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Immune responses against *Aspergillus fumigatus*: what have we learned?

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Abstract

Purpose of review—*Aspergillus fumigatus* causes invasive and allergenic disease. Host defense relies on the ability of the respiratory immune system to restrict spore germination into invasive hyphae and to limit fungus-induced or inflammation-induced damage in infected tissues. This review covers the molecular and cellular events that mediate innate and CD4 T-cell responses to *A. fumigatus* and fungal attributes that counter hostile microenvironments and, in turn, affect host responses.

Recent findings—Host recognition of fungal cell wall components is critical for fungal uptake, killing, and the formation of protective innate and CD4 T-cell effector populations. Beyond the known role of neutrophils and macrophages, circulating monocytes, dendritic cells, and natural killer cells contribute to optimal defense against *A. fumigatus*. Genetic and pharmacologic manipulation of *A. fumigatus* reveals that hypoxia adaptation, cell wall assembly, and secondary metabolite production in mammalian tissues contribute to fungal pathogenesis and the outcome of infection.

Summary—Greater understanding of the immune mechanisms that underlie protective responses and fungal pathways that promote microbial adaptation and growth in mammalian tissue provide a conceptual framework for improving current antifungal therapies.

Keywords

Aspergillus; fungus; immune response; inflammation; pathogenesis

Introduction

Aspergillus fumigatus is an airborne fungus that causes a range of disease states in humans (summarized in Table 1) [1]. Exposure to inhaled spores (conidia) is lifelong and, for most humans, symptomless. Invasive disease occurs in hosts with defects in myeloid cell number or function and results from spore germination into tissue-invasive hyphae. Allergic disease can develop in hosts with underlying inflammatory conditions, exemplified by asthma, atopy, and cystic fibrosis, and arises from dysregulated or exuberant immune responses to fungal antigens in colonized airways.

Following *A. fumigatus* spore inhalation, the respiratory immune system initiates a series of events that culminates in fungal clearance in immune-competent hosts. These include fungal particle recognition, uptake by opsonic and nonopsonic receptors, killing by reactive oxygen-dependent and oxygen-independent mechanisms, and the release of mediators that coordinate effector cell recruitment, activation, and function in the innate and adaptive phases of the response (for comprehensive reviews see [1,2]). Damage to host tissue can occur with unrestrained fungal growth or from the recruitment of inflammatory cells to infected sites. Although humans represent accidental hosts for airborne *A. fumigatus* spores, fungal attributes that have evolved for survival in its ecologic niche (decaying organic matter) can act to counter clearance mechanisms at distinct steps listed above and promote fungal pathogenesis and persistence in hostile tissue environments [3,4].

The fungal cell wall: the center of discourse

The fungal cell wall has a significant impact on the host immune response as it represents the first structure encountered by the host cells and contains a number of polysaccharides with immune activating and modulatory properties; these include β -1,3/1,4-glucan, α -1,3-glucan, chitin, galactomannan, and a unique polymer of galactosaminogalactan [5–7]. Fungal cell wall composition varies during the process of germination and hyphal growth and is influenced by the presence of antifungal drugs and local conditions in tissue microenvironments.

Cell wall interactions important for spore uptake

The surface of inhaled spores contains a proteinaceous layer that masks the underlying immunologically active polysaccharides [8•]. At this stage, a critical opsonic interaction is the binding of the collectin pentraxin-3, released primarily from neutrophil granules, to the spore surface [9]. Pentraxin-3^(-/-) mice are vulnerable to *A. fumigatus* infection [10], and this susceptibility has been associated with a defect in spore uptake, as neutrophils take up pentraxin-3-coated spores much more efficiently than uncoated spores. Pentraxin-3-dependent fungal cell uptake operates via a complement protein C3-dependent process that involves CD11b (CR3) and Fc γ R recruitment to the phagocytic cup. The protective function of pentraxin-3 administration is abolished in Fc γ R^(-/-) mice but not in SCID and Rag-2^(-/-) mice, excluding a role for antibody in pentraxin-3-dependent and FcR γ -dependent spore uptake process [11•]. Pentraxin-3 can also act as an endogenous inhibitor of neutrophil adhesion to vascular surfaces by binding to endothelial P-selectin [12]. Whether this regulatory mechanism is beneficial during respiratory fungal infection remains unclear, but may serve to limit inflammation-induced tissue damage.

A. fumigatus can counter opsonization and complement activation through secreted proteolytic activity. The alkaline protease Alp1 degrades human complement proteins C3, C4, and C5 [13]. Although the virulence of the Δ alp1 strain is similar to the wild-type strain in a pulmonary infection model [13], another study found that the complement-degrading proteolytic activity diminishes fungal uptake by brain microglial cells [14], suggesting a possible role in the pathogenesis of disseminated CNS aspergillosis.

Diverse signaling receptors recognize cell wall changes during germination

The process of spore swelling, the first step of germination, results in the obligate exposure of fungal ligands that bind cognate C-type lectin (CTL) and Toll-like receptors (TLRs) [1,2,15]. This process activates signaling pathways linked to the production of inflammatory cytokines and chemokines, prostaglandins, as well as the production of reactive oxygen. Germination is associated with an increase in fungal metabolism, which is important for the production of immunomodulatory factors such as primary and secondary metabolites and the

secreted protease mentioned above. Chief among these host–fungal interactions is the binding of the CTL receptor Dectin-1 to fibrillar β -(1,3)-glucan [16–18]. Dectin-1^(-/-) mice are more susceptible to high doses of *A. fumigatus* than control mice and produce diminished proinflammatory cytokine responses [19]. In allogeneic stem cell transplant patients, receipt of a T-cell-depleted allograft that contains a defective Dectin-1 allele (Dectin-1^{Y238X/+}) is associated with an increased risk of developing invasive aspergillosis [20•].

TLR2, TLR4, and TLR9 are implicated in *A. fumigatus* recognition and signaling through the common TLR adaptor protein MyD88 [1]. MyD88^(-/-) mice exhibit a higher lung fungal burden than control mice and mount dysregulated inflammatory responses [21]. In humans, the presence of a TLR4 polymorphism among donor cells increases the risk for invasive aspergillosis among hematopoietic stem cell transplant (HCT) recipients [22]. Thus, both Dectin-1-dependent and MyD88-dependent signals are crucial for host defense in mice and humans.

Germinating spores and hyphae also trigger interleukin (IL)-1 β release by a macrophage cell line [23] and *in vivo* [19]. Gene-silencing experiments implicate the NLRP3 inflammasome in caspase-1 activation [23]. The precise in-vivo role of the NLRP3 inflammasome and IL-1 signaling in host defense remains undefined, though downstream IL-1 receptor-dependent pathways may not be essential for survival [24].

New players on the block: traps, monocytes, and natural killer cells

Although neutrophil-mediated spore and hyphal killing is essential to defense [1], recent work indicates that other inflammatory cell populations, notably monocytes, dendritic cells, and natural killer (NK) cells, exert direct antifungal effector properties and, in the case of infection-induced dendritic cells, initiate the adaptive phase of the immune response.

Neutrophil extracellular traps

Beyond established effector mechanisms (reactive oxygen, phagosome proteolytic activity, and granule proteins such as lactoferrin), neutrophils can form extracellular traps upon encountering germinating *A. fumigatus* spores and hyphae in the test tube and lung [25,26]. Although their relative role in fungal killing remains unknown, Neutrophil extracellular trap formation was restored by gene therapy in a patient with chronic granulomatous disease and coincided with the resolution of preexisting pulmonary aspergillosis [27].

Role of monocytes, dendritic cells, and natural killer cells in host defense

The murine lung inflammatory cell infiltrate contains numerous Ly6C^{hi} monocytes (also known as inflammatory monocytes) recruited from bone marrow stores via CC chemokine receptor 2 (CCR2)-mediated mechanisms within 24h postinfection (Fig. 1) [28•]. Human CD14⁺ monocytes, murine Ly6C^{hi} monocyte counterparts, phagocytose spores and inhibit germination [29]. In infected lungs, Ly6C^{hi} monocytes rapidly differentiate into CD11b⁺CD11c⁺MHC class II^{hi} monocyte-derived dendritic cells (Mo-DCs) and transport spores to lung-draining lymph nodes, a process that is essential for the priming and expansion of *A. fumigatus*-specific CD4 T cells [28•].

The size of the lung Mo-DC population correlates with survival in neutropenic mice [30•] and is greater in neutropenic than in neutrophil-sufficient animals, even when mice are challenged with killed fungal products [31•]. In neutropenic mice, monocyte-dependent and Mo-DC-dependent tumor necrosis factor production enhances their CCR2-dependent pulmonary trafficking [31•]. Genetic deletion of the chemokine receptor CCR7 in hematopoietic cells enhances Mo-DC production from bone marrow precursors following

bone marrow transplantation [32] and diminishes Mo-DC lung egress to draining lymph nodes [30•], both of which increase the number of Mo-DCs in infected lungs and correlate with enhanced survival. Depletion strategies that predominately target monocytes and Mo-DCs in immune-competent mice (using ablation of CCR2⁺ cells) or that include Mo-DCs in neutropenic mice (using ablation of CD11c⁺ cells) result in delayed fungal clearance [28•] or in diminished survival [31•].

A recent study found a role for plasmacytoid dendritic cells during respiratory fungal infection [33]. Purified plasmacytoid dendritic cells damage *A. fumigatus* hyphae in a partially Zn²⁺-dependent mechanism that is consistent with a role for the granular protein calprotectin in fungal cell killing. Furthermore, mice defective in type I interferon production are susceptible to invasive disease. The finding that antibody-mediated plasmacytoid dendritic cell depletion recapitulates this phenotype, albeit in a milder form, is consistent with a role for plasmacytoid dendritic cell-dependent type I interferon production in host defense *in vivo*.

In neutropenic mice, the recruitment or adoptive transfer of NK cells to infected lungs is beneficial [34]. NK-cell-derived interferon (IFN)- γ activates macrophage-dependent fungal clearance *in vitro* and is essential for NK-cell-dependent host defense in neutropenic mice *in vivo* [35]. In-vitro studies demonstrate that human NK cells damage *A. fumigatus* hyphae [36]. Thus, a key translational question is to define a therapeutic role for NK cells, monocytes, and dendritic cells as effector cells in diseased patients, particularly as these cells can be harvested by leukopheresis and expanded *ex vivo*.

***Aspergillus fumigatus*-specific CD4 T cells**

A. fumigatus spore exposure leads to the development of diverse CD4 T-cell responses that include T helper (Th) 1, Th2, Th17, and regulatory T (Treg) cells [37]. Protection against invasive aspergillosis in mice [38,39] and humans [40] relies in part on fungus-specific CD4 T cells. In immune compromised patients, adoptive transfer of *A. fumigatus*-specific CD4 T cells can provide a benefit [41,42]. Consistent with the notion that Th1 responses are beneficial, neutralizing Th2-biased cytokines in neutropenic mice improves disease outcome [38].

Various factors contribute to the molecular and cellular mechanisms that regulate *A. fumigatus*-specific CD4 T-cell differentiation toward Th1, Th2, and Th17 effector cells *in vivo*. These include exposure frequency, antigen type, engagement of CTL and TLR signaling pathways, and infection-induced dendritic cell populations in the lung. In single exposure models, the *A. fumigatus*-specific CD4 T-cell airway response consists of a dominant Th1 and a smaller Th17 population [43•, 44]. Similarly, CD4 T-cell responses in healthy individuals are primarily Th1-biased [45], whereas *A. fumigatus*-specific Th2 CD4 T cells predominate in the response in allergic bronchopulmonary aspergillosis (ABPA) patients and contribute to disease progression [46•]. Repeated exposures to *A. fumigatus* spores in murine pulmonary infection models result in increased recruitment of Th2 and Th17 CD4 T cells [47]. In a vaccine model, distinct preparations of *A. fumigatus* antigens induce the selective expansion of various CD4 T-cell subsets with secreted antigens promoting the differentiation of Th2 cells, membrane components Th1 cells, and glycolipids Th17 cells [37]. Similarly, the protease activity of *A. fumigatus* allergens is crucial to their capacity to induce Th2 differentiation [48].

Role of Dectin-1 and Toll-like receptor signaling on the formation of fungus-specific CD4 effector cells

The induction of predominately Th1- or Th2-biased CD4 T-cell responses by live and heat-inactivated spores, respectively, indicates that innate receptor signaling activated by the process of spore germination is a critical determinant of CD4 T cell differentiation [49]. Innate TLR-MyD88 signaling is crucial for the initial induction of T-bet (Th1 transcription factor) in the lung-draining lymph node, while MyD88-independent and Dectin-1-independent mechanisms promote further T-bet expression in CD4 T cells that enter airways [43•, 44]. In turn, the induction of T-bet expression in fungus-specific CD4 T cells inhibits Th17 differentiation [43•], consistent with a role of T-bet in suppressing ROR γ t (Th17 transcription factor) induction [50] and similar to its known role in suppressing GATA-3 (Th2 transcription factor) induction [51,52]. Thus, T-bet expression is a critical switch in the regulation of *A. fumigatus*-specific CD4 T-cell differentiation.

Following *A. fumigatus* infection, Dectin-1 signaling and the presence of Mo-DCs influence T-bet expression levels in fungus-specific CD4 T cells. Dectin-1 signals act on innate cells to diminish the production of IL-12p35 and IFN- γ , leading to a reduction in T-bet expression by fungus-specific CD4 T cells. By regulating T-bet expression levels in CD4 T cells, Dectin-1 facilitates the development of Th17 effector cells and contributes to the establishment of a more diverse CD4 T-cell response [43•].

Beyond their role in fungal antigen transport to lung-draining lymph nodes, monocyte derivatives provide essential cues that shape CD4 T-cell differentiation [43•]. Depletion of Mo-DCs at various times after infection results in enhanced Th17 and diminished Th1 differentiation. The alteration in the Th1/Th17 balance correlates with a loss of T-bet expression in fungus-specific CD4 T cells. Thus, it appears that monocyte derivatives are important for the maintenance of T-bet expression in CD4 T cells and the development of a protective Th1 response [43•].

The infection microenvironment: a crossroad between fungal and host factors

The above studies illustrate our understanding of key immune system mechanisms to prevent invasive *A. fumigatus* infections primarily in the immunocompetent host. Future challenges will include learning how this understanding of fungal resistance can be translated into the immunocompromised patient populations that acquire *A. fumigatus* infections. However, once germination occurs in an immunocompromised patient, the relationship between the tissue microenvironment and fungal cell metabolic activity during the course of infection is largely unappreciated, but potentially important for disease outcome and evolution of host immune responses. A better understanding of how the host microenvironment influences fungal virulence and immunomodulatory factors, as well as what factors induce changes in the infection microenvironment itself, should improve our ability to design more effective therapeutics to manage invasive aspergillosis. With this in mind, we review recent advances in our understanding of the fungal response to the infection microenvironment encountered by *A. fumigatus* during colonization and invasive growth in immunocompromised hosts.

Fungal secondary metabolism

A unique aspect of *A. fumigatus* metabolism is the production of bioactive secondary metabolites, which are typically dispensable for completion of the fungal life cycle [53]. The most studied *A. fumigatus* secondary metabolite is the epipolythiodioxopiperazine gliotoxin [54–57] that is produced in murine models of invasive aspergillosis [58,59]. The role of

gliotoxin in invasive pulmonary aspergillosis (IPA) seems to be host microenvironment dependent, with the key factor being the method of immunosuppression. In murine models of IPA that utilize cyclophosphamide for neutrophil depletion, gliotoxin production was found to be dispensable for disease development [59–62]. However, in murine models that utilize corticosteroids for immunosuppression, gliotoxin was found to be a critical factor for disease development [62,63]. Although the exact mechanism for gliotoxin's differential role in *Aspergillus* virulence is unknown, its ability to induce apoptosis in neutrophils is thought to be a primary mode of action [62–64]. The massive myeloid cell depletion that occurs when cyclophosphamide is used as the immunosuppressant may mitigate any effects of gliotoxin on these target cells that are largely absent from the infection site.

In addition to gliotoxin's direct effect on neutrophils, recent data suggest an in-vivo link between secondary metabolite production and inhibition of host angiogenesis, as the *A. fumigatus* *laeA* null mutant that is deficient in secondary metabolite production does not inhibit angiogenesis to the same degree as the wild-type strain [65•]. Both gliotoxin and fumagillin are candidate secondary metabolites that may mediate the *LaeA*-dependent effect on host angiogenesis. Intriguingly, inhibition of host angiogenesis likely results in significant localized tissue hypoxia [66]. Deficiency of the *LaeA* transcriptional regulator also impacts the composition of the fungal cell wall, resulting in a diminished hydrophobic layer and enhanced phagocytosis of *ΔlaeA* spores by neutrophils [67].

Hypoxia adaptation

As mentioned, oxygen availability is an important feature of the infection microenvironment that is not well understood. Oxygen is a critical molecule for both host and pathogen cell metabolism and its absence places a significant stress on most eukaryotic cells. Recent studies confirm that in three immunologically distinct murine models of IPA (neutropenic, corticosteroid, and chronic granulomatous disease), hypoxia (O₂ 1.5%) occurs at sites of *A. fumigatus* invasive growth in the lung [68]. Importantly, the ability to overcome hypoxia appears to be a requirement for lethal disease, as *SrbA*, a fungal transcription factor in the sterol regulatory element-binding protein family, is required for *A. fumigatus* growth under hypoxia and for lethal invasive disease in multiple murine models of IPA [69]. Ongoing studies are exploring the mechanisms of *SrbA*-dependent hypoxia growth and its relation to invasive disease.

The presence of hypoxia at the site of infection is also likely to have a significant effect on the expression and composition of the fungal cell wall. In the yeast *Saccharomyces cerevisiae*, low-oxygen environments stimulate cell-wall remodeling that results in increased exposure or production of the *DAN/TIR* mannoproteins [70–72]. Although whole-genome gene expression studies reveal significant transcriptional changes in *A. fumigatus* cell-wall biosynthesis genes in response to hypoxia (B. Barker, R.A. Cramer, unpublished observation), the functional significance and exact nature of these changes remains to be elucidated, specifically in the context of clinically relevant immunosuppression regimens.

Immunopharmacologic effects

The presence of antifungal drugs used to treat *A. fumigatus* infections may also alter the infection microenvironment by affecting fungal metabolism and subsequent host responses. Echinocandin drugs, β -(1,3) glucan synthase inhibitors, alter the surface content of β -glucan on *A. fumigatus* hyphae resulting in enhanced macrophage and neutrophil inflammatory responses via a Dectin-1-dependent mechanism [73,74]. In a systemic candidiasis model, echinocandin-dependent β -(1,3)-glucan exposure occurs in infected tissues [75]. Thus, echinocandin drugs may act in part via an immunopharmacologic mechanism to benefit the

host, as they fail to inhibit *A. fumigatus* growth completely at any concentration. The effects of amphotericin B on the immune system are also an active area of investigation [76].

Conclusion

The past several years have witnessed significant advances in our understanding of host defense mechanisms against *A. fumigatus* and of fungal attributes that facilitate growth in hostile tissue microenvironments. Several of these findings have important clinical implications for patient care. First, dissection of host pathways involved in fungal recognition and killing has led to advances in the field of immunogenetics and the recognition of genetic risk for invasive aspergillosis in the context of bone marrow transplantation [77]. An important area of research is to incorporate these findings into patient care, for example, in prophylaxis strategies and allograft selection when multiple donors are available. Second, the recognition that monocytes, dendritic cells, and NK cells can act in host defense in neutropenic hosts opens new research directions for myeloid reconstitution and adoptive transfer strategies. Third, improved understanding of molecular cues that underlie CD4 T-cell differentiation has implications for modulating allergenic responses, as exemplified by the link between vitamin D levels and Th2 responses in ABPA patients [46••]. Fourth, research on the influence of the infection microenvironment on fungal metabolism may lead to new drug targets and a better understanding of which immunomodulatory techniques may be therapeutically viable. These areas of research provide fertile ground to build on improvements in clinical outcomes [78] and to reduce *Aspergillus*-related morbidity and mortality.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 397-398).

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Key points

- Dissecting the molecular mechanisms of *Aspergillus fumigatus* recognition in the lung has identified critical antifungal effector pathways and established that human genetic variants confer risk for invasive disease during hematopoietic stem cell transplantation.
- Monocytes, dendritic cells, and natural killer cells play important roles in *A. fumigatus* host defense and may be of particular significance in neutropenic patients.
- The formation of specific CD4 T-cell effector populations during invasive and allergenic disease depends in part on Toll-like receptor and C-type lectin signaling pathways in innate cells recruited to the lung.
- Understanding fungal metabolic pathways that are activated in hostile tissue microenvironments and in response to antifungal therapy point to novel microbial targets for drug development and a better understanding of the role of immunomodulatory therapies in these settings.

Figure 1. Model of monocyte function during pulmonary *Aspergillus fumigatus* infection
 Ly6C^{hi} CCR2⁺ monocytes rapidly infiltrate *Aspergillus fumigatus*-infected lungs, differentiate into monocyte-derived dendritic cells (Mo-DCs), and engulf fungal spores for transport to lung-draining lymph nodes. Monocyte and their derivatives generate inflammatory cues that are essential for the development of IFN- γ -producing CD4 airway effector cells. In neutropenic mice, Mo-DCs likely form a significant pool of innate antifungal effector cells that control fungal growth, although the precise mechanisms remain to be elucidated.

Table 1
Human disease associated with *Aspergillus fumigatus*

Disease	Pathogenesis	Patient groups
Acute aspergillosis (invasive aspergillosis)	Invasive, disseminates hematogenously, rapidly progressive (<1 month)	Hematologic malignancies or aplasia, bone marrow and lung transplant recipients, chronic granulomatous disease, hyper- IgE syndrome Invasive aspergillosis is associated with severely immune compromised states and myeloid cell dysfunction
Subacute aspergillosis (chronic necrotizing)	Invasive, rare dissemination, progressive (1–3 months), nodular and cavitary lesions	Chronic corticosteroid therapy, alcoholics, chronic obstructive pulmonary disease and underlying lung disease, cystic fibrosis Subacute aspergillosis is associated with a lower degree of immune compromise than patients with invasive aspergillosis
Chronic aspergillosis	Little to no fungal tissue invasion, slowly progressive (>3 months), cavitary and/or fibrosing lesions, fungus balls (aspergilloma)	Structural and/or underlying lung disease
Allergic aspergillosis	Fungal colonization, dysregulated inflammatory response to <i>Aspergillus</i> antigens	Allergic bronchopulmonary aspergillosis (ABPA), allergic fungal sinusitis