HHS Public Access

Author manuscript

Curr Opin Infect Dis. Author manuscript; available in PMC 2018 February 01.

Published in final edited form as:

Curr Opin Infect Dis. 2017 August ; 30(4): 364–371. doi:10.1097/QCO.0000000000000381.

Immune responses to invasive aspergillosis: new understanding and therapeutic opportunities

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Abstract

Purpose of review—Invasive aspergillosis is a worldwide disease that primarily affects immune-compromised patients, agricultural workers with corneal abrasions, individuals with structural lung disease, and patients with primary immune deficiency. The critical function of the immune system is to prevent the germination of airborne conidia into tissue-invasive hyphae. This review covers recent advances that shape our understanding of anti-*Aspergillus* immunity at the molecular and cellular level.

Recent findings—Host defense against conidia and hyphae occurs via distinct molecular mechanisms that involve intracellular and extracellular killing pathways, as well as cooperation between different myeloid cell subsets. The strength and efficacy of the host response is shaped by the tissue microenvironment. In preclinical models of disease, host immune augmentation strategies have yielded benefits, yet translating these insights into therapeutic strategies in humans remains challenging.

Summary—Although advances in early diagnostic strategies and in antifungal drugs have ameliorated clinical outcomes of invasive aspergillosis, further improvements depend on gaining deeper insight into and translating advances in anti-*Aspergillus* immunity.

Keywords

Aspergillus; cytokine; fungus; immunity; inflammation

INTRODUCTION

Invasive aspergillosis begins with inhalation or, occasionally, traumatic inoculation of conidia (vegetative spores) formed by *Aspergillus* species members [1–3]. Although the vast majority of these encounters conclude with asymptomatic clearance, humans with quantitative or qualitative myeloid cell defects are susceptible to invasive aspergillosis. Structural lung damage, due to anatomic or biochemical defects, and prior tuberculosis can predispose to acute and chronic *Aspergillus*-associated disease states (Table 1). Filamentous

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Conflicts of interest

There are no conflicts of interest.

hyphae that invade parenchymal tissues and can disseminate remotely is the pathologic hallmark of invasive aspergillosis. The sinopulmonary tract represents the most common site of disease in Western societies; in the developing world, agricultural workers who sustain corneal abrasions are at particular risk of fungal keratitis [4].

The prognosis of invasive aspergillosis patients has improved significantly with the introduction of mold-active triazoles (i.e., voriconazole, posaconazole, and isavuconazole) and with the advent of combination therapies with echinocandin drugs [1,5–7]. Similarly, fungal surrogate markers, in particular, the detection of *Aspergillus* galactomannan by ELISA of serum and bronchoalveolar lavage fluid, have enabled earlier detection in high-risk patient groups, specifically in patients with hematologic malignancies and in hematopoietic cell and lung transplant recipients [1]. Hence, overall and fungus-attributable mortality rates have improved significantly in high-risk patient groups [1,8]. For fungal keratitis, topically applied natamycin is the treatment of choice, with no added benefit of oral voriconazole [9]. The status of novel chemotherapeutic agents for aspergillosis in preclinical and early clinical trials is described in [10]. In this review, I survey the contemporary literature with emphasis on advances in understanding the determinants of host – Aspergillus encounters and implications for therapeutic approaches.

MECHANISMS OF *ASPERGILLUS* KILLING

Mouse models have been particularly insightful for elucidating host components that mediate the resistance to aspergillosis [11], a threat linked to the daily inhalation of ~10^3–10^5 conidia (in 10–15 m^3 of air). In humans, *Aspergillus fumigatus* represents the most common etiologic agent of aspergillosis and, in most surveys, accounts for 60–70% of invasive aspergillosis cases (Table 1). The vast majority of preclinical studies are performed with this species, although physicians have noted worse clinical outcomes with less prevalent non-*A. fumigatus* species [12].

Researchers typically administer 10^7–10^8 resting conidia into the murine respiratory system, usually in conjunction with global or conditional gene knockout mice, targeted host cell ablation, or blocking antibodies to host proteins involved in antifungal immunity [11]. In the lung of immune competent mice, the vast majority of conidia are inactivated prior to the formation of tissue-invasive hyphae. In the fungal keratitis model, 10^4–10^5 conidia are injected via the intraocular route and germinate into hyphae due to a relative delay in neutrophil recruitment to the infection site.

In immune competent mice, the pulmonary inoculum is cleared by the coordinated action of recruited neutrophils and $Ly6C^{hi}$ inflammatory monocytes [13–15]. Both of these myeloid cell populations engulf and inactivate conidia [14,16]. $Ly6C^{hi}$ monocytesand theirmonocyte-derived dendriticcell derivatives also condition the lung inflammatory environment to boost neutrophil conidial killing [14], though the precise mechanisms that underlie this intercellular crosstalk remain to be defined. Other myeloid cell populations, including alveolar macrophages, plasmacytoid dendritic cells, natural killer (NK) cells, innate NK T cells, and eosinophils contribute to fungal clearance, yet none are essential to prevent invasive aspergillosis in otherwise immune competent mice [3,17–20].

Conidial and hyphal killing occur via distinct mechanisms that vary based on the fungal morphotype and cell size encountered by innate immune cells [21]. For example, neutrophil conidial killing in the lung does not depend on calprotectin, an abundant neutrophil granule protein that sequesters Zn^{2+} and Mn^{2+} from fungal cells [22]. In contrast, hyphal growth inhibition in the eye depends on the presence of calprotectin [22]. The difference in size between conidia and hyphae induces distinct neutrophil antifungal programs. When neutrophils internalize 2–4 μm diameter conidia, azurophilic granules fuse with conidial phagosomes, sequester neutrophil elastase away from the nucleus, and prevent histone proteolysis in preparation for phagolysosomal killing [23].

In contrast, when neutrophilsencounterhyphae, typically more than 10 μm in length, neutrophil elastase translocates into the nucleus; this process permits histone proteolysis and chromatin remodeling in preparation for neutrophil extracellular trap (NET) formation (i.e., NETosis), an alternative antimicrobial program directed against hyphae [23]. NETs consist of a network of chromatin fibers, with DNA and histones as major constituents, together with calprotectin, pentraxin-3, and other proteinaceous cargo from neutrophil granules. Although NETs entrap and immobilize *A. fumigatus* hyphae [24], their direct role in hyphal killing *in vivo* remains controversial, in part because researchers lack genetic tools that interfere with their formation without affecting other neutrophil antimicrobial functions.

In murine lung neutrophils, conidial killing depends in part on cell-intrinsic NADPH oxidase activity [16], consistent with the ~40% lifetime incidence of invasive aspergillosis in humans with chronic granulomatous disease (CGD) [25]. In human neutrophils, a recent study reported that CGD neutrophils have the capacity to prevent conidial germination at a high effector-to-fungal cell ratio, consistent with the presence of compensatory NADPH oxidase-independent mechanisms, exemplified by iron sequestration via lactoferrin [21,26]. In the fungal keratitis model, topical application of lactoferrin or lipocalin-1 inhibits hyphal growth in the cornea by preventing fungal iron acquisition [27]. A recent study reported that the presence of neutrophils or iron chelation induces a pattern of evasive branching by *A. fumigatus* hyphae [28▪]; this growth pattern may increase the fragility of fungal cells and their susceptibility to immune-mediated clearance.

In macrophages, NADPH oxidase activity drives LC3-associated phagocytosis (LAP), a process in which components of the autophagy machinery conjugate lipidated LC3 to conidial phagosomes and prevent germination *in vitro* [29,30▪]. Conidial melanin inhibits LAP by sequestering the $p22^{phox}$ subunit from the phagosome [30▪]. Hence, its removal during the process of conidial germination enhances this noncanonical autophagy pathway. In support of this model, corticosteroid-treated hematopoietic *atg5* conditional knockout mice are susceptible to *A. fumigatus* challenge [30▪]. LAP is regulated further by death-associated protein kinase 1 (DAPK1) in an IFN-γ-responsive manner. DAPK1 also dampens NLRP3 activation and inflammasome activation that underlies fungus-induced IL-1β release [31]. A *dapk1* polymorphism increases susceptibility to invasive aspergillosis in hematopoietic cell transplantation (HCT) patients [31].

Neutrophil NADPH oxidase is essential for hyphal killing *in vitro* and in the pulmonary and ocular challenge models [32,33]. This may either reflect a requirement for NADPH oxidase

in NET formation and/or the direct extracellular action of NADPH oxidase independent of NETosis [34]. In contrast, products of inducible nitric oxide synthase and myeloperoxidase (MPO) do not appear essential for fungal clearance in the eye [33] or lung [35], consistent with infectious phenotypes in MPO-defective individuals [36]. Similarly, mice defective in neutrophil serine protease activation or in neutrophil elastase do not appear susceptible to respiratory *A. fumigatus* challenge, though fungal clearance may be delayed [37,38].

In human psoriatic lesions, skin extracts that contain disulfide-reduced psoriasin (i.e., S100A7) can inhibit *A. fumigatus* growth by sequestering Zn^{2+} ions, elevating fungal reactive oxygen species production, and inducing fungal programmed cell death [39▪▪]. In the murine lung, an inhaled treatment that combines Toll-like receptor (TLR)-2/6 and TLR-9 agonists (i.e., Pam2-ODN) induces a MyD88 signaling-dependent epithelial resistance that enhances murine survival upon subsequent challenge with *A. fumigatus* conidia in the setting of chemotherapy-induced neutropenia [40▪▪]. These preclinical observations indicate a role for epithelial-derived products that contribute to *Aspergillus* clearance independent of a functional myeloid cell compartment.

The composition of the *Aspergillus* cell wall (reviewed in [41]) may account for *Aspergillus* species-specific differences in host killing. *A. fumigatus* produces an exopolysaccharide, galactosamino-galactan (GAG), that is produced in high amounts and with a high *N*-acetylgalactosamine content in *A. fumigatus*, but to a lower extent in the less pathogenic species, *Aspergillus nidulans* [42]. Enforced GAG production in *A. nidulans* enhances fungal virulence and increases fungal resistance to NADPH oxidase-mediated killing in a murine corticosteroid model of aspergillosis and to the action of neutrophil extra-cellular traps *in vitro* [42]. The contribution of cell wall differences in fungal virulence and resistance to innate immune killing by a variety of pathogenic *Aspergillus* species remains to be surveyed in detail.

FUNGAL RECOGNITION AND INNATE IMMUNE ACTIVATION

The coupling of fungal recognition to cytokine production and to cell-intrinsic fungal killing, exemplified by NADPH oxidase activation, occurs by distinct signaling mechanisms. *A. fumigatus* conidial swelling and germination triggers inflammatory responses via morphotype-specific exposure of β-glucans and other ligands that activate the C-type lectin receptor dectin-1 (encoded by the *Clec7A* gene) and dectin-2 (*Clec4N* in mice, *CLEC6A* in humans) [3,18,43,44]. These spleen tyrosine kinase-coupled receptors activate caspase recruitment domain-containing protein 9 (CARD9) that forms a trimeric complex with BCL1 and MALT10 to activate canonical NF-κB subunits, resulting in the transcription of proinflammatory cytokines, including *il1b*, *il6*, *il12*, *cxcl1*, *cxcl2*, and *tnf* (reviewed in detail in [45,46]).

Conceptually, dectin-1 and CARD9 signaling contribute to innate host defense against aspergillosis by coordinating neutrophil recruitment to the infection site [16,47,48], but neither signal transducer is required in a neutrophil-intrinsic manner to kill conidia [21,49]. Humans with Mendelian defects in *Clec7a* do not spontaneously develop aspergillosis, consistent with functional redundancy at the level of fungal recognition. During allogeneic

HCT, *Clec7a* polymorphisms (in both donor and recipient genomes) increase the risk of pulmonary aspergillosis [50,51▪]. Thus, in the context of damage or injury to the immune system, dectin-1 signaling contributes to *Aspergillus* susceptibility.

In the lung, epithelial IL-1 receptor/MyD88 signaling mediates the early release of the neutrophil chemokine CXCL1 and initial neutrophil trafficking into infected airways [49], though MyD88 is dispensable for neutrophil-intrinsic conidial killing [49]. Both IL-1α [52] and IL-1β [53] contribute to this process; their relative roles may depend on the *Aspergillus* strain and morphotype as well as the use of exogenous immune suppression in murine models. Hematopoietic CARD9 signaling amplifies this chemotactic signal and sustains neutrophil recruitment to the lung during the ensuing phase of the infection [49]. In humans, Mendelian defects in CARD9 can lead to spontaneous extrapulmonary aspergillosis [54]; fungal abscesses in affected patients do not contain neutrophils. The lack of pulmonary disease described in affected individuals suggests that compensatory pathways, for example, by IL-1R/MyD88 signaling in mice, may adequately compensate for loss of CARD9-dependent lung neutrophil recruitment.

In a murine CGD model, IL-1 receptor signaling can lead to dysregulated inflammatory responses characterized by excess recruitment of neutrophils with impaired antifungal activity and tissue hypoxia. In this context, anakinra, a recombinant IL-1 receptor antagonist, modestly ameliorates murine survival [55]. Anakinra treatment does not improve experimental outcomes in *cxcr2*$^{-/-}$ mice, in which pulmonary neutrophil recruitment is delayed [55]. These findings underscore the importance of targeting interventions to the underlying mechanism of innate immune injury or dysfunction.

The soluble collectin pentraxin-3 coats *A. fumigatus* conidia in the alveolar spaces and enhances their uptake, in part via complement deposition and Fc gamma receptor IIA (CD32) and complement receptor 3 (CD11b/CD18) dependent phagocytosis [56]. Another study indicates that pentraxin-3 exerts biological effects via TLR4/MD-2 and TIR-domain-containing adapter-inducing interferon-β signaling [57]. *Md2*$^{-/-}$ mice are defective in neutrophil conidial uptake and killing *in vivo*. Consistent with these observations, human *ptx3* polymorphisms are associated with differences in human neutrophil conidial uptake and invasive aspergillosis risk in HCT patients [51▪,58].

Antibodies against or genetic deficiency of the β2 integrin subunit (i.e., CD18) of complement receptor 3 inhibit neutrophil NADPH oxidase activation by *A. fumigatus* hyphae [33,59]. This antifungal signaling pathway likely includes phosphatidylinositol-3-kinase β and δ isoforms and protein kinase B and is not affected by *CLEC7A or CARD9* genetic deletion [16,33,59]. However, in mice challenged with *A. fumigatus* conidia via the respiratory route, hematopoietic *cd18* deficiency was not associated with defective neutrophil conidial killing in the lung, unlike NADPH oxidase deficiency [16], suggesting that loss of CD18 can be functionally compensated. In the fungal keratitis model, CD18-dependent neutrophil trafficking to cornea is essential for host defense in the eye [33].

Patients with hyper-IgE syndrome (i.e., Job's syndrome; defect in *STAT3*) are susceptible to aspergillosis, typically in the third or fourth decade of life with concurrent bronchiectasis or

pneumatoceles [36]. S*tat3*-defective human neutrophils inhibit the growth of *A. fumigatus* swollen conidia and germlings and have normal chemotactic activity [60], consistent with the model that invasive aspergillosis susceptibility in these patients stems from anatomic lung defects associated with recurrent bacterial infections. Ruxolitinib, a small molecule JAK/STAT inhibitor, reduced *Aspergillus* killing by IL-6-stimulated and IL23-stimulated human neutrophils [61]. These data support the notion that JAK/STAT signaling contributes to neutrophil-mediated *Aspergillus* clearance though the precise mechanisms remain to be defined.

INSIGHTS INTO IL-17-DEPENDENT IMMUNITY

The role of the effector cytokine IL-17A (hereafter referred to as IL-17) has been the subject of significant investigation in murine models of aspergillosis. In murine models, innate sources of IL-17, dependent on dectin-1 signaling and IL-23 release, predominate at the early stage of infection [62]. Administration of anti-IL-17 antibodies delays fungal clearance [62], a finding consistent with reduced CXC-chemokine-mediated neutrophil recruitment to infected airways.

Following an initial exposure to *Aspergillus* antigens, RORγt-expressing neutrophils accumulate in the bone marrow and produce IL-17 [43]. Exposure to IL-6 and IL-23 is critical for *il17* and *il17rc* transcription in human and murine neutrophils, enabling IL-17 release and the expression of a functional IL-17 receptor [43]. Autocrine IL-17 – IL-17 receptor signaling enhances the neutrophil respiratory burst and protects against secondary challenge in an ocular and respiratory model of aspergillosis [43,63]. In humans, Mendelian defects in IL-17-mediated immunity predispose individuals to chronic mucosal fungal disease, particularly due to *Candida* spp. (reviewed in [36,64]), but not to pulmonary or extrapulmonary aspergillosis, consistent with the notion that IL-17-dependent host defense mechanisms can be functionally compensated in the lung and at deep tissue sites.

TRANSLATING RESEARCH PROGRESS INTO STRATEGIES FOR PROPHYLAXIS AND THERAPY

A series of murine studies conducted in the early 2000s found a protective role of myeloid progenitor transfusion on the outcome of invasive aspergillosis in a murine HCT and chemotherapy-induced neutropenia model of invasive aspergillosis [65,66]. More recently, a dose-dependent beneficial effect of transfusing granulocyte colony-stimulating factor (G-CSF)-mobilized isogenic neutrophils was observed on murine survival in a neutropenic model of aspergillosis [67]. In humans, the concept of myeloid cell transfusions to improve infectious outcomes was recently examined in a randomized trial using G-CSF/dexamethasone-mobilized granulocytes [68]. The authors did not observe a significant improvement in the outcome of any infection type examined, including invasive fungal infections. However, due to poor patient accrual, the power to detect a beneficial effect with granulocyte transfusion was low. The difficulty in obtaining large numbers of functionally competent granulocytes remains a significant barrier to investigate this form of cellular reconstitution as an adjunctive treatment modality. In a preclinical model, loading neutrophil-like HL-60 cells with the lipophilic drug pos-aconazole lowered the number of

fungal lesions in a neutropenic model of invasive aspergillosis [69]. Although these observations need to be extended to primary neutrophils in murine models and to human cells, it is possible that cellular therapies combined with antifungal agents achieve a higher level of antifungal activity *in situ* than with either compound alone.

To circumvent limitations associated with granulocyte transfusions, researchers have tested the concept of administering hematopoietic growth factors to enhance myeloid reconstitution during chemotherapy-induced neutropenia or following myeloablative conditioning and HCT. M-CSF, but not G-CSF or granulocyte-macrophage colony-stimulating factor (GM-CSF), can promote the myeloid lineage differentiation of hematopoietic stem cells via the transcription factor PU.1 independent of their survival or proliferative capacity [70]. In a murine isogenic HCT transplant model, M-CSF treatment protected mice from respiratory *A. fumigatus* challenge [71▪].

Following *A. fumigatus* challenge in immune competent mice, GM-CSF is rapidly produced in the lungs and potentiates the neutrophil respiratory burst in fungus-engaged cells *in vivo* [72]. Administration of recombinant GM-CSF accelerates *A. fumigatus* clearance in the lung of immunecompetent and immune suppressed mice [72,73]. In humans, a randomized double-blind prospective comparison of G-CSF, GM-CSF, and combined G-CSF and GM-CSF to prevent infections in individuals undergoing allogeneic HCT showed a lower incidence of invasive fungal infections, attributable to candidiasis, and transplant-related mortality in the two groups that received GM-CSF in their prophylaxis regimen [74▪▪].

Although lymphoid cells are dispensable for host defense against aspergillosis in otherwise immune competent mice [14], researchers have identified *Aspergillus*-specific human $CD4^+$ T cells [75] and, in one proof-of-principle study, administered these to invasive aspergillosis patients with beneficial effects [76]. CD4 T cell-dependent protection is ascribed to their production of IFN-γ and tumor necrosis factor; neutralization of either of these effector cytokines is associated with fungal disease in murine models (reviewed in [77]). More recent work has focused on delineating *Aspergillus* antigens that elicit IFN-γ-secreting Th1 CD4 T cells [78,79]. For example, *A. fumigatus* proteins Crf1, Gel1, and Pmp20 trigger robust Th1 responses in healthy persons and antigen-specific T cells to these antigens expand in patients with invasive aspergillosis [79]. In parallel, researchers have generated chimeric antigen receptor T cells that express the dectin-1 carbohydrate binding domain fused to the CD28 extracellular and transmembrane domains and to the CD3ζ signal transduction motif [80]. The resulting D-CAR^+ T cells inhibited *A. fumigatus* growth *in vitro* and in a murine cyclophosphamide-treated model of invasive aspergillosis [80].

CONCLUSION

The past decade has witnessed rapid advances in understanding protective immune responses against aspergillosis. Translating these advances into mechanism-based adjunctive cellular or molecular strategies that augment contemporary anti-fungal drugs or into risk stratification has been challenging. Limitations in measuring therapeutic responses during invasive aspergillosis, despite the availability of fungal surrogate antigen-based tests, and the difficulty in patient accrual have both contributed to the difficulty of testing novel

interventions. The recognition that the type of host innate immune injury (e.g., chemotherapy-induced neutropenia vs. corticosteroid-induced immune suppression vs. loss of the oxidative burst in CGD) can impact invasive aspergillosis pathogenesis indicates that targeted immune-based interventions are likely to be most efficacious when deployed in specific host contexts. Nevertheless, advances in understanding the genetic risk for invasive aspergillosis and in identifying growth factors and cytokines, epithelial products, and *Aspergillus*-specific T cells that are protective in preclinical models of disease and in early human trials suggest that immune-based interventions can facilitate further improvements in clinical outcomes.

Acknowledgments

I would like to acknowledge members of the T.M.H. laboratory for helpful discussions. I apologize to investigators whose work I could not cite due to space limitations.

Financial support and sponsorship

Investigations in the T.M.H. laboratory on anti-Aspergillus immunity is supported by NIH grant RO1 AI093808, a Burroughs Wellcome Fund Investigator in the Pathogenesis of Infectious Disease Award, and a grant from the Geoffrey Beene Foundation. NIH NCI Cancer Center grant P30 CA008748 (to MSKCC) provided support for this article.

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KEY POINTS

- Despite contemporary antifungal therapies, invasive aspergillosis remains a major cause of infectious morbidity and mortality in immune-compromised patient groups.
- Recent work into anti-*Aspergillus* immunity has delineated distinct immune mechanisms that kill conidia, the infectious propagules, and tissue-invasive hyphae.
- Epithelial products can inhibit *Aspergillus* growth *in vitro* and *in vivo*, even in the absence of functional leukocytes.
- Hematopoietic growth factors can stimulate anti-*Aspergillus* defense functions in leukocytes and may provide benefit for prophylactic and therapeutic antifungal strategies in humans

Table 1

Aspergillus -associated disease states

Disease	Clinical syndromes	Patient groups and notes
Invasive aspergillosis (IA): Most common species: *A. fumigatus, A. flavus, A. niger, A. terreus, A. ustus, A. versicolor*	Pneumonia and systemic disease; CNS disease most common site of dissemination; fungal keratitis	Induction-consolidation chemotherapy for hematologic malignancies and aplasia BM, lung, and heart transplant recipients Patients with graft vs. host disease, recipients of systemic corticosteroid therapy COPD and critically ill ICU patients Fungal keratitis in agricultural workers with corneal abrasions
Primary immune deficiencies associated with IA		*Affected gene*
Chronic granulomatous disease *A. fumigatus* and *A. nidulans* most common IA species in CGD	Pneumonia, deep tissue disease (e.g., osteomyelitis, brain abscess)	Defects in NADPH oxidase (e.g., in p22, p47, X-linked p91 subunits)
Job's syndrome (HIES)	Pneumonia, lung disease at sites of prior bacterial (staphylococcal) lung abscess	*STAT3*
MonoMAC syndrome	Pneumonia	*GATA2*
CARD9 deficiency	Extrapulmonary aspergillosis	*CARD9*
Several Congenital Neutropenia	Pneumonia and systemic disease	*ELA2, HAX1*
Leukocyte adhesion deficiency	Pneumonia and systemic disease	*CD18*
Pulmonary alveolar proteinosis	Pneumonia and systemic disease	Defect in GM-CSF signaling and surfactant catabolism
Chronic pulmonary aspergillosis (includes SAIA, CNPA, CCPA)	Pneumonia in patients with structural and/or cavitary lung disease; no angioinvasion and hyphae confined to preexisting lung cavity	Affects individuals previously treated for TB Aspergilloma refers to a fungus ball, a clump of hyphae in a pulmonary cavity
Tracheobronchial aspergillosis	Fungal disease of large airways without evidence of lung parenchymal involvement	Most common in lung transplant recipients and ICU patients
Allergenic disease	ABPA	Patients with underlying asthma and cystic fibrosis have defective fungal clearance and dysregulated immune responses Hypersensitivity to *Aspergillus* antigens can lead to progressive bronchiectasis
	Extrinsic allergic alveolitis	Typically observed after very high exposure to airborne conidia

ABPA, allergic bronchopulmonary aspergillosis; BM, bone marrow; CARD9, caspase recruitment domain-containing protein 9; CCPA, chronic cavitary pulmonary aspergillosis; CGD, chronic granulomatous disease; CNPA, chronic necrotizing pulmonary aspergillosis; CNS, central nervous system; GATA2, GATA binding protein 2; GM-CSF, granulocyte-macrophage colony-stimulating factor; HIES, hyper IgE syndrome; LAD, leukocyte adhesion deficiency; SAIA, subacute invasive pulmonary aspergillosis; SCN, severe combined neutropenia; TB, tuberculosis.