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RESEARCH ARTICLE

The Eng1 β-Glucanase Enhances *Histoplasma* Virulence by Reducing β-Glucan Exposure

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ABSTRACT **The fungal pathogen *Histoplasma capsulatum* parasitizes host phagocytes. To avoid antimicrobial immune responses, *Histoplasma* yeasts must minimize their detection by host receptors while simultaneously interacting with the phagocyte. Pathogenic *Histoplasma* yeast cells, but not avirulent mycelial cells, secrete the Eng1 protein, which is a member of the glycosylhydrolase 81 (GH81) family. We show that *Histoplasma* Eng1 is a glucanase that hydrolyzes β-(1,3)-glycosyl linkages but is not required for *Histoplasma* growth *in vitro* or for cell separation. However, *Histoplasma* yeasts lacking Eng1 function have attenuated virulence *in vivo*, particularly during the cell-mediated immunity stage. *Histoplasma* yeasts deficient for Eng1 show increased exposure of cell wall β-glucans, which results in enhanced binding to the Dectin-1 β-glucan receptor. Consistent with this, Eng1-deficient yeasts trigger increased tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) cytokine production from macrophages and dendritic cells. While not responsible for large-scale cell wall structure and function, the secreted Eng1 reduces levels of exposed β-glucans at the yeast cell wall, thereby diminishing potential recognition by Dectin-1 and proinflammatory cytokine production by phagocytes. In α-glucan-producing *Histoplasma* strains, Eng1 acts in concert with α-glucan to minimize β-glucan exposure: α-glucan provides a masking function by covering the β-glucan-rich cell wall, while Eng1 removes any remaining exposed β-glucans. Thus, *Histoplasma* Eng1 has evolved a specialized pathogenesis function to remove exposed β-glucans, thereby enhancing the ability of yeasts to escape detection by host phagocytes.**

IMPORTANCE **The success of *Histoplasma capsulatum* as an intracellular pathogen results, in part, from an ability to minimize its detection by receptors on phagocytic cells of the immune system. In this study, we showed that *Histoplasma* pathogenic yeast cells, but not avirulent mycelia, secrete a β-glucanase, Eng1, which reduces recognition of fungal cell wall β-glucans. We demonstrated that the Eng1 β-glucanase promotes *Histoplasma* virulence by reducing levels of surface-exposed β-glucans on yeast cells, thereby enabling *Histoplasma* yeasts to escape detection by the host β-glucan receptor, Dectin-1. As a consequence, phagocyte recognition of *Histoplasma* yeasts is reduced, leading to less proinflammatory cytokine production by phagocytes and less control of *Histoplasma* infection *in vivo*. Thus, *Histoplasma* yeasts express two mechanisms to avoid phagocyte detection: masking of cell wall β-glucans by α-glucan and enzymatic removal of exposed β-glucans by the Eng1 β-glucanase.**

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In contrast to many opportunistic pathogens, the fungal pathogen *Histoplasma capsulatum* is not controlled by the innate immune system. Macrophages of the innate branch of the immune system not only are ineffective in killing *Histoplasma* yeasts but also serve as the principal host cell for this intracellular pathogen and as the vehicle for dissemination (1). Once cell-mediated immunity is triggered, Th1-cytokine signals (e.g., tumor necrosis factor alpha [TNF-α] and gamma interferon [IFN-γ]) activate phagocytic cells and potentiate their antifungal mechanisms (2, 3). Central to the establishment of this protective cell-mediated immunity are cytokine signals that originate from host phagocytes. By limiting phagocyte detection and responses, *Histoplasma* creates a more permissive niche for proliferation.

Histoplasma yeasts are taken up by innate immune cells by phagocytosis following interactions between surface proteins on the yeast and phagocyte (4–8). Despite this close interaction, *Histoplasma* limits its detection by pattern recognition receptors (PRR) on the phagocyte. The C-type lectin receptor Dectin-1 is a major receptor for recognition of the β-glucans which comprise nearly all fungal cell walls (9, 10), including the walls of *Histoplasma* yeasts. However, most strains of *Histoplasma* yeasts produce a layer of α-linked glucan that overlies the β-glucans in the cell wall, significantly reducing β-glucan exposure and thus detection of yeast cells by Dectin-1 (11, 12).

Most *Histoplasma* virulence factors identified to date are characteristics of the yeast—but not of the mycelial phase (11, 13–16). Some of these have defined mechanisms, including the aforementioned α-glucan (17, 18), as well as the secreted Sod3 superoxide dismutase and CatB catalase (13, 14), which protect *Histoplasma* yeasts from phagocyte-produced reactive oxygen species (ROS).

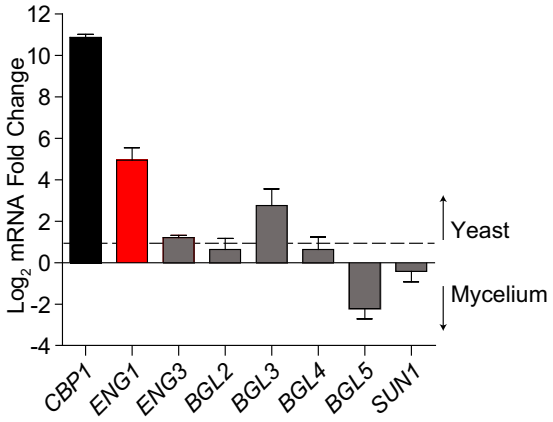

FIG 1 *Histoplasma ENG1* expression is enriched in cells in the pathogenic phase. Relative expression of putative *Histoplasma* endoglucanase genes in the virulent (yeast) and avirulent (mycelia) phases are indicated. Gene expression levels were determined by qRT-PCR of RNA harvested from wild-type G186A yeast-phase or mycelial-phase *Histoplasma* cultures. Gene expression levels were normalized to the level seen with the constitutively expressed *TEF1* gene, and the fold change in specific mRNA levels (yeast phase relative to mycelial phase) was determined. Yeast-phase enrichment was confirmed by expression of the *CBP1* yeast-phase-specific gene. Error bars indicate the standard deviations of results from biological replicates ($n = 3$).

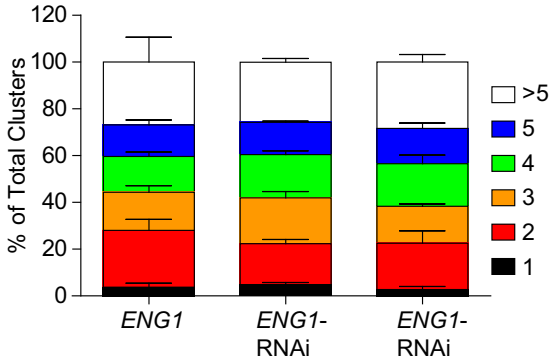

FIG 2 Depletion of Eng1 does not impair cell separation. Data represent distributions of the number of yeast cells per cluster for Eng1-producing (*ENG1*) and Eng1-deficient (*ENG1*-RNAi) yeasts from the G217B background in late-exponential-stage growth. The numbers of yeasts per cluster were quantified by microscopy ($n > 150$ clusters per strain). Data represent the average percentage of each class, and error bars represent the standard deviations of results from biological replicates for each line ($n = 3$).

To provide a more comprehensive catalog of the extracellular factors produced by *Histoplasma*, we used a proteomics approach to define the secreted proteome of cells in the pathogenic phase (19). Three yeast-phase secreted proteins, which included a chitinase (Cts2) and two predicted glycanases (Eng1 and Exg8), had high homology to glycoside hydrolases (GH). In this study, we showed that Eng1 encodes a β-(1,3)-glucanase that contributes to *Histoplasma* virulence by reducing exposure of yeast cell wall β-glucans, improving the ability of *Histoplasma* yeasts to avoid detection by phagocyte Dectin-1 receptors.

RESULTS

Identification of *Histoplasma* endoglucanases. Analysis of the secreted proteome of *Histoplasma* yeasts and bioinformatic examination of the *Histoplasma* genome indicated the yeast cells potentially produce multiple glycanase enzymes. One of these proteins, Eng1, is a putative endoglucanase (19). Six other *Histoplasma* genes (*ENG3*, *SUN1*, *BGL2*, *BGL3*, *BGL4*, and *BGL5*) were identified as putative endoglucanases in the *Histoplasma* transcriptome (20), corresponding to glycoside hydrolase (GH) families GH16, GH17, GH81, and GH132 (see Fig. S1 in the supplemental material) (21). Transcription of these genes by yeasts was compared to expression by mycelia to identify glucanases with potential yeast-phase-specific roles. Four genes (*ENG3*, *BGL2*, *BGL4*, and *SUN1*) are expressed at similar levels by both yeast and mycelial phases, suggesting that these have general functions in *Histoplasma* biology (Fig. 1). Only *ENG1* and *BGL3* have higher expression in the yeast phase (31-fold and 7-fold, respectively; Fig. 1). We focused our investigations on Eng1, since the Eng1 protein is abundantly produced by yeast cells (19) and *ENG1* transcription is highly enriched in yeasts, together suggesting a yeast-phase-specific role (i.e., pathogenesis).

***Eng1* is not required for cell growth or separation.** To study the functional roles of Eng1, RNA interference (RNAi) was used to deplete Eng1 from two phylogenetically distinct *Histoplasma* strains, strains G186A and G217B. Knockdown of *ENG1* expression was monitored by cosilencing of a green fluorescent protein (GFP) gene sentinel using *gfp*:*ENG1* chimeric RNAi vectors (22). Two independent RNAi isolates were generated in each GFP fluorescent G186A and G217B genetic background strain (see Table S1 in the supplemental material). In these lines, sentinel GFP fluorescence reduction indicated 81% to 91% depletion of Eng1 (see Fig. S2A and B in the supplemental material), which was confirmed by quantitation of the *ENG1* transcript levels in the G217B background by quantitative reverse transcription-PCR (qRT-PCR) (50-fold to 100-fold reduction in *ENG1* mRNA; see Fig. S2C). Depletion of *ENG1* by RNAi did not impair yeast growth in liquid medium relative to the Eng1-expressing yeasts, nor did it decrease the viability of the yeasts during broth culture (see Fig. S3).

Eng1 homologs in *Saccharomyces cerevisiae* and *Candida albicans* are required for cell separation during yeast budding (23, 24). To determine if *Histoplasma* Eng1 has a similar function, we examined the effect of Eng1 depletion on *Histoplasma* yeast cell separation. By microscopy, *Histoplasma* yeasts from exponential-phase growth are found in small clusters, rather than as individual yeasts as seen for *S. cerevisiae* or *C. albicans*. To assess cell separation in *Histoplasma*, yeasts from the G217B background (which grow dispersed in liquid culture) were used. Clusters of *Histoplasma* yeasts were examined by microscopy, and the number of yeasts comprising each individual cluster was determined and scored as 1, 2, 3, 4, 5, or greater than 5 yeasts per cluster. Loss of Eng1 function caused no statistically significant difference between Eng1-expressing and Eng1-deficient strains in the distribution of cluster compositions (Fig. 2). Expansion of this analysis to early and late stages of growth in liquid culture similarly showed no difference in cluster distributions between Eng1-producing and Eng1-deficient strains (data not shown). These data indicate that, unlike the *Saccharomyces* or *Candida* Eng1 proteins, Eng1 is not required for *Histoplasma* yeast cell separation.

Eng1 is a secreted β-(1,3)-glucanase. Unlike the *S. cerevisiae* and *C. albicans* Eng1 homologs which localize to the cell wall (23, 24), *Histoplasma* Eng1 is secreted from yeasts. In contrast to *S. cerevisiae* Eng1, *Histoplasma* Eng1 lacks a recognizable glycosyl-

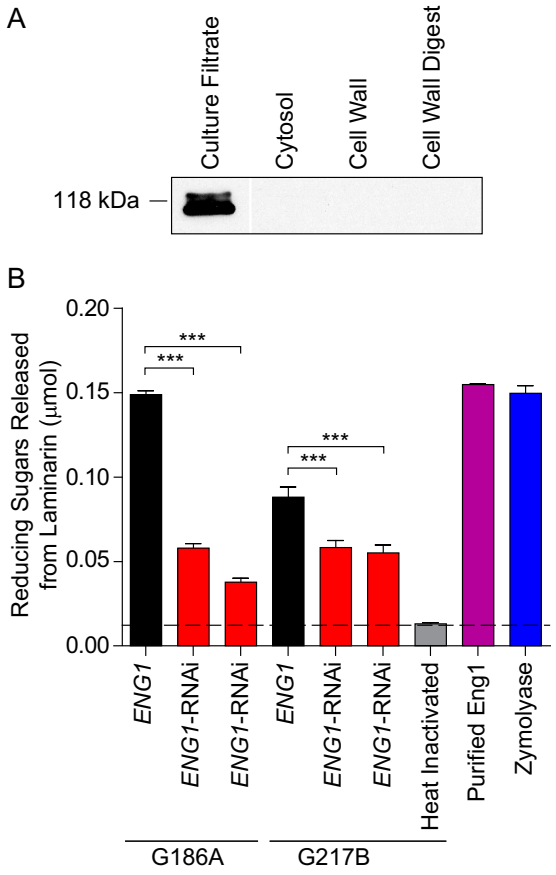

FIG 3 Eng1 is an extracellular β-(1,3)-glucanase. (A) Immunoblot of protein fractions representing yeast culture filtrate, cytosol, SDS extract of the cell wall ("cell wall"), and proteins solubilized by zymolyase digestion of the cell wall ("cell wall digest"). Protein fractions were prepared from wild-type *Histoplasma* yeasts expressing a FLAG epitope-tagged Eng1 protein, and the Eng1 protein was detected by anti-FLAG epitope immunoblotting. (B) Total β-(1,3)-glucanase activity in culture filtrates from Eng1-producing (*ENG1*; black bars) and Eng1-deficient (*ENG1*-RNAi; red bars) yeasts and purified Eng1 protein (purple bar). Glucanase activity was determined by incubating culture filtrates or protein with a β-(1,3)-glucan substrate (laminarin) and quantification of the released reducing saccharides. Reaction controls include zymolyase [a β-(1,3)-glucanase; blue bar] and heat-inactivated Eng1-producing culture filtrate (gray bar). The dashed line indicates the limit of detection. Data represent average glucanase activity ± standard deviations of results from replicates ($n = 3$). Asterisks denote the statistical significance of results of comparisons between Eng1-producing and Eng1-deficient yeasts as determined by one-tailed Student's *t* test (***, $P < 0.001$).

phosphatidylinositol (GPI)-anchor motif (see Fig. S1 in the supplemental material). Examination of the cellular and secreted fractions by immunoblotting for Eng1 demonstrated that the *Histoplasma* Eng1 protein is part of the soluble extracellular fraction but not the cytosolic or cell wall-associated fractions, including material solubilized by glucanase digestion of the cell wall (Fig. 3A). Examination of the insoluble material remaining after SDS extraction or zymolyase digestion of cell walls by immunofluorescence microscopy indicated that no Eng1 was associated with the insoluble cell wall fraction (data not shown).

To biochemically confirm that *Histoplasma* Eng1 is an active glucanase, β-glucanase activity produced by *Histoplasma* yeasts was measured with laminarin [a β-(1,3)-glucan substrate] and the amount of saccharides released from the glucan polymer quantified (Fig. 3B). Both G186A and G217B culture filtrates produce β-(1,3)-glucanase activity, and this activity is eliminated following heat treatment of the culture filtrate. RNAi-based depletion of Eng1 function reduced the extracellular β-(1,3)-glucanase activity, showing that Eng1 accounts for at least 61% of the total glucanase activity in the culture filtrate for G186A yeast and 33% of the G217B activity (Fig. 3B). Consistent with the Eng1 localization studies described above, no glucanase activity was found associated with the yeast cells themselves (data not shown). Purified *Histoplasma* Eng1 protein was sufficient to hydrolyze laminarin, similar to zymolyase [a known β-(1,3)-glucanase], confirming the glucanase activity of Eng1.

Eng1 is required for full virulence *in vivo*. Although no role for Eng1 was found for yeast growth *in vitro*, the enriched expression of *ENG1* in the yeast phase suggests that Eng1 may contribute to *Histoplasma* virulence. The pathogenesis requirement for Eng1 was investigated by infecting mice with wild type or Eng1-deficient *Histoplasma* yeasts and measuring proliferation of the yeasts *in vivo*. At 8 days postinfection (a time point reflecting acute pulmonary infection), levels of Eng1-producing yeasts were increased 45-fold to 200-fold over the inoculum level (Fig. 4A). In contrast, Eng1-deficient yeasts from two independent RNAi lines and both genetic backgrounds consistently showed a 4-fold reduction in lung infection compared to their respective Eng1-producing counterparts. Depletion of Eng1 function also reduced yeast dissemination to spleen tissue (see Fig. S4A in the supplemental material). Thus, Eng1 is required for the full virulence of *Histoplasma* in vivo.

To gain insight into how Eng1 acts to promote *Histoplasma* virulence *in vivo*, the kinetics of pulmonary infection in the absence of Eng1 were measured (Fig. 4B). At 4 days postinfection, there was no significant change in the proliferation of Eng1-deficient yeasts compared to Eng1-producing yeasts. By 8 days postinfection, the difference between Eng1-producing and Eng1-deficient yeasts was 4-fold to 5-fold, and this difference continued to increase at later time points. By days 12 and 16, Eng1-deficient yeasts showed 100-fold-to-1,000-fold-lower pulmonary infection than Eng1-producing yeasts (Fig. 4B). All the mice infected with Eng1-producing yeasts were moribund by day 14, but mice infected with Eng1-deficient yeasts survived and were efficiently clearing the fungal burden (Fig. 4B). The enhanced clearance of Eng1-deficient yeasts after day 8 coincides with the time point at which cell-mediated immunity had commenced. This enhanced clearance was not due to increased T-cell recruitment into the lung, as the Eng1-producing and Eng1-deficient yeasts elicited the movement of equivalent numbers of $CD4^+$ T cells into the lungs at 6 and 7 days postinfection (data not shown), although there was a 3-fold increase in the level of interleukin-17 (IL-17)-producing T cells in lungs infected with the Eng1-deficient strain.

Eng1 reduces β-glucan exposure and Dectin-1 recognition of *Histoplasma* yeasts. As Eng1 function is required for full virulence and as Eng1 acts biochemically on β-glucans, which can stimulate the immune response, we investigated the ability of Eng1 to reduce phagocyte detection of β-glucans of the *Histoplasma* cell wall. One of the major host receptors for β-glucan is Dectin-1, which, upon recognition of fungal glucan molecules, induces a proinflammatory response (10). To test if Eng1-deficient cells have greater exposure of β-glucan, Dectin-1 recognition of Eng1-producing yeasts was compared to Dectin-1 rec-

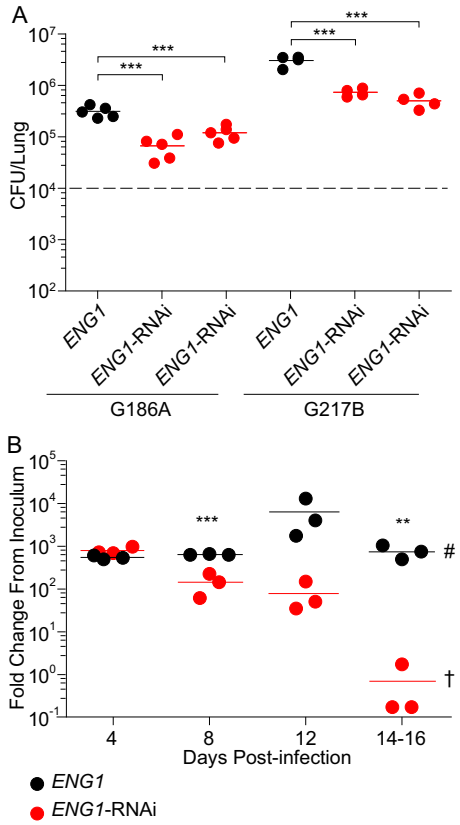

FIG 4 Eng1 promotes *Histoplasma* virulence *in vivo*. Wild-type C57BL/6 mice were infected intranasally with 1×10^4 Eng1-expressing (*ENG1*; black data points) or Eng1-deficient (*ENG1*-RNAi; red data points) yeast cells, and the fungal burden (CFU) in lungs was determined by plating of lung tissue homogenates. (A) *Histoplasma* burden in lungs 8 days postinfection with yeasts of the G186A or G217B genetic background. The dashed line indicates the inoculum level, and data points represent the *Histoplasma* CFU counts from each mouse ($n = 4$ to 5). (B) Kinetics of lung infection by *ENG1*-expressing and *ENG1*-deficient *Histoplasma* yeasts of the G217B background determined at 4, 8, 12, 14, or 16 days postinfection. Mice infected with Eng1-expressing yeasts were moribund at 14 days postinfection (# symbol), at which point lung tissue was harvested. Mice infected with Eng1-deficient *Histoplasma* remained alive, and lung tissue was harvested at 16 days postinfection († symbol). The data represent the fold change in CFU from the initial inoculum at each time point ($n = 3$ mice per strain). Horizontal bars represent means, and asterisks represent statistically significant differences between infections with Eng1-expressing and Eng1-deficient strains as determined by one-tailed Student's *t* test (**, $P < 0.01$; ***, $P < 0.001$).

ognition of Eng1-deficient yeasts. Yeast cells that lacked the Eng1 β-glucanase function showed 4-fold greater recognition by Dectin-1-expressing fibroblasts than the Eng1-producing yeast cells (Fig. 5A). Competition with excess soluble β-glucan (laminarin) eliminated yeast recognition, demonstrating that the enhanced recognition of Eng1-deficient yeasts was dependent on the β-glucan receptor.

To directly show that secreted Eng1 reduces β-glucan exposure, *Histoplasma* yeasts with exposed β-glucans (*ENG1*-RNAi yeasts) were pretreated with *Histoplasma* cell-free culture filtrates derived from either Eng1-producing strains (*ENG1*) or Eng1-deficient strains (*ENG1*-RNAi) and the recognition of the yeasts by Dectin-1 was quantified (Fig. 5B). Treatment of Eng1-deficient yeast with saline solution or with culture filtrates lacking Eng1 resulted in significant recognition of yeast cells by Dectin-1. In contrast, treating yeast cells with Eng1-containing culture filtrate reduced *Histoplasma* yeast detection by Dectin-1, similarly to treatment of cells with the β-glucanase enzyme zymolyase (Fig. 5B). Incubation of the yeasts with purified Eng1 protein also dramatically reduced detection by Dectin-1 (Fig. 5B). These data indicate that Eng1 can decrease β-glucan exposure on the cell surface, thereby decreasing Dectin-1 recognition.

The Eng1-dependent reduction in Dectin-1 recognition of *Histoplasma* yeasts is not due to large-scale alteration of cell wall composition and structure. Biochemical analysis of yeast cell walls for total glucose and mannose saccharide content demonstrated that yeast cells grown in the presence of Eng1 had no significant reduction in cell wall glucose content relative to the amount of mannose compared to yeasts grown in the absence of Eng1 (see Fig. S5 in the supplemental material). Although the absolute glycan compositions differed between the G186A and G217B backgrounds, there were no Eng1-dependent differences, indicating that the Eng1 glucanase does not cause any major changes in the glucan content of the yeast cell wall. By transmission electron microscopy, there were no large-scale abnormalities or notable differences in the ultrastructure or thickness of the cell walls between Eng1-producing and Eng1-deficient cells (see Fig. S6). Consistent with these data, Eng1-deficient *Histoplasma* yeast cells have no increased sensitivity to cell wall-destabilizing compounds (Calcofluor white, Congo red, or Uvitex), detergent (SDS), or antifungal drugs, including the β-glucan synthesis inhibitor caspofungin (see Table S2).

These findings suggest that the secreted Eng1 β-glucanase plays a role in fine scale hydrolysis of cell wall β-glucans, such as removal only of β-glucan segments that are surface exposed. As evidence for this, yeast cells were incubated with soluble Dectin-1 receptor (FcDectin-1) to visualize by immunofluorescence microscopy the β-glucan exposure on nonpermeabilized yeast cells. Consistent with the cell-based Dectin-1 binding assay (Fig. 5A), yeasts producing Eng1 had reduced labeling by FcDectin-1 compared to yeasts lacking Eng1 (Fig. 5C). Eng1-producing yeasts limit β-glucan exposure to the septum region of budding cells. Eng1-deficient yeasts also have enriched β-glucan at the septum between yeasts; however, FcDectin-1 binding is also abundantly present around the entire circumference of yeast cells. These data indicate that while Eng1 does not alter the gross cell wall structure, it effectively decreases β-glucan exposure from the surface of yeast cells.

The greater recognition of Eng1-deficient yeasts by Dectin-1 has consequences for pathogenesis, as greater recognition can translate to increased production of proinflammatory cytokines by macrophages and dendritic cells (DCs). Eng1-deficient yeast stimulated greater TNF-α (Fig. 6A) and IL-6 (Fig. 6B) production. Incubation of phagocyte populations with wild-type *C. albicans* yeasts similarly stimulated proinflammatory cytokines, often to levels even greater than those seen with Eng1-deficient yeasts. The increased cytokine response of macrophages to Eng1-deficient yeasts was negated by preincubation with a Dectin-1 blocking antibody, demonstrating dependence on Dectin-1 (see Fig. S7 in the supplemental material). Although Eng1-deficient yeasts had increased recognition by Dectin-1, their association with macrophages was equal to that of Eng1-producing yeasts (see Fig. S8A), indicating that Eng1 does not affect the ability of yeast to associate

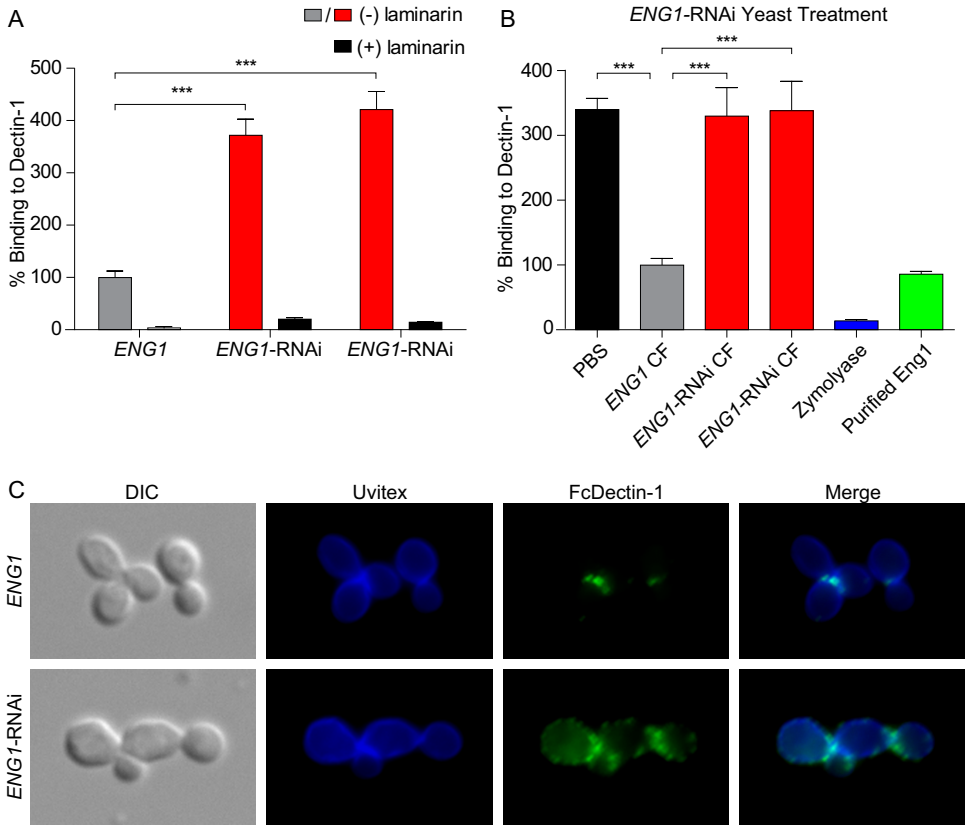

FIG 5 Eng1 decreases Dectin-1 recognition of *Histoplasma* yeasts. (A) Relative levels of binding of Eng1-expressing (*ENG1*; gray bar) and Eng1-deficient (*ENG1*-RNAi; red bars) yeasts to Dectin-1-expressing cells. Uvitex-labeled yeasts were added to Dectin-1-expressing 3T3 fibroblast cells. Adherent yeasts were released by lysis of the fibroblasts and the yeasts quantified by Uvitex fluorescence. Laminarin was added to the Dectin-1-expressing 3T3 cells as a competitive inhibitor (black bars) prior to addition of yeast cells. All data were normalized to the average level of binding of Eng1-expressing yeasts (*ENG1*) in the absence of laminarin. (B) Ability of Eng1-containing culture filtrates to reduce Dectin-1 recognition of yeasts. Eng1-deficient yeasts were treated with saline solution (PBS; black bar), Eng1-containing culture filtrate (*ENG1* CF; gray bar), Eng1-deficient culture filtrate (*ENG1*-RNAi CF; red bars), the β-glucanase zymolyase (blue bar), or purified Eng1 (green bar). Treated yeast cells were added to 3T3–Dectin-1 cells and the bound yeasts quantified. Data represent the relative amounts of bound yeasts after normalization to yeast cells treated with Eng1-containing culture filtrate. Error bars represent the standard deviations of results from replicate assays ($n = 3$), and asterisks represent statistically significant differences between Eng1-expressing and Eng1-deficient conditions as determined by one-tailed Student's *t* test ($n = 3$; ***, $P < 0.001$). (C) Representative images of *Histoplasma* yeasts showing surface-exposed β-glucans. Yeasts were fixed and exposed β-glucans detected by immunofluorescence following incubation with a soluble Dectin-1 molecule (FcDectin-1). Yeasts were visualized at ×600 magnification by differential interference contrast (DIC), Uvitex fluorescence (blue), and FcDectin-1 immunofluorescence (green) microscopy.

with other macrophage phagocytic receptors. Despite the increased recognition by Dectin-1, survival of Eng1-deficient yeast in macrophages was not affected (see Fig. S8B).

For *in vivo* confirmation that Eng1 reduction of exposed β-glucans enhances *Histoplasma* pathogenesis through Dectin-1 recognition of yeasts, we tested whether loss of Dectin-1 restores the virulence of Eng1-deficient yeasts. Pulmonary infections of wild-type and Dectin-1 knockout mice were established using Eng1-producing and Eng1-deficient strains, and virulence was assessed by quantitation of fungal burdens after 8 days. Eng1-deficient yeast showed a 5.8-fold reduction in fungal burden in the lungs compared to Eng1-producing yeasts when Dectin-1 is present (Fig. 7), consistent with earlier findings (Fig. 4A). Loss of Dectin-1 restored the virulence of the Eng1-deficient yeasts to a level matching that of Eng1-producing yeasts (Fig. 7). Dissemination of Eng1-deficient yeasts to splenic tissue was also comparable to that of Eng1-producing yeasts in the absence of Dectin-1 (see Fig. S4B in the supplemental material). These data show that the attenuation of β-glucan-exposed Eng1-deficient yeasts is dependent on the presence of Dectin-1 in the host and confirm the role of Eng1 in reducing β-glucan detection during infection.

Eng1 and α-glucan both reduce β-glucan exposure. The cell wall of most phylogenetic groups of *Histoplasma* contains α-glucan, which has been shown to mask cell wall β-glucans (12). To determine if Eng1 acts in addition to α-glucan production for minimizing β-glucan exposure, yeasts of the G186A background lacking Eng1 function or lacking α-glucan or lacking both Eng1 and α-glucan were tested for recognition by Dectin-1. Consistent with earlier tests, Eng1-deficient yeast showed a 4-fold to 5-fold increase in Dectin-1 recognition (Fig. 8). Lack of the α-glucan polysaccharide on *ags1* mutant (*ags1*Δ) yeast increased Dectin-1 recognition by 10-fold, consistent with α-glucan playing the major role in hiding yeasts from Dectin-1 (11). Loss of Eng1 function from yeasts also lacking α-glucan (*ags1*Δ/*ENG1*-RNAi double mutant yeasts) increased yeast recognition by Dectin-1 by an additional 30% (Fig. 8). These data suggest that Eng1 acts in addition

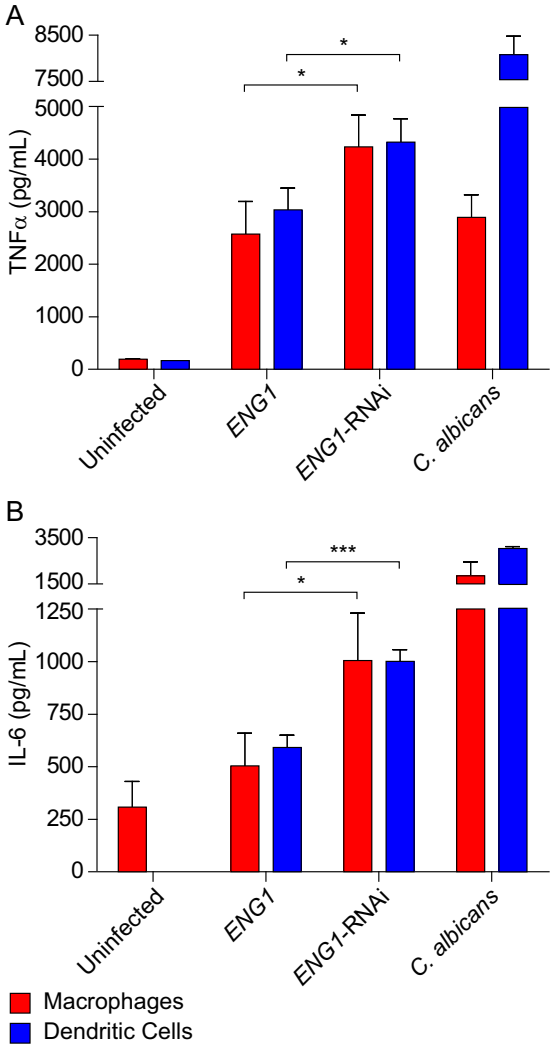

FIG 6 Eng1 activity decreases macrophage recognition and response to *Histoplasma* yeasts. Data represent cytokine production by phagocytes infected with Eng1-producing (*ENG1*) or Eng1-deficient (*ENG1-RNAi*) *Histoplasma* yeasts. Murine peritoneal macrophages (red bars) or bone marrow-derived dendritic cells (blue bars) were infected with *Histoplasma* or *Candida albicans* yeast cells for 8 h at an MOI of 0.5:1, and production of TNF-α (A) and IL-6 (B) was quantified by cytokine-specific ELISA of culture supernatants. Data indicate the average cytokine levels, and error bars represent the standard deviations of results from replicate infections ($n = 3$). Asterisks indicate statistically significant differences in cytokine stimulation between infections with Eng1-expressing and Eng1-deficient yeasts as determined by one-tailed Student's *t* test (*, $P < 0.05$; ***, $P < 0.001$).

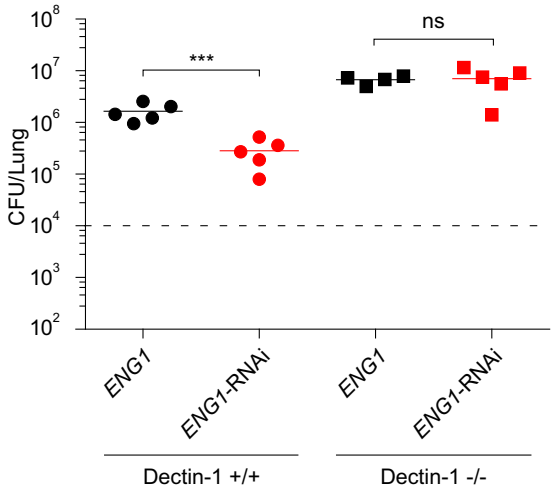

FIG 7 Dectin-1 mediates control of Eng1-deficient yeasts *in vivo*. Wild-type C57BL/6 (Dectin-1 +/+; circles) or Dectin-1 knockout (Dectin-1 −/−; squares) mice were infected intranasally with 1×10^4 Eng1-expressing (*ENG1;* black data points) or Eng1-deficient (*ENG1*-RNAi; red data points) yeast cells, and the fungal burden in lungs (CFU) (A) or spleens (B) was determined by plating of lung tissue homogenates. Data points represent the *Histoplasma* CFU counts from each mouse ($n = 4$ to 5) at 8 days postinfection. The dashed line indicates the inoculum level, and horizontal bars represent the mean CFU recovered. Asterisks represent statistically significant differences between infections with Eng1-expressing and Eng1-deficient strains as determined by one-tailed Student's *t* test (***, $P < 0.001$) ns, not significant.

to the α-glucan polysaccharide of α-glucan-producing strains to further reduce β-glucan exposure and minimize potential Dectin-1 recognition of *Histoplasma* yeasts.

DISCUSSION

The success of *Histoplasma* as a pathogen relies, in part, on its ability to avoid host pattern recognition receptors (PRR). By limiting recognition of fungal cell wall β-glucans by the Dectin-1 receptor, yeasts can curtail macrophage production of proinflammatory cytokines, which are necessary for robust activation of cell-mediated immunity. A major mechanism for this avoidance in some *Histoplasma* strains is the production of α-glucan, which covers the β-glucan layer to limit the yeast cell β-glucan exposure (12). *Histoplasma* strains of the North American type 2 phylogenetic group do not produce α-glucan and yet are fully virulent and are still able to restrict β-glucan exposure (11). In this study, we identified a glucanase (Eng1) which contributes to reduction of cell wall β-glucan exposure. In α-glucan-producing strains (e.g., G186A yeast), α-glucan is responsible for two-thirds of the combined reduction and Eng1 contributes about one-third of the reduction as determined by analysis of single and double mutants. In *Histoplasma* strains naturally lacking α-glucan (e.g., G217B yeast), β-glucan exposure increases when Eng1 is removed as well, likely supplementing other as-yet-undefined mechanisms. For both strain backgrounds, full virulence requires production of Eng1 by infecting yeasts.

In contrast to α-glucan, which minimizes β-glucan exposure by a concealment mechanism, the Eng1 glucanase acts by removal of exposed β-glucans. While we cannot rule out other substrates for Eng1, given that the fungal cell wall is rich in β-glucans, Eng1 likely acts on the *Histoplasma* yeast cell wall. Even though *Histoplasma* Eng1 has homology to *S. cerevisiae* and *C. albicans* Eng1 proteins, *Histoplasma* Eng1 differs in critical aspects. *Histoplasma* Eng1 is secreted, whereas the *S. cerevisiae* Eng1 is localized to the septum, consistent with the absence versus the presence of a GPI anchor motif, respectively. The *S. cerevisiae* Eng1 is necessary for cell separation (23–25), but the *Histoplasma* Eng1 protein is not. While a β-glucanase could potentially function in large-scale glycan remodeling, our data indicate that *Histoplasma* Eng1 functions on a smaller scale. Loss of Eng1 does not cause gross alteration in cell wall composition, structure, function, or integrity as indicated by biochemical, ultrastructural, and chemical sensitivity analyses. These results do not rule out smaller structural changes,

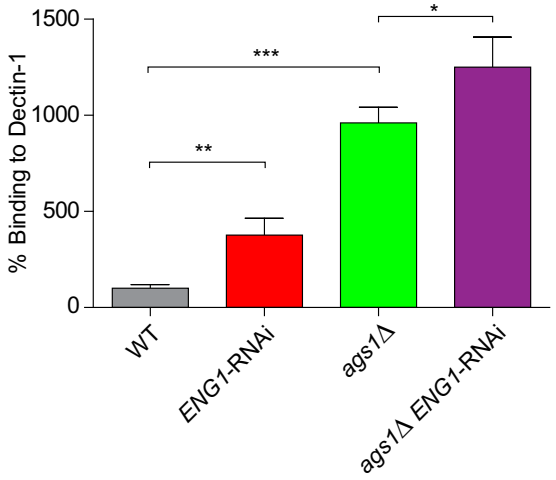

FIG 8 Eng1 and α-glucan combine to reduce yeast β-glucan exposure. Data represent Dectin-1 recognition of G186A-background yeasts lacking Eng1 function (*ENG1*-RNAi; red bar) or α-glucan (*ags1*Δ; green bar) or both factors (*ags1*Δ *ENG*1-RNAi; purple bar). Uvitex-labeled yeasts were added to Dectin-1-expressing 3T3-fibroblasts, and adherent yeasts were quantified by Uvitex fluorescence. Data indicate the average number of yeasts bound by Dectin-1 relative to the number of bound wild-type yeasts (WT; gray bar). Error bars represent the standard deviations of results from replicates ($n = 3$). Asterisks represent statistically significant differences in recognition as determined by one-tailed Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

and our data indicate that *Histoplasma* Eng1 appears to fine-tune the cell wall; we suggest a model in which *Histoplasma* Eng1 is secreted from yeasts, enabling the glucanase to reduce levels of exposed β-glucans on the cell wall surface and not just at the septum of budding cells or throughout the bulk of the cell wall. Consistent with this, cells expressing Eng1 lack Dectin-1-detectable β-glucans on the cell periphery but Eng1-deficient yeasts have Dectin-1-recognizable glucans around the yeasts. Together, these differences in Eng1 structure and localization suggest that the *Histoplasma* Eng1 β-glucanase has been relocalized and repurposed from septum degradation to promotion of *Histoplasma* pathogenesis by trimming away exposed cell wall β-glucans around the periphery of yeast cells.

Prevention of β-glucan recognition by phagocytes is critical for the virulence of *Histoplasma* yeasts. Dectin-1 recognition of *Histoplasma* yeasts is increased without Eng1 function, which translates into increased macrophage production of the proinflammatory cytokines TNF-α and IL-6. Eng1-deficient yeasts also do not stimulate IL-12 release from macrophages (data not shown), but IL-12 production has been shown to be prevented by interaction of *Histoplasma* yeasts with macrophage CR3 (26). This highlights the complexity of the interactions between the yeast and macrophage cell surfaces, the combination of which stimulates different phagocyte outputs. Nonetheless, these studies show that limitation of levels of exposed β-glucans is necessary for reducing proinflammatory cytokine production by macrophages.

Eng1-deficient yeasts consistently showed reduced infectivity *in vivo* which became more pronounced during the adaptive immune response stage. Mice infected with Eng1-producing *Histoplasma* yeasts succumb to the infection, but mice infected with Eng1-deficient yeast efficiently clear the fungal burden. Experiments with cultured phagocytes suggest that Eng1 reduction in exposed β-glucans is not essential for yeast survival in macrophages but is important for reducing proinflammatory cytokine production by phagocytes. Combining the *in vivo* infection kinetics of Eng1-deficient yeasts and the *in vitro* phenotypes of Dectin-1 recognition and cytokine production by cultured phagocytes leads to a model in which Eng1-based reduction in β-glucan exposure results in decreased production of proinflammatory cytokines by phagocytes. Without this mechanism, the increased β-glucan exposure on *Histoplasma* yeasts stimulates a more effective immune response, leading to enhanced control of Eng1-deficient *Histoplasma* yeasts *in vivo*. Thus, Eng1 promotes full *Histoplasma* virulence by removing exposed cell wall β-glucans, thereby reducing host recognition of yeasts and enhancing their ability to survive defenses of the immune system.

MATERIALS AND METHODS

***Histoplasma* strains and cultures.** *Histoplasma capsulatum* strains were derived from the wild-type strains G186A (ATCC 26029) and G217B (ATCC 26032) and are listed in Table S1 in the supplemental material. *Histoplasma* yeasts were grown in *Histoplasma*-macrophage medium (HMM) (27). For growth of uracil auxotrophs, HMM was supplemented with 100 μg/ml uracil. Yeast cultures were grown with continuous shaking (200 rpm) at 37°C. Growth rates of yeasts in liquid culture were determined by measurement of culture turbidity (optical density at 595 nm). Strains derived from G186A yeast were treated with 1 M NaOH to disperse clumps before the optical density was read. Cultures were grown to the late exponential phase or the early exponential phase unless otherwise indicated. Hemacytometer counts were used for precise enumeration of yeasts. For growth on solid medium, HMM was solidified with 0.6% agarose supplemented with 25 μM $FeSO_4$.

Quantitative RT-PCR. Transcriptional profiles for the identified *Histoplasma* endoglucanase genes were determined using quantitative reverse transcription-PCR (qRT-PCR) with SYBR green-based visualization of product amplification (Bioline). RNA was isolated from G217B yeast or mycelia by mechanical disruption in Ribozol reagent (AMRESCO, Inc.) and reverse transcribed with Maxima reverse transcriptase (Thermo Scientific) primed with random pentadecamers. Cycle thresholds were normalized to expression of the transcription elongation factor gene (*TEF1*), and differences between the yeast and mycelial phases were quantified using the threshold cycle ($\Delta\Delta C_T$) method (28). Reduction of *ENG1* transcripts by RNA interference was similarly quantified using RNA from OSU247 (GFP gene-RNAi) or OSU248 (*ENG1*-RNAi). For these, reverse transcription was primed with a 22-mer poly(T) primer and results for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene (control gene) and *ENG1* mRNA were normalized to transcript levels of the ribosomal small-subunit gene (*RPS15*) before comparison between Eng1-producing and Eng1-deficient strains was performed.

Epitope-tagged Eng1 localization and Eng1 purification. The *ENG1* gene was amplified by high-fidelity PCR (Phusion; NEB) from G217B genomic DNA and cloned into *URA5*-based *Histoplasma* expression plasmids (containing the constitutive *Histoplasma* H2B promoter). The *ENG1* CDS was fused to the FLAG epitope (pCR628 [20]) or a hexahistidine tag (pCR493) at the C terminus. *Histoplasma* strain WU15 was transformed with *ENG1* expression plasmids via *Agrobacterium tumefaciens* (29), and transformants were screened for secretion of epitope-tagged protein by immunoblotting of transformant culture filtrates with antibodies to the FLAG epitope (Sigma) or the hexahistidine tag (GenScript).

For subcellular fractionation, 1×10^8 yeast cells expressing FLAG-tagged Eng1 were separated from culture filtrates by centrifugation and filtration. Cellular lysates were prepared by mechanical disruption. The cytosolic fraction was separated from cellular debris by centrifugation (10 min at 14,000 $\times$ *g*). Insoluble material was treated with 1% SDS and 0.1 M dithiothreitol (DTT) to extract cell wall-associated proteins or incubated with 3 mU/μl of zymolyase (GBiosciences) to release embedded cell wall proteins. Solubilized material was separated from the insoluble

fraction by centrifugation (10 min at 14,000 × *g*). The remaining insoluble material was examined by immunofluorescence microscopy after incubation of the cellular debris with the anti-FLAG epitope antibody and Cy3-conjugated secondary antibody (Pierce). Subcellular fractions representing material from 1×10^7 yeasts were probed for the FLAG epitope by immunoblotting after separation of the proteins by electrophoresis through 10% polyacrylamide with SDS (SDS-PAGE) and transfer to nitrocellulose.

For purification of Eng1, yeasts expressing Eng1 with the hexahistidine tag were grown to saturation. The culture filtrate was concentrated 100-fold by ultrafiltration (10-kDa-cutoff membrane). Tagged-Eng1 protein was purified by affinity chromatography (HisPur Co^{2+} resin; Thermo Fisher Scientific).

Depletion of gene function by RNAi. Eng1 function was depleted from *Histoplasma* yeasts by RNA interference (RNAi) (22). The ENG1-RNAi vector was created by PCR-based amplification of nucleotides 445 to 2091 of the *ENG1* coding region (CDS). Vectors for GFP gene-RNAi or *ENG1*-RNAi were transformed by *Agrobacterium*-mediated transformation (22) into GFP gene-expressing sentinel strains OSU22 (G186A background) or OSU194 (G217B background). Ura^+ transformants were recovered, and the sentinel GFP gene fluorescence was quantified using a modified gel documentation system (22) and ImageJ software (v1.44p; http://imagej.nih.gov/ij). *ENG1*-RNAi depletion in the *ags1*Δ mutant was performed by transformation of *ags1* mutant yeasts with the *ENG1*-RNAi plasmid. In the absence of the GFP gene sentinel, silencing of *Eng1* was confirmed by the reduction in extracellular glucanase activity.

Dectin-1 recognition of the *Histoplasma* cell wall. Soluble Dectin-1 (FcDectin-1) was collected from HEK293T cells transformed with the pSecTag2 expression vector containing the Dectin-1 carbohydrate recognition domain fused with the Fc region of human IgG1 (30). Washed *Histoplasma* yeast cells were fixed in 3% paraformaldehyde, and FcDectin-1-containing culture medium was added directly to yeast cells. FcDectin-1 binding was visualized using Alexa Fluor 488-conjugated anti-IgG-Fcγ antibody (Jackson ImmunoResearch). Yeast cells were costained with 0.1% Uvitex 3BSA (CIBA-Geigy). DIC and fluorescent images were collected using an Eclipse-Ti eipfluorescence microscope (Nikon) with a 1.4 megapixel charge-coupled-device (CCD) camera (CoolSnap HQ2; Photometrics).

Assay of glucanase activity. Cell-free supernatant and yeast cells were separated by centrifugation (2,000 × *g*) and the supernatants filtered (0.2-μm-pore-diameter membrane) and concentrated by ultrafiltration. Culture filtrate supernatant (equivalent to supernatant from 5×10^6 cells), 3 ng of purified Eng1, or zymolyase was incubated with laminarin (5 mg/ml) at 37°C for 90 min. Hydrolysis of laminarin was measured by the production of reducing sugar ends, which were quantified by adding 3 volumes of dinitrosalicylic acid (DNS) solution (0.687% 3,5-dinitrosalicylic acid, 1.28% phenol, 19.92% potassium-sodium-tartarate, 1.226% sodium hydroxide) and incubating at 95°C for 5 min (31, 32). Reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid was quantified by absorbance at 540 nm and compared to a standard curve created from glucose.

Murine model of respiratory and disseminated histoplasmosis. C57BL/6 mice (Charles River) or C57BL/6-Dectin-1 knockout mice (Dectin-1 −/−) were infected with *Histoplasma* by intranasal delivery of approximately 1×10^4 yeast cells to mice under anesthesia. Actual levels of inocula delivered were determined by plating serial dilutions of the inoculum suspensions for enumeration of CFU. At 4, 8, 12, or 16 days postinfection, mice were euthanized and lungs and spleens collected. Organs were homogenized, and serial dilutions of the homogenates were plated on solid HMM to determine the fungal burden (CFU) in each organ.

Mammalian cell culture and primary cell isolation. Murine peritoneal macrophages, bone marrow-derived dendritic cells (BMDCs), and Dectin-1-expressing 3T3 fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Mammalian cells were cultured at 37°C in 5% CO_2/95% air. Peritoneal macrophages were obtained from C57BL/6 mice by peritoneal lavage with phosphate-buffered saline (PBS). For elicitation of macrophages, peritoneal injection of 3% protease peptone was performed 4 days prior to lavage. Bone marrow cells were isolated from femurs of C57BL/6 mice and differentiated by being cultured in 1,000 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) for 7 days to obtain dendritic cells (33) followed by removal of nonadherent cells from plastic dishes. Cells were enumerated by hemacytometer and seeded at the appropriate density for the respective assays. Animal experiments were performed in compliance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at Ohio State University (2007A0241).

Phagocyte infections and cytokine profiling. Macrophages and dendritic cells were infected with *Histoplasma* yeasts by coincubation with yeast cells in 96-well microtiter plates. The numbers of phagocytes per well were 2×10^5 (peritoneal macrophages) and 1×10^5 (BMDCs). *Histoplasma* yeast cells were added at a multiplicity of infection (MOI; yeasts/phagocytes) of 0.5:1 for yeast survival and for cytokine profiling. Yeast survival was determined by hypotonic lysis of phagocytes in water and plating of serial dilutions of the phagocyte lysate to enumerate *Histoplasma* CFU. For cytokine analysis, culture supernatants were collected after 8 h of incubation at 37°C. TNF-α and IL-6 cytokine production was determined by cytokine-specific enzyme-linked immunosorbent assays (ELISAs) (R&D Systems). Cytokine concentrations were calculated by comparison of absorbance results to TNF-α and IL-6 standard curves. For Dectin-1 blocking, 30 μg/ml of either anti-Dectin-1 monoclonal antibody (InvivoGen; catalog no. mabg-mdect) or isotype control monoclonal antibody (specific for *Escherichia coli* β-galactosidase) (InvivoGen; catalog no. mabg2a-ctlrt) was added to the macrophages for 1 h prior to infection with yeast.

Dectin-1 binding assay. Dectin-1-expressing 3T3 fibroblasts (9, 11) were adhered to wells of a 24-well plate at 3×10^4 cells/well and incubated overnight. Yeast cells were stained with 0.1% Uvitex–PBS and added to the 3T3–Dectin-1 cells for 2 h at 37°C at an MOI of 50:1 (yeast/3T3 cells) followed by removal of unbound yeasts. Associated yeasts were released by lysing the 3T3–Dectin-1 cells with 1% Triton X-100 and quantified by Uvitex fluorescence (375-nm excitation, 435-nm emission) using a FluoroMax-3 spectrofluorimeter (Horiba Jobin Yvon) (11). Competition of yeast with laminarin was performed by preincubating the 3T3–Dectin-1 cells with 1 mg/ml laminarin. For treatment of *ENG1*-RNAi yeast prior to Dectin-1 binding, *Histoplasma* strain OSU248 yeasts were washed with PBS and resuspended in a 1× volume of Eng1-containing culture filtrate (derived from strain OSU247), Eng1-deficient culture filtrate (derived from strain OSU248 or OSU249), 3 mU of zymolyase, 1.5 ng of purified Eng1, or PBS. Yeast cells were treated for 3 h at 37°C before addition to the 3T3–Dectin-1 cells.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01388-15/-/DCSupplemental.

Figure S1, PDF file, 0.2 MB.
Figure S2, PDF file, 0.3 MB.
Figure S3, PDF file, 0.3 MB.
Figure S4, PDF file, 0.3 MB.
Figure S5, PDF file, 0.3 MB.
Figure S6, PDF file, 1.6 MB.
Figure S7, PDF file, 0.3 MB.
Figure S8, PDF file, 0.3 MB.
Table S1, PDF file, 0.04 MB.
Table S2, PDF file, 0.1 MB.

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