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The Role of Phagocytes in Immunity to *Candida albicans*

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Additional information is available at the end of the chapter

Abstract

Body clearance of fungi such as *Candida albicans* involves phagocytosis by fixed tissue macrophages as well as infiltrating monocytes and neutrophils. Through phagocytosis, the fungi are confined and killed by the oxidative and non-oxidative anti-microbial systems. These include oxygen derived reactive species, generated from the activation of the NADPH oxidase complex and granule constituents. These same mechanisms are responsible for the damage to hyphal forms of *C. albicans*. Complement promotes phagocytosis, through their interaction with a series of complement receptors including the recently described complement receptor immunoglobulin. However, it is also evident that under other conditions, the killing of yeast and hyphal forms can occur in a complement-independent manner. Phagocytosis and killing of *Candida* is enhanced by the cytokine network, such as tumour necrosis factor and interferon gamma. Patients with primary immunodeficiency diseases who have phagocytic deficiencies, such as those with defects in the NADPH oxidase complex are predisposed to fungal infections, providing evidence for the critical role of phagocytes in anti-fungal immunity. Secondary immunodeficiencies can arise as a result of treatment with anti-cancer or other immunosuppressive drugs. These agents may also predispose patients to fungal infections due to their ability to compromise the anti-microbial activity of phagocytes.

Keywords: *Candida albicans*, macrophages, neutrophils, complement, innate immunity, phagocytosis, fungal killing mechanisms, cytokines, trained immunity, immunodeficiency, immunopharmacology

1. Introduction

C. albicans is considered to be the most common fungus causing both skin and disseminated disease, particularly in immunodeficient and immunocompromised patients. Phagocytes, particularly neutrophils, play an important role in clearing candidal infections. The importance of neutrophils in immunity to *C. albicans* is clearly evident from the increased rate of infection seen in patients with severe neutropenia [1].

In neutrophils, the major response associated with phagocytosis of microbial pathogens is the oxygen-dependent respiratory burst and the generation of reactive oxygen species (ROS). Several decades ago it became evident that neutrophils displayed a unique respiratory burst in the absence of mitochondria, where the generation of ATP comes mainly from glycolysis (reviewed in [2]). It also became apparent that the majority of the oxygen consumed was converted to superoxide ($O_2^{\cdot-}$) which is then converted to further oxygen intermediates, including singlet oxygen and H_2O_2. The enzyme which catalyses the conversion of O_2 to $O_2^{\cdot-}$ is assembled in the phagocytic vacuole membrane, facilitating its release into the bacteria or fungus-containing vacuole. In neutrophils, the release of the azurophilic granule content simultaneously into the phagocytic vacuole leads to the generation of HOCl, a highly potent anti-microbial agent, as a result of the action of myeloperoxidase on H_2O_2 in the presence of chloride ions. In addition, ingestion of microbial pathogens and their confinement to the vacuolar environment may restrict the supply of essential nutrients necessary for growth.

The NADPH oxidase complex is responsible for the respiratory burst and consists of a number of different proteins which assemble in the neutrophil vacuole membrane following cell stimulation. This is typically initiated during phagocytosis of bacteria and fungi [3]. The complex consists of the oxidase-specific phox proteins gp91^{phox}, p22^{phox}, p40^{phox}, p47^{phox}, p67^{phox} and the small GTPases, Rac1 and Rac2. Cell activation leads to the assembly of these components in the membrane and the initiation of enzymatic activity.

The non-oxidative microbicidal system complements the respiratory burst. Components of the azurophilic granules in neutrophils have been shown to have anti-microbial activity. These include defensins, serprocidins and bactericidal/permeability increasing protein (BPI). Defensins are cationic peptides with broad spectrum antimicrobial activity [2]. The seroprodins, elastase, azurocidin and cathepsin G have antimicrobial activity independent of their enzymatic activity [2].

As with neutrophils, most bacteria and fungi are confined and killed within phagosomes by macrophages [4], involving a variety of agents such as toxic metabolites, peptides and enzymes. These may act either alone or synergistically. In addition, macrophages can produce ROS which have anti-microbial action but unlike monocytes, macrophages lack MPO. Most striking is the marked heterogeneity of macrophages enabling these leukocytes to perform functions relevant to specific tissues in which they are located.

The extrusion of neutrophil extracellular traps (NETs) is also considered to be a defence mechanism against microbial pathogens. NETs are structures composed of DNA as well as anti-microbial substances, elastase, calprotectin and MPO [5]. NETs not only trap the microbial pathogens, but also kill them. Interestingly, it has been reported that the formation of NETs requires the presence of ROS [6].

Effective recognition of microbial pathogens by neutrophils and macrophages requires receptors which bind peptides deposited on bacterial and fungal surfaces which have been generated through the activation of complement, namely C3b and iC3b. Receptors recognising iC3b include CR3 (CD18/CD11b) and CR4 (CD18/CD11c), which are present on both neutrophils and macrophages. Recently, another complement receptor type, complement immunoglobulin receptor (CRIg), expressed only by a subpopulation of macrophages has been described, which binds both iC3b and C3b (reviewed in [7]). It has been shown that this receptor plays an important role in clearance of bacteria from the circulation by liver Kupffer cells [8] and may also be a pattern recognition receptor, facilitating clearance of bacteria in the absence of complement [9].

Antibody bound to microbial pathogens also promotes phagocytosis through the Fcγ receptors, FcγRI (CD64), FcγRIIA (CD32) and FcγRIIIB (CD16), all of which engage the Fc domain of Immunoglobulin G (IgG). The FcαRI which binds the Fc domain of Immunoglobulin A (IgA) also promotes microbial phagocytosis and killing [2].

Apart from the integrins and FcγRs, neutrophils and macrophages express a range of pattern recognition receptors (PPR) which recognise conserved microbial pathogen structures, such as lipoteichoic acid, β-glucans and lipopolysaccharide. Families of PPRs include those found in serum (pentraxins, collectins, complement), those which are membrane bound (classic C-type lectins, non-classic C-type lectin leucine-rich proteins, scavenger receptors) and those which are located intracellularly (NODs, interferon induced proteins).

2. Complement dependent and independent phagocytosis of *C. albicans*

Despite the importance of complement-independent mechanisms for host anti-candidal immunity, it is evident that complement is required for optimal resistance to fungal infection [10–12]. It was also evident in these studies that complement could be activated by *C. albicans* by the alternative pathway. Activation of complement leads to the generation of chemotactic peptides and C5a, which attracts neutrophils to the site of candidal infection [13, 14]. Thong and Ferrante [11], in their studies on the generation of chemotaxis promoting factors by serum treated with *C. albicans*, showed that this activity was totally dependent on heat-labile factors and activation of complement via the alternative pathway. Chemotaxis of neutrophils towards fungus-treated serum was abolished when the serum was either heated at 56°C for 20 min or was C2 deficient (where the alternative but not the classical pathway can be activated). The subsequent step, phagocytosis, was also highly dependent on heat labile opsonins [12]. However, while the chemotactic response was totally dependent on serum complement, the heat labile opsonins only acted to enhance other phagocytosis-promoting mechanisms. Thus, significant phagocytosis was still observed in the presence of heat-inactivated serum. In both of these studies on chemotaxis and phagocytosis-promoting activity of serum, it was shown that these principles applied to a wide-range of clinical isolates of *C. albicans* from patients and both including Serotypes A and B [11, 12].

Zymosan A is a yeast cell wall glucan and, like *C. albicans* derived β(1,3) (1,6)-glucan, is an agonist to TLR2 and Dectin-1 [15]. Using commercially available labelled zymosan A bioparticles

which are non-fluorescent outside of the cell and fluoresce once taken into acidic phago-somes, we showed that neutrophils require opsonising conditions to phagocytose particles efficiently (**Figure 1**). This supports the findings of [16], and demonstrates that like monocytes and macrophages, neutrophils require complement for the rapid phagocytosis of yeast par-ticles. Interestingly, the complement dependency of phagocytosis diminished at incubation times of 45–60 min, where complement-independent mechanisms of phagocytosis become more prominent (**Figure 1C**).

The classical complement pathway is likely to be activated by mannan-specific antibod-ies found in human serum [19] whereas the lectin pathway is activated by the binding of mannose-binding lectin to mannan on the cell wall of the fungus [20]. However, it has also been shown that *C. albicans* can bind the complement regulatory protein, C4b-binding protein (C4BP), thereby inactivating C4b and hence preventing complement activation on the yeast surface. As a result, the microbial pathogen will evade complement activation via the classical and lectin pathways, but the alternative pathway remains operative, generating chemotac-tic factors and opsonins. Furthermore, *C. albicans* has the ability to regulate the alternative pathway and factors H and FHL-1 [21]. The binding of these regulators is seen with both the cellular and hyphal forms of *C. albicans* [22].

The unique complement receptor CRIg is a member of the transmembrane protein of the type 1 immunoglobulin superfamily, encoded by *VSIG4*. Although two spliced forms of CRIg have been described, a long (L) and short (S) form [8], we have recently identified five forms based on expression of transcripts and western blot analysis [23]. The receptor is expressed selectively by a subpopulation of macrophages, probably of the M2 type, and is abundant in fixed tissue macrophages such as liver Kupffer cells and resident peritoneal macrophages [24, 25]. Unlike CR3 and CR4 which require prior activation, CRIg is naturally active and its activity is controlled by its recycling pattern from the endoplasmic reticulum [8]. Our studies have demonstrated that cytokines alter CRIg expression in human macrophages and this was associated with a corresponding change in ability of neutrophils to phagocytose *C. albicans* in a complement-dependent manner [23, 26, 27].

While CR3 and FcRγ mediate phagocytosis of complement and antibody opsonised *C. albi-cans*, in the absence of these opsonins, adherence and phagocytosis by neutrophils and mac-rophages is promoted by C-Type Lectin Receptors (CLRs), in particular Dectin-1 [28–31]. The targets for Dectin-1 are β-1,3 glucan polymers, major components of the fungal cell wall. In *C. albicans* hyphae, this polymer is masked and appears to be different in the yeast form [32].

Cells of the phagocytic system are able to recognise *C. albicans* through multiple classes of receptors [33]. These include pattern recognition receptors (PRRs) such as Toll-like receptor (TLRs) 4 and 2 [34, 35], and CLRs such as Dectin-1 and the mannose receptor [36]. While these receptors are able to induce phagocytosis independently of complement, efficiency of uptake in both macrophages and neutrophils can be significantly increased when *C. albicans* is opsonised [16]. Under these conditions CR3 present on phagocytes is able to recognise iC3b deposited on the fungal cell surface and promote phagocytosis. In macrophages, this process is also able to occur through CRIg [27]. Agents such as dexamethasone that promote the upregulation of CRIg protein expression are also able to induce increased levels of phago-cytosis of *C albicans* [23], suggesting that CRIg rather than CR3 plays an important role in the phagocytosis of *C. albicans* in macrophages.
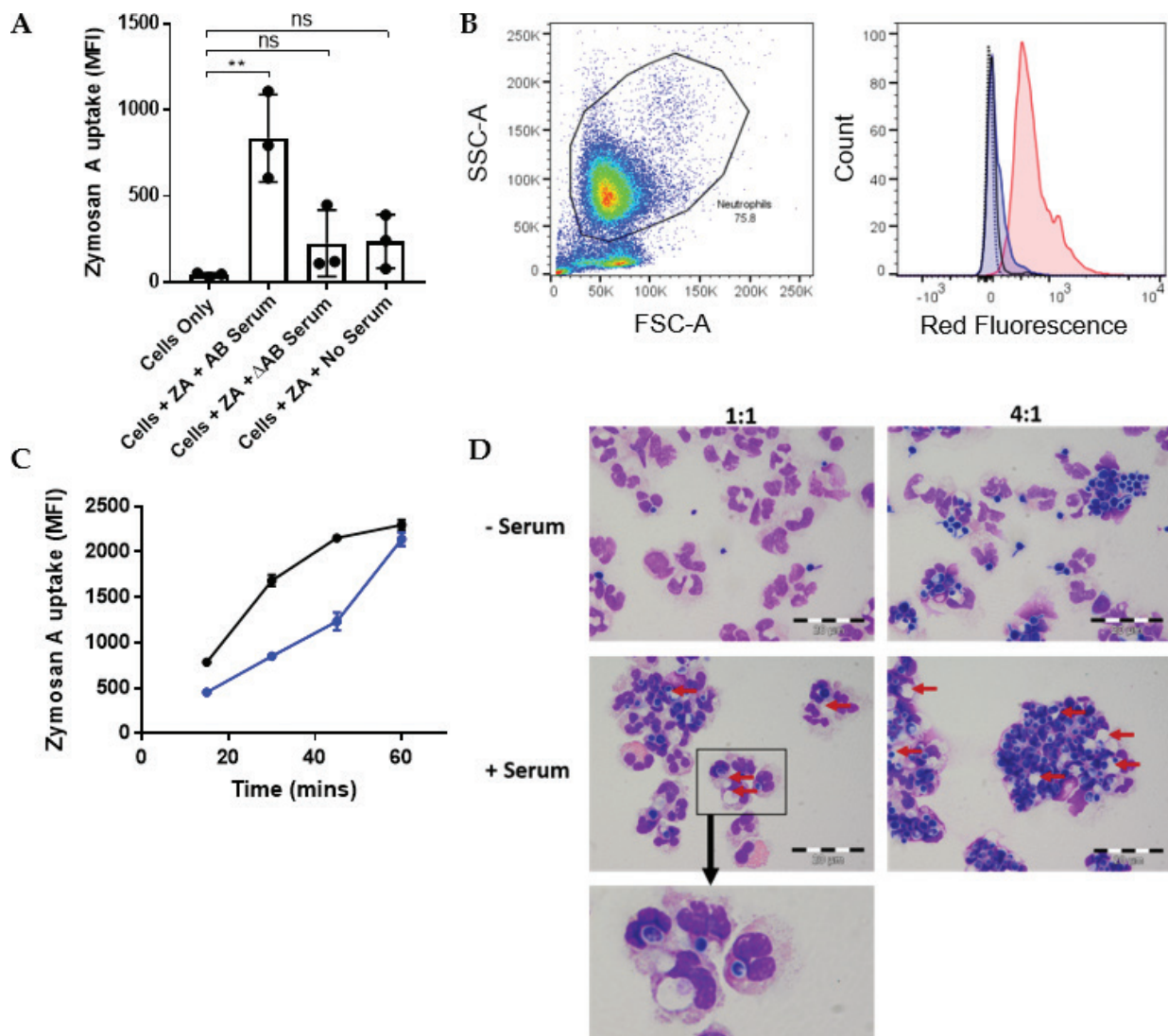
Figure 1. Complement-dependent and -independent phagocytosis of zymosan A bioparticles by human neutrophils. (A) Phagocytosis of *C. albicans* under the different treatment conditions indicated in the x-axis. Results are the mean ± SD of three experiments. (B) Representative histogram/gating strategy for these experimental runs. In these experiments the reaction was terminated at 30 min. Neutrophils only are shown by the dashed line, no serum in black, heat-inactivated serum shown in blue, and native AB serum shown in red. (C) Phagocytosis kinetics over a 60 min incubation period in the presence or absence of serum. Results are presented as mean ± SD of triplicate reactions. Neutrophils were prepared from human peripheral blood from healthy volunteers, using the high density gradient method [17]. Phagocytosis was assayed using pHrodo™Red Zymosan A bioparticles (ThermoFisher, Walter MA, Cat no. P35364) as described previously [18]. Human AB serum was prepared from peripheral blood of healthy volunteers. The serum was shown to have normal levels of complement activity using the CH50 assay. AB serum heated at 56°C for 20 min was confirmed to lack complement activity. The cell samples were analysed using a FACSCanto I flow cytometer (BD). The work was approved by the Human Research Ethics Committee of the Women's and Children's Hospital Network, Adelaide. Statistical analyses were carried out by ANOVA followed by Dunnet's post hoc test. (D) Photomicrographs of the interaction of *C. albicans* with neutrophils in the presence or absence of serum at a 1:1 and 1:4 neutrophil:fungal ratio. Red arrows indicate phagocytic vacuoles following the digestion of the yeast or non-degraded yeast particles (following 30 min of incubation).

Neutrophils recognise *C. albicans* through the PRRs TLR2, TLR4 and Dectin-1, and also under opsonising conditions through FcγR and CR3 [37]. Similar to macrophage phagocytosis of *C. albicans*, uptake of isolated fungal zymosan A is more efficient in opsonising conditions, with phagocytosis after a 15-min incubation time being three times higher in reactions with complement compared to no serum and heat-inactivated serum controls (**Figure 1**).

C. albicans is able to exist in multiple forms, as a single-celled budding yeast or in pseudohyphal or hyphal filamentous forms [38]. While in its unicellular form, the fungus can be tolerated as a commensal organism by the oral or vaginal epithelium. However, when it converts to its hyphal form, the fungus displays pathogenic properties. The host is able to discriminate against the potential danger [37] through MAPK-based recognition in the epithelial cells [39], which leads to mitogen-activated protein kinase phosphatase 1 (MKP1) and c-Fos activation. Neutrophils also play a role in this protection through TLR4-mediated recognition [40].

3. Trained macrophage immunity in anti-fungal immunity

Trained immunity (TI) refers to the ability of innate immune cells to exhibit 'memory' and prevent reinfection of previously encountered invading pathogens [41]. Termed by Netea and colleagues [42], TI induces a state of enhanced antimicrobial action in cells of the innate immune system, particularly monocytes and macrophages, which is distinct from both typical innate immunity and the memory of the adaptive immune system. Alternatively, TI refers to the enhanced response to reinfection against the initial invading microorganisms and cross-protection against different pathogens. Although the concept of TI is relatively new, the phenomenon of protection afforded by previous infection in a manner distinct from adaptive immunity has long been known, particularly in plant and insect systems [43, 44].

TI has been shown to have a role in infection and immunity against *C albicans.* Bistoni et al. [45] demonstrated that not only did injection with a non-pathogenic strain of *C. albicans* induce protection against reinfection, but also cross-protected against the other pathogens *Candida tropicalis* and *Staphylococcus aureus*. This protection was determined to be macrophage-dependent, as transfer of adherent splenic cells from mice administered with the non-pathogenic strain conferred protection to the recipient mice. Two decades later, Quintin et al. [46] expanded on this concept, demonstrating that mice injected with low doses of *C. albicans* showed increased survival rates when administered lethal infection loads, and increased proinflammatory cytokine production upon secondary exposure. This protection was also shown in mice deficient in T and B cells and not in mice lacking *CCR2*, indicating that similar to the results of Bistoni et al. [45], the observed protection was monocyte-dependent. It was also shown that training of monocytes could be induced through purified β-glucan, a polysaccharide that makes up the cell wall of selected bacteria and fungi [47]. The group further investigated the mechanisms behind this protection by analysis of the genome-wide binding pattern of the methylation marks on histone 3 lysine 4 (H3K4me3) and on histone 3 lysine 27 (H3K27me3), and concluded that protection was controlled at the epigenetic level through H3K4me3 in known genes involved in innate immunity. Furthermore, mRNA levels of TNF and IL-6 were higher in trained monocytes compared with non-trained control cells.

While other molecules such as fungal chitin have also been shown to induce TI [48], β-glucan remains the most well-studied molecule with respect to *C. albicans*, which has been shown to induce TI in both human and murine systems [46, 49, 50]. Along with its antimicrobial priming, β-glucan-induced TI has also been investigated in anti-tumour immunity [51].

4. Killing of *C. albicans* by neutrophils and macrophages

Ferrante [52] demonstrated that killing of yeast forms of *C. albicans* and *Candida glabrata* was associated with release of the ROS, superoxide, and constituents of azurophilic granules and specific granules. During this interaction the generation of HOCl occurred, another potent anti-fungal agent. The importance of ROS was demonstrated by the finding that inhibitors of superoxide and H_2O_2 decreased intracellular killing of *C. albicans* [53]. Further proof of the role of ROS generation in the killing of *C. albicans* came from the demonstration that neutrophils and macrophages from patients with chronic granulomatous disease (CGD) (who have defective NADPH oxidase activity), were unable to effectively kill the fungi [54]. However, whether ROS *per se* are responsible for the killing of *C. albicans* remains to be established [55]. The reaction of H_2O_2 with MPO, in the presence of chloride ions, forms a very potent anti-microbial system. We have previously demonstrated that opsonised *C. albicans* induces the release of both H_2O_2 and MPO, thereby establishing an anti-candidal environment [52]. The importance of MPO in the killing of *C. albicans* is supported by the finding that neutrophils and monocytes from MPO deficient patients failed to kill *C. albicans* [56, 57].

In vivo the absence of MPO in macrophages may be overcome by the cells incorporating MPO released by neutrophils at infection sites. Thus, resident peritoneal mouse macrophages in the presence of recombinant MPO caused an increase in intracellular killing of *C. albicans* [58]. However, it is noteworthy that using mouse models of X-linked CGD and MPO deficiency, susceptibility was most evident in the former, suggesting that ROS are the major mediators of candidicidal activity [59]. In comparison, the neutrophil-mediated damage to *C. albicans* pseudohyphae was found to be mediated by the oxidative burst and MPO [60]. Interestingly, this neutrophil-mediated damage occurred in the absence of serum complement.

Two distinct mechanisms for human neutrophil-mediated killing have been documented, depending on the state of fungal opsonisation. Using *in vitro* fungicidal assays, Gazendam et al. [61] showed that killing of un-opsonised *C. albicans* was dependent on CR3 and phosphatidylinositol-3-kinase (PI3K) signalling, but was independent of NADPH oxidase activation. However, the killing of antibody opsonised *C. albicans* by neutrophils was dependent on Fcγ receptors and protein kinase C (PKC) in addition to NADPH.

5. Intracellular signalling required for killing of *C. albicans*

Approximately two decades ago it was demonstrated that human neutrophil-mediated killing of *C. albicans*, in a complement-dependent manner, required the activation of the extracellular signal-regulated protein kinase cascade [62]. More recently it has been reported that PKCδ activation downstream of the receptors Dectin-1 and Mac-1 is important in the neutrophil-mediated resistance to *C. albicans* and fungi-induced ROS generation [63]. In contrast, while PKCδ deficiency in macrophages prevented the stimulation of production of ROS induced by *C. albicans*, this did not affect the killing of the fungus. It has been demonstrated that BTK and

Vav1 are Dectin-1 interacting proteins [64]. These were found to be recruited to phagocytic cups containing yeast or hyphae of *C. albicans*, at the less mature stage of phagosome development. These contribute to the Dectin-1 dependent phagocytosis of *C. albicans*.

In comparison, Gazendam et al. [61] demonstrated that neutrophils display two different mechanisms in the killing of *C. albicans* by evaluating patients with Dectin-1 deficiency, CARD9 deficiency or NADPH deficiency. One of these mechanisms was CR3, PI3K and CARD9 dependent, but independent of ROS generation. The other was selectively dependent on Fcγ, PKC and ROS generation. Both of these candidicidal pathways required Syk tyrosine activation but were independent of Dectin-1.

6. Neutrophil extracellular traps in immunity to *C. albicans*

C. albicans has been shown to induce NET extrusion in phagocytes, particularly in neutrophils. While the formation of this structure is considered as part of cell death or NETosis [65], Byrd et al. [66] reported that the rapid extrusion of NETs in response to *C. albicans* occurs in the absence of cell death. However, others have demonstrated that the yeast forms of *C. albicans* stimulated NETs through autophagy and ROS generation in the early stage of the interaction (first 15 min) [67]. However, with the hyphal forms, NET formation occurred via autophagy and not ROS generation. In the longer term (4 h), only the hyphae stimulated NETs. Interestingly, they found less killing of yeast forms by NETs compared to the high level of damage to the hyphae forms. Other strategic functions of extracellular protrusions of neutrophils have been demonstrated for *Plasmodium falciparum*. Here, the neutrophils were observed to 'throw out' protrusions which penetrated the parasitophorous vacuole containing the intraerythrocytic stage of the parasite and withdrawing the parasite without damaging the erythrocyte [68].

7. Cytokine priming in phagocyte-mediated killing of *C. albicans*

Over three decades ago it became evident that neutrophil responses to microbial pathogens could be significantly increased if the cells were pre-sensitised with products released by activated lymphocytes and macrophages [69], a process dependent on the presence of TNF [70, 71]. The importance of cytokine priming in killing of *C. albicans* by neutrophils was also observed [72]. Thus, neutrophil mediated killing of *C. albicans* and a related fungus, *Candida glabrata* was significantly increased if the phagocytes had been pre-treated with either TNF or GM-CSF [52, 73]. The TNF treatment also increased the candida-induced release of ROS and MPO, consistent with the increased anti-fungal activity induced by the cytokines [52]. The mechanism by which TNF primes neutrophils for increased killing of *C. albicans* has not been studied. However, these mechanisms can be inferred from studies with other microbial pathogens. Kowanko et al. [74] demonstrated that the TNF-induced effects responsible for increased microbial killing could be mediated by both oxygen-dependent and oxygen-independent mechanisms, with respect to killing of opsonised *S. aureus* and *Plasmodium falciparum* infected erythrocytes, respectively. Furthermore, studies with the pathogenic soil amoeba,

N. fowleri have shown that the TNF-enhanced killing requires a functional H_2O_2-MPO-halide system [75]. The priming of neutrophils by TNF is reflected by an increase in expression of CR3 and CR4 on the surface of these cells. The enhanced killing of *S. aureus* was dependent on these receptors, given that this was not seen upon the addition of anti-CD11b and -CD11c monoclonal antibodies [76].

The use of TNF to enhance immunity against various microbial infections has not been considered appropriate because of the highly toxic and tissue damaging effects of TNF. In an effort to harness the anti-infective properties of TNF and exclude some of its tissue damaging properties, we synthesised short peptides representative of the TNF sequence [77]. One of these elevenmer peptides, TNF_{70-80}, was found to activate neutrophils and macrophages to increase microbial killing both *in vitro* and *in vivo* [77–81].

Our studies with *C. albicans* demonstrated that TNF_{70-80} also protected against infections with this fungus (**Tables 1** and **2**). In the first set of experiments, the effect of administering either TNF or TNF_{70-80} to mice infected with *C. albicans* was examined. The recovery of fungi from

Treatment	No. mice/group	Log CFU/g kidney (M ± SD)
PBS	23	7.3 ± 0.6
Amphotericin B	15	2.7 ± 2.4[***]
TNF (0.1 mg/kg)	29	5.6 ± 1.2[***]
TNF_{70-90} (4 mg/kg)	9	5.75 ± 1.7[**]

Eight week old Balb/c mice were challenged with 5×10^5 CFU *C. albicans* intravenously. Treatment of mice commenced 24 h prior to infection, and continued with daily administration until 2 days post-infection. Mice were sacrificed on day 2 and kidney preparations plated on Sabouraud agar. The degree of infection was determined by enumeration of the number of organisms in the kidney at the time of euthanisation ([**]$p < 0.01$, [***]$p < 0.001$, 1-way ANOVA, SNK test). The research received approval from the Women's and Children's Hospital Animal Ethics Committee.

Table 1. The effect of TNF and TNF_{70-80} on *C. albicans* infection in mice.

Treatment	Route	Dose (mg/kg)	Survivors 10 days post-infection
Vehicle control	IP	—	8
Cyclophosphamide	PO	30	2[*]
TNF_{70-80} + cyclophosphamide	IP	100	7[ns]
TNF_{70-80} + cyclophosphamide	IP	10	4[ns]
TNF_{70-80} + cyclophosphamide	IP	1	4[ns]
TNF_{70-90} + cyclophosphamide	IP	0.1	2[*]
Azimexone + cyclophosphamide	IP	100	6[ns]

Balb/c mice (10/group) were treated with 3 doses of oral (OP) cyclophosphamide (30 mg/kg) and infected with *C. albicans* as described in **Table 1**. Mice were also treated with three doses of TNF_{70-80} at the schedule described in **Table 1**. Azimexone (used as a positive control) was administered intraperitoneally (IP) (n = 10 mice, [*]$p < 0.05$, ns: not significant, one-sided Fisher's exact test). The research received approval from by Women's and Children's Hospital Animal Ethics Committee.

Table 2. Effect of TNF_{70-80} on *C. albicans* infection in immunocompromised mice.

the kidneys of these mice was significantly lower than in non-treated control mice (**Table 1**). In the second experimental set-up, mice treated with cyclophosphamide became highly susceptible to *C. albicans* with the survival of mice dropping from 80 to 20%, 10 days after infection. If the mice had been treated with $TNF_{70–80}$, survival was increased with 70% survival observed at the highest dose (**Table 2**).

Cytokines also influence the ability of macrophages to phagocytose and kill fungi. Human monocyte-derived macrophages (MDMs) treated with interferon gamma showed increased ability to phagocytose and kill yeast forms of *C. albicans* [82]. The cytokine treated cells showed a corresponding increase in ROS production when challenged with the fungus. This effect of interferon gamma was evident with non-opsonised *C. albicans* and was independent of CR3. These effects of interferon gamma were reproduced with mouse peritoneal macrophages [83]. M-colony stimulating factor has also been shown to increase macrophage phagocytosis and killing of *C. albicans* yeast forms and cause damage to hyphae [84].

From the described studies, it is evident that when considering killing of microbial pathogens including *C. albicans*, this needs to be interpreted in terms of the cytokine milieu generated during the infection. It is evident from other published work that several cytokines regulate phagocyte-mediated microbial killing properties, including interferon gamma, lymphotoxin and interleukin-1 [71, 85].

8. Primary immunodeficiency diseases associated with susceptibility to fungal infection

Primary immunodeficiency diseases (PID) are a heterogeneous group of inborn errors of immunity. Affected individuals develop severe, unusual or recurrent infections, and may also develop features of immune dysregulation with autoimmune manifestations. There are currently over 320 described molecular genetic causes of PID, which can be categorised according to presenting phenotypic features [86]. The International Union of Immunological Sciences (IUIS) classify PID into the following disease categories: immunodeficiencies affecting cellular and humoral immunity, combined immunodeficiencies (CID) with associated or syndromic features, predominantly antibody deficiencies, diseases of immune dysregulation, congenital defects of phagocyte number, function or both, defects in intrinsic and innate immunity, auto-inflammatory disorders, complement deficiencies and phenocopies of PID [86].

Intact immunological processes and pathways are required to mount an effective immune response against fungi, incorporating both innate and adaptive components [87]. Several immune cells and immunological mediators such as cytokines are of critical importance to maintenance of anti-fungal immunity. These include phagocytes, dendritic cells, T cells (particularly T helper 1 (TH1) and T helper 17 (TH17) cells) [87]. The importance of these effectors is evidenced by patients with PID affecting cellular or phagocytic immunity developing severe, invasive or recurrent fungal infections [1].

Primary phagocytic disorders result from mutations in genes encoding key proteins that are essential for normal phagocytic development and function. These disorders may be classified

according to whether phagocyte number, function or both are affected, and by the presence or absence of associated syndromic features [86]. These disorders and their underlying, causative genetic abnormality are summarised in **Table 3**.

Congenital defects of phagocytic number, function or both			
Associated with syndromic features		**Not associated with syndromic features**	
Disorder	**Gene(s)**	**Disorder**	**Gene(s)**
Shwachman-Diamond syndrome	*SBDS, DNAJC21*	Elastase deficiency (SCN1)	*ELANE*
G6PC3 deficiency (SCN4)	*G6PC3*	Kostmann disease (HAX1 deficiency; SCN3)	*HAX1*
Glycogen storage disease type 1b	*G6PT1*	GFI1 deficiency (SCN2)	*GFI1*
Cohen syndrome	*COH1*	X-linked neutropaenia/myelodysplasia WAS GOF	*WAS*
Barth syndrome (3-methylglutaconic aciduria type II)	*TAZ*	G-CSF receptor deficiency	*CSF3R*
Clericuzio syndrome (poikiloderma with neutropaenia)	*C16ORF57 (USB1)*	Neutropaenia with combined immune deficiency	*MKL1*
VPS45 deficiency (SCN5)	*VPS45*		
P14/LAMTOR2 deficiency	*LAMTOR2*		
JAGN1 deficiency	*JAGN1*		
3-methylglutaconic aciduria	*CLPB*		
SMARCD2 deficiency	*SMARCD2*		
WDR1 deficiency	*WDR1*		
HYOU1 deficiency	*HYOU1*		
Congenital defects of phagocytic function			
Associated with syndromic features		**Not associated with syndromic features**	
Disorder	**Gene(s)**	**Disorder**	**Gene(s)**
Cystic fibrosis	*CFTR*	Chronic granulomatous disease	*CYBB, NCF1, CYBA, NCF4, NCF2*
Papillon-Lefevre syndrome	*CTSC*	Rac2 deficiency	*RAC2*
Localised juvenile periodontitis	*FPR1*	G6PD deficiency Class 1	*G6PD*
Leukocyte adhesion deficiency (LAD) 1	*ITGB2*	GATA2 deficiency (MonoMac syndrome)	*GATA2*
Leukocyte adhesion deficiency (LAD) 2	*SLC35C1*	Specific granule deficiency	*C/EBPE*
Leukocyte adhesion deficiency (LAD) 3	*FERMT3*	Pulmonary alveolar proteinosis	*CSF2RA, CSF2RB*

Adapted from [86].

SCN = severe congenital neutropaenia, WAS = Wiskott-Aldrich Syndrome, GOF = gain of function.

Table 3. Primary immunodeficiency diseases affecting phagocytic number and/or function.

Of the described primary immunodeficiency diseases of phagocytic number or function, recurrent or invasive candidal disease has been reported in cases of chronic granulomatous disease and myeloperoxidase deficiency [1] and GATA2 deficiency [88]. Candidosis is reported but tends to be less common in leukocyte adhesion deficiency and congenital neutropaenic syndromes [1].

Chronic granulomatous disease (CGD) occurs as a result of defects in components of the NADPH oxidase system, resulting in defective neutrophil oxidative burst and susceptibility to a narrow range of organisms, particularly those which are catalase-producing. As well as the predisposition to infection, patients with CGD develop a hyperinflammatory response and granuloma formation [89]. X-linked CGD occurs due to mutations in the *CYBB* gene which encodes the NADPH oxidase complex component gp91^{phox} [86]. Autosomal recessive forms of CGD are less common, and occur due to mutations in the *NCF1*, *CYBA*, *NCF4* or *NCF2* genes, which encode for other components of the complex, namely p47^{phox}, p22^{phox}, p40^{phox} and p67^{phox}, respectively [86, 89].

Candidosis is well described in CGD patients, with candidal species implicated in episodes of meningitis, fungaemia, suppurative adenitis, pneumonia, subcutaneous abscesses and liver abscess reported in a cohort of 368 patients with CGD [90]. Although the majority of these infections were expected to be due to underlying, impaired phagocytic function, additional factors such as steroid use likely increase the risk of invasive candidiasis. Candidal oesophagitis, keratitis and disseminated infection (particularly affecting young infants) have also been described, however mucocutaneous candidiasis is uncommon in CGD patients [1].

Patients with gp40^{phox} mutations have been noted to have a distinct clinical phenotype as compared with those with other forms of CGD, with a milder clinical course and lower frequency of invasive fungal infection [91]. There is no impairment in the ability of the neutrophils of affected patients to kill candida, suggesting residual NADPH oxidase activity and a potential gp40^{phox}-independent process for reactive oxygen species production. Furthermore, monocyte and monocyte-derived macrophage NADPH oxidase generation appears to occur independently of gp40^{phox} [91]. In patients with CGD, a correlation has been shown between residual production of reactive oxygen intermediates (ROI) and improved long-term survival [92]. The specific mutation in NADPH oxidase predicts the amount of residual production of ROI [92].

CGD may be conservatively managed with antibiotic and antifungal prophylaxis, along with adjunctive therapies including subcutaneous interferon therapy. CGD is curable by haematopoietic stem cell transplantation (HSCT), and trials are underway to evaluate the role of gene therapy as an alternative definitive management strategy [93].

MPO deficiency is autosomal recessive with variable penetrance, may be complete or partial, and has an estimated incidence of between 1:2000 and 1:4000 individuals [94]. Most individuals are clinically asymptomatic, although severe infections are reported in around 5% of those affected. MPO-deficient phagocytes have an impaired capacity to kill *C. albicans*, as evidenced by severe infection in MPO-deficient mice.

GATA2 encodes a zinc finger transcription factor which is critical for haematopoetic cell development [95]. Mutations in this gene give rise to a syndrome also known as 'MonoMac', which

refers to the monocytopaenia and predisposition to mycobacterial infection which are characteristic of this condition [95, 96]. In addition, affected patients have other haematological anomalies including thrombocytopaenia and neutropaenia, predisposition to haematological malignancy and severe mycobacterial, fungal and human papilloma viral infections [88, 96]. In a recent study of 79 French and Belgian patients with *GATA2* mutations, 16 patients were reported to have had 18 episodes of fungal infection, 5 of which were candidoses [88]. Eight of the 18 infections were associated with chemotherapy or HSCT. The neutrophils from some *GATA2* deficient patients were noted to have reduced granularity [97]. When stimulated with PHA (phytohaemagglutinin), patient PBMCs (peripheral blood mononuclear cells) demonstrated reduced lymphocyte proliferative and cytokine production capacity, which normalised after addition of monocytes [96], highlighting the important role of these cells in eliciting an effective immune response.

In addition to the critical role of phagocytes in anti-fungal immunity, defects in other immune cells and immunologic pathways also give rise to susceptibility to infection with candida and other fungi. A range of single-gene inborn errors of immunity resulting in severe or recurrent superficial or invasive candidiasis have been described [86, 98]. Cell-mediated immunity is essential for anti-fungal immunity. This is evidenced by the predisposition to severe fungal infection in infants with severe combined immunodeficiency (SCID), a life-threatening condition manifested by low, absent or severely dysfunctional T cells [86]. Other forms of combined immunodeficiency, for example, those due to deficiencies in CD25, NEMO/IKBG, DOCK8, TCR-α, ORAI1, MST1/STK4, MHC Class II, along with *IKBA* gain of function mutations and idiopathic CD4+ T cell lymphopaenia are associated with chronic mucocutaneous candidiasis (CMC) [98]. In addition, CMC is a feature of several PID with syndromic features, including STAT3 deficiency (autosomal dominant hyper-immunoglobulin E syndrome), APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dysplasia), also known as APS-1 (autoimmune polyglandular syndrome type 1) which occurs due to mutations in the *AIRE* gene), and deficiencies of IL12Rβ, IL-12p40 and CARD9 [98, 99]. The importance of the TH17 pathway and IL-17 signalling in anti-candidal immunity has become apparent [100, 101], with severe CMC described in patients with deficiencies of IL-17RA, IL-17F, RORC and *STAT1* gain of function mutations [98, 102]. In particular, AIRE has been demonstrated to have a key role in anti-candidal immunity, as evidenced by its role in fungal synapse formation which is required for initial macrophage recognition of fungal hyphae [103]. AIRE, along with Dectin-1, Dectin-2, Syk and CARD9 are required for formation of the fungal synapse upon stimulation of macrophage-like THP-1 cells after stimulation with *C. albicans* [103].

9. Secondary immunodeficiency diseases associated with disorders of phagocyte number or function

Immunosuppression is a well-described risk factor for infection with candida and other fungal species [98]. Corticosteroids are commonly used in the management of a range of inflammatory and malignant conditions, and use of these agents is a known risk factor for fungal infection [104]. The precise mechanisms by which corticosteroids lead to impaired anti-candidal

immunity remain unclear, and this is likely multifactorial [105]. In terms of phagocytic cell function, corticosteroids appear to alter leukocyte differentiation programs. They induce monocytes and macrophages to adopt an anti-inflammatory phenotype. This is modulated by the cytokine environment (including increased IL-10 expression on macrophages), increased apoptotic activity and induction of transcription of anti-inflammatory genes which impact upon chemotaxis, phagocytosis and resistance to oxidative stress [105]. However, despite these observations it has been recently shown that dexamethasone increases the expression of CRIg on human MDMs but not CR3 or CR4, and that this increase was associated with an increase in phagocytosis of complement opsonised *C. albicans* [23, 26, 27].

Cancer patients are at an increased risk of systemic candidiasis, and *C. albicans* is reported to be one of the most common causes of sepsis in this patient group [104]. This predisposition to fungal infection is multifactorial, and may be due to a secondary immunodeficiency caused by the underlying malignancy itself, or due to the effects of chemotherapeutic agents. Chemotherapeutic drugs may induce neutropaenia or affect neutrophil function, thereby impairing anti-candidal immunity. Neutrophil function may be impaired as a result of reduced trafficking, chemotaxis or phagocytic activity. For example, chemotherapeutics targeting microtubule structures likely impair cytoskeletal processes and actin polymerisation, thereby reducing neutrophil chemotaxis and phagocytosis. Chemotherapeutic agents can also interfere in NETosis, which is important for antimicrobial activity. Some drugs may also induce monocytopaenia and impaired monocytic function, further increasing the risk of candidal infection [104].

Patients with liver disease are at an increased risk of fungal infection. Those with cirrhosis have been found to have reduced complement levels and impaired monocyte activation and neutrophil mobilisation [106]. Patients with liver disease are at risk for infectious peritonitis, and *C. albicans* and *C. neoformans* were amongst the main species isolated in these cases. Renal disease is also a risk factor for invasive fungal disease [104]. Neonatal candidal sepsis has been reported in association with jaundice [107]. Interestingly, unconjugated bilirubin in hyperbilirubinemia has also been linked to reduced phagocytic cell function; phagocytosis and killing of fungi [108, 109]. Burns patients are at increased risk of fungal infection owing to a breached skin barrier and use of antimicrobial agents, with candidal infection in particular being associated with increased morbidity and mortality in these patients [106]. In addition to these disease states, other physical factors, alone or in combination, such as the use of intravenous catheters and mechanical ventilation also increase the risk of invasive fungal disease [98, 104].

Finally, it is also evident that anti-fungal drugs *per se* can compromise immunity [109–111]. Several of the imidazoles were found to inhibit neutrophil functions, chemotaxis, phagocytosis and microbial killing of bacteria and candida [110].

Acknowledgements

Conflicts of interest

Authors AGS and JRK declare no conflicts of interest. Authors DAR and AF declare that they are inventors on patent relating to TNF_{70-80} technology.

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