit{B. dermatitidis}. We were surprised to discover that \textit{B. dermatitidis} yeast cells do not detoxify nitric oxide when grown alone in the presence of the nitric oxide-generating compound DETA NONOate. Because we observed a requirement for macrophage contact in the ability of yeast to reduce nitric oxide levels, we devised a system in which the yeast could come into contact with activated macrophages to activate the molecular system that would detoxify the nitric oxide generated by DETA NONOate. Unexpectedly, the yeast still failed to detoxify nitric oxide and remove it from the coculture supernatant. This indicated that, despite the presence in the \textit{B. dermatitidis} genome of nitric oxide reductase (data not shown), \textit{B. dermatitidis} yeast cells in coculture with macrophages do not reduce nitric oxide levels by detoxification. Instead, \textit{B. dermatitidis} yeast cells must impair the ability of activated macrophages to generate nitric oxide.

Suppression of nitric oxide has also been postulated to occur during infection with other fungal pathogens. During infection with \textit{P. brasiliensis}, upregulation of regulatory cytokines, like interleukin 10 (IL-10) causes reduced expression of iNOS and production of nitric oxide (23). Additionally, it has been suggested that \textit{C. immitis} is able to induce production of the host enzyme arginase, reducing arginine availability for iNOS and subsequently reducing the ability of the host to produce nitric oxide (13). Although a specific mechanism has not been identified in either of these cases, they point to two intriguing ways that pathogens are able to manipulate the host nitric oxide system.

We tested the ability of \textit{B. dermatitidis} to reduce iNOS levels within activated macrophages. Interestingly, iNOS transcript and protein levels in \textit{B. dermatitidis}-exposed macrophages were not significantly reduced compared to control activated macrophages. In fact, levels of iNOS protein were slightly elevated in \textit{B. dermatitidis}-exposed macrophages. We also investigated the possibility
of arginine limitation in B. dermatitidis-exposed macrophages. We were surprised to discover that although arginine uptake, presumably by the CAT2B transporter that supplies arginine to iNOS, remains intact in B. dermatitidis-exposed macrophages, supplementation of the cocultures with additional arginine was unable to restore nitric oxide production to the level of control activated macrophages. These experiments ruled out two likely mechanisms by which B. dermatitidis yeast cells could suppress nitric oxide production by activated macrophages.

The results of this study are compatible with the hypothesis that B. dermatitidis yeast cells suppress macrophage nitric oxide production by inhibiting the activity of the iNOS enzyme. Because inducible nitric oxide synthase is a complex enzyme that requires the cooperation of binding partners, binding of cofactors, homodimerization, and proper intracellular localization to function optimally (1), the precise mode by which B. dermatitidis yeast cells are able to suppress its function remains unresolved. Nevertheless, the finding that B. dermatitidis is one of a number of fungal pathogens that have been shown to inhibit the production of nitric oxide by macrophages argues that this pathogen attribute plays a fundamental role in fungal virulence.

Altogether, our work establishes that B. dermatitidis yeast cells suppress the production of nitric oxide by activated alveolar macrophages through potential inhibition of iNOS activity. Our findings lay the foundation for elucidating the precise mechanism by which B. dermatitidis yeast cells disrupt nitric oxide production and the virulence factor that mediates this effect.

ACKNOWLEDGMENTS

This work was supported by funds from a Microbes in Health and Disease NIH T32 training grant (N.M.R.) and by NIH grants R37 AI135681 and AI40996 (B.S.K.).

REFERENCES