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Neutrophil extracellular traps in fungal infection

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Abstract

Fungal infections are a continuously increasing problem in modern health care. Understanding the complex biology of the emerging pathogens and unraveling the mechanisms of host defense may form the basis for the development of more efficient diagnostic and therapeutic tools. Neutrophils play a pivotal role in the defense against fungal pathogens. These phagocytic hunters migrate towards invading fungal microorganisms and eradicate them by phagocytosis, oxidative burst and release of neutrophil extracellular traps (NETs). In the last decade, the process of NET formation has received unparalleled attention, with numerous studies revealing the relevance of this neutrophil function for control of various mycoses. Here, we describe NET formation and summarize its role as part of the innate immune defense against fungal pathogens. We highlight factors influencing the formation of these structures and molecular mechanisms employed by fungi to impair the formation of NETs or subvert their antifungal effects.

Keywords

Neutrophil; neutrophil extracellular trap (NET); immunology; *Candida*; *Aspergillus*

1. Introduction

The innate immune system comprises an effective shield against fungal organisms that may otherwise invade tissues of our bodies. Physical, chemical, and microbial barriers cooperate with myeloid immune cells to recognize and eliminate fungal pathogens. Among these cells, polymorphonuclear leukocytes (neutrophils) play a decisive role. The importance of neutrophils for prevention and clearance of invasive fungal infections is widely recognized (1, 2). Patients with neutropenia are at high risk for contracting fatal fungal infections and prolonged neutropenia is associated with poor outcome. However, aspects of how neutrophils control the growth of this diverse group of pathogens, which are often too large

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to be phagocytosed, remained somewhat of a mystery until the discovery of neutrophil extracellular traps (NETs) (3). NET formation provides a means for neutrophils to kill fungi extracellularly, and the significance of this process in host immunity has been described for a variety of fungal infections, including candidiasis and aspergillosis (4–6). In this article, we review the neutrophil response to fungal pathogens, with a focus on the role of NETs. We describe the current understanding of factors stimulating NET release and mechanisms employed by fungi to resist killing by NETs.

2. Neutrophils and NETs

Neutrophils outnumber other white blood cells in circulation and are recruited in large numbers to sites of infection by chemokine gradients, where they serve as a first line of defense (7). In infected tissues, neutrophils activate their granule-stored weaponry via pattern recognition and cytokine receptor engagement, either by secretion of granule contents extracellularly, or by fusion of granules with pathogen-containing vesicles, so called phagosomes (8). The granules contain a variety of antimicrobial substances, including short antimicrobial peptides (AMPs) and proteolytic or nucleolytic enzymes (9). Upon stimulation of pattern recognition receptors, downstream kinase activation and Ca^{2+}-mediated signaling trigger neutrophils to assemble a large protein complex known as Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or phagocyte oxidase (Phox) (10). Components of this complex are both cytoplasmic (p40, p47 and p67) and membrane-bound (gp91 and p22). Assembly occurs on plasma and granular membranes, resulting in a functional enzymatic multimeric protein that reduces molecular oxygen to superoxide anion (11). The neutrophil enzymes superoxide dismutase and myeloperoxidase (MPO) further convert these highly reactive radicals to hydrogen peroxide and hypochloric acid, respectively. Collectively known as reactive oxygen species (ROS), the mix of these intermediates act both as efficient antimicrobials and as short-lived signaling molecules. Reactive oxygen species actively support the elimination of ingested microbes and promote the activation of pro-inflammatory processes (12). Although transcriptionally less active than other myeloid cells, neutrophils launch specific pathogen-tailored transcriptional responses upon microbial contact. Transcriptional regulation of cellular transport and cytokine production prepares neutrophils for battle and prompts the recruitment of additional immune cells (13).

In addition to degranulation, phagocytosis, ROS generation, and cytokine production, pathogen-induced activation of neutrophils also initiates cellular processes to expel chromatin to the exterior for NET release. Before release, these chromatin threads are decorated with antimicrobial components from granules and the cytoplasm (4). The cationic nature of histones and other antimicrobial proteins promote their attachment to DNA. The NET fibers then bind to the anionic surfaces of microbes, trapping and entangling them (Figure 1). Neighboring antimicrobials can also arrest growth or kill captured microbes (14). This extracellular chromatin meshwork has been implicated as an extracellular defense mechanism (3).

Since the discovery of NETs in 2004, several release mechanisms have been described. The first reported general mechanism involves chromatin decondensation accompanied by

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disassembly of the nuclear envelope, which is followed by release of NETs upon plasma membrane rupture (15). This process generally lasts several hours and, as it results in neutrophil death, is referred to as “suicidal NETosis” (16). This form of NETosis is dependent on a functional Phox, as neutrophils from immune-deficient chronic granulomatous disease patients lacking functional Phox do not release NETs to either bacteria or phorbol 12-myristate 13-acetate (PMA), an activator of Protein Kinase C (15). As a consequence of kinase activation and Phox assembly, granules become leaky and allow serine protease neutrophil elastase (NE) to enter the nucleus, where it cleaves histones (17). This cleavage in turn supports chromatin decondensation, which is further propelled by MPO entering the nucleus. A membrane-associated, MPO-containing complex of cationic granule proteins enables NE release from granular vesicles in the absence of membrane fusion. In the cytoplasm, NE degrades actin filaments to arrest cellular movement and facilitate NE’s nuclear translocation (18).

Later, a mechanism of NET formation by living, rather than dead neutrophils, was described (19). During this process, neutrophils expel their mitochondrial DNA by a catapult-like mechanism which had earlier been described for eosinophil DNA trap release (20). Upon priming with GM-CSF and stimulation with LPS or anaphylatoxin, mitochondrial DNA traps are released rapidly, within 15 minutes of exposure. Similar to suicidal NETosis, this process appears to be Phox-dependent (19). In contrast, a ROS-independent mechanism of NET release occurs upon stimulation with certain bacteria. Here, nuclear DNA is packaged into vesicles, which fuse with the plasma membrane and release their DNA content to the exterior (21). In doing so, remaining neutrophil ghosts can still migrate and phagocytose (22). Bacterial toxins that create pores in host cell membranes can also induce NET-like structures. This mechanism appears to be independent of ROS (23, 24), and similar to induction of NETs by the activation of calcium channels. Consecutive calcium influx induces NETs in the absence of a functional Phox system (25). In contrast to PMA-induced NETosis, fewer mechanistic studies have shed light on how the process of fast DNA release, whether ROS-dependent or not, may occur. However, downstream of ROS-dependent and –independent mechanisms, enzymatic histone modification appears to be essential for chromatin decondensation and subsequent release of NETs (26). The protein arginine deaminase 4 (PAD4) catalyzes the conversion of arginine residues to citrulline, mainly on core histones. The importance of PAD4 for NET release has been implicated in several models of infection and other diseases (27). A recent review article, however, suggests that several cellular processes that expel DNA may only mimic NET release. Proposed mimics include expelled mitochondrial DNA following cytokine or anaphylotoxin stimulation and excreted nuclear DNA resulting from pore-forming toxins or calcium ionophors (28). The authors suggest that pore-forming toxins induce calcium influx and subsequent activation of PAD4 leading to hypercitrunillation. As a consequence of histone citrullination, chromatin undergoes decondensation and is excreted. Conversely, ROS-dependent NETosis and release of mitochondrial DNA do not appear to involve PAD4 activation or histone citrullination.

Other myeloid cells, including eosinophils and mast cells, have also been described to release DNA traps. However, neutrophils seem to be able to release the structures more efficiently than other cell types, arguing for a specific relevance of NETs among the neutrophil defense mechanisms (20, 29, 30). Given the versatility of neutrophils and the

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wide range of host niches where these granulocytes are required to function flawlessly, it is likely that they respond differently to a variety of stimuli. After all, the existence of redundant yet differentially-triggered pathways may ensure full neutrophil functionality in any possible host milieu.

3. Relevance of NETs during mycoses

3.1 Candidiasis

Candida spp. are microbial commensals of the gastrointestinal tract that can colonize the genitourinary tract and skin (31, 32). However, in the face of immunosuppression, *Candida* spp. frequently can cause mucosal disease or more severe invasive infection. Neutropenia, often due to chemotherapy or hematologic malignancy, places patients at particularly high risk for life-threatening disseminated candidiasis (33). *Candida* spp. rank as the third most common bloodstream infection in hospital setting, with *C. albicans* as the predominant pathogen (31). A role for NETs in the response to fungi was first described for this model pathogen *in vitro*, with subsequent investigations revealing the importance of this process for control of candidiasis *in vivo* (Figure 1) (4, 34).

Neutrophils release NETs in response to *C. albicans* in murine models of localized and disseminated candidiasis (4). In a subcutaneous abscess model of infection, neutrophils are recruited to the site of infection and align on the periphery of fungal foci. Imaging reveals the presence of web-like structures of DNA that co-localize with NET-associated proteins, including MPO, histones, and calprotectin. Similar structures can also be found in the lungs of mice with disseminated candidiasis (4, 6). Much of the importance of this neutrophil process for control of *Candida* resides in the delivery of the antimicrobial protein calprotectin (4). This protein complex (S100A8/A9) is a divalent metal ion chelator with potent activity against a variety of fungal pathogens, including *C. albicans*, *C. neoformans,* and *Aspergillus* spp. (4, 35, 36). Once in close proximity, calprotectin exerts antifungal activity through depletion of Zn^{2+} and/or Mn^{2+}, which are essential for proliferation of these pathogens (4). Mice deficient in the production of calprotectin fail to deliver NET-associated calprotectin and exhibit a more rapid progression of subcutaneous *C. albicans* abscesses. The phenotype of calprotectin-deficiency is even more pronounced in a disseminated candidiasis model, where mice succumb to disseminated candidiasis twice as quickly as wildtype mice. These findings highlight the necessity of NETs for control of both superficial and invasive candidiasis, through their role in calprotectin release and delivery.

In the host, *C. albicans* displays multiple morphotypes, including yeast, hyphae, and pseudohyphae. While yeast forms are actively engulfed by phagocytosis, NETs appear to be critical for the killing and containment of the larger hyphal forms (6, 37). In one model of disseminated candidiasis, NETs are released in response to wildtype *C. albicans*, which produces filamentous forms during the course of pulmonary infection (6). However, NETs are not produced during infection with a yeast-locked mutant (*hcg1Δ*). Furthermore, NET production is a requirement for immune control of filamentous growth *in vivo*. Mice deficient in NET production through disruption of either MPO or Phox succumb to invasive candidiasis. In contrast, neutrophil attack against yeast morphotypes appears to function independent of NETosis, as MPO-deficient mice are capable of clearing infection caused by

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the yeast-locked mutant, presumably through phagocytosis. In addition, NETs are able to remodel the cell wall composition of *C. albicans* upon contact, leading to unmasking of β-glucan and enhanced recognition by Dectin-1-positive immune cells (38). In a model of disseminated candidiasis, these neutrophil-induced cell wall changes appear to be governed by activation of the *C. albicans* MAP kinase signaling pathway. Together, these studies demonstrate neutrophil-*C. albicans* interactions are influenced by fungal morphology and that subsequent neutrophil responses provoke fungal cell wall alterations, which in turn influence immunity.

While the majority of studies have focused on *C. albicans*, work with several non-*albicans Candida* spp. has revealed a likely role for NETs during candidiasis caused by these pathogens as well (39, 40). Although *C. dubliniensis* is capable of inducing NETs, the degree of NET induction is greatly reduced from that observed for *C. albicans* (39). This difference may be attributed to a difference in filamentation, as *C. dubliniensis* generates fewer hyphal forms. Surprisingly, *C. glabrata*, which lacks the ability to filament, is capable of eliciting NETs through a phagocytosis-dependent pathway (40). The mechanism underpinning why neutrophils respond differently to yeast forms of *C. albicans* and *C. glabrata in vitro* remains a mystery. Given the distinct neutrophil responses to these pathogens, further investigation of the role of NETs for control of candidiasis caused by non-*albicans* spp. would be of great interest.

3.2 Aspergillosis

Aspergillus spp. are ubiquitous environmental fungi that release spores, which are continuously inhaled and cleared by people with healthy immunity (41). However, patients with impaired immunity who do not efficiently eradicate these spores from their lungs develop invasive aspergillosis, a life-threatening angioinvasive infection (41–43). A large cohort at risk includes patients with neutropenia or hematologic malignancy, for whom *A. fumigatus* represents the most common pathogen (41). A second group of patients at risk are those who suffer from chronic granulomatous disease (CGD) (44). These patients have impaired Phox function, resulting in poor NET production and reduced neutrophil activity (15). In patients with this inherited disorder, *A. nidulans* emerges as a major pathogen, often resulting in refractory, disseminated disease (45).

Clinical studies and investigation with animal models of aspergillosis have shed light on the significance of NETs for containment and clearance of both of these pathogenic species (5, 36, 46, 47). In a clinical study involving a patient with CGD suffering from refractory invasive *A. nidulans* infection, Bianchi *et al*. linked the production of NETs to the resolution of invasive aspergillosis (46). *In vitro*, the Phox-deficient neutrophils lacked activity against *A. nidulans* conidia and hyphae. Restoration of Phox function by genetic complementation restored both NET production and antifungal activity. Furthermore, administration of gene therapy providing Phox activity rapidly cured the patient with treatment-refractory *A. nidulans* infection. Subsequent investigation revealed calprotectin (S100A8/A9) as the key antifungal component of NETs accounting for the activity against *A. nidulans* (36). By chelating Zn^{2+}, calprotectin inhibits *A. nidulans* growth and can induce irreversible zinc starvation at higher concentrations.

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In a murine model of pulmonary aspergillosis, neutrophils induce NETs upon encounter with *A. fumigatus* (Figure 2) (5). Observations made by 2-photon microscopy show that NETs form in conjunction with developing clusters of fungi with outgrowing hyphae. NETs are produced by newly recruited neutrophils, approximately 3–4 hours after migration to the site of infection. In contrast, conidia are engulfed by neutrophils. The formation of NETs *in vivo* to the larger hyphal forms of *A. fumigatus* is consistent with *in vitro* studies demonstrating a more robust NET release to hyphae over conidia, which would presumably be adequately cleared by phagocytosis (5, 35). *In vitro*, NETs exhibit fungistatic activity and are hypothesized to prevent fungal dissemination (5, 35). Similar to *A. nidulans*, *A. fumigatus* is inhibited by the NET-associated calprotectin (35). NETs also appear to modulate host immunity to *A. fumigatus* through release of long pentraxin (PTX) 3, a pattern recognition receptor that activates complement and facilitates pathogen recognition (48). The triggering of NETs in response to *A. fumigatus* is Phox-dependent (47). In a murine model of pulmonary aspergillosis, $p47^{phox-/-}$ mice deficient in Phox fail to generate NETs and ultimately develop progressive pneumonia. *In vitro* studies similarly show a requirement for Phox during induction of NETs by *A. fumigatus*.

3.3 Other mycoses

While the majority of investigations examining the relevance of NETs in fungal mycoses have focused on candidiasis and aspergillosis, NETs are anticipated to be a player in other fungal infections as well. For example, NETs can be visualized in the corneal scrapings from patients with fungal keratitis, a sight-threatening infection caused by a variety of fungal species, including *Aspergillus*, *Fusarium*, *Candida*, and *Alternaria* (49). The degree of NET release appears to vary with regard to the infecting pathogen and may correlate with clinical cure. However, little is yet known about the influence of NET release on inflammation and the clinical course of fungal keratosis.

Recently, investigations have been performed to determine the role of NETs in the host response to paracoccidioidomycosis (50–52). *Paracoccidioides* spp. are dimorphic environmental pathogens endemic to many areas of Latin America. After inhalation of spores, *Paracoccidioides* spp. can propagate as yeast, establishing pulmonary infection which may progress to disseminated disease (53). One of the hallmarks of dissemination is cutaneous paracoccidioidomycosis. Using histopathological samples collected from patients with these cutaneous lesions, Della Coletta *et al.* revealed the formation of NETs (52). However, the importance of NETs for the clearance of paracoccidioidomycosis remains unclear. While both conidia and yeast induce NETs *in vitro*, *P. brasiliensis* is not susceptible to attack by NETs (50–52). Thus, it seems plausible that NETs function to contain *P. brasiliensis* during infection, preventing dissemination.

A role for NETs in the control of cryptococcosis has also been explored (4, 54, 55). This environmental pathogen propagates in the host as a yeast capable of causing pulmonary disease and disseminated disease with meningitis (56). Patients at particular risk include those with immunity impaired by either human immunodeficiency virus (HIV) or transplant-related immunosuppressant medications (56). While NETs can be induced in response to *C. neoformans* under some conditions, the capsule of *C. neoformans* is a potent inhibitor of

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NETosis (54). In contrast, *C. gattii* appears to trigger the release of NETs, but possesses virulence factors that resist killing by neutrophils (55). Whether NETs form in response to *Cryptococcus* spp. *in vivo* and the importance of this neutrophil process in cryptococcosis are areas of interest.

4. NETs and fungal pathogens

4.1 Induction of NETs by fungi

There have been many attempts to describe induction of NETs by different fungal pathogens (table 1). However, a one ligand-to-one pattern recognition receptor concept seems unlikely given the diversity of reports. NET induction by a fungus was first observed for *C. albicans,* a multi-morphic fungal pathogen (34). While relatively little is known about chlamydospore and pseudohyphal development in *C. albicans*, the transition from yeast growth to hyphal filamentation has been extensively studied. Invasion of and damage inflicted on epithelium and other host cells largely correlates with hyphal growth of *C. albicans* (57). Both yeast and hyphal forms induced NETs and were susceptible to inhibition by NETs (34). It is tantalizing to assume that the hyphal filaments, often too large to be ingested, may constitute a main target of NETs, in an attempt to compensate for the less efficient phagocytic uptake. Indeed, hyphae are more prone to trigger NET release (Figure 3) (58). This observation seems to be dependent on ROS, since hyphae induce increased ROS production by neutrophils when compared to yeast forms. This is in line with other reports showing that *C. albicans* yeasts can suppress ROS production by phagocytes (59) and that large amounts of yeasts are more efficient in ROS scavenging than hyphae (60).

A recent report shed light on neutrophil signaling pathways involved in the more robust NET release observed in response to *C. albicans* hyphae when compared to yeast forms (6). Examination of a yeast-locked mutant strain revealed that one mechanism underlying this size-sensing programming involves the engagement of Dectin-1 and the initiation of phagocytosis. This process downregulates NE translocation to the nucleus, which prevents chromatin decondensation and NET release. The pathway is suggested to inhibit NET release when organisms can be effectively eliminated by phagocytosis, ultimately preventing uncontrolled NETosis and associated tissue damage. The relevance of cell size for NET induction has also been shown for *A. nidulans* (46) and *A. fumigatus* (5). Similar to *C. albicans,* long *Aspergillus* filaments readily induce NET release, whereas the relatively small conidia are poor inducers (5).

While numerous investigations have considered the influence of fungal morphology on NET induction, the neutrophil receptors triggered by fungal ligands and subsequent signaling pathways that initiate NET release remain more obscure. In the presence of the extracellular matrix protein fibronectin, neutrophils respond faster to *C. albicans* and release NETs more rapidly as compared to the suicidal NETosis induced by PMA (61). This fast induction can be induced by β-glucan particles and soluble β-glucan molecules via complement receptor 3 (CR3) mediated signaling as elucidated using blocking anti-CR3 antibodies. Additionally, this form of NET formation appears to be independent of Phox-derived ROS. Subsequent study has confirmed that particulate β-glucan triggers NETs and that this induction is dependent on Syk kinase, as demonstrated by the use of pharmacological inhibition (62).

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However, diverging engagement of neutrophil signaling pathways upon glucan particles and the various *C. albicans* morphotypes is likely to be observed, considering the influence of fungal size and morphology on NET production.

A recent study has explored the identities of neutrophil cell surface receptors that might be involved in the triggering of NETosis following fungal recognition. As may be expected, numerous *C. albicans* ligands exhibit the potential to induce NET formation. According to Zawrotniak and colleagues, *C. albicans* derived β-glucan, mannan, and certain cell wall proteins are all individually capable of inducing NETs (63). In this investigation, the β-glucan triggering of NETs could be partially blocked by specific antibodies directed against the cell surface lectin Dectin-1, the prime receptor for β-glucan. This is somewhat in contradiction to the size-sensing mechanism described by Branzk *et al*. (6), which reported that Dectin-1 activation during phagocytosis inhibited NET formation, whereas Dectin-1 blockage induced NETs. The reason why Dectin-1 inhibition results in differing neutrophil responses under these experimental conditions remains unclear.

In addition to cell wall polysaccharides, secreted aspartic proteases (SAPs) of *C. albicans* are capable of triggering NETs (63). *C. albicans* produces ten different SAPs, with expression patterns dependent on environmental conditions. All SAPs, with the exception of SAP3, 5 and 7, can induce NETosis. In contrast to β-glucans, for which Dectin-1 mediates the signal for NET triggering, SAPs appear to engage mainly the β-2 integrin CR3 (63) in accordance with the previously mentioned study (61). However, little is known about how these SAPs may trigger NETosis and if they play a role in immunity through NET induction *in vivo*. Further investigation in this area will be of interest.

Recently, Moyes *et al*. discovered the first cytolytic peptide toxin produced by *C. albicans*, which has been termed candidalysin (64). As bacterial cell-lytic toxins induce NET-like structures when exposed to human neutrophils (23), it seems logical that candidalysin may contribute to the NET release we observe when neutrophils and *C. albicans* are co-cultured. To date, however, it remains to be determined whether candidalysin exposure can trigger NET release in neutrophils. Furthermore, it is unclear if other pathogenic fungi produce similar cell-lytic toxins that may influence NET production.

4.2 Fungal strategies for modulation and inhibition of NET induction

Although NETs can provide targeted delivery of calprotectin and exhibit potent antifungal activity, a variety of fungi have developed strategies which can either modulate or block the release of NETs. *C. albicans*, *C. neoformans*, and *A. fumigatus* possess virulence traits to suppress NET release upon neutrophil encounter (5, 54, 65). Impairment of NET production by *C. neoformans* correlates with capsule production, which is typically observed during infection (54). However, for *C. albicans* and *A. fumigatus*, NET inhibition is growth phase-dependent (biofilm for *C. albicans*, conidia for *A. fumigatus*) (5, 65). Both *C. neoformans* and *C. albicans* engage inhibitory pathways that are not rescued by potent stimulators of NETosis, such as PMA. The mechanisms underpinning the impairment of NET release by these pathogens are discussed below.

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In vitro investigation shows that neutrophils fail to release NETs in response to *C. neoformans* (54). This inhibition is linked to the production of a polysaccharide capsule, as a *cap67Δ* mutant defective in capsular production triggers NET release (54). The capsule of *C. neoformans* is largely comprised of a unique polysaccharide, glucuronoxylomannan, a key virulence factor with multiple immunomodulatory activities (66–69). The purified polymer alone, which consists of linear α-(1–3)-mannan substituted with β-(1–2)-glucopyranosyluronic acid and β-(1–4)-xylopyranosyl, suppresses NET release in response to PMA, consistent with a role for this capsule polysaccharide in NET impairment (54). NET inhibition may contribute to immune evasion *in vivo*. Neutrophils induced to form NETs, by stimulation with either PMA or the acapsular mutant, exhibit antifungal activity against the wildtype *C. neoformans*. This suggests NETs could be an effective method of killing if their formation were not otherwise inhibited.

Many fungal infections, including candidiasis, involve the formation of biofilms, communities of adherent cells growing within an extracellular matrix (70–77). Biofilms commonly propagate on surfaces of medical devices, such as vascular catheters, urinary catheters, and ventricular fluid shunts (76–79), as well as mucosal surfaces (80, 81). Given the large size of these aggregates, NETs would appear to be an ideal method to control these infections. However, very few NETs form upon exposure to *C. albicans* biofilms (65, 82). Inhibitory pathways induced by *C. albicans* biofilm persist in the presence of potent inducers, such as PMA (65). This impairment of NET release is thought to account for the resilience of biofilms to neutrophil attack, as *C. albicans* biofilms are up to 5-fold more resistant to killing by neutrophils when compared to free-floating planktonic organisms (65, 83–85). However, neutrophils pre-induced to form NETs (by PMA) are capable of inhibiting biofilms, suggesting NET release may be an effective mechanism of controlling biofilm infections, if the process were not otherwise blocked by the mature biofilm (65).

Impairment of NET release by *C. albicans* biofilms requires the presence of an intact extracellular matrix (65). Physical disruption of the biofilm both induces NET release and increases susceptibility to killing by neutrophils (65, 85). Inhibition of NETs appears to correlate with the production of matrix polysaccharides that are distinct from cell wall polysaccharides (65). A genetic screen identified a mutant strain (*pmr1Δ/Δ*) capable of inducing NETs while growing as a biofilm. *PMR1* encodes a transporter required for cell wall mannosylation during planktonic growth. During biofilm growth, this enzyme is critical for production of α-mannans of the matrix, which assemble with β-glucans extracellularly to form a mannan-glucan complex (86, 87). This unique polysaccharide complex is postulated to contribute to NET inhibition during *C. albicans* biofilm formation. *C. glabrata* biofilms also appear to inhibit NET formation, although to lesser extent when compared to *C. albicans* biofilms (40). However, it is unknown if a similar mechanism of impairment by extracellular matrix is employed by *C. glabrata* biofilms.

Conidia of *A. fumigatus* suppress NET formation. *In vivo* imaging of the neutrophils in a murine model of pulmonary aspergillosis reveals distinct neutrophil responses to hyphal and conidial forms (5). While time-lapse imaging shows that NETs are released in response to hyphal forms, resting or swollen conidida trigger fewer NETs. Modulation of NET release is linked to hydrophobin RodA, a major conidial surface component that also impairs adaptive

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immunity (88). Conidia of a *rodAΔ* mutant, which lack this surface component, trigger NETosis, even to a greater level than that observed for hyphal forms (5). The specific mechanism underlying the ability of RodA to impair NET production is unknown, but one theory is that it exerts its activity by masking pathogen-associated molecular patterns of the conidia cell wall.

4.3 Mechanisms employed by fungi to escape NETs

A variety of fungi have developed defenses to resist killing by NETs. For example, *A. fumigatus C. gattii* and *P. braziliensis* induce NET release, but the NETs exhibit minimal fungicidal activity against these pathogens (51, 55, 89). While NETs may not be capable of eradicating these fungi, the structures may function to prevent dissemination. Several studies have shed light on the factors contributing to the resistance to NET attack for *A. fumigatus* and *C. gattii*. The most well-defined mechanism of protection from NETs has been described for hyphae in *A. fumigatus* biofilms.

During pulmonary infection, *A. fumigatus* forms microcolonies of hyphae encased in an extracellular matrix (75). One of the most abundant and well-described components is galactosaminogalactan (GAG), an α-1,4-linked linear heteroglycan composed of variable combinations of galactose and N-acetyl-galactosamine (GalNAc) (75, 90, 91). Disruption of GAG attenuates both biofilm formation and virulence (92, 93). While GAG exhibits multifactorial influence on immunity, one of its key roles is providing protection from killing by neutrophils, shielding *A. fumigatus* from the antifungal activity of NETs (89).

While *Aspergillus* spp. commonly produce GAG, the relative proportion of galactose and GalNAc varies among species and strains (94–97). Lee *et al*. capitalized on the differences in GAG composition between *A. fumigatus* and *A. niger* to further characterize the activity of GAG (89). While *A. fumigatus* produces GalNAc-rich GAG, *A. niger* produces GalNAc-poor GAG with 5-fold higher levels of galactose. In comparison to *A. fumigatus*, *A niger* is less virulent and more susceptible to neutrophil attack. To test a role for GalNAc-rich GAG in virulence, *A. niger* was induced to produce GalNAc-rich GAG by heterologous expression of *Uge3,* an *A. fumigatus* gene encoding an epimerase required for its synthesis. The *A. niger* strain manipulated to produce GalNAc-rich GAG exhibited enhanced virulence and increased resistance to neutrophil attack, similar to *A. fumigatus*. Therefore, protection from NETs links closely to GalNAc-rich GAG. The protective effect of GAG, a partially deacetylated and polycationic glycan, likely resides in its positive charge, which is theorized to inhibit the binding of the cationic antimicrobial peptides or histones in NETs.

C. gattii, an emerging cause of cryptococcosis, is another pathogen protected from NETs. The basidiomycete inhabits tropical and temperate regions in association with trees (98). To investigate fungal-plant interactions, Springer *et al*. utilized an *Arabidopsis thaliana* wound model and discovered that *C. gattii* produces unique extracellular fibrils when growing on plants or plant-derived media (55). Fibril production is dependent on capsule formation, as a capsular mutant (*cap59Δ*) lacks these structures (55, 99). Wildtype *C. gattii* initially grown in the wound model or on plant-derived media demonstrates hypervirulence when used in murine models of both pulmonary and disseminated cryptococcosis, as compared to yeast that had initially been grown in standard conditions. This difference in virulence correlates

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with decreased susceptibility to neutrophil attack, with *C. gattii* growing in plant-derived media exhibiting an approximately 2-fold higher resistance to neutrophil killing. As *C. gattii* grown under these conditions induces NETs, the extracellular fibrils produced by *C. gattii* are proposed to physically impair fungal entrapment and killing.

In addition to evading killing by NETs, fungi may also combat NETs through degradation of their DNA backbone. Numerous bacterial pathogens escape NETs through release of extracellular endonucleases capable of destroying NETs (100–105). Recent investigation of several *C. albicans* isolates revealed secretion of DNase into their culture supernatants (106). While these enzymes are hypothesized to degrade NETs and prevent fungal death, further studies are needed to define the role of secreted DNases in the protection of C. *albicans* from neutrophil killing.

4.4 Possible host-derived regulators of NET formation in the context of fungal infections

Neutrophils keep a potentially dangerous arsenal which can effectively eradicate intruders, but at the same time can harm the host's own tissue (7). Therefore, neutrophil activity is tightly controlled. Given the fact that NETs expose membrane-damaging peptides, proteolytic enzymes and pro-inflammatory molecules directly to host tissue, it seems obvious that NET induction and half-life would need to be meticulously regulated. It is believed that blood-borne host-derived DNases significantly contribute to degradation of NETs both in circulation as well as in tissue (107). Smaller NET debris and neutrophil remnants are then likely ingested by macrophages and cleared by the liver. Presumably, there may be additional host factors that negatively regulate NET induction. Extensive studies of *C. albicans* in a whole blood model system did not reveal signs of NET formation, such as extracellular DNA or neutrophil cell death, during incubation times of several hours (108). These findings argue for the existence of factors circulating in blood that may limit the release of NETs, presumably to ensure blood flow and to avoid premature clotting of vessels, which may have fatal consequences for the host. In extreme scenarios, such as bloodstream infection with sepsis, the regulatory circuits seem to be overruled, as reports reveal extensive NET release in septic animal models (as reviewed by (109, 110)). In these extreme situations, thrombosis, which is also promoted by NETs (111), may be employed as a last resort to confine pathogen spread.

Recent studies have begun to uncover host-derived modulators of NET release for several infection models. Endothelial p33, a kininogen-binding and complement-related protein, has been shown to downregulate NET formation caused by danger-associated molecular pattern signaling (112). This protein is detectable in patients with fasciitis caused by *Streptococcus pyogenes,* where it co-localizes with MPO, an inducer and constituent of NETs. Another example is human immunodeficiency virus-1 (HIV-1) infection, where IL-10 released by dendritic cells contributes to the downregulated release of NETs, which are important for binding and neutralizing virus particles (113). In response to leishmanial parasites, neutrophils can release NETs capable of modulating immunity. During a protective Th1 response, IL-4 and GM-CSF drive monocytes to differentiate into dendritic cells. However, in the presence of NETs, this differentiation is skewed, leading to the development of anti-inflammatory macrophages which in turn release high amounts of IL-10 (114). While we

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have only scarce information about how NET formation is controlled during viral, bacterial and parasitic infections, knowledge about host-derived, negative regulators of NET release during fungal infections is virtually absent.

5. Outlook

Since their discovery in 2004, NETs have received increasing attention. Investigations have revealed diverse stimuli, signaling pathways, and release mechanisms for these DNA traps. In addition to their importance for control of numerous pathogens, their impact appears to reach far beyond antimicrobial immunity. NETs are suggested to be key players in autoimmune diseases, such as lupus erythematosus (107) and small vessel vasculitis (115). In addition, they can influence cancer pathology by promoting metastases (116) or aggravating cancer-associated thrombosis (117). Even possible contributions to brain-degenerative diseases, such as Alzheimer's disease, have been attributed to NETs (118). It seems likely that the involvement of NETs in even more inflammatory disorders will be uncovered, as a myriad of these diseases exist. When reporting about inflammation, immunologists are unable to pass over neutrophils, since these granulocytes are recruited in large numbers to virtually any inflamed site and serve as a hallmark for an inflammatory milieu (119).

As neutrophils have the potential to trigger and release NETs, it is logical to assume that these structures fulfill a task and are relevant for the outcome of infection. However, we should keep in mind that neutrophils are efficient hunters with several loaded weapons, and NETs are just one piece of a bigger puzzle. Undoubtedly, neutrophils play a central role in the defense against many fungal pathogens (120) and thus one may mistakenly assume that NETs are equally relevant for eradication of all mycoses. As an example, *A. fumigatus* hyphae trigger NETs, but are not susceptible to NET attack (5). Nevertheless, people with healthy immunity do not acquire aspergillosis despite the inhalation of hundreds of *A. fumigatus* spores every day. Instead, neutrophils efficiently eradicate these inhaled spores primarily by ingestion and ROS-dependent triggering of apoptosis-like cell death in the fungus (121), indicating that NETs are less important in this particular situation. However, while NETs may be dispensable to prevent *A. fumigatus* infection in the immunocompetent host, they have been demonstrated to be critical for control of infection caused by the closely related pathogen *A. nidulans* (46). These differences convincingly illustrate that more studies are required to understand individual contributions of neutrophil weaponry to eradicate diverse types of mycoses. Ultimately, detailed knowledge should promote development of more efficient diagnostic and therapeutic tools which are urgently required to cure emerging fungal infections.

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Abbreviations

AMP	antimicrobial peptide
CGD	chronic granulomatous disease
CR3	complement receptor 3
GAG	galactosaminogalactan
GalNAc	N-acetyl-galactosamine
GM-CSF	granulocyte-macrophage colony stimulating factor
HIV	human immunodeficiency virus
IL	interleukin
LPS	Lipopolysaccharide
MPO	myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	neutrophil elastase
NET	neutrophil extracellular trap
PAD4	protein arginine deaminase 4
PMA	phorbol 12-myristate 13-acetate
Phox	phagocyte oxidase
PTX3	pentraxin 3
ROS	reactive oxygen species
SAP	secreted aspartic protease

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Highlights

- Neutrophils release extracellular DNA traps to ensnare invading microbes
- Neutrophil extracellular traps (NETs) contribute to innate immune responses in mycoses
- Many human fungal pathogens induce NETs and are susceptible to their attack
- Release of NETs is regulated by size and morphology of fungal pathogens

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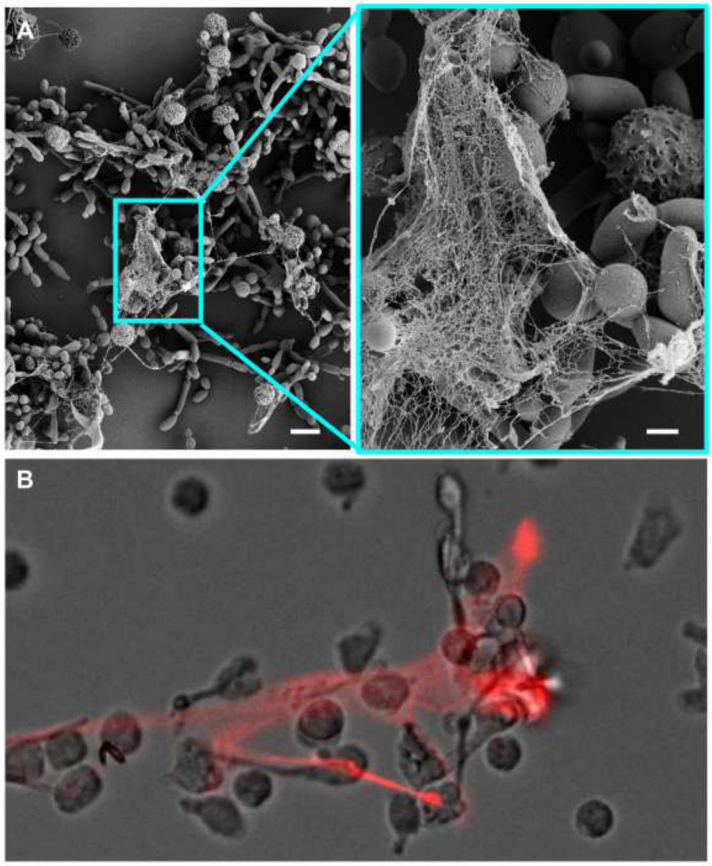

Figure 1. Neutrophils release NETs in response to *C. albicans*

A) Scanning electron microscopy images reveal the formation of NETs following a 4 h incubation of human neutrophils with *C. albicans*. Measurement bars represent 10 μm and 2 μm for images obtained at 2,000 (left) and 10,000x (right), respectively. (B) Propidium iodide (red) staining shows the extracellular DNA of NETs released by human neutrophils upon exposure of human neutrophils to *C. albicans*.

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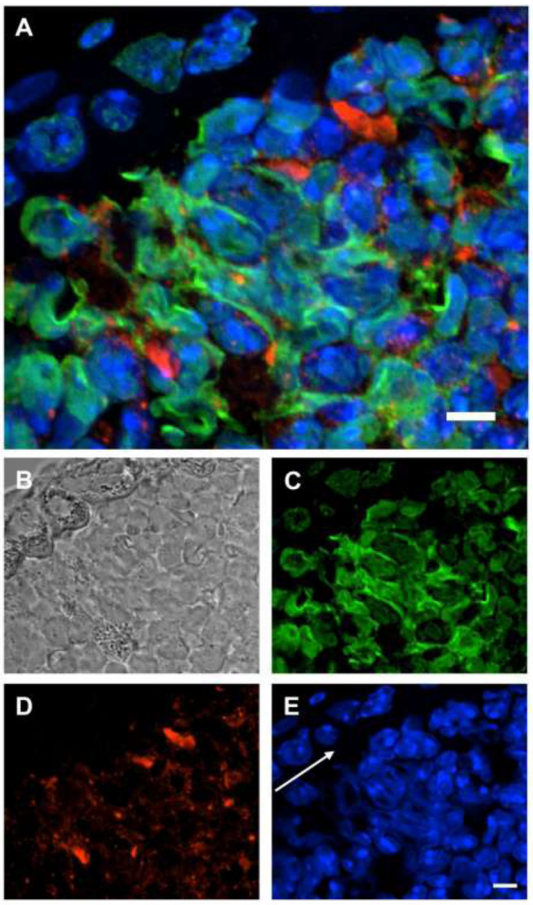

Figure 2. Neutrophils release NETs during *A. fumigatus* infection

Immunohistochemistry was performed on bronchioles of mice with invasive pulmonary aspergillosis induced by nasal infection with hyphal filaments. MPO (red) and histone (green) were stained with specific antibodies and fluorescently-labelled secondary antibodies. The nuclear contents of cells are stained with DAPI (blue). In patchy areas NET-associated proteins MPO, histone and DAPI co-localize extracellularly as depicted by superimposition of all three fluorescent channels (A), phase contrast (B), histone (C), MPO (D) and DAPI (E). White arrow indicates direction of epithelial layer. Size bars represent 5 μM.

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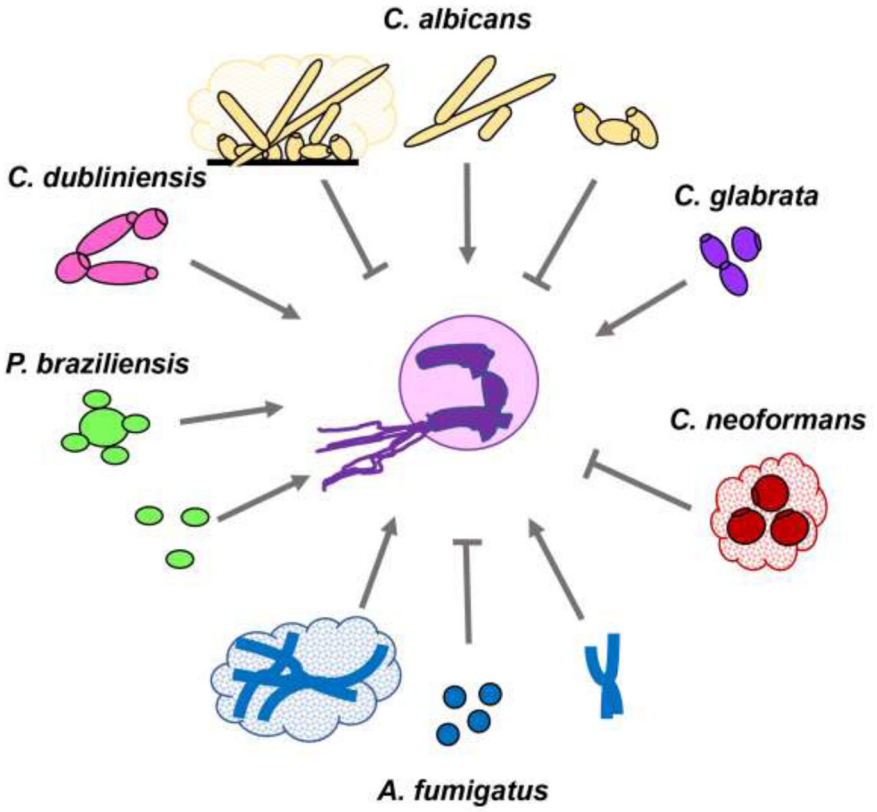

Figure 3. Induction and inhibition of NET release in response to fungal pathogens based on morphology

C. albicans, *C. dubliniensis*, and *C. glabrata* induce NET formation. Yeast forms of *C. albicans can* inhibit NET release through engagement of Dectin-1 and subsequent induction of phagocytosis. In addition, yeast efficiently scavenge ROS which is required for NET induction. The capsule of *C. neoformans* inhibits NET release. *A. fumigatus* hyphae and biofilms trigger NET release. However, *A. fumigatus* conidia inhibit formation and are engulfed by phagocytosis. Both conidia and yeast forms of *P. braziliensis* induce NETs.

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Table 1

Summary of fungi inducing / inhibiting NETs and their respective susceptibility to NET attack.

Fungal organism	NET induction		Susceptibility to NETs	Reference
	Potency	Comment		
Aspergillus fumigatus	++	Hyphae better inducers than conidia, GAG on cell surface shields off NETs	−	(5, 35, 89)
Aspergillus nidulans	++	Hyphae better inducers than conidia	++	(36, 46)
Arthroderma benhamiae	++	Conidia and hyphae induce NETs to similar extent	unknown	(122)
Candida albicans	++	Hyphae better inducers than yeast Yeast-locked mutants fail to induce Biofilms inhibit NETs	++	(6, 34, 58, 65)
Candida dubliniensis	+	Decreased NET induction compared to *C. albicans*	++	(39)
Candida glabrata	++	Yeast induce NETs more than biofilms	+	(40)
Cryptococcus neoformans	−	Capsule prevents NET formation	++	(54)
Cryptococcus gattii	++	Extracellular fibrils induce NETs and prevent NET attack	−	(55)
Paracoccidioides brasiliensis	++	Both conidia and yeast induce NETs	−	(52)