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Neutrophil Responses to Aspergillosis: New Roles for Old Players

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Abstract Neutrophils are professional phagocytic cells that play a crucial role in innate immunity. Through an assortment of antifungal effector mechanisms, neutrophils are essential in controlling the early stages of fungal infection. These mechanisms range from the production of reactive oxygen intermediates and release of antimicrobial enzymes to the formation of complex extracellular traps that aid in the elimination of the fungus. Their importance in antifungal immunity is supported by the extreme susceptibility to infection of patients with primary (e.g., chronic granulomatous disease) or acquired (e.g., undergoing immunosuppressive therapy) neutrophil deficiency. More recently, common genetic variants affecting neutrophil antifungal capacity have also been disclosed as major risk factors for aspergillosis in conditions of generalized immune deficiency. The present review revisits the role of neutrophils in the host response against *Aspergillus* and highlights the consequences of their deficiency in susceptibility to aspergillosis.

Keywords Aspergillosis · Neutrophils · Innate immunity · Antifungal effector function

Introduction

Neutrophils are professional phagocytes of the innate immune system that are essential to control bacterial and fungal infection. Clinical evidence has long suggested a prominent role for these cells in the defense against invasive aspergillosis (IA). Patients suffering from chronic granulomatous disease (CGD) have a dramatically increased risk of IA, and after the

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introduction of bone-marrow transplantation, it was soon realized that the duration of neutropenia is one of the key determinants of risk for IA in the respective patients. Over the last decades, experimental studies have revealed a prominent role of neutrophils in engulfing and killing *Aspergillus fumigatus* through a variety of effector mechanisms. In addition, recent advances in genetics of fungal infection have provided further insights into effector functions with important impact on the antifungal activity of neutrophils. This review highlights the role of neutrophils in the host immune response to *A. fumigatus* and addresses the consequences of their qualitative or quantitative insufficiency to susceptibility to aspergillosis.

Fungal Sensing and Immune Activation of Neutrophils

Humans routinely inhale small fungal spores from *A. fumigatus*—mostly without adverse consequences, except perhaps for allergic sensitization. The first line of resistance against invasion by the fungus is conferred by the physical barriers of the respiratory tract. In the event that fungal spores escape the ciliated epithelium and reach lung alveoli, they will then be challenged by cells of the innate immune system, including resident alveolar macrophages and dendritic cells. Consequently, everyday inhalation of *Aspergillus* spores will normally not result in inflammation and recruitment of inflammatory cells.

Neutrophils have long been recognized as an essential innate cell defense against IA, as the duration and extent of neutropenia as well as qualitative defects in neutrophil function (e.g., patients with CGD) are the most pervasive risk factors for the development of IA [1]. These findings are recapitulated in animal models in which antibody-mediated depletion of neutrophils [2] or absence of functional nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is required for generating reactive oxygen intermediates, [3] leads to uncontrolled fungal growth in the lung and mortality from aspergillosis.

Unlike macrophages, neutrophils are only rarely found in the lungs of naïve mice. However, this situation changes dramatically within a few hours after infection with *A. fumigatus*, as neutrophils are rapidly recruited to the site of infection [4]. This has been demonstrated to occur mostly due to the release of a subset of the CXC family of chemokine ligands by alveolar macrophages [5]. Moreover, the proinflammatory cytokine interleukin (IL)-1β, which is produced upon infection, directly induces chemokine release by lung epithelial cells [6]. The production of chemokines in the lung also correlated with the accumulation of monocytes/macrophages and expression of T helper (Th) 1-associated cytokines at the site of infection, suggesting an association between neutrophil recruitment and chemokine production by other cell types [5]. This notion is further supported by the finding that inflammatory monocytes, by conditioning the lung inflammatory milieu, modulate the conidiocidal activity of neutrophils [7].

Neutrophils express a vast repertoire of pattern recognition receptors (PRRs) that sense pathogen-associated molecular patterns (PAMPs) on the surface of conidia and drive the secretion of proinflammatory cytokines and chemokines that arbitrate immune responses and antifungal activity [8]. Fungal sensing is further assisted by the action of collectins, ficolins, pentraxins, and complement components that act as opsonins and facilitate the interaction of neutrophils with fungi. Similar to other phagocytes, neutrophils recognize and internalize fungal spores upon recognition by PRRs such as toll-like receptor (TLR)2, TLR4, or dectin-1 [9]. Of interest, mutations in caspase-associated recruitment domain 9 (CARD9), a signaling molecule downstream dectin-1, have been associated with impaired neutrophil killing and susceptibility to severe *Candida* infection [10]. Whether CARD9 deficiency can also predispose to aspergillosis remains to be elucidated. Despite the fact that signaling PRRs are present on neutrophils [11], evidence demonstrating cytokine and chemokine release by neutrophils is scarce. A few exceptions include the in vitro release of chemokine (C-X-C motif) ligands 1, 2, and 3 and chemokine (C–C motif) ligands 2, 3, and 4 by artificially stimulated neutrophils [12] and the upregulation of IL-8 production assessed after TLR2- and TLR4-dependent stimulation of neutrophils with *A. fumigatus* antigens [13].

One of the other important aspects regarding neutrophil activation has been provided recently. In a model of *A. fumigatus*-induced keratitis, neutrophils were found to express the transcription factor retinoic acid-related orphan receptor γt (RORγt) and induced the expression of IL-17A, IL-17 receptor C (IL-17RC), and dectin-2 [14]. This revealed the existence

of a novel neutrophil population with autocrine IL-17 activity that likely contributes to the inflammatory response against the fungus. In contrast, *A. fumigatus* is endowed with the ability to suppress neutrophil-dependent inflammatory responses. The hydrophobin layer of fungal spores, by masking dectin-1- and dectin-2-dependent recognition, restrains neutrophil infiltration and cytokine production [15, 16]. In addition, galactosaminogalactan (GAG), an immunosuppressive polysaccharide of the fungal cell wall produced by glucose epimerases [17], inhibits neutrophil infiltration and promotes fungal development even in immunocompetent mice [18]. Moreover, GAG functions as the dominant adhesin of *A. fumigatus*, mediating adherence and suppressing host inflammatory responses, in part through masking cell wall β-glucans from recognition by dectin-1 [19]. The immunomodulatory properties of GAG have also been attributed to its ability to induce IL-1 receptor antagonist (IL-1Ra) [20]. Ultimately, these findings suggest that targeting IL-1Ra in IA might be a therapeutic possibility to be exploited in the future.

Antifungal Effector Mechanisms of Neutrophils

Phagocytosis

Like macrophages, neutrophil granulocytes are "professional" phagocytic cells and one main antimicrobial function of this cell type is the killing of microbes after internalization (Fig. 1). The phagocytic activity of neutrophils relies on the opsonization of fungal spores. Among the opsonins identified to bind *A. fumigatus*, the long pentraxin 3 (PTX3) has been demonstrated to play a nonredundant role in antifungal host defense [21]. More importantly, PTX3 is stored in the granules of neutrophils in a ready-made form and undergoes release in response to fungal recognition and inflammatory signals [22]. This enables the formation of complexes on the conidial surface, thus enhancing recognition and phagocytosis through mechanisms that depend on Fcγ receptor, CD11b, and complement activation [23]. This pivotal role of PTX3 in response to *A. fumigatus* has also been recently confirmed in humans. A functional donor haplotype in the human *PTX3* gene has been demonstrated to increase the risk of IA among recipients of allogeneic hematopoietic stem cell transplantation [24]. This susceptibility phenotype was associated with a qualitative defect in the ability of newly reconstituted neutrophils to ingest and kill the fungus and not directly with the neutrophil counts.

Accumulating evidence suggests that neutrophils also communicate strongly with other components of the immune system. It has long been known that the activation of the complement system is closely linked to neutrophil activation. Fungal surfaces are triggers of complement activation, whereas fungi have evolved several mechanisms to escape complement activity, suggesting that a main function of complement activation may be the stimulation of neutrophils [25, 26]. Neutrophils have also been shown to interact with natural killer (NK) cells, which are activated by *A. fumigatus*. *Aspergillus*-induced activation of NK cells can lead to secretion of cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and granulocyte macrophage colony-stimulating factor (GM-CSF) which in turn directly stimulate neutrophil activation [27–29]. On the other hand, it is noteworthy that GAG is able to induce neutrophil apoptosis through a NK cell-dependent mechanism [30], suggesting that NK cells may play a role during neutropenia or autoimmune diseases. In a recent study, a receptor on the surface of NK cells recognizing fungi has been identified for the first time [31]. These data suggest that there is likely to be more to neutrophils function than just killing of the invading pathogen. For this reason, elucidating the role of neutrophils in IA is crucial for an in-depth understanding of the immunobiology of this disease.

Reactive Oxygen Species (ROS)

Each phagocytic event results in the formation of a phagosome into which ROS and hydrolytic enzymes are released after its fusion with lysosomes. Indeed, patients with CGD owing to an inborn deficiency of NADPH oxidase are highly susceptible to IA [32]. Neutrophils of these patients show markedly deficient NADPH oxidase activity and are unable to adequately produce superoxide anions and other reactive oxygen intermediates. These findings have been confirmed in animal models of CGD, either $gp91^{phox}$- or $p47^{phox}$-deficient [3, 33]. The fact that $gp91^{phox}$-deficient alveolar macrophages, but not neutrophils, efficiently controlled the growth of *A. fumigatus* conidia [33] suggests that neutrophils rely on ROS production to

eliminate the fungus. However, it is also true that CGD patients are exposed to *Aspergillus* conidia every day, yet considerable numbers of them survive years without infection, thus suggesting that neutrophils from these patients may also use ROS-independent mechanisms to counter the fungus. Consequently, ROS may be exclusively relevant in activating vacuolar enzymes [34, 35], suggesting that these enzymes may be responsible for the direct killing effect. One such example is the myeloperoxidase enzyme that critically relies on the presence of an oxidative environment for its proper function. Indeed, when released in the context of the oxidative burst, it generates the potent antimicrobial hydrochlorous acid from hydrogen peroxide and chloride anion [36]. Therefore, it is not surprising that myeloperoxidase-deficient mice are also susceptible to IA, though relatively less than NADPH oxidase-deficient animals [37]. Another emerging finding regards the possibility of neutrophil cluster formation and that formation of these oxidative-active aggregates may represent a novel antimicrobial mechanism preventing the germination of *A. fumigatus* conidia [33].

Granule Enzymes

Besides their phagocytic activity, neutrophils possess a very unique ability to counter invading pathogens. Following recognition, these cells are able to secrete antimicrobial compounds stored in their granules during a process called degranulation. This provides the cell with an effective mechanism to injure pathogens that are impossible to phagocytose, such as the large hyphae of *A. fumigatus* [38]. Indeed, neutrophils were initially thought to exclusively promote hyphal damage. However, recent studies showed that neutrophils are also able to eliminate spores from the infected lungs of macrophage-depleted mice [39].

There is also in vitro evidence to suggest that neutrophils can mediate antifungal activities via nonoxidative mechanisms. For example, knockout mice lacking neutrophil proteases cathepsin G and elastase that do not require ROS for their proper function were found to be susceptible to *A. fumigatus* infection [40]. This has been nonetheless challenged by the finding that neutrophil proteases may have a redundant role in antifungal host defense [41]. In addition, neutrophils release lactoferrin as part of their degranulation when interacting with *A. fumigatus* conidia [42]. The antifungal function of lactoferrin appears to rely on the sequestration of iron and inhibition of conidial growth [43]. In this regard, cell-free supernatants of degranulated ROS-deficient neutrophils (which had abundant lactoferrin) were able to suppress conidial growth. Further adding to the complexity of this mechanism, human neutrophils have been found to synthesize lipocalin-1, which by sequestering fungal siderophores restricts fungal growth [44]. This points to the existence of specific host iron-chelating and fungal iron-acquisition mediators regulating fungal growth. Finally, PTX3 is also stored in neutrophil granules, and besides its role as opsonin, PTX3 localizes to neutrophil structures known as neutrophil extracellular traps (NETs) (discussed below) upon activation [22].

NETosis

NETosis is a recently discovered neutrophil-dependent defensive system. During this process, dying neutrophils actively secrete their nuclear DNA and throw it on extracellular pathogens resulting in NET formation [45]. The process of NET formation depends on the induction of a ROS-mediated signaling cascade in neutrophils that ends up in the disintegration of the nuclear envelope and granular membranes [46]. Indeed, NADPH oxidase has been found to be required for generation of NETs in vivo, suggesting that the modulation of NETosis by NADPH oxidase enhances antifungal host defense and promotes resolution of inflammation upon infection clearance [47]. This is further supported by the finding that restoration of NADPH oxidase activity in CGD patients has been shown to reestablish NETosis and restore fungal defense against *A. nidulans* [48]. Pathogens coming in contact with the NETs are immobilized, thereby limiting the spread of infection. In addition, NETs also facilitate pathogen elimination, as they contain a number of antibacterial proteins, including PTX3 [22], defensins, the cathelicidin LL37 [49], histones [45], or calprotectin [50]. Although NET formation in response to *A. fumigatus* has been demonstrated in vitro [22] and in vivo in a lung infection model [4, 9], a direct effect of these structures in fungal killing has not yet been demonstrated. Of note, the RodA hydrophobin of *A. fumigatus*, previously shown to inhibit immune cell activation [15], has also been shown to inhibit NET formation, thus revealing the

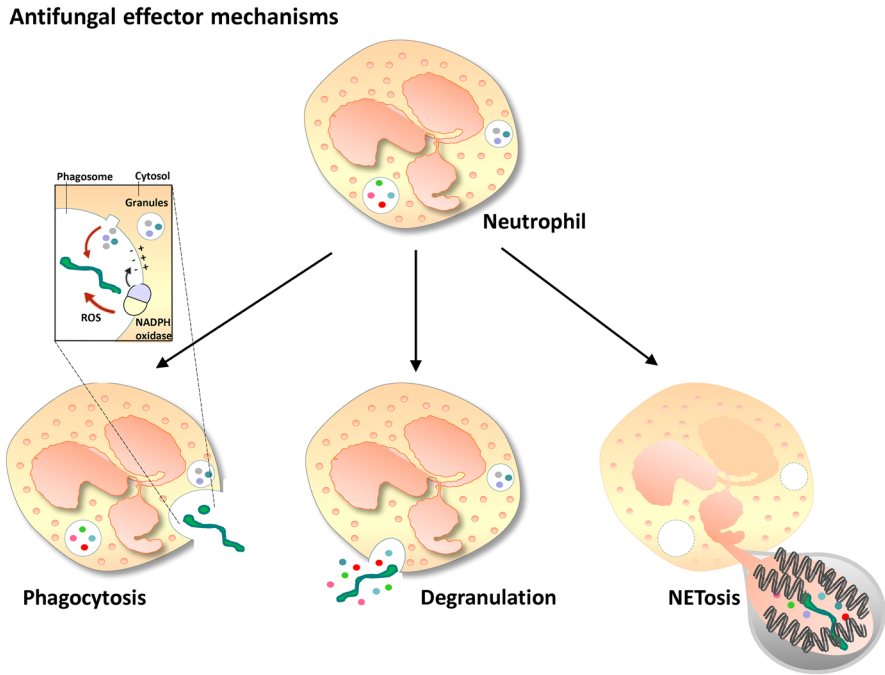

Fig. 1 Major antifungal effector mechanisms of neutrophils. Neutrophils can eliminate *Aspergillus* by multiple means, both intra- and extracellular. When neutrophils come into contact with the fungus, they phagocytose and kill it using NADPH oxidase-dependent mechanisms or antibacterial proteins. The antibacterial proteins are released from the neutrophil granules either into phagosomes or into the extracellular milieu. Highly activated neutrophils can also eliminate extracellular fungi by releasing NETs. These structures immobilize fungi, preventing their spreading but also facilitating their subsequent phagocytosis

first molecularly defined fungal escape strategy from NETs.

Future Perspectives

Current evidence demonstrates the importance of neutrophils in controlling fungal clearance in *Aspergillus* infections. Insufficient neutrophil counts (e.g., in patients undergoing hematopoietic stem cell transplantation) and specific qualitative defects owing to primary immunodeficiency (e.g., in CGD patients) have historically been considered the most important risk factors for severe *Aspergillus* infections. However, for most individuals at risk, propensity to aspergillosis will have a polygenic source. A genetic variant by itself has a negligible effect, and only in combination with other remarkable predisposing variants (e.g., profound immunosuppression), sizeable phenotypic effects will arise. Indeed, our own studies have recently identified common genetic variants influencing neutrophil function that predispose to fungal infection in particular conditions of immune dysfunction such as those observed in patients undergoing hematopoietic stem cell transplantation [24]. Genetic deficiency of signal transducer and activator of transcription 3 (STAT3) has also been found to increase risk of IA in patients with hyper-IgE syndrome [51]. However, in this case, it does not seem to affect neutrophil function and is instead likely related to impaired lung epithelial homeostasis and defense. The dissection of the genetic bases underlying susceptibility to infectious diseases inspires a very active, yet complex, field of research [52]. As the awareness of the individual genetic makeup may hold the key to expose unsuspected risk factors for these diseases, the current understanding of the immunological network involved in the immune response to *Aspergillus* needs to be addressed also from a genetic point of view. In addition, as shown for PTX3 deficiency [24], targeting neutrophil function (e.g., exogenous administration of lacking or deficient factors) may prove an interesting approach to be validated in the future.

Given the efforts carried out aiming at the detection of accurate prognostic markers and currently ongoing genome-wide studies [53], it is expected that novel genetic variants affecting neutrophil function may be identified in the future. These may provide important implications regarding patient outcome and healthcare costs in a near future by allowing the discrimination of patients that require enhanced surveillance for fungal disease and/or alternative antifungal therapies.

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