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Fungal killing by mammalian phagocytic cells

André Moraes Nicola, Arturo Casadevall and David L Goldman

Phagocytes are considered the most important effector cells in the immune response against fungal infections. To exert their role, they must recognize the invading fungi, internalise, and kill them within the phagosome. Major advances in the field have elucidated the roles of pattern-recognition receptors in the innate immunity sensing and the importance of reactive oxygen and nitrogen species in intracellular killing of fungi. Surprising exit mechanisms for intracellular pathogens and extracellular traps have also been discovered. These and several other recent breakthroughs in our understanding of the mechanisms used by phagocytes to kill fungal pathogens are reviewed in this work.

Addresses
Albert Einstein College of Medicine, 1300 Morris Park Avenue, Forchheimer Building, Room 411, Bronx, NY 10461, USA

Corresponding author: Nicola, André Moraes (anicola@aecom.yu.edu), Casadevall, Arturo (casadeva@aecom.yu.edu) and Goldman, David L (dgoldma@aecom.yu.edu)

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Introduction

A diverse group of fungi is known to infect humans. These organisms range from small unicellular yeasts to those that produce long filamentous hyphae and come from several different phyla, indicating the great evolutionary distance between them. The diseases they cause are equally diverse, ranging from simple self-limited, subclinical flu-like illnesses and superficial skin or mucosal infections to life-threatening systemic mycoses. Despite this great variability, fungal infections share a common theme with respect to the central role of phagocytes in the host response.

The incidence of fungal infections has been steadily rising in the past decades owing to a variety of factors, including the AIDS epidemic. *Cryptococcus neoformans*, *Pneumocystis jirovecii*, and *Histoplasma capsulatum* are major pathogens for patients with AIDS. Improvements in healthcare, such as the advent of immunosuppressive therapy for transplant recipients, novel immunotherapies for rheumatologic conditions and cancer chemotherapy, have also led to an increase in fungal infections.

Fungi infect humans via several different routes, including: attachment and invasion of damaged skin, inhalation and deposition in the respiratory tract, and direct inoculation into deep tissues. Regardless of the route of infection, macrophages play a primary role in the initial interaction between host and pathogen. Other phagocytic cells, such as neutrophils and dendritic cells (DCs), are also intimately involved in the initial host–pathogen interaction.

The increased incidence of fungal diseases has led to a surge of interest in their pathogenesis, a topic that has been the subject of extensive reviews [1,2]. The objective of this article is to review the most recent studies on the role of phagocytes in immunity to fungi.

The interaction between phagocytes and fungi can be divided into fungal recognition, phagocytosis, and intracellular killing. In addition, phagocytes have evolved mechanisms for phagocytosis-independent killing of fungi. Each of these subjects will be reviewed in more detail.

Recognition of fungi

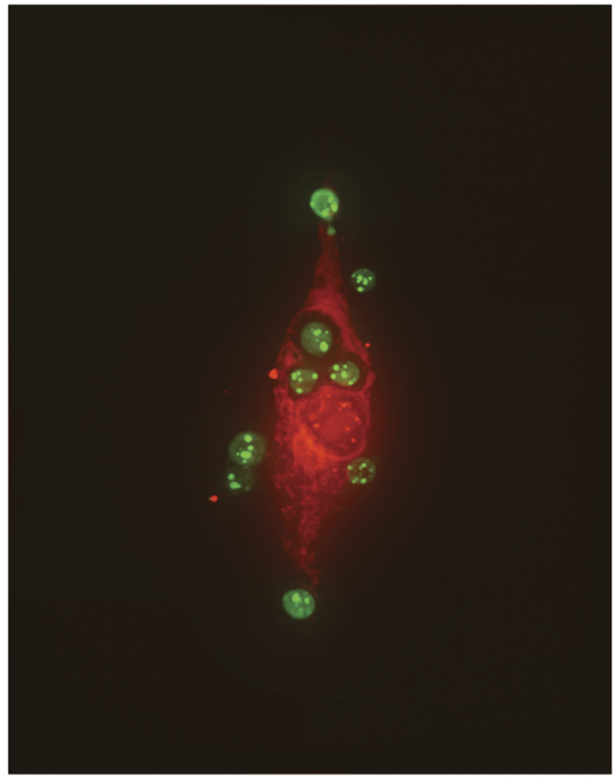
Macrophages, neutrophils, and DCs are innate immune system phagocytic cells, and as such, non-specific immune effectors. This paradigm has been questioned by the discovery of pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs) and lectin receptors (LR). These receptors recognize pathogen-associated molecular patterns (PAMPs) that are commonly found in a wide range of pathogens but not on the mammalian host. As a group, fungi share surface structural features including β-glucans, chitin, and mannoproteins that could allow recognition by a common set of receptors. The engagement of TLR and LR by fungi leads to phagocytosis, generation of anti-fungal molecules, and cytokine production.

A single fungal species can be recognized by different PRRs. *Candida albicans*, for instance, has been shown to bind to TLR1, TLR2, TLR4, TLR6, TLR9, mannose receptor (MR), Dectin-1, Dectin-2, galectin 3, as well as to the lectin domain of complement receptor 3 (CR3) (reviewed in reference [3]). Binding of cell-wall β-glucan to Dectin-1 on the surface of macrophages induces production of both anti-inflammatory interleukin 10 (IL-10) and pro-inflammatory tumor necrosis factor-alpha (TNF-α) cytokines [4]. It may also be involved in induction of eicosanoid inflammation mediators in macrophages [5] and NADPH oxidase activation in DCs [6], generating fungicidal reactive oxygen species (ROS). The importance of Dectin-1 in the host response to *C. albicans*, though, is unclear. Two studies using Dectin1-knockout mice reported contradicting results in susceptibility to *C. albicans* infection [7,8].

The role for mannan receptors TLR2 and TLR4 has also been under intense scrutiny. TLR2 was proposed to be important in immunity against *C. albicans*, while TLR4 was not [9]. However, studies with knockout mice and mutant *C. albicans* strains have shown the importance of TLR4 [10••]. Galectin-3 is a β-1,2 mannan receptor that specifically recognizes the pathogenic yeast *C. albicans* but not the non-pathogenic *Saccharomyces cerevisiae* [11] and exerts direct fungicidal effect [12]. TLR1 and TLR6, known to form heterodimers with TLR2, have been recently shown to have no or mild effect on macrophage recognition of *C. albicans* [13].

Another pathogen for which PRRs recognition is extensively studied is the filamentous mold *Aspergillus fumigatus*. TLR2 and TLR4 bind *A. fumigatus* cell-wall components and induce cytokine expression in a MyD88-dependent fashion [14]. TLR2 and Dectin-1 have been implicated in the differential recognition of resting conidia and germ tubes [15] and in the phagocytosis by macrophages [16]. However, studies with knockout mice have shown that phagocytes derived from immunocompetent hosts can still control infection with conidia in TLR2, TLR4, and MyD88 knockouts [17]. *A. fumigatus* has also been shown to contain unmethylated CpG DNA sequences that bound TLR9 and induce secretion of pro-inflammatory cytokines by DCs [18].

Figure 1



Phagocytosis of *C. neoformans* murine macrophage-like J774 cells and *C. neoformans* labeled with cell-tracer dyes were incubated in the presence of opsonizing antibody. Some fungal cells have already been internalized, while others are only attached to the cell membrane.

Binding to PRRs has been also documented with other fungi. *C. neoformans* activates dendritic cells via TLR9 [19] and DC-SIGN [20]. In contrast to other fungi, it does not induce signaling through Dectin-1 [21] or TLR4 [22] and only mildly affects cytokine expression via TLR2 [22]. *P. jirovecii*, on the contrary, requires Dectin-1 [7], TLR2, [23] and MR, to induce cytokine release by phagocytes.

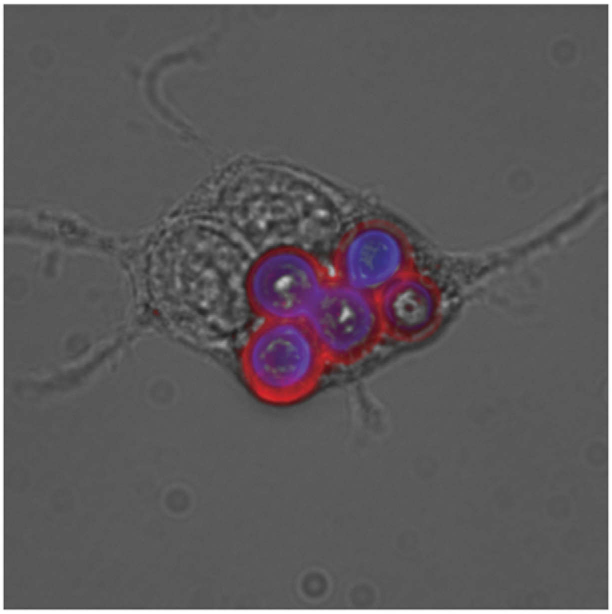
Phagocytosis

Following recognition of fungi as non-self, phagocytes attempt to internalize these organisms and transfer them into phagosomes (Figure 1). This allows local delivery of microbicidal molecules and restriction of essential nutrients leading to pathogen death while minimizing damage to neighboring cells.

The first step in phagocytosis is the attachment of the pathogen to the phagocyte. This attachment can be mediated either directly via PRRs or indirectly through opsonins, molecules that bind to the pathogen and are recognized by surface receptors in the phagocyte. The most studied opsonins are complement proteins and immunoglobulins (Ig), although recent reports also highlight the role of mannose-binding lectin (MBL) and surfactant protein A (SP-A) in opsonization of fungal cells.

MBL binds mannans in the cell walls of *C. albicans* both *in vitro* and *in vivo* [24], leading to complement deposition via the lectin pathway and subsequent phagocytosis [24,25]. By contrast, MBL binding to *Blastomyces dermatitidis* masks 1,3-beta-glucan recognition by macrophages, hindering the secretion of TNF-α [26]. In *C. neoformans*, mannans recognized by the MBL are concealed by the capsule [27], which also hides SP-A binding sites [28]. However, SP-A binding to encapsulated *C. neoformans* is facilitated by IgG, an effect that does not appear to be significant to immunity because SP-A knockout in mice is not disease-enhancing [28]. The cryptococcal capsule (Figure 2), one of its most important virulence factors, also hides cell-wall-associated complement binding sites, inhibiting complement-mediated phagocytosis [29].

Figure 2



Phagocytosis of encapsulated *C. neoformans* by J774 cells. Murine macrophage-like J774 cells were infected with IgG-opsonized *C. neoformans* and stained with anti-capsule antibody (red) and cell-wall-binding Uvitex 2B (blue).

In addition to innate immunity opsonins, adaptive antibodies arise during the course of fungal infections. These proteins have a greater versatility in binding specificities and are gaining increasing attention owing to evidences of their importance in immunity to fungal infections [30]. Antibody-coated *C. albicans* yeasts and germ tubes are internalized and killed more effectively than non-opsonized cells [31]. Antibody-mediated *in vitro* phagocytosis of *C. neoformans* has been linked to macrophage cell cycle progression [32].

Intracellular killing

Fungicidal molecules in the phagosome can be classified as oxidative (e.g. hydrogen peroxide, nitric oxide (NO), and oxygen- and nitrogen-derived oxidants) and non-oxidative (e.g. anti-fungal peptides and enzymes). While these mechanisms have been known for a long time [1,33], some interesting findings have been recently reported. In general, suppression of nitric oxide generation has been associated to impaired anti-fungal defense. Fernandes *et al.*, though, have recently shown that it has a beneficial effect in *Sporothrix schenckii* murine infection [34••]. Also, the absence of ROS resulting from phagocyte NADPH oxidase depletion reduced fungal dissemination and protected mice against *C. neoformans* infection [35••]. By contrast, mice deficient in neutrophil myeloperoxidase, an enzyme that generates toxic hypohalous acids from H_2O_2 and halides, exhibited marked increase in dissemination and death caused by *C. neoformans* [36•].

Acidification of the macrophage phagosome is also an important tool in killing fungal pathogens. Newman *et al.* have shown that murine macrophages require phagosomal acidification to kill *H. capsulatum* cells, whereas human macrophages do not [37]. Acidification has also been shown to be necessary for recruitment of CD63, a molecule that participates in antigen presenting by class II major histocompatibility complex (MHC), to *C. neoformans*-containing phagosomes [38].

The microbicidal effects of toxic molecules in the phagosome are augmented by the restriction of essential nutrients to the pathogen [39]. The most studied of these nutrients is iron, which is essential for growth by all microorganisms. Very low amounts of free iron are usually available in tissue fluids, with the element being largely bound to storage proteins. Phagocytes use additional iron-binding proteins to further reduce the iron availability. One of these proteins, lactoferrin, has been recently shown to be one of the fungicidal tools used by PMNs to control *A. fumigatus* [40]. Recent studies on the transcriptional response of fungi to phagocytosis have also demonstrated the lack of other nutrients as well. Engulfed *C. neoformans* cells induce expression of 19 sugar, phosphate, vitamin, purine, ammonium, aminoacid, and iron transporters, as well as the glyoxylate cycle [41], necessary for the utilization of alternative carbon sources. Studies with *Paracoccidioides brasiliensis* demonstrate induction of amino acid synthesis enzymes uptake transporters, as well as the glyoxylate cycle in cells recovered from *in vitro* infected macrophages [42,43]. The importance of oxygen depletion has also been recently stressed by studies with *C. neoformans* mutants sensitive to hypoxia, which were hypovirulent [44,45].

In response to all of the tool phagocytes use to promote intracellular killing, fungi evolved a long list of escape mechanisms. A recent addition to this list is the phenomenon of phagosomal extrusion or expulsion [46•,47•], in which internalized *C. neoformans* is expelled from macrophages and both cells remain alive.

Non-phagocytic killing

Phagocytes can also kill fungi using phagocytosis-independent mechanisms. This is readily apparent in the case of filamentous fungi, in which a single hypha is much larger than the phagocyte itself and cannot be ingested. Neutrophils are most frequently associated with extracellular killing mechanisms that involve the release of large amounts of ROS and granule components in the extracellular medium (reviewed in reference [2]). A recent report with *A. fumigatus* and *Rhyzopus oryzae* hyphae has shown that this process is probably regulated by pathogen recognition systems. Human PMNs produced equivalent amounts of superoxide anion in response to both, but released larger quantities of ROS when challenged with *A. fumigatus* [48].

Two novel mechanisms of extracellular neutrophil-mediated immunity have been recently discovered. Bonnet *et al*. have shown that neutrophils form aggregates around *A. fumigatus* conidia and that this aggregation inhibited conidial germination in a NADPH oxidase-dependent manner [49]. Another mechanism, named neutrophil extracellular trap (NET), has been described in defense against bacteria [50]. These NETs are composed of chromatin-based web of fibers studded with toxic components of the PMN granules, which restricts the pathogen in a highly toxic environment. NETs have been identified in immunity to several bacteria, in auto-immunity and even in fertility (reviewed in reference [51]). So far, *C. albicans* is the only fungal pathogen known to induce the formation of NETs that mediate killing of both yeast and hyphal forms [52].

Conclusions

Our review of the literature reveals a striking diversity in the interactions between phagocytic and fungal cells. Several different mechanisms are used by phagocytes to kill and/or inhibit pathogens. The past two years have produced great advances in our knowledge about how pathogens are recognized through PRRs and how this recognition shapes intracellular killing and cytokine secretion. A large body of evidence has emerged in studies with *C. albicans* and *A. fumigatus*, but information for other important fungal pathogens is still scant. In parallel, technical advances in genetic engineering, genomics, and cell biology have also contributed to extending our understanding of phagocytosis and subsequent pathogen killing.

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