Innate immune evasion strategies against Cryptococcal meningitis caused by *Cryptococcus neoformans* (Review)

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Abstract. As an infectious fungus that affects the respiratory tract, *Cryptococcus neoformans* (*C. neoformans*) commonly causes asymptomatic pulmonary infection. *C. neoformans* may target the brain instead of the lungs and cross the blood-brain barrier (BBB) in the early phase of infection; however, this is dependent on successful evasion of the host innate immune system. During the initial stage of fungal infection, a complex network of innate immune factors are activated. *C. neoformans* utilizes a number of strategies to overcome the anti-fungal mechanisms of the host innate immune system and cross the BBB. In the present review, the defensive mechanisms of *C. neoformans* against the innate immune system and its ability to cross the BBB were discussed, with an emphasis on recent insights into the activities of anti-phagocytotic and anti-oxidative factors in *C. neoformans*.

Contents

1. Introduction

Cryptococcus neoformans (*C. neoformans*) is the most common fungus to cause meningoencephalitis in the central nervous system (CNS) worldwide (1). Each year, an estimated 1 million cases of cryptococcal meningitis are reported, with a >60% mortality rate within the first 3 months of infection (2,3). *C. neoformans* is typically acquired by inhaling spores or desiccated yeast from the environment (3). Following an initial asymptomatic pulmonary infection, the organism is carried in the bloodstream and subsequently disseminated to target organs, including lung, skin and bone, which typically results in lymphocytic meningitis (2-9). Results from experimental mouse models and human cases of cryptococcal meningitis have indicated that *C. neoformans* infection may also spread to the brain (4-9). While the lungs are considered a common site of infection, *C. neoformans* predominantly targets the brain; however, this is dependent on its ability to overcome the innate immune system of the host and cross the blood-brain barrier (BBB) in the initial phase of infection (10).

To defend against *C. neoformans* infection in the initial phase, the host employs several types of innate immune cells, including macrophages, dendritic cells (DCs) and neutrophils, which phagocytize invading fungi and generate reactive oxygen species (ROS), nitrogen species (RNS) and chlorine species to aid in host protection (11,12). In response to the host innate immune response, *C. neoformans* activates virulence factors, including polysaccharide capsules and melanin pigment, to resist phagocytosis and avoid clearance (13,14). Furthermore, *C. neoformans* induces the activation of antioxidant enzymes, including superoxide dismutase (SOD) and catalases, and the synthesis of antioxidants, such as glutathione (GSH), to adapt to oxidative attack (14,15). These anti-oxidative factors have been demonstrated to be important for ROS and RNS resistance, repair of damage caused by oxidative attack and survival in the host (13-15).

The present review summarized the current understanding of the anti-innate immune response strategies utilized by *C. neoformans* and the mechanism involved in cryptococcal BBB traversal.

2. Innate immune system

Innate immune responses restrict the growth and invasion of *C. neoformans* in mammalian hosts (16). Innate immune cells are the first cells to encounter fungi and are the primary effector cells in the destruction and clearance of cryptococcal

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infection (17-26) (Table I). Furthermore, the generation of oxidative products by phagocytic cells may directly destroy the invading fungi (12).

Macrophages. Macrophages are critical phagocytic cells within the host innate immune system (17). Complement and mannose receptors on the surface of macrophages mediate the phagocytosis of *C. neoformans* (17). In addition, macrophages release high levels of reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), superoxide and nitric oxide, which damage DNA and a number of chemical moieties (11). Furthermore, macrophages promote Th1-like responses to induce fungal clearance (18,19). However, *C. neoformans* is able to proliferate within the macrophage phagosome, and may pass between macrophages (18-20).

Neutrophils. Similar to macrophages, neutrophils capture and degrade pathogens and serve a specific role in the initiation of inflammation in response to infection (21). Neutrophils enhance granuloma formation to destroy *C. neoformans* by oxidative and non-oxidative mechanisms (21). Furthermore, a previous study indicated that myeloperoxidase, which is located primarily in neutrophils, produces a strong oxidant hypochlorous acid from hydrogen peroxide and chloride ions (22). This is another predominant mechanism that neutrophils use to defend against fungal infection (22). In addition, neutrophils contain defensins 1-4, which are cytotoxic to *C. neoformans* (23).

DCs and natural killer (NK) cells. DCs phagocytize *C. neoformans* via complement or anti-capsular antibody-mediated opsonization, which leads to fungal internalization and destruction, ultimately resulting in tumor necrosis factor-α secretion and DC activation (24). Once phagocytized, cryptococci are degraded through oxidative and non-oxidative mechanisms following passage through lysosomes (24). A previous study indicated that NK cells bind and inhibit the growth of *C. neoformans in vitro* and induce fungal clearance in mice (25). In addition, previous results suggest that NK cells may directly destroy *C. neoformans* when mediated by perforin (26).

3. Innate immune system evasion

C. neoformans has a number of established virulence factors, including polysaccharide capsules and melanin, which function as non-enzymatic factors that potently influence the overall pathogenicity and phagocytic resistance of *C. neoformans* (27). A variety of extracellular proteins, including phospholipases, proteases and ureases (27), and enzymatic components of the redox system, including thioredoxins (Trxs), glutaredoxins (Grxs), peroxiredoxin (Prxs) and catalases, serve as enzymatic factors for *C. neoformans* survival during innate immune attack (27-66) (Table II).

Roles of virulence factors in defense against the innate immune response

Polysaccharide capsules. The polysaccharide capsule, which is composed of 90-95% glucuronxylomannan and 5% galactoxylomannan, is among the most important virulence factors of *C. neoformans* that aids the fungus to avoid recognition and phagocytosis by host phagocytes (28). The capsule prevents the phagocytosis of *C. neoformans* and resists phagosome digestion to preserve *C. neoformans* survival (28,29). Once engulfed by macrophages, the *C. neoformans* capsule may release polysaccharides into vesicles surrounding the phagosome that accumulate in the host cell cytoplasm, which promotes macrophage dysfunction and lysis (29). In addition, the capsular material suppresses the migration of phagocytes (30), interferes with cytokine secretion (31), directly inhibits T-cell proliferation (32), induces macrophage apoptosis (33) and delays the maturation and activation of human DCs (34). By contrast, acapsular mutants of *C. neoformans* may be effectively recognized and induce pro-inflammatory cytokine secretion in macrophages (29).

Melanin. Melanin is a negatively charged, hydrophobic pigment that is located in the cell walls of *C. neoformans,* and serves a key role in virulence and survival (34). A melanin gene disruption study has indicated that wild-type melanin-producing *C. neoformans* are more virulent (34). Melanin is composed of aggregates of small particles or granules and exogenous substrates (34,35). In the natural environment, melanin protects fungi from ultraviolet light, high temperatures, freezing and thawing (36). The potent antioxidant activity of melanin provides protection against oxidant concentrations similar to those produced by stimulated macrophages (37). The oxidative burst that follows phagocytosis is an important mechanism by which immune effector cells mediate antimicrobial action, which suggests that melanin may enhance virulence by protecting fungal cells against immune system-stimulated oxidative attack (37).

Roles of extracellular proteins in defense against the innate immune response

Phospholipases. Phospholipases are a heterogeneous group of enzymes that cleave phospholipids to produce various biologically active compounds, which alter the infection microenvironment and may favor the survival of *C. neoformans* in the host (38). The action of phospholipases may result in the destabilization of membranes, cell lysis and release of lipid second messengers (39). The secretion of PLB has been reported to promote the survival and replication of *C. neoformans* in macrophages *in vitro* (38). In addition, a previous study demonstrated that disruption of the *PLB1* gene led to reduced virulence *in vivo* and growth inhibition in a macrophage-like cell line (40).

Proteinase. Environmental and clinical isolates of *C. neoformans* possess proteinase activity that has been demonstrated to degrade host proteins, including collagen, elastin, fibrinogen, immune-globulins and complement factors (41). Replication of *C. neoformans* inside macrophages is accompanied by the production of enzymes, including proteinases and phospholipases, which damage the phagosomal membrane (42). Therefore, cryptococcal proteinases may cause tissue damage, providing nutrients to the pathogen and protection from the host.

Ureases. As a nitrogen-scavenging enzyme, urease catalyzes the hydrolysis of urea to ammonia and carbonate, and is an

Table I. Primary functions of host innate immune cells.

Cells	Primary function	(Refs.)
Macrophages	Phagocytosis, production of ROI, RNI, superoxide and nitric oxide	(17-20)
Neutrophils	Phagocytosis, production of ROI, RNI, myeloperoxidase, defensins 1-4 and lysozymes	(21-23)
Dendritic cells	Fungal internalization and destruction	(24)
Natural killer	Direct destruction	(25,26)

ROI, reactive oxygen intermediates; RNI, reactive nitrogen intermediates.

Table II. Primary function of *C. neoformans* antioxidant factors against host innate immune cells.

Antioxidant factor	Function against host innate immune cells	(Refs.)
Polysaccharide capsules	Inhibition of phagocytosis and resistance to phagosome digestion	(27-34)
Melanins	Scavenging ROS and reactive nitrogen intermediates	(27,34-37)
Phospholipases	Promotion of survival and replication in macrophages	(38-40)
Proteinase	Promotion of replication in macrophages and damage to phagosomal membranes	(41,42)
Ureases	Scavenging nitrogen	(33-35,43-45)
Peroxiredoxins	Metabolism of peroxides and/or peroxynitrite	(46-48)
Thioredoxins, glutaredoxins	Metabolism of ROS, reduction of oxidized sulfhydryl groups and maintenance of cellular redox homeostasis	(46,49-54) (46,49,58-60)
Superoxide dismutases	Conversion of superoxide to hydrogen peroxide	(15,61-63)
Catalases	Conversion of hydrogen peroxide to water and molecular oxygen	(64)
Cytochrome *c* peroxidases	Degradation of hydrogen peroxide	(65)
Alternative oxidase genes	Interaction with the classic oxidative pathway	(66)

ROS, reactive oxygen species.

important virulence factor of *C. neoformans* (43). *C. neoformans* may utilize urease to invade the CNS via the BBB and cause life-threatening meningoencephalitis (43,44). A previous study suggested that urease promoted the sequestration of cryptococcal cells in the microvasculature of the brain, while urease-negative strains seldom penetrated the CNS or caused disease (45). Although the specific role of urease protein in BBB invasion is unknown, it has been suggested that the extracellular enzymatic degradation of urea to toxic ammonia may damage endothelial cells and lead to an increase in barrier permeability (33-35).

Roles of antioxidant systems in defense against the innate immune response. Resistance to RNS and ROS through antioxidant defense systems has been correlated with virulence in *C. neoformans* clinical isolates, and has been associated with *in vitro* and *in vivo* oxidative stress resistance (46). There are several enzymatic anti-oxidant systems that have been identified in *C. neoformans*, including the Prx, Trx and Grx systems (Fig. 1).

Prx systems. The Prx system is important for cellular processes associated with disulfide bond formation, the anti-oxidative stress response and pathogenesis of *C. neoformans* infection (47). Prxs, also known as thiol peroxidases, are 20-30 kDa-sized molecules that provide antioxidant protection by removing peroxides (47). Prxs may be classified into 1-Cys and 2-Cys subgroups (48). Following peroxidation, typical 2-Cys Prxs form homodimers through an intersubunit disulfide bridge, whereas atypical 2-Cys Prxs form an intramolecular disulfide bridge (48). By contrast, 1-Cys Prxs assume a monomeric form with a single active cysteine site (48). In a previous study, two Prxs, TSA1 and TSA3, were discovered in *C. neoformans*, of which TSA1 is highly conserved (48). In addition, the findings of Missall *et al* (48) indicated that Prxs were induced under oxidative and nitrosative stress and were critical for *C. neoformans* virulence in mice. Furthermore, their study demonstrated that deletion of *TSA1*, but not *TSA3*, abolished virulence of the pathogen, which indicated that the TSA1-mediated Prx system is a core antioxidant system in *C. neoformans* (48).

Trx systems. The downstream component of Prx is the Trx system, which is comprised of NADPH, Trx and thioredoxin reductase (TrxR), and is involved in the regulation of DNA synthesis, gene transcription, cell growth and apoptosis (49-51). Trx is a small dithiol oxidoreductase that serves as a major carrier of redox potential in cells (52) and a cofactor for essential enzymes, and is involved in protein repair via methionine sulphoxide reductase (53) and the

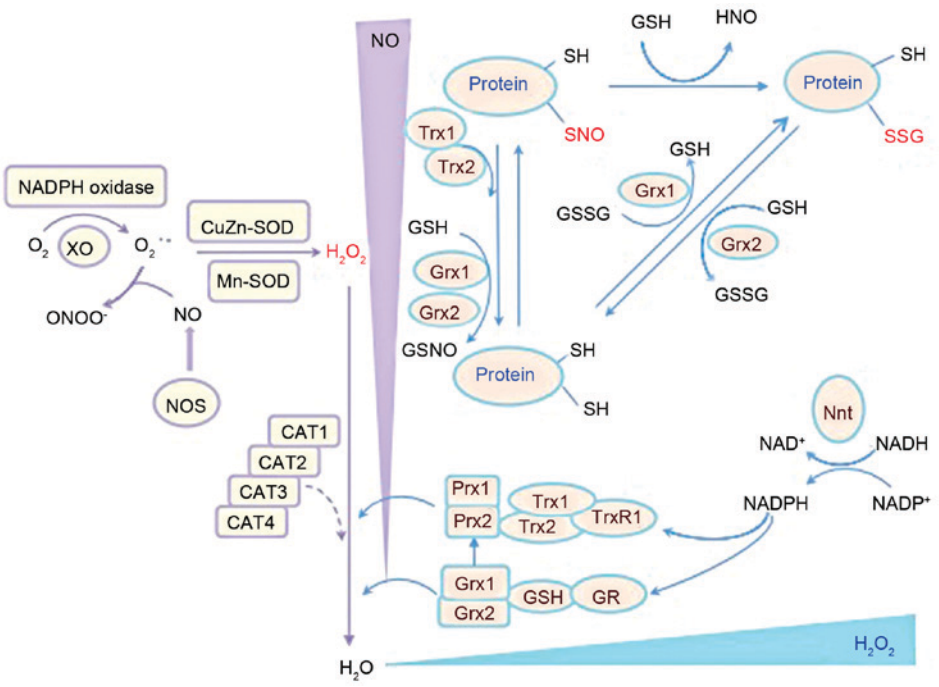

Figure 1. Schematic representation of the antioxidant chain of *C. neoformans* and the primary antioxidant enzymes involved. Prx, Trx and Grx systems are major enzymatic antioxidant systems in *C. neoformans* that regulate redox balance (49-51) that are associated with 2 SODs, Cu/Zn-SOD (SOD1) and Mn-SOD (SOD2), which may convert superoxide to hydrogen peroxide (61). *C. neoformans*, *Cryptococcus neoformans*; CAT, catalase; GR, glutathione reductase; Grx, glutaredoxin; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; NADPH, nicotinamide adenine dinucleotide phosphate; Nnt, nicotinamide nucleotide transhydrogenase; NO, nitric oxide; NOS, nitric oxide synthase; protein-SNO, protein nitrosylation; protein-SSG, protein glutathionylation; Prx1/2, peroxiredoxin 1/2; SH, thiol; SOD, superoxide dismutase; Trx, thioredoxin; TrxR, thioredoxin reductase.

reduction of protein disulphides (54). This redox control of the Trx system is considered to regulate the expression of multiple stress defensive enzymes and protect cells against oxidative stress (54).

C. neoformans contains two Trx proteins (TRX1 and TRX2) and one TrxR protein (TRXR1) (50), which are involved in the reduction and recycling of the oxidized, inactive form of Prxs (55). TRX1 promotes normal growth and a healthy oxidative state (50,53). TRX2, though dispensable for vegetative growth, is important for resistance to nitrosative stress (50,53). TRXR1 is stimulated during oxidative stress induced by hydrogen peroxide and nitrosative stress induced by nitric oxide (56). In *C. neoformans*, deletion of these genes renders them sensitive to oxidative stress and results in decreased survival in macrophage culture (50,57).

Grx systems. The GSH/Grxs system in *C. neoformans* is another major thiol-dependent antioxidant system that participates in the defense against oxidization (49). GSH is an important antioxidant for fungi. Strains that lack or possess altered GSH are sensitive to particular types of oxidative stress components, including peroxides, superoxide anions and the toxic products of lipid peroxidation (49). Exposure of yeast cells to hydrogen peroxide caused a reduction in GSH levels and a shift in the redox balance towards an oxidized state (49). In *C. neoformans*, Grxs are small heat-stable proteins encoded by two Grx genes (*GRX1* and *GRX2*), and are reduced in a manner similar to Prx (49). GSH and Grxs may regulate protein function by reversible protein S-glutathionylation under oxidative stress (58). *C. neoformans* contains two glutathione peroxidases (GPX1 and GPX2) that respond differently to various stressors (59). A strain of *C. neoformans* that lacked the *GRX1* gene was sensitive to oxidative stress induced by the superoxide anion, whereas a *GRX2* mutant was sensitive to oxidative stress generated by hydrogen peroxide (59). Furthermore, *GPX1* and *GPX2* deletion mutants have been reported as only mildly sensitive to oxidant-induced destruction by macrophages and exhibited no change in virulence in a murine model (59). GPX1 and GPX2 are involved in the defense against organic peroxides, including tert-butyl hydroperoxide (59). Although both Gpx proteins are required for survival in macrophages, deletion of *GPX1* and *GPX2* does not affect the virulence of *C. neoformans* in mice (57), suggesting that other peroxidases or antioxidant systems may compensate for the loss. In addition, the GSH Prxs and GSH S-transferases are involved in the breakdown of organic hydroperoxides with GSH as a reductant or in the conjugation of toxic lipophilic compounds to GSH, respectively (60).

Additional antioxidant systems. The primary function of SOD is to convert superoxide to hydrogen peroxide (61). There are four classes of SODs: Mn, Fe, Ni and Cu/Zn (15). *C. neoformans* has two SODs, Cu/Zn-SOD (SOD1) and Mn-SOD (SOD2) (61). Cytosolic SOD1 has been demonstrated to serve a pivotal role in the defense against ROS and contributed to virulence in a mouse model (61). Furthermore, in a previous study, a *C. neoformans SOD1* mutant strain exhibited slower growth in macrophages and greater susceptibility to neutrophil destruction (62). In addition, Narasipura *et al* (63) reported that a *C. neoformans SOD1* mutant strain exhibited

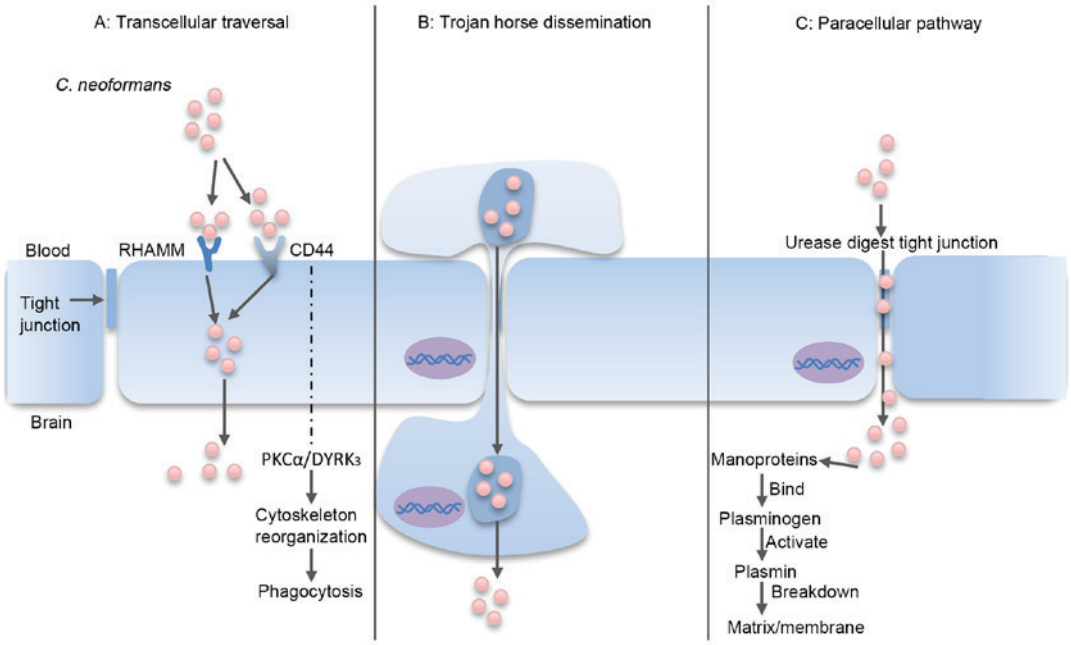

Figure 2. *C. neoformans* traversal of the BBB. (A) Transcellular traversal: *C. neoformans* binds to receptors on the endothelial cell, which triggers cellular endocytosis (72-79). (B) Trojan horse dissemination: *C. neoformans* is phagocytosed by a macrophage, which is able to cross the BBB, resulting in pathogen transportation into the brain (85-92). (C) Paracellular pathway: *C. neoformans* damages and weakens the intercellular tight junctions, which facilitates passage of the organism between the endothelial cells (80-82). RHAMM, receptor of hyaluronan-mediated motility; CD44, cluster of differentiation 44; PKCα, protein kinase Cα; DYRK3, dual-specificity tyrosine phosphorylation-regulated kinase 3; BBB, blood-brain barrier; *C. neoformans*, *Cryptococcus neoformans*.

defects in phospholipase, urease and laccase expression, which severely attenuated virulence and the anti-phagocytotic activity of the pathogen.

Catalases are antioxidant metalloenzymes that promote the conversion of hydrogen peroxide to water and molecular oxygen (64). *C. neoformans* contains four catalases (CAT1, CAT2, CAT3 and CAT4), among which *CAT2* and *CAT4* are the closest orthologs of yeast *CAT1* and *CAT3* respectively (64). However, previous results have suggested that deletion of all four *CAT* genes did not affect the sensitivity to ROS or virulence of the pathogen (64), indicating that other peroxide-detoxifying systems may have a complementary role in *C. neoformans*. In addition, *C. neoformans* contains other enzymatic factors that protect against oxidative stress. For instance, cytochrome *c* peroxidase (CCP1) may degrade hydrogen peroxide (65). Furthermore, alternative oxidase gene (AOX1) is an enzyme that forms part of the electron transport chain in the mitochondria in *C. neoformans*, and its AOX1 mutant strain was demonstrated to be more sensitive to the oxidative stressor tert-butyl hydroperoxide; however, its target in the classic oxidative pathway remains unknown (66).

4. Traversal of the BBB

After surviving phagocytosis and oxidative attack initiated by the innate immune system, *C. neoformans* may be carried in the bloodstream and disseminated to target organs, including the brain (67,68).

The BBB ensures that the brain is protected, and provides limited access to circulating macromolecules and microorganisms (67,68). In order to infect the brain, *C. neoformans* may use one of three potential traversal pathways to cross the BBB: Transcellular traversal, the paracellular pathway and Trojan horse dissemination (69-71) (Fig. 2).

Transcellular traversal. Transcellular traversal refers to the penetration of *C. neoformans* through barrier cells via the exploitation of cellular endocytosis (72,73). Transcellular traversal of the BBB has been widely studied using *in vitro* models, which have demonstrated an ability of *C. neoformans* to adhere to one or more receptors on the endothelial cell barrier (67,74).

Previous *in vitro* and *in vivo* results have demonstrated that the glycoprotein cluster of differentiation (CD)44 receptor on the surface of brain endothelial cells has a key role in transcellular traversal invasion of *C. neoformans* (75,76). CD44 is the endothelial cell receptor for hyaluronic acid, and is located in lipid rafts/caveolae on the endothelial cell surface (77,78). When hyaluronic acid in the *C. neoformans* capsule binds to CD44, a downstream signaling pathway mediated by protein kinase Cα and dual-specificity tyrosine phosphorylation-regulated kinase 3 is initiated, which triggers actin cytoskeleton reorganization and phagocytosis (5,71,79-81). Notably, Jong *et al* (79) documented that $CD44^{-/-}$ mice exhibited only a 2-fold reduction in cryptococcal meningitis compared with wild-type mice following intravenous injection of *C. neoformans*. Furthermore, knockdown of CD44 and the hyaluronan-mediated motility (RHAMM) receptor in mice conferred significantly higher protection and inhibited the invasion of *C. neoformans* in the brain when compared with knockdown of either receptor alone (79). These results suggest that CD44 and RHAMM serve as receptors for *C. neoformans* on the surface of brain endothelial cells through binding to hyaluronic acid. In addition, Maruvada *et al* (40) reported that *C. neoformans* PLB1 may

interact with lipid mediators in the endothelial cell membrane of the brain to convert GDP-Ras-related C3 botulinum toxin substrate 1 (Rac1) to GTP-Rac1, which may then associate with signal transducer and activator of transcription 3 (40). A recent study also indicated that metalloprotease 1, belonging to the M36 class of proteases, promoted the migration of *C. neoformans* across the brain endothelium and into the CNS by facilitating cryptococcal attachment to the endothelium surface, thus underscoring the critical role of M36 proteases in BBB permeation (80).

Paracellular pathway. The paracellular pathway involves the entry of *C. neoformans* through damaged or weakened tight junctions between the intercellular spaces of endothelial cells (81,82). Neuropil edema is a characteristic of endothelium damage (83,84), and sequestration of *C. neoformans* in the brain microvessels and cryptococcal binding to the endothelium has been demonstrated to induce tight junction alterations (83,84). In addition, cryptococcal mannoproteins bind and activate host plasminogens (82). Activated plasmins may subsequently bind and break down the extracellular matrix and membrane, which increases the likelihood of paracellular invasion (82).

A standard *in vivo* approach to investigate the mechanisms involved in the paracellular pathway is intravenous incubation of mice with *C. neoformans* and imaging of the blood vessels in the brain (67). Shi *et al* (71) imaged the cerebral blood vessels of mice infected with a fluorescently labeled *C. neoformans* urease mutant strain, and identified a markedly reduced capacity of the mutant to traverse to the brain (71). It is possible that urease participates in the enzymatic digestion of tight junctions between endothelial cells, and thus facilitates brain invasion via the transcellular route (43). Collectively, these findings indicate the possibility that *C. neoformans* uses a paracellular entry mechanism by weakening the brain endothelial tight junctions.

Trojan horse dissemination. The Trojan-horse dissemination of *C. neoformans* traversal involves the transport of pathogens into the brain within parasitized phagocytes (67,85). Previous results support the existence of a Trojan horse mechanism for BBB traversal (85). *C. neoformans* is a facultative intracellular pathogen that may survive and multiply inside phagocytes (86,87). Furthermore, *C. neoformans* may infect other phagocytes following their escape from phagocytic cells by vomocytosis, leaving the host macrophage unharmed (19,88). This direct cell-to-cell spread potentially explains how cryptococci may exploit phagocytes to penetrate the BBB in a Trojan horse manner (89). In previous studies, *C. neoformans* was identified inside phagocytes on the outer side of a meningeal capillary, which suggests that *C. neoformans* may have been transported within circulating phagocytes (90-92). These findings suggest that *C. neoformans* may use the Trojan-horse dissemination model to traverse into the brain.

5. Conclusions

The successful traversal of *C. neoformans* across the BBB relies on failure of the defense mechanisms imposed by the host innate immune system in the first phase of infection. Various innate immune constituents, including macrophages and neutrophils, contribute to phagocytosis, oxidative stress and clearance of *C. neoformans* (27). However, *C. neoformans* contains redundant layers of anti-phagocytic and anti-oxidative factors to resist the innate immune cells of the host, including polysaccharide capsules, melanin, phospholipases, proteases, ureases, TSA1, TSA3, TRX1, TRX2, TRXR1, GRX1, GRX2, GPX1, GPX2, SOD1, SOD2, CAT1, CAT2, CAT3, CAT4, CCP1 and AOX1.

Following its subversion of the innate immune response, *C. neoformans* may disseminate to the brain. However, entry into the highly protected environment of the brain requires *C. neoformans* to overcome the BBB. *C. neoformans* may gain entry through direct engulfment by endothelial cells (transcellular traversal), inducing damage to tight junctions (paracellular pathway) or hiding within phagocytes (Trojan-horse dissemination). Following successful evasion of the innate immune response, the fungi may proliferate (67). Results have indicated that >0.6 million cryptococcal meningitis cases each year result in mortality within 3 months of infection, even with treatment. In light of the present findings, the primary challenge in the field will be to obtain sufficient resources to identify *Cryptococcus* biomarkers for the improved determination of disease risk, treatment and prevention using therapies and vaccines, which may boost the immunity of the host to *C. neoformans*.

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References

1. Loyse A, Thangaraj H, Eastervrook P, Ford N, Roy M, Chiller T, Govender N, Harrison TS and Bicanic T: Cryptococcal meningitis: Improving access to essential antifungal medicines in resource-poor countries. Lancet Infect Dis 13: 629-637, 2013.
2. Beardsley J, Wolbers M, Kibengo FM, Ggayi AB, Kamali A, Cuc NT, Binh TQ, Chau NV, Farrar J, Merson L, *et al*: Adjunctive dexamethasone in HIV-associated cryptococcal meningitis. N Engl J Med 374: 542-554, 2016.
3. Fang W, Fa Z and Liao W: Epidemiology of *Cryptococcus* and cryptococcosis in China. Fungal Genet Biol 78: 7-15, 2015.
4. Liu TB, Kim JC, Wang Y, Toffaletti DL, Eugenin E, Perfect JR, Kim KJ and Xue C: Brain inositol is a novel stimulator for promoting *Cryptococcus* penetration of the blood-brain barrier. PLoS Pathog 9: e1003247, 2013.
5. Jong A, Wu CH, Prasadarao NV, Kwon-Chung KJ, Chang YC, Ouyang Y, Shackleford GM and Huang SH: Invasion of *Cryptococcus neoformans* into human brain microvascular endothelial cells requires protein kinase C-alpha activation. Cell Microbiol 10: 1854-1865, 2008.
6. Panackal AA, Wuest SC, Lin YC, Wu T, Zhang N, Kosa P, Komori M, Blake A, Browne SK, Rosen LB, *et al*: Paradoxical immune responses in non-HIV Cryptococcal meningitis. PLoS Pathog 11: e1004884, 2015.
7. Graciela Agar CH, Orozco Rosalba V, Macias Ivan C, Agnes F, Juan Luis GA and José Luis SH: Cryptococcal choroid plexitis an uncommon fungal disease. Case report and review. Can J Neurol Sci 36: 117-122, 2009.
8. Kumari R, Raval M and Dhun A: Cryptococcal choroid plexitis: Rare imaging findings of central nervous system cryptococcal infection in an immunocompetent individual. Br J Radiol 83: e14-e17, 2010.

9. Schwerk C, Tenenbaum T, Kim KS and Schroten H: The choroid pleus-a multi-role player during infectious diseases of the CNS. Front Cell Neurosci 9: 80, 2015.
10. Ngamskulrungroj P, Chang Y, Sionov E and Kwon-Chung KJ: The primary target organ of *Cryptococcus gattii* is different from that of *Cryptococcus neoformans* in a murine model. MBio 3: e00103-e00112, 2012.
11. Nathan C and Shiloh MU: Reactive oxygen and nitrogen inter-mediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci USA 97: 8841-8848, 2000.
12. Almeida F, Wolf JM and Casadevall A: Virulence-associated enzymes of *Cryptococcus neoformans*. Eukaryot Cell 14: 1173-1185, 2015.
13. de Jesús-Berríos M, Liu L, Nussbaum JC, Cox GM, Stamler JS and Heitman J: Enzymes that counteract nitrosative stress promote fungal virulence. Curr Biol 13: 1963-1968, 2003.
14. Kwon-Chung KJ, Fraser JA, Doering TL, Wang Z, Janbon G, Idnurm A and Bahn YS: *Cryptococcus neoformans* and *Cryptococcus gattii*, the etiologic agents of cryptococcosis. Cold Spring Harb Perspect Med 4: a019760, 2014.
15. Upadhya R, Campbll LT, Donlin MJ, Aurora R and Lodge JK: Global transcriptome profile of *Cryptococcus neoformans* during exposure to hydrogen peroxide induced oxidative stress. PLoS One 8: e55110, 2013.
16. Voelz K, Lammas DA and May RC: Cytokine signaling regulates the outcome of intracellular macrophage parasitism by *Cryptococcus neoformans*. Infect Immun 77: 3450-3457, 2009.
17. Redlich S, Ribes S, Schütze S, Eiffert H and Nau R: Toll-like receptor stimulation increases phagocytosis of *Cryptococcus neoformans* by microglial cells. J Neuroinflammation 10: 71, 2013.
18. Chrisman CJ, Albuquerque P, Guimaraes AJ, Nieves E and Casadevall A: Phospholipids trigger *Cryptococcus neoformans* capsular enlargement during interactions with amoebae and macrophages. PLoS Pathog 7: e1002047, 2011.
19. Alvarez M and Casadevall A: Cell-to-cell spread and massive vacuole formation after *Cryptococcus neoformans* infection of murine macrophages. BMC Immunol 8: 16, 2007.
20. Rohatgi S and Pirofski LA: Host immunity to *Cryptococcus neoformans*. Future Microbiol 10: 565-581, 2015.
21. Mambula SS, Simons ER, Hastey R, Selsted ME and Levitz SM: Human neutrophil-mediated nonoxidative antifungal activity against *Cryptococcus neoformans*. Infect Immun 68: 6257-6264, 2000.
22. Winterbourn CC, Vissers MC and Kettle AJ: Myeloperoxidase. Curr Opin Hematol 7: 53-58, 2000.
23. Alcouloumre MS, Ghannoum MA, Ibrahim AS, Selsted ME and Edwards JE Jr: Fungicidal properties of defensin NP-1 and activity against *Cryptococcus neoformans* in vitro. Antimicrob Agents Chemother 37: 2628-2632, 1993.
24. Wozniak KL and Levitz SM: *Cryptococcus neoformans* enters the endolysosomal pathway of dendritic cells and is killed by lysosomal components. Infect Immun 76: 4764-4771, 2008.
25. Islam A, Li SS, Oykhman P, Timm-McCann M, Huston SM, Stack D, Xiang RF, Kelly MM and Mody CH: An acidic microenvironment increases KN cell killing *Cryptococcus neoformans* and *Cryptococcus gattii* by enhancing perforin degtanulation. PLoS Pathog 9: e1003439, 2013.
26. Marr KJ, Jones GJ, Zheng C, Huston SM, Timm-McCann M, Islam A, Berenger BM, Ma LL, Wisenman JC and Mody CH: *Cryptococcus neoformans* directly stimulates perforin production and rearms NK cells for enhanced anticryptococcal microbicidal activity. Infect Immun 77: 2436-2446, 2009.
27. Rodrigues ML, Nakayasu ES, Oliveira DL, Nimrichter L, Nosanchuk JD, Almeida IC and Casadevall A: Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence. Eukaryot Cell 7: 58-67, 2008.
28. Doering TL: How sweet it is! Cell wall biogenesis and polysaccharide capsule formation in *Cryptococcus neoformans*. Annu Rev Microbiol 63: 223-247, 2009.
29. Leopold Wager CM, Hole CR, Wozniak KL, Olszewski MA and Wormley FL Jr: STAT1 signaling is essential for protection against *Cryptococcus neoformans* infection in mice. J Immunol 193: 4060-4071, 2014.
30. Haynes BC, Skowyra ML, Spencer SJ, Gish SR, Williams M, Held EP, Brent MR and Doering TL: Toward an integrated model of capsule regulation in *Cryptococcus neoformans*. PLoS Pathog 7: e1002411, 2011.
31. Villena SN, Pinheiro RO, Pinheiro CS, Nunes MP, Takiya CM, Dosreis GA, Previato JO, Mendonça-Previato L and Freire-de-Lima CG: Capsular polysaccharides galactoxylomannan and glucuronoxylomannan from *Cryptococcus neoformans* induce macrophage apoptosis mediated by Fas ligand. Cell Microbiol 10: 1274-1285, 2008.
32. Yauch LE, Lam JS and Levitz SM: Direct inhibition of T-cell responses by the *Cryptococcus* capsular polysaccharide glucuronoxylomannan. PLoS Pathog 2: e120, 2006.
33. Lupo P, Chang YC, Kelsall BL, Farber JM, Pietrella D, Vecchiarelli A, Leon F and Kwon-Chung KJ: The presence of capsule in *Cryptococcus neoformans* influences the gene expression profile in dendritic cells during interaction with the fungus. Infect Immun 76: 1581-1589, 2008.
34. Chatterjee S, Prados-Rosales R, Itin B, Casadevall A and Stark RE: Solid-state NMR reveals the carbon-based molecular architecture of *Cryptococcus neoformans* fungal eumelanins in the cell wall. J Biol Chem 290: 13779-13790, 2015.
35. Sundaram C, Shantveer GU, Umabala P and Lakshmi V: Diagnostic utility of melanin production by fungi: Study on tissue sections and culture smears with Masson-Fontana stain. Indian J Pathol Microbiol 57: 217-222, 2014.
36. Nosanchuk JD, Rosas AL, Lee SC and Casadevall A: Melanisation of *Cryptococcus neoformans* in human brain tissue. Lancet 355: 2049-2050, 2000.
37. Mauch RM, Cunha Vde O and Dias AL: The copper interference with the melanogenesis of *Cryptococcus neoformans*. Rev Inst Med Trop Sao Paulo 55: 117-120, 2013.
38. Santangelo R, Zoellner H, Sorrell T, Wilson C, Donald C, Djordjevic J, Shounan Y and Wright L: Role of extracellular phospholipases and mononuclear phagocytes in dissemination of cryptococcosis in a murine model. Infect Immun 72: 2229-2239, 2004.
39. Evans RJ, Li Z, Hughes WS, Djordjevic JT, Nielsen K and May RC: Cryptococcal phospholipase B1 is required for intracellular proliferation and control of titan cell morphology during macrophage infection. Infect Immun 83: 1296-1304, 2015.
40. Maruvada R, Zhu L, Pearce D, Zheng Y, Perfect J, Kwon-Chung KJ and Kim KS: *Cryptococcus neoformans* phospholipase B1 activates host cell Rac1 for traversal across the blood-brain barrier. Cell Microbiol 14: 1544-1553, 2012.
41. Rawson RB: The site-2 protease. Biochim Biophys Acta 1828: 2801-2807, 2013.
42. Desalermos A, Tan X, Rajamuthiah R, Arvanitis M, Wang Y, Li D, Kourkoumpetis TK, Fuchs BB and Mylonakis E: A multi-host approach for the systematic analysis of virulence factors in *Cryptococcus neoformans*. J Infect Dis 211: 298-305, 2015.
43. Morrow CA and Fraser JA: Is the nickel-dependent urease complex of Cryptococcus the pathogen's Achilles' heel? MBio 4: e00408-e00413, 2013.
44. Singh A, Panting RJ, Varma A, Saijo T, Waldron KJ, Jong A, Ngamskulrungroj P, Chang YC, Rutherford JC and Kwon-Chung KJ: Factors required for activation of urease as a virulence determinant in *Cryptococcus neoformans*. MBio 4: e00220-13, 2013.
45. Olszewski MA, Noverr MC, Chen GH, Toews GB, Cox GM, Perfect JR and Huffnagle GB: Urease expression by *Cryptococcus neoformans* promotes microvascular sequestration, thereby enhancing central nervous system invasion. Am J Pathol 164: 1761-1771, 2004.
46. Breitenbach M, Weber M, Rinnerthaler M, Karl T and Breitenbach-Koller L: Oxidative stress in fungi: Its function in signal transduction, interaction with plant hosts, and lignocellulose degradation. Biomolecules 5: 318-342, 2015.
47. Toledano MB, Delaunay-Moisan A, Outten CE and Igbaria A: Functions and cellular copartmentation of the thioredoxin and glutathione pathways in yeast. Antioxid Redox Signal 18: 1699-1711, 2013.
48. Missall TA, Pusateri ME and Lodge JK: Thiol peroxidase is critical for virulence and resistance to nitric oxide and peroxide in the fungal pathogen, *Cryptococcus neoformans*. Mol Microbiol 51: 1447-1458, 2004.
49. Benhar M, Shytaj IL, Stamler JS and Savarino A: Dual targeting of the thioredoxin and glutathione systems in cancer and HIV. J Clin Invest 126: 1630-1639, 2016.
50. Missall TA and Lodge JK: Function of thioredoxin proteins in Cryptococcus neoformans during stress or virulence and regulation by putative transcriptional modulators. Mol Microbiol 57: 847-858, 2005.

51. Benhar M: Nitric oxide and the thioredoxin system: A complex interplay in redox regulation. Biochim Biophys Acta 1850: 2476-2484, 2015.
52. Netto LE and Antunes F: The roles of peroxiredoxin and thioredoxin in hydrogen peroxide sensing and in signal transduction. Mol Cells 39: 65-71, 2016.
53. Lu J and Holmgren A: The thioredoxin antioxidant system. Free Radic Biol Med 66: 75-87, 2014.
54. Yoshioka J: Thioredoxin superfamily and its effects on cardiac physiology and pathology. Compr Physlol 5: 513-530, 2015.
55. Ianiri G and Idnurm A: Essential gene discovery in the basidiomycete *Cryptococcus neoformans* for antifungal drug target prioritization. MBio 6: e02334-14, 2015.
56. Missall TA and Lodge JK: Thioredoxin reductase is essential for viability in the fungal pathogen *Cryptococcus neoformans*. Eukaryot Cell 4: 487-489, 2005.
57. Kalinina EV, Chernov NN and Novivhkova MD: Role of glutathione transferase, and glutaredoxin in regulation of redox-dependent processes. Biochemistry (Mosc) 79: 1562-1583, 2014.
58. Lu J and Holmgren A: The thioredoxin antioxidant system. Free Radic Biol Med 66: 75-87, 2014.
59. Grant CM: Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions. Mol Microbiol 39: 533-541, 2001.
60. Hanschmann EM, Godoy JR, Berndt C, Hudemann C and Lilling CH: Thioredoxins, glutaredoxins, and peroxiredoxins-molecular mechanisms and health significance: From cofactors to antioxidants to redox signaling. Antioxid Redox Signal 19: 1539-1605, 2013.
61. Narasipura SD, Chaturvedi V and Chaturvedi S: Characterization of *Cryptococcus neoformans* variety gattii SOD2 reveals distinct roles of the two superoxide dismutases in fungal biology and virulence. Mol Microbiol 55: 1782-1800, 2005.
62. Cox GM, Harrison TS, McDade HC, Taborda CP, Heinrich G, Casadevall A and Perfect JR: Superoxide dismutase influences the virulence of *Cryptococcus neoformans* by affecting growth within macrophages. Infect Immun 71: 173-180, 2003.
63. Narasipura SD, Ault JG, Behr MJ, Chaturvedi V and Chaturvedi S: Characterization of Cu, Zn superoxide dismutase (SOD1) gene knock-out mutant of *Cryptococcus neoformans* var. gattii: Role in biology and virulence. Mol Microbiol 47: 1681-1694, 2003.
64. Giles SS, Stajich JE, Nichols C, Gerrald QD, Alspaugh JA, Dietrich F and Perfect JR: The *Cryptococcus neoformans* catalase gene family and its role in antioxidant defense. Eukaryot Cell 5: 1447-1459, 2006.
65. Giles SS, Perfect JR and Cox GM: Cytochrome *c* peroxidase contributes to the antioxidant defense of *Cryptococcus neoformans*. Fungal Genet Biol 42: 20-29, 2005.
66. Akhter S, McDade HC, Gorlach JM, Heinrich G, Cox GM and Perfect JR: Role of alternative oxidase gene in pathogenesis of *Cryptococcus neoformans*. Infect Immun 71: 5794-5802, 2003.
67. Vu K, Eigenheer RA, Phinney BS and Gelli A: *Cryptococcus neoformans* promotes its transmigration into the central nervous system by inducing molecular and cellular changes in brain endothelial cells. Infect Immun 81: 3139-3147, 2013.
68. Zou LL, Ma JL, Wang T, Yang TB and Liu CB: Cell-penetrating peptide-mediated therapeutic molecule delivery into the central nervous system. Curr Neuropharmacol 11: 197-208, 2013.
69. Tseng HK, Liu CP, Price MS, Jong AY, Chang JC, Toffaletti DL, Betancourt-Quiroz M, Frazzitta AE, Cho WL and Perfect JR: Identification of genes from the fungal pathogen *Cryptococcus neoformans* related to transmigration into the central nervous system. PLoS One 7: e45083, 2012.
70. Stie J and Fox D: Blood-brain barrier invasion by *Cryptococcus neoformans* is enhanced by functional interactions with plasmin. Microbiology 158: 240-258, 2012.
71. Shi M, Li SS, Zheng C, Jones GJ, Kim KS, Zhou H, Kubes P and Mody CH: Real-time imaging of trapping and urease-dependent transmigration of *Cryptococcus neoformans* in mouse brain. J Clin Invest 120: 1683-1693, 2010.
72. Charlier C, Chrétien F, Baudrimont M, Mordelet E, Lortholary O and Dromer F: Capsule structure changes associated with *Cryptococcus neoformans* crossing of the blood-brain barrier. Am J Pathol 166: 421-432, 2005.
73. Chang YC, Wang Z, Flax LA, Xu D, Esko JD, Nizet V and Baron MJ: Glycosaminoglycan binding facilitates entry of a bacterial pathogen into central nervous systems. PLoS Pathog 7: e1002082, 2011.
74. Vu K, Weksler B, Romero I, Couraud PO and Gelli A: Immortalized human brain endothelial cell line HCMEC/D3 as a model of the blood-brain barrier facilitates in vitro studies of central nervous system infection by *Cryptococcus neoformans*. Eukaryot Cell 8: 1803-1807, 2009.
75. Jong A, Wu CH, Gonzales-Gomez I, Kwon-Chung KJ, Chang YC, Tseng HK, Cho WL and Huang SH: Hyaluronic acid receptor CD44 deficiency is associated with decreased *Cryptococcus neoformans* brain infection. J Biol Chem 287: 15298-15306, 2012.
76. Jong A, Wu CH, Chen HM, Luo F, Kwon-Chuang KJ, Chang YC, Lamunyon CW, Plaas A and Huang SH: Identification and characterization of CPS1 as a hyaluronic acid synthase contributing to the pathogenesis of *Cryptococcus neoformans* infection. Eukaryot Cell 6: 1486-1496, 2007.
77. Huang SH, Long M, Wu CH, Kwon-Chung KJ, Chang YC, Chi F, Lee S and Jong A: Invasion of *Cryptococcus neoformans* into human brain microvascular endothelial cells is mediated through the lipid rafts-endocytic pathway via the dual specificity tyrosine phosphorylation-regulated kinase 3 (DYRK3). J Biol Chem 286: 34761-34769, 2011.
78. Long M, Huang SH, Shackleford GM and Jong A: Lipid raft/caveolae signaling is required for *Cryptococcus neoformans* invasion into human brain microvascular endothelial cells. J Biomed Sci 19: 19, 2012.
79. Jong A, Wu CH, Shackleford GM, Kwon-Chung KJ, Chang YC, Chen HM, Quyang Y and Huang SH: Involvement of human CD44 during *Cryptococcus neoformans* infection of brain microvascular endothelial cells. Cell Microbiol 10: 1313-1326, 2008.
80. Vu K, Tham R, Uhrig JP, Thompson GR III, Na Pombejra S, Jamklang M, Bautos JM and Gelli A: Invasion of the central nervous system by *Cryptococcus neoformans* requires a secreted fungal metalloprotease. MBio 5: e01101-e1114, 2014.
81. Kim KS: Mechanisms of microbial traversal of the blood-brain barrier. Nat Rev Microbiol 6: 625-634, 2008.
82. Stie J, Bruni G and Fox D: Surface-associated plasminogen binding of *Cryptococcus neoformans* promotes extracellular matrix invasion. PLoS One 4: e5780, 2009.
83. Charlier C, Chrétien F, Lortholary O and Dromer F: Early capsule structure changes associated with Cryptococcus neoformans crossing of the blood-brain barrier. Med Sci (Paris) 21: 685-687, 2005 (In French).
84. Chen SH, Stins MF, Huang SH, Chen YH, Kwon-Chung KJ, Chang Y, Kim KS, Suzuki K and Jong AY: *Cryptococcus neoformans* induces alterations in the cytoskeleton of human brain microvascular endothelial cells. J Med Microbiol 52: 961-970, 2003.
85. Charlier C, Nielsen K, Daou S, Brigitte M, Chretien F and Dromer F: Evidence of a role for monocytes in dissemination and brain invasion by *Cryptococcus neoformans*. Infect Immun 77: 120-127, 2009.
86. Alvarez M and Casadevall A: Phagosome extrusion and host-cell survival after *Cryptococcus neoformans* phagocytosis by macrophages. Curr Biol 16: 2161-2165, 2006.
87. Coelho C, Souza AC, Derengowski Lda S, de Leon-Rodriguez C, Wang B, Leon-Rivera R, Bocca AL, Gonçalves T and Casadevall A: Macrophage mitochondrial and stress response to ingestion of *Cryptococcus neoformans*. J Immunol 194: 2345-2357, 2015.
88. Sorrell TC, Juillard PG, Djordjevic JT, Kaufman-Francis K, Dietman A, Milonig A, Combes V and Grau GE: Cryptococcal transmigration across a model brain blood-barrier: Evidence of the Trojan horse mechanism and differences between *Cryptococcus neoformans* var. grubii strain H99 and *Cryptococcus gattii* strain R265. Microbes Infect 18: 57-67, 2016.
89. Ma H, Croudace JE, Lammas DA and May RC: Direct cell-to-cell spread of a pathogenic yeast. BMC Immunol 8: 15, 2007.
90. Davis MJ, Eastman AJ, Qiu Y, Greorka B, Kozel TR, Osterholzer JJ, Curtis JL, Swanson JA and Olszewski MA: *Cryptococcus neoformans*-induced macrophage lysosome damage crucially contributes to fungal virulence. J Immunol 194: 2219-2231, 2015.
91. Liu TB, Perlin DS and Xue C: Molecular mechanisms of cryptococcal meningitis. Virulence 3: 173-181, 2012.
92. Alanio A, Vernel-Pauillac F, Sturny-Leclère A and Dromer F: *Cryptococcus neoformans* host adaptation: Toward biological evidence of dormancy. MBio 6: e02580-14, 2015.