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The role of neutrophils in host defense against invasive fungal infections

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Abstract

Purpose of Review: Invasive fungal infections caused by the commensal yeast *Candida* and the ubiquitous, inhaled mold *Aspergillus* have emerged as major causes of morbidity and mortality in critically ill and immunosuppressed patient populations. Here, we review how neutrophils contribute to effective immunity against these infections.

Recent Findings: Studies in mouse models of invasive candidiasis and aspergillosis, and observations in hematological patients with chemotherapy-induced neutropenia and in patients with primary immunodeficiency disorders that manifest with these infections have highlighted the critical role of neutrophils and have identified key immune factors that promote neutrophil-mediated effective host defense against invasive fungal disease.

Summary: Neutrophils are crucial in host protection against invasive candidiasis and aspergillosis. Recent advances in our understanding of the molecular cues that mediate protective neutrophil recruitment and effector function against these infections hold promise for developing immune-based strategies to improve the outcomes of affected patients.

Keywords

neutrophils; recruitment; effector function; fungal killing; *Candida*; *Aspergillus*

Introduction

Neutrophils are the most abundant leukocytes in human blood with an estimated production in the bone marrow of approximately 10^{11} cells daily [1]. When acute infection develops, upon their recruitment from the blood into the infected tissue, neutrophils exert a variety of effector functions, which include binding, phagocytosis and intracellular killing of microorganisms via oxidative and non-oxidative cytotoxic mechanisms, extracellular degranulation of antimicrobials that are pre-stored in specialized granules, formation of neutrophil extracellular traps (NETs), and generation of pro-inflammatory and anti-inflammatory cytokines, chemokines and other mediators [2, 3].

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Neutrophils represent the first line of innate immune defense against invasive infection caused by certain fungal pathogens such as *Candida* and *Aspergillus* species, among which *Candida albicans* and *Aspergillus fumigatus* are the most common species infecting humans and will be the focus on our review. Instead, other fungi such as *Cryptococcus neoformans*, *Pneumocystis jirovecii*, and the endemic dimorphic fungi *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Coccidioides immitis* do not rely on neutrophils but depend on an effective CD4⁺ T cell-macrophage cross-talk for optimal host defense [4–9]. Indeed, patients with acquired and inherited quantitative and qualitative neutrophil defects are at heightened risk for developing invasive candidiasis and aspergillosis (but not cryptococcosis, pneumocystosis or endemic dimorphic fungal disease) and experiencing worse outcomes from these infections [4, 5, 8].

In this review, we outline recent advances in immunological knowledge that pertains to the mechanisms by which neutrophils are mobilized and become activated in the fungal-infected tissues derived from mouse models of invasive candidiasis and aspergillosis and from patient cohorts at risk for developing these infections.

The role of neutrophils in host defense against candidemia and invasive candidiasis

Neutrophils are indispensable for effective host defense during invasive candidiasis in mice and neutropenia is a well-known predisposing factor for development of candidemia and invasive candidiasis and for increased mortality after infection in humans [4, 10–13].

Protective neutrophil trafficking into *Candida albicans*-infected tissues

Mouse studies have shown that early neutrophil recruitment to the site of infection is of critical importance for *Candida albicans* growth control [14, 15]. Of note, mouse tissues such as the spleen and liver that promptly recruit large numbers of neutrophils within the first 24–48 hours post-infection are able to successfully control *Candida albicans* proliferation [16]. In the infected kidneys, the glycoprotein ICAM-1 (intercellular adhesion molecule 1) which binds to integrins, is important for mediating neutrophil adhesion and diapedesis, is induced and in agreement, *Icam1*^{–/–} mice are susceptible to systemic candidiasis and show decreased neutrophilic infiltrates in kidney histological sections [17, 18]. Additionally, a large number of CC- and CXC- families of chemokines, other chemoattractants, and pro-inflammatory mediators are highly and rapidly induced in the *Candida albicans*-infected kidney, however, mobilization of neutrophils into the kidney is sluggish and this recruitment delay is associated with an ineffective immune response and inexorable fungal invasion within the renal parenchyma [19, 16, 20]. Thus far, the molecular cues that are responsible for early neutrophil trafficking into the *Candida albicans*-infected kidney *in vivo* remain elusive.

Important insights into organ-specific neutrophil accumulation during invasive candidiasis have been recently derived from CARD9 (Caspase recruitment domain-containing protein 9) deficiency, a rare autosomal recessive primary immunodeficiency disorder (PID) that manifests with fungal-specific infection susceptibility without predisposition to non-fungal

infections, malignancy, atopy or autoimmunity [21••–24]. Strikingly, CARD9-deficient patients develop invasive candidiasis that has a unique tropism for involvement of the central nervous system (CNS) while typically sparing the kidney, liver or spleen that are commonly affected in patients with iatrogenic immunosuppression who develop invasive candidiasis [24, 13].

CARD9 is an adaptor protein that relays signals downstream of several C-type lectin receptors (CLRs) such as dectin-1, dectin-2, dectin-3, mincle and others that recognize carbohydrates on the fungal cell wall [25, 26, 22]. CARD9-deficient mice and humans exhibit a CNS-specific and fungal-specific inability to mobilize neutrophils during infection, whereas neutrophil recruitment into the fungal-infected kidney and bacterial-infected CNS are intact in CARD9 deficiency [21••]. The defect in neutrophil accumulation in the CARD9-deficient *Candida albicans*-infected CNS is attributed to defects in the production of the CXC chemokines CXCL1, CXCL2, CXCL5 and CXCL8 (IL-8) in the *Candida albicans*-infected CNS by resident glial cells and recruited myeloid phagocytes, while cell-intrinsic neutrophil chemotaxis and survival are intact [21••]. In addition to the significantly impaired trafficking to the infected CNS, the small numbers of neutrophils that are recruited into the tissue exhibit a defect in killing of unopsonized *Candida albicans* yeast forms, which further contributes to infection susceptibility in these patients [27, 21••]. Recent clinical reports indicated that a small number of CARD9-deficient patients benefited from adjunct immunotherapy with granulocyte-macrophage colony stimulating factor (GM-CSF) or granulocyte-colony stimulating factor (G-CSF) [28–30]; because these colony stimulating factors are known to exert pleiotropic effects on recruitment and/or effector function of neutrophils and other myeloid phagocytes including microglia, future work will be needed to examine whether the benefit seen in these patients relates to overcoming the aforementioned neutrophil recruitment and/or function defects.

Anti-*Candida albicans* neutrophil effector functions

Assembly of the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex at the neutrophil phagosomal membrane and activation of myeloperoxidase (MPO) result in generation of reactive oxygen species (ROS), which along with the NADPH oxidase-induced K^+ -flux-mediated activation of neutrophil proteases within the phagosome are thought to be responsible for pathogen (including *Candida albicans*) killing [31, 32]. Moreover, calcineurin-mediated, NFAT (Nuclear factor of activated T cells)-independent signaling, and Mac-1/Vav/PKC δ (protein kinase delta) activation, both dependent on the CLR Dectin-1, contribute to ROS formation in mouse neutrophils [33–36]. In addition to the Dectin-1 dependent activation, Mac-1 (also known as integrin $\alpha_M\beta_2$ or CR3) can also directly bind to *C. albicans* and regulates phagocytosis and intracellular *Candida* killing [37, 38]. Consonant with the important roles of oxidative killing mechanisms, NADPH oxidase- and MPO-deficient mouse and human neutrophils exhibit impaired *Candida albicans* killing capacity and patients with chronic granulomatous disease (CGD) caused by mutations in any of the five subunits of the NADPH oxidase complex and those with complete MPO deficiency occasionally develop spontaneous invasive candidiasis [39, 32, 40, 41]. In addition to ROS, reactive nitrogen species (RNS, such as peroxynitrite ONOO $^-$) are also candidacidal as has been shown for macrophages in response to opsonized and

nonopsonized *C. albicans* [42]. Neutrophils also produce peroxynitrite in response to bacterial components and cytokines [43, 44]. However, whether *C. albicans* induces peroxynitrite production by neutrophils requires further investigation. However, the vast majority of CGD and MPO-deficient patients who lack ROS production by neutrophils never develop the infection despite lifelong ubiquitous exposure to *Candida albicans* commensal organisms, implying that lack of phagocyte ROS production in humans can be largely compensated *in vivo* by non-oxidative killing mechanisms of *Candida albicans*. Recent studies have uncovered two important molecular signals involved in non-oxidative *Candida albicans* killing. The endoplasmic reticulum transmembrane protein Jagunal homolog 1 (JAGN1) that modulates neutrophil N-glycosylation and the chemokine receptor CXCR1 were both shown to mediate cell-intrinsic neutrophil granulogenesis, degranulation and non-oxidative *Candida albicans* killing without affecting neutrophil recruitment from the blood into *Candida albicans*-infected organs [45••, 46]. Of importance, similar to Cxcr1-deficient neutrophils, the dysfunctional *CXCR1-T276* allele was found to mediate degranulation and non-oxidative *Candida albicans* killing in human neutrophils and was an independent risk factor for development of disseminated candidiasis in intensive care unit patients who suffered from candidemia [45••].

Two independent phagolysosomal mechanisms for *Candida albicans* killing were recently characterized in human neutrophils as a function of *Candida albicans* opsonization by evaluating patients with various PIDs [47••, 48]. On one hand, killing of opsonized *Candida albicans* is dependent on the NADPH oxidase system as well as Fc γ receptors and protein kinase C (PKC). On the other hand, killing of unopsonized *Candida albicans* occurs independently of the NADPH oxidase system and relies on complement receptor 3 (CR3), CARD9 and phosphoinositide-3-kinase (PI3K). While both killing mechanisms in human neutrophils depend on functional Syk activity, the CLR Dectin-1 is dispensable. This observation is in keeping with the absence of invasive candidiasis in patients with *CLEC7A* mutations that result in functional Dectin-1 deficiency and underscores the differences in molecular factors involved in fungal recognition and killing between mouse and human neutrophils [49]. Of interest, autophagy appears largely dispensable for *Candida albicans* killing [50], while the precise role of NETs in regulating the balance between anti-*Candida albicans* resistance and immunopathology *in vivo* requires further investigation [51, 52].

Elegant recent studies have uncovered an intricate cross-talk between tissue-resident and recruited mononuclear phagocytes and NK cells that boosts neutrophil fungicidal activity in *Candida albicans*-infected tissues via the production of GM-CSF by NK cells. On one hand is IL-23p19 produced by resident dendritic cells via activation of Syk [53, 54] and on the other hand is IL-15 produced by recruited inflammatory Ly6C^{hi} monocytes via type I interferon activation [55••, 56], which both promote the production of GM-CSF by NK cells that leads to enhanced neutrophil candidacidal activity. In fact, a recent randomized clinical trial indicated that administration of GM-CSF in recipients of allogeneic hematopoietic stem cell transplantation (HSCT) may protect from invasive fungal infection (primarily invasive candidiasis)-related mortality [57].

Neutrophil-mediated immunopathology during invasive candidiasis

Although neutrophils are crucial for host defense during candidemia and invasive candidiasis, neutrophil-mediated control of *Candida albicans* may come at the cost of immunopathology and tissue injury. In agreement with that, excessive neutrophil accumulation in mouse renal tissue is detrimental in the late phases of the infection [14], and leukotriene B₄-driven intravascular neutrophil clustering and occlusion in mouse lung tissue results in neutrophil-mediated capillaritis, pulmonary hemorrhage and hypoxia [58•]. Pathogenic neutrophil effects may be seen in patients with renal candidiasis and in a subset of neutropenic patients with hepatosplenic candidiasis during neutrophil recovery; strikingly, these patients occasionally require administration of corticosteroid therapy given the worsening of their clinical status [59, 60]. The chemokine receptor CCR1, the TEC tyrosine kinase, the endoribonuclease MCPIP1, the interleukin IL-17C, and the suppressor of TCR signaling (STS) phosphatases are implicated in neutrophil-mediated immunopathology in infected tissue [61, 19, 62–66], while galectin-3 signaling is deleterious via cell-intrinsic impairment in neutrophil ROS formation [67]. These data indicate that pharmacological inhibition of these pathways may represent targeted therapeutic strategies in selected patients with invasive candidiasis.

The role of neutrophils in host defense against aspergillosis and other invasive mold infections

Neutrophil depletion in mice renders them highly susceptible to invasive pulmonary aspergillosis [4, 7]. In keeping with these experimental observations, neutropenia is a well-established risk factor for development of invasive aspergillosis and suffering a worse outcome after infection in hematological malignancy patients and HSCT recipients [68]. In fact, patients with prolonged neutropenia and treatment-refractory invasive fungal infections (including aspergillosis) are occasionally treated with granulocyte transfusions, which when given in high doses may protect from infection-related mortality [69]. In addition, G-CSF and GM-CSF have been used extensively in neutropenic hematology patients to decrease the duration of neutropenia and ameliorate its impact on infection (including fungal) susceptibility, although convincing data on the impact of this intervention in improving patient survival are lacking [70]. Recent evidence in mice indicates that macrophage colony-stimulating factor (M-CSF), but not G-CSF, instructs myeloid commitment in hematopoietic stem cells via direct activation of the myeloid transcription factor PU.1, and results in earlier enhanced production of mature myeloid donor cells post-transplantation and improved survival of transplanted mice when infected with *Aspergillus fumigatus* [71•]. These preclinical findings show promise for the potential use of M-CSF to decrease the duration of neutropenia and the incidence of invasive aspergillosis (and other infections) in HSCT recipients, and clinical studies are warranted to examine the efficacy of this intervention in patients.

Protective neutrophil trafficking into *Aspergillus fumigatus*-infected tissues

Mouse studies have defined the molecular factors that mediate protective neutrophil recruitment in the *Aspergillus fumigatus*-infected lungs. Elegant studies from the Hohl and

Obar labs have defined two distinct waves of signaling events that drive trafficking of neutrophils into the lungs [72•, 73•]. Early on, the first wave involves MyD88 expression on lung epithelial cells, which promotes the production of the CXC chemokines CXCL1 and CXCL5. Operating upstream of MyD88 signaling to recruit neutrophils is the IL-1 α -IL-1 β /IL-1R axis, not Toll-like receptors [72•–74]. The second wave involves CARD9 expression on hematopoietic cells in the lung, which drives the production of the CXC chemokines CXCL1 and CXCL2. Both neutrophils and CCR2-expressing monocytes contribute to CXC chemokine production during lung infection. In line with the critical role of the production of CXC chemokines in promoting protective neutrophil recruitment are the findings by Mehrad and colleagues, which showed that neutralization of these CXC chemokines impairs neutrophil trafficking and *Aspergillus fumigatus* growth control in the lung, and that over-expression of CXCL1 in mice protects against invasive pulmonary aspergillosis [75]. Consonant to these findings in the lung, CXCL1 was also shown to be critical for protective neutrophil recruitment in the *Aspergillus fumigatus*-infected cornea [76]. Another chemoattractant signal that was recently shown to mediate protective neutrophil trafficking into the *Aspergillus fumigatus*-infected lung is eicosanoid leukotriene B₄ (LTB₄) via binding to its receptor LTB₄R1 [77]. Specifically, LTB₄ is produced early on during *Aspergillus fumigatus* infection by radiosensitive hematopoietic cells in the lung via a pathway that is, at least in part, dependent on hypoxia inducible factor 1 α (HIF-1 α).

The reliance on both IL-1R/MyD88 and CARD9 signaling for neutrophil recruitment into the *Aspergillus fumigatus*-infected lung may account for the clinical observation that invasive pulmonary aspergillosis is not seen in patients with inherited MYD88 or CARD9 deficiency, as each of these pathways may be able to compensate for the lack of the other in humans [23, 78, 79]. Of note, CARD9-deficient patients have been reported to develop extrapulmonary aspergillosis involving the CNS and intra-abdominal tissues while sparing the lungs [80]. This observation identifies CARD9 deficiency as the first known inherited or iatrogenic condition that predisposes to strictly extrapulmonary aspergillosis. Interestingly, CARD9-deficient neutrophils do not exhibit impaired anti-*Aspergillus fumigatus* effector function. Instead, impaired accumulation of neutrophils in the extrapulmonary infected tissue was evident in affected patients, indicative of a neutrophil mobilization defect. Because CARD9-deficient patients with extrapulmonary aspergillosis do not have peripheral neutropenia nor do their neutrophils have cell-intrinsic chemotaxis defects [80•], the aforementioned observations suggest that impaired production of neutrophil-targeted chemoattractant molecules (CXC chemokines and/or other) in extrapulmonary tissue may drive susceptibility to aspergillosis in CARD9 deficiency.

Anti-*Aspergillus fumigatus* neutrophil effector functions

Following their recruitment into the *Aspergillus fumigatus*-infected infected tissue, neutrophils uptake fungal conidia for intracellular destruction and inhibit the extracellular growth of larger fungal hyphal elements that cannot be internalized. Pentraxin-3 (PTX3) is a soluble collectin that covers the surface of *Aspergillus fumigatus* conidia in the alveolar spaces and promotes their uptake by mouse neutrophils and control of aspergillosis in mice [81]. Mechanisms include a) deposition of complement and phagocytosis via CR3 and Fc γ receptor 2A (CD32), and b) activation of myeloid differentiation protein 2 (MD-2) and TIR-

domain-containing adapter-inducing interferon- β signaling (TRIF) [82, 83]. In keeping with the mouse findings, dysfunctional *PTX3* polymorphisms in humans are associated with impaired neutrophil uptake of *Aspergillus fumigatus* conidia and increased risk for development of invasive aspergillosis in HSCT and solid organ transplant recipients [84–86].

Neutrophils employ distinct mechanisms for *Aspergillus fumigatus* conidial and hyphal killing. For instance, in the mouse lung where rapid neutrophil deployment prevents conidial germination to hyphal elements, conidial killing does not depend on the neutrophil granule protein calprotectin (S100A8/A9), which acts to sequester zinc and manganese from *Aspergillus fumigatus* cells. Instead, in the mouse eye, germination of conidia to hyphae occurs, at least in part due to the sluggish mobilization of neutrophils to the infected tissue, and neutrophil-mediated inhibition of *Aspergillus fumigatus* hyphal growth requires calprotectin [87].

In mouse lung neutrophils, *Aspergillus fumigatus* conidial killing depends at large on neutrophil-intrinsic NADPH oxidase activity that results in induction of fungal apoptosis-like programmed cell death via modulation of the *Aspergillus fumigatus* anti-apoptotic protein, AfBIR1, a homolog of human *SURVIVIN* [88••]. In humans, CGD is the “signature” PID that underlies *Aspergillus fumigatus* infection susceptibility as these patients have a ~40% lifetime risk for developing the infection; a unique predisposition has been observed for infection with *Aspergillus nidulans*, a species of *Aspergillus* that is not seen in patients with iatrogenic immunosuppression, for reasons that remain largely unknown [78]. In contrast to NADPH oxidase-dependent ROS production, neutrophil MPO or serine protease activation do not appear essential for anti-*Aspergillus fumigatus* neutrophil killing in mice and, in agreement with that, MPO-deficient patients and patients with Papillon-Lefèvre syndrome who are deficient in cathepsin C do not develop invasive aspergillosis [4, 78, 89, 39].

Importantly, compensatory killing mechanisms do exist in phagocytes in the absence of NADPH oxidase, which is reflected in the clinical observation that ~60% of CGD patients never develop invasive aspergillosis despite ubiquitous daily exposure to airborne *Aspergillus* conidia. One of these non-oxidative burst-dependent pathways involves iron sequestration by lactoferrin, which is present within neutrophil secondary granules [90]. Of note, the pattern of mold infection susceptibility in CGD patients has also unveiled the mold-specific dependence on neutrophil oxidative versus non-oxidative cytotoxicity for effective host defense; indeed, while CGD patients are at high risk for aspergillosis, they rarely develop infection by the ubiquitous molds *Rhizopus* or *Fusarium* species, indicating that these fungi can be effectively controlled by neutrophil non-oxidative cytotoxic mechanisms in the absence of oxidative burst [78]. In *Rhizopus* species and other *Mucorales* fungi, which cause infections in patients with neutropenia but also characteristically infect patients with diabetic ketoacidosis (DKA), it was recently shown that ketone bodies impair the anti-*Rhizopus* killing capacity of neutrophils; this neutrophil function defect along with the ketone body-, hyperglycemia- and acidosis-induced up-regulation of fungal CotH and endothelial cell GRP78 that collectively promote *Rhizopus* angioinvasion shed light to the unique propensity of patients with DKA to develop mucormycosis, while they are not susceptible to other mold infections [91–93].

Mouse neutrophils express ROR γ t upon *Aspergillus fumigatus* exposure, which requires IL-6 and IL-23 signaling and is critical for expression of IL-17A, Dectin-2 and IL-17RC by neutrophils. IL-17A/IL-17RC act in an autocrine manner to promote neutrophil oxidative cytotoxicity and to protect against *Aspergillus fumigatus* keratitis in mice [94]. In humans, inherited deficiency in IL-17-dependent immunity, such as that seen with mutations in *IL17F*, *IL17RA*, *IL17RC* or *ACT1*, is dispensable for anti-*Aspergillus* host defense. Instead, human IL-17 deficiency impairs immunity at the mucocutaneous barrier and predisposes to chronic mucocutaneous candidiasis, cutaneous staphylococcal disease, and pulmonary bacterial infections [95, 96].

Recent elegant studies in the Rivera lab uncovered a critical role for CCR2-expressing monocyte and neutrophil cross-talk in the *Aspergillus fumigatus*-infected mouse lung for priming of ROS production and fungicidal activity by neutrophils [97, 98]. Specifically, the type III interferons IFN- λ s are produced *in vivo* by recruited inflammatory Ly6C^{hi} monocytes via the generation of type I interferon and act on neutrophils to promote their antifungal effector functions in the infected lung. In agreement, mice with neutrophil-specific deletion of IFNLR1 are highly susceptible to invasive aspergillosis and adoptive transfer of CCR2⁺ monocytes or exogenous administration of recombinant IFN- α and IFN- λ rescues the impaired neutrophil effector function seen in CCR2-depleted mice, which lack monocyte influx in the infected lung [97, 98]. Therefore, type I/III interferons orchestrate monocyte-neutrophil crosstalk to prime neutrophil fungicidal activity during pulmonary aspergillosis [97, 98], reminiscent of the cross-talk between NK cells and neutrophils that is orchestrated by IL-15/IL-23/GM-CSF for priming neutrophil fungicidal activity during renal candidiasis [55, 54, 53].

Independent mechanisms for killing of *Aspergillus fumigatus* conidia versus hyphae were recently characterized in human neutrophils by evaluating patients with various PIDs [48, 99]. Sensing of *Aspergillus fumigatus* conidia involves CR3 but not Dectin-1, which drives PI3K-dependent non-oxidative intracellular conidial killing. When conidia escape from killing and germinate into hyphae, their extracellular destruction requires antibody-mediated opsonization, sensing via Fc γ receptors, and signaling via Syk, PI3K, and PKC to drive NADPH oxidase-mediated ROS production. Of interest, although *Aspergillus fumigatus* hyphae induce NET formation in human neutrophils, which depends on intact NADPH oxidase, NETs do not contribute to *Aspergillus fumigatus* killing, in agreement with the dispensable role of NETs in host defense in a mouse model of ocular aspergillosis (Eric Pearlman, personal communication, manuscript submitted).

Conclusions

Invasive infections by *Candida* and *Aspergillus* species have emerged as significant causes of infection-related mortality in vulnerable patients with acute illness and iatrogenic immunosuppression [100, 7, 68]. The high fatality rates of these infections despite administration of antifungal therapy and the continuously expanding patient populations at risk for such infections highlight the unmet medical need for development of better diagnostic and therapeutic interventions in order to improve the prognosis of these infections [101, 12, 102, 68]. Neutrophils play a critical role in host defense against invasive

candidiasis and aspergillosis via their rapid deployment to the site of fungal invasion and by mediating fungal destruction using a panoply of effector mechanisms. Better understanding of the molecular cues that instruct recruitment and effector function of neutrophils to the fungal-infected tissues should help devise immune-based strategies with a goal to complement conventional antifungal therapy and improve the outcome of infected patients.

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