Brain Inositol Is a Novel Stimulator for Promoting Cryptococcus Penetration of the Blood-Brain Barrier

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Abstract

Cryptococcus neoformans is the most common cause of fungal meningitis, with high mortality and morbidity. The reason for the frequent occurrence of Cryptococcus infection in the central nervous system (CNS) is poorly understood. The facts that human and animal brains contain abundant inositol and that Cryptococcus has a sophisticated system for the acquisition of inositol from the environment suggests that host inositol utilization may contribute to the development of cryptococcal meningitis. In this study, we found that inositol plays an important role in Cryptococcus traversal across the blood-brain barrier (BBB) both in an in vitro human BBB model and in in vivo animal models. The capacity of inositol to stimulate BBB crossing was dependent upon fungal inositol transporters, indicated by a 70% reduction in transmigration efficiency in mutant strains lacking two major inositol transporters, Itr1a and Itr3c. Upregulation of genes involved in the inositol catabolic pathway was evident in a microarray analysis following inositol treatment. In addition, inositol increased the production of hyaluronic acid in Cryptococcus cells, which is a ligand known to binding host CD44 receptor for their invasion. These studies suggest an inositol-dependent Cryptococcus traversal of the BBB, and support our hypothesis that utilization of host-derived inositol by Cryptococcus contributes to CNS infection.

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Introduction

Cryptococcus neoformans is a basidiomycetous yeast pathogen that often causes life-threatening infections. It causes the most common fungal infection of the central nervous system (CNS) in HIV-infected persons and may present as encephalitis, meningitis, or cerebral-space-occupying lesions [1,2,3,4,5,6]. Cryptococcal CNS infections are uniformly fatal in the absence of treatment [1,7]. A recent survey suggests that each year there are around 1 million new cases of cryptococcal meningitis, which result in over 600,000 deaths annually [2]. Despite its medical importance and significant research efforts [3,8,9,10], the molecular basis of cryptococcal CNS infection and host factors affecting disease development are poorly understood, which complicates efforts for rapid diagnosis and effective treatment. Hence, there is an urgent need to understand the molecular basis of cryptococcal CNS infection to allow the discovery and development of safer and more effective antifungal drugs.

C. neoformans is a globally ubiquitous organism, which is commonly associated with certain environmental niches, including plant debris and soil contaminated from plant surfaces to complete its sexual cycle [11]. Inositol is essential for cellular structure and regulation of intracellular signaling in all eukaryotes. Recent studies showed that the enzymes involved in inositol metabolism and inositol sphingolipid biosynthesis play a central role in the pathogenesis of C. neoformans [12,13]. The inositol phosphoceramide synthase 1 (Ipc1) protein, an enzyme of the fungal sphingolipid pathway, activates protein kinase C (PKC), which regulates the cell wall integrity of Cryptococcus and manifestation of its virulence factors [12,14,15]. Moreover, although it prefers to grow on media containing fermentable sugars such as glucose, C. neoformans can utilize free inositol as a sole carbon source [16,17]. Consistent with the central importance of inositol in its development and virulence, Cryptococcus has developed a sophisticated inositol acquisition system that contains an unusually large inositol transporter gene (ITR) family with more than ten members, which contrasts with the one or two members found in most other fungi [18,19,20,21]. We also demonstrated that these ITRs are required for cryptococcal infection in murine models [11,22,23]. In addition, Cryptococcus can utilize intracellular glucose to produce inositol in a multi-step de novo inositol biosynthetic pathway in which inositol 1-phosphate synthase (Ips1) is the rate-determining enzyme [19,24].
Inositol Promotes Cryptococcus Brain Invasion

Author Summary

Cryptococcus neoformans is an AIDS-associated human fungal pathogen that annually causes over 1 million cases of meningitis world-wide, and more than 600,000 attributable deaths. Cryptococcus often causes lung and brain infection and is the leading cause of fungal meningitis in immunosuppressed patients. Why Cryptococcus frequently infects the central nervous system to cause fatal meningitis is an unanswered critical question. Our previous studies revealed a sophisticated inositol acquisition system in Cryptococcus that plays a central role in utilizing environmental inositol to complete its sexual cycle. Here we further demonstrate that inositol acquisition is also important for fungal infection in the brain, where abundant inositol is available. We found that inositol promotes the traversal of Cryptococcus across the blood-brain barrier (BBB), and such stimulation is fungal inositol transporter dependent. We also identified the effects of host inositol on fungal cellular functions that contribute to the stimulation of fungal penetration of the BBB. We propose that inositol utilization is a novel virulence factor for CNS cryptococcosis. Our work lays an important foundation for understanding how fungi respond to available host inositol and indicates the impact of host inositol acquisition on the development of cryptococcal meningitis.

Cryptococcus invasion and traversal of the blood-brain barrier (BBB) are prerequisites for CNS infection, the major cause of morbidity and mortality in people with cryptococcosis. There are evidences for both direct invasion of the endothelial cells lining the brain vasculature [25,26] and for a “Trojan horse” mechanism whereby cryptococci enter the CNS after macrophage ingestion [27,28,29]. Several factors, including urease [30,31], phospholipase B1 [32,33], as well as host plasmin [34], have been reported to be involved in the Cryptococcus invasion of the BBB. It was recently reported that Cryptococcus interacts with lipid rafts of human brain microvascular endothelial cells (HBMECs) to promote invasion in a glycoprotein CD44-dependent manner [35]. Hyaluronic acid produced by the fungus has been found to function as a ligand for the CD44 receptor during the fungal-host cell interaction [36,37]. However, the molecular basis for the highly frequent occurrence of cryptococcal CNS infection remains poorly understood. Human and animal brains contain high concentrations of free inositol, and inositol can be used as a carbon source for Cryptococcus. Inositol is one of the most abundant metabolites in the human brain; it is located mainly in glial cells, and functions as an osmolyte. Inositol is present in the human cerebellum (5.1 mM) at 200-fold higher concentrations than in plasma (0.02 mM) [38]. Even higher inositol concentrations (>8 mM) are detected in astrocytes that directly associate with the BBB, and inositol can be rapidly released during hyperosmolarity [38,39]. It is believed, because of the tight interaction of astrocytes with brain microvascular endothelial cells of the BBB, that the inositol concentration around the BBB is much higher than in plasma, although the inositol level in the parenchyma around cerebral vasculature has not been precisely determined. Together with the importance of ITRs in fungal infection, we hypothesize that brain inositol is an important host factor for the development of cryptococcal meningitis. We further hypothesize that fungal inositol transporters are important for sensing and/or transporting host inositol during disease progression.

In this study, we utilize an in vitro human BBB model and in vivo murine models to dissect the role of fungal inositol transporters and host inositol in the traversal of Cryptococcus across the BBB and in the development of cryptococcal meningitis. Our results showed that addition of inositol can facilitate Cryptococcus transmigration in an in vitro BBB model in an ITR-dependent manner. These observations tie inositol to the fungal infection in the brain. This work provides a framework explaining the role of inositol in enabling Cryptococcus to cross the BBB.

Results

Inositol enhances C. neoformans transmigration across an HBMEC monolayer

Previous studies demonstrate that the route by which C. neoformans gains access to the CNS is through traversal across the BBB [37,40,41]. The high abundance of inositol in human brain is suggested to be a host factor that promotes the high rate of cryptococcal meningitis [22,42]. To understand whether inositol plays a role in the development of cryptococcal CNS infection, we investigated the role of free inositol in C. neoformans transmigration across the BBB, using an in vitro human BBB system. Our in vitro BBB system is composed of human brain microvascular endothelial cells (HBMECs) grown on Transwell membranes to confluence, separating the top compartment (blood side) and bottom compartment (brain side) as described in Materials and Methods. We initially performed transmigration assays with C. neoformans in the presence of inositol. Inositol was added to the bottom compartment of the Transwells 30 min prior to addition of Cryptococcus cells to the top compartment to mimic the situation in the brain. As shown in Fig. 1A, the number of C. neoformans var. grubii (strain H99) cells that transmigrated across the HBMEC monolayers was not different from control transmigration (no inositol) at inositol concentrations up to 0.5 mM. However, at inositol concentrations greater than 1 mM, transmigration of C. neoformans was increased 3-fold compared to controls without inositol treatment, indicating that inositol enhances cryptococcal traversal in a dose-dependent manner.

Because the binding of fungal cells to HBMEC monolayers is the first step in transmigration, association assays were carried out under the same inositol treatment. The results showed a significantly better association between cryptococcal cells and the brain endothelial cells when the bottom chamber contained 1 mM or higher concentration of inositol, indicating that inositol can promote Cryptococcus binding (Fig. 1B).

Because the tissue culture medium for HBMEC is rich in nutrients including sufficient glucose (5 mM) for optimal fungal growth, all Cryptococcus strains should grow well in this medium. To address the concern that fungal cells may proliferate better in the presence of additional inositol, which might account for the apparent increase in fungal cells in the bottom compartment in the presence of high inositol, the growth rate of H99 in the bottom culture medium was determined in the presence or absence of 1 mM inositol. The results showed that H99 proliferated at the same rate with or without inositol (Fig. 1C). The average replication time for H99 in both media was about 2.2 hours. Therefore, the increased number of Cryptococcus cells in the bottom compartment at higher inositol levels could not be due to increased proliferation, and must be the result of increased transmigration. Thus, these results demonstrate that inositol stimulates traversal of cryptococcal cells across the BBB.

The effect of inositol on fungal transmigration is Cryptococcus specific

To determine whether the inositol effect is strain specific, the transmigration assay was also carried out with C. neoformans var.
C. neoformans var. grubii strains are in general more virulent than var. neoformans strains even though both varieties are able to cause systemic cryptococcosis and meningitis [43]. The number of transmigrated B3501 cells was comparable to that of strain H99, demonstrating that inositol enhances transmigration of C. neoformans regardless of strain origins (Fig. 1D). In addition, the inositol effect on transmigration was examined in Candida albicans, another yeast pathogen that occasionally crosses the BBB through direct transcytosis to cause CNS infection in humans [44,45]. C. albicans contains one inositol transporter, Itr1, that is not required for fungal virulence [20]. Transmigration of C. albicans occurred at a much lower rate and was not enhanced by inositol (Fig. 1D).

Subsequently, the transmigration assays were performed in the presence of another inositol isomer scyllo-inositol, or other monosaccharides such as galactose and mannose. Myo-inositol elevated the efficiency of cryptococcal transmigration exhibiting 2-fold greater number of transmigrated fungal cells than the untreated control after 3, 6 and 9 hrs of incubations (Fig. 1E). However, the cryptococcal transmigration remained unchanged in the presence of other sugars. This result indicates that myo-inositol is a specific effector for promoting cryptococcal traversal across the BBB. Taken together, our findings indicate that inositol specifically increases BBB traversal by C. neoformans.

Cryptococcus-HBMEC interactions lead to increased inositol permeability of the HBMEC monolayer

To understand how inositol in the bottom compartment affects Cryptococcus cells that are present in the top compartment, we measured the concentration of inositol in the top chamber by using an enzymatic method [46]. The medium alone contains around 0.18 mM inositol. In the absence of Cryptococcus in the top compartment, the addition of 1 mM inositol in the bottom compartment did not result in a measurable increase in the inositol levels in the top compartment after 3 hr incubation and only an increase to 0.25 mM inositol after 6 hr incubation, indicating that the HBMEC monolayer maintained high integrity and that inositol diffusion was very slow (Fig. 2A). In contrast, when 10^5 Cryptococcus cells were added in the top compartment, the inositol level in the top compartment reached 0.47 mM after 3 hr and 0.84 mM after 6 hr (Fig. 2A). These results demonstrated that inositol can diffuse through the HBMEC monolayer from the bottom compartment.
increased inositol permeability without causing major damage in the integrity of the HBMEC monolayer. To investigate whether such modification leads to the alteration of the integrity of the HBMEC monolayer, we also measured the transendothelial electrical resistance (TEER) of the monolayer. Our results showed a similar TEER readout in the absence or presence of yeast cells in the upper chamber (Fig. S1), suggesting there was no major change to the integrity of the monolayer, which is consistent with previous studies [25,47].

**Inositol transporter genes of C. neoformans are required for its traversal across the HBMEC monolayer.**

Our previous studies demonstrated that *C. neoformans* has an unusually large inositol transporter (*ITR*) gene family, and established that *ITRs* were required for the full virulence of *Cryptococcus* in *in vivo* murine models [11,22,23]. Among them, *Itr1a* and *Itr3c* are two major *ITRs* for inositol uptake and fungal virulence [22]. To determine whether the attenuated brain infection of *Itr* gene deletion mutants was due to their defective ability to cross the BBB, we examined the transmigration ability of *C. neoformans* *itr1aA itr3cA* double mutants lacking these two major inositol transporter genes in our *in vitro* BBB model. In the absence of additional inositol, transmigration of the *itr1aA itr3cA* double mutant was decreased by 30% at 3 and 6 hr incubation periods compared to the wild type, indicating that *ITR* genes are required for cryptococcal crossing of the BBB. With inositol treatment, cryptococcal traversal was enhanced regardless of presence of *ITR* genes; however, a more significant defect of transmigration ability of *itr1aA itr3cA* mutants was evident. The number of transmigrated wild type H99 strain increased by 2.7 and 2-fold in the presence of inositol at 3 and 6 hr incubation, respectively, compared to transmigration in the absence of added inositol. In contrast, the *itr1aA itr3cA* double mutant exhibited approximately 1.7 and 1.3-fold increased transmigration rates after 3 and 6 hr incubation, respectively, with the result that the number of transmigrated *itr* double mutant cells was a third of that of the wild type strain H99 (Fig. 3A). The reduction in transmigration ability of the *itr1aA itr3cA* double mutant was fully restored by complementation.

Because inositol can be used as the carbon source for growth, there is a logistic concern that fungal cells with intact *ITRs* could grow better in medium with additional inositol. To address the concern, we compared the growth rate of wild type and the *itr1aA itr3cA* double mutant in the medium with or without addition of inositol. The results showed that all tested strains have similar growth rates regardless of the presence of inositol or *ITR* genes (Fig. 1C). Thus, the data are consistent with the interpretation that *Itr*1a and *Itr*3c play an important role in responding to inositol availability and contribute to cryptococcal traversal across the HBMEC monolayer.

We next pre-incubated *Cryptococcus* cells with 1 mM inositol (0.5, 1 and 3 hr), and then removed inositol by thorough washing with PBS before assessing transmigration (Fig. 3B). *Cryptococcus* cells pre-incubated for 30 min showed comparable transmigration to the untreated control, whereas longer pre-incubations of 1 or 3 hr enhanced the number of transmigrated fungal cells by 20% and 40%, respectively. However, similar enhancement in fungal transmigration was not detected with the *itr1aA itr3cA* double mutants following inositol pre-incubation (Fig. 3B). Association assays were then carried out with fungal cells pre-incubated with inositol to compare the ability of the wild type strain and the *itr1aA itr3cA* double mutant to associate with the HBMEC. The number of associated H99 cells was not changed after 30 min pre-incubation but was significantly increased after 3 hr compared to that of the untreated control (Fig. 3C). *Cryptococcus* cells pre-

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**Figure 2. Determination of myo-inositol concentration in the top compartment.** (A) *In vitro* human BBB model was prepared with 1 mM inositol in the bottom compartment and incubated with or without 10⁵ *Cryptococcus* cells in the top compartment. After 3 and 6 hr incubations, the medium in the top compartment was collected and inositol concentration was determined. (B and C) Confluent HBMEC monolayers grown on the collagen-coated glass coverslips were incubated with medium only (B) or with the wild type H99 (C) for 1 hr. The cells were incubated with ZO-1 antibody, followed by Alexa488-conjugated secondary antibody. The coverslips were mounted on slides with Vectashield mounting solution containing DAPI and observed under a fluorescence microscope. ZO-1 (green) and nuclei (blue) are shown. Bar indicates 50 μm.

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Incubation and transmigration across the BBB.

Transmigration assays were performed with H99, the itr1a wild type strain, the association rate of the incubated for 1 hr exhibited a modest increase. Unlike the H99 mutant, and its complemented strain in the absence or presence of 1 mM inositol in the bottom compartment. (B and C) H99 and the itr1a itr3c mutant were pre-incubated with 1 mM inositol for the indicated times and their abilities to associate with the HBMEC monolayer and to transmigrate were determined by transmigration and association (C) assays, respectively. Pre-incubated Cryptococcus cells were washed three times with the experiment medium to remove any residual inositol before assays. These assays were repeated three times independently. Cn: Cryptococcus neoformans. * Denotes statistical significance (P<0.001).

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Involvement of ITRs in BBB crossing in vivo

To further extend the results obtained from the in vitro human BBB model, we assessed infection with the itr1aA itr3cA double mutant in a murine model via intravenous injection. Infected mice were sacrificed at 1, 6, 24, 48, or 72 hr post-inoculation; brains and lungs were isolated and yeast CFUs were determined. Our results demonstrated that there was a significant difference in fungal burden in the brain between mice infected by wild type H99 or the itr1aA itr3cA double mutant after 24 hr post-infection. However, there was no significant difference in CFU at earlier time points (Fig. 4A). On the other hand, the fungal burden was similar in lungs infected either by wild type or the mutant at all time points except 72 hr (Fig. 4B). Our results thus demonstrate that inositol transporters Itr1a and Itr3c are required for fungal cells to either cross the BBB or grow in the brain after transmigration. It has been reported that although fungal crossing of the BBB occurred quite early after inoculation, the rate of traversal of Cryptococcus cells across the BBB increases dramatically 24 hr post-injection via tail vein [48]. We hypothesize there is a threshold effect with respect to time or number of cryptococcal cells accumulating on the endothelial monolayer before they can effectively penetrate the barrier. Fungal burdens at 72 hr post-injection were reduced in both brains and lungs infected by the mutant. However, the reduction is much greater in the brain (7-fold) compared to the reduction in the infected lung (4-fold) (compare 72 hr time point in Fig. 4). To investigate whether the mutant has a defect in survival in macrophage, we performed Cryptococcus-macrophage interaction assays using the macrophage-like cell line J774. Our results showed that wild type and the double mutant had similar response to phagocytosis and macrophage killing (Fig. S2).

To further understand the brain fungal infection at early time points post inoculation, we examined the presence of Cryptococcus within the CNS. To perform these studies, animals were infected with H99 or the itr1aA itr3cA double mutant for 48 hr before they were sacrificed to obtain the brain. Staining and subsequent confocal microscopy of 30–50 μm brain tissue sections was performed for polysaccharide glucuronoxylomannan (GXM) antibody to visualize the transmigration of cryptococcal cells into the CNS parenchyma. Our results showed that brains infected with H99 resulted in cryptococcal cells invasion of the CNS (5 to 7 lesions for each brain, especially in the cortex area). Transmigration of Cryptococcus cells was associated with large lesions (Fig. 5C). Animals infected with the itr1aA itr3cA mutant present fewer lesions and cryptococcal cells within the CNS (Fig. 5C, an average of 2 lesions by brain analyzed). The outcomes from fungal CFU counts and from immunofluorescent staining further support our hypothesis that fungal ITRs play a positive role in Cryptococcus traversal of the BBB in CNS infection. All these results are consistent with our CFU counts of infected brains (Fig. 4A).

Itr1a and Itr3c are not required for fungal growth in infected brains

To address the possibility that the double mutant may grow slower once inside the brain, thereby leading to lower CFU recovery, we tested the growth of the itr1aA itr3cA mutant on an in vitro cerebral spinal fluid (CSF) medium that has been successfully used to identify strains with a growth defect in the CNS compartment [49]. There was no significant growth defect exhibited by the itrA single and double mutants on CSF medium (Fig. 6A).
during brain infection (Fig. 6B). Fungal cells, indicating that there is no growth difference between wild type H99 or the double mutant contain similar amount of CFUs from mouse brains were measured at 1, 3, and 7 days post-injection. Our results showed that at all three time points, brains (n = 4) infected by either the wild type or the mutant strain (Fig. 6C). This result comparable numbers of CFUs were isolated from rabbits infected 10 days post-inoculation. Overall, these results showed that the rabbit subarachnoid space were measured 3, 7, and 10 days post-infection. CFU from mouse intravenous model. Error bars indicate standard deviations. "*" indicates statistical significance (P < 0.001). doi:10.1371/journal.ppat.1003247.g004

To further confirm the in vitro growth results on CSF medium, we tested the itr1a Δ itr3c Δ double mutant or its complemented strain at indicated times post-inoculation. CFU values are expressed as percentages of the initial inoculum. Error bars indicate standard deviations. "**" indicates statistical significance (P < 0.001). doi:10.1371/journal.ppat.1003247.g004

Figure 4. ITRs are required for traversal crossing of the BBB in a mouse intracerebral infection model. CFUs were recovered from either the brain (A) and the lung (B) of mice injected with H99, the itr1a Δ itr3c Δ double mutant or its complemented strain at indicated times post-inoculation. CFU values are expressed as percentages of the initial inoculum. Error bars indicate standard deviations. 

Genes involved in inositol catabolism are highly upregulated by inositol incubation

Cryptococcus phospholipid composition is related to inositol treatment

Inositol is a precursor for the production of phospholipids, which are essential for cellular functions in eukaryotes. Because large amounts of inositol diffused from the bottom compartment into the top in our in vitro system, Cryptococcus can utilize the inositol available in the medium for its cellular functions. One possible explanation for the inositol effect on Cryptococcus transmigration is a change in phospholipid composition. To interrogate this hypothesis, we performed 2-dimensional thin layer chromatography assays (2D-TLCs) to evaluate the production of phospholipids in Cryptococcus. Our results showed that when yeast cells were grown on synthetic medium containing 5 mM inositol, the same phospholipid species were present in both H99 and the itr1a Δ itr3c Δ double mutant, but the production of phosphatidylinositol (PI) was two-fold lower in the mutant strain than in the wild type. Production of one unidentified lipid species was also significantly reduced in the mutant strain with inositol treatment (Fig. 7). Production of other major phospholipid species, e.g. phosphatidylcholine (PC), phosphorylcholine (PS), and phosphatidylethanolamine (PE), were similar between these two strains (Fig. 7). Hence, phospholipid composition, especially the difference in PI composition, could play a role in the dramatic reduction of transmigration in the double mutant. Furthermore, when we compared the phospholipids in H99 treated or not treated with inositol, we observed a dramatic difference in overall phospholipid levels, and addition of inositol significantly induced the production of all detected phospholipid species (Fig. 7). These results indicate that inositol plays a significant role in fungal phospholipid production, and could be part of the explanation for the inositol effect on Cryptococcus interaction with the HBMEC monolayer.

To further understand how Cryptococcus cells respond to inositol, we analyzed transcriptional profiles of Cryptococcus that were treated or not treated with inositol to identify genes that are regulated by inositol. Cryptococcal cells were treated with 5 mM inositol for 24 hrs. Total RNA was prepared for hybridization with Cryptococcus 70-mer whole genome array chips. Our results showed that roughly 50 genes were significantly upregulated (>2 fold) (Table 1; Fig. S3), which is similar to the number of genes upregulated during mating under inositol induction conditions [23], while more than 300 genes were significantly downregulated (>2 fold) (Table 2). The complete list of genes with greater than two-fold change is shown in Table S1. Among upregulated genes, inositol oxygenase and beta-glucuronidase homologs were highly induced by inositol treatment (Table 1). Quantitative RT-PCR analyses were performed for six genes to confirm the microarray results (Fig. S3). The results demonstrated that the expression of two (myo)-inositol oxygenase genes (MIO1 and MIO2), as well as two beta-glucuronidase genes (CBG1 and CBG2) were indeed highly upregulated.

Cryptococcus can use inositol as a carbon source. Conversion of inositol to glucuronic acid by inositol oxygenases (MIO6) is the first step of the only known pathway for inositol catabolism: the oxygenase controls the utilization of inositol as an energy source [50,51]. C. neoformans has three MIO homologues (CNAG_06623, CNAG_03277, and CNAG_05316), which is another unusual
Multiple copies of the MIO gene is unique to Cryptococcus among the animal and fungal kingdoms [51]. Beta-glucuronidase is a member of the glycosidase family that catalyzes the breakdown of complex carbohydrates by releasing the glucuronic acid residues from polysaccharides [52]. In addition, a few inositol transporters are upregulated by inositol, while the gene encoding inositol 1-phosphate synthase (INO1) is significantly downregulated, suggesting, as we expected, that these two inositol acquisition pathways are regulated by inositol (Table 1 & 2).

Inositol treatment induces the production of hyaluronic acid in C. neoformans

Cps1 is identified as the hyaluronic acid synthase in Cryptococcus [53,54]. Hyaluronic acid has been reported to be a Cryptococcus ligand that can bind to the CD44 glycoprotein in HBMECs [55]. Using a quantitative RT-PCR analysis, we also detected high induction of CPS1 gene expression following treatment with inositol (Fig. 8A). The overproduction of hyaluronic acid likely increases the association and transmigration of Cryptococcus. Therefore, we measured hyaluronic acid production using a hyaluronic acid ELISA kit (Corgenix, Colorado, AZ). The results showed that the production of hyaluronic acid in wild type H99 was significantly increased (P<0.001) when the medium contained 1 mM inositol, confirming that inositol regulates hyaluronic acid production in C. neoformans, leading to an increased rate of fungal association and transmigration. In addition, the itr1Δ itr3cΔ double mutant also produced a reduced level of hyaluronic acid compared to H99 in the presence or absence of inositol in the medium, but the reduction is less significant (P = 0.12) (Fig. 8B). This outcome suggests that inositol regulates the production of hyaluronic acid, but additional mechanisms may also be involved in fungal transmigration.

Treatment with an inositol transporter inhibitor blocks Cryptococcus transmigration

Fungal inositol transporters are proton-dependent, which is different kinetically and pharmacologically from the sodium-dependent myo-inositol transporters (SMITs) in mammalian cells [56]. Thus, fungal inositol transporters have potential as attractive drug targets. Dinitrophenol (DNP) is a protonophore that dissipates transmembrane proton gradients and has been shown to effectively block inositol uptake in Candida albicans [56]. We treated Cryptococcus wild type strain H99 with DNP or with the human sodium-dependent inositol transport inhibitor phloretin before performing inositol uptake assays. The inositol uptake...
assays revealed that DNP produced a pronounced inhibitory effect on inositol import in *Cryptococcus*, while phloretin had no obvious effect (Fig. 9). Subsequently, DNP or phloretin pretreated H99 cells were used in fungal transmigration assays in the *in vitro* BBB model. The results demonstrated that the DNP pretreatment leads to a significant reduction in cryptococcal transmigration stimulated by the addition of inositol, compared to those treated with a vehicle (DMSO) or phloretin. These findings further confirm that cryptococcal uptake of host inositol is through proton-dependent inositol transporters, and is required for assisting *Cryptococcus* traversal across the HBMEC monolayer. Due to the nature of protonophore, DNP treatments may cause effects on cryptococcal cells in addition to inhibiting inositol uptake, such as effects on mitochondria function. To address the concern that DNP may alter the growth of cryptococcal cells, we compared the growth rates of H99 cells treated with or without either DNP or phloretin. The results showed that the different treatments did not affect growth curves (Fig. S4), indicating that the decreased cryptococcal transmigration was due to reduced inositol uptake in H99 rather than an effect on other fungal physiology. Taken together, these findings further confirm that host inositol plays a positive role in *Cryptococcus* traversal across the BBB.
The mechanisms for the frequent occurrence of Cryptococcal CNS infection remain unclear. In this study, we explored our hypothesis that the high abundance of inositol in human brain contributes to virulence of Cryptococcus and the development of cryptococcal meningitis. In our in vitro model of BBB using the HBMEC monolayer, we observed that inositol promotes an increase in Cryptococcus association with and transmigration through the HBMEC monolayer. This increase is dependent upon fungal inositol transporters (ITRs), demonstrated by the fact that mutation of two major ITRs, ITR1A and ITR3C, partially abolishes the stimulation of Cryptococcus transmigration by inositol. Our results indicate that inositol plays a role in the traversal of Cryptococcus across the BBB, and showed that fungal cells can respond to inositol availability in the brain. Because we measured the transmigration rate by counting yeast cell numbers in the bottom chamber in our in vitro model, one concern in interpreting our results is whether addition of inositol to the bottom chamber has an effect on the proliferation of yeast cells. We measured the proliferation rate of Cryptococcus cells in HBMEC medium with or without addition of inositol, and observed a similar growth rate in all strains tested, confirming that the difference in cell numbers in the bottom compartment reflected a difference in yeast transmigration. Cryptococcus can use inositol as a carbon source although it is not a preferred source; Cryptococcus grows very slowly on medium containing inositol as the sole carbon source. Glucose is a much better carbon source for Cryptococcus. The HBMEC medium is enriched in nutrients and contains sufficient glucose for optimal fungal growth, which would explain why inclusion of 1 mM inositol did not affect cell growth. In addition, the association assays demonstrate that inositol promotes the association of Cryptococcus with the HBMEC monolayer. Because a better association often leads to increased transmigration, this effect could explain the increase in transmigration. In addition, because inositol is highly abundant in human and animal brains, for example, the astrocytes that directly interact with the BBB contain over 8 mM inositol that can be rapidly released [38,39], we believe the inositol level in the

| Protein ID | Description | Average fold changes |
|------------|-------------|----------------------|
| **JEC21 ID** | **H99 ID** | **CN01750** | **CNAG_04394** | Beta-glucuronidase (Cbg1) | 33.11 |
| **CNF03950** | **CNAG_06623** | Inositol oxygenase (Mio1) | 32.63 |
| **CNF04890** | **CNAG_06530** | Inositol 2-dehydrogenase (Idh1) | 5.45 |
| **CN01540** | **CNAG_04374** | Beta-glucuronidase (Cbg2) | 3.65 |
| **CNM02480** | **CNAG_06247** | Glycosyl transferase | 2.34 |
| **CN00130** | None | Polyadenylation factor | 2.33 |
| **CN03010** | **CNAG_03277** | Inositol oxygenase (Mio2) | 2.21 |

**Signal transduction**

| CNG04220 | CNAG_03143 | Heat shock protein | 2.38 |
| CNO05900 | CNAG_00030 | Fungal specific transcription factor | 2.25 |
| CNO03740 | CNAG_06642 | Phosphatidylinositol 3-kinase | 2.22 |
| **CNO02200** | **CNAG_06220** | Allergen | 2.24 |

**Inositol transporters**

| CND00070 | CNG_00867 | Itr3a | 1.99 |
| CNO08809 | CNAG_00097 | Itr1 | 1.66 |
| CNO03060 | CNAG_05667 | Itr3b1 | 1.44 |
| CNG02980 | CNAG_03357 | Itr2a | 1.26 |
| CNO05060 | CNAG_04024 | Itr5 | 1.08 |
| CNO09990 | CNAG_05381 | Itr3c | 1.23 |
| CNO29900 | CNAG_05662 | Itr4 | 0.95 |
| CND00020 | CNAG_00864 | Itr2 | 0.83 |

**Proteins with unknown function**

| CN03670 | CNAG_05261 | Cryptococcus specific protein, unknown (isp1) | 2.42 |
| CN03330 | CNAG_07840 | Cryptococcus specific protein, unknown (isp2) | 2.42 |
| CN02130 | CNAG_01873 | Fungal specific protein, unknown | 2.27 |
| CN03390 | CNAG_04919 | Unknown | 2.18 |

Discussion

The mechanisms for the frequent occurrence of Cryptococcal CNS infection remain unclear. In this study, we explored our hypothesis that the high abundance of inositol in human brain contributes to virulence of Cryptococcus and the development of cryptococcal meningitis. In our in vitro model of BBB using the HBMEC monolayer, we observed that inositol promotes an increase in Cryptococcus association with and transmigration through the HBMEC monolayer. This increase is dependent upon fungal inositol transporters (ITRs), demonstrated by the fact that mutation of two major ITRs, ITR1A and ITR3C, partially abolishes the stimulation of Cryptococcus transmigration by inositol. Our results indicate that inositol plays a role in the traversal of Cryptococcus across the BBB, and showed that fungal cells can respond to inositol availability in the brain. Because we measured the transmigration rate by counting yeast cell numbers in the bottom chamber in our in vitro model, one concern in interpreting our results is whether addition of inositol to the bottom chamber has an effect on the proliferation of yeast cells. We measured the proliferation rate of Cryptococcus cells in HBMEC medium with or without addition of inositol, and observed a similar growth rate in all strains tested, confirming that the difference in cell numbers in the bottom compartment reflected a difference in yeast transmigration. Cryptococcus can use inositol as a carbon source although it is not a preferred source; Cryptococcus grows very slowly on medium containing inositol as the sole carbon source. Glucose is a much better carbon source for Cryptococcus. The HBMEC medium is enriched in nutrients and contains sufficient glucose for optimal fungal growth, which would explain why inclusion of 1 mM inositol did not affect cell growth. In addition, the association assays demonstrate that inositol promotes the association of Cryptococcus with the HBMEC monolayer. Because a better association often leads to increased transmigration, this effect could explain the increase in transmigration. In addition, because inositol is highly abundant in human and animal brains, for example, the astrocytes that directly interact with the BBB contain over 8 mM inositol that can be rapidly released [38,39], we believe the inositol level in the...
brain side of the BBB is high and the inositol concentrations we used in in vitro assays are physiologically relevant.

Our in vivo study using a murine model revealed a significant difference in numbers of CFUs recovered from mouse brains 24 hr post tail vein injection between mice infected by wild type versus the \( \text{itr1a} \ \text{itr3c} \) double mutant, confirming that the double mutant has a defect in brain infection. In the mutant, the observation of lower fungal burden in brains but not in lung

| Protein ID | Description | Average fold changes |
|------------|-------------|----------------------|
| JEC21 ID   | H99 ID      |                      |
| Metabolism |
| CNH03280   | CNAG_05303  | Isocitrate lyase      | −5.27 |
| CNA00470   | CNAG_00057  | Fructose-1,6-bisphosphatase | −5.02 |
| CNA04370   | CNAG_00457  | Glutamine synthetase  | −4.41 |
| CNF03900   | CNAG_06628  | Aldehyde dehydrogenase | −4.31 |
| CN02480    | CNAG_01745  | sn-Glycerol-3-phosphate dehydrogenase | −3.52 |
| CNF04450   | CNAG_07660  | Pyruvate dehydrogenase | −3.51 |
| CNA01050   | CNAG_00115  | Sorbitol dehydrogenase | −3.33 |
| ‘CN0095’   | CNAG_04659  | Pyruvate decarboxylase | −3.23 |
| CNG0600    | CNAG_07745  | Mannitol-1-phosphate dehydrogenase | −3.00 |
| CNG0440    | CNAG_01539  | Inositol-1-phosphate synthase (Ino1) | −2.79 |
| CNE01560   | CNAG_02399  | Glutathione reductase  | −2.72 |
| CND00180   | CNAG_00879  | Glutamate dehydrogenase | −2.63 |
| CNA04610   | CNAG_07363  | Isocitrate dehydrogenase | −2.59 |
| CNL04840   | CNAG_05138  | Exo-beta-1,3-glucanase | −2.54 |
| CN02410    | CNAG_01737  | Methyl sterol oxidase  | −2.54 |
| CNB00190   | CNAG_04756  | 2-Oxoglutarate dehydrogenase complex | −2.53 |
| CNE02710   | CNAG_07639  | Triacylglycerol lipase  | −2.50 |
| Signal transduction |
| CNA01180   | CNAG_00130  | Serine/threonine protein kinase | −5.11 |
| CN05690    | CNAG_04090  | bZip transcription factor | −3.13 |
| CNB00230   | CNAG_00621  | Cyclophilin A           | −2.96 |
| CNH00140   | CNAG_05348  | Small GTPase_CDC42      | −2.88 |
| CNH00970   | CNAG_05431  | Transcription factor PacC-related | −2.76 |
| CNE03320   | CNAG_02209  | SIK1                   | −2.43 |
| CNB01720   | CNAG_03673  | Serine/threonine protein kinase | −2.39 |
| CN00140    | CNAG_01591  | Secretory pathway gdp dissociation inhibitor | −2.30 |
| CNA01820   | CNAG_00193  | Transcription factor ScGAT | −2.25 |
| CNO04340   | CNAG_02817  | Rab/GTPase              | −2.17 |
| CN04890    | CNAG_04005  | Negative regulator of differentiation 1 | −2.12 |
| CN04600    | CNAG_05392  | ROK1 related            | −2.08 |
| CN03330    | CNAG_04197  | Yak1 homolog            | −2.06 |
| Membrane proteins |
| CNE02570   | CNAG_02288  | Succinate/fumarate mitochondrial transporter | −3.22 |
| CNO07060   | CNAG_00727  | Cation transporter-like protein | −2.62 |
| CN04770    | CNAG_03991  | GPCR                    | −2.46 |
| CNA00570   | CNAG_00066  | Membrane protein        | −2.39 |
| CNL04850   | CNAG_05137  | Putative KDEL receptor   | −2.14 |
| CN00490    | CNAG_07874  | Sugar transporter        | −2.06 |
| CNE00290   | CNAG_03060  | Multiple drug resistance protein | −2.04 |

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Table 2. Genes downregulated by inositol.
during early infection suggests a defect in traversal across the BBB, consistent with our results from the in vitro system. We did observe a significant reduction in CFU in mutant-infected lungs 3 days post-inoculation, although the reduction was much less pronounced than in infected brains. Because the itr1aΔ itr3cΔ double mutant exhibits a moderate melanin defect [22], the reduced fungal burdens in the mutant infected lungs over time may be partially caused by reduced laccase activity, a known virulence factor [57]. However, these additional effects cannot account for the observed reduction in transmigration of cells carrying the double mutation, especially during early infection. Our confocal images of immunofluorescent staining further confirmed that Cryptococcus cells transmigrate into the brain by a mechanism that depend in part on inositol transporters, because strains lack of Itr1a and Itr3c have reduced fungal invasion in the brain.

The outcome of our animal studies on transmigration could be influenced by the impact of the CNS environment on yeast growth and survival after transmigration. To address this biological artifact on transmigration analysis, we examined growth and/or survival of H99 and the double mutant within relevant biological fluids. Both in human CSF and in animal models of cryptococcal meningitis there was no apparent influence of the inositol transporters on yeast growth or survival in vivo, supporting their early impact on transmigration rather than direct effect on CNS compartmental survival and growth. However, we could not

Figure 8. Inositol plays a positive role in hyaluronic acid production. The expression of hyaluronic acid synthase, CPS1, was determined by qRT-PCR (A), and hyaluronic acid production was measured using the HA-ELISA kit (Corgenix, Colorado) (B) for strains including wild type H99 and the itr1aΔ itr3cΔ double mutant that are either treated by 5 mM inositol (+) for 24 hrs or without treatment (−). The results shown are from at least three independent replicates. Error bars indicate the standard deviations. *** indicates statistical significance (P<0.001).

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Figure 9. Blockage of inositol uptake inhibits Cryptococcus transmigration of the HBMEC monolayer. (A) Inositol uptake assays for H99 strain were performed with the 3H-labeled myo-inositol in the presence of cold myo-inositol or inositol transporter inhibitors dinitrophenol (DNP) and phloretin. (B) C. neoformans (H99) was incubated with either vehicle (DMSO), DNP (0.3 mM) or Phloretin (0.3 mM). After 30 min incubation, H99 was washed with experiment medium to remove inhibitors and added to Transwells. Transmigration assays were performed for 3 hr and the number of H99 crossing the HBMEC monolayers were determined by counting CFU. Each assay was set up in triplicate and independently performed twice. *** indicates statistical significance (P<0.001).

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Inositol Promotes Cryptococcus Brain Invasion
Inositol Promotes Cryptococcus Brain Invasion

completely rule out that there may be a growth difference in certain microenvironment during brain infection, such as certain parts of the brain parenchyma, which is bypassed in the intracerebral injection model. In addition, we have shown that the presence of inositol transporters did reduce survival of the host in a murine intracerebral infection model [22]. Therefore, Itr1a and Itr3c are necessary for full virulence at this CNS site of infection. In fact, our preliminary results from a RNA-SEQ analysis of infected brains indicated that the double mutant caused a more active host defense response than wild type, a potential explanation of the virulence attenuation of the double mutant during the establishment of the CNS cryptococcosis (our unpublished data). Therefore, inositol utilization likely influences fungal transmigration across the BBB as well as subsequent disease development in the CNS.

Furthermore, studies have shown that yeast cells cross the BBB early after infection, but that a dramatic increase occurs 24 hr post-infection [48]. Thus, a significant defect in the transmigration may not be detectable before 24 hr. We hypothesize that there is a threshold effect with respect to time or number of cryptococcal cells associated with brain microvascular endothelial cells before they can effectively penetrate the BBB in vivo. This is not without precedent; it has been reported that a bacteremia approaching 10^3 cells per milliliter in bloodstream is a prerequisite for meningitis [58]. Alternatively, it is also possible that Cryptococcus K1 to cross the BBB [58,59].

In the in vitro BBB model used in this study, inositol added in the bottom compartment can diffuse to the top compartment to form an inositol concentration gradient. Our analysis showed that there was a time-dependent increase of inositol concentration in the top compartment with the presence of Cryptococcus, while very little increase of inositol level at the top without incubation with Cryptococcus. Furthermore, Cryptococcus incubation induces the modulation of the tight junction, as shown by the ZO-1 dislocation. These results suggest that the Cryptococcus-HBMEC interaction is required to modulate the permeability of the HBMEC monolayer to inositol. It has been reported that Cryptococcus invasion causes a modification of tight junctions [40]. In addition, inositol uptake by the host cells may contribute to the inositol level increase in the top compartment, since host cells also have inositol transporters. Our studies have shown that inositol alone is able to stimulate HBMEC and its uptake by HBMEC enhances the Cryptococcus-mediated phosphorylation of host signaling proteins and the permeability of dextran across the HBMEC monolayer in the presence of Cryptococcus (Kim et al., unpublished). Alternatively, it is also possible that Cryptococcus and HBMECs may release some amount of inositol into the medium during their interactions. However, despite the increase in inositol permeability and ZO-1 dislocation, TEER measurement of the HBMEC monolayer was not significantly changed during incubation with Cryptococcus, suggesting that inositol diffuses through the monolayer without causing major damage to the BBB, which is consistent with previous reports [25,47]. Further characterization on the inositol effects on the Cryptococcus-mediated modulation of HBMEC barrier is required to precisely understand the mechanisms for inositol diffusion.

Because both Itr1a and Itr3c are major inositol transporters, Cryptococcus may be able to import inositol for its cellular function. It is possible that inositol uptake through inositol transporters modulates Cryptococcus cells to enhance their association with HBMEC and transmigration across the BBB. Our 2D-TLC analysis demonstrated that, in both the wild type and the itr1aA itr3cA double mutant, incubation with inositol significantly increases the amount of phospholipids produced (Fig. 7). Interestingly, among phospholipids, the amount of phosphoinositide (PI)
detected after inositol treatment was much lower in the itr1aA itr3cA mutant than in the wild type. PI is a precursor in the production of inositol phospholipid and other downstream inositol metabolites that are essential for cellular function. Changes in PI production could profoundly affect Cryptococcus cellular signaling regulation and modification of cell surface dynamics. Phospholipids, including phosphatidylycholine (PC), have recently been identified to play a role in the capsule enlargement [60]. In our TLC results, PC production is highly induced by inositol treatment. The role of the capsule in Cryptococcus transmigration remains controversial in that some studies have demonstrated that capsule is involved [48,61], while other reports have suggested a capsule-independent brain invasion process [26,62]. Although we did not detect an obvious difference between the mutant and wild type in capsule size based on microscopy, it is possible that inositol may play a role in capsule structure or density, which may also influence cryptococcal transmigration and/or other functions during Cryptococcus-host interactions.

Microarray analysis also revealed that inositol treatment induces upregulation of genes related to inositol catabolism and metabolism. Converting inositol to glucuronic acid by inositol oxygenases is the first step of the only known inositol catabolism pathway [50]. Cryptococcus has three genes encoding oxygenase enzymes and the expression of two of them is highly induced by addition of inositol, suggesting that the addition of inositol stimulates the production of glucuronic acid. The upregulation of beta-glucuronidase genes by inositol may indicate that yeast cells have increased carbohydrate turnover, resulting in overproduction of polysaccharides due to inositol supplementation. Whether inositol-converted glucuronic acid can be used to produce UDP-glucuronic acid, as one substrate of the polysaccharide capsule, remains uncertain. Previous studies have shown that UDP-glucuronic acid in C. neoformans is exclusively produced by the glycolytic pathway from glucose [63,64]. However, it remains a possibility that under certain culture conditions, such as when inositol is used as a sole carbon source, glucuronic acid can also be a precursor of UDP-glucuronic acid.

UDP-glucuronic acid is also one substrate for the synthesis of hyaluronic acid, a Cryptococcus ligand that interacts with CD44 on host cells during fungal invasion and transmigration of the BBB [36,65]. Interestingly, we have found the gene encoding hyaluronic acid synthase, CPS 1, is upregulated by inositol treatment and leads to the induction of hyaluronic acid production. The increased production of hyaluronic acid indicates that inositol may enhance the association between Cryptococcus and brain endothelial cells. Therefore, the changes induced in Cryptococcus through inositol uptake may play a positive role in fungal association as well as transmigration. The fact that the itr1aA itr3cA double mutant only showed a marginal reduction in hyaluronic acid production compared to wild type is consistent with results demonstrating that this mutant can invade the BBB and cause infection although less efficiently. Additional factors, such as the influence of inositol on the cellular lipid rafts, caveolin-1, cytoskeleton, etc., could also be in play to alter the transmigration. As well, additional redundant ITRs may contribute and/or partially compensate for losses in the double mutant background. A mutant strain lacking the whole ITR gene family would be valuable for understanding the complete role of ITRs. We are in the process of generating such a mutant strain.

In addition, inositol itself is an important signaling molecule that affects many biological functions. Inositol stimulates the long distance sodium uptake from root to leaf in plants [66], and many species of caterpillar can sense inositol on plants for feeding [67,68]. Cryptococcus may sense the concentration gradient of

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inositol and promote its association with endothelial cells and increase the transcytosis in response to higher inositol concentration, a phenomenon similar to chemotropism. The role of inositol as a potential chemoattractant to stimulate Cryptococcus to associate and penetrate the BBB needs to be further investigated.

Overall, we have demonstrated that brain inositol affects fungal cells to promote the traversal across the BBB as presented in our model (Fig. 10). Cryptococcal cells disrupt tight junctions of the BBB allowing leakage of inositol from the brain to the bloodstream to generate an inositol concentration gradient; Cryptococcus senses the presence of inositol gradient via inositol sensors that remain to be identified, and takes up inositol using these inositol transporters. Inositol import leads to modifications in fungal physiology such as hyaluronic acid production. The inositol-mediated changes on fungal cells lead to enhanced yeast binding to and transmigration of the BBB, resulting in cryptococcal brain infection and disease development. This model suggests that there is a complex interaction between fungal cells and host cells that involves host inositol and fungal inositol transporters.

Because the transmigration and subsequent disease establishment in the brain is a continuous process that is difficult to separate definitely, we cannot rule out the possibility that in addition to its involvement in the fungal transmigration, inositol utilization could also play an important role in the establishment of cryptococcal meningitis in the CNS. In fact, this hypothesis is supported by our unpublished data on the difference in host immune response between brains infected by the wild type and the itrD mutant.

Despite the importance of host inositol, we do aware that inositol may be only one of multiple factors that affect the development of the CNS cryptococcosis. Additional factors and mechanisms likely also exist to stimulate the fungal cell transmigration and the establishment of fungal infection in the brain. In fact, several factors, such as capsule [48,61], urease [30,31], phospholipase B1 [32,33], and host copper utilization [69], have been reported to play a role in cryptococcal dissemination and/or brain infection. Nevertheless, our discovery of the involvement of host inositol and fungal inositol transporters in the development of
cryptococcal CNS infection leads to a better understanding of this complex host-pathogen interaction during the development of cryptococcal meningitis.

Material and Methods

Ethics statement

The animal studies conducted at Duke University and University of Medicine and Dentistry of New Jersey (UMDNJ) were in full compliance with all of the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) and in full compliance with the United States Animal Welfare Act (Public Law 98–198). The Duke and UMDNJ IACUCs approved all of the vertebrate studies. The studies were conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Strains, media, and growth conditions

*C. neoformans* var. *grubii* (serotype A) H99 and its isogenic mutant strains (itr1Δ, itr3Δ, itr1Δ itr3Δ) have been previously described [22,23]. *C. neoformans* var. *neoformans* (serotype D) strain B-3501 was kindly provided by Dr. David Perlin (UMDNJ). Yeast cells were grown on YPD (1% yeast extract, 2% peptone, 2% glucose) agar plates and synthetic (SD) media. *C. grubii* strain ATCC90028 was kindly provided by Dr. Kyung J. Kwon-Chung (NIAID). The *C. neoformans* strain (serotype D) strain B-3501 was kindly provided by Dr. David Perlin (UMDNJ). Yeast cells were grown on YPD (1% yeast extract, 2% peptone, 2% glucose) agar plates and synthetic (SD) medium at 30°C and stored at 4°C until use. The Anti-GXM antibody 18B7 was kindly provided by Dr. Arturo Casadevall (Albert Einstein College of Medicine). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were obtained from Invitrogen (Grand Island, NY). Dinitrophenol and inositol isomers were purchased from Sigma-Aldrich (St. Louis, MO).

Human brain microvascular endothelial cells (HBMEC)

Primary isolates of HBMEC were cultured as previously described [70]. HBMEC were routinely grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 10% Nu-serum, 2 mM glutamine, 1 mM sodium pyruvate, penicillin (100 units/ml), streptomycin (100 μg/ml), essential amino acids, and vitamins. The cells were incubated at 37°C in a humidified incubator with 5% CO2. Before each experiment, the culture medium was replaced with experimental medium containing Hams-F12/M199 (1:1, v/v), supplemented with 5% heat-inactivated fetal bovine serum.

*C. neoformans* association and transmigration assay

Total yeast cells associated with HBMEC were determined as previously described [41,71]. Briefly, HBMEC were grown in 24-well tissue culture plates (or Transwell tissue-culture insert plates with a pore diameter of 8.0 μm (Corning Costar)) until confluence. Inocula of 10⁵ *Cryptococcus* cells in experimental medium were added to each well (or top compartments in Transwells), and then incubated for 3 hr at 37°C. Free unbound yeast cells were removed by washing 3 times with PBS. The HBMEC were lysed with sterile distilled water and the lysates were diluted and plated onto sheep blood agar plates. The colonies were counted and results were presented as the total number of yeast cells per monolayer. Each set was triplicated and repeated at least three times independently.

The *in vitro* human blood-brain barrier (BBB) model was generated and used for fungal transmigration assays as previously described [70]. HBMEC were seeded on Transwell polycarbonate tissue-culture inserts with a pore diameter of 8.0 μm (Corning Costar) and cultured until their transendothelial electrical resistance (TEER) reached over 350 Ω/cm², as measured by an Endohm volt/ohm meter in conjunction with an Endohm chamber (World Precision Instruments). The medium was replaced with experiment medium before each experiment. Yeast cells were washed with phosphate-buffered saline (PBS) and resuspended in HBMEC culture medium. 10⁷ *Cryptococcus* cells were added to the top compartment and then incubated at 37°C. At 3, 6, and 9 hr, the medium in bottom compartments was collected and immediately replaced with fresh medium. Fungal cells numbers in the collected medium were addressed by CFU counts to determine the number of transmigrated viable yeast cells. To determine the specificity of myo-inositol, the transmigration assay was performed in the presence of myo-inositol, scylo-inositol, D(+)-galactose or D(+)-mannose (1 mM each) in the bottom compartments prior to addition of *Cryptococcus* cells (10⁷) in the top compartment of Transwells. Results are presented as the total number in the bottom chamber. Each set was triplicated and repeated three times independently. The statistical analysis of the data from our *in vitro* studies was done with a two-tailed Student t test. Statistical significance was determined at P<0.001.

Measurement of myo-inositol concentration

The inositol concentration in medium was determined as previously described with minor modifications [46]. Briefly, the medium containing inositol collected from the top compartments of the BBB model was incubated with hexokinase to reduce interference from glucose by phosphorylation. The mixture was then incubated with 4.1 U/ml myo-inositol dehydrogenase for 15 min. Subsequently, 100 μl medium was mixed with an equal volume of detection reagent. The inositol concentration was determined by measuring optical density at 492 nm with a microplate reader (BioTek, Winooski, VT). Each assay was triplicated and repeated three times independently.

In vivo virulence studies

Virulence of the *C. neoformans* strains was assessed using both a murine intravenous infection model and a rabbit CSF model of cryptococcosis as previously described [22,72,73]. For virulence study in a murine intravenous injection model, *Cryptococcus* strains were grown at 30°C overnight and cultures were washed twice with 1× PBS buffer by centrifugation, and resuspended at a final concentration of 5×10⁷ cells/ml. Groups of 15 female A/Jcr mice (NCI-Frederick, MD) were used for each infection. Mice were infected with 5×10⁴ yeast cells of each strain in 100 μl PBS through tail vein injection [22,74]. At each time point, 3 mice infected with either H99 or the mutant were sacrificed after 1, 6, 24, 48, and 72 hr post-infection. Fungal burden in infected brains was analyzed by CFU counts. Data from the murine experiments were statistically analyzed between paired groups using the long-rank test and the PRISM program 4.0 (GraphPad Software) (P values of <0.01 were considered significant). For the murine intracerebral injection model, mice were sedated with a Ketamine-Xylazine combination and the top of the head was sterilized using antiseptic. A total of 500 yeast cells in 50 μl were directly injected into the cerebrum as previously described [22]. For Rabbit infection, male New Zealand White (NZW) rabbits were treated with cortisone via daily injection and intrathecal inoculation into the subarachnoid space with 10⁶ cells of each *C. neoformans* strain (3 rabbits per group). Fungal burden in brain was analyzed by CFU counts.

Microarray analyses for genes regulated by inositol

H99 overnight culture was washed with dH₂O twice. Equal amount cells were inoculated on SD medium with or without 5 mM inositol and incubated for 24 hr before cells were collected for total RNA purification. Total RNAs were extracted using Trizol
Detection of phospholipids in Cryptococcus using Thin Layer Chromatography (TLC) analysis

Overnight cultures of Cryptococcus were washed with dH2O twice and re-incubated to 5 ml SD with or without 5 mM inositol and shaken for 2 hrs; 25 μCi/ml of 32P-phosphorus was added and the cultures were incubated with shaking overnight. Yeast cells were collected and concentrations were determined by hemocytometer counts. Steady-state labeling of phospholipid with 32P and lipid extraction were performed as described previously [77]. Lipids were dried in a SpeedVac apparatus, and resuspended into 500 μl methanol. Five microliter aliquots were removed to measure the radioactivity in a scintillation counter. The remaining lipids were chloroform/methanol/glacial acetic acid/water (32:4:5:1, v/v) as the solvent system for dimension one resolution using two-dimensional silica gel TLC plates (EMD, Gibbstown, NJ) following the instructions provided by the manufacturer. Expression of candidate genes and GAPDH were analyzed with the competitive C T method using SYBR green QPCR reagents (Clontech) as described previously [23].

Immunofluorescence microscopy

HBMECs were grown on coverslips coated with type-I collagen from rat tail (Millipore, Billerica, MA) until confluence. After incubation with C. neoformans (H99) or 1 hr, HBMECs were washed three times with PBS and processed for immunofluorescent staining as previously described [78]. Briefly, the cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.5% Triton X-100 for 5 min and then incubated with ZO-1 antibody, followed by AlexaFluor 488-conjugated secondary antibody (Invitrogen) to visualize. The coverslips were mounted with Vectashield mounting solution with DAPI (Vector Laboratory, Burlingame, CA) and observed with a Nikon fluorescence microscope. Images were taken with a MetaMorph Microscopy Automation & Image Analysis Software.

Supporting Information

Figure S1 The Cryptococcus-HBMEC interaction does not change the integrity of the monolayer based on the transendothelial electrical resistance. The in vitro human BBB model was prepared and incubated with Cryptococcus (H99 or the itr1A itr3cA double mutant) in the absence (−) or presence (+) of inositol for 9 hr. Transendothelial electrical resistance (TEER) was measured with endohm/EVOM as described in transmigration assay. Each set was triplicated. (TIF)

Figure S2 The itr1A itr3cA double mutant exhibits normal phagocytosis and intracellular growth inside macrophages. Phagocytosis assays were performed in 48-well plates containing 5×10⁵ J774 macrophages. Total 2×10⁵ PBS washed C. neoformans strains H99, an itr1A itr3cA double mutant and its complemented strain were added to the macrophages and incubated for 2 hrs at 37°C in 10% CO₂. Extracellular yeast cells were removed by washing the cells with fresh DMEM medium. Macrophages containing Cryptococcus were further incubated for 0, 2, and 22 hrs before macrophages were lysed by dH₂O. Yeast cell suspensions were plated on YPD for CFU counts to determine the total alive yeast cells. Error bars indicate the standard deviations. (TIF)
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Author Contributions

Conceived and designed the experiments: TBL, JCK, JRP, EE, KJK CX. Performed the experiments: TBL, JCK, YW DLT EE, KJK CX. Analyzed the data: TBL, JCK, DLT EE, JRP, KJK CX. Contributed reagents/materials/analysis tools: EE, JRP KJK CX. Wrote the paper: KJK CX.

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