Hyaluronic Acid Receptor CD44 Deficiency Is Associated with Decreased Cryptococcus neoformans Brain Infection*

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Background: Previous studies suggest that the C. neoformans hyaluronic acid and host CD44 interaction could be the initial step of brain invasion.

Results: CD44 knock-out mouse model verified the pathophysiology of C. neoformans brain invasion.

Conclusion: The CD44 receptor on membrane lipid rafts during invasion is involved.

Significance: The mechanism of invasion and a potential clinical intervention strategy were identified.

Cryptococcus neoformans is a pathogenic yeast that can invade the brain and cause meningoencephalitis. Our previous in vitro studies suggested that the interaction between C. neoformans hyaluronic acid and human brain endothelial CD44 could be the initial step of brain invasion. In this report, we used a CD44 knock-out (KO or CD44Δ/Δ) mouse model to explore the importance of CD44 in C. neoformans brain invasion. Our results showed that C. neoformans-infected CD44 KO mice survived longer than the infected wild-type mice. Consistent with our in vitro results, the brain and cerebrospinal fluid fungal burden was reduced in CD44-deficient mice. Histopathological studies showed smaller and fewer cystic lesions in the brains of CD44 KO mice. Interestingly, the cystic lesions contained C. neoformans cells embedded within their polysaccharide capsule and were surrounded by host glial cells. We also found that a secondary hyaluronic acid receptor, RHAMM (receptor of hyaluronan-mediated motility), was present in the CD44 KO mice. Importantly, our studies demonstrated an in vivo blocking effect of simvastatin. These results suggest that the CD44 and RHAMM receptors function on membrane lipid rafts during invasion and that simvastatin may have a potential therapeutic role in C. neoformans infections of the brain.

Cryptococcus neoformans invades the brain and causes meningoencephalitis, primarily in immunocompromised patients and sporadically in normal hosts. It is the most common fungal infection of the central nervous system and is one of the major causes of death in AIDS patients (1, 2). C. neoformans is encompassed by a polysaccharide capsule composed mainly of glucuronoxylomannan (GXM) (3); this capsule is known to be the major virulence factor of this yeast (4–6). As such, the role and biogenesis of the capsule has become an area of focus for research in this pathogen (7). Previously, we characterized the C. neoformans gene CPS1 (capsule polysaccharide synthase 1) (8). Deletion of CPS1 from C. neoformans cells causes alterations in the ultrastructure between the cell wall and capsule, and the deletant shows a reduced ability to associate with human brain microvascular endothelial cells (HBMEC). Polysaccharide hyaluronic acid (HA; also known as hyaluronan) can be detected in the wild-type C. neoformans strain but not in cps1Δ cells. Testing of C. neoformans strains with different concentrations of HA demonstrated that the ability of yeast to bind to HBMEC is proportional to their HA content. Subsequent analysis indicated that the CPS1 gene encodes an HA synthase (9). Taken together, these findings indicate that the C. neoformans CPS1 gene product, HA, plays a role as an adhesion molecule during interaction with endothelial cells.

Several HA-binding proteins (or HA receptors) localized in the membranes, such as CD44, RHAMM, Ivd4, LEC receptor, and others, have been identified in different cell types (10, 11). The most common HA receptor is CD44, which plays different roles in different cell types. As CD44 is a major membrane HA receptor, it is conceivable that C. neoformans HA directly engages with the HBMEC CD44 as a part of its invasion mechanism. Indeed, we have demonstrated that C. neoformans HA interacts with CD44 on HBMEC, a primary receptor in C. neoformans infection (12). For example, an anti-CD44 neutralizing antibody treatment significantly reduces C. neoformans association with HBMEC. Association of C. neoformans with HBMEC is also found to be considerably impaired either in CD44 knockdown HBMEC or HA-deficient C. neoformans.

The abbreviations used are: GXM, glucuronoxylomannan; CSF, cerebrospinal fluid; HBMEC, brain microvascular endothelial cell; GFAP, glial fibrillary acidic protein; HA, hyaluronic acid or hyaluronan; HBMEC, human brain microvascular endothelial cell; RHAMM, receptor of hyaluronan-mediated motility; YPD, yeast extract, peptone, and dextrose.

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strains. Likewise, overexpression of CD44 in HBMEC increases the association of C. neoformans with HBMEC. Furthermore, confocal microscopic images show that CD44 is enriched at and around the C. neoformans adhesion sites. Finally, upon C. neoformans and HBMEC engagement, a subpopulation of CD44 and β-actin are translocated to the host membrane lipid rafts. Our results highlight the interactions between C. neoformans HA and host CD44. The dynamic results of these interactions may represent events during the adhesion and entry of C. neoformans at HBMEC membrane rafts by eliciting downstream events of the entry process at HBMEC membrane rafts. Subsequently, membrane signaling may relay through CD44 to the actin cytoskeleton inside the HBMEC cells. Scanning electron microscopic images reveal the course of invasion: internalization by the microvilli embrace of C. neoformans, followed by a zipper-like mechanism in which the host cell plasma membrane encompasses the invading yeasts (13). This mechanism requires C. neoformans cell-induced HBMEC cytoskeletal rearrangements for the accumulation of actin at the site of C. neoformans entry. The C. neoformans cell may then be drawn progressively into the host cell.

Pathogens usually use multiple mechanisms to invade their host(s). Several studies have suggested that C. neoformans may use different routes to enter the brain. For example, transmission electron microscopic images showed that C. neoformans cells embedded inside macrophages could be found in the crossing site of the brain microcapillaries, suggesting a “Trojan horse” mechanism (14). It has also been reported that urease is required for brain invasion (15, 16). The roles of Ure1 and CPS1 in brain invasion are not necessarily mutually exclusive. However, their relevant functions are yet to be elucidated, and our understanding of their complicated invasion process has just begun. Recently, it has been documented that the lipid raft-dependent endocytosis process mediates C. neoformans internalization into HBMEC and that the CD44 protein of the hosts, cytoskeleton, and intracellular kinase DYRK3 are involved in this process (17). Taken together, a transcellular mechanism seems to be the predominant route for C. neoformans brain invasion (13, 17).

Previously, we had used the in vitro blood-brain barrier model to demonstrate that the C. neoformans HA-HBMEC CD44 interaction plays an initial role during the fungal invasion across the blood-brain barrier (12). The CPS1 deletion strain (C559) is temperature-sensitive (unable to grow at 37 °C), precluding in vivo studies. In this report, we used a mouse model to investigate C. neoformans brain invasion in vivo. In the virulent studies, the CD44 knock-out (KO) mice survived longer than the wild-type mice. Compared with the wild-type mice, the fungal load in the brain and CSF was lower in infected CD44 KO mice. Similarly, the CD44−/− mouse brain contained smaller and fewer cystic lesions. These results suggested that host CD44 is required for C. neoformans brain invasion. Immunofluorescence microscopic images of the brains sections showed that a mass of C. neoformans cells within the lesions are imbedded in their capsule material; these lesions were surrounded by many reactive astrocytes displaying a unique pattern. We also found that, in both the wild-type and CD44−/− mice, a secondary HA receptor, RHAMM, was present in the brain. Finally, we demonstrated the in vivo blocking effect of the hypolipidemic drug simvastatin. Our results suggest that simvastatin may remove receptor function on the membrane lipid raft or yet identified action(s), providing a cue for a novel anticytoscoccal intervention.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Cultures**—C. neoformans strains B-4500FO2 and C559 (cps1Δ) are isogenic wild-type and CPS1 gene-deleted strains, respectively (8, 9). Yeast cells were grown aerobically at 30 °C in 1% yeast extract, 2% peptone, and 2% dextrose (YPD broth) or in Sabouraud medium (Difco Laboratories, Detroit, MI). Cells were harvested at an early log phase, washed with phosphate-buffered saline (PBS), and then resuspended in Ham’s F-12/M199 (1:1, v:v), 5% heat-inactivated fetal bovine serum (experimental medium), and 1% human serum. The Cryptococcus cell number was determined by direct counting from a hemocytometer (18). Mouse C57LB/6 strain was used as an in vivo model for C. neoformans pathogenesis studies. The CD44−/− knock-out (stock No. 5085) and its control strain (stock No. 0664) derived from the C57LB/6, were purchased from the Jackson Laboratory for our brain invasion studies.

**Mouse Survival Study**—Mouse survival measurements were performed as described previously (13). Mouse strain C57LB/6 has been widely used as an in vivo model for C. neoformans pathogenesis studies. The derived CD44−/− knock-out strain was used in parallel for these studies. Two groups (wild type versus CD44−/−) of 18 female mice were infected individually with 106 B-4500FO2 yeast cells via lateral tail vein injection. Their survival was monitored daily, and moribund mice were sacrificed by CO2 inhalation.

**Tissue Burden**—Groups of three to five C57LB/6 mice were infected with 105 yeast cells via lateral tail vein injection. After 24 h, the brain was removed and washed with 2 ml of PBS. The tissues were homogenized and plated onto triplicate YPD plates for cfu counting.

**Measurement of Infected Cryptococcus Cells in Cerebrospinal Fluid (CSF)**—The presence of Cryptococcus cells in CSF is an indicator of cryptococcal meningitis. We used the published method (19) with slight modifications. Briefly, groups of 8–10 C57LB/6 female mice were infected with 106 yeast cells via lateral tail vein injection. After 24 h, the animal was perfused with 20 ml of PBS to remove blood circulating yeast cells. The skull was opened for CSF collection. The brain is suspended in CSF inside the skull, so this fluid was collected first, and the brain was then removed and washed with 500 μl of PBS. In the meantime, the cranial cavity was washed four times with 100 μl of PBS. The washing solutions were combined. After centrifugation, the pellet was resuspended in 50 μl of PBS, designated as the CSF fraction. An aliquot was used for counting erythrocytes (RBCs). Samples were discarded if the CSF was contaminated with blood (>25 RBCs/μl present in the sample). The uncontaminated samples were used for Cryptococcus cell counting.

**Immunofluorescence Microscopy and Histological Stains**—For histological staining, hematoxylin and eosin (H&E; purchased from Sigma-Aldrich) was used to stain mouse brain sections. The infected yeast cells in cystic lesions from five random
sections of wild-type and CD44−/− mouse brain sections were counted and averaged. The result is presented in Fig. 3, C and D. ImageJ software was used to measure the hollow areas and followed by statistical analyses. The calculated result is presented in Fig. 3E. Samples for immunofluorescence microscopy were prepared as follows. Brain section slides were blocked with 5% nonfat milk/PBS for 30 min, and C. neoformans cells were stained with anti-GXM monoclonal antibody (18b7) and rhodamine-conjugated second antibody displaying a red color, anti-GFAP monoclonal antibody (Cell Signaling Technology, 1:200 dilution), and FITC-conjugated second body displaying a green color (shown in Figs. 4 and 7B). The MetaMorph program associated with our fluorescence microscope was used to scan images with 5–10 random fields, and then the statistical package GraphPad Prism 5 was used to quantify the readings (Fig. 4G). For HBMEC studies, ~5 × 104 cells were seeded onto one coverslip 24 h before the experiment. HBMEC were prewashed four times with PBS and then fixed with 2% formaldehyde/PBS (v:v) for 30 min at room temperature. The samples were blocked with 5% nonfat milk/PBS for 30 min and then incubated with anti-CD44 monoclonal antibody (Santa Cruz Biotechnology, 1:500 dilution) at 4 °C overnight. The coverslips were then washed four times with PBS, and propidium iodide (1 µg/ml) (Sigma) in 1% BSA/PBS and/or anti-mouse IgG-FITC conjugate (1:100 dilution) was added into each well for 1 h at 4 °C. After three additional washes with PBS, the coverslips were sealed onto slides. As shown in Fig. 5, crude brain vessels were isolated as described (12). The samples were fixed, diluted, and cytospun onto the slide. Anti-RHAMM monoclonal antibody (sc-25488, Santa Cruz Biotechnology) and rhodamine-conjugated second antibody displaying a red color were then applied. All samples were examined under a fluorescence microscope at the Congressman Dixon Cellular Imaging Core Facility, Children’s Hospital Los Angeles.

siRNA Treatment—Anti-CD44 siRNA (sc-35534) and anti-RHAMM siRNA (sc-40182) were purchased from Santa Cruz Biotechnology, and a control oligonucleotide (sc-36869) was used in parallel. The siRNA treatment was performed according to the manufacturer’s protocol. GenMute™ siRNA transfection reagent (SignaGen Laboratories, Gaithersburg, MD) was used. Briefly, 20 pmol of siRNA was transfected into HBMEC. After 5 h, the culture was replaced with fresh medium and grown for 24 h. The HBMECs were then washed with PBS three times for the in vitro adhesion assay as described previously (12).

Effect of Simvastatin on C. neoformans Brain Invasion—Simvastatin (Merck, Sharp & Dohme, Ltd., Enfield, Middlesex, UK) was prepared as a 4 mg/ml stock. Briefly, 4 mg of simvastatin was dissolved in 100 µl of ethanol and 150 µl of 0.1 N NaOH and incubated at 50 °C for 2 h, after which the pH was adjusted to 7 and the total volume was corrected to 1 ml. The stock solution was diluted to the appropriate concentration in sterile PBS immediately before use. Simvastatin (0–20 mg/kg) was given by intraperitoneal injection overnight. After 12 h, the mice were boosted with the same amount of simvastatin and immediately inoculated with 106 B-4500FO2 cells via lateral tail vein injection. After 24 h, the mice were sacrificed and the brain homogenized and used for cfu counting. The control mice received only PBS. The doses of simvastatin used were comparable to those used previously in murine studies in vivo and are significantly higher than those used in humans, because in rodents, there is a significant up-regulation of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase with statin treatment (20).

Statistical Analysis—The statistical analysis of the data obtained from our studies was performed from samples of wild-type versus CD44−/− mice. Raw data were entered into EXCEL files and automatically converted to the compatible format for the statistical analysis package of the GraphPad Prism 5 program followed by two-tailed t tests to determine the statistical significance between the control and treatment groups. p < 0.05 was considered significant.

RESULTS

In Vivo Virulence Studies of Wild-type and CD44−/− KO Mice—Our previous studies suggested that the CD44 of HBMEC may play a critical role as the host receptor during the fungal traversal across the blood-brain barrier in vitro (12). Here, we performed in vivo studies to test whether C. neoformans is less virulent in CD44−/− mice by conducting the survival assay in a murine systemic infection model. C. neoformans cells (B-4500FO2, 106) were injected intravenously via the lateral tail vein into two groups, CD44+/+ and CD44−/− of C57BL/6 mice, with 18 mice/group. The health of the mice and their survival were monitored daily. Fig. 1 shows the survival rate of the infected mice. The wild-type mice infected with C. neoformans cells started to succumb to infection within 38 days post-inoculation. In contrast, the CD44−/− mice infected with C. neoformans remained generally healthy but some eventually succumbed to cryptococcosis. At least 33% (6 of 18) of the CD44−/− mice survived for more than 60 days, by which time all wild-type mice had passed away. Similar results were obtained with serotype A strain H99, a more virulent strain (data not shown). Thus, it is concluded that knock-out of CD44 results in higher resistance to C. neoformans infection (p = 0.039).

Brain Invasion by C. neoformans Is Reduced in CD44 Knock-out Mice—To explore the reasons for the longer survival of CD44−/− mice, we examined the brain fungal loads of the wild-type and CD44−/− mice. In the wild-type mice, the fungal load
of the brain was proportional to the injected fungal cells at 7 days post-injection (Fig. 2A, gray columns). The increased cell number was presumed to represent the propagation of cells that might cross the blood-brain barrier into the brain cortex. In the infected brains of CD44−/− mice, lower levels of fungal cells was observed (⁎, p < 0.05; **, p < 0.01) (Fig. 2A, white columns). C. neoformans cells were also found in the CSF at 24 h post-infection, an indication of cryptococcal meningoencephalitis (Fig. 2B). A similar result was obtained from both serotype A strain H99 and serotype D strain B-4500FO2, in which the numbers of invaded C. neoformans cells were lower in the CSF of CD44−/− mice compared with the wild-type mice (p < 0.01) (Fig. 2B).

C. neoformans cells in the brain could also be counted directly under the immunofluorescence microscope (see “Experimental Procedures”). Five sections were randomly chosen from the brains of the wild-type and CD44−/− mice, and C. neoformans cells were counted; the average numbers are presented in Fig. 2C. The result again showed that there are more C. neoformans cells inside the brain cortex in the wild-type brain sections (p < 0.05) (Fig. 2C). Overall, these in vivo results are consistent with our previous in vitro studies (12) demonstrating that CD44 plays an important role during C. neoformans brain infection.

Brain Sections of C. neoformans-infected Wild-type and CD44−/− Mice—Compared with the isogenic wild-type strain, CD44−/− mice exhibited reduced C. neoformans infection in CSF and a lower fungal load in the brain (Fig. 2). To explore the cryptococcal brain infection further, we compared the histopathological features of the brain sections obtained from infected CD44+/+ and CD44−/− C57BL/6 mice using H&E staining. Representative images are presented in Fig. 3. In the infected mice, cystic lesions (small holes) were observed throughout the brain sections (for example, see Fig. 3, A and B, arrows). An enlarged image of a cystic lesion marked by the arrow in Fig. 3A is shown in Fig. 3C. This image (Fig. 3C), along with the one in Fig. 4B, leads us to believe that the large unstained central hollow region may be filled with capsule components. Compared with the CD44−/− brain (Fig. 3, C versus D, for example), the lesions are generally larger and more abundant in the wild-type mouse brain. Images of the degraded areas were scanned and subjected to statistical analysis. The results show that the wild-type mouse brains have a larger damaged area (p < 0.01) (Fig. 3E). In the infected brain, C. neoformans cells can easier be detected in the brain venules, between the meninges and cortex of the wild-type mice, and to a lesser extent in the CD44 KO mouse (Fig. 3F). Another pathological feature is the presence of obvious abnormal morphology such as vacuolization around the lesion sites of edema. Overall, the CD44−/− mouse brain shows less damage, i.e., the cerebral
architecture is usually preserved (Fig. 3B). Although *C. neoformans* cells can be observed inside the vessels as well as cystic lesions of the CD44<sup>+/−</sup> mouse brain, lesions are usually smaller in size. Thus, the CD44<sup>+/−</sup> mice are less vulnerable to *C. neoformans* brain invasion.

**Mouse Glial Cells Surround the Infected *C. neoformans* Colonies**—To further examine the colony region and the host responses, we used cell markers to examine the infected brain sections. In a paraffin section (5–7 µm thick) the GXM signals can be detected in large areas of the *C. neoformans* colonies grown within the infected wild-type versus CD44<sup>+/−</sup> mouse brain (Fig. 4, B versus E). When an anti-GFAP antibody (a glial cell marker) is used, a unique pattern of glial cells associated with cryptococcal colonies is observed (Fig. 4, C and F). Statistical analysis of the relative GXM signals shows that the wild-type brain has a higher GXM intensity than that of the CD44<sup>+/−</sup>-infected brains (p < 0.05) (Fig. 4G). In the uninfected control brain, as shown in Fig. 4, H (glial cells) and I (glial cells and DAPI stain), no *C. neoformans* GXM could be detected. Although some scattered glial cell signals are observed, these signals are in general weaker than those seen in the infected brain sections (Fig. 4, C and F). Furthermore, in the infected brain sections, the GFAP-positive reactive astrocytes with prominent cytoplasmic processes surround a pool of mucoid-like materials imbedded among *C. neoformans* cells (shown in Fig. 4, C and F). Almost no inflammatory infiltrate was observed in the parenchymal lesions. At 2 days post-infection, the cystic lesions positive for GXM stain were found throughout the infected brains, but the brain sections showed little or no positive staining for mouse CD11a (marker of lymphocytes, monocytes/macrophages, and granulocytes) or CD11b (marker of granulocytes, monocytes/macrophages, NK cells, and some T and B cells) (data not shown). This suggests that inflammation is not evident when *C. neoformans* invades the brain.

Possibly because of a distinctive immune response of the host, we only rarely observed the formation of cryptococcoma (a mass of *C. neoformans* with inflammatory response and variable fibrosis) or in some cases granulomatous inflammation with *C. neoformans* present inside multinucleated cells in wild-type brain sections. These changes are seen both in meninges and in brain parenchyma. In summary, our images show that accumulation of the host glial cells (or reactive astrocytes), but not lymphocytes or macrophages, is the primary response around the cystic lesions; such
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**FIGURE 5.** Expression of RHAMM in CD44 KO mouse brains. A, brain microvessels were isolated from wild-type (upper panel) versus CD44−/− (lower panel) C57BL/6 mice, respectively. Anti-CD44 and anti-RHAMM antibodies were used to detect the presence of CD44 (green) and RHAMM (red) molecules by immunofluorescence microscopy. DAPI staining is shown in blue. Last column, overlaid images. B, isolated vessel extracts from three individual mouse brains (wild type versus CD44−/−) were separated in a 12.5% SDS-PAGE to detect CD44 and RHAMM molecules by Western blot. β-Actin was used as the loading control. C, intensity of protein bands in samples (Fig. 5A) were separated in a 12.5% SDS-PAGE to detect CD44 and RHAMM molecules by Western blot. β-Actin was used as the loading control. C, intensity of protein bands in samples (Fig. 5B) was scanned and quantified using ImageMaster 2D Platinum 6.0 software (***, p < 0.001).

Responses may be unique during C. neoformans-induced granulomatous development.

**Presence of RHAMM in CD44−/− Mice**—Despite the attenuation of brain infection and virulence in CD44−/− mice, cells of C. neoformans are still found in the infected brains (Figs. 2–4). The trapping of the C. neoformans cells in the microvessels might not be sufficient to attribute those backgrounds to CD44 KO brains (Figs. 2 and 3). These observations raise the possibility of another route of C. neoformans brain invasion when the CD44 route is blocked. One possibility may be the existence of another route of C. neoformans brain invasion and quantified using ImageMaster 2D Platinum 6.0 software (***, p < 0.001) but no major difference in the RHAMM levels. The presence of RHAMM suggests a possible backup mechanism in the CD44−/− mouse model (Fig. 5C). Similar observations of RHAMM compensation have been documented in collagen-induced arthritis CD44 KO mouse models (21). Together, our results suggest that the presence of RHAMM may partially compensate for deficient CD44 functions for C. neoformans brain invasion in CD44−/− mice.

**Association of C. neoformans with Mouse BMEC Revealed by siRNA Treatment**—C. neoformans CPS1 wild-type strain contains HA. In CPS1 knock-out mutant strain C559 (cps1Δ), surface HA is diminished and its association with HBMEC is significantly reduced in vitro (8, 9). Here, we used the parental strain (B-4500FO2) and CPS1 deletion strain C559 (cps1Δ) for the association assay. The association efficiency of these strains was tested on mouse BMEC (MBMEC) with and/or without anti-CD44 and anti-RHAMM siRNA treatments. Without siRNA treatment, C. neoformans strain B-4500FO2 (CPS1 wild type) had a high association activity toward MBMEC (Fig. 6A, leftmost dark gray column), whereas the strain C559 has low level association activity (leftmost light gray column). In this assay, non-associated fungal cells were washed...
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away before the counting of colony-forming units. Some basal association could be detected because the washing must not be too harsh in order to preserve the attached BMEC. Therefore, the reading obtained with C559 is most likely because of the nonspecific trapping of *C. neoformans* cells in the assay. In our siRNA treatment of MBMEC (5 h), ~70–80% of the target proteins (CD44 and RHAMM) were knocked down (Fig. 6B). In the case of CD44 knockdown MBMEC, the association ability of B-4500FO2 cells was reduced to a significantly lower level (p < 0.01) (Fig. 6A, second set of columns). This result suggests that CD44 is a potent HA receptor for *C. neoformans* association. In anti-RHAMM siRNA-treated MBMEC, the association activity was slightly lower than that of the controls (p < 0.05) (Fig. 6A, third set of columns). When MBMEC was treated with both anti-CD44 and anti-RHAMM siRNA (CD44−/− and RHAMM−/−), an additive effect was observed, exhibiting a low level close to that of C559 (p < 0.05) (Fig. 6A, rightmost columns). Thus, *C. neoformans* association activity was significantly reduced by a lack of: 1) *C. neoformans* adhesion molecule (presumably HA, based on the *cps I Δ* studies); 2) the host HA receptor CD44; and 3) the secondary receptor, RHAMM. These results suggest that the association between *C. neoformans* cells and MBMEC requires a HA-CD44 interaction and, to a lesser extent, a HA-RHAMM interaction.

*C. neoformans* Invasion Is Impaired after Simvastatin Treatment in the Mouse Model—Evidence suggests that the HA receptors (CD44 and RHAMM) exert their functions on the membrane lipid rafts; for example, it has been demonstrated that CD44 is diminished in filipin-treated HBMEC and the *C. neoformans* association declines in filipin-treated HBMEC in vitro (12). Filipin is too toxic for *in vivo* study. As with filipin, it has been reported that simvastatin reduces the association of membrane receptors to lipid rafts (22). Therefore, we addressed the question of whether simvastatin can perturb the CD44 and RHAMM receptor functions on the membrane raft resulting in a reduction of *C. neoformans* brain invasion in *in vivo*. Mice were treated intraperitoneally with different amounts of simvastatin (from 0 to 20 μg/gm weight). After 12 h, the mice were boosted with the same amount of simvastatin and immediately inoculated with 10^8^ B-4500FO2 cells by i.v. injections. Brains were harvested after a 24-h infection. Quantitative cultures were performed by plating the brain homogenates on YPD medium. The culture data from each sample were averaged and analyzed with a paired Student’s *t* test. Data expressed as cfu were the means of triplicates of the inoculated plates ± S.D. from three experiments (*, p < 0.05; **, p < 0.01). B, an immunofluorescence microscope was used to detect CD44 (green) on the surface of cultured HBMEC in the presence of 0, 5, and 10 μg of simvastatin/sample in the chamber slide. Bar: 100 μm. C, the MetaMorph program associated with the fluorescence microscope was used to scan images with 5–10 random fields from B, and then GraphPad Prism 5 was used to quantify the readings (**, p < 0.01).

**DISCUSSION**

*C. neoformans* has a predilection for the brain and causes devastating cryptococcal meningoencephalitis. However, the mechanism of its brain invasion, resulting in central nervous system dysfunction, is still largely unknown. From our previous *in vitro* studies (12), we found that *C. neoformans* uses the host CD44 as the receptor and HA as the ligand in their association. To verify this finding *in vivo*, we used CD44−/− mice to explore the role of CD44 during *C. neoformans* invasion. Our results show that CD44−/− mice are more resistant to *C. neoformans* infection (Fig. 1). This is most likely because *C. neoformans* cells are less effective in crossing the blood-brain barrier in CD44−/− mice. In support of this hypothesis, the fungal loads of the brain as well as the cfu counts of CSF are lower in CD44−/− mice than in wild-type mice (Fig. 2). Thus, the CD44−/− mouse model is useful for *in vivo* *C. neoformans* brain infection studies. As a control, we used a pathogenic *Escherichia coli* E44 strain to perform the experiments; we observed no difference in invasion (data not shown). Thus, CD44 specifically is required for *C. neoformans* brain invasion. In complement, several CD44 functions have been documented in CD44−/− murine models from various studies. For example, studies with CD44−/− mice demonstrate that CD44 is a macrophage binding site for *Mycobacterium tuberculosis* (23). It has also been demonstrated that CD44 plays a critical role in the progression of atherosclerosis through the recruitment of

![fig7_1.png](http://www.jbc.org/content/327/19/15304/suppl/DC1/Figure7/Figure7_1.jpg)
macrophages to the atherosclerotic lesion (24). Moreover, CD44$^{-/-}$ mice injected with ConA exhibited a more severely acute supplicative hepatitis, suggesting that activated T cells use CD44 to undergo apoptosis and that dysregulation in this pathway could lead to increased pathogenesis in hepatitis (25). Roles for CD44 in the resolution of lung injury (26) and E. coli urinary tract infection (27) have also been demonstrated. Our studies with CD44$^{-/-}$ mice also clearly demonstrate that CD44 plays a role during C. neoformans invasion (Figs. 1–3). We further used anti-GFAP antibodies to examine the distribution of glial cells in infected brain sections. Interestingly, the staining of GFAP-positive glial cells (or reactive astrocytes) with prominent cytoplasmic processes showed a unique pattern, i.e. surrounding a mass of C. neoformans cells (Fig. 4). On the other hand, inflammation is not evident when C. neoformans invades the brain, which substantiates how C. neoformans, in general, could cause chronic meningoencephalitis.

However, the use of a CD44$^{-/-}$ murine model is not without limitations. One puzzling occurrence is that there still remain some C. neoformans cells in the brains of CD44$^{-/-}$ mice (Fig. 2). Although CD44 is known to play a wide variety of roles, mice deficient in CD44 do not exhibit defects in development or neurological functions (28). One possibility is that a second HA receptor may at least partially compensate for CD44 functions in the CD44$^{-/-}$ mice. Indeed, we have observed that another HA receptor, RHAMM, is present in the CD44$^{-/-}$ brain. The expression level of RHAMM is approximately the same in both wild-type and CD44$^{-/-}$ mouse brains (Fig. 5), and its ability to associate with C. neoformans is weaker than that of CD44 in vitro (Fig. 6). Similar observations of RHAMM compensation have been documented in a collagen-induced arthritis CD44 knock-out mouse model (21). Based on the knowledge gained from our studies, we favor the possibility that CD44 is a primary HA receptor and that RHAMM, in the case of a CD44$^{-/-}$ background, partially compensates for the lack of CD44 (Fig. 6). The complexity of in vivo models may hinder a further pursuit. For example, a double KO of CD44 and RHAMM may induce expression of another HA receptor or may even cause a lethal phenotype. Additionally, pathogens may use multiple mechanisms to invade hosts and thus complicate the issue further. For example, it has been reported that the Trojan horse mechanism is another possible route for C. neoformans brain invasion (14). However, ample evidence shows that a transcellular mechanism may be the major one for C. neoformans brain invasion, as a free C. neoformans cell can be detected inside the brain a short time after i.v. injection (~0.5 h) (13). Although macrophages containing C. neoformans cells near the capillary in the brain parenchyma have been reported, it is unclear whether the inclusion of C. neoformans cells in macrophages occurred after C. neoformans crossed the blood-brain barrier. Macrophages or monocytes alter their morphology significantly when crossing the BMEC, and thus the Trojan horse mechanism may be suitable for viruses but not appropriate for the large size of C. neoformans (>5 μm in diameter plus its rigid cell wall). Overall, the complexity of the in vivo model prompts us to inquire, under certain pathophysiology conditions, as to what is the main mechanism of C. neoformans invasion rather than what specific mechanism(s) C. neoformans uses to invade the brain.

Despite the limitations of the in vivo CD44$^{-/-}$ model, it is evident that HA receptors may exert their function(s) on surface membrane rafts. Sufficient evidence indicates that the CD44 (and RHAMM) function on membrane lipid rafts (12) and the endocytic pathway (17) are crucial for C. neoformans brain invasion. Blocking this pathway can be an effective way of attenuating the fungal infection. Indeed, our results (Fig. 7) show that simvastatin significantly reduced the brain fungal load. One possible mechanism is that simvastatin may exert its role on the membrane lipid rafts, disturbing CD44 and RHAMM functions and consequently attenuating cryptococcal meningitis (Fig. 7, B and C). Notably, it has been shown that statins have other actions (29); for example, administration of simvastatin in injured rat brains improves the outcome through attenuating the NF-κB-mediated inflammatory response (30). Thus, the exact mechanisms by which simvastatin could offer protection against C. neoformans brain invasion requires further investigation. In any case, simvastatin is an FDA-approved drug with low toxicity. It is significant that simvastatin can drastically reduce the C. neoformans brain burden after treatment. Taken together, our studies support the view that inhibitors of lipid rafts (Ref. 12 and Fig. 7), or other regulators of endocytic function (17) administered in combination with antifungal drugs, could be an effective anti-cryptococcal intervention.

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