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INNATE ANTIFUNGAL IMMUNITY: THE KEY ROLE OF PHAGOCYTES

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Abstract

Fungal diseases have emerged as significant causes of morbidity and mortality, particularly in immune compromised individuals, prompting greater interest in understanding the mechanisms of host resistance to these pathogens. Consequently, the last few decades have seen a tremendous increase in our knowledge of the innate and adaptive components underlying the protective (and non-protective) mechanisms of anti-fungal immunity. What has emerged from these studies is that phagocytic cells are essential for protection, and that defects in these cells compromises the hosts ability to resist fungal infection. This review covers the functions of phagocytes in innate anti-fungal immunity, along with selected examples of the strategies that are used by fungal pathogens to subvert these defences.

Keywords

pattern recognition receptor; phagocytosis; inflammation; oxidative and non-oxidative microbial killing; Th17

INTRODUCTION

There are well over a million species of fungi but only a few can be considered to be truly pathogenic and capable of causing disease in individuals with intact immune systems. However, many fungal species (including saprophytes, such as Aspergillus, or commensals, such as Candida) are opportunistic pathogens which will cause disease when there is an alteration in immune status or if the physical barriers of the host are breached. Indeed, infections with this latter group have increased significantly over the last few decades as a direct result of modern medical interventions, immunosuppressive therapies and AIDS. There are, for example, an estimated one million new infections with cryptococcus every year, and Candida is now ranked fourth on the list of nosocomial agents of sepsis (1, 2). Furthermore, despite the availability of anti-fungal agents, systemic infections with these organisms have a poor prognosis and high rates of mortality. Infections with Aspergillus, for example, can have a mortality rate exceeding 80%, and are one of the most feared complications in patients with haematological malignancies (3).

Interest in the immunology of fungal infections has lagged behind those of other pathogens, perhaps because historically there has been little medical need. However, with the considerable increase in the prevalence and severity of fungal diseases there has been a

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greater focus in recent times on understanding the host response to these pathogens. The last two decades, in particular, have been incredibly exciting, with major discoveries providing substantial new insights into the mechanisms underlying innate and adaptive anti-fungal immunity. Central to all of these studies is the demonstration that phagocytic cells (neutrophils, monocytes, macrophages and dendritic cells) are essentially required for protection against fungal pathogens, and loss of these cells, or defects in their anti-fungal effector functions, results in susceptibility.

While an innate response mediated primarily by neutrophils and macrophages is sufficient for controlling infections with some fungi, full protection against most pathogens also requires an adaptive response, that is initiated and directed by dendritic cells (DC) with contributions from other cells, such as monocytes (4). This ultimately generates an immune response involving several fungal-specific components, but the augmentation of the anti-fungal activities of phagocytes (through the actions of IFN-γ for example) is required for protection in all cases. In this review, I will explore the mechanisms of innate antifungal immunity mediated by phagocytic cells; covering the mechanisms they use to recognise, ingest and kill these pathogens, as well as the mechanisms they use to induce inflammation and to initiate the development of adaptive immunity. Selected examples of strategies that are employed by fungi to overcome or subvert these defences will also be discussed.

RECOGNITION OF FUNGI

The innate recognition of fungi by phagocytes is achieved largely through the sensing of cell wall constituents, although other internal components (such as fungal DNA) can also be detected (Table 1). The cell wall is composed predominantly of carbohydrate polymers which provide a rigid framework that gives the organism its shape and protection from the environment, and although not well studied for many species, is thought to consist of an inner meshwork of β-glucans and chitin, covered by an outer layer of mannosylated proteins (mannan). Some of the internal components may, however, be exposed in specific areas on the cell surface, such as the bud scar in *C. albicans* (5). Furthermore, the cell wall is a dynamic structure and able to change significantly, particularly during the morphological transitions that many fungi undergo. For example, *C. albicans* can reversibly switch between yeast and filamentous forms, an activity that is thought to contribute to the virulence of this pathogen (6). The composition of the cell wall also varies between different fungal species and between the different morphological forms of the same species.

Fungal components are detected by membrane-bound pattern recognition receptors (PRRs), which enable the direct (or non-opsonic) recognition of fungi by the phagocyte. However, recognition of these organisms can also be indirect, where soluble secreted PRRs (which can be produced by non-phagocytic cells) coat or “opsonise” the pathogen allowing recognition through membrane-bound opsonic receptors. A good example is complement, which is activated through several mechanisms by all fungi, including exposed β-glucan, allowing recognition of the opsonised organisms by phagocyte complement receptors (CR), such as CR3 (7). The production of fungal-specific antibodies, following the initiation of the adaptive response, also enhances opsonic recognition, although the role of antibodies in antifungal immunity is not clear for all pathogens.

Of all the infectious agents, fungi are recognised by the widest range of membrane-bound and soluble PRRs (Table 1). However, no cytoplasmic intracellular receptors for fungi have yet been described, although they are able to activate the inflammasome (discussed later). While the role of many of these receptors are poorly understood, or have only been studied with a limited number of fungal species, they have been shown to mediate binding of the organism to the phagocyte and/ or trigger intracellular signalling pathways which induce

cellular responses, such as phagocytosis, anti-microbial effector functions, and the production of cytokines and chemokines. Polymorphisms in several of these receptors have been linked to susceptibility in humans (see Table 1 and below).

The interaction of intact fungi with host phagocytes is complex, involving multiple PRRs, and is not very well understood. All of the major fungal cell wall carbohydrates can be recognised by the innate immune system, although the actual structurally-defined fungal ligands that are recognised by the various PRRs are largely unknown. Furthermore, while receptors, such as the Toll-like receptors (TLRs) and Dectin-1, are involved in the sensing of many, but not all, fungal species, there is evidence to suggest that particular receptors are required for sensing distinct organisms. Examples include the C-type lectin, Mincle, which has preference for species of Malassezia, and Galectin-3, which allows phagocytes to discriminate between *C. albicans* and non-pathogenic yeast (8, 9).

To add further complexity, the same fungal species may be sensed by discrete subsets of receptors that are expressed on different phagocytic cells, and this may be influenced by the morphological form of the fungus. For example, the recognition of mannan on Candida yeast appears to be mediated primarily by the mannose receptor (MR) on macrophages, whereas the MR, Dectin-2 and DC-SIGN, contribute to the recognition of these structures on DC (10). In contrast, mannan on Candida hyphae may be preferentially sensed by Dectin-2 on both cell types (11-13).

Fungal Evasion of Recognition

Fungal pathogens employ several strategies to evade recognition by phagocytes, but a common mechanism involves the concealment of the cell wall pathogen associated molecular patterns (PAMPs). One of the best examples is the extracellular polysaccharide capsule of *Cryptococcus neoformans*, which is intimately linked to the virulence of this pathogen and covers the cell wall, preventing recognition and uptake by host cells (14). Other pathogens utilise components that are not recognised by PRRs, such as the outer cell wall α-glucan layer of *Histoplasma capsulatum,* or the external hydrophobin layer of *A. fumigatus* resting conidia, both of which mask underlying PAMPS, including β-glucan (15, 16). Concealment can also be associated with the morphological form of the pathogen, such as the hyphae of *C. albicans* which, unlike yeast, do not display β-glucans on the surface (5). There is now interest in developing therapeutic approaches, including the use of established anti-fungal drugs such as echinocandins, to “unmask” cell-wall PAMPs so as to enhance innate fungal recognition and clearance (17, 18).

Fungi also utilise other mechanisms to subvert recognition, such as the modulation of complement activation or the targeting of “permissive” host receptors or cells. Fungi such as Aspergillus and Candida, can prevent complement opsonisation by recruiting host-derived regulatory proteins to the fungal surface (Factor H and C4 binding protein, for example), or by secreting fungal-derived complement degrading proteases and inhibitory factors (19, 20). Other fungi, including *Blastomyces dermatitidis*, actively target “permissive” phagocyte receptors, such as CR3, thereby avoiding or inhibiting the activation of anti-fungal effector mechanisms (21). Finally, several pathogenic fungal species, such as Cryptococcus, Candida and Aspergillus, can induce their uptake into endothelial and epithelial cells, which is likely to help these organisms escape recognition by phagocytes (22).

FUNGAL UPTAKE

The recognition of fungi by receptors on phagocytes leads to their internalization through the actin-dependent process of phagocytosis, whereby cellular membranes enclose the fungal particle resulting in the formation of an intracellular vacuole called the phagosome.

Although only examined in limited detail for a few species, the ingestion of fungi by phagocytes appears to involve several different mechanisms which depend on the host cell type, presence of opsonins, fungal cell wall composition, and the morphological form of the fungus. In DC, for example, the ingestion of Candida yeasts or Aspergillus conidia occurs through coiling phagocytosis, whereas the hyphae of both species are ingested by a distinct "zipper-type" mechanism (23, 24). Certain fungi can induce the formation of actin-based cellular structures that are thought to be involved in their uptake, such as the "ruffle-like" structures which resemble macropinosomes that are induced by Aspergillus conidia in macrophages (25), or the recently described "fungipods" that are induced in DC and macrophages by zymosan (a particulate cell wall extract of *S. cerevisiae*) and *C. parapsilosis*, but not by other Candida species (26). Uptake can also be influenced by the anatomical location in which the phagocyte-fungal interaction occurs, such as on mucosal surfaces or in tissues (27).

Fungal phagocytosis is most efficient when the fungi are opsonised with serum opsonins, but several non-opsonic PRRs (including the mannose receptor, DC-SIGN, Dectin-1, and SCARF1) can mediate fungal uptake, although there is still some debate about the phagocytic abilities of some of these PRRs, such as the MR (28). While the processes involved in phagocytosis by the opsonic receptors are reasonably well described, less is known about the mechanisms utilised by the non-opsonic receptors, which involve distinct signalling pathways and can vary between different cell types (see (29) for a recent review). Dectin-1, for example, triggers phagocytosis through a cytoplasmic immunoreceptor tyrosine based activation (ITAM)-like motif and (like traditional ITAM-coupled opsonic Fc receptors) requires Syk kinase for uptake in DC, yet this kinase was found to be dispensable for Dectin-1-mediated phagocytosis in macrophages (30, 31).

The TLRs, although not phagocytic receptors in their own right, also influence fungal phagocytosis. These receptors possess extracellular leucine-rich repeat ligand-binding domains and a conserved intracellular toll/IL-1R (TIR) signaling domain, which induces specific signaling cascades through intracellular TIR containing adaptors, such as MyD88. The effects of these receptors on fungal phagocytosis are still unclear, but appear to vary depending on the host cell type and fungal species involved. For example, the uptake of Aspergillus conidia by macrophages has been shown to involve TLR2, but not MyD88, whereas in neutrophils this process required MyD88 and TLR4, but not TLR2 (32-34). In contrast, the uptake of Candida by macrophages required MyD88, but no specific TLR (33, 34)

Phagosome Maturation

Following internalization, the phagosome matures through a number of sequential steps involving extensive vesicle fission and fusion events, largely with components of the endosomal network, leading to the development of the phagolysosome, a compartment with potent anti-microbial activities in which the ingested fungus is killed and digested. An important outcome of this process, particularly in DC, is the generation and presentation of fungal antigens, which along with selected cytokines, leads to the induction and shaping of adaptive immunity (see later). The anti-microbial activities are acquired during phagosome maturation, and involve acidification through the actions of vacuolar ATPase (V-ATPase) proton pumps, and the acquisition of a variety of oxidative and non-oxidative effector mechanisms, as discussed below.

The factors controlling the kinetics of phagocytosis and phagosome maturation are incompletely understood, but appear to be be regulated during fungal ingestion. In macrophages, for example, the phagocytosis of live or heat-killed Candida results in the rapid accumulation of maturation markers to the fungal phagosome (35). These effects are

likely to be mediated by the receptors involved in fungal recognition, including the TLRs (36). Although the role of the TLRs in phagosome maturation is still controversial (37), there is evidence that they influence fungal phagocytosis and that this may be linked to their ability to engage the autophagy pathway (38). Recent evidence suggests that the activation of cellular enzymes, such as group V secretory phospholipase A_2, by opsonic and non-opsonic receptors also plays an important role in the control of phagosome maturation in response to fungi (39).

The recognition of extracellular pathogens by PRRs is generally thought to occur at the cell surface or early phagosomes, but fungal recognition can also occur during phagosome maturation. The MR for example, was found to be recruited to maturing phagosomes following the uptake of Candida, and was not involved during the initial recognition and ingestion of these microbes (40). Recognition may also occur in the phagolysosome, although the evidence for this is indirect. In mature macrophage phagosomes, for example, the transition of Candida yeast to hyphae induces actin polymerization (41), whereas the swelling of Aspergillus conidia stimulates the respiratory burst (42); responses which are suggestive of receptor mediated recognition and signaling.

Fungal Evasion of Phagosomal Maturation

Fungal pathogens use a variety of strategies to survive following uptake by phagocytes, including inhibiting phagosomal maturation, escaping from the phagosome, and resisting the degradative phagolysosomal environment. Two fungal species, *H. capsulatum* and *C. albicans,* are capable of altering phagosomal maturation, although the underlying mechanisms by which this is achieved are unknown. *H. capsulatum* can have several effects on maturation, including inhibition of phagosome-lysosome fusion and modification of the intra-phagosomal pH (which is achieved in part through exclusion of the V-ATPase), however the magnitude of these activities depend on the type of host phagocyte (43). *C. albicans* has similar effects on maturation, and although phagosomes rapidly acquire markers of maturation following uptake (discussed above), these markers are then recycled out of phagosomes containing live fungi at later time points (44). *C. albicans*, and *A. fumigatus*, can also escape from the phagosome by generating hyphae, which leads to disruption of the vesicle and ultimately the destruction of the host cell. Interestingly, this only occurs in certain phagocytes (such as selected macrophage and monocyte populations), whereas neutrophils are able to block hyphal development in both species, in part through the formation of cellular aggregates (45). In addition, *C. albicans* may also be able to escape by inducing host cell apoptosis using mechanisms which are unknown, but possibly involve Candida proteases (46).

Cryptococcus neoformans is also able to escape from the phagosome and the mechanism used by this organism is noteworthy as it does not lead to host-cell death, but involves a fungal-directed expulsion from the phagocyte that is likely to facilitate infection of adjacent cells (47). *C. neoformans* has also adapted to survive within the phagolysosomal vacuole and appears to require this environment in order to replicate, which it does so rapidly, leading to lysis of the host cell (48). However, this pathogen does alter the integrity of the phagolysosomal membrane, allowing access to the cytoplasmic contents and leading to the accumulation of fungal-derived polysaccharide-containing vesicles within the phagocyte (49).

FUNGAL KILLING BY PHAGOCYTES

Phagocytes utilize a number of oxidative and non-oxidative mechanisms which work synergistically to kill extracellular and internalized fungi. These activities are strongly influenced by the state of cellular activation, which is controlled largely, but not exclusively,

through the actions of cytokines and other soluble mediators, which can both enhance (such as IFN-γ) or suppress (such as IL-10) the anti-fungal effector mechanisms. Different phagocytes vary in their ability to kill fungi or restrict their growth, and these activities are dependent on the fungal species involved. With *C. albicans*, for example, neutrophils are the most potent effector cells, followed by monocytes, macrophages and then DC (50). In contrast, DC possess fungicidal activity towards *H. capsulatum*, whereas neutrophils are only fungistatic, and non-activated macrophages and monocytes are permissive for intracellular fungal growth (51). Neutrophils are thought to be essential for resistance to infections with most fungal pathogens, but they may actually contribute to susceptibility to infection with *C. neoformans*, despite the fact that these cells show fungicidal activity towards this pathogen in vitro (52).

Oxidative Anti-Fungal Mechanisms

The Respiratory Burst—The production of reactive oxygen intermediates (ROI), also termed the respiratory burst, is thought to be a major component of the anti-fungal defense mechanism of phagocytes and is mediated through a multi-component protein complex, the phagocyte NADPH oxidase (phox). This complex, which includes the phagocyte component NOX2 (gp91^{phox}), assembles at the phagosomal or plasmalemma membrane (in response to non-ingestible particles) upon activation by cellular receptors, and transfers electrons from cytoplasmic NADPH to O_2, resulting in the production of superoxide. Superoxide itself has limited, if any, toxicity, but is converted through processes such as the Haber-Weiss reaction, to toxic reactive oxygen intermediates, including hydroxyl radicals and hydrogen peroxide (53). Other effective fungicidal oxidants that can be produced are hypochlorous and hypoiodous acid, which are generated from hydrogen peroxide by myeloperoxidase (MPO), an enzyme located in the azurophilic granules of neutrophils and in lysosomes of monocytes (54). Although macrophages are deficient in this enzyme, they can scavenge MPO through mannose receptors and traffic it to phagolysosomes where it can contribute to fungal killing (55).

The importance of the phagocyte NADPH oxidase is exemplified by Chronic Granulomatous Disease (CGD), an inherited disease resulting in an inability to generate superoxide. Patients with this disease suffer from chronic inflammatory conditions and recurrent, and often lethal fungal (and bacterial) infections, particularly with invasive aspergillosis, but also with a variety of other fungi, including Candida (56). There are also mutations which result in the loss of MPO which increases the risk of infection with Candida, however, the majority of individuals with MPO deficiency are asymptomatic (56). Gene-deficient mouse models have recapitulated the importance of these systems for several fungi, including Candida, Cryptococcus and Aspergillus (57, 58).

The antifungal activities of the respiratory burst are generally considered to arise from the toxic effects of the reactive oxygen species produced, but several lines of evidence suggest that the fungicidal activity of this response is more complex. In neutrophils, for example, the respiratory burst was found to induce a potassium influx and rise in phagosomal pH, which was required for the release and activation of antifungal proteases from the proteoglycan matrix of neutrophil granules (59). More recently, the susceptibility of CGD mice to aspergillosis was shown to be due to a block in tryptophan catabolism, catalyzed by the superoxide-dependent indoleamine 2,3-dioxygenase (IDO), which caused inappropriate IL-17-mediated neutrophil activation and inflammation, and resulted in defective anti-Aspergillus immunity (60).

Several soluble and membrane bound PRRs are capable of triggering the respiratory burst, including Galectin-3, Dectin-1, Fcγ receptors and the TLRs. Galectin-3 can induce the respiratory burst in activated neutrophils through undefined mechanisms, although this

activity has not yet been associated with fungi (61). Dectin-1 and the Fcγ receptors are capable of directly triggering the respiratory burst through their cytoplasmic ITAM sequences and intracellular signaling via Syk kinase, however, the ability of Dectin-1 to induce this response may be restricted to specific subset(s) of phagocytes that are able to activate Syk (62). The TLRs can also directly induce the respiratory burst in leukocytes, through a MyD88 and Vav dependent pathway, and can prime these cells for an enhanced respiratory burst upon recognition of fungal particles by other PRRs, including Dectin-1 (63, 64).

Reactive Nitrogen Intermediates—The production of reactive nitrogen intermediates (RNI) by the inducible nitric oxide synthase (iNOS or NOS2) is another oxidative system of phagocytes that is thought to possess fungicidal activity, although its importance in humans is still uncertain. This system can be induced by the TLRs and cytokines (such as TNF and IFN-γ), leading to the production of nitric oxide (NO) through the oxidative deamination of L-arginine. NO, which itself has little antimicrobial activity, reacts further with superoxide to produce peroxynitrite, which can kill fungi (53). There is a large body of literature (some of it contradictory) examining the role of RNI in the control of various fungal pathogens in vitro, but data from iNOS-deficient mice suggests that this pathway may only be essential for the control of selected pathogens, such as *C. neoformans* (65). Although not required for the in vivo control of fungi, such as Aspergillus or Candida, the role of iNOS has yet to be fully explored under all experimental contexts (eg: oral versus systemic infection) (42, 66). Indeed, mice deficient in both iNOS and $gp91^{phox}$ were found to be extremely susceptible to candidiasis but, importantly, phagocytes from these mice did not show any defect in their capacity to kill Candida in vitro, highlighting the effectiveness of the non-oxidative fungicidal systems that are also present in these cells (67).

Non-Oxidative Anti-Fungal Mechanisms

Phagocytic cells, particularly neutrophils, possess a number of non-oxidative mechanisms which are very effective at killing intra- and extracellular fungi or restricting their growth, including antimicrobial peptides, hydrolases, and components designed to restrict access to essential nutrients. Several of the better characterized systems will be discussed here, but the roles of many of the non-oxidative antimicrobial components, such as neutrophil gelatinase-associated lipocalin, bactericidal/permeability-increasing protein (BPI), and Galectin-3 for example, have only been briefly examined or not at all (68, 69). A number of soluble PRRs, such as Galectin-3 and the surfactant proteins, also contribute to fungal killing or growth inhibition, but the mechanisms by which this is achieved are poorly understood (69, 70).

Antimicrobial peptides (AMP)—Mammals express a large variety of anti-fungal AMP that are secreted by epithelial cells or produced in secretions, such as the β-defensins and histatin 5, and a number of AMP that are produced in phagocytic cells. The α-defensins (HNP1-HNP4), for example, are small cationic peptides that are found in the azurophilic granules of human, but not murine, neutrophils. These peptides can kill numerous fungal pathogens, including *C. albicans*, *A. fumigatus*, *H. capsulatum* and *C. neoformans*, but their fungicidal mechanisms are unclear. Studies with *C. albicans* have suggested that these AMP bind to specific sites on the fungal membrane, inducing nonlytic permeabilization and the release of intracellular ATP (71).

The cathelicidins are a group of AMP characterized by the presence of a conserved N-terminal cathelin domain that is proteolytically cleaved from the highly heterogeneous C-terminal cationic peptide, which possesses the antimicrobial activity. Humans (and mice) only possess one cathelicidin, termed hCAP-18, which is found in neutrophil granules, monocytes, natural killer (NK) cells, lymphocytes and epithelial cells, and in a number of

secretions (such as sweat, seminal fluid, and plasma). The cleavage of hCAP-18 produces LL-37 and several other differentially cleaved peptides which possess antifungal activity (72). LL-37 and other cathelicidins can kill Candida and Cryptococcus by disrupting their cellular membranes, although they do not appear to be effective against filamentous fungi, such as Aspergillus (73, 74). However, the physiological role of these AMP is still unclear, as deficiency of the mouse cathelicidin (mCRAMP) was found to have no effect on in vivo resistance to Candida (75).

Hydrolases—Lysozyme is a cationic anti-microbial enzyme that is found in the granules and lysosomes of granulocytes, monocytes and macrophages, but also in blood and a number of secretions. Although traditionally associated with anti-bacterial activity, lysozyme also has activity against fungi and has been shown to kill or inhibit the growth of Candida, Cryptococcus, Histoplasma, Aspergillus, and Paracoccidioides (76). The effects of lysozyme on fungi are unclear, but may involve enzymatic hydrolysis of N-glycosidic bonds within the fungal cell wall and / or injury to the cell membrane (77).

The serprocidins are a family of serine proteases stored within neutrophil granules which possess antifungal activity, but which also perform many other cellular functions including the processing of cathelicidins (78). The importance of these proteases, including proteinase-3, cathepsin G and elastase, has been demonstrated for a number of pathogens, such as Histoplasma, Aspergillus and Candida (59, 68, 79). The activation of these proteases by the respiratory burst (described above) has been proposed as the primary mechanism of microbial killing (80). However, azurocidin, a serprocidin homologue which lacks protease activity is still fungicidal, suggesting that the antimicrobial activities of these proteases may not solely be related to their enzymatic activity (81).

Limitation of nutrients—The limitation of nutrients is an effective anti-fungal mechanism which is achieved by containment of ingested fungi within the phagosome and by active restriction of nutrient availability. Limiting iron, for example, is essential for controlling many fungal infections and is achieved through a number of mechanisms. This includes sequestration by lactoferrin, an iron-binding protein which also possesses direct antimicrobial activity that is found in neutrophil granules and most exocrine secretions (including breast milk, intestinal mucus, saliva and tears) (82). Iron levels can also be controlled intracellularly following phagocytosis, by the down regulation of transferrin receptors, which reduces iron delivery to the phagosome through the endosomal-recycling network, and by transporter proteins, such as natural resistance-associated macrophage protein-1 (Nramp-1), which removes iron and other divalent cations from the phagosome (83).

Another effective mechanism of nutrient limitation is the sequestration of zinc by the dimeric molecule calprotectin (also termed MRP8/MRP14 or S100A8/S100A9). Calprotectin, whose antifungal activity is activated by oxidative stress, is a major cytoplasmic component of neutrophils, but is also found in monocytes and epithelial cells, and although not actively secreted, is released following neutrophil death induced by fungi and at sites of inflammation (84, 85). Indeed, calprotectin mediates the anti-fungal activity of neutrophil extracellular traps or NETs; extracellular anti-microbial structures which are generated by a novel ROS-dependent death pathway. NETs consist of DNA, histones and granule proteins and have been shown to trap and kill both yeast and hyphal forms of *C. albicans* (86). NETs also contain PTX3, a soluble PRR that is stored in neutrophil granules which is essential for the recognition, uptake and killing of *A. fumigatus* by these cells (87). In fact, the restoration of NET formation has been proposed to reestablish resistance to Aspergillus infections in CGD patients following gene therapy (88).

Fungal Evasion of Phagocyte Mediated-Killing

I have already discussed some mechanisms by which fungi can avoid the antimicrobial activities of phagocytes, such as escaping from the phagosome, masking cell-wall PAMPS, or targeting "permissive" phagocyte receptors for uptake. But fungi also employ several strategies to cope with oxidative and nitrosative stress, including active suppression of these responses, such as occurs with Candida and Cryptococcus, although the underlying mechanisms are unclear (44, 89, 90). Many fungi resist these stresses by producing anti-oxidant enzymes, such as catalase and superoxide dismutase, and by inducing protective responses, such as DNA damage repair systems and heat shock proteins, that help the fungal cell cope with oxidative damage (91). Other non-enzymatic mechanisms of resistance include, for example, capsule enlargement by Cryptococcus and the production of scavengers of ROI and RNI, such as melanin, mannitol or trehalose (14, 92).

Fungi can resist the non-oxidative antimicrobial actions of phagocytes and the acquisition of host nutrients, particularly iron, has been an area of specific interest. Some pathogens, such as Aspergillus and Histoplasma, synthesize and secrete siderophores to capture iron and although fungi, such as Candida and Cryptococcus, do not produce these high-affinity chelators, they do express siderophore transporters and high-affinity iron permeases (93). Furthermore, some fungi, including Candida, Cryptococcus and Histoplasma, can obtain iron by attacking host iron-binding proteins, such as transferrin, ferritin or haemoglobin (93). These pathogens also change their metabolism to respond to nutrient deprivation in the host, such as occurs with Candida following ingestion by phagocytes, which results in upregulation of the fungal amino-acid biosynthesis and nitrogen assimilation machinery, and a shift from the glycolytic pathway to the glyoxylate cycle and gluconeogenesis (94).

FUNGI, PRRs AND THE INDUCTION OF SOLUBLE MEDIATORS

The recognition of fungi by phagocyte PRRs induces intracellular signaling pathways that results in the production of numerous cytokines, chemokines, eicosanoids and other soluble mediators which initiate and modulate inflammatory responses and help shape the development of adaptive immunity. These responses are crucial and deletion or polymorphisms within these PRRs can result in susceptibility. Intracellular signaling giving rise to these responses is mediated by TLR and non-TLR PRRs, including Dectin-1, mannose receptor, the Fcγ-coupled receptors (Dectin-2 and MINCLE), DC-SIGN, and the scavenger receptors (CD36 and SCARF1), and these receptors collaborate to initiate optimal antifungal immunity.

TLRs

The TLRs were first identified based on their ability to control fungal infections in Drosophila, and five mammalian TLRs have subsequently been implicated in the recognition of most fungal pathogens; namely TLR2, which heterodimerizes with TLR1 or TLR6, TLR4 and TLR9. Recognition of fungi by these receptors triggers the induction of numerous cytokines and chemokines, and mice deficient in the intracellular adaptor MyD88 are highly susceptible to infections with *C. albicans*, *P. brasiliensis*, *A. fumigatus* and *C. neoformans*, although defects in IL-1 receptor signaling were also probably contributing to these phenotypes (33, 95, 96). What is less evident, however, is the role of the individual TLR receptors in anti-fungal immunity, as there is contradictory literature for almost every TLR and fungal pathogen (96). TLR2 deficient mice, for example, have been shown to be more susceptible to infection with *C. albicans*, as a result of reduced proinflammatory cytokine production and neutrophil recruitment (97). However, TLR2 deficient mice have also been shown to be more resistant to infection with this pathogen, as a result of decreased IL-10 and increased production of IL-12 and IFN-γ (33, 98). The reasons for these

discrepancies are still unclear, but may be related, at least in part, to the experimental model and strain of fungus tested (99).

In humans, several polymorphisms in the TLRs have been linked to a susceptibility to fungal infection. A polymorphism in TLR4 (Asp229Gly) has been associated with increased susceptibility to invasive and chronic cavitary pulmonary aspergillosis, as well as bloodstream infections with Candida (100). A polymorphism in the promoter of TLR9 (T-1237C) has been associated with the development of allergic bronchopulmonary aspergillosis, and polymorphisms in TLR1 (Arg80Thr) and in both TLR1 (Asn248Ser) and TLR6 (Ser249 Pro) have been associated with invasive aspergillosis in transplant patients (100). Despite all of these associations, however, no increased susceptibility or defects in antifungal immunity have been found in patients with defects in MyD88 or other critical downstream signalling components, such as IRAK-4 (101).

Dectin-1

The β-glucan receptor Dectin-1 possesses a single extracellular C-type lectin-like domain (CTLD) that recognizes a variety of fungal species, including Candida, Coccidoides, Pneumocystis, Aspergillus, and spores of *C. neoformans* (102, 103) . In response to fungi, Dectin-1 induces intracellular signaling via its cytoplasmic ITAM-like motif through a pathway involving Syk kinase, PLCγ2 and Card9, and through the Raf-1 kinase pathway (102, 104). Dectin-1 signaling is also thought to mediate the activation of calcineurin (the target of cyclosporine A), a protein phosphatase whose activity is critical for the control of fungal infections in both mice and humans (105). Induction of these pathways results in the production of numerous cytokines and chemokines, including GM-CSF, TNF, MIP-2, IL-2, IL-10, IL-6, IL-23 and IL-1β, as well as arachidonic acid release and prostaglandin production. In all phagocytes, Dectin-1 can collaborate with the TLRs to modulate cytokine production (discussed below), however, the ability of Dectin-1 to induce these responses directly is cell-type dependent (102). For example, Dectin-1 can directly trigger cytokine production in DC, but not in macrophages, yet this receptor mediates fungal binding and uptake in both cell types (106, 107).

The production of IL-1β is of particular interest, as the generation of this cytokine requires activation of the inflammasome following sensing by cytoplasmic PRRs. Indeed, activation of the NLRP3 inflammasome and IL-1β production are induced in response to *C. albicans* and *A. fumigatus*, and this requires signaling through the Dectin-1 /Syk and Card9 pathway (95, 108). However, the mechanism by which these fungi actually activate the cytosolic NLRP3 inflammasome is still unclear, although it does involve fungal uptake, the respiratory burst and a potassium efflux.

Dectin-1 plays an imporant role in antifungal immunity, and mice deficient in this receptor have enhanced susceptibility to *C. albicans* (in one model) and *A. fumigatus*, which resulted from insufficient inflammatory responses and fungal killing, as well as slight defects in resistance to *P. carinii* (109-111). Furthermore, individuals homozygous for a polymorphism in human Dectin-1 (Y238X), which causes receptor misfolding and loss of expression from the cell surface, were susceptible to mucocutaneous infections with *C. albicans* and *Trichophyton rubrum* (112). This susceptibility was due to defects in cytokine production, but not fungal uptake and killing, explaining why these individuals did not succumb to systemic infections. Individuals with mutations in Card9, and Card9 deficient mice, were also susceptible to fungal infections (113, 114).

Mannose Receptor

The MR (CD206) was one of the first fungal PRRs to be identified and has been a focus of considerable interest, although many of the earlier studies are likely to be inaccurate as they were based on using fungal mannan as an inhibitor, which is also recognized by several other PRRs (Table 1). The MR possesses eight CTLDs through which it recognizes a number of fungi including *C. neoformans*, *C. albicans* and *P. carinii*, and despite lacking classical signaling motifs within its cytoplasmic tail, the MR has been implicated in mediating the production of a number of cytokines, including IL-6, TNF, MCP-1, GM-CSF, IL-1β, IL-12 and IL-10 (115). Although a soluble form of the MR is generated by proteolytic cleavage and shed into the serum, the majority is located intracellularly within the endocytic pathway, and the recognition of fungi by this receptor may occur only following uptake, as described above (40). Deficiency of the MR in mice did not significantly alter the resistance of these animals to infection with *C. albicans* or *P. carinii*, although there were minor effects on fungal burdens and pulmonary pathology, respectively (116, 117). However, the MR knockout mice have increased susceptibility to infection with *C. neoformans,* and this was related to defects in the development of adaptive responses to this pathogen (118).

Fcγ-coupled receptors (Dectin-2 and Mincle)

Dectin-2 and Mincle both possess a single extracellular CTLD and a short cytoplasmic domain that lacks consensus signaling motifs, but these receptors associate with the ITAM-containing adaptor molecule, Fcγ, inducing intracellular signaling through the Syk / Card9 pathway. Dectin-2 can recognize a number of fungal pathogens, including *H. capsulatum*, *P. brasiliensis*, unencapsulated *C. neoformans, C. albicans*, *T. rubrum*, and *Microsporum audounii*, although this receptor is thought to preferentially recognize hyphal forms of the latter three pathogens (11). Fungal recognition by Dectin-2 and signaling through the Fcγ chain, can induce the production of numerous cytokines, including TNF, IL-1Ra, IL-2, IL-10, IL-6, IL-1β, IL-12 and IL-23, and possibly cysteinyl leukotrienes (11-13, 119). Dectin-2 deficient animals were shown to be susceptible to infection with *C. albicans*, but not with *C. neoformans*, and although the underlying reasons for this susceptibility are unclear, it is thought to be linked to defective induction of Th17 type adaptive immunity (see below) (13).

Like Dectin-2, fungal recognition by Mincle induces the production of cytokines, including MIP-2, KC, IL-10 and TNF (8, 120). Despite initially being described as a receptor involved in the recognition of *C. albicans*, Mincle was subsequently shown to preferentially recognize Malassezia species, including *M. furfur* and *M. pachydermatis* (8, 120). Limited analysis of the Mincle knockout mice have suggested that they do have defects in their ability to control *C. albicans* infections, at least at an early time point after systemic infection, as well as having defective inflammatory responses to *Malassezia* (8, 120).

DC-SIGN

Human DC-SIGN (CD209) possesses a single extracellular CTLD, a stalk region which enables multimerization of the receptor, and a cytoplasmic tail containing internalization motifs. DC-SIGN recognizes several species of Candida, *A. fumigatus* conidia, *Chrysosporium tropicum* and possibly *C. neoformans* (121-123). There are several murine homologs of this receptor, but only SIGNR1 and SIGNR3 can recognize fungi (124). DC-SIGN can induce intracellular signaling through the Raf-1 kinase pathway, but the receptor does not appear to be able to directly induce cytokine production in phagocytes. Rather, DC-SIGN appears to modulate TLR-mediated cytokine production (125).

Scavenger receptors (CD36 and SCARF1)

The scavenger receptors CD36 and SCARF1 were recently identified as PRRs for *C. albicans* and *C. neoformans* and were capable of inducing cytokine production, although the intracellular signaling pathways leading to these responses are unknown (126). CD36, in particular, was found to be required for the production of IL-1β, TNF, IL-12p40, MIP-2, MIP-1α, MIP-1β and RANTES, in response to these fungal pathogens, and loss of CD36 resulted in increased susceptibility to systemic infection with *C. neoformans* (126).

Collaboration between PRRs in response to fungi—Fungi possess a diverse array of PAMPS and the recognition of intact organisms will involve interactions with many PRRs. There are now numerous examples where the interactions between PRRs, particularly the TLR and non-TLRs, have been shown to modulate responses to fungi, although our understanding of the underlying molecular mechanisms is still poor. Cooperative signaling between Dectin-1 and MyD88-coupled TLRs, for example, synergistically induces the production of cytokines such as IL-10, IL-6, TNF and IL-23, while simultaneously repressing the production of IL-12 (102). The interaction of DC-SIGN with the TLRs induces the production of IL-10, but also stimulates or represses the induction of cytokines, such as IL-12 and IL-6, depending on the nature of the carbohydrate ligand (125, 127). Other examples of cooperation between TLRs and non-TLR PRRs include the interactions between TLR2 and galectin-3 (*Candida*), SCARF1 (*C. neoformans*), and CD36 (*C. neoformans*) (9, 126). Although virtually nothing is known about the interactions between multiple PRRs, optimal responses to *Candida* were shown to require (at least) TLR2, TLR4, Dectin-1 and the MR (128).

Fungal Modulation of Inflammatory Responses—In addition to the various strategies such as morphogenic switching that are involved in masking cell wall PAMPS and preventing recognition by specific PRR, fungi have developed several other mechanisms to avoid, modulate and/ or suppress the induction of protective inflammatory responses. *P. carinii*, for example, may avoid MR-mediated recognition by secreting a MR-binding glycoprotein and by enhancing shedding of the MR from phagocytes (129). Fungi, such as *C. albicans,* can modulate inflammatory responses by targeting receptors, including DC-SIGN, FcγR, CR3 and TLR2, to induce the production of immunosuppressive cytokines, such as IL-10. Indeed, mice deficient in FcγR, CR3 and TLR2 (at least in some models) were found to be more resistant to infection with *C. albicans* (98, 125, 130).

Other fungi have evolved virulence factors which actively suppress inflammatory responses, such as BAD-1 of *B. dermatitidis,* which targets CR3 to inhibit the production of TNF (21). Another example is the polysaccharide glucuronoxylomannan (GXM), a major component of the capsule of *C. neoformans*, which induces the production of IL-10 by targeting inhibitory Fc receptors on monocytes (14). Interestingly, GXM (which is also shed by the fungus) stimulates the production of inflammatory cytokines in neutrophils, but inhibits their migration, in part, by inducing L-selectin shedding (14). *C. neoformans* also produces melanin, a virulence factor found in many other fungi, that has been associated with the suppression of proinflammatory cytokines, although the underlying mechanisms are unclear (131).

THE INDUCTION OF ANTI-FUNGAL IMMUNITY

Protection against fungal infections requires recruitment and activation of phagocytes, which is mediated through the induction and sensing of inflammatory cytokines and chemokines, and the development of T_H1-type adaptive immunity. This is supported by several lines of evidence, particularly for disseminated infections, from both human patients and animal models. Mice deficient in the T_H1 cytokines, IL-12 or IFN-γ, for example, are susceptible to

infection with many fungi, including *C. neoformans*, *A. fumigatus*, *C. albicans*, *H. capsulatum* and *P. brasiliensis,* and humans with defects in these pathways are susceptible to disseminated infections with *H. capsulatum* and *P. brasiliensis* (100, 132). There are also data (mouse knockout, gene polymorphism and /or clinical studies) supporting a role for other inflammatory cytokines, chemokines and chemokine receptors in protective antifungal immunity, including TNF, IL-1β, CXCL10, CCR2, CXCR2, and IL-6 (45, 100, 132-134).

In contrast, the production of immunosuppressive cytokines (such as IL-10) or the development of T_H2-type adaptive immunity is generally thought to contribute to fungal susceptibility, by repressing the antifungal activities of phagocytes. Indeed, patients with polymorphisms resulting in the upregulation of IL-4 or IL-10 are at increased risk of developing infections with fungi, such as Aspergillus or Candida (100). However, studies from murine models have suggested that the role of these cytokines is more complex. IL-4, for example, was found to be required for the induction of protective antifungal T_H1 responses, whereas IL-10 helped to limit inflammatory pathology and promote long-term memory immunity, in part through the actions of regulatory T cells (135, 136).

More recently, T_H17-type adaptive immunity has been implicated in the control of fungal infections, particularly at the mucosa, although this may only be relevant for specific pathogens (ie: mucocutaneous forms of candidiasis and not pulmonary Aspergillus infections) (137, 138). Indeed, defective T_H17 responses have now been linked to the fungal susceptibility that has been observed in diseases such as hyper-immunogolublin E syndrome and autoimmune polyendocrine syndrome 1 (138). Evidence from mouse models also suggests that Th17 responses may protect against disseminated infections (139). In contrast to these reports, however, there is also contradictory evidence suggesting that T_H17 responses may actually promote fungal susceptibility, by driving inappropriate neutrophil activation and suppression of T_H1 immunity (140). Although the reasons for these discrepancies are unclear, they may be related to the experimental models employed (eg: oral versus gastric candidiasis).

The interaction of fungi with phagocyte PRRs plays a key role in determining the cytokine and chemokine profiles which shape and influence the resultant immune response. The interaction of *C. albicans* with TLR4, for example, was found to promote T_H1 responses, whereas the interaction with Fcγ R or TLR2 promoted T_H2 responses (130, 141). More recently, the interaction of *C. albicans* with Dectin-1, Dectin-2 and the MR, was shown to induce T_H17 responses (12, 13, 142, 143). However, as discussed above, each PRR individually induces a complex pattern of cytokines (and other cellular responses), and how these are all integrated into a coordinated immune response is still unknown. Furthermore, the PAMPS displayed on the fungal surface, and the strategies utilized by these pathogens to alter the availability of these structures, such as morphogenic switching, may have significant effects and there is experimental evidence to support this. In mice, for example, the interaction of *C. albicans* yeast with DC was shown to induce T_H1-type responses, whereas hyphae induced T_H2-type responses (23).

CONCLUSION

Recent discoveries have shed significant new insights into the underlying mechanisms that provide resistance to fungal infections. Phagocytes are the central players in host resistance, and deficiencies in these cells or their antifungal effector mechanisms, result in susceptibility to infection. Conversely, fungal pathogens have evolved strategies to overcome these host defense mechanisms. A better understanding of the mechanisms utilized by both host and pathogen will hopefully enable the development of novel strategies for vaccination, immunotherapy and antifungal drugs.

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ACRONYMS

PRR	pattern recognition receptor
PAMP	pathogen associated molecular pattern
TLR	toll-like receptor
MR	mannose receptor
T_H	T helper
IL	interleukin
Card	caspase recruitment domain-containing protein
MPO	myeloperoxidase
CGD	chronic granulomatous disease
ROI	reactive oxygen intermediates
RNI	reactive nitrogen intermediates

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FUTURE ISSUES

1. Determining the differences in the recognition of yeast, hyphal and other fungal morphotypes (including cell wall composition and PAMP exposure) and how this affects the development of immunity.
2. Obtaining a better understanding of the roles of the various PRRs in directing the development of antifungal immune responses in vivo.
3. Understanding how the recognition by multiple PRRs is integrated, and how fungi can influence these responses.
4. Improving our understanding of the role of the immune response in commensalism with opportunistic pathogens, such as Candida, and what changes shift this towards pathogenesis.
5. Determining the immunological factors governing resistance and susceptibility at different anatomical sites, and particularly the role of epithelia in antifungal immunity.
6. Exploring the mechanisms of host resistance to poorly studied mycoses, particularly those endemic in developing countries.

TABLE 1

Fungal Pattern Recognition Receptors

Location	PRR	Selected Fungal PAMP(s) / Ligands
soluble	surfactant protein A[a]	mannan, glycoprotein A[b,Pc]
	surfactant protein D	mannan, β-glucan
	galectin-3	β-1,2-mannosides[Ca]
	mannose binding lectin[a]	mannan
	pentraxin-3	galactomannan[Af]
	ficolin-2	β-1,3 glucan, GlcNAc[c]
	complement	fungal surfaces, mannan, β-1,6 glucan
	c-reactive protein	phosphocholine
membrane	TLR1[a]	unknown
	TLR2[a]	mannan, phospholipomannan[Ca], GXM[d,Cn]
	TLR4	mannan, O-mannan[Ca], GXM[Cn]
	TLR6[a]	unknown
	TLR9[a]	fungal DNA
	Dectin-1[a]	β-1,3-glucan
	Dectin-2	α-mannan
	CR3	β-glucan, mannan, BAD-1[Bd], HSP60[Hc], GXM[Cn]
	DC-SIGN	mannan, galactomannan[Af]
	mannose receptor	mannan, N-mannan[Ca], GlcNAc, glycoprotein A[Pc]
	CD14	mannan, GXM[Cn]
	FcγR	mannan, GXM[Cn]
	Mincle	α-mannose
	SCARF	mannan, β-glucan
	CD36	β-glucan
	CD5	β-glucan
	very late antigen-5	cyclophilin A[Hc]
	langerin	mannan, β-glucan
unknown	unknown	chitin

[a] Polymorphisms in encoding genes that have been associated with susceptibility to fungal infections in humans.

[b] Fungal specific PAMPs: Pc, *P. carinii*; Ca, *C. albicans*; Af, *A. fumigatus*; Cn, *C. neoformans*; Bd, *B. dermatitidis*; Hc, *H. capsulatum*.

[c] GlcNAc, N-acetyl-D-glucosamine (monomer)

[d] GXM, glucuronoxylomannan