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REVIEW

Mechanisms of fungal dissemination

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Abstract

Fungal infections are an increasing threat to global public health. There are more than six million fungal species worldwide, but less than 1% are known to infect humans. Most of these fungal infections are superficial, affecting the hair, skin and nails, but some species are capable of causing life-threatening diseases. The most common of these include *Cryptococcus neoformans, Aspergillus fumigatus* and *Candida albicans.* These fungi are typically innocuous and even constitute a part of the human microbiome, but if these pathogens disseminate throughout the body, they can cause fatal infections which account for more than one million deaths worldwide each year. Thus, systemic dissemination of fungi is a critical step in the development of these deadly infections. In this review, we discuss our current understanding of how fungi disseminate from the initial infection sites to the bloodstream, how immune cells eliminate fungi from circulation and how fungi leave the blood and enter distant organs, highlighting some recent advances and offering some perspectives on future directions.

Keywords Fungus · *Cryptococcus neoformans* · *Aspergillus fumigatus* · *Candida albicans* · Trojan horse · Transcytosis · Paracellular crossing · Meningoencephalitis · Invasive aspergillosis · Invasive candidiasis · Fungal pathogenesis

Introduction

The prevalence of fungal infections has been steadily increasing over the last three decades due to the increased use of immunosuppressants, as well as the increased number of patients with HIV/AIDS [1–3]. It is estimated that there are more than 6 million fungal species worldwide, but only a small fraction (less than 600) are capable of causing diseases in humans [3, 4]. While the majority of these fungal infections are superficial, some fungal species are capable of causing life-threatening infections [3]. The most common of these include *Cryptococcus neoformans, Aspergillus fumigatus* and *Candida albicans* [5].

C. neoformans and *A. fumigatus* are found throughout the environment and are inhaled into the lungs, where they initially cause pulmonary infections [1, 6]. *C. albicans* on the other hand is a human commensal and commonly colonizes mucosal tissues but can also be acquired in healthcare settings [1, 3, 7]. When these opportunistic pathogens disseminate from sites of initial infection, they can cause serious diseases including meningoencephalitis, invasive aspergillosis and invasive candidiasis, respectively. Together, these and other fungal infections affect over one billion people each year and kill more than 1.5 million annually [8]. In the USA alone, fungal diseases are estimated to cost upwards of $7.2 billion each year, a trend that is expected to continue to rise as advances in medical care for immunocompromised patients continue to be made [9]. As such, fungal infections impose a considerable economic burden on healthcare systems worldwide and represent an increasing threat to global public health [9]. Furthermore, current antifungal treatment options remain limited and are threatened by the continued emergence of resistant fungal strains [10, 11]. For these reasons, it is critical that we understand the pathogenesis of fungal diseases, specifically the ways by which mycopathogens disseminate, in order to develop alternative antifungal therapies. In this review, we discuss how *C. neoformans, A. fumigatus* and *C. albicans* disseminate from initial sites of infection to distant organs.

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Dissemination of *C. neoformans*

C. neoformans is an encapsulated yeast fungus found globally in soil and avian excrement [6]. Infectious propagules in the form of desiccated yeast or spores are inhaled into the respiratory tract on a daily basis [12]. Due to their small size, these organisms are able to avoid cough and mucociliary clearance and penetrate deep into alveolar spaces [1]. Here, *C. neoformans* encounters lung resident immune cells. In healthy, or immunocompetent, individuals, these fungi are either successfully cleared or establish long-term, latent infections [1]. Alternatively, in the case of immunocompromised individuals, including AIDS patients, organ transplant recipients and patients treated with immunosuppressive therapies, *C. neoformans* can establish symptomatic pulmonary infections resulting in pneumonia, acute respiratory distress syndrome, and subsequent extrapulmonary dissemination [13]. Disseminated *C. neoformans* enters the bloodstream and can infect distant organs, but preferentially targets the central nervous system (CNS) [12]. If these fungal cells cross the blood–brain barrier (BBB) and enter the brain parenchyma, they can cause meningoencephalitis [14]. The global incidence of cryptococcal meningoencephalitis is estimated to be more than 220,000 cases annually and results in approximately 180,000 deaths each year [15].

Extrapulmonary dissemination

There are a number of proposed routes by which *C. neoformans* is thought to escape the lungs. These include an intracellular route within phagocytes known as the "Trojan horse" mechanism, transcellular crossing through epithelial cells, paracellular crossing between epithelial cells and an extracellular route in which free fungi escape through damaged epithelial barriers [12] (Fig. 1).

Trojan horse

Following pulmonary infection, one of the first immune cells *C. neoformans* encounters are lung resident alveolar macrophages. These professional phagocytes rapidly ingest cryptococcal cells for degradation [16, 17]. However, *C. neoformans* is a facultative intracellular pathogen and is equipped to survive and replicate within these cells [16]. This enables viable yeast to be transported out of the lungs within migrating phagocytes, a process known as the Trojan horse mechanism of dissemination [12].

Early studies provided indirect evidence supporting this mechanism, by demonstrating that the inability to survive within macrophages correlates with decreased extrapulmonary dissemination [18–20], while adoptive transfer studies confirmed that macrophages could indeed facilitate hematogenous dissemination to the brain [18, 21]. In addition, depletion of macrophages using clodronate liposomes significantly diminished dissemination to the CNS, suggesting that these cells may contribute to the extrapulmonary spread of *C. neoformans* [21–23]. It was later determined that $CD11c^+$ lung resident cells, primarily alveolar macrophages and to a lesser extent dendritic cells, are responsible for internalizing and transporting cryptococci to the lymph system following intranasal instillation [24]. This event occurs early during infection, as fungal burdens were detected in mediastinal lymph nodes in as little as 24 h post-infection [24], and indicates that transport of *C. neoformans* may be an unintended consequence of the antigen-presenting function of these phagocytes. Depletion of these $CD11c^+$ populations in transgenic mice resulted in a complete loss of dissemination [24], further supporting the role of these cells in Trojan horse transport.

In order to utilize phagocytes as vehicles for dissemination, *C. neoformans* must successfully survive within and exit from these cells following internalization. This requires that *C. neoformans* defends against acidic, oxidative and nitrosative stresses commonly encountered within macrophages [12]. Unlike *A. fumigatus* and *C. albicans*, which inhibit phagolysosomal maturation and must escape to the cytoplasm in order to establish an intracellular niche, *C. neoformans* can survive and replicate within phagosomes [25, 26]. The ability to grow in those acidic pHs found within mature phagolysosomes is dependent on *C. neoformans* expression of inositol phosphosphingolipid–phospholipase C 1 (Isc1) [20, 27]. Protection against oxidative and nitrosative stresses on the other hand is conferred by the cryptococcal capsule (a complex polysaccharide structure composed primarily of glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal)) [28–34], the iron oxidase laccase [35, 36] and the antioxidant melanin [19, 35–38]. In addition, phospholipase B1 (Plb1) is thought to alter macrophage activation through the production of immune regulatory eicosanoids in order to further promote intracellular survival and replication [39–41].

Once phagocytosed, *C. neoformans* can exit macrophages following intracellular replication. Cryptococcal replication within phagocytes is accompanied by the formation of a leaky phagosome and the accumulation of polysaccharide-containing vesicles in the cytoplasm and eventually culminates in the lysis of host cells [42]. This event triggers local inflammation and leaves extracellular *C. neoformans* exposed to host immune responses [43]. Conversely, *C. neoformans* can exit macrophages via non-lytic exocytosis, or vomocytosis. Vomocytosis is mediated by phagosomal extrusion: a process in which mature phagosomes containing cryptococcal cells fuse with the plasma membrane and release fungi into the extracellular milieu [43–46]. The

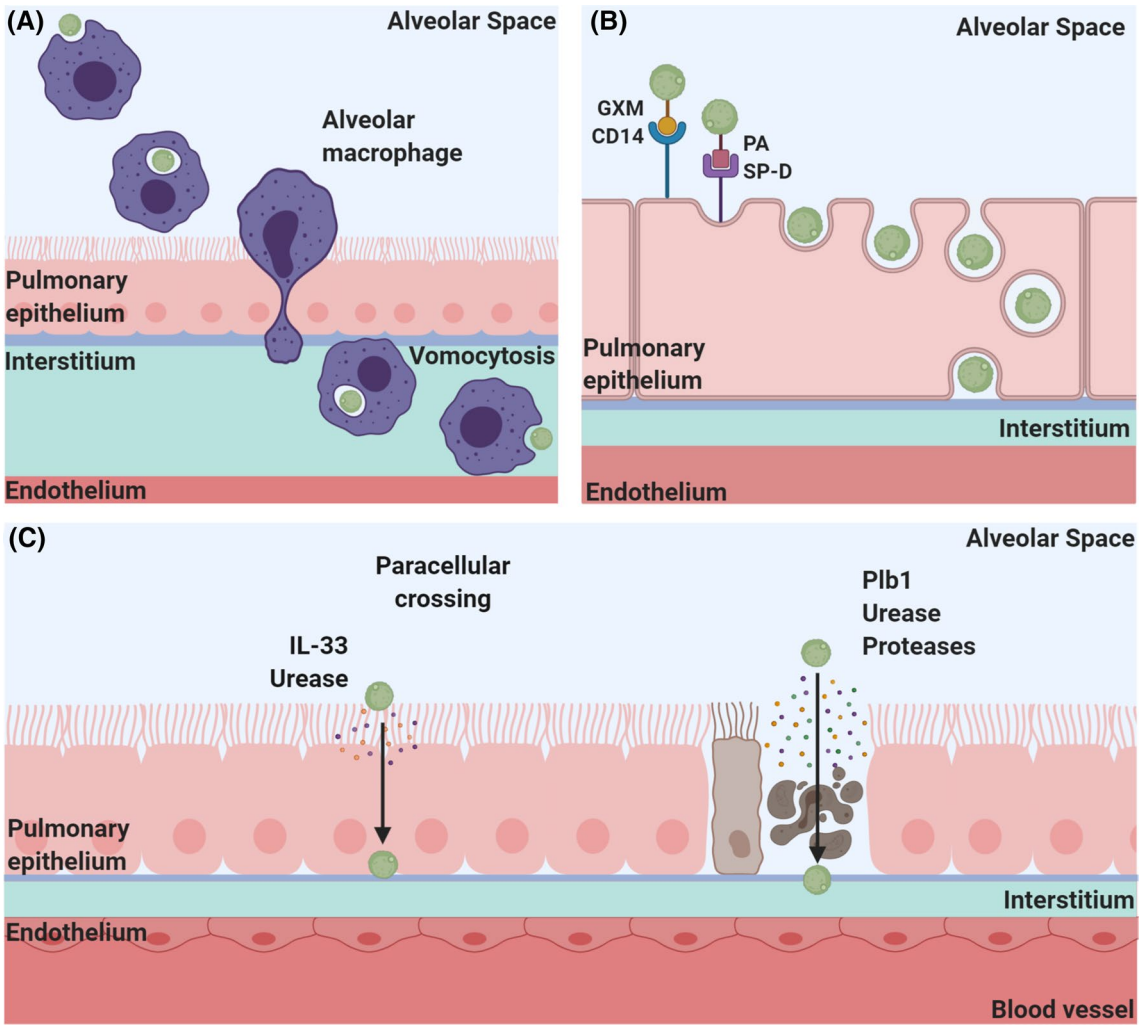

Fig. 1 Mechanisms mediating extrapulmonary dissemination of *C. neoformans* **a** Trojan horse: following phagocytosis, *C. neoformans* (green) is able to survive within alveolar macrophages (purple) and is transported across the epithelium inside these migrating phagocytes where they can access the interstitium, lymph system and/or bloodstream. *C. neoformans* can exit macrophages through vomocytosis and disseminate throughout the host as free fungi or be taken up by peripheral monocytes in the bloodstream and transported to the vasculature of various organs including the brain. **b** Transcytosis: *C. neoformans* adheres to epithelial cells through interactions between glucuronoxylomannan (GXM) and CD14 as well as palmitic acid (PA) and surfactant protein-D (SP-D) receptors. Following adherence, epithelial cells endocytose yeast, transporting them across the epithelium. **c** Paracellular crossing and loss of barrier integrity: *C. neoformans* induces the production of IL-33 (peach), which along with urease (purple) disrupts tight junctions and allows fungal cells to pass between epithelial cells. *C. neoformans* also secretes Plb1 (green), urease (purple) and proteases (yellow) which damage epithelial barriers, allowing fungi to cross the epithelium and access the bloodstream

cellular and molecular mechanisms governing vomocytosis remain poorly understood, but are reported to involve both host and pathogen factors [45, 47, 48]. After the expulsion event, both phagocytes and fungi remain viable, allowing *C. neoformans* to stealthily exit macrophages without triggering cell death-associated inflammation [43]. *C. neoformans* promotes vomocytosis through its expression of urease [49]. Urease catalyzes the hydrolysis of urea to yield the weak base ammonia [50, 51], which acts as a buffer and raises the pH within phagosomes [49]. For other pathogens, this activity can inhibit acid-induced damage; however, in the case of *C. neoformans,* this increase in pH results in yeast entering into a quiescent state, in which intracellular replication is delayed [25, 49]. This prolongs intracellular residence of *C. neoformans* and increases the likelihood that fungal cells will be transported outside of the lung prior to the non-lytic expulsion of dormant yeast.

Transcytosis

In addition to intracellular means of escape, *C. neoformans* can exit the lungs as free fungi via a number of extracellular

routes. One such pathway is transcellular crossing, also known as transcytosis. This mechanism involves exploitation of host cell endocytic pathways to facilitate fungal traversal of epithelial barriers and grants *C. neoformans* access to the lung interstitium, vasculature or lymph system [12].

Transcytosis is preceded by fungal cell adherence to the epithelium. To date, a limited number of adhesins have been identified as mediating cryptococcal–epithelial interactions. Initial findings demonstrated that both encapsulated and acapsular strains of *C. neoformans* adhere to A549 lung epithelial cells in vitro [52, 53]. In the case of encapsulated *C. neoformans*, the major capsule component GXM is responsible for mediating adherence by binding to CD14 on epithelial cells [54, 55]. As for acapsular strains, the mannoprotein MP84 was determined to bind A549 cells through interactions with a yet identified host cell receptor [56]. In addition, a temperature-sensitive adhesin was reported to facilitate adherence independently of GXM and was determined to be cryptococcal Plb1 [52, 54, 57]. Plb1 possesses multiple enzymatic activities that allow it to break down phospholipids found in host cell membranes and lung surfactant [40, 58, 59]. Degradation of dipalmitoylphosphatidylcholine (DPPC), the main component of lung surfactant, results in the release of palmitic acid (PA) which is thought to mediate adherence by binding to surfactant protein D (SP-D) receptors on A549 cells [41, 57]. This is supported by the dose-dependent increase in adherence that occurs when PA is added to co-cultures of A549 cells and a Plb1-deficient strain of *C. neoformans* [53, 57].

Adherence to epithelial cells results in the internalization of *C. neoformans* as well as eventual epithelial cell lysis [52, 54]. While little is known about the mechanisms involved in this process, it is likely actin-dependent, similar to those facilitating *C. neoformans* invasion of brain endothelial cells [60, 61].

Paracellular crossing and loss of barrier integrity

In addition to traveling through epithelial cells, *C. neoformans* can pass between epithelial cells as the result of mechanical or biochemical disruption of tight junctions, a process known as paracellular crossing or paracytosis [12]. During pulmonary infection, *C. neoformans* induces the production of interleukin (IL)-33 by alveolar type 2 epithelial cells [62]. IL-33 promotes type 2 innate immune responses and suppresses the expression of the tight junction protein E-cadherin which may allow fungal cells to pass through weakened tight junctions. Similarly, urease activity is thought to disrupt the integrity of the pulmonary epithelium. While the effects of cryptococcal urease on epithelial tissues have yet to be fully examined, its activity has been extensively studied in the context of the bacterial pathogen *Helicobacter pylori* and was reported to increase phosphorylation of myosin regulatory light chains (MLCs) in epithelial cells, which can in turn disrupt tight junctions and result in a loss of barrier function [63]. In this way, *C. neoformans* may disrupt tight junctions allowing for free fungi to pass between epithelial cells.

Extracellular *C. neoformans* can also pass freely through weakened or damaged epithelial barriers [12]. This model is supported by the observation that prolonged incubation of *C. neoformans* with A549 cells results in significant amounts of host cell death [54] and the fact that *C. neoformans* produces a number of enzymes capable of disrupting host tissues [64]. Of these, urease, phospholipases and proteases have been implicated in contributing to fungal dissemination [65].

In addition to disrupting epithelial tight junctions, urease activity was found to be toxic to host cells [50, 51]. Ammonia derived from *H. pylori* urease was reported to cause local tissue damage and induce host cell death [50, 51]. Urease has also been implicated in causing immune-mediated injury to host tissues, as it can act as a chemotactic factor and cause local inflammation as well as result in the production of inducible nitric oxide synthase (iNOS) which may also have cytotoxic effects [50, 51]. It is possible that cryptococcal urease may function similarly during pulmonary infection and could damage the epithelium, thereby promoting fungal dispersal and subsequent dissemination [65, 66].

Plb1 activity is also thought to contribute to the disruption of epithelial barriers. Once *C. neoformans* is in proximity to the epithelium, secreted or cell wall-associated Plb1 can access and degrade epithelial cell membranes [47, 67]. Disruption of these membranes can result in the lysis of host cells and lead to a weakened barrier through which extracellular yeast can pass [18, 65].

Finally, *C. neoformans* produces one or more unnamed serine proteases [68, 69]. These enzymes are capable of degrading extracellular matrix (ECM) and basement membrane elements [68]. Together, these proteolytic activities can assist *C. neoformans* in tissue invasion and dissemination by degrading structural components of the epithelium [68]. Supporting their role in the breakdown of host barrier tissues is the report that cryptococcal serine proteases mediate disruption of the BBB [69]. It is possible that within the lungs, these proteases could act similarly to disrupt epithelial barriers.

Intravascular clearance

Once outside of the lungs, cryptococcal cells can enter into the bloodstream resulting in blood infections known as fungemia [18, 19]. Early studies have demonstrated that brain infections occur following fungemia and that there is a direct correlation between the severity of the infection and the magnitude of fungemia [70–72]. As such, intravascular recognition and clearance of *C. neoformans* represent an

important step in preventing dissemination to the central nervous system (Fig. 2).

Neutrophils

Neutrophils are the most abundant phagocytes in the bloodstream and are typically the first immune cells to be recruited to sites of infection [73]. To date, their role in defense against *C. neoformans* remains controversial. In vitro, neutrophils were found to effectively kill cryptococci [74–81], although it was also reported that *C. neoformans* can negatively regulate the extracellular killing activity of these cells [82, 83]. In vivo, augmentation of neutrophil activity enhanced anticryptococcal activity [84–86], while impaired neutrophil activity significantly compromised survival during cryptococcal infection [87, 88]. These suggest that neutrophils may be protective. By contrast, depletion of neutrophils markedly reduced pulmonary fungal burdens following infection with *C. neoformans* [89], and in AIDS patients with cryptococcosis, enhanced blood neutrophil counts are associated with mortality [90], indicating instead that neutrophils may play a detrimental role during cryptococcal infection. Nevertheless, neutrophils have been shown to contribute to the intravascular clearance of disseminating *C. neoformans* through the efficient phagocytosis and removal of fungal cells from the microvasculature of organs as demonstrated by intravital imaging in both a mouse and zebrafish model [91, 92].

As *C. neoformans* circulates throughout the host, they can become mechanically trapped in the microvasculature of organs with closed capillary networks, including the brain [93]. Here, cryptococci activate the complement system [93–97] which induces neutrophil migration to arrested fungal cells [98–100]. Neutrophils then bind and ingest opsonized *C. neoformans* [91, 98] and, in turn, can secrete leukotriene B4 (LTB4), which attracts additional neutrophils to sites of fungal infection [101, 102]. Following phagocytosis, neutrophils can kill yeast through oxidative [74, 77–80] and nonoxidative mechanisms [81]. In this way, neutrophils help to limit dissemination by directly killing and/or removing fungal cells from the endothelium [91, 92, 98, 100].

This ability for neutrophils to clear intravascular cryptococci is less efficient in the brain as compared to other organs such as the lungs [100]. This is likely due to the fact that brain vasculature structure is different and lacks complement, which may explain the lower rates of neutrophil recruitment to the brain and the increased susceptibility of the CNS to infections by *C. neoformans* [99, 103]. Indeed, enhancing the recruitment of neutrophils to the brain vasculature significantly improves intravascular clearance of *C. neoformans* in the brain [100], further supporting the role of neutrophils in the clearance of disseminating *C. neoformans* in the blood.

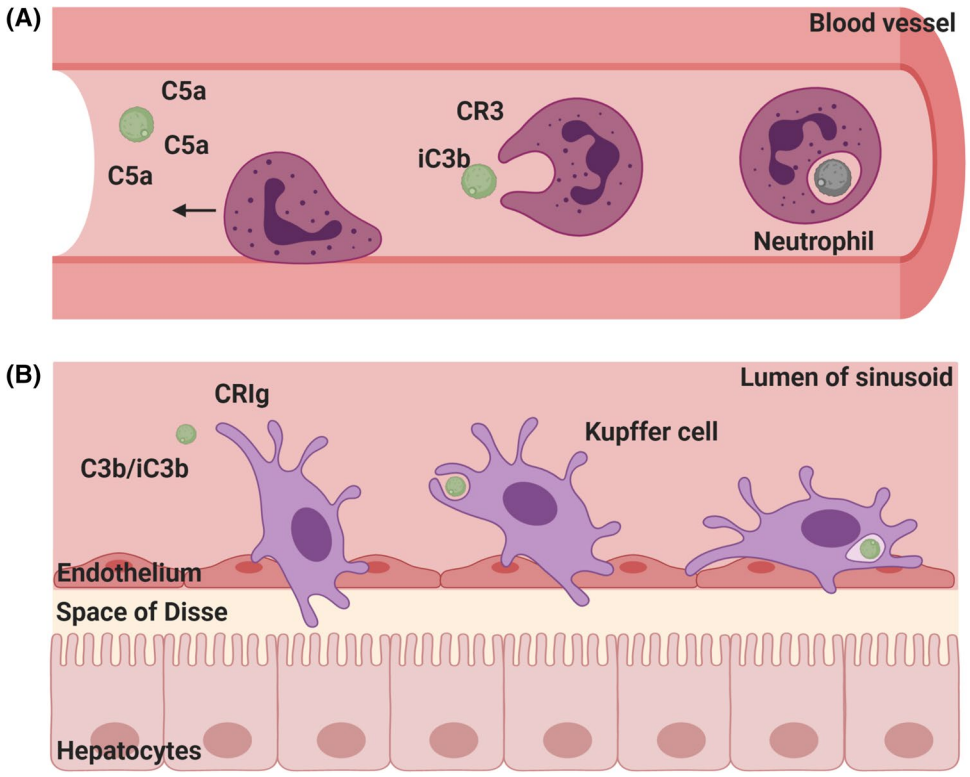

Fig. 2 Clearance of intravascular *C. neoformans* **a** Neutrophils (dark purple) are recruited to intravascular *C. neoformans* (green) in a C5a-dependent manner. Fungal cells are phagocytosed by neutrophils via iC3b–CR3 interactions and are killed through oxidative and nonoxidative mechanisms. **b** Liver-resident Kupffer cells (light purple) recognize and bind C3b/iC3b on circulating *C. neoformans* through CRIg receptors. Following phagocytosis, Kupffer cells can inhibit cryptococcal growth and limit fungal dissemination to target organs

Kupffer cells

In addition to neutrophils, liver-resident macrophages, known as Kupffer cells (KCs), have been shown to play a role in clearing intravascular *C. neoformans*. The liver is the largest internal organ, receiving 30% of the total volume of blood in the body each minute, and contains approximately 90% of the total tissue macrophages in the body [104, 105]. Here, KCs scan passing blood and remove potentially harmful substances [104–109] and have been identified as playing an important role in maintaining blood sterility through the capture and removal of intravascular bacteria and parasites [107–110]. Although the liver is not the target organ of *C. neoformans*, evidence suggests that liver disease is a risk factor for cryptococcosis [111–114]. In this context, recent results revealed that the liver plays a prominent role in filtering disseminating *C. neoformans* and *C. albicans* out of circulation through KCs [115, 116].

Intravital imaging showed that disseminating *C. neoformans* is captured in the liver sinusoids [116]. This capture is mediated by KCs through the recognition of complement C3 on fungal cells by complement receptor CRIg (complement receptor of the immunoglobulin superfamily). As such, depletion of KCs significantly reduces the fungal burden in the liver, leading to enhanced fungemia in the blood and increased deposition of fungi in other organs [116]. Following capture, KCs phagocytose *C. neoformans* and can inhibit fungal growth in a manner independent of IFN-γR signaling [116]. This demonstrates an important role for KCs in reducing fungal dissemination to other organs by directly removing organisms from circulation [116] and likely explains the association between liver disease and increased susceptibility to fungal infections [112–114].

Brain invasion

If *C. neoformans* avoids intravascular clearance, circulating fungal cells are preferentially deposited in the brain vasculature and eventually invade the brain [117]. Once inside the brain, *C. neoformans* begins to proliferate and causes fatal meningoencephalitis. Thus, although cryptococcal infection starts in the lungs, the most devastating event occurs when the fungus crosses the BBB and migrates to the brain parenchyma [117, 118]. The mechanism by which *C. neoformans* invades the brain remains to be completely understood. To date, there are a number of competing hypotheses that have been proposed for brain invasion by *C. neoformans* and include the Trojan horse mechanism, transcytosis, paracellular crossing and free entry through damaged endothelial barriers [118, 119] (Fig. 3).

Trojan horse

It has been well reported that phagocytes can transmigrate from the blood to the brain parenchyma across the BBB [73], and as described, *C. neoformans* can survive within phagocytes [16]. This raised the possibility that, following phagocytosis of fungal cells, phagocytes can escort *C. neoformans* to the brain parenchyma through the so-called Trojan horse mechanism.

To directly visualize the dynamics of brain invasion by *C. neoformans* using Trojan horse crossing, live cell imaging was performed in vitro using human endothelial cell lines [120, 121]. The results confirmed that both *C. neoformans* and *C. gattii* can use the Trojan horse mechanism to cross endothelial cell layers [120]. Phagocytes derived from human monocytic cell lines containing *C. neoformans* were also seen to cross endothelial cell layers in real time [121]. Interestingly, transcellular transmigration (through endothelial cells) of monocytes harboring *C. neoformans* is the major pathway for Trojan horse crossing [121]. In contrast, paracellular transmigration (between endothelial cells) likely represents a small percentage of Trojan horse crossing as transendothelial electrical resistance (TEER) values remained stable during in vitro assays, indicating little to no disruption of endothelial tight junctions [121]. In vivo, monocytes containing *C. neoformans* were observed in the perivascular space of cortical post-capillary venules in the brains of infected mice [71, 122]. This is thought to be the major site of Trojan horse entry [122]. Once here, *C. neoformans* can escape the phagocyte and access the brain parenchyma and cerebrospinal fluid (CSF) [123], further confirming a role for these cells in dissemination to the CNS.

In addition to carrying yeast across the BBB, it has been proposed that phagocytes transport *C. neoformans* to the brain vasculature where they instead pass them directly to endothelial cells [124]. This cell-to-cell spread, known as lateral transfer or dragotcytosis, is an actin-dependent process that requires donor and acceptor cells to be in physical contact [124–126]. Originally, lateral transfer was observed between two macrophages [124, 125], but was later reported to occur between monocytes and endothelial cells in vitro [121]. Alternatively, phagocytes may carry fungal cells to the brain, where they then escape and cross the BBB alone through an extracellular mechanism [121].

Blood-derived monocytes have been implicated in the transport of *C. neoformans* across the BBB. In humans, these monocytes exist in two major populations: $CD14^{hi}CD16^{-}$ and $CD14^{low}CD16^{hi}$ monocytes [127]. The corresponding populations in mice are $CCR2^{+}Ly6C^{hi}$ and $CX3CR1^{+}Ly6C^{low}$ monocytes, respectively [128]. During intravenous infection with *C. neoformans*, $CX3CR1^{+}Ly6C^{low}$ monocytes are recruited to the brain vasculature starting at 12 h post-infection [129]. Through

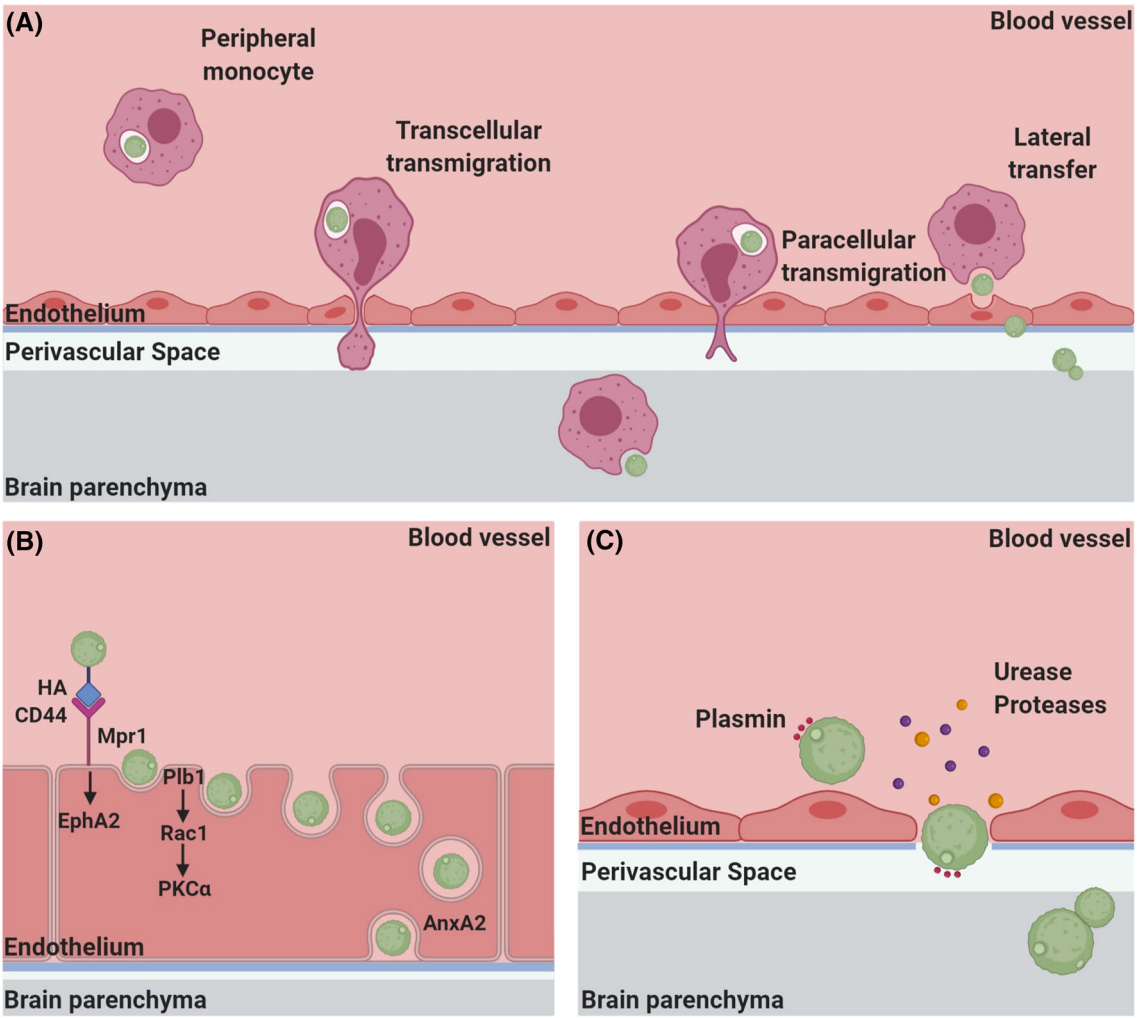

Fig. 3 Mechanisms mediating brain invasion by *C. neoformans* **a** Trojan horse: peripheral monocytes (dark pink) ingest intravascular *C. neoformans* (green) and transport them to the brain vasculature. Monocytes can carry yeast across the BBB through transendothelial pores (transcellular transmigration), or can paracellularly transmigrate between endothelial cells. In addition, monocytes can transfer *C. neoformans* directly to endothelial cells (red) (lateral transfer), facilitating transmigration of cryptococci across the BBB. **b** Transcytosis: hyaluronic acid (HA)–CD44 interactions along with Mpr1 promote *C. neoformans* adherence to brain endothelial cells. Engagement of CD44 activates EphA2, while cryptococcal Plb1 activates host cell Rac1 which in turn activates PKCα. Activation of these pathways induces endocytosis of *C. neoformans*. Following internalization, *C. neoformans* engages host AnxA2 via Mpr1 which facilitates exit of endothelial cells and successful crossing of the BBB. **c** Paracellular crossing and loss of barrier integrity: *C. neoformans* disrupts the BBB through the utilization of host plasmin (pink) and secretion of urease (purple) and other proteases (yellow) and migrates across the endothelium into the brain parenchyma

the use of intravital microscopy, these cells were observed to engulf *C. neoformans* in brain vasculature and carry the yeast as they crawled on and adhered to the luminal wall of brain vasculature and migrated to the brain parenchyma [129], suggesting the involvement of these monocytes in cryptococcal crossing of the BBB. $CCR2^{+}Ly6C^{hi}$ monocytes on the other hand were observed to accumulate in the brain starting 14 days after intravenous infection, indicating that these cells are not involved in the early transport of *C. neoformans* to the CNS [130]. Instead, $CCR2^{+}Ly6C^{hi}$ monocytes promote brain inflammation, leading to lethal immune pathology in the brain in mice as well as in humans [131, 132].

Transcytosis

In addition to Trojan horse crossing, extracellular *C. neoformans* cells can directly transmigrate across the BBB and invade the brain via transcytosis. Indirect evidence supporting this comes from a mouse model of *C. neoformans* infection, in which yeasts were detected in the cytoplasm of endothelial cells of small capillaries, suggesting that

endothelial cells internalize *C. neoformans* in vivo [71]. Direct evidence on the other hand is derived from in vitro studies using human brain microvascular endothelial cells (HBMECs) [60, 133, 134]. In this model, free *C. neoformans* cells were observed to have crossed endothelial monolayers via a transcellular pathway that did not affect monolayer integrity [133].

Similar to the lungs, transcytosis in the brain involves exploitation of host cell endocytic pathways in order to traverse the BBB [119] and is dependent on cryptococcal adherence to and internalization by brain endothelial cells [133, 135]. Adherence of *C. neoformans* to endothelial cells occurs independently of capsule expression and is mediated by hyaluronic acid (HA) [136–139]. HA is the product of an HA synthase encoded by the gene *CPS1* and binds primarily to CD44 receptors on brain endothelial cells, although the RHAMM receptor has also been reported to play a minor role in binding HA [140]. Interestingly, in response to the high levels of inositol found in mammalian brains, *C. neoformans* upregulates its expression of *CPS1*, leading to an increased production of HA and an enhanced association of cryptococcal cells and HBMECs [141].

Upon binding, *C. neoformans* is internalized by endothelial cells in an actin-dependent manner [60, 61, 142, 143]. Cryptococcal Plb1 activates host cell Rac1 (Ras-related C3 botulinum toxin substrate 1) which in turn activates the actin reorganizing protein, protein kinase C-α (PKCα) [138, 144, 145]. The mechanism by which this occurs is not well understood, though it has been suggested that Plb1 activity might generate lipid mediators required to facilitate Rac1 activation [145, 146]. In addition, engagement of CD44 by *C. neoformans* activates ephrin type-A receptor 2 (EphA2). This receptor is involved in various signaling pathways that regulate cytoskeleton remodeling [147] and has been reported to be utilized by a number of pathogens in order to invade host cells suggesting that *C. neoformans* could also use this pathway to enter endothelial cells [147].

Lipid rafts also play a role in the internalization of *C. neoformans* by endothelial cells. Supporting this is the report that the HA receptor CD44 co-localizes with the lipid raft marker ganglioside GM1 on the plasma membrane of endothelial cells and that *C. neoformans* adheres to host cells in areas where GM1 is enriched [143]. CD44 was also found to co-localize with and activate the caveolae membrane marker caveolin-1 (Cav1) upon engagement with *C. neoformans* [148]. These demonstrate that transcytosis of *C. neoformans* across the BBB occurs through a lipid raft/caveolae-dependent endocytotic process [143, 148].

C. neoformans can further promote internalization by endothelial cells, through secretion of *C. neoformans*-derived extracellular microvesicles (CnMVs). These vesicular compartments, referred to as "virulence bags," contain polysaccharides, lipids and cytoplasmic proteins and can fuse with host cells independently of CD44 [149, 150]. This interaction increases lipid raft activity and promotes CD44 migration to membrane rafts [149]. In this way, CnMVs may enhance fungal adherence and endocytosis.

Transcytosis was also found to occur independently of CD44. *C. neoformans* further promotes its adherence to endothelial cells through expression of the metalloprotease, Mpr1 [151, 152]. Once bound, Mpr1 engages host annexin A2 (AnxA2), an important signaling protein involved in endocytosis that was found to be essential for the transmigration of cryptococcal cells across the endothelium [152, 153]. While the absence of AnxA2 in HBMECs had no effect on adherence or internalization of cryptococci, yeast cells were unable to exit endothelial cells and enter into the parenchyma following endocytosis, indicating that AnxA2-Mpr1-mediated interactions promote successful crossing of the BBB [152].

Paracellular crossing and loss of barrier integrity

Additional mechanisms of brain invasion by extracellular *C. neoformans* are paracellular crossing between brain endothelial cells as well as migration across damaged endothelial barriers [119]. These pathways involve the entry of free yeast through a damaged or weakened BBB and are supported by reports that accumulation of *C. neoformans* in the brain results in severe damage to endothelial cells as well as tight junction alterations [60, 121, 136]. In this context, *C. neoformans* produces several degradative enzymes capable of disrupting the BBB.

Cryptococcal urease has been identified as a major virulence factor during brain infection [154]. Initial studies reported that urease promotes cryptococcal sequestration within brain microcapillaries [155]. Later reports suggest a more active role for urease in cryptococcal penetration of the BBB [93]. While the mechanism remains unknown, it is possible that urease-derived ammonia may have toxic effects on endothelial cells which weakens the integrity of the BBB and promotes opening of tight junctions leading to transmigration of *C. neoformans* [93, 154–156]. This is supported by the fact that urease-positive cryptococcal strains cause a reduction in levels of the tight junction protein ZO1 in HBMECs, while urease-deficient strains have no effect on monolayer integrity [156].

C. neoformans also secretes a number of proteases including serine proteases, which were found to degrade key components of ECM and the basement membrane [64, 68, 69, 157, 158]. This activity was reported to increase BBB permeability during infection with *C. neoformans* both in vitro and in vivo [68, 69]. In addition to its own proteases, *C. neoformans* is capable of utilizing host proteases. Cryptococcal cells can bind and activate host plasminogen, a plasma protein and central component of the fibrinolytic system.

This interaction promotes the conversion of plasminogen to the serine protease plasmin via the urokinase-type plasminogen activator (uPA) [159–161]. Plasmin digests components of the BBB, as well as activates matrix metalloproteinases, which are capable of damaging tight junction components [159].

In addition to those factors expressed by *C. neoformans,* it is possible that host behaviors can also lead to the disruption of endothelial barriers and promote cryptococcal neuroinvasion. For example, abuse of methamphetamines was reported to enhance dissemination of *C. neoformans* to the CNS [162] and alter BBB integrity through the modified expression of tight junction and adhesion molecules [163]. This pharmacological disruption to the endothelium promotes transmigration of yeast into the brain parenchyma, demonstrating that external factors can also aid in dissemination of *C. neoformans* to the brain [163]. Regardless of entry mechanism, once inside the brain, *C. neoformans* can rapidly proliferate and cause life-threatening cases of meningoencephalitis.

Dissemination of *A. fumigatus*

A. fumigatus is a ubiquitously distributed saprophytic fungus that produces small spores known as conidia. On average, individuals inhale upwards of 200 conidia per day [164, 165]. By virtue of their size, conidia are able to avoid cough and mucociliary clearance and enter deeper into the lungs. In healthy individuals, these conidia are rapidly cleared, but in the case of immunocompromised patients, conidia can germinate and develop filamentous hyphae [1, 166]. This fungal growth can result in invasive aspergillosis: a severe and aggressive fungal disease characterized by tissue damage, necrosis and hypoxia [8, 164, 167]. Although the target organ of *A. fumigatus* is the lungs, *Aspergillus* is known to be angiotropic and has an affinity for host vasculature. As growing hyphae penetrate pulmonary tissues, they can invade the endothelial lining of nearby blood vessels and break off into circulation, causing sepsis and dissemination accompanied by thrombosis, hemorrhagic infarction and invasion of distant organs [8, 164, 167]. It is estimated that there are approximately 250,000 cases of invasive aspergillosis worldwide each year, with an associated mortality rate ranging from 30 to 80% [8].

The primary route by which *A. fumigatus* is thought to traverse the pulmonary epithelium is transcytosis. As with *C. neoformans, A. fumigatus* adheres to and invades epithelial cells [164, 168–170]. *A. fumigatus* adherence to the epithelium is mediated by several fungal factors, including sialic acid (SA), galactosaminogalactan (GAG) and β-1,3-glucan binding to Dectin-1 [171–173]. Following adherence, *A. fumigatus* is endocytosed in an actin-dependent manner [174–178]. The fungal invasin calcineurin A (calA) promotes this internalization through interactions with integrin α5β1 on host cells [179]. In addition, gliotoxin, a major mycotoxin of *A. fumigatus*, as well as the cell wall component β-1,3-glucan, activates actin cytoskeleton rearranging proteins in host epithelial cells, including cofilin-1 and phospholipase D (PLD) [175, 177, 180, 181], further promoting internalization. Inside these cells, a portion of engulfed conidia are killed, while approximately 3% remain viable [174, 176]. Of those, one-third of surviving conidia germinate and form extracellular hyphae without lysing host cells [170, 174, 176]. These organisms can continue to grow and invade pulmonary tissues and nearby blood vessels.

A. fumigatus also encodes an array of degradative enzymes which may facilitate migration across the epithelium [182–184]. Serine and cysteine proteases, including the serine protease AF-ALF, disrupt host epithelial tissues [182–184]. The enzyme elastase is required for the development of invasive disease suggesting a role in dissemination [164, 185, 186]. In addition, a number of secondary metabolites, including gliotoxin, fumagillin, helvolic acid, and verruculogen, have been implicated in modifying the epithelium [164, 187, 188]. This combination of proteolytic activity may contribute to the disruption of host tissues and enable *A. fumigatus* to penetrate the epithelium and enter the bloodstream directly.

As for the Trojan horse mechanism, there is evidence suggesting that *A. fumigatus* may be able to utilize host phagocytes in order to escape the lung. Firstly, *A. fumigatus* can survive within phagocytes [189, 190]. Secondly, there are reports that monocyte-derived $CD11b^+$ lung dendritic cells internalize and transport *A. fumigatus* to mediastinal lymph nodes [128]. This event is reminiscent of the transport of *C. neoformans* to the lymph system by alveolar macrophages. Once inside of the lymph node, *Aspergillus* could escape from dendritic cells and disseminate via the bloodstream. As *A. fumigatus* circulates throughout the host, it can become stopped in host microvasculature and can invade various organs including the liver, kidneys, spleen and brain [167]. To date, the mechanism by which *A. fumigatus* penetrates the BBB is not well understood, though studies have shown that gliotoxin (GTX) is able to damage the endothelium which may facilitate transmigration [191].

Dissemination of *C. albicans*

C. albicans is a polymorphic fungus that can transition from a yeast phase to a hyphal phase and is commonly found as a commensal in the human body, asymptomatically colonizing the gastrointestinal, respiratory and reproductive tracts, as well as skin of approximately 30–80% of people [1, 3, 7]. While a considerable proportion of *C. albicans* infections arise from indwelling medical devices [192], studies

have identified the intestinal population of *C. albicans* as the main source of endogenous infection, especially following immunosuppression [193]. These organisms can cause lethal bloodstream infections (candidemia) and lead to invasive disease when they manage to cross the intestinal epithelium [193]. Approximately 700,000 cases of invasive candidiasis are diagnosed worldwide each year, with mortality rates of up to 40% [8, 194].

As with other fungi, invasion of epithelial barriers by *C. albicans* is preceded first by adherence to the intestinal epithelium. *C. albicans* expresses multiple surface moieties that mediate this adherence. These adhesins exhibit differential expression in yeast and hyphal forms and promote binding by different mechanisms. In the case of yeast, adherence is due to passive forces such as hydrophobic and/or electrostatic interactions as well as agglutinin-like sequence (Als) 5 [2, 195]. In addition, yeast β-glucan is also recognized by the nonclassical pattern recognition receptor EphA2 [195]. These epithelial–yeast interactions stimulate germination, exposing several hyphal-associated adhesins that further promote adherence. These include a number of proteins from the Als family, particularly Als3, as well as hyphal wall protein 1 (Hwp1) [2, 195–198].

Following adherence, *C. albicans* invades the epithelium through induced endocytosis or active penetration. Induced endocytosis involves interactions between fungal invasins and host cell proteins. *C. albicans* invasins include Als3 and the heat shock protein Ssa1 [195, 199, 200]. These invasins bind epithelial cell E-cadherin and initiate endocytosis through an actin-dependent mechanism that requires clathrin [199, 201–205]. Once internalized, *C. albicans* prevents endolysosomal maturation and continues to grow. Intracellular hyphal extension is dependent on the expression of *EED1* (epithelial escape and dissemination 1) [206], and continued growth of hyphae results in the piercing of epithelial cells and subsequent dissemination.

Active penetration on the other hand is a separate mechanism that occurs at later time points than endocytosis. It requires viable fungi and results from hyphal extension and invasion in between or through epithelial cells. To date, the process of active penetration remains poorly understood, and it is unclear which fungal components are involved, although secreted aspartic proteinases (Saps) (especially Sap3), lipases and phospholipases are thought to play a role through degradation of the epithelial tight junction protein E-cadherin [2, 198, 199, 207].

In addition to Saps, *C. albicans* secretes a number of other proteases and phospholipases which may contribute to fungal-induced epithelial damage and enable passage across the intestinal epithelium [198]. Similar to cryptococcal Plb1, candidal Plb1 promotes penetration of the epithelium by breaking down host cell membranes [208]. The cytolytic peptide toxin candidalysin was also found to be essential for *C. albicans* to damage host enterocytes [193]. In addition, a recent report indicates that *C. albicans* is also able to utilize phagocytes and the Trojan horse mechanism to disseminate throughout the host [209].

Once the intestinal epithelium has been breached, *C. albicans* can invade local tissues and nearby blood vessels and disseminate throughout the host. The primary target organs of disseminated *C. albicans* are the kidneys and brain [210–213]. Approximately 50% of patients with disseminated candidiasis have CNS fungal invasion, which can cause meningoencephalitis and has an associated mortality rate of up to 90% [213]. Kidneys on the other hand are the most heavily colonized organ following *Candida* infection [210, 214]. These fungal burdens correlate with mortality, suggesting that the kidney is the critical target organ during candidiasis [215]. Inside the kidneys, candidal infection can result in tissue damage and eventual organ failure [216].

Invasion into these distant organs requires fungal cells first adhere to endothelial cells prior to migrating into tissues [217]. *C. albicans* binding to the endothelium is not well understood, but similar to binding of the epithelium, endothelial binding involves adhesins and invasins that are differentially expressed during yeast and hyphal phases. Some such molecules include integrin-like proteins, members of the Als family and fungal cell wall components [217]. For example, invasins Als3 and Ssa1 bind to the heat shock protein gp96 and an unknown endothelial receptor respectively, and induce endocytosis into brain endothelial cells [211, 218]. Additionally, Als3 has also been reported to promote fungal cell internalization into endothelial cells by binding N-cadherin [201, 217, 219, 220].

Alternative methods of transmigration across the endothelium include Trojan horse transport and paracellular crossing between adjacent endothelial cells [217]. Lastly, fungal cell migration across damaged endothelial barriers also occurs and is facilitated by the host protease plasmin. Similar to *C. neoformans, C. albicans* binds and activates host plasminogen to plasmin via a number of cell wall proteins, including enolase [221, 222]. Surface-bound plasmin can degrade components of the endothelium and enhance the ability of *C. albicans* to invade tissues [222].

Conclusion

The most medically important fungal diseases are cryptococcosis, aspergillosis and candidiasis. Fungal infections are usually limited to initial sites of infection in immunocompetent individuals. However, if fungi disseminate from initial infection sites to the bloodstream, they can invade virtually any organ and cause fatal infections which account for more than one million deaths worldwide each year. Thus, fungal dissemination is a critical step in the development of invasive

fungal diseases. Although the mechanism(s) involved in fungal dissemination remains incompletely understood, increasing evidence suggests that fungi can hijack phagocytes from initial sites of infection to enter the bloodstream, a process called Trojan horse crossing. Alternatively, free fungal cells can also directly enter into circulation through transcytosis, paracellular crossing or across damaged barrier tissues. During fungal dissemination, neutrophils and Kupffer cells have been shown to play a role in filtering circulating fungi out of the vasculature. Interestingly, fungi in the blood can also use similar strategies to those they use to escape from the site of initial infection to invade distant organs. The mechanism(s) involved in fungal dissemination reflects complicated interactions between the host and the pathogen, and an understanding of this mechanism(s) is fundamental for the development of therapeutic strategies (Table 1).

Future directions

In vitro studies utilizing HBMECs monolayers as a BBB model have been extensively used to study the migration of *C. neoformans* and other pathogens across the BBB. These studies have made great contributions to our understanding of those mechanisms governing the transcytosis [133, 138, 141, 145, 151, 223] and Trojan horse pathways [120, 121]. However, these BBB models cannot recapitulate some important in vivo features in the brain microvasculature, such as shear stress, cell–ECM interactions and cylindrical geometry characteristics. In addition, the BBB is composed of not only endothelial cells, but also pericytes and astrocytes. Therefore, what occurs in vivo in terms of fungal dissemination could be different to what we have observed in those in vitro models [224]. To overcome this weakness and to achieve physiological barrier function, three-dimensional self-organized microvascular models of the BBB containing endothelial cells, pericytes and astrocytes have been recently developed [225–228]. It is expected that these three-dimensional models of the BBB would provide us a better platform to study fungal dissemination in a way more closely related to what happens in vivo.

In vivo models of mouse infection with *C. neoformans* have also been used to study fungal dissemination. In line with in vitro findings, histological studies support both the Trojan horse mechanism [71, 122] and transcytosis crossing [71, 133]. However, fungal dissemination is a dynamic process, and histological studies are not an optimal approach to address dynamic events. In this regard, intravital microscopy has been recently used to study fungal dissemination through the imaging of dynamic interactions between fungal cells and endothelial cells as well as phagocytes [17, 91–93, 116, 129, 229]. However, the limitation of intravital microscopy is that only a small area can be visualized and that a limited number of fungal cells can be analyzed [230]. As such, it is unknown whether the observations reflect most of the fungal cells. To complement histology and intravital microscopy, colony-forming unit (CFU) determination has been used to study fungal dissemination in vivo [139, 141, 145, 151]. However, CFU analysis fails to distinguish between fungal cells residing within the vasculature bed and those in the brain parenchyma after crossing the BBB. More recently, flow cytometry has been used to determine the invasion of brain endothelial cells by the protozoan parasite *Toxoplasma gondii* [231]. This technique is likely a powerful tool that can be used for future studies on fungal dissemination by quantifying the frequency of endothelial cells containing fungal cells.

Lastly, fungal cells can disseminate using numerous different mechanisms, namely Trojan horse crossing, transcytosis, paracellular crossing and passage through damaged tissues [232]. Although there is evidence to support all of these mechanisms, an important aspect of fungal dissemination

Table 1 Summary of dissemination mechanisms of three fungal pathogens

Fungal species	*C. neoformans*	*A. fumigatus*	*C. albicans*
Initial site of infection	Lungs	Lungs	Mucosal tissues, indwelling devices
Hematogenous dissemination	Yes	Yes	Yes
Target organ	Brain/CNS	Lungs	Kidneys, brain
Trojan horse escape	Yes	Yes	Yes
Transcytosis escape	Yes	Yes	Yes
Paracellular crossing/loss of barrier integrity escape	Yes	Yes	Yes
Intravascular clearance by neutrophils	Yes	?	?
Liver capture	Yes	?	Yes
Trojan horse entry	Yes	?	Yes
Transcytosis entry	Yes	Yes	Yes
Paracellular crossing/loss of barrier integrity entry	Yes	Yes	Yes

left unanswered is the relative frequency of each mechanism during infection. Determining the major pathway of fungal dissemination is fundamental for developing therapeutic strategies for invasive fungal diseases. It is a challenge to determine which pathway is the predominant route for fungal dissemination. To address this issue, an in vivo animal model of fungal infection is likely required, and when one mechanism is analyzed the other two mechanisms must be properly blocked simultaneously. With the emergence of new technologies and scientific approaches, we believe that we will have a better understanding of fungal dissemination in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The experiments comply with the current laws of the country in which they were performed.

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