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.

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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
infection (17-26) (Table 1). 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 catalasases, 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 PLBI 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
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 sulfoxide reductase (53) and the

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**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 sulphydryl groups and maintenance of cellular redox homeostasis | (46,49-54) |
| 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.
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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 oxidation (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...
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 (72-79). 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 *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).

**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*.
interact with lipid mediators in the endothelial cell membrane of the brain to convert GDP-Ras-related C3 botulinum toxin substrate 1 (Racl) 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 mannanproteins 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, CCPI 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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