5 Macrophages in the Immune Response Against *Cryptococcus*

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I. Introduction

Cryptococcus neoformans is a pathogenic fungus that causes disease in humans and other mammals. The first recorded case of human *C. neoformans* infection was found in a subcutaneous skin lesion (Mitchell and Perfect 1995), but infection more commonly occurs in the lungs following inhalation. Although infection begins in the lungs, life-threatening symptoms only develop if the fungus disseminates to the central nervous system (CNS); fatal dissemination occurs almost exclusively in individuals with pre-existing immune deficiencies. In the decades following the discovery of *C. neoformans*, cases of fatal cryptococcosis were rare because immune deficiency syndromes were uncommon. Medical advances in the twenty-first century such as organ transplantation and cancer treatment (which require or result in immune deficiency) increased cryptococcosis rates slightly but it was not until the spread of HIV in the 1980s that *C. neoformans* truly emerged as a major global pathogen.

Cryptococcosis currently afflicts about one million people around the world annually, mainly within HIV-infected populations. In sub-Saharan Africa, where the HIV epidemic hit hardest, complications caused by cryptococcosis account for up to 44% of HIV-related deaths (Park et al. 2009). Ominously, the rise of cryptococcosis within immune-suppressed populations may foreshadow a wider emergence of disease within healthy populations in the future. Of particular note is an ongoing outbreak of infections in immunocompetent residents of the Pacific Northwest, caused by a particularly virulent lineage of *Cryptococcus gattii* – a newly designated *Cryptococcus* species closely related to *C. neoformans* (Bartlett et al. 2008; MacDougall et al. 2007; Byrnes et al. 2009, 2010).

Cryptococcus is a genus of basidiomycete, containing at least 40 recognised species. Almost all cases of human infection are caused by two species: *C. neoformans* and *C. gattii*. These two species have been further classified according to variations in the capsule polysaccharide (Fig. 5.1) between isolates (Ikeda et al. 1982). Variants are split into five serotypes: serotype A, otherwise known as *C. neoformans var. grubii*; serotype D, otherwise known as *C. neoformans var. neoformans*; serotypes B and C, which collectively make up the newly classified *C. gattii* species; and serotype AD, which is a hybrid of

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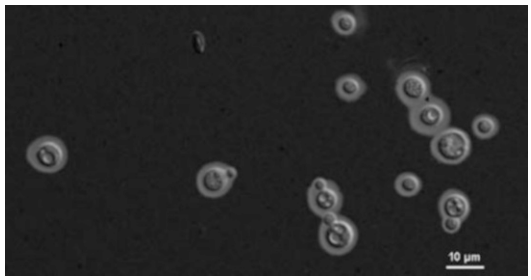

Fig. 5.1. India ink stain of *C. neoformans var. grubii* strain H99, revealing the characteristic polysaccharide capsule surrounding the fungal cells as a white halo. Note also that a number of the cells in the image are in the process of budding to produce new daughter cells

serotypes A and D (Lengeler et al. 2001). This chapter will mainly refer to *C. neoformans* serotypes A and D unless otherwise stated.

C. neoformans is primarily an environmental organism, which is widespread in soil, bird guano and rotting wood (Kronstad et al. 2011). *C. neoformans* can reproduce asexually, via budding, or sexually via a teleomorph form. The sexual (teleomorph) state is called *Filobasidiella neoformans* and forms in response to certain environmental and nutritional conditions. Sexual reproduction occurs between two distinct mating types, a and α, producing basidiospores, which may be the main infective vectors in cryptococcosis (Giles et al. 2009; Hull and Heitman 2002).

II. The Pathogenesis of Cryptococcosis

Cryptococcal infection in the lungs begins following inhalation of infectious cells or spores (Giles et al. 2009). There are no documented cases of human-to-human spread of cryptococcosis and thus infections are thought to result almost exclusively from environmental exposure. Following inhalation, *C. neoformans* initially colonises the extracellular alveolar space, where it comes into contact with the host innate immune system. As part of the innate immune response, alveolar macrophages migrate to the site of infection and attempt to clear the infection by engulfing fungal cells via phagocytosis (see Fig. 5.2). Following phagocytosis, however, ***C. neoformans* is able to survive and grow within the intracellular niche provided by the macrophage.** Initial respiratory infection occurs in both healthy and immunocompromised individuals, but **subsequent disseminated infection is far more likely if an immune deficiency is present.**

Disease outcome following *C. neoformans* infection depends on the strength of the host immune response. Broadly there are **three possible outcomes:**

1. Fungal cells in the alveolar space are phagocytosed by alveolar macrophages and infiltrating neutrophils; following phagocytosis the fungi are killed, resulting in **total clearance of infection.**
2. Fungal cells are phagocytosed, but not destroyed, by alveolar macrophages. The fungi reproduce in alveolar macrophages, eventually **disseminating to the CNS where they cause fatal meningoencephalitis.**
3. Fungal cells in the alveolar space are phagocytosed by alveolar macrophages; the immune system is unable to fully clear infection but manages **to contain the fungi within the lungs in a latent state within granulomatous structures.**

For *C. neoformans*, fatal disseminative disease (ii) occurs almost exclusively in individuals with pre-existing immune deficiencies whereas total clearance (i) and latent disease (iii) can manifest in individuals who are immune competent.

Epidemiological studies can never truly determine the extent of *C. neoformans* infection within immune competent populations because the majority of infections are asymptomatic and go unreported. To address this issue, studies have used anti-cryptococcal immunoglobulin titres within immune competent populations as a sign of past infection. These studies conclude that cryptococcal infection is common in the normal adult population (Deshaw and Pirofski 1995; Houpt et al. 1994) and most individuals are probably exposed to the fungus during childhood (Abadi and Pirofski 1999; Houpt et al. 1994). These conclusions seem reasonable considering the ubiquity of *C. neoformans* within

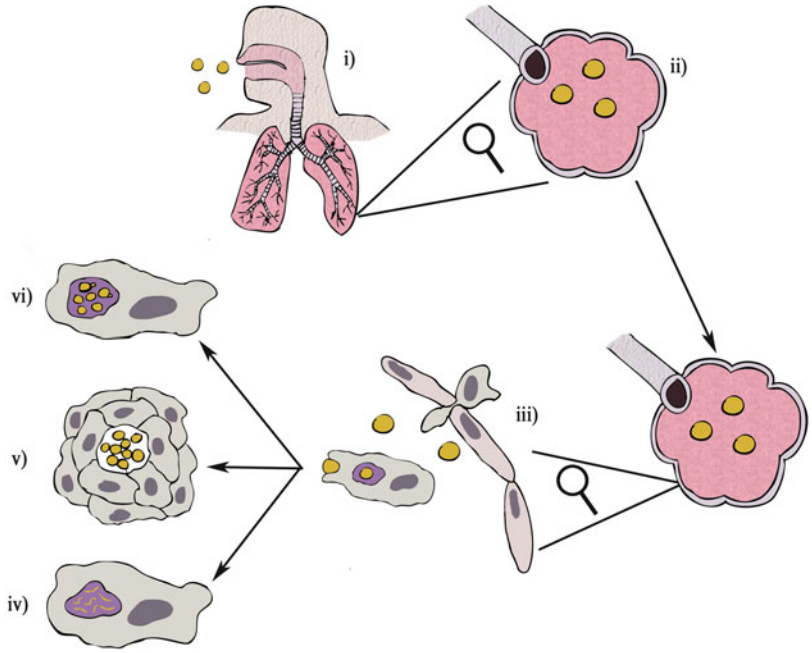

Fig. 5.2. *Cryptococcosis* pathogenesis. (*i*) Desiccated *C. neoformans* cells or infectious spores are breathed into the respiratory tract following environmental contact. (*ii*) The fungi move down the respiratory tract until they reach the alveoli where they initially grow extracellulary. (*iii*) Immune phagocytes such as macrophages are attracted to the site of infection and proceed to engulf extracellular fungi via phagocytosis. Following phagocytosis there are three possible outcomes for fungi in the macrophage: (*iv*) the macrophages succeeds in killing the invader and the fungus is destroyed; (*v*) the macrophage cannot kill the fungus but is able to keep it contained within a granuloma in a dormant state; or (*vi*) the macrophage cannot control fungal growth and the fungi begin to replicate within the macrophage, escape from the macrophage and may disseminate to the central nervous system

the natural environment, thus it is likely that **individuals encounter *C. neoformans* many times during their lifetime but are protected by a healthy immune system.**

Some studies show that latent infection contained by the immune system may re-emerge if an immune deficiency later develops. Evidence for this phenomenon comes from a study by Garcia-Hermoso et al., which determined the genetic origin of clinical *C. neoformans* isolates taken from a cohort of cryptococcosis sufferers in France. This analysis found that a number of immunocompromised African immigrants in the study, who had lived in France for many years before developing immune deficiency, were infected with *C. neoformans* strains originating in Africa. Garcia-Hermoso et al. concluded that these patients may have developed a latent form of cryptococcosis before moving to France and that the infection re-emerged when immune deficiency later developed (Garcia-Hermoso et al. 1999).

III. The Macrophage

Macrophages are innate immune phagocytic cells that patrol the body's tissues searching for signs of infection. When a macrophage detects a foreign cell, such as a pathogenic microorganism, it becomes activated and will attempt to engulf the invader via a process called phagocytosis (see Fig. 5.3). Phagocytosis begins when phagocytic receptors on the cell surface of the macrophage encounter ligands that are only found on foreign cells. Following receptor ligation, signalling pathways are activated inside the macrophage, which trigger cytoskeletal actin

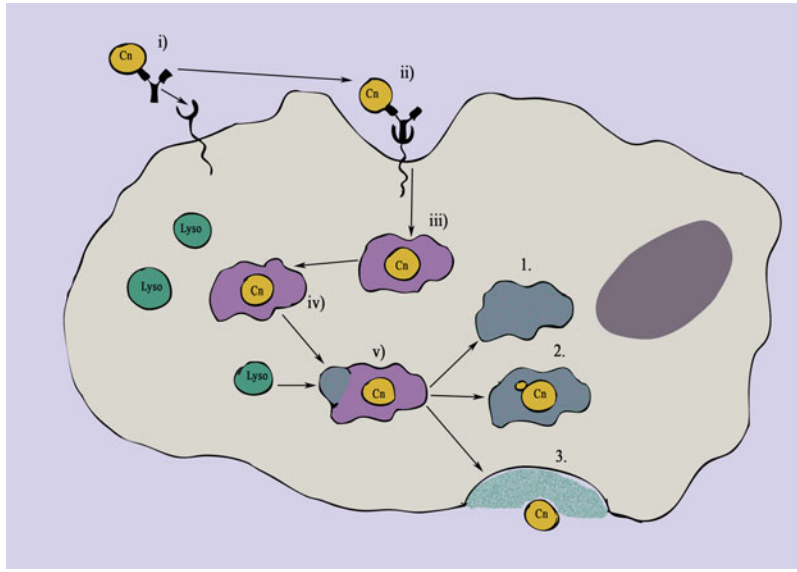

Fig. 5.3. Possible outcomes following *C. neoformans* phagocytosis. (*i*) Opsonised *C. neoformans* (in this case opsonised with antibody) binds to phagocytic receptors on the cell surface of the macrophage. (*ii*) Ligation of phagocytic receptors induces cytoskeletal rearrangements within the macrophage resulting in invagination of the membrane and the formation of phagocytic pseudopodia around the bound cell. (*iii*) *C. neoformans* is contained inside the macrophage within a membrane-bound compartment called the phagosome. (*iv*) Over time the phagosome matures, beginning with the recruitment of v-ATPase, NADPH oxidase and NOS to the phagosome membrane, resulting in the lowering of pH and the production of reactive oxygen species within the phagosome. (*v*) Phagosomal maturation culminates when lysosomes fuse with the phagosome; this releases digestive proteolytic enzymes into the phagosome (which is now termed the phagolysosome). There are three possible outcomes for *C. neoformans* following formation of the phagosome: (*1*) Biocidal conditions within the phagosome destroy the fungus, which is then digested; (*2*) *C. neoformans* resists the conditions within the phagolysosome and begins to replicate; or (*3*) *C. neoformans* escapes from the macrophage via a process called vomocytosis (either following, or independently of, replication)

rearrangements around the receptor, leading to internalisation of the particle into a membrane-bound compartment termed the phagosome. Over time, conditions within the phagosome are altered by the macrophage to create an environment that is destructive to microorganisms, a process called phagosomal maturation. Via a series of vesicle fusion events, membrane proteins such as NADPH oxidase, V-ATPase and NOS are delivered to the phagosome. Together, these create a highly antimicrobial environment, for instance via the production of reactive oxygen species (ROS) by the NADPH oxidase, or lowering of the phagosomal pH by the V-ATPase. Finally, the phagosome fuses with a specialised cytosolic vesicle called the lysosome, which contains an array of digestive enzymes. These digestive enzymes, which work best at low pH, kill any remaining organisms in the phagosome and then break up the dead cell into its constituent parts.

Phagocytosis can clear infection directly but it also triggers signals that enhance the immune response. Upon sensing infection, macrophages can release proinflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β; the inflammation that these cytokines produce attracts other immune cells to the site of infection, ultimately engaging the adaptive immune system if the infection is not cleared rapidly.

IV. Phagocytosis of *C. neoformans* by Macrophages

Macrophages must detect the presence of foreign organisms before they can initiate phagocytosis.

To facilitate detection, macrophages express an array of cell surface phagocytic receptors that bind to ligands present on foreign cells but not host cells. Phagocytic receptors can be categorised according to the nature of their ligands. Non-opsonic phagocytic receptors or pathogen recognition receptors (PRRs) bind to molecular ligands (pathogen-associated molecular patterns or PAMPs) such as polysaccharide residues or lipid species found on the surface of foreign cells. In contrast, opsonic receptors bind to opsonising proteins, such as antibodies or complement proteins, which coat foreign cells following activation of the humoral immune system.

Capsule expression has long been implicated in cryptococcal virulence since it was observed that *Cryptococcus* acapsular mutant strains are avirulent (Fromtling et al. 1982; Chang and Kwon-Chung 1994). The capsule has a number of properties that contribute to virulence during infection, but the most important seems to be its **anti-phagocytic quality**, which was first realised when acapsular strains were found to be phagocytosed by macrophages more readily than were capsular strains (Kozel and Gotschlich 1982; Cross and Bancroft 1995). Capsule growth can be stimulated by CO_2 levels and pH conditions similar to those found during host infection (Granger et al. 1985) and has also been observed to occur in vivo (Feldmesser et al. 2001). Synthesis of the capsule is controlled by at least four genes, designated *CAP64*, *CAP59*, *CAP10* and *CAP60* (Buchanan and Murphy 1998; Ma and May 2009). In a series of experiments, Chang et al. found that deletion of each CAP gene produced an acapsular mutant that lacked virulence until the gene was reconstituted (Chang et al. 1996; Chang and Kwon-Chung 1994, 1999, 1998). **The capsule is composed of two key polysaccharide components: glucuronoxylomannan (GXM) and galactoxymannan (GalXM)** (Bose et al. 2003), with a ratio of approximately 9:1 by mass in favour of GXM (Idnurm et al. 2005).

One of the main ways that the capsule protects *C. neoformans* against phagocytosis is by providing a shroud around the cell, which interferes with macrophage detection. This shrouding effect blocks uptake via both opsonic and non-opsonic phagocytic receptors although it appears to have a greater inhibitory effect on non-opsonic uptake because non-opsonised *C. neoformans* cells are almost completely protected from phagocytosis (Mukherjee et al. 1996).

A. Non-opsonic Uptake

Phagocytosis of yeast-like fungi that do not produce a polysaccharide capsule, such as *Candida albicans* and *Saccharomyces cerevisiae,* often occurs via non-opsonic phagocytic receptors such as Dectin-1 and the Mannose receptor (MR) (Gantner et al. 2005; Brown and Gordon 2001; Porcaro et al. 2003; Giaimis et al. 1993). Both phagocytic receptors bind to cell wall constituents found in the fungal cell wall; Dectin-1 binds to β-1,3- and β-1,6-linked glucan residues (Brown and Gordon 2003) whereas MR binds to mannoproteins. **Macrophages can easily phagocytose acapsular *C. neoformans* mutants via Dectin-1 or MR ligation, but they struggle to phagocytose encapsulated cells** (Cross and Bancroft 1995). In wild-type capsular strains, the ligands for each receptor are obscured by the overlying capsule, thus it is thought that non-opsonic phagocytic receptors such as Dectin-1 and MR may contribute to the phagocytosis of recently inhaled cryptococcal cells (which have a minimal capsule), but once capsule growth is stimulated non-opsonic uptake becomes redundant.

B. Opsonic Uptake

Under most conditions, the cryptococcal capsule conceals cell wall ligands detected by non-opsonic receptors so **the main route of phagocytosis by macrophages is via opsonic phagocytic receptors** (Mukherjee et al. 1996). Opsonisation is more effective at facilitating the detection and phagocytosis of *C. neoformans* because opsonins bind to capsular components and thus are more accessible to macrophage phagocytic receptors than the non-opsonic receptor ligands. Two main classes of opsonic phagocytic receptors are expressed by macrophages: **complement receptors**, which recognise activated complement proteins, and **Fc receptors**, which recognise the Fc regions of immunoglobulin (antibody) molecules.

Complement can be activated via two pathways – the classical pathway and the alternative pathway. Classical activation requires the presence of antigen-specific immunoglobulin bound to the surface of the foreign cell, whereas alternative activation does not require antigen specificity. Antibody produced during cryptococcal infection is thought to be ineffective at activating classical complement cascades (Houpt et al. 1994) and thus **the alternative complement cascade is considered more important to opsonisation during infection**. Regardless of the route of activation, the end result of the complement cascade is the deposition of the complement fragment C3b, which can be recognised and phagocytosed by the macrophage complement receptor CR3.

Although complement deposition facilitates macrophage detection of *C. neoformans,* the pathogen is still able to reduce the efficiency of complement-mediated uptake by modifying the makeup of its capsule. Consequently, cryptococcal cells producing a capsule that is thick (Zaragoza et al. 2003) or has a low density (Gates and Kozel 2006) are harder for macrophages to detect, since both of these properties increase the likelihood that complement proteins will bind within the capsule, where they are obscured, as opposed to at the surface.

The production of antigen-specific antibody against a newly encountered pathogen requires activation of the adaptive immune response, although secondary exposure to an antigen evokes a quicker response due to the persistence of immunological memory. Antibodies can improve the phagocytic uptake of foreign cells in two ways once they have bound to the cell. They can amplify complement opsonisation by activating the classical complement pathway, but they can also directly stimulate phagocytosis via ligation of macrophage Fc receptors, which bind to the non-variable region of antigen-bound antibodies. **During *C. neoformans* infection, most antibody produced is against capsular components such as GXM. Anti-capsular GXM antibody titres are often used as markers of acquired immunity to *C. neoformans* in healthy individuals** (Deshaw and Pirofski 1995; Houpt et al. 1994); however, the protective role of anti-GXM antibodies during infection is debateable. For example, a 1994 study by Houpt et al. found that the sera of healthy subjects contained IgG and IgM antibodies reactive against cryptotoccal GXM, but that these antibodies were ineffective at activating the classical complement cascade (Houpt et al. 1994).

C. Capsule-Independent Antiphagocytic Factors

The capsule is a major antiphagocytic mechanism deployed by *C. neoformans*, but capsule-independent antiphagocytic mechanisms also exist. A study by Liu et al. identified a GATA family transcription factor called **Gat201**, which appears to control such a mechanism (Liu et al. 2008). A recent study of Gat201 revealed that knockout of its gene affected the transcription of ~1,100 genes in *C. neoformans*, although only 62 of these genes are thought to be regulated by direct binding of Gat201. **Phenotypic analysis of the *GAT201* knockout strain showed reduced capsule size and increased uptake by macrophages.** The susceptibility to phagocytosis observed was greater than that of acapsular mutants alone, suggesting that capsule-independent antiphagocytic mechanisms are also controlled by Gat201 potentially via two downstream genes – *BLP1* and *GAT204* (Chun et al. 2011).

One capsule-independent antiphagocytic mechanism is the secreted protein **APP1**. This protein is expressed by *C. neoformans* during infection. APP1 can block complement-dependent phagocytosis by binding to and subsequently **blocking macrophage CR3 receptors** (Luberto et al. 2003).

D. Titan Cell Formation

Several studies have reported the presence of abnormally large cryptococcal cells in the lungs during in vivo infection (Zaragoza et al. 2010; Cruickshank et al. 1973; Feldmesser et al. 2001; Love et al. 1985) that appear to be **resistant to phagocytosis** (Okagaki et al. 2010). These giant cells are now recognised as a distinct cell morphology during infection and are now often

called "Titan cells" in the literature. The size of reported Titan cells differs between studies and ranges from around **25 μm to 100 μm, making their volume up to 900 times greater than that of normal cryptococci** (Zaragoza et al. 2010). The underlying mechanism or stimuli behind Titan cell development is not known, although the presence of macrophages, or spent media used to grow macrophages, can induce the morphology (Okagaki et al. 2010) suggesting that interactions with the macrophage play at least some role in the process.

Titan cells appear to be more resistant to phagocytosis than normally sized cells. The most obvious defence a Titan cell has against a macrophage is its physical size. However, recent work has suggested that the presence of **Titan cells during infection may also protect neighbouring wild-type cells from phagocytosis, suggesting the presence of a secreted inhibitory factor** (Okagaki et al. 2010; Okagaki and Nielsen 2012).

In summary, the detection and phagocytosis of *C. neoformans* cells is an important part of the immune response against the fungus. To protect itself from phagocytosis, *C. neoformans* relies heavily on its polysaccharide capsule but also deploys a range of other antiphagocytic strategies. Such mechanisms appear effective at helping some cells evade phagocytosis, but nonetheless the infection of macrophages in vitro and in vivo is often observed, indicating that phagocytosis of *C. neoformans* is far from rare.

V. Life Within the Phagosome

To grow inside a macrophage following phagocytosis, an intracellular pathogen must successfully avoid the killing mechanisms that develop in the phagosome during phagosomal maturation. A number of mechanisms are employed by pathogens to do this, including inhibition of V-ATPase to prevent phagosomal acidification as seen with *Mycobacterium tuberculosis* infection (Huynh and Grinstein 2007) and escape from the phagosome into the cytoplasm as seen with *Listeria monocytogenes* infection (Hamon et al. 2006). In contrast to such pathogens, *C. neoformans* is able to survive within the phagosome seemingly without modifying the maturation process. Instead, it is able to **resist and overcome the destructive conditions it encounters in the phagosome and can even grow within this harsh environment**.

The first barrier to cryptococcal growth in mammalian macrophages is the higher temperature within the host than in the environment. The complete molecular mechanisms that allow *C. neoformans* to grow at 37 °C are not fully known, but a number of potential pathways have been implicated. For instance, higher temperatures induce upregulation of TSP1 and TSP2, which together lead to higher levels of the polysaccharide trehalose, protecting cellular proteins from denaturation (Petzold et al. 2006).

Inside the phagosome, the largest barrier to cryptococcal growth is phagosomal maturation, i.e. the production of oxygen free radicals accompanied by the acidification of the phagosome. During infection most *C. neoformans* strains synthesise **melanin**, which is a dark-coloured pigment and an antioxidant (Wang et al. 1995) that is thought to protect the fungi from reactive oxygen species. In addition to melanin, **capsule enlargement** during macrophage infection may also be protective against oxygen free radicals by creating a barrier between the cell body and phagosomal contents (Zaragoza et al. 2008); furthermore, GXM within the capsule may act as an antioxidant (Monari et al. 2006; Vecchiarelli et al. 1996). Interestingly, however, the phagosomal acidification that occurs during maturation may actually be beneficial to *C. neoformans* because neutralising phagosomal pH can block intracellular replication (Levitz et al. 1999).

While in the phagosome, *C. neoformans* secretes a number of proteins that potentially influence the macrophage. One such protein, which is well known to contribute to cryptococcal virulence, is the enzyme **phospholipase B (Plb)**. Plb is a lipid-modifying enzyme that has multiple enzymatic activities. It possesses phospholipase B activity, which can cleave the ester bonds linking the glycerol headgroup in

a phospholipid to the two fatty acid chains at both the sn1 (phospholipase A1 activity) and sn2 positions (phospholipase A2 activity) to produce a free fatty acid and a lysophospholipase. As well phospholipase B activity, Plb also has lysophospholipase and lysophospholipid transacetylase activity; these two activities modify the lysophospholipid products of sn1 or sn2 cleavage, resulting in further degradation or re-synthesis of the original substrate (Shea et al. 2006).

Previous studies have reported that Plb-deficient strains of *C. neoformans* are avirulent during murine infection, displaying reduced fungal burden in the lungs, possibly due to a reduced ability to proliferate within macrophages (unpublished observations from our group), and a lower propensity to disseminate to the central nervous system (Chayakulkeeree et al. 2011; Noverr et al. 2003; Chen et al. 1997; Cox et al. 2001). The molecular mechanism behind Plb-mediated virulence is not fully known. However, a compelling theory is that Plb activity metabolises *Cryptococcus* or macrophage-derived phospholipids to produce arachidonic acid (AA), which is a precursor to immunoregulatory eicosanoids. Members of the eicosanoid family include prostaglandins, leukotrienes and lipoxins. Both macrophages (Harizi et al. 2008) and *C. neoformans* itself (Noverr et al. 2001) can produce eicosanoids, meaning that either or both types of cell could potentially make use of the AA produced.

Eicosanoids might aid *C. neoformans* survival by directly suppressing the antimicrobial mechanisms of the infected macrophage; in addition they may also alter the immune response during cryptococcosis at a wider level. For example, PGE_2 (one of the most ubiquitous prostaglandins during inflammation) can produce a Th2 $CD4^+$-biased immune response by downregulating IL-12 release from macrophages (van der Pouw Kraan et al. 1995). With this in mind, it has been observed that the development of **Th1 adaptive immune responses is protective against *C. neoformans* infection, whereas the Th2 adaptive immune response is not** (Hoag et al. 1997; Voelz et al. 2009; Wormley et al. 2007).

VI. Escape from the Macrophage

Pathogens that are specialised in the parasitism of macrophages often possess mechanisms that allow them to escape the intracellular niche when the time is right. *C. neoformans* is no different in this respect and possesses an escape mechanism called non-lytic phagosomal extrusion or vomocytosis.

Vomocytosis is a mechanism first observed by our group (Ma et al. 2006) and others (Alvarez and Casadevall 2006) that allows *C. neoformans* to escape from infected macrophages without causing host cell lysis. Exiting without causing harm to the macrophage is relatively rare for a phagocytic escape mechanism and is beneficial to *C. neoformans* because it produces lower levels of inflammation than host cell lysis. The cellular events driving vomocytosis are not fully understood, although it appears to occur via fusion of *Cryptococcus*-loaded phagosomes with the macrophage outer membrane following prior permeabilisation of the phagosome membrane (Johnston and May 2010). Interestingly, host cells appear to attempt to block vomocytosis by initiating repeated actin polymerisation/depolymerisation cycles (actin flashing) around the phagosome following permeabilisation (Johnston and May 2010).

VII. Macrophages as a "Trojan Horse"

Dissemination from the lungs to the CNS is a crucial escalation point during cryptococcosis because CNS infection is almost always fatal unless treated. **To enter the CNS, *C. neoformans* must cross the blood–brain barrier (BBB),** whose normal function is to prevent such passage. There are **two main routes** that *C. neoformans* potentially uses to cross the BBB: (**a**) extracellular *C. neoformans* cells in the blood during fungemia **pass independently across the BBB** by an as-yet-undefined mechanism or (**b**) **macrophages infected with *C. neoformans*** in the lungs pass back into circulation and subsequently cross the BBB and disgorge their fungal cargo – this is popularly termed the "Trojan horse" theory of dissemination (Fig. 5.4).

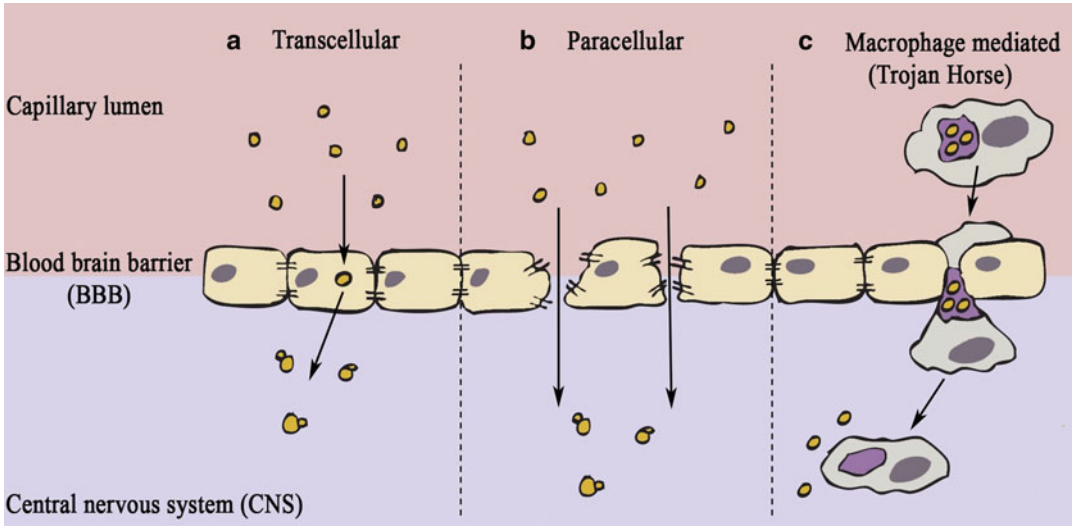

Fig. 5.4. Possible routes of dissemination across the blood brain barrier for *C. neoformans*. There are three routes that *C. neoformans* is thought to use to cross the blood–brain barrier (BBB). (*a*) Transcellular route: fungal cells pass directly through endothelial cells lining capillaries in the brain and are taken up at the apical side and exit on the basal side. (*b*) Paracellular route: fungal cells pass between endothelial cells following disruption of cell-to-cell contacts; this disruption could be due to factors produced by the fungi or to inflammation. (*c*) "Trojan horse" dissemination: macrophages infected with *C. neoformans* cells pass from the circulation through the BBB via diapedesis; the fungi within the macrophage then escape into the extracellular space

Evidence to support both routes of entrance can be found in the literature, suggesting that the mechanisms may not be mutually exclusive and in fact may co-exist. In this respect, a study that examined disseminative cryptococcosis in an in vivo mouse model found that cryptococcal cells could be found within the brain both extracellulary or associated with monocytes and endothelial cells (Chrétien et al. 2002). Intravital imaging of *C. neoformans* in mouse brain capillaries found that extracellular fungi in the capillary lumen could cross the epithelial layer following sudden stopping. In this study, arrest in the lumen appeared to be purely via mechanic trapping when the size of *Cryptococcus* cells exceeded that of the capillary, whereas crossing of the epithelial layer was an active process requiring fungal viability (Shi et al. 2010).

In support of the Trojan horse theory, infected macrophages from the alveolar space can re-enter circulation and transport *C. neoformans* to other parts of the body whereas infected macrophages injected directly into the blood lead to increased fungal burden in the brain (Charlier et al. 2009).

VIII. How Has the *C. neoformans*–Macrophage Interaction Evolved?

A significant challenge for the field is to explain why an environmental opportunistic pathogen that is not dependent on a host has nonetheless evolved a battery of virulence factors that seem specific for mammalian hosts. The most likely explanation is that ***C. neoformans* did not evolve its virulence factors to cause infections in animals but rather that such factors were selected in response to attack by soil predators**. Considerable support for this model comes from investigations looking at the interaction between *C. neoformans* and soil-dwelling amoebae such as *Acanthamoeba castellanii* and *Dictyostelium discoideum*. Both of these species can engulf C. *neoformans* and, excitingly, many aspects of the *Cryptococcus*/macrophage relationship, e.g. intracellular proliferation and the production of

exopolysaccharides, were mirrored in the amoebal host following phagocytosis. Further confirmation of the similarities between the two models were provided by the observations that avirulent mutants in the macrophage, such as acapsular or phospholipase-deficient cryptococcal strains, were also more vulnerable to predation (Steenbergen et al. 2001; Steenbergen and Casadevall 2003; Casadevall et al. 2003).

IX. Conclusion

To summarise, the role of the macrophage during antifungal immune responses is of central importance. Not only is the macrophage responsible for detecting infection and shaping subsequent immune responses, it is also required to directly kill and clear fungal cells. The behaviour of the macrophage during *C. neoformans* infection is of even greater importance than for most fungal infections because while the macrophage seeks to clear infection the fungus itself relies on the macrophage as safe niche for intracellular proliferation and possibly as a vehicle for CNS dissemination.

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