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Cryptococcus neoformans Promotes Its Transmigration into the Central Nervous System by Inducing Molecular and Cellular Changes in Brain Endothelial Cells

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***Cryptococcus* spp. cause fungal meningitis, a life-threatening infection that occurs predominately in immunocompromised individuals. In order for *Cryptococcus neoformans* to invade the central nervous system (CNS), it must first penetrate the brain endothelium, also known as the blood-brain barrier (BBB). Despite the importance of the interrelation between *C. neoformans* and the brain endothelium in establishing CNS infection, very little is known about this microenvironment. Here we sought to resolve the cellular and molecular basis that defines the fungal-BBB interface during cryptococcal attachment to, and internalization by, the human brain endothelium. In order to accomplish this by a systems-wide approach, the proteomic profile of human brain endothelial cells challenged with *C. neoformans* was resolved using a label-free differential quantitative mass spectrometry method known as spectral counting (SC). Here, we demonstrate that as brain endothelial cells associate with, and internalize, cryptococci, they upregulate the expression of several proteins involved with cytoskeleton, metabolism, signaling, and inflammation, suggesting that they are actively signaling and undergoing cytoskeleton remodeling via annexin A2, S100A10, transgelin, and myosin. Transmission electronic microscopy (TEM) analysis demonstrates dramatic structural changes in nuclei, mitochondria, the endoplasmic reticulum (ER), and the plasma membrane that are indicative of cell stress and cell damage. The translocation of HMGB1, a marker of cell injury, the downregulation of proteins that function in transcription, energy production, protein processing, and the upregulation of cyclophilin A further support the notion that *C. neoformans* elicits changes in brain endothelial cells that facilitate the migration of cryptococci across the BBB and ultimately induce endothelial cell necrosis.**

Immunocompromised populations worldwide are at risk for developing a devastating and life-threatening infection of the brain that is caused by *Cryptococcus neoformans*, a pathogenic fungus (1–7). Among the AIDS population, *C. neoformans* causes higher mortality than tuberculosis in sub-Saharan Africa (8), and ongoing outbreaks of cryptococcal disease in healthy individuals by the sibling species *Cryptococcus gattii* have raised the threat of this emerging pathogen (9, 10). Why *C. neoformans* has this remarkable tropism for the central nervous system (CNS) is not clear, in part because our understanding of the processes enabling dissemination of cryptococci from the primary site of infection, the lung, to the CNS is incomplete.

It is known that the neurotropic behavior of *C. neoformans* during disseminated cryptococcosis is likely influenced by fungemia since it is the bloodstream that must mediate the movement of *C. neoformans* from the lung to the blood-brain barrier (BBB) (11). The BBB functions to maintain brain homeostasis by acting as a protective shield against circulating components in the blood that could harm brain function. The endothelial cells are the main elements of the BBB that form the brain capillaries and the tight junctions between these cells; however, the astrocytes, pericytes, and basal lamina also form an integral part of the BBB (12). The brain endothelium (or BBB) differs both morphologically and functionally from endothelial cells of the peripheral vasculature primarily because of the tight junctions (12). It is the specialized brain endothelial cells that serve as the central route penetrated by *C. neoformans* during cryptococcal meningoencephalitis.

The migration of *C. neoformans* across the brain endothelium is supported by two distinct mechanisms. The phagocytosis-mediated (Trojan horse mechanism) pathway involves the passive migration of fungal cells into the CNS inside emigrating monocytes, while the transcellular mechanism is a receptor-mediated, active process that allows the internalization of fungal cells (11, 13–18). Paracellular migration of *C. neoformans* can also occur but only following mechanical or biochemical disruption of the junctions within the brain endothelium (16, 19, 20).

To fully resolve the mechanism used by *C. neoformans* to transmigrate across the brain endothelium and invade the CNS, it is necessary to examine the role of brain endothelial factors that facilitate this process. The fungus-host interaction at the brain endothelium is a dynamic and complex process, and consequently very little is known about the physiological response of the brain endothelium to fungal invasion. Recently, however, CD44, an adhesion protein expressed in brain endothelial cells, was shown to mediate the attachment of cryptococci via hyaluronic acid, an inner component of the capsule (17). This association involves a kinase (DYRK3)-mediated redistribution of CD44 to membrane rafts in brain endothelial cells upon exposure to *C. neoformans* (21). The lipid rafts were proposed to function as a recruitment point for actin and other components that ultimately promote

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fungal attachment to the brain endothelium (21). Plasmin has also been found to mediate the association between the BBB and *C. neoformans* (22).

Here, we sought to resolve the molecular and cellular response of the BBB to the attachment and internalization of *C. neoformans* by examining the changes in protein expression levels in the brain endothelium. It had previously been reported that brain endothelial cells incubated with *C. neoformans* underwent changes in cell ultrastructure, but the molecular basis for these changes was unclear (16). In this study, we provide evidence suggesting that endothelial cells exposed to *C. neoformans* express protein changes that are indicative of significant alterations in cell signaling, cell permeability, cytoskeleton, metabolism, and organelle function. Transmission electron microscopy (TEM) analysis and the translocation of HMGB1 (high-mobility group box 1 protein; a marker for cell injury) revealed significant structural and molecular changes in mitochondria, endoplasmic reticulum (ER), nuclei, and the plasma membrane of endothelial cells exposed to *C. neoformans*, supporting the notion that not only do cryptococci promote endothelial cell stress but also internalization of cryptococci leads to significant damage to the BBB. These results suggest that *C. neoformans* engages the cytoskeleton and ultimately induces the demise of brain endothelial cells as a means to facilitate its own transmigration to the CNS.

MATERIALS AND METHODS

hCMEC/D3 cells and fungal cells. Cells were grown on a collagen-coated surface in EGM-2 medium (1×). Once cells reached confluence, the strength of the medium was reduced to 0.5× or 0.25× to facilitate differentiation into an endothelium monolayer as previously explained (13, 14). The hCMEC/D3 cells were used between passages 25 and 35. A strain of *Cryptococcus neoformans* (H99 *MAT*α serotype A) was recovered from 15% glycerol stocks stored at −80°C prior to use. The strain was maintained on yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% dextrose). Fungal cells were cultured in YPD medium at 30°C overnight. Cells were collected, washed in phosphate-buffered saline (PBS), suspended in PBS, and added to hCMEC/D3 cells. Fungal cells and hCMEC/D3 cells were incubated together at 37°C and 5% CO_2 for 1.5 h or 8 h.

Growth and processing of hCMEC/D3 cells for MS analysis. hCMEC/D3 cells were grown in EGM-2 medium on glass coverslips to confluence. About 2×10^6 cells of H99 strain or PBS alone (control) was added to each coverslip for 1.5 h or 8 h (multiplicity of infection [MOI], ~3). To prepare cells for mass spectrometry (MS) analysis, coverslips were washed 3 times with PBS and flash frozen in liquid nitrogen. Afterwards, the coverslips were placed in a solution consisting of 1 ml of 100 mM ammonium bicarbonate (AMBIC) plus 2 μg of trypsin. The coverslips were then incubated for 4 h at room temperature with shaking. After the incubation period, a cell culture scraper was used to scrape cells that were still attached to the coverslip after the 4-h trypsin treatment. The cell lysates were transferred to 1.5-ml microtubes, heated to 60°C for 30 min, cooled, and then dried down with a speed-vac. Both control and treatment groups were done in triplicate. Dried broken cells were solubilized in 50 mM AMBIC and then reduced and alkylated with 10 mM TCEP (3,3′,3′′-phosphanetriyltripropanoic acid) (Pierce BondBreaker) at 90°C and 15 mM iodoacetamide (IAA) at room temperature, respectively. The IAA was quenched with 5 mM dithiothreitol. Samples were then digested overnight with 0.5 μg of Promega modified sequencing grade trypsin, after which an equal amount of dichloromethane was added to each tube; this was vortexed vigorously for 1 min and then centrifuged 2 min at 10,000 rpm on a microcentrifuge to separate phases. The upper aqueous phase (containing peptides) was retained, and the lower phase and interface (lipids) were discarded. Samples were dried again in a speed-vac and then suspended in 2% acetonitrile (ACN)–0.1% trifluoroacetic acid (TFA). Samples were normalized by first using the A280 program on an ND-1000 Nanodrop spectrometer for a rough quantification and then loading equal peptide amounts for liquid chromatography-tandem MS (LC-MS/MS).

Mass spectral analysis. Digested peptides were analyzed by LC-MS/MS on an LTQ with Michrom Paradigm LC and CTC Pal autosampler. Peptides were separated with a 90-min gradient using a Michrom 200-μm by 150-mm Magic C_{18} AQ reversed-phase column at 2 μl/min. Peptides were directly loaded onto an Agilent ZORBAX 300SB C_{18}, reversed-phase trap cartridge, which, after loading, was switched in-line with a Michrom Magic C_{18} AQ 200-μm by 150-mm C_{18} column connected to a Thermo-Finnigan LTQ ion trap mass spectrometer through a Michrom Advance Plug and Play nanospray source. Peptides were separated using a 90-min-long gradient (1 to 10% buffer B for 5 min, 10 to 35% buffer B for 65 min, 35 to 70% buffer B for 5 min, 70% buffer B for 1 min, 1% buffer B for 14 min) at a flow rate of 2 ml min^{-1} for the maximum separation of tryptic peptides. MS and MS/MS spectra were acquired using a top 10 method whereby the top 10 ions in the MS scan were subjected to automated low-energy collision-induced dissociation (CID). All MS/MS samples were analyzed using X! Tandem (www.thegpm.org; version TORNADO [2008.01.01.1]). X! Tandem was set up to search a hybrid Uniprot database of both human and *Cryptococcus neoformans* proteins; Uniprot complete proteome sets (12152011), assuming the digestion enzyme trypsin. X! Tandem was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 1.8 Da. Iodoacetamide derivative of cysteine was specified in X! Tandem as a fixed modification. Oxidation of methionine was specified in X! Tandem as a variable modification.

Statistical analysis for SC. Scaffold (version Scaffold_3.5.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability as specified by the Peptide Prophet algorithm (23–25). Protein identifications were accepted if they could be established at greater than 80.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (23–25). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Unweighted spectral counts (SC) and Fisher's exact test or the Student *t* test were used as criteria for differences in proteins between the treated and control sets. An equal number of decoy sequences were searched. Protein decoy false discovery rate (FDR) and peptide decoy FDR for the 8-h samples were 0.9% and 0.05%, respectively, at 80/80 confidence levels. For the 1.5-h samples, the protein FDR was 0.7% and the peptide FDR was 0.01% at the same confidence levels. Four replicates were analyzed for control and treated (in the presence of *C. neoformans*) samples for the 8-h time point. Three replicates were analyzed for control and treated samples for the 1.5-h time point. Fold changes were also calculated. Proteins were considered significant in Scaffold if Fisher's exact *P* values were <0.05 and Student's *t* test *P* values were <0.05. Proteins were considered significantly different if QSpec reported a Bayes factor of >10, which corresponds to an FDR of approximately 5% (26).

Immunofluorescence. hCMEC/D3 cells were grown on a coverslip as explained above and exposed to a wild-type strain of *C. neoformans*. The medium was discarded, and coverslips were washed with 1× PBS followed by the addition of 4% paraformaldehyde for 20 min at room temperature. Fixed cells were washed with ice-cold 1× PBS, and cells were either not permeabilized or permeabilized with 0.25% Triton-PBS for 10 min. hCMEC/D3 cells were incubated in blocking solution (1× PBS, 1% bovine serum albumin [BSA], and 0.5% Tween) for 1 h. Primary antibody for annexin A2 and HMGB1 (purchased from abCAM) was added (1:500) for 1 h at room temperature, and coverslips were subsequently washed 3 or 4 times in 1× PBS–0.2% Tween. The appropriate secondary antibody

was then added (1:1,000), cells were washed, and coverslips were placed on a microscope slide for microscope analysis.

Ultrastructural analysis. TEM was performed as follows: hCMEC/D3 cells were grown to confluence on an 8-well Permanox slide and infected with H99 cells for 8 h as described above. Cells were washed with PBS and fixed in Karnovsky's fixative. Samples were then submitted to the University of California—Davis Microscopy Lab for further processing, where samples were cut vertically for TEM viewing.

RESULTS

The aim of this study was to resolve the cellular and molecular signatures that define the interactions between *C. neoformans* and the BBB. In order to do this from a systems-wide perspective, protein changes that occurred in human brain endothelial cells during attachment and internalization of cryptococci were characterized. We used an immortalized human brain endothelial cell line (hCMEC/D3) that has been well established and shown to recapitulate features of primary brain endothelial cells (27–31). It had previously been shown that cryptococci exposed to the hCMEC/D3 cell line actively transmigrate via a predominately transcellular mechanism and induce formation of microvilli, similar to what occurs in primary cells (14). These results underscored the robustness of the hCMCE/D3 cell line and its usefulness in resolving invasion tactics of cryptococci into the CNS (14). By eliminating the challenges and limitations associated with primary brain endothelial cells, the hCMEC/D3 cell line has facilitated studies aimed at understanding pathogen-host interactions at the BBB.

***C. neoformans* induced the expression of a variety of proteins in brain endothelial cells.** In order to establish how human brain endothelial cells respond to the presence of cryptococci, protein expression profiles of the endothelium were examined following 1.5 h and 8 h of incubation with *C. neoformans* var. *grubii*. This particular species causes disease primarily in immunocompromised individuals. The two time points were chosen because previous studies using an *in vitro* model of the BBB have shown that cryptococci adhere to the surface of brain endothelial cells within the first 1 to 2 h and by 8 h cryptococci are internalized and are in the process of transmigrating across the brain endothelium (13, 14). The second time point is in line with *in vivo* studies that have shown that cryptococci can invade the brain parenchyma within ~8 h of a tail vein inoculation (19, 20). With these two time points, the protein changes at the BBB-cryptococcus interface corresponding to cryptococcal attachment and internalization could be analyzed.

Label-free LC-MS/MS analysis of brain endothelial cells exposed to *C. neoformans* indicated significant changes in protein expression (Fig. 1). Of the 806 endothelium proteins identified (protein FDR of 0.9%) at the 8-h time point, approximately 3.11% were found by SC to be differentially expressed in the presence of *C. neoformans* (Table 1). Of the 425 endothelium proteins identified at the 1.5-h time point (protein FDR of 0.7%), approximately 2.35% were found to be differentially expressed in the presence of *C. neoformans* by SC. We corroborated protein changes identified by SC with quantitative reverse transcription-PCR (qRT-PCR) and found a significant correlation between changes in protein expression and in transcript levels in endothelial cells exposed to *C. neoformans* (data not shown). Although SC is a relatively new approach, it has been successfully used in different systems to identify and quantify protein changes (32, 33).

In this study, SC was used to analyze protein changes in brain

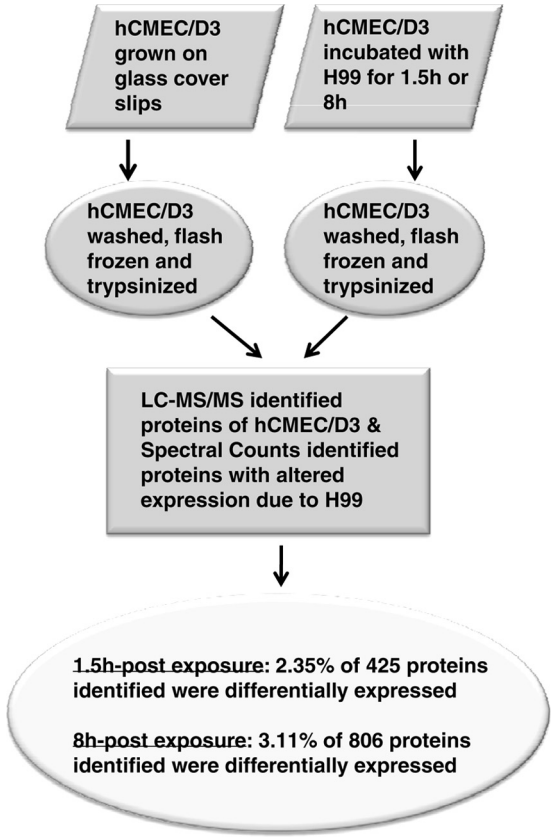

FIG 1 Schematic representation of the approach followed and the label-free shotgun methodology used to identify and quantify changes in protein expression of human brain endothelial cells exposed to *C. neoformans* var. *grubii* (H99).

endothelial cells that had associated with cryptococci. The data suggest that by 1.5 h significant protein changes had occurred. Following 8 h, endothelial cells had 29 proteins with altered expression compared to endothelial cells that were not incubated with *C. neoformans* (Fig. 2). The greatest fold changes at the 8-h time point were detected in mitochondrion-related proteins and proteins involved in signaling, transcription, transport, immune system, and protein processing (Table 1). Interestingly, *C. neoformans* induced an ~2-fold decrease in the expression of all mitochondrial proteins in endothelial cells, including anion channels VDAC1 and 2. Similarly, a 2-fold to 5-fold decrease in protein expression was observed in 6 proteins associated with the ER, including ribophorin II, calumenin, and reticulocalbin (Table 1). In contrast, all proteins associated with cell signaling displayed an ~2-fold to 4-fold increase in expression compared to unexposed endothelial cells.

Five proteins were identified with altered expression levels at both time points (1.5 h and 8 h) (Table 2). These proteins were lactate dehydrogenase (LDH), anion channel VDAC1, disulfide isomerase, calumenin, and S100A10 (Table 2). Both calumenin (down 2-fold) and S100A10 (up ~2-fold) are Ca^{2+} binding proteins, so the changes in expression levels early on may reflect their response to immediate fluctuations in intracellular Ca^{2+} levels upon activation of brain endothelial cells resulting from attachment of *C. neoformans*. The 2.4-fold upregulation of the

TABLE 1 Functional groups of brain endothelial proteins with altered expression as identified by spectral counting[a]

Functional group and protein name	Bayes factor	Fold change	Protein expression direction[b]	Time point(s) (h)	*P* value (time point in h) by: Student's *t* test	Fisher's exact test
Cytoskeleton						
Myosin	16.465	1.77	1	8	0.018 (8)	0.013 (8)
Transgelin	3.932	62.5	−1	1.5		0.0049 (1.5)
Immune response						
Cyclophillin A	3.7×10^4	1.31	1	8		
MHC- HLA class 1	54	3.52	1	8		
Metabolism						
Lactate dehydrogenase	16.793	2.42	1	8 and 1.5		0.028 (8), 0.0064 (1.5)
Phosphoglycerate kinase		1.30	1	8	0.0035 (8)	0.005 (8)
Phosphoglycerate mutase		1.91	1	1.5		0.0054 (1.5)
Mitochondrion related						
Anion channel, VDAC1	112	2.31	−1	8 and 1.5		0.0002 (1.5)
Anion channel, VDAC2	731	1.66	−1	8		
Inner membrane protein		1.58	−1	1.5		0.0100 (1.5)
Aspartate aminotransferase		1.47	−1	1.5		0.0085 (1.5)
Protein processing/ER related						
Reticulocalbin	20.202	2.07	−1	8		
Protein disulfide isomerase	10.693	4.18	−1	8 and 1.5		0.0057 (1.5)
Calumenin	29.872	2.01	−1	8 and 1.5		0.0001 (1.5)
Ribophorin I	14.413	2.79	−1	8		
Ribophorin II	59	1.65	−1	8	0.013 (8)	
Glycosyltransferase	4.767	2.33	−1	8 and 1.5		0.010 (8), <0.0001 (1.5)
Signaling						
Annexin A2	37.836	3.06	1	8		
S100A10-calcium binding	23.63	1.67	1	8 and 1.5		<0.0001 (1.5)
Fibrinogen beta chain	46.243	1.21	1	8		
Fibrinogen alpha chain	25.582	1.81	1	8		
Transport						
Hemoglobin alpha	14.669	1.98	1	8		
Hemoglobin beta	13.599	1.42	1	8		
Transcription/nuclear related						
Histone H3	293	1.62	−1	8		
Histone H3.1	6.0×10^7	1.56	−1	8		
Histone H4	1.6×10^5	3.12	1	8		
Histone H32	6.1×10^4	1.87	−1	8		
Histone H2B		1.71	1	8	0.012 (8)	0.0087 (8)
Importin		1.27	−1	8	0.029 (8)	0.039 (8)

[a] Proteins were analyzed by Scaffold and QSpec (23–26). In Scaffold, protein differences assigned a *P* value of <0.05 with the Student *t* test and Fisher's exact test were considered significant (23–25). For QSpec, a Bayes factor of >10 was used for significant difference (26). All time points are indicated as 8 or 1.5 h.
[b] A decrease in protein expression in treated endothelial cells (relative to untreated endothelial cells) is indicated by −1, and 1 indicates an increase.

glycolysis-related protein LDH may suggest a role as an indicator of initial BBB injury, given that high levels of LDH are found in cerebrospinal fluid during bacterial and viral meningitis, its release likely due to tissue breakdown. VDAC1, its expression downregulated 2.3-fold, is a voltage-dependent anion-selective channel that regulates mitochondrial membrane potential, suggesting that exposure to *C. neoformans* may disrupt ion homeostasis in mitochondria. Protein disulfide isomerase (PDI; downregulated 4-fold) is an ER protein that in addition to assisting with protein processing and folding also appears to load antigenic peptides onto major histocompatibility complex (MHC) class1 molecules in the ER.

TEM micrographs reveal cellular features in infected brain endothelial cells consistent with cell stress and cell injury. The proteomic analysis identified several changes in protein expression that suggested likely alterations in the overall cellular and/or organelle structure of the brain endothelial cells. To examine this notion further, ultrastructural analysis of brain endothelial cells exposed to *C. neoformans* was performed using transmission electron microscopy (TEM). TEM micrographs of endothelial cells

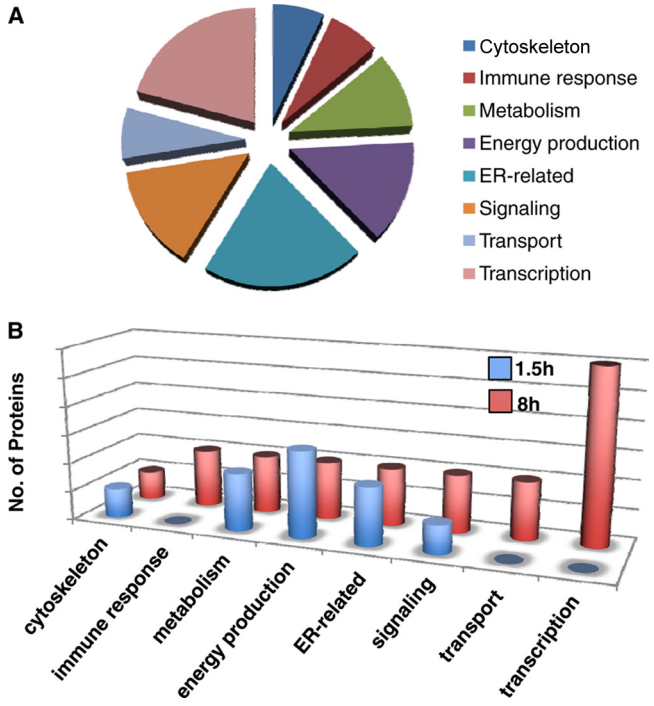

FIG 2 *C. neoformans* induces protein changes in human brain endothelial cells (hCMEC/D3). (A) Label-free LC-MS/MS analysis and spectral counts (SC) identified changes in protein expression of brain endothelial cells that were specifically induced by *C. neoformans*. The proteins identified are grouped according to the known or predicted function of each protein as shown in the pie graph. (B) Of the ~800 endothelium proteins identified at the 8-h time point, approximately 3.11% were found by SC to be differentially expressed in endothelial cells exposed to *C. neoformans*. Approximately 2.35% of the 425 proteins identified were differentially expressed in endothelial cells following a 1.5-h exposure to *C. neoformans*. The cylinders in the graph correspond to protein changes detected at 1.5 h (blue) or 8 h (red) following exposure to *C. neoformans*. The functional groups for each protein are shown on the *x* axis, and the *y* axis corresponds to the number of protein changes within each functional group.

exposed to *C. neoformans* for 8 h revealed significant structural changes in several organelles (Fig. 3A, C, and E). These changes were not observed in brain endothelial cells not exposed to *C. neoformans* but grown in otherwise identical conditions (Fig. 3B, D, and F). Among the most severe physical changes observed were shrunken nuclei with condensed chromatin. These features were typically observed only throughout endothelial cells that were exposed to *C. neoformans*. The change in chromatin packing from relaxed in control endothelial cells to condensed in exposed endothelial cells was consistent with the proteomic analysis that identified changes in protein expression of several histones (Fig. 3A).

Interestingly, the most striking changes were those observed in mitochondria. In exposed endothelial cells, mitochondria were severely swollen and misshapen, likely due to discontinuity in the outer membrane (Fig. 3C). Electron-dense material was also a common feature in these mitochondria. The appearance of these physical changes suggests that mitochondria were stressed and likely not functioning normally. This notion is consistent with the ~2-fold decrease in protein expression of two key components of the outer mitochondrial membrane: anion channels VDAC1 and VDAC2 (Table 1).

Cryptococci also induced structural changes in the endoplasmic reticulum (ER). The smooth ER appeared swollen with distended and bloated ends only in brain endothelial cells exposed to *C. neoformans* (Fig. 3E). Consistent with this observation, endothelial cells exposed to *C. neoformans* revealed that expression of several ER-related proteins were decreased 2-fold to 5-fold. Among the proteins identified were ribophorin I and II, reticulocalbin, calumenin, disulfide isomerase, and glycosyltransferase (Table 1).

Taken together, the TEM micrographs and the protein expression profiles point to specific changes in cellular structures and organelle function of brain endothelial cells that were specifically induced by *C. neoformans*. These results are indicative of activated cells that are likely stressed and have sustained injury due to the transmigration of *C. neoformans*.

Brain endothelial cells exposed to *C. neoformans* lost plasma membrane integrity. Among the signaling proteins that demonstrated increases in expression were annexin A2 and its cellular ligand, S100A10 (Table 2). Annexin A2 resides primarily in the cytoplasm and plays a role in many different cellular processes, including recruitment of factors that regulate the actin cytoskeleton and modulation of endosomal functions (34–38). Based on the TEM results and proteomic analysis, we sought to establish whether *C. neoformans* altered the integrity of the plasma mem-

TABLE 2 Expression profile of brain endothelial proteins following exposure to *C. neoformans*

Protein	Accession no. (UniProtKB/Swiss-Prot)	Altered expression identified at time point(s): 1.5 h	8 h	1.5 and 8 h
Myosin	tr|G3V1V0|		×	
Transgelin	sp|P37802|	×		
Cyclophilin A	sp|P62937|		×	
MHC-HLA class 1	sp|P10316|		×	
Lactate dehydrogenase	sp|P00338|	×	×	×
Phosphoglycerate kinase	sp|P00558|		×	
Phosphoglycerate mutase	sp|P18669|	×		
Anion channel, VDAC1	sp|P21796|	×	×	×
Anion channel, VDAC2	sp|P45880|		×	
Inner membrane protein	sp|Q16891|	×		
Aspartate aminotransferase	sp|P00505|	×		
Reticulocalbin	sp|Q15293|		×	
Protein disulfide isomerase	sp|P30101|	×	×	×
Calumenin	sp|O43852|	×	×	×
Ribophorin I	sp|P04843|		×	
Ribophorin II	tr|QJYR6|		×	
Glycosyltransferase	sp|P04844|	×		
Annexin A2	sp|P07355|		×	
S100A10-calcium binding	sp|P60903|	×	×	×
Fibrinogen alpha chain	sp|P02671|		×	
Fibrinogen beta chain	sp|P02675|		×	
Hemoglobin alpha 1	sp|P69905|		×	
Hemoglobin beta	sp|P68871|		×	
Histone H3.1	sp|P68431|		×	
Histone H3	tr|Q5TEC6|		×	
Histone H4	sp|P62805|		×	
Histone H2B	sp|O60814|		×	
Histone H32	sp|Q71Dl3|		×	
Importin	sp|Q14974|		×	

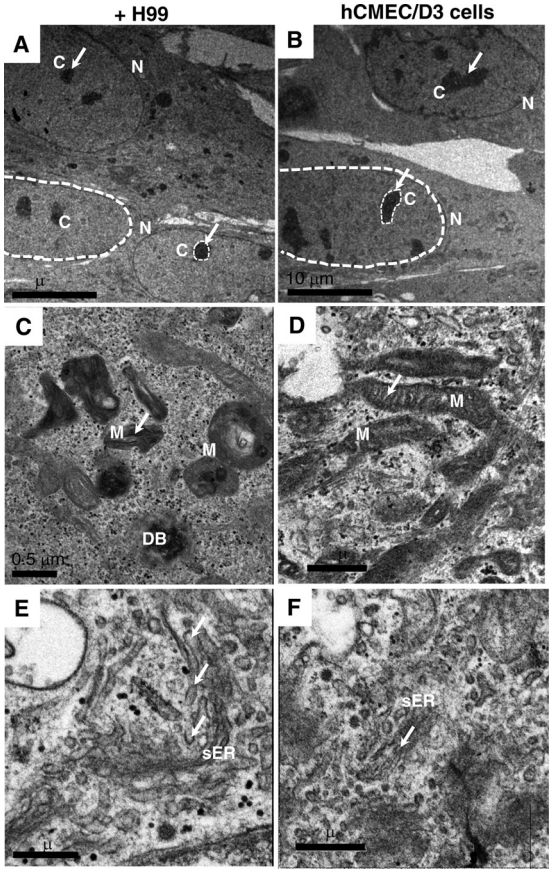

FIG 3 *C. neoformans* promotes dramatic changes in organelle structure of brain endothelial cells. Micrographs of transmission electron microscopy (TEM) reveal changes in cellular structures of human brain endothelial cells exposed to cryptococci (A, C, E) that are consistent with cellular necrosis. TEM micrographs shown in panels A, C, and E revealed condensed chromatin (C) in shrunken nuclei (N), swollen mitochondria (M), and bloated, distended ends in smooth ER (sER) (all indicated by white arrows). These ultrastructural changes were not observed in brain endothelial cells not exposed to *C. neoformans* (B, D, F). White line tracings were added to aid with visual clarification.

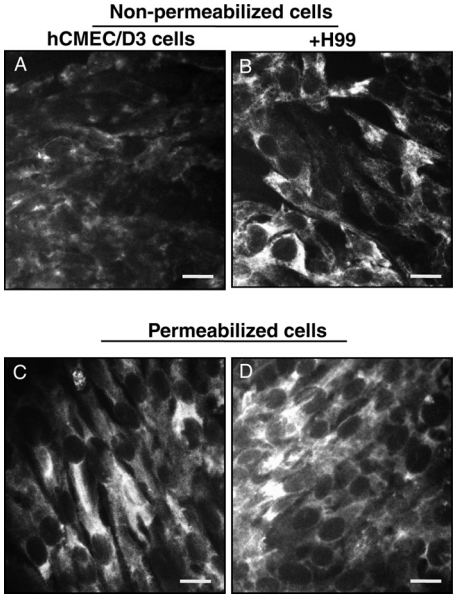

FIG 4 Brain endothelial cells display compromised plasma membrane integrity in the presence of *C. neoformans*. A cytosolic protein, annexin A2, was specifically detected in nonpermeabilized brain endothelial cells exposed to cryptococci (B) in contrast to control nonpermeabilized cells (A), suggesting that cryptococci damage the plasma membrane, thus allowing the annexin A2 antibody to enter cells. Specifically permeabilizing control endothelial cells (C) prior to the addition of antibodies resulted in a localization pattern for annexin A2 similar to the *C. neoformans*-treated endothelial cells (nonpermeabilized). A polyclonal rabbit antibody to annexin A2 and a Texas Red-conjugated secondary antibody were used to visualize annexin A2 by confocal microscopy.

brane and whether this contributed to endothelial cell damage. We used immunofluorescence to track the localization of annexin A2 in endothelial cells. We reasoned that intact, nonpermeabilized endothelial cells could not permit the binding of antibodies to an intracellular protein like annexin A2 due to the presence of an undamaged plasma membrane whereas cells that had lost plasma membrane integrity would show specific labeling of annexin A2. We found that endothelial cells exposed to cryptococci (but not chemically permeabilized) clearly showed the localization of annexin A2 throughout the cytoplasm but nonexposed and nonpermeabilized endothelial cells did not (Fig. 4A and B). In contrast, specifically permeabilizing control endothelial cells prior to the addition of antibodies resulted in a localization pattern for annexin A2 similar to that seen in treated endothelial cells (Fig. 4C). These data suggested that *C. neoformans* appeared to have compromised the integrity of the plasma membrane of endothelial cells (Fig. 4).

***C. neoformans* induced the translocation of HMGB1 in brain endothelial cells.** Collectively, the data suggested that *C. neoformans* not only activated endothelial cells as determined from the protein expression profiles but also appeared to induce cell injury as indicated by the metabolic, cellular, and structural changes. In order to explore this notion further, we examined the expression of a cellular biomarker, HMGB1, whose translocation out of the nucleus is indicative of cellular damage. HMGB1 was previously thought to function only as a nuclear factor that enhanced transcription, but recently it was discovered to be a crucial cytokine that becomes active during infection, injury, and inflammation (39–41). The passive release of HMGB1 to the cytoplasm and eventually into the extracellular space is indicative of cell damage and/or injury.

HMGB1 was identified among the proteins detected in endothelial cells exposed to *C. neoformans* for 1.5 h and 8 h, suggesting that HMGB1 is a resident protein of human brain endothelial cells. However, HMGB1 did not show any differences in expression between treated endothelial cells and cells that had not been exposed to *C. neoformans* (Tables 1 and 2).

Confocal microscopy revealed a clear and specific localization of HMGB1 in the nuclei of control (nonexposed) endothelial cells at 8 h, 24 h, and 30 h (Fig. 5C, E, and G). This localization pattern for HMGB1 was consistent with the nucleus-specific DAPI (4′,6-diamidino-2-phenylindole) staining as previously reported, and the differential inference contrast (DIC) image revealed an intact brain endothelium (Fig. 5A and B) (39). In contrast, the presence of *C. neoformans* altered the behavior of HMGB1 dramatically. Endothelial cells exposed to *C. neoformans* revealed a translocation of HMGB1 from nuclei to the surrounding cytoplasm and to the surface of endothelial cells at >24 h (Fig. 5H). This is consistent with extensive cellular injury and suggests that internalization

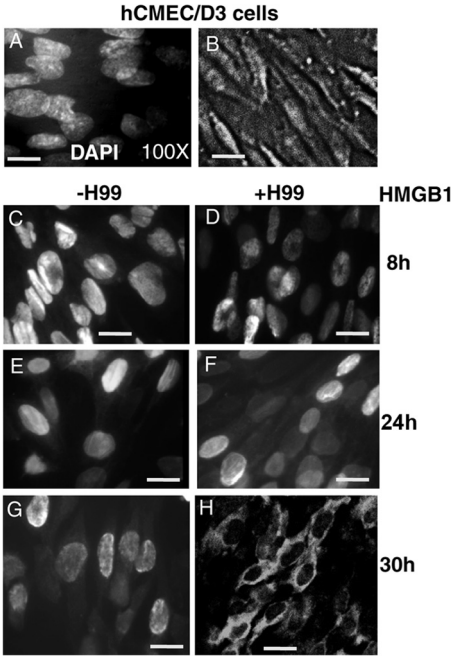

FIG 5 *C. neoformans* induces the translocation of HMGB1 in brain endothelial cells. Confocal microscopy revealed that prolonged exposure of brain endothelial cells to cryptococci resulted in the translocation of HMGB1 out of the nucleus and into the surrounding cytoplasmic space (H). Endothelial cells that had not been exposed to *C. neoformans* revealed a nuclear localization of HMGB1 (C, E, G) consistent with DAPI staining (A). A polyclonal rabbit antibody to HMGB1 and a Texas Red-conjugated secondary antibody were used to visualize HMGB1.

of *C. neoformans* damages brain endothelial cells and likely promotes the eventual breakdown of the BBB. Interestingly, the release of HMGB1 was not observed at earlier time points, suggesting that HMGB1 may be released only from cells that are significantly necrotic.

DISCUSSION

It is known that *C. neoformans* enters the brain by a predominately transcellular mechanism that involves a direct association with the brain endothelium (also known as BBB). Establishment of fungal disease in the CNS is the result of a complex interplay between cryptococci and the brain endothelial cells. We sought to resolve the molecular basis for the cellular and molecular changes imposed on human brain endothelial cells by *C. neoformans*. We used spectral counting with a label-free shotgun protein identification approach to identify and quantify protein changes in brain endothelial cells that had been challenged (for 1.5 h or 8 h) with a species of *C. neoformans* that causes disease in immunocompromised patients. We reasoned that protein changes detected at the earlier time point (1.5 h) would reflect the BBB's response to the attachment of cryptococci to the endothelium—an event required in order for *C. neoformans* to invade the brain parenchyma. Protein changes identified at the later time point (8 h) would instead reflect how the endothelial cells responded to the internalization of cryptococci across the brain endothelium.

Cryptococci transmigrate across the BBB by engaging the cytoskeleton of brain endothelial cells. Our analysis demonstrated significant changes in protein expression of factors known to associate with the cytoskeleton. The 4-fold to 16-fold differences in the expression of cytoskeleton-related proteins like myosin and transgelin were likely due to the reorganization of the cytoskeleton in brain endothelial cells upon the attachment and internalization of *C. neoformans*. This is consistent with the 3-fold increase in the expression of annexin A2 and the ~2-fold increase in S100A10, proteins involved in recruiting factors to the cytoskeleton (42). This notion is supported by previous results demonstrating that transmigration of *C. neoformans* often involves a transcellular process that is dependent on cytoskeleton rearrangement (16, 43). Indeed, it has been shown that actin reorganization via the dephosphorylation of cofilin plays a significant role in the migration of cryptococci across the blood-brain barrier, and the downregulation of S100A10 in murine brain endothelial cells inhibited the internalization of cryptococci (16, 43). Accordingly, we found that S100A10 was upregulated during both attachment and internalization, further suggesting that S100A10 facilitates the migration of *C. neoformans* across the human brain endothelium. We did not identify changes in the tight junction proteins of the brain endothelial cells. This is consistent with the notion that the paracellular pathway (through the tight junctions) is likely not a primary route for migration of cryptococci across the BBB (11, 13, 14, 16, 17, 19, 21, 44).

Interestingly, studies of the brain endothelium challenged with the encapsulated bacterium *Neisseria meningitidis* revealed changes in the expression of genes encoding cytoskeletal proteins, likely indicating a similar reorganization of the cytoskeleton as observed with *C. neoformans* (45). *N. meningitidis* is a common cause of bacterial meningitis, and disruption of the BBB is a hallmark event in this disease (45). Changes in protein expression of cytoskeleton-related proteins were detected as early as 1.5 h following *C. neoformans* exposure. This is consistent with previous results that examined the physical association of *C. neoformans* with brain endothelial cells by ultrastructural analysis and found that endothelial cells produce microvilli within 1 h of exposure to cryptococci (13). The formation of microvilli is observed at the site of *C. neoformans*'s attachment and is thus likely required for internalization and migration of *C. neoformans*. Studies focused on interactions between *N. meningitidis* and endothelial cells (human umbilical vein endothelial cells [HUVECs]) have revealed a similar formation of microvillus-like structures and have further shown that CD44 and actin polymerization colocalize within these structures (46). Given the involvement of CD44 and actin in the attachment of *C. neoformans* to the endothelium, it is likely that microvilli play a crucial role in the invasion of *C. neoformans* into the CNS.

Increased permeability in the brain endothelium following traumatic brain injury has been associated with increased levels of albumin, cofilin, and cyclophilin A. In this case, cyclophilin A appeared to play a protective role in maintaining blood-brain barrier integrity and reducing tissue damage during ischemia or oxidative stress (47, 48). It has also been demonstrated that lipopolysaccharide (LPS)-treated macrophages and HUVECs secrete cyclophilin A and that it acts as a mediator of endothelial cell activation and also in inflammation (49, 50). In the case of *C. neoformans*, the induced increase of cyclophilin A at 8 h postexposure may reflect a change in BBB permeability likely due to the involvement of the cytoskeleton, which would promote a more permeable brain endothelium and thus enhance the transcellular migration of *C. neoformans* across the BBB. Alternatively, cyclo-

philin A may facilitate the migration of cryptococci across the BBB by simply acting as a signaling mediator or regulator of other key proteins in brain endothelial cells. Taken together, the evidence demonstrates that *C. neoformans* engages the cytoskeleton of the brain endothelium and in doing so promotes its own migration across the brain endothelium.

Internalization of *C. neoformans* leads to brain endothelial cell damage. Our results indicate that brain endothelial cells challenged with *Cryptococcus* displayed changes in protein expression and morphology that are consistent with endothelial cell stress and cell damage. It is known that the brain microvascular endothelium maintains a high concentration of mitochondria, and the pathophysiology of brain endothelial cells is closely associated with mitochondrial dysfunction (51, 52). In this study, the TEM analysis showed severe morphological changes in mitochondria, and the proteomic analysis found the expression of key mitochondrial enzymes altered. This is consistent with mitochondrial stress that can impair its function, leading to decreased ATP production, increased reactive oxygen species (ROS) production, and ultimately, compromised function of the brain endothelium (51). Also, endothelial cells responded to *C. neoformans* by increasing the expression (2-fold to 52-fold) of metabolic proteins, suggesting that *C. neoformans* induced an increase in energy demand and this led to an upregulation of glycolysis-associated proteins in the brain endothelial cells.

The decrease in the expression of several proteins associated with the ER reported here likely indicates impaired ER activity and further supports the notion that *C. neoformans* promotes brain endothelial cell stress. It is interesting that the MHC-HLA class 1 protein whose function is associated with peptide recognition on the ER was upregulated 3.5-fold in contrast to all other ER-related proteins. Normally, the ER ensures proper folding and posttranslational modification of proteins and also plays a role in maintaining Ca^{2+} homeostasis; however, perturbations in ER function lead to changes in protein processing, cell stress, and eventually cell death (53, 54). We and others have found that endothelial cells begin to show signs of injury as early as 4 h to 8 h postexposure to *C. neoformans* and internalization is required for cell damage to occur (16, 31, 55). *In vivo* studies have shown that exposure (>24 h) to *C. neoformans* can result in extensive damage to the endothelial barrier and basal lamina (20). Similarly, by monitoring the *in vitro* translocation of HMGB1 (an indicator of cellular injury), we found that endothelial cells sustained significant cellular damage during prolonged exposure to *C. neoformans*, suggesting that *C. neoformans* induces the eventual breakdown of the brain endothelium.

Taken together, the loss of plasma membrane integrity, the mitochondrial dysfunction, the morphological changes in organelles, the translocation of HMGB1, and the changes in protein expression of key proteins would suggest that brain endothelial cells not only are actively stressed during internalization of cryptococci but also sustain significant damage that is consistent with key features of the process of cell necrosis (52, 56). The endothelial cell necrosis reported here may be representative of the programmed cell death known as necroptosis (in contrast to apoptosis) (52, 56, 59). Although plasma membrane disruption, swelling of organelles, ultrastructural changes to nuclei and chromatin, and HMGB1 release (as we observed) are indicative of necroptotic cell death, since no unique biochemical marker for necroptosis exists, further analyses would be required to confirm that cryptococci specifically induce necroptosis of brain endothelial cells (52, 57, 58). Interestingly, the release of HMGB1 was also reported during pathogen-induced cellular necrosis involving *Neisseria gonorrhoeae*, *Porphyromonas gingivalis*, *Klebsiella pneumoniae*, and *Shigella flexneri* (57, 58). The process of necroptosis is thought to be advantageous for the host, since necrosis has been shown to be vital in defending against viral and bacterial infections and possibly also against cancer (58, 59). It is intriguing to consider that cryptococci may have evolved a particular mechanism of entry that specifically activates the cell necrosis pathway in the brain endothelium. This would destroy the integrity of brain endothelial cells and promote disruption of the BBB, which would further facilitate the movement of *C. neoformans* into the CNS.

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