

Pathogenesis of allergic bronchopulmonary aspergillosis in cystic fibrosis: current understanding and future directions

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Allergic bronchopulmonary aspergillosis (ABPA) is an allergic disease characterized clinically by wheezing, pulmonary infiltrates, bronchiectasis, and fibrosis that affects patients with asthma and cystic fibrosis (CF). Although this disease has been characterized by a Th2 immune response to *Aspergillus*, the disease has some features such as central bronchiectasis which is not seen in other Th2 driven lung diseases such as atopic asthma. Here we will review the current pathophysiology of ABPA in CF and highlight new molecules that may affect immune responses against *Aspergillus* and ABPA disease pathogenesis.

Keywords *Aspergillus*, cystic fibrosis, allergy

Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is an allergic disease characterized clinically by wheezing, pulmonary infiltrates, bronchiectasis, and fibrosis that affects patients with asthma and cystic fibrosis (CF). In patients with ABPA, immunological responses to a variety of *Aspergillus fumigatus* antigens result in a heightened Th2 response, eosinophilia, and an elevated immunoglobulin E (IgE) level. ABPA has a relatively high prevalence in the setting of CF, affecting 2–15% of patients [1]. The infectious agent contributing to ABPA pathology, *A. fumigatus* is a ubiquitous opportunistic fungal pathogen, commonly inhaled into the airways and in healthy individuals rapidly cleared by alveolar macrophages utilizing oxidase-dependent killing mechanisms. However, in individuals with CF these organisms are often persistent and are found in the sputum of approximately 80% of CF patients at one time or another. Although the immunological phenotype of CF patients with ABPA has been well-

described, the mechanism whereby some patients remain tolerant to *A. fumigatus* whereas others develop a chronic allergic inflammatory response remains unknown. However, it is likely that an individual's risk of an allergic response to *A. fumigatus* depends on both innate and adaptive immune responses.

It has been recently demonstrated by several groups that Dectin-1, a β -glucan receptor expressed by dendritic cells, macrophages, and neutrophils is involved in innate recognition and shapes proinflammatory cytokine and chemokine responses against swollen conidia, a form of *Aspergillus* that precedes hyphal development [2–4]. The role of Dectin-1 in controlling immune tolerance versus Th2 responses to different forms of *Aspergillus* remains to be determined. We have recently reviewed the prevalence of ABPA at the Antonio J. and Janet Palumbo CF Center at the University of Pittsburgh which follows approximately 440 patients ranging in age from infancy to 56 years. Of these 440 patients, 181 (41%) patients have grown *A. fumigatus* in their airway in at least two cultures in the last three years. Of the 181 culture-positive patients, 31 patients have confirmed ABPA using the recently published consensus criteria of Stevens and colleagues [1] (Table 1). The large number of patients with *A. fumigatus* colonization but not ABPA suggests that either anergy or active immune tolerance may suppress the development of ABPA. This article will review the current understanding of the pathogenesis of ABPA, describe

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Table 1 Criteria for the diagnosis of classic ABPA in CF*

1. Acute or subacute clinical deterioration (cough, wheeze, exercise intolerance, exercise-induced asthma, change in pulmonary function, or increased sputum production) not attributable to another etiology.
2. Total serum IgE concentration of 1000 IU/ml (1200 ng/ml).
3. Immediate cutaneous reactivity to *A. fumigatus* (prick skin test wheal of >3 mm in diameter with surrounding erythema, while the patient is not being treated with systemic antihistamines) or *in vitro* demonstration of IgE antibody to *A. fumigatus*.
4. Precipitins to *A. fumigatus* or *in vitro* demonstration of IgG antibody to *A. fumigatus*.
5. New or recent abnormalities on chest radiography (infiltrates or mucus plugging) or chest CT (bronchiectasis) that have not cleared with antibiotics and standard physiotherapy.

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ongoing research at the Antonio J. and Janet Palumbo CF Center to better understand the immunology of ABPA and discuss mechanisms of immune tolerance to Af, as well as highlight future areas of needed investigation to prevent or advance treatment of ABPA.

Cystic fibrosis

As ABPA is prevalent in patients with Cystic Fibrosis (CF) and mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (Cftr) are an independent risk factor in asthmatics that go on to develop ABPA, it is pertinent to understand the basic defect that causes CF. CF is the most common severe life-shortening genetic disease in Caucasians, affecting approximately 30,000 people in the United States [5,6]. Patients with CF demonstrate pathophysiology in many organ systems, primarily the respiratory tract, the gastrointestinal tract (including the liver and pancreas), the skin, and the endocrine system. Despite the multisystem nature of the disease, lung disease causes the overwhelming majority of morbidity and mortality in CF. CF lung disease is a result of decreased chloride secretion and increased sodium absorption at the apical membrane of airway epithelial cells [5,6]. This change in ion transport results in decreased airway surface liquid (ASL) height and altered mucociliary clearance leading to chronic bacterial infection, airways inflammation, and damage to lung tissue [5,6]. It is likely that reduced ASL height and decreased mucociliary clearance leads to retention of bacterial and fungal antigens which in turn leads to activation of both innate and adaptive immune responses against these antigens. However, due to inadequate clearance mechanisms this immune response contributes to the development of lung injury and bronchiectasis over time and is the greatest cause of morbidity and mortality in these patients [7].

Epidemiology and diagnosis of allergic bronchopulmonary aspergillosis in cystic fibrosis

Among fungal organisms colonizing the respiratory tract of patients with CF, the environmental mold *A. fumigatus* is the most prevalent. In fact, in one study, up to 80% of patients with CF demonstrate IgG antibody to Asp f1, an immunodominant *Aspergillus* peptide antigen, by an early age [8]. The presence of *A. fumigatus* in a patient's sputum and immune recognition may or may not manifest in overt clinical disease. However, when *A. fumigatus* does cause clinical symptoms they are most often along the spectrum of ABPA, which is characterized clinically by wheezing, pulmonary infiltrates, bronchiectasis, and pulmonary fibrosis. The relatively high prevalence of ABPA in CF patients may be further increasing due to the use of inhaled corticosteroids, a treatment that is relatively common in CF patients [8,9]. Therefore, it is critical to understand the immunological etiology of ABPA so that novel, specific therapies can be devised and clinical strategies that potentially contribute to allergic inflammation can be avoided.

Aspergillus fumigatus is found in the sputum of many more patients than meet classical criteria for the diagnosis of ABPA (Table 1), and ABPA may be clinically suspected even in the absence of a respiratory tract culture positive for *A. fumigatus* [1]. Therefore, diagnosis of ABPA is often made on the basis of clinical impression, which may be one reason for the disparate prevalence rates seen when comparing ABPA rates across CF centers. This variation in diagnostic criteria creates a potential problem in prospectively studying ABPA.

There are many advantages of studying the mechanisms underlying ABPA in the cohort of patients with CF. First, the CF population is genetically more homogenous than a broader population with other allergic diseases. Second, studies are restricted to a defined set of antigens with high exposure prevalence.

Third, despite the high prevalence of documented antigen exposure, the CF population contains patients that develop allergic sensitization and patients that are tolerant or anergic. Understanding these dichotomous immune responses will not only inform on strategies for the treatment of ABPA in CF, but will improve our understanding of sensitization and tolerance in the broader population with allergies.

Host defense and pulmonary aspergillosis

Clinical and experimental data support a role for neutrophils in host defense against *A. fumigatus*, particularly in invasive pulmonary aspergillosis (IPA) [10]. Although part of the innate arm of the immune system, neutrophils are not part of the first-line defense system of the lungs. Instead, this function falls to the alveolar macrophage, the major resident phagocytic cell in the pulmonary microenvironment. Upon inhalation of airborne *A. fumigatus*, alveolar macrophages are the first to ingest and destroy inhaled conidia in an attempt to prevent germination into hyphae, which is the invasive form of *A. fumigatus* that allows it to disseminate to extrapulmonary sites. It is the invasive form of *A. fumigatus* where neutrophils are thought to play the most important role in defense, a second-tiered attack involving the release of oxidants and granule components in response to hyphae that have escaped alveolar macrophage effector function. Resident lung dendritic cells have also been shown to be early effector cells against *Aspergillus*, serving to endocytose both conidial and hyphal forms of the organism leading to subsequent activation of the adaptive T cell response [11]. As with most fungal infections, numerous reports investigating T cell mediated defense against aspergillosis have shown that Th1 and Th2 responses correlate with resistance and susceptibility, respectively, to infection [11]. Recently a new lineage of T-cells, Th17 cells, has been described in mouse and humans that express IL-17A, IL-17F, and IL-22 [12,13]. The development of these cells controlled by Stat3 and the transcription factor retinoic acid-related orphan receptor (ROR) γ T and ROR α as well as the cytokines, TGF- β , IL-6, IL-23, IL-21, and IL-1 β [12,14]. IL-23, IL-17A and IL-17F have been found to be elevated in the sputum at the time of pulmonary exacerbation in patients with CF [15]. In the lung IL-17 is critical for neutrophil recruitment to a variety of stimuli [13] and IL-22 regulates epithelial barrier function and host defense against the gram negative bacteria *Klebsiella pneumoniae* [16]. It remains unclear whether a Th17 response in CF is protective or contributes to tissue injury. Using the agarose bead model of airways infection with

Pseudomonas aeruginosa showed that mice deficient in IL-23 had less inflammation and lower levels of matrix metalloproteinase 9 in lung tissue compared to control mice [17]. Despite this, there was no difference in control of the bacterial challenge in this model. It has also been shown that a immune response characterized by both IL-23 and IL-17 production is also associated with increased tissue pathology in response to *A. fumigatus* [18]. This tissue pathology occurs in both wild-type mice [18] as well in mice deficient in NADPH oxidase, a mouse model of chronic granulomatous disease [19]. The chemokines that are responsible for Th17 cell migration to the lung remains to be determined but it has been reported that human Th17 cells express chemokine (C-C motif) receptor 6 (CCR6) and to a lesser extent CCR4 [20]. Thus it is interesting to note that Hartl *et al.* has shown that the expression of chemokine (C-C motif) ligand 17 (CCL17), a CCR4 ligand is significantly elevated in CF patients with ABPA compared to controls which included atopic CF patients without ABPA or *A. fumigatus* colonized CF patients without ABPA [21]. The contribution of IL-17A, IL-17F, or IL-22 producing T-cells to human ABPA pathology remains to be determined.

Innate recognition of *A. fumigatus*: role of TLRs and non-TLRs

An intense area of investigation is how *A. fumigatus* is recognized by innate effector cells and what is produced from this interaction. Receptors in the Toll-like receptor (TLR) family are likely candidates for these recognition events and of these, TLR2 and TLR4 are the most studied. The data on TLR2 is conflicting in that there are reports both in support [22] and against [10] its importance in host defense. Studies have also shown a role for TLR4 in the inflammatory response to *A. fumigatus* conidia, but not hyphae [23]. Other studies have attempted to clarify a role for TLRs by employing mice deficient in the TLR signaling adaptor MyD88 $^{-/-}$, which is shared by the TLR and IL-1 receptor pathway. However, this is again not without controversy as macrophages from MyD88 $^{-/-}$ mice exposed to *A. fumigatus* hyphae, but not conidia, have preserved TNF- α production [10,24]. Interestingly, in the latter study, TNF- α production was unaffected in macrophages from either TLR2 $^{-/-}$ or TLR4 $^{-/-}$ mice [10]. Recently it has been demonstrated that mice deficient in MyD88 (a downstream adaptor from TLR and IL-1 receptor signaling) challenged with *A. fumigatus* have reduced levels of interleukin-1 β (IL-1 β), IL-6, KC, and interferon- γ early after challenge, which is also associated with higher fungal burdens but by 72

hours both wild type and mutant mice cleared the organisms [10]. These data clearly show that pathways independent of MyD88 are important in fungal clearance. Finally a recent study in humans has shown a significant association between allele G on Asp299Gly (TLR4) and chronic cavitary pulmonary aspergillosis (odds ratio [OR], 3.46; $P=0.003$). In contrast, susceptibility to ABPA was associated with allele C on T-1237C in TLR9 (OR, 2.49; $P=0.043$) [25].

Non-TLRs are important in innate recognition of *A. fumigatus*. The cell wall of *Aspergillus* contains galactomannan moieties that are thought to be covalently linked to the non-reducing ends of β -1,3-glucan side chains [26,27]. Accordingly, mannose- or mannan-specific receptors have been implicated in the uptake of *A. fumigatus* conidia by phagocytic cells [28]. Recent studies have identified the C-type lectin dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) as being involved in the binding of *A. fumigatus* conidia to human macrophages and dendritic cells [29]. As one might hypothesize given that linear and branched β -glucan moieties comprise a large portion of fungal cell walls, as seen in *Aspergillus*, receptors identifying β -glucans such as complement receptor type 3 (CR3) [11], are involved in *Aspergillus* recognition. Disease caused by *A. fumigatus* is initiated when conidia, termed 'resting conidia' (RC) are inhaled into the lung. Upon entering the lung, RC go through genotypic and phenotypic changes that lead to swelling, now termed 'swollen conidia' (SC), followed by germination into hyphae. Several labs have shown that the Dectin-1 interactions with β -glucan are crucial for interactions of *A. fumigatus* with murine macrophages and human DCs that initiate the typical inflammatory response to the pathogen both *in vivo* and *in vitro* [2–4]. These responses included influencing macrophage production of TNF- α , IL-1 α , IL-1 β , and IL-6, and chemoattractants and growth factors such as MIP-2, MIP-1 α , G-CSF and GM-CSF [2,3]. Additionally, it has been reported that the interaction of Dectin-1 on human dendritic cells with *A. fumigatus* germ tubes is important for the production of IL-12 which may promote Th1 over Th2 responses [30]. Collectively, these data suggest that multiple innate recognition pathways involving distinct signaling machinery are involved in DC and macrophage recognition of *A. fumigatus* at different growth stages. These recognition pathways may have distinct functions in enhancing or suppressing the signaling cascades promoting allergic inflammation.

Additionally, soluble pattern recognition receptor proteins play an important role in *A. fumigatus* recognition, and may function in concert with antigen present-

ing cells. For example, surfactant proteins A (SP-A) and D bind multiple glycosylated *A. fumigatus* antigens and *A. fumigatus* glycoproteins, preventing specific IgE recognition of *A. fumigatus* antigens, while enhancing neutrophil and macrophage killing of *A. fumigatus in vitro* [31,32]. Individuals with polymorphisms in one of the SP-A genes, SP-A2, showed significant association with ABPA patients, and further, two specific polymorphisms in exonic sequences of SP-A2 together are predictive for both increased association with ABPA disease and worsened clinical correlates of ABPA severity [33]. Of note, the polymorphisms are located in the collagen tail of SP-A molecule, which interacts with multiple receptors on APCs. A recently described soluble pattern recognition receptor important for host defense against *A. fumigatus* is Pentraxin-3, which is made by macrophages and dendritic cells, binds *Aspergillus*, and facilitates pulmonary DC production of IL-12 and MCP-1 [34,35]. It is currently unknown which ligands Pentraxin-3 identifies on *A. fumigatus*, though galactomannan incubation inhibits its recognition of *A. fumigatus* [34]. It is also currently unclear the mechanism whereby *A. fumigatus* -Pentraxin-3 complexes interact with DC's to influence their priming and polarity.

Immune responses to *A. fumigatus* in CF

As stated above, up to 80% of patients with CF demonstrate sensitization to Asp f1, the immunodominant *Aspergillus* peptide antigen, by an early age [8]. However, which of these patients will go on to have clinical ABPA cannot be predicted presently as the clinical responses of CF patients to *A. fumigatus* range from diagnostically established ABPA, i.e., meeting all four criteria listed in Table 1, to immediate cutaneous response to *A. fumigatus* despite the absence of *A. fumigatus* in sputum cultures [36]. In patients with diagnostically established ABPA, immunological responses to a variety of *A. fumigatus* antigens, including Asp f1, result in a heightened allergic, inflammatory response mediated by a Th2 cell response and characterized by an elevated immunoglobulin E (IgE) level [37].

From an adaptive immunity standpoint, one can hypothesize that patients with CF and ABPA have elevated Th2 responses because of fundamentally different interactions between dendritic cells and T-cell responses. For example polymorphisms in inducible co-stimulator molecule (ICOS), IL-4 or IL-4 receptor alpha gene have been associated with Th2 diseases such as asthma [38–41]. In the case of ABPA, it has been demonstrated that Asp f1-specific T-cell clones from

patients with ABPA produce IL-4 only in response to antigen presented by HLA-DR2 or HLA-DR5 [42] and that allelic frequency of HLA-DR2 is higher in patients with ABPA than those with cutaneous reactivity to *A. fumigatus* but no ABPA [43], suggesting that there are genetic modifiers on the antigen presenting cell side, that contribute to the development of ABPA. Two new additional molecules also deserve investigation in regulating Th2 immunity to *A. fumigatus*. IL-25 or IL-17E has been shown to drive Th2 immune responses in the lung [44] and recently it has been demonstrated that epithelial expression of IL-25 can drive Th2 immune responses [45]. Furthermore blockade of IL-25 can attenuate both inflammation and airways hyperreactivity in a murine model of asthma [46]. Another molecule made by epithelial cells that can drive Th2 responses is thymic stromal lymphopoietin (TSLP). TSLP can signal to both dendritic cells [47] and mast cells [48] to augment Th2 cellular differentiation and cytokine production. The role of these two molecules in the development of ABPA have not been defined as of yet. As mentioned above CCL17, a CCR4 ligand is significantly elevated in CF patients with ABPA compared to controls, however the potential use of this as a discriminating bio-marker would need to be replicated in additional cohorts of patients.

Less work has been done to investigate the role of immune regulation, but recent work in a mouse model of airway antigen sensitization has begun to elucidate the mechanisms by which immune tolerance to inhaled antigens is generated. This work by Ostroukhova *et al.* demonstrates that antigen-induced tolerance to OVA antigen is dependent on a CD4+CD25+ population of regulatory T-cells (Tregs) that co-express membrane bound TGF- β and the transcription factor FOXP3 [49]. Indeed in preliminary studies we have observed an increase in CD4+ Foxp3+, surface TGF- β positive cells in CF patients that are colonized with *A. fumigatus* but do not have elevated *A. fumigatus*-specific IgE or detectable *A. fumigatus* specific Th2 cells in peripheral blood. These data suggest that a subpopulation of CF patients at risk for ABPA may be protected from disease by the development of regulatory T-cells. In fact CD4+, CD25+ regulatory cells have been shown to limit Th2 responses in mice challenged with *A. fumigatus* conidia [50].

Future directions

With the recent discovery of the Th17 T-cell lineage it will be important to determine if this lineage is involved in tissue protection or tissue injury in patients with ABPA. Furthermore, as CF is a disease of the

epithelium, epithelial products such as TSLP or IL-25 could be dysregulated in CF patients that are susceptible to ABPA. Moreover the role of Tregs needs to be defined in humans with ABPA. If Tregs are critical for maintaining tolerance, than one could envision Treg mobilization strategies to prevent or treat ABPA. Lastly due to the relatively high prevalence of ABPA in patients with CF and the fact that many patients receive excellent coordinated care through CF centers, one could envision a whole genome single nucleotide polymorphism array study to determine gene modifiers that predict ABPA in CF. Susceptibility alleles for the development of Inflammatory Bowel Disease include the innate sensor nucleotide-binding oligomerization domain containing 2 (NOD2) and the IL-23 receptor and these far exceed Class II MHC polymorphisms in determining risk of disease [51,52]. It is possible that genes, such as SP-A2, influencing innate and adaptive immune responses against *A. fumigatus*, could be critical in determining the susceptibility to ABPA, and could potentially be of greater associative or predictive value than current MHC II allelic variations associated with ABPA risk [53].

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