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The Unappreciated Intracellular Lifestyle of Blastomyces dermatitidis

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Blastomyces dermatitidis, a dimorphic fungus and the causative agent of blastomycosis, is widely considered an extracellular pathogen, with little evidence for a facultative intracellular lifestyle. We infected mice with spores, that is, the infectious particle, via the pulmonary route and studied intracellular residence, transition to pathogenic yeast, and replication inside lung cells. Nearly 80% of spores were inside cells at 24 h postinfection with 10⁴ spores. Most spores were located inside of alveolar macrophages, with smaller numbers in neutrophils and dendritic cells. Real-time imaging showed rapid uptake of spores into alveolar macrophages, conversion to yeast, and intracellular multiplication during in vitro coculture. The finding of multiple yeast in a macrophage was chiefly due to intracellular replication rather than multiple phagocytic events or fusion of macrophages. Depletion of alveolar macrophages curtailed infection in mice infected with spores and led to a 26-fold reduction in lung CFU by 6 d postinfection versus nondepleted mice. Phase transition of the spores to yeast was delayed in these depleted mice over a time frame that correlated with reduced lung CFU. Spores cultured in vitro converted to yeast faster in the presence of macrophages by 6 d postinfection versus nondepleted mice. Thus, although advanced B. dermatitidis infection may exhibit extracellular residence in tissue, early lung infection with infectious spores reveals its unappreciated facultative intracellular lifestyle. The Journal of Immunology, 2015, 194: 000–000.

Blastomyces dermatitidis is the causative agent of blastomycosis, a potentially deadly fungal infection. The fungus is considered a primary pathogen that can infect immune-competent individuals, yet B. dermatitidis can also reactivate in previously infected patients that become immunocompromised (1, 2). B. dermatitidis is one of six dimorphic fungi that are collectively responsible for most systemic fungal infections in the United States (3). Infections with the dimorphic fungi represent a growing public health problem, particularly in immunocompromised patients (4), and limited measures are available to prevent their acquisition.

Blastomycosis is commonly reported in endemic regions of the United States, Canada, Africa, and the Middle East (5–8). B. dermatitidis, similar to the other dimorphic fungi, grows in the soil or a similar environmental substrate as a mold, which bears spores (conidia). Primary pulmonary infection is initiated when spores are inhaled into the lungs of a susceptible host (9). There, spores enter alveoli and undergo a morphological transition into budding yeast. The phase transition to yeast is essential for pathogenesis and replication during the early pathogenesis of disease (10). Yeast cells are more resistant than spores to killing mediated by host immune cells such as neutrophils, macrophages, and monocytes (11). Moreover, deletion of a global regulator of phase transition, dimorphism-regulating kinase 1, locks the fungus in the mold form and abrogates virulence (12).

B. dermatitidis is generally thought of as an “extracellular” pathogen. Histological sections of infected lung tissue and extrapulmonary sites following fungal dissemination support this premise by showing that most of the yeast are found in the extracellular space. Owing to the long incubation period and the frequent delay in diagnosing blastomycosis, these data are often collected from human patients well after infection is initiated (13–15). This circumstance leaves a gap in the knowledge about the early stage of infection. Some evidence points to the intracellular residence of B. dermatitidis. Sections of infected tissue have reported yeast inside of phagocytes (13, 15). In vitro, phagocytes quickly and efficiently internalize the small (2–5 μm) spores. The larger yeast (10–30 μm) are also phagocytosed, but to a lesser extent and at a slower rate (11). Whereas spores are more vulnerable to killing by phagocytes, yeast can replicate in vitro in their presence, and electron microscopy has revealed multiple yeast inside human monocytes during coculture (16).

Although prior work supports the idea that B. dermatitidis may grow inside of host phagocytes in vitro (16), this and other work did not exclude the possibilities that phagocytes may repeatedly internalize yeast from the extracellular environment, nor that phagocytes may fuse upon exposure to yeast. Each of these events could also result in the presence of multiple yeast in phagocytes and give the erroneous conclusion of intracellular replication. Additionally, the in vitro studies above were done with yeast and not spores, the infectious particles that initiate infection. If spores are indeed rapidly internalized and highly sensitive to killing by leukocytes, this raises the question of where and how inhaled spores convert into yeast and replicate during early infection to establish disease. To our knowledge, studies of pulmonary blastomycosis have not been conducted with spores to initiate lung infection and interrogate their intracellular residence, transition, and replication during the early pathogenesis of disease.
In this study, we investigated the early pathogenesis of pulmonary blastomycosis in a model involving infection with spores. We investigated the host–pathogen interaction with emphasis on elucidating intracellular residence and replication of the fungus. We tackled several questions. First, are spores taken into phagocytes in the lung, and, if so, what are the cells and time course? Second, do spores convert to yeast inside lung phagocytes? Third, do yeast replicate inside these cells, and to what extent are multiple intracellular yeast due to replication, multiple phagocytic events, or cell fusion? Finally, if \textit{B. dermatitidis} replicates inside host lung phagocytes, do the phagocytes constrain or permit progression of early infection? We report that spores are rapidly taken up into alveolar macrophages where they convert to yeast and replicate intracellularly. Moreover, intracellular residence and replication in macrophages is required for initiation of disease.

Materials and Methods

\textbf{Mice}

C57BL/6 wild-type (WT) mice were obtained from the National Cancer Institute. CD45.1 C57BL/6 mice were obtained from Taconic. CD11c–diphertheria toxin (DTx) receptor (DTR) mice were obtained from The Jackson Laboratory and bred in-house. Mice were housed and cared for according to guidelines from the University of Wisconsin Animal Care and Use Committee, who approved this work. Their guidelines are in compliance with Health and Human Services Guide for the Care and Use of Laboratory Animals.

\textbf{Reagents and cell culture}

Bone marrow was collected to generate bone marrow–derived macrophages (BMMs) and chimeric mice. Marrow was collected from femurs and tibias by rimming and disruption through a 26-gauge needle and filtering via a 40-µm filter. BMMs were differentiated in culture at 37°C using a 1:10 dilution of L929 supernatant, and adherent cells were collected after 1 wk. Alveolar macrophages were collected from exsanguinated mice by repeated lavage of the lung alveoli through a cannula with 0.6 mM EDTA in PBS at 37°C. The lavage fluid was then placed on ice. BRCs were lysed using ammonium chloride/potassium bicarbonate buffer. The murine alveolar-like macrophage cell line AMJ2-C11 was obtained from the American Type Culture Collection. Cells were counted on a hemocytometer using trypan blue (Sigma-Aldrich) to assay viability. Cells were cultured at 37°C and 5% CO2 and maintained in RPMI 1640 medium (Gibco) with 10% heat-inactivated (56°C for 30 min) FBS (Atlanta Bioculture). Cells were counted on a hemocytometer using trypan blue (Sigma-Aldrich) to assay viability. Cells were cultured at 37°C and 5% CO2 and maintained in RPMI 1640 medium (HyClone) with 10% heat-inactivated (56°C for 30 min) FBS (Atlanta Biologicals), 100 U penicillin, and 100 µg streptomycin (HyClone).

\textbf{Fungi}

\textit{B. dermatitidis} yeast from strains 26199 and 14081 (American Type Culture Collection) were taken in log-phase growth and suspended in PBS. Yeast were aspirated through a 26-gauge needle and passed through a 40-µm filter to reduce aggregated yeast. In some experiments, yeast were heat-killed at 65°C for 1 h in PBS. Yeast CFU were counted from a wire on a 45° plane. Mice were intubated with a BioLite intubation system. Spores were delivered intratracheally through a cannula in a 20 µl suspension in PBS.

\textbf{Flow cytometry}

Lungs were diced by pressing them through a 40-µm filter with the plunger of a 5-ml syringe. Homogenates were digested using 1 mg/ml collagenase D (Roche) and 10 ng/ml DNAse I (Sigma-Aldrich) for 20 min at 37°C. RBCs were lysed with ammonium chloride/potassium bicarbonate buffer and the remaining cells were washed with 2 mM EDTA and 0.5% BSA in PBS (FACS buffer). Staining was done in 100 µl FACS buffer for 20 min at 4°C in the dark. Stained cells were washed with FACS buffer, fixed with 2% paraformaldehyde solution for 20 min, washed again, and suspended in FACS buffer for analysis. All centrifugation was done at 1500 rpm for 5 min and 4°C in an Eppendorf 5825 centrifuge. Events were gated on forward light scatter and side light scatter to exclude debris and Live/Dead fixable yellow stain (Molecular Probes) was used to exclude dead cells. Alveolar macrophages were defined phenotypically as CD11c+; Mac3–; CD11b+; CD103–; Ly6g–, and autofluorescent. Neutrophils were defined as CD11b+; Ly6g+ (clone IA8), and CD11c+. Dendritic cells (DCs) were defined as CD11c+; MHC class II+ with the following subsets: neutrophil-derived DCs were CD11c+, CD11b+, and Ly6g+; inflammatory DCs were CD11c+, CD11b+, and Ly6g–; and resident DCs were CD103+ and CD11c+ (19–21). Abs were conjugated to the fluorophores FITC, PE, PerCP-Cy7, allophyocyanin, Alexa Fluor 700, or allophyocyanin-Cy7 and were from BD Bioscience, eBioscience, and BioLegend.

Spores and yeast were identified in FACS by low and high expression, respectively, of mCherry fluorescence as noted above and illustrated in Results. Extracellular fungi were stained with 10 µg/ml Uvitex 2B (Poly-Sciences). The percentage of fungi that were intracellular was defined as the number of mCherry+/Uvitex 2B+ events divided by the total number of fungi that stained with either intracellular dye. Data were collected on an LSR II cytometer (BD Biosciences) and analyzed by FlowJo software (Tree Star).

\textbf{Microscopy}

To characterize yeast inside of AMJ2-C11 macrophages, cells were cultured for 24 h with 26199 yeast in a 24-well plate with cRPMI. The medium was removed and cells were stained with 10 µg/ml Uvitex 2B to identify extracellular yeast and 12 µM ethidium bromide to identify dead cells in PBS. Washed cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% saponin (Sigma-Aldrich) for 20 min. Cells were stained with anti–BAD-1-FITC to identify yeast. Anti–BAD-1 mAb (DD5-CB4) (22) was made from ascites, ammonium sulfate precipitated, purified on an A/G agarose column (Pierce Chemical), and then labeled with FITC (Molecular Probes) and purified by dialysis. Differential fluorescence microscopy was performed with an Olympus BX60 microscope. Images were captured with an EXI Aqua Camera (QImaging) and QCapture Pro 6.0 software. Images were processed using Adobe Photoshop. Live imaging was done on an Observer Z1 microscope with a humidified incubation chamber kept at 37°C and 5% CO2, and data were collected using AxioVision software (Zeiss). Videos were processed using iMovie.

\textbf{Intracellular replication}

In vitro intracellular replication was quantified by culturing 7 × 10^5 BMMs or alveolar macrophages with 7 × 10^5 yeast at a multiplicity of infection of 0.1 on glass coverslips in a 24-well plate. We used yeast that express either red (mCherry) or green (GFP) fluorescence for these assays. We added the yeast at a 1:20 ratio of red to green yeast to reduce the likelihood of multiple yeast within a single macrophage arising from multiple phagocytic events. After allowing the yeast to be phagocytosed for 4 h, free yeast were washed away with PBS. At 4, 24, 48, and 72 h time points, samples were collected by washing the coverslips with PBS and staining with either Live/Dead fixable violet (Molecular Probes) or Alamar Blue (BioVision). Abs were collected on an LSR II cytometer (BD Biosciences) and analyzed by FlowJo software (Tree Star).

\textbf{Macrophage fusion}

BMMs were stained with 15 µM 5-chloromethylfluorescein diacetate (Invitrogen) or PKH26 (Sigma-Aldrich), as per the manufacturers’ instructions, and equal parts of the stained cells were aliquoted into a 24-well plate containing glass coverslips for a total of 7 × 10^5 cells per well, as described above for quantification of intracellular replication. Fusion
was stimulated with $7 \times 10^3$ yeast or, as a positive control, with the addition of 10 ng/ml IL-4 (PeproTech) and 1 µg/ml GM-CSF (R&D Systems) (23).

**Bone marrow chimeras**

Administration of DTx (Sigma-Aldrich) to CD11c-DTR mice is lethal after more than two treatments of 100 ng (see Fig. 4). Therefore, we generated bone marrow chimeric mice as previously described (24). Briefly, chimeric mice were generated by lethally irradiating CD45.1 mice with two doses of 550 rad 4 h apart on an X-RAD 320 (Precision X-ray). Mice were injected with $1 \times 10^7$ bone marrow cells i.v. from either CD45.2 WT or CD11c-DTR donor mice. Chimeric mice were maintained on 0.5 mg/ml Baytril 100 (Bayer) and 2 mg/ml neomycin sulfate (Sigma-Aldrich) in their drinking water for 2 wk. Alveolar macrophages were allowed to reconstitute for 12 wk. Mice were injected with 100 ng DTx i.p. every other day starting 2 d before infection with $3.2 \times 10^5$ spores. Lungs were collected every 2 d for 12 d and analyzed by FACS and for CFU.

**Neutrophil depletion**

Neutrophils were depleted by i.v. injection every other day with 250 µg anti-Ly6g (clone 1A8; Bio X Cell). Rat IgG was used as a control. Mice were treated with DTx and anti-Ly6g 1 d before infection and lungs were collected 2 d postinfection.

**Statistical analysis**

Experimental conditions were compared with controls using an unpaired Student t test, a Mann–Whitney U test, or ANOVA with a Tukey’s multiple comparison test where appropriate. A p value <0.05 was considered statistically significant. Analysis was performed with Prism software (GraphPad Software). Data are presented as means, and error bars represent SEM.

**Results**

Interaction between spores and leukocytes early during infection

Pulmonary infection with *B. dermatitidis* begins with inhalation of infectious particles. Thus, we introduced spores into the lungs of mice to mimic the natural infection. Alveolar macrophages (CD11c$^{\text{high}}$, CD11b$^-$, Mac3+) are the predominant leukocyte present in the lungs of naive mice, with $\approx 5 \times 10^5$ cells, and they

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**FIGURE 1.** Most spores reside in alveolar macrophages early during infection. Mice were infected with mCherry 14081 spores by intubation. (A) At 24 h postinfection, lung homogenates were analyzed for the total number and lineage of leukocytes. The gating markers and strategy are described in Materials and Methods and illustrated in Supplemental Fig. 1. AM, alveolar macrophages; Neu, neutrophils. DC subsets (inlay) are resident (Res), neutrophil-derived (Neu), and inflammatory (Inflam). (B) The proportion of total spores (mCherry$^+$ events) in the lungs that are intracellular (Uvitex 2B$^+$) at 1 and 24 h postinfection was measured at a range of inocula. Results are representative of three experiments, each with five mice per group. (C) Intracellular residence of spores within alveolar macrophages from lung homogenates of mice infected with mCherry spores for 12 h. Alveolar macrophages were identified with anti–CD11c-FITC. Uvitex 2B stain of chitin was used to determine whether the spores were extracellular (only extracellular spores bind the dye). Scale bar, 25 µm. (D) Distribution of intracellular spores (mCherry$^+$, Uvitex 2B$^-$) 24 h after mice were infected with $1 \times 10^5$ spores. A flow plot of a representative mouse illustrating intracellular spores (red dots) overlaid on total leukocytes (gray) with representative gates for alveolar macrophages (top left), DCs (top right), and neutrophils (bottom right) is shown. Distribution of spores within leukocytes (left panel) is based on the gating strategy shown in Supplemental Fig. 1; group mean ± SEM was analyzed with FACS (right panel). (E) The total number of lung leukocytes that contain spores 24 h postinfection. Results shown in (D) and (E) are representative of three experiments with five mice per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
also accounted for most of the leukocytes in the lungs early during infection (Fig. 1A, Supplemental Fig. 1). Infection with spores induced inflammation and the influx of other leukocytes (Fig. 1A) compared with naive mice, which have $\approx 1 \times 10^5$ neutrophils (CD11b+, CD11c−, Ly6g+) and $5 \times 10^5$ DCs (CD11c+, MHC class II+) in their lungs (data not shown). Higher inocula induced a more prominent influx of these cells. Infection with spores also led to the influx of a rarely described DC subset, neutrophil-derived DCs (CD11c+, CD11b+, Ly6g+) (Fig. 1A, inset, Supplemental Fig. 1), which are barely detected in naive mice (25).

We used *B. dermatitidis* that expresses mCherry protein fluorescence to let us track (dimmly fluorescent) spores in the lungs of infected mice. We analyzed the intracellular residence of spores by identifying extracellular spores with the membrane-excludable, chitin-specific stain Uvitex 2B. We found a dose-dependent effect on intracellular residence, with lower spore inocula showing more intracellular infection than did higher inocula (Fig. 1B). The lower limit of reliable detection by FACS was with an inoculum of $10^3$ spores. At this inoculum, 70–80% of spores were located inside leukocytes by 24 h postinfection. Although spores could not be reliably tracked at a lower inoculum, intracellular residence increased as the inoculum was reduced and thus could be higher at inocula of $<10^4$ spores.

We sought to identify the cells in which spores reside early during infection. Spores were readily identified inside alveolar macrophages upon microscopic analysis of homogenized lung tissue (Fig. 1C). To quantify the distribution of spores, we further analyzed whole-lung homogenates by flow cytometry (Fig. 1D). Independent of the inoculum, most (>70%) intracellular spores were detected in alveolar macrophages after 24 h of infection, with only minor proportions in DCs and neutrophils (Fig. 1D). The selective association of spores with alveolar macrophages remained strong even at a higher inoculum of $10^5$ spores, which leads to greater numbers of neutrophils in the lung. At $10^6$ spores, there were ~4700 alveolar macrophages with intracellular spores versus only 700 neutrophils and 250 DCs (Fig. 1E). Thus, most *B. dermatitidis* spores enter leukocytes early during infection and these spores reside predominantly within alveolar macrophages, not neutrophils or DCs.

**Fate of intracellular spores and yeast**

The high percentage of spores found inside alveolar macrophages suggests a likely role for macrophages early during infection, either in restricting growth of the fungus or providing a locale for intracellular replication and establishment of infection. We therefore investigated the ability of the fungus to survive, convert from spores to yeast, and replicate in alveolar macrophages. To monitor the phase transition of spores to yeast, we exploited the *B. dermatitidis* reporter strain. In this strain, the yeast phase–specific gene promoter (BAD-1) upregulates mCherry fluorescence during the phase transition from mold or spore to yeast (18). Hyphae have no fluorescence, spores show dim expression, and yeast highly express mCherry (Fig. 2A). Spores of this strain were rapidly internalized by alveolar macrophages during in vitro coculture, with

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**FIGURE 2.** *B. dermatitidis* spores survive and replicate as yeast in alveolar macrophages. (A) Bright-field and fluorescence microscopy of spores and yeast of reporter strain 14081 that upregulates mCherry under the control of a yeast phase–specific BAD-1 promoter. mCherry fluorescence is quantified by FACS in Fig. 6A. (B) Uptake of mCherry spores by macrophages in vitro. Spores were cultured with primary alveolar macrophages at a multiplicity of infection (MOI) of 0.2. Uptake was quantified during 4 h and analyzed by FACS. Extracellular spores stained Uvitex 2B+. Results are the means of triplicate wells and are representative of three experiments. (C) Live imaging of mCherry 14081 spores cultured with primary alveolar macrophages at an MOI of 0.2. Images shown are every 5 h. The full video is available online. (D) Mice were infected with mCherry 14081 spores and 3 d later bronchoalveolar lavage fluid was collected and stained with anti-CD11c-FITC to identify alveolar macrophages and Uvitex 2B to distinguish intracellular and extracellular yeast. Inlay in the left panel shows a budding yeast cell overlying another yeast. (E) Alveolar macrophage cell line AMJ2-C11 was cultured for 48 h with 14081 yeast on coverslips. Cultures were treated with Uvitex 2B to stain the extracellular yeast, and with ethidium bromide to ascertain macrophage membrane integrity and cell viability. Cultures were fixed and then permeabilized to identify yeast with anti–BAD-1-FITC. Scale bars, 25 μm.
95% of spores inside macrophages by 2 h (Fig. 2B). During live cell imaging of these spores cultured with primary alveolar macrophages, we observed intracellular transition of the spores to yeast and ensuing replication (Fig. 2C, Supplemental Video 1). We also detected budding yeast within individual alveolar macrophages found in lavage fluid from spore-infected mice (Fig. 2D), consistent with intracellular replication in vivo. Moreover, when yeast were cultured with AMJ2-C11 macrophages in vitro, some macrophages contained 30 or more yeast after 2 d of culture (Fig. 2E). Thus, spores can survive, germinate, and even replicate as yeast inside of alveolar macrophages shortly after infection.

Intracellular replication of yeast inside macrophages

Because *B. dermatitidis* is not considered a facultative intracellular pathogen that replicates inside macrophages, we further investigated the extent to which replication occurs intracellularly. The finding of multiple yeast in a single macrophage could be the result of multiple phagocytic events, fusion of macrophages, or replication of yeast inside of a macrophage. To maximize the likelihood of quantifying only intracellular replication events, we cocultured macrophages in vitro with an inoculum containing red (red fluorescent protein) and green (GFP) yeast in a ratio of 1:20. By interrogating macrophages that harbor only the less common red yeast, we greatly increased the probability that multiple, intracellular red yeast arose from replication in macrophages and not from multiple phagocytic events or macrophage fusion (Fig. 3A). For example, the probability that four red yeast in a macrophage arose from multiple phagocytic events is low, that is, ~1/1.6 × 10^5. Briefly, we cultured BMMs with yeast in vitro for 4 h to enable phagocytosis and then removed the nonadherent yeast. We incubated cultures for an additional 24 h to allow yeast to replicate. Then, we counted the number of red yeast inside macrophages. We excluded extracellular yeast that stained positive for Uvitex 2B and macrophages that contained green yeast.

Macrophages cultured with live yeast had more red yeast per macrophage than did macrophages cultured with heat-killed yeast (Fig. 3B). Because only cocultures with live yeast resulted in three or more red yeast per macrophage (cultures with heat-killed yeast had only one to two red yeast per macrophage), we concluded that three or more yeast per macrophage was likely due to intracellular replication, not multiple phagocytic events. Some macrophages had seven or more intracellular red yeast. The percentage of BMMs that contained three or more red yeast grew steadily over time during 72 h of incubation (Fig. 3C). Similar trends were observed in cocultures of live yeast with primary alveolar macrophages (data not shown). The increasing proportion of macro-

### FIGURE 3.

Intracellular replication by *B. dermatitidis* yeast. (A) Probability of only red yeast being phagocytosed when they comprise 5% of the yeast inoculum. (B) BMMs were cultured for 24 h with two yeast strains, one expressing red fluorescent protein and the other, GFP (ratio of 1:20, respectively); both strains in an assay were either live or heat-killed. The number of yeast per macrophage was enumerated for the BMMs that contained only red yeast. Five hundred yeast-containing macrophages were enumerated per condition; results are the means ± SEM of five experiments. (C) BMMs containing three or more red yeast were enumerated. Five hundred yeast-containing macrophages were counted per time point; results are the means ± SEM of three experiments. (D) The fusion of a red macrophage with a green one results in a yellow, multinucleated giant cell. (E) Fusion between PKH26 (red)-stained and 5-chloromethylfluorescein diacetate (green)–stained BMMs (1:1) was enumerated for cells cultured 24 h with or without yeast (strain 26199) or with IL-4 and GM-CSF (control). Results are the means ± SEM of four experiments in which >400 macrophages were counted per condition. (F) A fused, yellow, multinucleated giant cell from a culture with yeast that does not contain intracellular yeast. (G) Red and green BMMs were cultured with yeast over time. The proportion of fused BMMs with three or more intracellular yeast is depicted. Results are the means ± SEM of three experiments in which >400 macrophages were counted per condition. *p < 0.05, **p < 0.001.
phages with three or more yeast over time suggests that yeast frequently replicate inside macrophages.

The ratiometric analysis above reduced the chance that repeated phagocytic events could account for the finding of multiple intracellular red yeast, but fusion of macrophages could not be entirely excluded. We therefore assayed whether yeast induce fusion of macrophages and the extent to which this event explained our finding of multiple yeast inside a macrophage. In this assay, BMMs were stained with red or green fluorescent dye and mixed together in equal proportions; fusion of the two yielded a yellow, multinucleated giant cell (Fig. 3D). Yeast did induce macrophage fusion, but it occurred in only \( \approx 2\% \) of macrophages (Fig. 3E). This amount of fusion was similar to that found for BMMs exposed to IL-4 and GM-CSF, which are known to induce fusion (26).

Nevertheless, not all fused macrophages harbored intracellular yeast (Fig. 3F). In fact, fusion accounted for only a minority (\( \approx 20\% \)) of BMMs with three or more yeast (Fig. 3G). Thus, although coculture with \( B. \ dermatitidis \) induces macrophage fusion, the phenomenon does not contribute substantially to the finding of multiple yeast inside macrophages.

Role of macrophages during progressive pulmonary infection

Because we observed that most of the spore inoculum entered alveolar macrophages early during infection, and that these particles convert to yeast that replicate intracellularly, we sought to distinguish whether alveolar macrophages are required to constrain the early infection or, alternatively, enable replication and progression of infection. We sought to use CD11c-DTR mice to address the role of macrophages in the pathogenesis of early infection. We found that uninfected CD11c-DTR mice are too sensitive to DTx to survive multiple doses (Fig. 4A). Thus, we created bone marrow chimeric mice in which WT CD45.1 mice were lethally irradiated and reconstituted with bone marrow from CD45.2 WT or CD45.2 CD11c-DTR mice (Fig. 4B) (24). Chimeric CD11c-DTR mice tolerated multiple doses of DTx, similar to WT mice (Fig. 4A). Although CD11c is expressed on alveolar macrophages as well as DCs, toxin treatment reduced the macrophages by 21-fold and the DCs by only 2.6-fold (Fig. 4C). Depletion of CD11c\(^+\) cells in chimeric mice that were challenged with \( B. \ dermatitidis \) spores resulted in a surprising 26-fold reduction in CFU by day 6 postinfection, compared with WT chimeric mice treated with DTx. This trend was seen across multiple time points (Fig. 4D). Toxin treatment of WT mice and DTR expression in the absence of toxin treatment did not independently reduce lung CFU (Fig. 4D). Thus, CD11c\(^+\) cells in the lung were essential for propagation and pathogenesis of infection, and their elimination unexpectedly stemmed the extent of pulmonary disease after infection with spores.

Role of neutrophils during spore infection

Although we show above that yeast can replicate inside alveolar macrophages, it is also possible that residence inside these phagocytes protects the fungus from other more potent leukocyte effectors. Neutrophils are more effective than macrophages at killing \( B. \ dermatitidis \) spores and yeast (11, 27). Neutrophils also comprise the next largest population of leukocytes (after alveolar macrophages) in the lungs of spore-infected mice (Fig. 1A). We also observed that the proportion and number of lung neutrophils increased significantly after infection in toxin-treated versus untreated control mice (Fig. 5A, 5B). This neutrophilia upon toxin treatment is consistent with previous work with these mice (28).
Toxin-treated CD11c-DTR mice showed a >6-fold increase in the percentage of leukocyte-associated spores that were associated with neutrophils (9 versus 56%) (Fig. 5D, left panel); numbers showed corresponding trends (Fig. 5D, right panel).

We hypothesized that if macrophages offer a relatively “protected” locale for spores against neutrophils, then an increased exposure to neutrophils in toxin-treated mice might explain the reduced lung CFU after depletion of CD11c+ cells. To test this hypothesis, we depleted neutrophils with an Ly6g-specific Ab in toxin-treated CD11c-DTR mice before infection with spores (Fig. 5A, 5B). Neutrophil depletion did not affect the number of alveolar macrophages (compared with rat IgG treated controls) (Fig. 5C). Whereas the lung CFU postinfection rose slightly in neutrophil-depleted WT mice versus untreated mice, lung CFU did not increase significantly after neutrophils were depleted from toxin-treated CD11c-DTR mice (Fig. 5E). Spores did show a small increase in association with DCs in toxin-treated CD11c-DTR mice that were depleted of neutrophils (Fig. 5F). Thus, exposure of *B. dermatitidis* to neutrophils did not appear to explain the sharply reduced lung CFU in mice depleted of CD11c+ cells, that is, chiefly alveolar macrophages. This implies that *B. dermatitidis* spores may benefit directly from the growth environment inside macrophages.

**Effect of macrophages on phase transition of spores to yeast**

Because spores are more vulnerable than yeast to killing by leukocytes (11), the rapidity of phase transition would likely offer a selective advantage to the fungus in survival early during infection. By flow cytometric analysis, transition reporter yeast display a 10-fold higher mCherry fluorescence than do spores (Fig. 6A). Using this reporter strain during infection, we found that the phase transition of spores to yeast is significantly delayed in the first week of infection in chimeric mice that are depleted of CD11c+ cells (Fig. 6B); this delay in transition correlated with the time frame in which lung CFU are sharply curtailed in these mice.

We also used the reporter strain for in vitro studies with macrophages. The percentage of spores that demonstrated phase transition to yeast in vitro during 4 d nearly tripled during coculture with BMMs (Fig. 6C) and alveolar macrophages (data not shown) compared with culture in medium alone. During this time frame, little to no replication of the fungus occurred in vitro as determined by CFU analysis (data not shown). These data indicate that infectious spores undergo significantly faster transition to pathogenic yeast in vivo in the lungs of mice in which CD11c+ cells, that is, mainly alveolar macrophages, are present. Spores also evince more rapid transition to yeast when they are cocultured in vitro with macrophages.

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**FIGURE 5.** Neutrophils do not account for reduced lung CFU in CD11c-depleted mice. CD11c-DTR mice were treated with rat IgG, 100 ng DTx i.p., anti-Ly6g Ab i.v., or both DTx and Ab. Mice were infected with mCherry 14081 spores and lung homogenates were analyzed 2 d later. (A) Flow plots showing the proportion of lung neutrophils in representative mice. (B) Neutrophil numbers were quantified by FACS and hemocytometer count. (C) The number of alveolar macrophages (AM) in mice given DTx alone or together with neutrophil-depleting Ab. (D) Flow plot of the distribution of intracellular spores (mCherry+, Uvitex 2B2) denoted as black dots with respect to leukocytes (gray) and alveolar macrophages (AM; top left gate), DCs (top right), and neutrophils (Neu; bottom right) in representative PBS- versus toxin-treated CD11c-DTR mice (left panel). The right panel shows the number of intracellular spores associated with each cell type. (E) Lung CFU in mice corresponding to (A)–(C). (F) The distribution of spores among leukocytes in depleted or control mice evaluated by FACS. All results are representative of three independent experiments with five mice per group. *p < 0.05, **p < 0.01, ****p < 0.0001.
Intracellular Lifestyle of B. dermatitidis

Discussion

B. dermatitidis is generally considered an extracellular pathogen. Histological sections of infected patient tissues typically show a high proportion of the yeast in the extracellular space. However, most data from patients represent the late stage of infection once large numbers of yeast occupy tissue and neutrophils dominate. The large size of budding yeast can be hard for neutrophils to ingest, perpetuating the notion of extracellular residence. The role of intracellular residence in early pathogenesis and initiation of infection with spores has not been investigated.

In this study, using a murine model of infection with spores, we observed that a high proportion of the spores that reach the lungs are located inside of alveolar macrophages within 24 h of infection. Alveolar macrophages contained most of the spores after pulmonary infection despite a rapid influx of neutrophils, inflammatory DCs, and even neutrophil-derived DCs. This finding contrasts with reports of late-stage infection dominated by yeast and pyogranulomas (13). We observed that the lowest inoculum yielded the greatest association of spores with alveolar macrophages, approaching 80%. Although our flow cytometric analysis was not sensitive enough to reliably detect leukocyte interactions with <10^4 spores, others have reported that as few as 70 spores are sufficient to cause disease and death in mice (29). Although the inoculum of spores that causes disease in humans is unknown, our findings demonstrate that low inocula most of the infectious spores enter alveolar macrophages, a niche in which they undergo morphogenesis to initiate infection and suppress innate host defense such as NO and TNF-α production (30, 31).

We used live cell imaging to investigate the uptake of spores by naive alveolar macrophages, phase transition to yeast and intracellular replication in real time. Although spores are vulnerable to killing by macrophages, and infection has been thought to arise from extracellular spores (32), we found that >95% of spores were taken up by 2 h of incubation, and that spores converted to yeast, which replicated inside alveolar macrophages. Although we are currently unable to monitor spore transition in vivo in real time, our imaging in vitro established intracellular transition and replication inside primary alveolar macrophages. Based on our findings that three or more yeast within a single macrophage is most likely due to replication, and not multiple phagocytic events or macrophage fusion, our finding of three or more yeast and budding within single alveolar macrophages from the bronchoalveolar lavage fluid of infected mice supports the premise that intracellular replication occurs in vivo.

Previous investigators have sought to evaluate intracellular replication of B. dermatitidis yeast within macrophages in vitro (16, 33). Their findings of an increase in CFU may have been confounded by the potential growth of extracellular or partially internalized yeast. Our live imaging data demonstrate unequivocal intracellular replication by yeast, and they are further supported by our ratiometric studies. Using quantitative analysis involving red and green yeast at a defined ratio of 1:20, we established that the increasing number of live yeast per macrophage was due to intracellular replication over time. We chose the ratiometric method of investigation over an alternate method of measuring the budding index, where the proportion of budded yeast is quantified to identify growth under different conditions (34–36). We found that regardless of the intracellular or extracellular location of yeast, a high proportion of them (>70%) are budding (data not shown). This prevented us from observing a change in the proportion of yeast that bud under different conditions and using the budding index to evaluate intracellular replication.

We considered the possibility that our quantitation of intracellular replication could be confounded by cell fusion and formation of giant cells. Multinucleated giant cells are observed during histological analysis of B. dermatitidis–infected human lungs. These cells are commonly seen in proximity to yeast and occasionally with intracellular yeast (14). We did find evidence that yeast induce macrophage fusion and multinucleated giant cells. However, this was a rare occurrence and did not alter our conclusions because the vast majority of macrophages with three or more yeast were not fused. Furthermore, ratiometric analysis reduced the likelihood that fusion confounded our analysis: a macrophage containing a red yeast was 20-fold more likely to fuse...
with a macrophage containing a green yeast than one with another red yeast, and macrophages that contained the more prevalent green yeast were excluded from analysis.

In view of finding most spores within alveolar macrophages in vivo, and strong in vitro evidence of intracellular phase transition and replication, we tested whether macrophages constrain or facilitate early pathogenesis of infection. CD11c-DTR mice were useful to address this question, although the intolerance of these mice to multiple toxin treatments required the generation of chimeric mice. Depletion of CD11c<sup>high</sup> cells sharply curtailed lung CFU in the toxin-treated chimeric mice. We interpret this finding as evidence that the entry of spores into alveolar macrophages permits initiation of infection. We sought to restore lung macrophages by adoptive transfer back into depleted mice, but we were unsuccessful in reconstituting the numbers; thus, we lack formal proof for the role of alveolar macrophages per se. Cells other than alveolar macrophages express CD11c, for example DCs, and they are also depleted in CD11c-DTR mice. However, DCs are unlikely to account for the reduction in CFU we observed. There are many more alveolar macrophages in the lungs and spores chiefly associated with them and not DCs. Moreover, DTx was 10-fold more efficient in depleting alveolar macrophages than DCs. Finally, some monocyte-derived DCs are insensitive to depletion by this toxin (37). Still, we cannot exclude that DCs might play a role in the outcome we observed in toxin-treated mice.

Neutrophils can kill B. dermatitidis, particularly the spore form (32). One explanation for the reduction in lung CFU after depletion of CD11c<sup>high</sup> cells is that intracellular residence inside macrophages protects spores from attack by other leukocytes such as neutrophils. Neutrophils are the next most prevalent leukocyte in the lungs after alveolar macrophages. Furthermore, toxin treatment of CD11c-DTR mice induces an influx of neutrophils (28). However, depleting neutrophils at the same time as CD11c<sup>high</sup> cells did not restore CFU to the levels of untreated mice. It is possible that other leukocytes compensated and killed spores in the absence of neutrophils and macrophages. For example, spores associated more with DCs than these double-depleted mice. Thus, enhanceduptake by DCs could contribute to reduced CFU in these mice. Alternatively, the intracellular environment in alveolar macrophages may independently foster better phase transition or growth of B. dermatitidis.

Spores converted to yeast more rapidly in vivo in wild-type mice than in mice depleted of CD11c<sup>high</sup> cells. These in vivo data are supported by our in vitro findings in which spore transition was hastened by coculture with macrophages versus culture in medium alone. These findings support the idea that delayed phase transition to yeast may have contributed to the reduction in CFU we observed in CD11c-depleted mice. Faster transition to the yeast form offers a selective growth advantage to B. dermatitidis because this form is much more resistant to elements of host defense (11).

Our work offers strong evidence that B. dermatitidis is capable of intracellular survival, phase transition, and replication within alveolar macrophages, and that these events direct the pathogenesis of early infection. Thus, B. dermatitidis displays a facultative intracellular lifestyle. In recent years, Cryptococcus neoformans has been reclassified as a facultative intracellular pathogen of macrophages (36). This reevaluation of C. neoformans has led to new insight about survival strategies of the fungus in the mammalian host and inside macrophages (36, 38–40). The entry of B. dermatitidis spores into alveolar macrophages likewise gives the fungus a clearcut advantage during the early pathogenesis of blastomycosis. Our findings set the stage for further work into the mechanisms for intracellular survival and replication and the elevated rate of spore transition of B. dermatitidis within naive macrophages.

In summary, we provide new insight about the early stages of B. dermatitidis infection after inhalation of spores. Although intracellular residence and replication appear to be integral parts of early infection, the extent to which such events play a role in late infection remain unknown. Intracellular residence could enable B. dermatitidis to spread from the lungs to extrapulmonary tissue. This “Trojan Horse” method of dissemination has been proposed for other intracellular fungi such as C. neoformans (41–45), where in a murine model of cerebral infection yeast have been detected in monocytes circulating in blood and in leptomeningeal capillaries of brain sections. New insight into how B. dermatitidis grows in host cells may likewise improve our understanding of how the fungus establishes itself in the lung and disseminates to visceral sites.

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Disclosures

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References


Figure S1. Gating strategy for lung leukocytes. Mice were infected by intubation with 10^4 mCherry 14081 spores. After 3 days, lung leukocytes were analyzed. Debris was excluded on the forward and side scatter and dead cells were excluded based on the uptake of a membrane excludable viability dye. CD11c- events were analyzed for Ly6g and CD11b expression to identify neutrophils. CD11c+ events were analyzed by Ly6g to identify neutrophil derived DCs. The CD11c+, Ly6g- events were analyzed by CD103 to identify residential DCs. Finally, the CD11c+, Ly6g-, CD103- events were analyzed by Mac-3 and CD11b to identify the alveolar macrophage and inflammatory DCs.
MOVIE LEGEND

Movie 1. Spore transition and replication as yeast within alveolar macrophages.

The movie shows mCherry 14081 spores cultured for 42 hours with primary alveolar macrophages. Phase contrast images were taken every 15 minutes. Note the budding and replication of *B. dermatitidis* inside of macrophages.