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Interaction of pathogenic yeasts with phagocytes: survival, persistence and escape

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Pathogenic yeasts, either from the environment or the normal flora, have to face phagocytic cells that constitute the first line of defence during infection. In order to evade or counteract attack by phagocytes, pathogenic yeasts have acquired a repertoire of strategies to survive, colonize and infect the host. In this review we focus on the interaction of yeasts, such as *Candida*, *Histoplasma* or *Cryptococcus* species, with macrophages or neutrophils. We discuss strategies used by these fungi to prevent phagocytosis or to counteract phagocytic activities. We go on to describe the strategies that permit intracellular survival within phagocytes and that may eventually lead to damage of and escape from the phagocyte.

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Introduction

Pathogenic yeasts are either commensals of the normal microbial flora or environmental yeast, which incidentally infect the human host. Amongst the commensal fungal pathogens are *Candida albicans* and *Candida glabrata*, which colonize different body locations. They do not usually elicit overt immune recognition and inflammation, but may occasionally face immune cells. Environmental fungal pathogens include *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis* or *Blastomyces dermatitidis*, and grow as saprophytes in environmental niches, which can sometimes reflect certain aspects of the host environment. These pathogens may enter the human host by inhalation of spores or mycelial fragments, which then convert into parasitic forms inside the body where they, by applying strategies adapted in their environmental niches, may cause disease. In the host, potentially pathogenic yeasts can be killed by phagocytes; however, certain species have developed mechanisms to avoid such killing. In this review, we focus on the evasion strategies of pathogenic yeasts from killing by phagocytic cells (Figure 1).

Prevention of phagocytosis

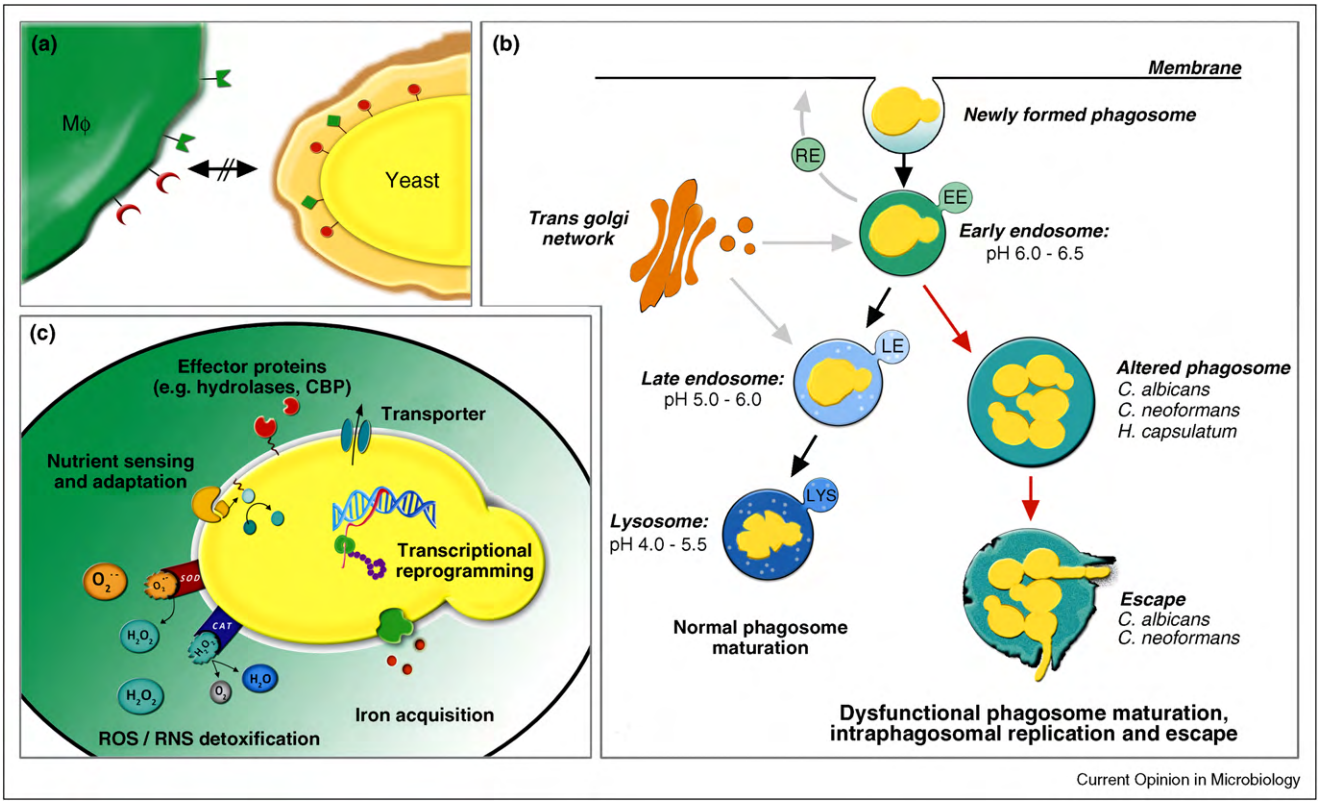
Pathogenic fungi have evolved efficient strategies to overcome killing by effector cells of the innate immune system. One possibility is simply to avoid contact with immune cells by evading recognition, often mediated by masking of immunostimulatory surface molecules. Another tactic is to prevent phagocytosis by either the use of physical barriers, inhibiting deposition of host opsonic molecules or by exerting influence on the cytoskeleton-based phagocytic machinery of immune effector cells.

Avoiding immune recognition: shielding PAMPs and physical barriers

The first step in induction of an antifungal innate immune response depends on recognition of conserved fungal structures by front-line immune cells. These so-called pathogen-associated molecular patterns (PAMPs) can be either secreted or on the fungal surface. Fungal pathogens harbour a variety of PAMPs including β-glucan, chitin and mannoproteins that are detected by a corresponding set of host transmembrane pattern-recognition receptors (PRRs). The resulting combination of PRR–ligand interactions will determine a customized response depending on the pathogen and the type of immune effector cell. Recognition normally initiates signalling cascades, resulting in phagocytosis, secretion of microbicidal compounds and production of pro-inflammatory mediators.

Avoiding such detection can be considered as the simplest immune evasion strategy. For example, *C. albicans* shields its immunostimulatory β-glucan, known to be recognized by the PRR Dectin-1, with a mannoprotein coat, thereby making itself less visible to the host. A recent study, working with gene deletion strains, reconfirmed the importance of O-linked and N-linked mannans in avoiding phagocytosis [1]. However, the same report also implicated immunostimulatory PAMPs, like phosphomannan, in enhancing the uptake by macrophages. Additionally it should be noted that mannoproteins themselves are potent PAMPs, recognized by

Figure 1



Model for immune evasion and modulation strategies used by pathogenic fungi. **(a)** A protective layer (e.g. mannoproteins of *C. albicans*, the capsule of *C. neoformans* or α-glucan of *H. capsulatum* and *P. brasiliensis*) masks immunostimulatory pathogen-associated molecular patterns (PAMPs). **(b)** The phagosome maturation process within phagocytes normally leads to the formation of a phagolysosomes (by fusion with early endosomes [EE], late endosomes [LE] and lysosomes [LYS]), where microbes are killed and digested (black arrows). Some pathogenic fungi are able to alter this process, yielding a compartment in which they can survive, replicate and finally escape (red arrows). **(c)** Within the phagosome, fungal pathogens use mechanism to adapt to the hostile environment or counteract antimicrobial activities.

several PRRs. Therefore, the recognition of *C. albicans*, appears to be a complex multiple-level process involving specific receptor systems [2]. Nevertheless, shielding β-glucan with mannoproteins seems to benefit the fungus. By switching morphology from yeast to hyphae *C. albicans* circumvents β-glucan exposure at birth scars, which appear during normal yeast growth [3]. However, during murine infection, no differences in β-glucan exposure between yeast form and hyphal cells were observable at later stages [4••]. Of note, only the minor cell wall component β-1,6-glucan efficiently induced engulfment of *C. albicans* by human neutrophils, in contrast to the predominant overlaying β-1,3-glucan [5].

A similar mechanism enables *H. capsulatum* and *P. brasiliensis* to avoid detection: an α-(1,3)-glucan layer overlays the immunostimulatory β-glucan signatures of *H. capsulatum*, whilst *P. brasiliensis* modifies its cell wall glucan polymer linkage from a β-1,3-glucan to an α-1,3-glucan during transition to the pathogenic yeast form [3]. Moreover, *P. brasiliensis* is characterized by a multiple budding phenotype and polymorphic cell growth, leading to the formation of cells with extreme variations in shape and size. A reduction in cell size renders *P. brasiliensis* completely avirulent and more susceptible to phagocytosis by macrophages [6].

The most prominent virulence factor of *C. neoformans* is an extensive polysaccharide capsule with antiphagocytic properties, which even increases in size during infection (reviewed in [7]). Accordingly, hypermucoid variants are more resistant to phagocytosis and therefore may promote immune evasion during establishment of chronic infections [8•]. Despite the inhibitory role of the capsule, phagocytosis can still occur, but then mainly depends on opsonization by complement proteins or antibodies. Mutants lacking the capsule, on the other hand, can be phagocytosed in the absence of opsonins. More recently, novel capsule-independent mechanisms of *C. neoformans* to avoid phagocytosis have been identified. In particular, a GATA family of transcription factors have been

described which are involved in avoiding recognition by immune cells [9••].

Modulating complement-mediated uptake

Phagocytosis can either be directly mediated by PRRs or indirectly through host opsonins. The complement system consists of some 30 proteins, either circulating in blood plasma or residing on cell surfaces; the deposition of these proteins on foreign surfaces not only promotes recognition and phagocytosis, but also the recruitment of inflammatory cells. Because the complement system has the potential to severely damage host tissues, complement activation must be tightly controlled by specific regulators. Remarkably, certain fungal pathogens have taken advantage of these regulators, achieving complement evasion.

C. albicans, for example, possesses two proteins, the pH-regulated antigen 1 (Pra1) and a phosphoglycerate mutase (Gpm1), which can bind host complement regulators (either Factor H or FHL-1 binding protein) — thus providing a protective layer which shields against complement deposition [10,11]. Similarly, *C. neoformans* possesses the antiphagocytic protein, App1, which binds to complement receptors 3 and 2 thereby inhibiting phagocytosis by alveolar macrophages [12]. In addition to complement proteins, collectins, such as mannose-binding lectin (MBL) and surfactant protein A (SP-A), are host molecules which opsonize fungal cells. *B. dermatitidis*, however, binds MBL, thereby masking its immunogenic β-glucan, preventing recognition and tumour necrosis factor-α secretion by macrophages [13]. In contrast, capsulated *C. neoformans* cells are resistant to MBL binding [14] and SP-A binding sites are concealed by the cryptococcal capsule (even though SP-A binding to encapsulated *C. neoformans* can be facilitated by immunoglobulin G) [15]. As already mentioned, the capsule of *C. neoformans* itself is antiphagocytic and its enlargement can additionally function in inhibiting deposition of opsonins [7].

Intracellular survival: manipulating the host, detoxifying antimicrobial compounds and gaining nutrients

Shielding of stimulatory PAMPs or complement evasion represent effective fungal strategies for circumventing the host innate immune system and phagocytosis. Moreover, *C. albicans* and *C. glabrata*, for instance, secrete metabolites which impair the uptake efficiency of macrophages, probably by interfering with the host cell cytoskeleton [16]. However, despite the mechanisms described here, fungal pathogens cannot completely elude phagocytosis.

Following recognition of a microbe, phagocytes initiate engulfment leading to the formation of a phagosome. This vesicle then undergoes a series of fission and fusion events, associated with alterations of the surrounding membrane and vacuolar content, ultimately leading to the formation of a specialized organelle: the phagolysosome. The degradative and microbicidal microenvironment of this organelle is associated with a reduction in pH, the presence of hydrolytic enzymes, defensins as well as other antimicrobial peptides and the generation of toxic oxidative compounds. Moreover, it exhibits severe nutrient and trace element limitation. Nevertheless, several fungi have developed strategies to survive within phagocytes. These strategies include: activities or attributes which alter the phagolysosome maturation process, scavenging of toxic compounds; escape from the phagolysosome; or adaptation to and survival within the harsh environment within the phagocyte.

Manipulating phagosome maturation

More than 10 years ago, it was discovered, that *H. capsulatum* can survive phagocytosis and even replicate within macrophages. Phagolysosomes containing *H. capsulatum* maintain a pH of about 6.5, thereby minimizing the activity of lysosomal hydrolases, but still allowing iron release from host transferrin. The exact mechanism underlying this remains unclear, but it is known that the fungus inhibits accumulation of V-ATPase, the proton pump of the phagolysosomal membrane [17]. Additionally, the *H. capsulatum* saposin-like calcium binding protein (CBP), expressed specifically by the pathogenic yeast form, may play a key role in altering the phagosomal environment [18]. CBP has a high structural homology to mammalian saposin-B and presumably interacts with host lipids of the phagosome [19••]. Although these phenomena depend on the host cell type [20] it appears that *H. capsulatum* can actively manipulate the phagosome composition and the host killing machinery.

C. albicans can also manipulate phagosome maturation, resist killing by macrophages and replicates intracellularly [21]. Although the ability to block phagosome maturation does not require the dimorphic transition, comparison with a non-filamentous mutant showed that the filamentous form is more capable of controlling phagosomal composition [22].

C. neoformans was originally described as an intracellular pathogen that resides in acidified phagolysosomes and does not avoid lysosomal fusion [23]. However, further studies revealed that fungal replication takes place in enlarged phagosomes and is associated with the accumulation of shredded fungal polysaccharides and the formation of leaky membranes, providing access to nutrients of the cytoplasm [24]. Furthermore, *C. neoformans* infection of macrophages is accompanied by the formation of a large number of vesicles [24] that originate from the phagosome. Besides fungal lipids and polysaccharide, these vesicles contain a variety of virulence-associated enzymes, for example laccase, urease and phosphatase

[25] and have been recently shown to be involved in proper fungal melanization [26]. These so-called 'virulence bags' may function in compromising host cell survival during infection.

Interestingly, such a mechanism of transcellular vesicular secretion is also used by *H. capsulatum* and other ascomycetes, suggesting a more generally applicable fungal activity [27].

Transcriptional reprogramming within phagocytes

Once phagocytosed, fungal pathogens exhibit dramatic transcriptional and translational reprogramming, reflecting survival strategies and adaptation to the harsh internal environment of the phagocyte.

Comprehensive transcriptional and proteomic studies have provided insights into the response of *C. albicans* to phagocyte ingestion [28]. Immediately following macrophage phagocytosis, fungal yeast cells experience a shift to alternative carbon sources, reflected by induction of the glyoxylate cycle, gluconeogenesis and fatty acid degradation, together with repression of glycolysis and translation, suggesting that glucose is scarce in macrophages. Alongside this starvation response, substantial non-metabolic transcriptional profiles were also detected. These included the induction of oxidative stress response, DNA damage repair, arginine biosynthesis, peptide utilization and an abundant number of *C. albicans*-specific genes [29]. Similar transcriptional patterns were also detected in *C. neoformans*, Both, *C. albicans* and *C. neoformans* appear to be exposed to carbon limitation, as suggested by the upregulation of glucose and other carbohydrate transporter genes, whereas genes encoding key enzymes of the glyoxylate cycle and gluconeogenesis were only upregulated by *C. albicans*. In contrast, *C. neoformans* induces genes encoding extracellular lipases, which may digest host lipids, fatty acids transporters and enzymes involved in β-oxidation and peroxisome formation [30].

Degradation and recycling of endogenous cellular components (autophagy) represents another strategy of adaptation when nutrients are limited. This process is applied by *C. neoformans* and *C. glabrata* within macrophages [31,32], but does not seem to play an important role for *C. albicans* survival [33].

In summary, phagocytosed fungal cells have the ability to precisely sense the surrounding environment and appropriately modify their transcriptional profile.

Counteracting the oxidative burst

The oxidative stress that fungal pathogens experience within the phagosome is presumably because of a toxic cocktail of reactive oxygen (ROS), nitrogen (RNS) and chloride species generated by phagocytes and the consequent damage of DNA, proteins and lipids contributes to killing of fungal cells. Therefore, in order to survive phagocytosis, a rapid response and induction of protective mechanisms by fungal pathogens is necessary.

Recent *in vitro* studies indicate that *C. albicans* and *C. glabrata* actively suppress ROS production in phagocytes, whereas *S. cerevisiae* does not [34]. Furthermore, transcriptional and proteomic studies of *C. albicans* and *C. glabrata* exposed to exogenous ROS revealed an activation of detoxifying enzymes with antioxidant function including catalases, superoxide dismutases and components of the thioredoxin and glutaredoxin systems [35–38]. Inactivation of detoxifying enzymes, such as superoxide dismutases, in *C. albicans* accordingly lead to severe attenuations in virulence and viability inside immune cells [39,40].

C. albicans cells exposed to nitric oxide induce another specific detoxification programme which includes the induction of nitric oxide dioxygenase, catalases and Yhb1, a flavohemoglobin that converts NO to harmless nitrate [41,42]. However, mice with defects in the inducible nitric oxide synthase (NOS2) are not more susceptible to *C. albicans* infection than wild type mice, suggesting that RNS play a minor role for fungal killing *in vivo* (at least in mice) [41].

Similar to *C. albicans*, the release of RNS by pre-stimulated macrophages is suppressed by *C. neoformans* in a capsule-independent manner [43]. *C. neoformans* therefore uses enzymes involved in nitric oxide detoxification and damage repair [44]. Several studies have identified individual proteins of the remarkably robust and redundant antioxidant system of *C. neoformans*. These include four catalases, two superoxide dismutases, glutathione peroxidases, thioredoxin proteins, the inositol phosphosphingolipid-phospholipase C1 (Isc1) and the protein kinase C (Pkc1), which are essential for surviving the oxidative environment of macrophages [45–50]. In addition to capsule-independent mechanisms, the enlargement of the capsule size during infection has been shown to provide additional protection against oxidative stress and antimicrobial peptides [51].

Moreover, pigment production of *C. neoformans*, *P. brasiliensis* or *C. glabrata* is believed to scavenge ROS produced by phagocytes [52,53].

Different virulence factors with importance for adaptation and survival

In addition to nutritional adaptation and detoxification of ROS or RNS, pathogenic fungi possess a variety of further factors and activities which can counteract phagosomal killing.

For example, production of trehalose [54,55] and glycosylphosphatidylinositol-linked aspartic proteases [56] are

crucial for survival of *C. albicans* or *C. glabrata* after ingestion by macrophages. Furthermore, Vps41, which functions in the alkaline phosphatase and carboxypeptidase Y sorting pathways, appears to be important for response to starvation and is essential for survival of *C. neoformans* in macrophages [57].

Microarray data indicate a central role of the Gpa1–cAMP–PKA pathway for growth of *C. neoformans* in macrophages. This pathway not only coordinates transcription of genes involved in capsule and melanin production, but also responses to nutrient limitation within phagosomes. In accordance, mutants of Gpa1 or Pka1 display reduced growth in macrophages [30]. In general, the ability to deal with nutrient limiting conditions can be considered as a key attribute for intracellular survival.

This is also true for indispensable micronutrients such as iron. *H. capsulatum*, for example, gains iron via three different strategies. Preventing complete phagosomal acidification allows gradual iron release from transferrin [58]. Another strategy relies on the secretion of siderophores; consequently, the disruption of *SID1*, encoding the enzyme that catalyzes the first step in siderophore biosynthesis, leads to a significant growth defect in murine bone-marrow-derived macrophages and attenuation in a mouse infection model [59]. The third strategy depends on cell surface-bound or secreted enzymes with iron reducing activity. A recent study presented evidence for a role of a secreted γ-glutamyltransferase (Ggt1) in extracellular enzymatic iron reduction and its importance for survival during co-incubation with macrophages [60].

Other activities which contribute to survival of *C. neoformans* within macrophages are transport activities [61,62] and utilization of host lipid components for production of cryptococcal eicosanoids, which inhibit macrophage fungistatic activity [63].

Damaging phagocytes and escape

Fungal exit strategies

Although microbial intracellular survival strategies allow persistence within phagocytes, pathogens eventually escape to spread and infect distant tissue. Studies on fungal exit strategies are mainly focussed on *C. albicans* and *C. neoformans*.

The yeast-to-hyphae transition of *C. albicans* plays a pivotal role in facilitating escape from phagocytes [64]. Within macrophages, production of carbon dioxide induces hyphal growth allowing piercing and killing of macrophages [65•].

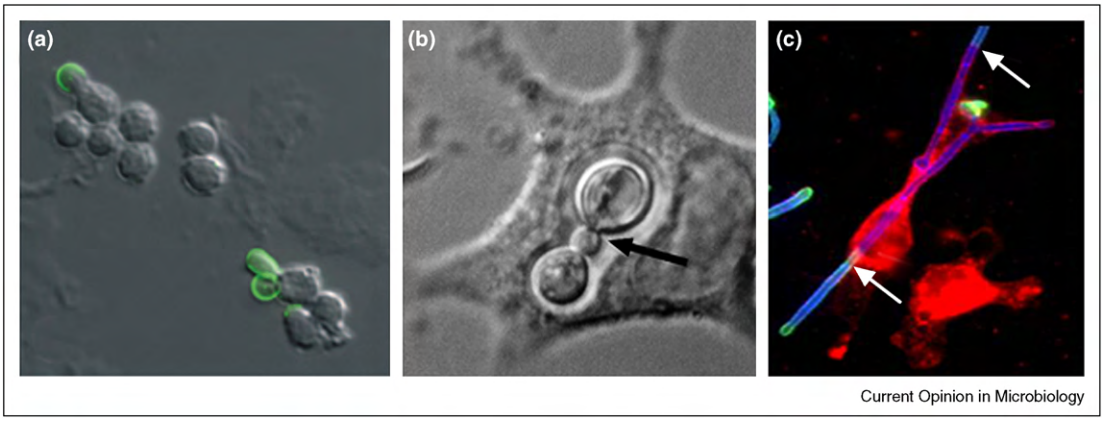
C. neoformans can induce host cell apoptosis [66]; alternatively, this fungus can persist asymptomatically until the onset of infection. In the latter situation, it is beneficial for the fungus to escape from the phagocyte without triggering host cell death and inflammation. Extrusion (or expulsion) represents a novel escape mechanism employed by *C. neoformans* [67,68]. During this process, intra-phagosomal replication and homotypic phagosomal fusion leads to the formation of massive vacuoles, followed by extrusion of the phagosomes and release of the pathogen. Macrophages and expelled yeast cells appear morphologically normal and remain viable. In addition to exiting to the extracellular milieu, the fungus can also undergo lateral transfer from infected donor cells to uninfected neighbouring cells, which may allow continuous concealment from the immune system [69,70]. Interestingly, another strategy involves macrophage mitosis. It has recently been shown, that macrophages resume proliferation, especially during inflammation. Because of the fusion of *C. neoformans*-containing phagosomes the fungus affects intracellular post-mitotic delivery, promoting unequal sorting into daughter cells, which appears to enhance exocytosis. Another intriguing observation is the fusion of two daughter macrophages after the cell division, which is limited to yeast infected cells [71].

Dissemination within the host

Exiting phagocytes without triggering inflammation, lateral transfer to neighbouring cells, as well as avoidance of a pro-inflammatory immune response, not only mediate survival of *C. neoformans*, but may also contribute to prolonged periods of latency until impaired immunity of the infected individual permits symptomatic onset of disease (Figure 2).

It has recently been suggested that *C. neoformans* may promote its own uptake by monocytes or macrophages, using the immune cell as a 'Trojan horse' for distribution within the host, whilst remaining undetected by the immune system. This was supported by the observation that binding of the host surfactant protein D (SP-D) not only enhances *C. neoformans* uptake by macrophages but also protects fungal cells from intracellular killing [72•]. However, this conflicts with the described antiphagocytic properties of the predominant capsular polysaccharide glucuronoxylomannan: indeed, the presence of the capsule is inversely related to SP-D binding. It has been hypothesized that initial infections are caused by desiccated yeast cells or basidiospores with a reduced capsule size. This would permit opsonization with SP-D and initiate phagocytosis by alveolar macrophages, allowing fungal cells to gain access to intracellular niches. Different stages of infection might therefore be influenced by different levels of fungal virulence and defence attributes. For example, whilst a thick capsule enhances fungal survival within macrophages, a reduction in capsule size has been shown to allow better dissemination to the brain [73]. Additionally, using a mouse model of dissemination, together with bone-marrow-derived monocytes, pre-infected with *C. neoformans*, it was demonstrated that the fungus may hijack monocytes in

Figure 2



Different virulence mechanisms of pathogenic yeasts during interactions with phagocytes. **(a)** During the transition from conidia to yeast, *H. capsulatum* produces α-(1,3)-Glucan (green) exclusively around the yeast buds (from Rappleye *et al. PNAS* 2007 **104**:1366–1370 with permission from the National Academy of Sciences, U S A). **(b)** Intracellular budding of *C. neoformans* strain 24067 in J774 macrophages (arrows indicate budding site) (from Tucker *et al. PNAS* 2002 **99**:3165–3170 (Figure 1b) with permission from the National Academy of Sciences, U S A). **(c)** Yeast-to-hyphae transition of *C. albicans* plays a pivotal role in facilitating escape from phagocytes (arrows indicate piercing sites).

order to be transported across the blood–brain barrier [74•].

Immune evasion via concealment in intracellular niches is also employed by *H. capsulatum* and *P. brasiliensis*. Both organisms delay host cell death of neutrophils and also, in the case of *H. capsulatum*, mononuclear cells, by inhibiting apoptosis [75,76]. Such immune evasion strategies might support fungal survival by maintaining an environment to which these fungi are pre-adapted. Nevertheless, these results must be interpreted with caution, as it is also possible that delayed death is also favourable for the host, permitting enough time to produce antimicrobial mediators and finally combat the fungus. This exemplifies the fact that strategies employed by fungal pathogens to overcome host innate immunity are always in close competition with a successful host defence response.

Conclusions

Some of the strategies of opportunistic pathogenic yeasts to avoid or survive attack from phagocytes, to persist within phagocytes or to escape from phagocytes reviewed here are also employed by bacteria and parasites; however, some seem to be specific for fungi — for example, the role of morphology for *C. albicans* escape from phagocytes. Certain pathogenic yeasts can multiply inside of phagocytes, a mechanism which may protect them during the early stages of infection, but which may also be a strategy for persistence. Indeed, intracellular survival of pathogenic yeasts may be more common than anticipated and may enable some fungal cells to persist within hosts for long periods. Such unrecognized persistence in the human body may represent a source of disease when the immune system becomes attenuated. This may include yeasts not previously described as facultative intracellular fungi, for example *C. glabrata*, which can persist in mice for long periods without causing disease [77]. The strategies employed by such fungi to evade or survive attack by phagocytes remain to be investigated.

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