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Host defense mechanisms against *Aspergillus fumigatus* lung colonization and invasion

Joseph J Mackel and Chad Steele

The human lung is continually exposed to airborne conidia of the fungus *Aspergillus fumigatus* (AF) and related species. The innate immune system efficiently eliminates inhaled AF conidia from the lung in normal individuals, but immunocompromised patients are at risk for highly lethal invasive aspergillosis (IA). Some individuals not at risk for IA may still suffer from failed clearance of AF in the form of noninvasive colonization associated with conditions such as allergic bronchopulmonary aspergillosis. Understanding of normal innate immune function against AF as well as failures of these functions will enable better treatment of these patient groups. In this review, we will focus on recent research that elucidates mechanisms of host defense and their failures resulting in colonization as well as tissue invasion.

Address
Department of Microbiology and Immunology, Tulane University, New Orleans, LA, United States

Corresponding author: Steele, Chad (csteele4@tulane.edu)

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Introduction

Invasive aspergillosis (IA) is tissue damaging infection caused by the opportunistic fungal pathogen *Aspergillus fumigatus* (AF) and is a hazard to patients with suppressed immune function commonly associated with hematopoietic stem cell transplant, organ transplant, cancer therapy, and select genetic mutations. Innate immune defects, including neutropenia, alveolar macrophage dysfunction, and impaired STAT3 and NAPDH oxidase activity allow difficult to treat fungal growth that results in high mortality. Other groups of patients with less severe or altogether lacking patent immunosuppression are at risk of noninvasive colonization with AF. These patients include individuals with cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and allergic bronchopulmonary aspergillosis (ABPA) [1,2]. Distinguishing between colonized patients and those with IA is complicated by diagnostic limitations. Measurement of a component of the host innate immune response, pentraxin 3 levels, was found to be an effective method of discriminating between these two groups, indicating the exposure to the immune system or the capacity of the response is different between the two conditions [3]. Although IA is clearly a result of defects in immunity, recent research indicates that colonization may also be the result of less apparent immune defects, such as failures of epithelial integrity.

Epithelial defense

The lung epithelium is the initial site of host–fungus interaction. Recent work has underscored its active role in host defense against *A. fumigatus*. Gago *et al.* reported that ABPA patients heterozygous for a single nucleotide polymorphism in the transcription factor zinc finger protein 77 (ZNF77), which normally functions in epithelial integrity, carried higher fungal load than patients without the mutation. *In vitro* studies of bronchial epithelial cells carrying the mutation exhibited decreased epithelial integrity and *A. fumigatus* conidia adhered to the mutant cells at higher rates and germinated more quickly than on WT cells. Mass spectrometry secretome analysis revealed increased synthesis of adhesive extracellular proteins, such as ficolins, by mutant cells suggesting that wild-type ZNF77 protects against fungal adhesion by regulating synthesis of proteins used by *A. fumigatus* for adherence. The authors hypothesized that failures of the epithelium such as this may allow for sensitization to fungal allergens [4••]. Another report found that human bronchial epithelial cells inhibit germination of extracellular *A. fumigatus* conidia in a contact-dependent manner and was partially dependent on PI3-kinase. The effect also required an interaction between a yet to be identified host ligand and the fungal lectin FleA [5]. In opposition to these mechanisms of epithelial control, recent research has also uncovered mechanisms of *A. fumigatus* evasion of epithelial defenses. Liu *et al.* reported that the *A. fumigatus* protein CalA induces endocytosis in epithelial and endothelial cells by binding integrin $\alpha5\beta1$. A CalA mutant strain was less virulent *in vivo*, inducing less mortality and fungal burden in an immunosuppressed model of IA [6]. *A. fumigatus* hyphae were also observed to breach bronchial epithelial cell layers *in vitro* by manipulation of host actin without causing cell damage [7]. Together, these studies underscore the critical role of the lung epithelium in preventing fungal germination and keeping *Aspergillus* spp. in the air space where mechanisms such as

mucocilliary clearance and alveolar macrophage phagocytosis may clear them with minimal inflammation. Two recent studies demonstrated that nutrient availability is also a key determinant of invasion. Zinc chelation *in vivo* significantly decreased fungal burden and improved host survival during IA [8]. Using a mouse model of airway transplantation, Hsu *et al.* presented multiple lines of evidence showing that iron availability promotes fungal tissue invasion over colonization [9•]. It is thought that once angioinvasion occurs and induces hemorrhage, the scale tips in favor of the pathogen, further highlighting the importance of epithelial defense mechanisms to halting fungal growth before tissue invasion.

PRRs and cell wall components

Innate immune recognition of AF cell wall components has been an area of intense research and multiple families of pattern recognition receptors (PRRs) including C-type lectin receptors (CLRs) and Toll-like receptors are known to mediate recognition of cell wall components of swollen conidia and hyphae. In an interesting recent report, Stappers *et al.* found that a CLR, MelLec, recognizes melanin in the cell wall of dormant conidia and protects mice from fungal dissemination following intravenous challenge [10••]. Human monocyte derived macrophages from individuals with a single nucleotide polymorphism (SNP) in the gene encoding MelLec produced less IL-1β and IL-8 upon AF stimulation. HSCT transplant recipients from donors carrying the SNP had a greater risk of IA [10••]. A soluble C-type lectin, surfactant protein D, was also shown to bind melanin in resting conidia as well as galactomannan and galactosaminogalactan on swollen conidia/mycelia. This binding increased phagocytosis and inflammatory cytokine levels in human monocyte derived macrophages [11]. Recognition of melanin in dormant conidia is particularly striking because AF was previously considered to be immunologically inert in this state. Another report found the CLR CD23 (CLEC4J) to recognize α-mannan and β-glucan and protect from IA by induction of iNOS via NF-κβ [12]. Finally, a member of the immunoglobulin superfamily, CD56, was found to mediate AF activation of NK cells. Blocking CD56 on NK cells ablated AF induced activation and cytokine secretion *in vitro* was decreased, although the importance of this PRR during infection was not demonstrated [13]. Chitin is a major AF cell wall component that has garnered considerable recent interest because of its diverse impacts on immune responses. Mammals express chitinases, which cleave chitin, as well as chitinase-like-proteins (CLPs), which bind chitin but are catalytically inactive. Both of these protein families could conceivable have antifungal activities or modulate the amount or form of chitin that other host factors encounter. Larger particles of chitin from AF appear to have a proinflammatory effect during IA. An AF mutant with increased chitin and decreased beta glucan exposure elicited a hyperinflammatory host response. Compared to mice infected with WT AF, mutant infected mice exhibited increased inflammatory cell recruitment associated with increased production of MIP1α, CXCL1, and TNFα and decreased fungal burden [14]. Investigations in our laboratory found that mice lacking acidic mammalian chitinase (AMCase), which presumably have more chitin due to lack of AMCase chitinolytic activity, produced higher levels of IL-1α, IL-1β, IL-17A, and IL-22 during IA, which was associated with improved fungal clearance [15]. Amarsaikhan *et al.* reported that treatment with caspofungin, which inhibits β-glucan synthesis and thus increases AF chitin exposure, caused increased eosinophil recruitment during IA. This study also utilized SPAM mice, which constitutively express AMCase, and saw this effect of caspofungin reversed, supporting the prospect that AMCase may degrade or otherwise decrease the inflammatory capacity of AF chitin [16]. Chitinases and CLPs are also players in chronic exposure and colonization. We have reported that AMCase negatively affects lung function and inflammation during fungal asthma, with mice deficient in the chitinase exhibiting lower levels of proinflammatory factors CCL17, CCL22, IL-17A, and IL-22 as well as lower airway resistance [15]. Weigt *et al.* reported that expression of the chitinase chitotriosidase and the CLP chitinase 3-like-1 in AF-colonized lung transplant patients was associated with progression to chronic lung allograft dysfunction [17].

A. fumigatus employs multiple immune evasion strategies to support its growth in the lung despite the ability of the host to recognize its cell wall. The cell wall protein CcpA was demonstrated to contribute to virulence by masking other cell surface proteins from immune recognition. A CcpA mutant elicited higher reactive oxygen species (ROS), inflammatory cytokines, and epithelial damage *in vitro*. Nonneutropenic mice infected with the CcpA mutant experienced greater mortality than those infected with WT AF; however, this effect was lost in neutropenic mice [18]. AF also employs multiple offensive strategies against innate immune effector functions. AF binds plasmin via Aspf2, which damages epithelia and evades complement by binding regulatory factors [19]. AF also releases proteases to cleave innate immune proteins. Mep1p is secreted by conidia, and it cleaves not only complement proteins C3, C4, and C5 but also the activating proteins properdin, MBL, and ficolin-1. Mep1p also inhibited phagocytosis *in vitro*. Infection with a Mep1p mutant did not result in a significant difference in survival compared to WT AF, but the data suggested that a longer observation period may have revealed improved survival in the mutant infected mice [20]. Similarly, culture filtrates from the highly virulent AF strain CEA10 were found to cleave the PRR Dectin-1 and the activity was ablated by a serine protease inhibitor [21]. Melanin in the AF conidial cell wall, which as noted above is recognized by the CLR MelLec, is well known to protect the fungus from host ROS. A recent work has now

added that melanin also enables conidia to evade phagocytosis by human monocytes via sequestration of calcium ions, which blocks activation of LC3-associated phagocytosis [22]. Host factors may also negatively impact host defense against *A. fumigatus*. Gresnigt *et al.* recently reported that the intracellular PRRs NOD1 and NOD2 are maladaptive during IA. *Nod1−/−* mice are resistant to IA both in terms of survival and fungal burden. Murine bone marrow derived macrophages genetically deficient in NOD1, as well as human monocyte derived macrophages treated with NOD1 siRNA, exhibited increased ROS production and fungal killing *in vitro*. These changes were associated with NOD1 suppression of *Clec7A* (Dectin-1) [23]. Similarly, *Nod2−/−* mice exhibited improved survival and fungal clearance during a model of IA. NOD2 also negatively regulates *Clec7A* expression, inflammatory cytokine production, and phagocytosis. Furthermore, donor SNPs in *NOD2* decreased risk of IA in HSCT patients [24•]. As NOD1/2 function as intracellular sensors of bacterial ligands, their regulation by Dectin-1 might be viewed as an attempt to tailor inflammatory responses towards intracellular infections at the expense responses that promote fungal clearance. The *A. fumigatus* ligand or ligands responsible for the deleterious activation of NOD1/2 during IA is yet to be defined, leaving the possibility that an *A. fumigatus* virulence factor may exploit the immunosuppressive effects of this pathway.

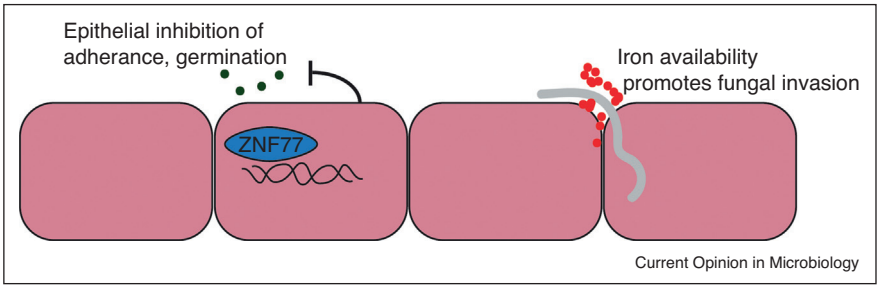
Orchestration of innate immunity to AF

The signals received from PRRs must be integrated into a coordinated immune response. Briard *et al.* uncovered an elegant mechanism of this organization in which Dectin-1 and TLR signaling coordinately regulate inflammasome priming and activation [25]. A separate report described a similarly nuanced pathway in which IFN-γ restrains inflammation via inhibition of NLRP3 activation and mediates fungal clearance via LC3-associated phagocytosis, both through activation of death-associated protein kinase 1 (DAPK1) [26]. Our laboratory has a long-standing interest in the regulation of IL-22 production during IA. We have recently reported that the IL-1 family member IL-33 negatively regulates IL-17A and IL-22 production during IA. These studies also revealed a role for the eicosanoid PGE_2 in the promotion of IL-22 [27]. In a separate report, we found that the common γ-chain cytokines IL-7 and IL-21 support, while IL-15 regulates, IL-22 production. Here, we identified iNKT cells, γδ T cells and type 3 innate lymphoid cells as innate cells sources of IL-22 during IA [28]. An interesting report comparing inflammatory responses among AF strains found that more virulent, rapidly germinating isolates induce more IL-1α, which is critical for clearance but also induces greater lung damage [29]. The essential role of neutrophils in clearance of AF makes understanding of their localization and function during IA a research priority. The eicosanoid LTB_4 was found to promote neutrophil as well as eosinophil recruitment during IA. Mice lacking this signal succumbed to infection at higher rates [30]. Type III interferon produced largely by inflammatory monocytes is critical for neutrophil function including ROS [31]. Shlezinger *et al.* reported an unconventional mechanism of fungal clearance whereby neutrophils kill AF conidia by activating programmed cell death in the fungus [32]. Production of reactive oxygen species is a critical antifungal effector function of neutrophils. Protein kinase C-δ (PKC-δ) in neutrophils was shown to be activated by *Candida albicans in vitro* via Dectin-1 and Mac-1, which were required for optimal ROS production. Mice deficient in PKC-δ demonstrated higher fungal burden during IA, presumably due to activation of the same pathway [33]. Our laboratory has previously shown that in addition to neutrophils, eosinophils also contribute to *in vivo* host defense against IA and kill AF *in vitro* in a manner reproducible in both a transwell system and with eosinophil lysates [34]. Investigation of eosinophil extracellular traps (EETs) detected the presence of EETs in bronchial mucus plugs of ABPA patients and observed their release *in vitro* by human eosinophils stimulated in AF. Strikingly, EETs were not fungicidal, suggesting that they may mediate nonproductive inflammation and host damage in AF colonized patients [35].

Cellular and molecular consequences of immunosuppression

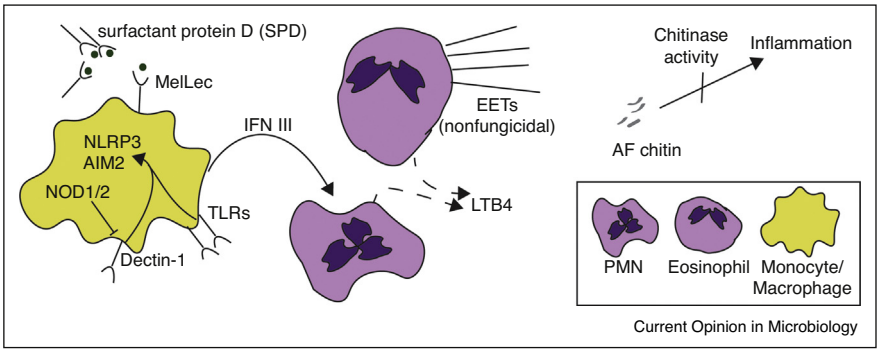
Although immunosuppressed models of IA are common, their use has largely focused on the elucidation of WT antifungal mechanisms and not the effects of various immunosuppressive agents. Because use of immunosuppressive agents is a primary risk factor for development of IA, more precise understanding of how they mediate susceptibility to IA may be key to developing strategies to mitigate their damage. Kalleda *et al.* compared the responses of immunocompetent mice to mice immunosuppressed via cyclophosphamide and corticosteroid treatment (CCT) or corticosteroid treatment alone (CT). CCT treatment decreased numbers of multiple critical cells types including PMNs, monocytes, macrophages, eosinophils, and dendritic cells during IA. Proinflammatory and anti-inflammatory cytokine levels were decreased below the limit of detection in both naïve and infected CCT mice. CT treatment had no effect on recruitment of PMNs or macrophages, but diminished recruitment of dendritic cells and monocytes. The authors demonstrated that adoptive transfer of CD11b+ cells protected cyclophosphamide treated mice from IA, but this transfer was not protective if the mice were also treated with corticosteroid [36•]. Calcinuerin inhibitors (CI) are another common class of immunosuppressive agents used in transplant recipients. A recent report found that the CI cyclosporine reduces neutrophil NET production and inhibition of AF [37]. Another investigation of this pathway found that macrophages undergoing

Figure 1



Epithelial antifungal defense.
The intact lung epithelium inhibits adherence and germination of *A. fumigatus* conidia, whereas iron availability resulting from tissue damage promotes fungal invasion.

Figure 2



Activation and coordination of antifungal immunity.
Innate immune activation is mediated by recognition of *A. fumigatus* cell wall components including melanin (by MelLec and SPD) and chitin, which may promote production of inflammatory factors unless digested by chitinases. CLRs and TLRs coordinately activate the NLRP3 and AIM2 inflammasomes, while NOD1 and NOD2 suppress inflammation by supression of *Clec7A*(Dectin-1) transcription. The eicosanoid LTB_4 mediates recruitment of neutrophils and eosinophils, while type III interferon activates neutrophils.

necrosis due to germination of internalized AF transfer endosomes containing AF to other macrophages, which was inhibited by the CI tacrolimus and resulted in greater fungal germination [38]. Deletion of calcinuerin in CD11c+ cells decreases resistance to IA when challenged intravenously. This decreased resistance was associated with decreased expression of the antifungal protein pentraxin 3 [39]. In contrast, a third mouse study found that cyclosporin A inhibited recruitment of lymphoid but not myeloid cells and thus did not leave the mice susceptible to IA when challenged intranasally [40]. The epithelium is an important innate immune effector that may be left intact during immunosuppressive targeting of the hematologic compartment. Leiva-Juarez *et al.* reported that treatment of chemotherapeutically immunosuppressed mice with a combination of TLR2/6 and TLR9 agonists before infection with *A. fumigatus* profoundly improved survival and decreased fungal burden compared to untreated mice [41]. This effect was attributed to antimicrobial activity of lung epithelial cells, highlighting the antifungal capacity of these cells and providing proof of concept for stimulating epithelial responses to protect against IA. Finally, in the absence of anti-AF antibodies, which may occur in immunosuppressed patients, the complement pathway was found to efficiently compensate by activation via the lectin, rather than classical, pathway [42]. The redundancy of the host response in this area potentially explains why *in vivo* animal survival phenotypes associated with complement components are not well-reported.

Conclusion

Recent research has revealed new details of host defense against AF and fungal evasion strategies against these mechanisms. Early events following host exposure including epithelial defenses likely lead to clearance of many AF exposures before germination (Figure 1). When germination does occur, numerous innate immune mechanisms

recognize AF and mediate clearance (Figure 2). Ultimately, understanding the function or dysfunction of these mechanisms in the context of immunosuppressive treatments is critical to developing remedies to restore antifungal immunity in susceptible patients.

Conflict of interest statement

Nothing declared.

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