

# Immune Response, Diagnosis and Treatment of Allergic Bronchopulmonary Aspergillosis in Cystic Fibrosis Lung Disease

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**Abstract:** Patients with cystic fibrosis (CF) suffer from chronic infective lung disease, which determines morbidity and mortality. While bacteria, such as *Pseudomonas aeruginosa*, are well-known to contribute to pulmonary pathology, the relevance of fungi in CF airways remains poorly understood. The best studied fungus in CF is *Aspergillus fumigatus*, which frequently colonizes CF airways and causes a disease condition termed allergic bronchopulmonary aspergillosis. This review aims to provide an update on the immunological mechanisms, diagnostic approaches and therapeutic strategies for allergic bronchopulmonary aspergillosis and other *Aspergillus fumigatus*-mediated phenotypes in CF lung disease.

**Keywords:** Cystic fibrosis, Allergic Bronchopulmonary Aspergillosis (ABPA), Immune response.

## CYSTIC FIBROSIS LUNG DISEASE

Cystic fibrosis (CF) is a systemic disease caused by mutations within the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1, 2]. Due to the broad expression of CFTR, disease pathology is present in a variety of organs, but morbidity and mortality arise nowadays mainly from chronic airway disease, where inflammation and infection are perpetuated and irreversibly damage the pulmonary tissue. In CF, the inflammatory response is driven substantially by chronic bacterial infections, especially *Pseudomonas aeruginosa* species, which leads to tissue breakdown and severe lung damage [3]. Pro-inflammatory mediators in CF airway fluids are increased and reflect an ongoing chronic inflammation and remodeling process [4]. In particular, the airway inflammatory response in CF is persistently neutrophilic, marked by an up-regulation of neutrophil chemotactic mediators such as interleukin 8 (IL-8/CXCL8) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>), resulting in the continuous recruitment of neutrophils from the circulation into the airways and the uncontrolled and unbalanced release of pro-inflammatory, oxidative and proteolytic mediators from the neutrophil, such as serine and matrix metalloproteases (MMPs), reactive oxygen species (ROS) and others [5-8]. Inhaled and systemic antibiotics are employed to control the bacterial colonization and infections within the CF airways and have contributed significantly to improved survival in the last decades [9]. Besides bacterial pathogens, it becomes clear that fungal species, especially those related to *Aspergillus* species, are increasingly detectable in CF airway fluids [10], probably due to the advent of intensified inhaled antibiotic therapy [11, 12], raising the awareness for fungal species in CF airway fluids, especially those related to *Aspergillus* species [13, 14]. In addition to *Aspergillus*, recent studies underscore the potential role of *Candida* species for CF lung disease. We refer to a recent review for this topic [15]. Allergic bronchopulmonary aspergillosis (ABPA) is an allergic disease against *Aspergillus fumigatus* occurring mainly in patients with CF but also in asthmatics. Since the first description of ABPA in the 1950s [16], there has been a tremendous effort to understand the characteristics and immunopathogenesis of ABPA. However, while the pathogenicity and disease

relevance of *Aspergillus fumigatus* in CF still remain poorly understood, recent data provide limited evidence that not only the full clinical presentation of ABPA, but also the colonization with and sensitization to *Aspergillus fumigatus* may be associated with CF disease parameters, such as hospital admissions [17-19]. However, studies on the relationship of *Aspergillus fumigatus* and clinical disease parameters in CF are still limited and some studies do not support the concept that *Aspergillus fumigatus* colonization or colonization has an effect on lung function [11, 13, 20, 21].

This review aims to summarize and highlight recent findings on the underlying immune response to *Aspergillus fumigatus*, the potential value of biomarkers for the diagnosis of ABPA and current and future therapeutic options for ABPA or *Aspergillus fumigatus* colonization in CF lung disease. Since this field is extensive, we have focused on recent studies and do not raise the claim to completely cover the whole area.

## IMMUNE RESPONSE TO ASPERGILLUS

*Aspergillus fumigatus* is a ubiquitous fungal organism found commonly in house dust, water damaged walls or ceilings, and decomposing organic material. Its conidia are approximately 2-3.5 µm in diameter, which allows deposition in terminal airways and alveoli [22]. *Aspergillus fumigatus* represents, besides its relevance for CF lung disease, currently the most frequent pathogen of invasive fungal infections in immunocompromised hosts, for instance after stem cell transplantation [23]. In general, *Aspergillus fumigatus* is associated with several disease entities in humans, from life-threatening infections to allergic manifestations. Colonization and/or infection with this fungus cause a broad range of clinical entities, including invasive blood-stream infections, direct lung infections including the forming of pulmonary aspergillomas, allergic bronchopulmonary aspergillosis (ABPA), hypersensitivity pneumonitis, allergic chronic sinusitis, severe asthma with fungal sensitization (SAFS) and others [24-27].

Several hundreds of *Aspergillus fumigatus* conidia are inhaled from a human lung per day [23]. Despite this fact, manifest diseases caused by *Aspergillus fumigatus* are surprisingly very rare. Accordingly, the human pulmonary immune system is efficiently able to prevent the invasion of *Aspergillus fumigatus* [28]. From this point of view it is understandable, that *Aspergillus fumigatus*-associated diseases occur almost exclusively in immunodeficient patients, and the individual immune status of the host determines the resulting *Aspergillus* disease manifestation [29]. The immune response to

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*Aspergillus fumigatus* starts basically with recognition of the fungus through pattern recognition receptors (PRRs) (C-type lectin receptors, mainly dectin-1, and Toll-like receptors, mainly TLR2, 4 and 6), which detect conserved *pathogen associated molecular patterns* (PAMPs) on the surface of *Aspergillus fumigatus*, followed by a pro-inflammatory response of the innate immune system and, later on, by activation of the adaptive arm of immunity. In addition to cell-associated PRRs, humoral PRRs, such as the surfactant proteins A (SP-A) and SP-D [30-32] and the recently appreciated pattern recognition molecule long Pentraxin 3 (PTX3) are essential in fungal recognition [33, 34]. *Aspergillus fumigatus* PAMPs mainly comprise the cell wall constituents  $\beta$ -glucans, galactomannan and chitin [35]. In resting conidia the  $\beta$ -glucans are hidden by a shield of surface hydrophobins preventing their efficient immune recognition [36]. In swelling conidia or germ tubes the remodeling of the cell wall exposes  $\beta$ -glucans on the cell surface and leads to immune recognition through the host. This morphotype specific recognition seems beneficial in preventing unnecessary and harmful inflammatory reactions. Subsequent activation of PRRs leads to maturation of antigen presenting cells (APCs), mainly dendritic cells and alveolar macrophages, that further direct the adaptive T cell response (see Fig. 1). An efficient Th1 response is crucial for the prevention of an invasive mycosis [37], whereas an exaggerated Th2 response is associated with ABPA [38]. Importantly, the Th2-associated chemokine CCL17 / TARC orchestrates Th2 cell priming, activate Tregs and impair macrophage activities. In parallel, the Th17 axis is

activated. Further downstream responses include eosinophil activation, B cell activation, IgE production and mast cell degranulation. Released chemokines (such as CXCL8) promote neutrophil (PMN) diapedesis / transmigration and maintain neutrophilic tissue inflammation. The recruited phagocytic cells of the innate immune system are highly effective in destroying *Aspergillus fumigatus* conidia by (opsono)phagocytosis, the delivery of reactive oxygen species (ROS) and neutrophil extracellular trap (NET) formation [39]. Additionally, neutrophils gather around the fungus and discharge defensins, calprotectin, lactoferrin and other proteins from their primary and secondary granula to combat the fungus [40, 41]. This type of defense, in concert with NETosis, is especially important against large hyphal forms of *Aspergillus fumigatus* which cannot be phagocytosed [23].

In general, inflammation is fundamentally important to limit spreading of the agent. A dysregulated response, however, might lead to chronic inflammatory diseases or autoimmune / auto-inflammatory manifestations. It has been shown that besides direct combating the fungus, the adaptive system constrains the innate inflammatory response to prevent host tissue damage through overwhelming inflammation / auto-inflammation [28, 42]. The recently recognized Th17 cells seem to have considerable adverse effects in *Aspergillus fumigatus* infections, since they were found to cause an ineffective hyperinflammatory response to the fungus [43, 44]. Several mechanisms have already been described that limit inflammation towards *Aspergillus fumigatus* including ROS distri-

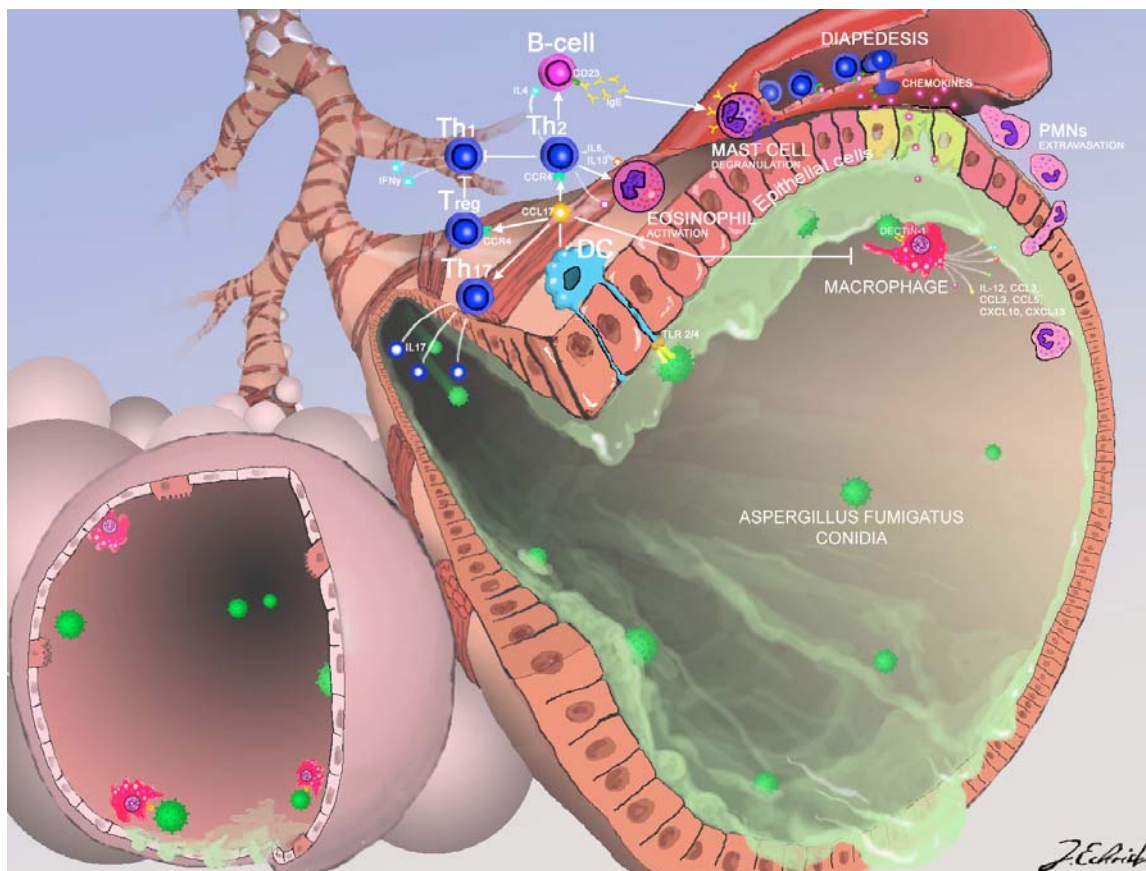


**Fig. (1). Host-fungus interactions in the airways.** When *Aspergillus fumigatus* conidia (green) enter the airways, they are recognized by pattern recognition receptors (mainly TLRs and dectin-1), expressed by macrophages, dendritic cells and, to a lesser extent, airway epithelial cells. This interaction triggers early pro-inflammatory responses, such as cytokine and chemokine release. Chemokines, such as the Th2-associated chemokine CCL17 / TARC orchestrate Th2 cell priming, recruit Tregs and impair macrophage activities (such as phagocytosis). In parallel, the Th17 axis is activated. Further downstream responses include eosinophil activation, B cell activation, IgE production and mast cell degranulation. Released chemokines (such as CXCL8) and IL-17 promote neutrophil (PMN) diapedesis / transmigration and maintain neutrophilic tissue inflammation.

bution, indoleamine 2,3-dioxygenase (IDO) activity and/or activation of the TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF) pathway [28]. The exact interactions, however, between physiologic anti-fungal inflammation, hyperinflammatory host damage, chronic infection or commensal survival are just beginning to be dissected.

The host-*Aspergillus fumigatus* interaction in CF lung disease is peculiar in several aspects: (i) the fungus chronically colonizes the CF airways without apparently triggering systemic / invasive infections, (ii) only a subset of colonized CF patients mount a robust allergic response / sensitization towards the fungus and (iii) only a subset of those sensitized patients develop significant pulmonary symptoms leading to the diagnosis of ABPA. The underlying reasons seem to be complex, are modulated by CFTR, non-CFTR genetic immune host susceptibility / gene modifiers, patient's age, sex, atopy, microbial interactions, medication and others and are discussed in the sections below.

### ASPERGILLUS LIKES COMPANYY

While *Aspergillus fumigatus* is probably the most relevant fungus in CF lung disease, *Pseudomonas aeruginosa* represents the most common and most relevant bacterial pathogen in CF patients beyond infancy. Consequently, the question arises, how these two chronic colonizers of the CF airway tract relate to each other. Several studies report an association between detection of *Aspergillus fumigatus* and *Pseudomonas aeruginosa* in CF airway fluids samples [45-47] and there are indications that *Aspergillus fumigatus* and *Pseudomonas aeruginosa* have a synergistic/ interactive effect on lung function [17] and sensitization to *Aspergillus fumigatus* has been reported to be associated with [48] or preceded by *Pseudomonas aeruginosa* infection [49].

Besides *Pseudomonas aeruginosa* other microbes reported to be associated with *Aspergillus fumigatus* include *Alternaria* and *Candida* [46, 50-52], *Stenotrophomonas maltophilia* [46-48], atypical mycobacteria [47], *Burkholderia cepacia* [46] and *Scedosporium apiospermum* [47, 53]. Interestingly, in patients colonized by *Scedosporium apiospermum*, ABPA-like symptoms have been observed [54, 55]. The potential spectrum of *Aspergillus fumigatus*-microbe interactions has been further extended by recent studies on the pulmonary microbiome in CF patients [56-58], a research area warranting to be explored, especially regarding the pulmonary microbiome.

When viewed in combination, these studies support the view that *Aspergillus fumigatus* colonization and / or sensitization is favored by the presence of other bacterial or fungal microbes in the CF airway microenvironment. The underlying microbe-to-microbe communication and microbe-to-host interaction mechanisms remain to be uncovered, but may involve (i) pathogen-mediated damage of airway epithelial cells paving the way for colonization of other secondary microbes; (ii) *Pseudomonas aeruginosa*-mediated Th2 shift [38], as also discussed below in detail, that may favor pro-allergic responses and sensitization to *Aspergillus fumigatus*, (iii) CF lung disease severity-, microbial colonization-, patient age-, and medication-dependent phenomena, which overlap within the course of disease and should be controlled for by adjustment for confounders [20], and others.

### ABPA IN CYSTIC FIBROSIS

Healthy individuals, when exposed to *Aspergillus fumigatus*, rapidly clear it in contrast to patients with CF, where *Aspergillus fumigatus* persists and chronically infects the respiratory tract [59]. Colonization of the respiratory tract by *Aspergillus fumigatus* is common in CF. It is thus reasonable to prevent the inhalation of large numbers of *Aspergillus fumigatus* spores in CF patients and therefore to advise patients with ABPA to avoid exposure to places high spore counts, such as damp basements, barns, and compost heaps [60]. The prevalence of *Aspergillus fumigatus* in CF lungs

shows a broad range depending on culture frequency, methodology, duration of monitoring, climatic conditions and patient population [61, 62]. In Cystic Fibrosis Foundation (CFF) accredited US care centers reported prevalence increased from 6 to 13% from 1995 to 2005. Other investigations found prevalences from 6 to 57% [51, 55, 63], emphasizing the meaning of special culture media selective for fungi and inhibition of bacterial overgrowth [64]. Culture independent diagnostic approaches with DNA based techniques direct to even higher prevalence rates [65]. However, the exact reasons for these intercenter differences and longitudinal prevalence changes, beyond increasing use of inhaled antibiotics, are still to be defined. In general, colonization with *Aspergillus fumigatus* is defined as the presence of *Aspergillus fumigatus* in respiratory secretions, and is different from the most severe clinical manifestation of *Aspergillus fumigatus* in CF, namely ABPA. Prior to the development of ABPA, colonization of the respiratory tract can trigger immunological sensitization to *Aspergillus fumigatus*. Adapting the Leeds criteria for chronic *Pseudomonas aeruginosa* infection [66], persistent *Aspergillus fumigatus* infection has to be taken into account in case of at least two annually positive cultures, whereas transient infection can be defined if there is one *Aspergillus fumigatus* positive culture per year. However, only a subset of *Aspergillus fumigatus* infected CF patients will develop ABPA [67-69].

ABPA is a pulmonary disease that results from hypersensitivity to *Aspergillus* antigens, mostly due to *Aspergillus fumigatus*. The diagnosis of ABPA is complicated and therefore the definition of clinical ABPA is vague. Since the late 1970s, the diagnosis of ABPA is mainly based on the Nelson criteria, which include clinical aspects like wheezing and new pulmonary infiltrates together with laboratory findings like elevated total IgE serum levels, elevated specific IgE and IgG serum levels against *Aspergillus fumigatus*, and the detection of *Aspergillus fumigatus* in sputum [70]. Although these criteria underwent some minor modifications over time, they are still considered to be the gold standard for ABPA diagnosis and are used by many CF clinicians in daily practice. If related to the pathophysiological picture described earlier, the Nelson criteria combine the stages of *Aspergillus fumigatus*-specific sensitization, activation of Th2 response, maintenance of the allergic process and disabled resolution of the inflammatory response [71]. Potential therapeutic interventions act at the above mentioned cornerstones of colonization/ infection, sensitization, activation of inflammatory response [62, 67, 68] and initiation of resolution [72, 73]. The common disputable 'hen and egg' question what comes first in CF lung disease, the infection with bacteria or the inflammation of the airways, can also be asked in terms of colonization of the respiratory tract with *Aspergillus fumigatus* conidia and airway inflammation. This may be explained by different recommendations regarding the diagnostic criteria for ABPA among physicians, centers and countries [46, 68, 74]. As reviewed by Hafén *et al.* [71] such differences are illustrated by the use of different definitions used in North America and Europe. For the Epidemiologic Study of CF, conducted in the USA and Canada, at least two features out of wheezing, peripheral blood eosinophilia, history of pulmonary infiltrates, elevated specific IgE against *Aspergillus fumigatus*, clinical response to steroids or *Aspergillus fumigatus* in sputum; and two of the following three criteria were required for diagnosis of ABPA: immediate cutaneous reaction to *Aspergillus fumigatus*, serum precipitating antibodies to *Aspergillus fumigatus* and total serum IgE of more than 1000 IU/ml [45]. For the European CF Registry the following four criteria were required for diagnosis of ABPA: physician's suspicion of ABPA based on at least one clinical feature out of wheezing, pulmonary infiltrates, peripheral eosinophilia, response to steroids or *Aspergillus fumigatus* in sputum, immediate cutaneous reaction to *Aspergillus fumigatus*, serum precipitating antibodies to *Aspergillus fumigatus*, and total serum IgE of over 1000 IU/ml [46]. The lack of a sustainable definition of clinical ABPA points to the importance of reliable biomarkers as discussed below. Even though it is well known that ABPA is associated with

deterioration of lung function, the effects of *Aspergillus fumigatus* colonization on lung function in the absence of ABPA are not clear [62]. Some studies suggest an effect on disease severity, hospital admissions and/or lung function [17-19, 49, 75], others not [13, 20]. A retrospective cohort single-center study of the effect of *Aspergillus* on lung function and hospitalization in CF patients in 230 pediatric patients showed higher *Pseudomonas aeruginosa* infection rates, lower baseline lung function and a higher ABPA rate in chronically *Aspergillus fumigatus* infected patients compared with other patients [17]. These data unmask patients with persistent *Aspergillus fumigatus* in sputum cultures to suffer by real infection that worsens CF lung disease. In addition to *Aspergillus fumigatus* infection and ABPA, sensitization to *Aspergillus fumigatus* may also cause adverse effects, like worse lung function [18, 19, 21, 75]. However, *Aspergillus* colonization seems to be much higher than sensitization. Interestingly, there does not appear to be a clear correlation between *Aspergillus* load in the environment and the development of ABPA [76]. Apart from ABPA, other *Aspergillus* associated disease entities namely, *Aspergillus fumigatus* colonization, *Aspergillus fumigatus* sensitization, fungal asthma and the even more severe form severe asthma with fungal sensitization (SAFS) [77] may occur in and affect the course of CF lung disease. These disease entities play also a critical role in terms of lung function and bronchial inflammation.

In order to prevent the development of ABPA it is essential to determine risk factors triggering ABPA and its preceding disease entities as early as possible. So far, a few studies have evaluated the prevalence of ABPA and have investigated various risk factors, mainly in adult CF populations [10, 11, 45, 46, 48, 68, 69]. Recently published work by Jubin *et al.* [50] investigated in a retrospective study the risk factors for *Aspergillus* colonization and ABPA in children with CF. Even though studying a small sample size they found a strong association between long-term azithromycin therapy and *Aspergillus* colonization, whereas poor nutritional status was associated with ABPA. The underlying mechanisms remain elusive, but a poor nutritional status may favor a Th2 response against *Aspergillus* species that might facilitate the switch from *Aspergillus* colonization to ABPA [50]. Prophylactic oral and inhaled antibiotics against *Staphylococcus aureus* or *Pseudomonas aeruginosa* have been associated with an increased risk of *Aspergillus fumigatus* colonization in CF in some studies, while others found no association [11, 12, 63]. Some previous studies have further reported that the frequency of *Aspergillus* colonization increases with age [21, 69] and correlates with increased disease severity [69], as assessed by clinical and radiographic scores. This is in accordance with reports that ABPA occurs preferentially in older and male CF patients with atopic symptoms and deteriorated lung function [45, 46, 69, 78]. Furthermore, higher cumulative doses of inhaled corticosteroids have been described to be associated with either ABPA [46] or *Aspergillus fumigatus* sensitization [48], but data is not conclusive [63]. Interestingly, also prolonged rhDNase therapy was found to be associated with ABPA [46, 50]. This association between rhDNase therapy and *Aspergillus* colonization and ABPA could be partly due to the effect of prolonged ( $\geq 6$  months) rhDNase therapy on pulmonary host defense mechanisms, in particular by inhibiting neutrophil elastase activities and IL-8 concentrations in the bronchoalveolar lavage [79] and the sputum [80] of CF patients.

#### DIAGNOSIS OF ABPA: POTENTIAL BIOMARKERS

The diagnosis of ABPA is hampered by the fact that its clinical presentation overlaps with (i) common features of CF lung disease (exacerbations due to other pathogens, radiographic changes/abnormalities, changes in IgG and IgE levels) and (ii) features of *Aspergillus fumigatus* colonization / sensitization, that occur much more frequently in CF patients than ABPA and are therefore sometimes hard to categorize as ABPA or non-ABPA in daily clinical routine. Accordingly, biomarkers are warranted to (i) iden-

tify ABPA flares to initiate treatment as soon as possible to detain disease progression and, even more, to (ii) identify CF patients early, who are at risk for developing ABPA later on in their course of disease. The latter approach requires prognostic biomarkers that are capable of stratifying CF patients based on their immunological phenotype into ABPA-high versus ABPA-low risk patients longitudinally. In general, biomarkers can be subcategorized into (i) diagnostic, (ii) severity, (iii) risk assessment and (iv) drug response / monitoring biomarkers. Related to ABPA, the most preferable biomarker would be one for early risk assessment, whereas markers for disease severity and monitoring already exist, such as IgE. Thus, novel putative biomarkers have to be compared with the gold-standard of IgE. Reported potential biomarkers that have been published include (i) total serum IgE, (ii) specific IgE / IgG to *Aspergillus fumigatus* (iii) specific IgE to recombinant *Aspergillus fumigatus* antigens, (iv) CCL17 / TARC serum levels and (v) the basophil release assay. Here we will focus on the potential role of recently described biomarkers, that are (iv) – (v), namely CCL17 / TARC serum levels and the basophil release assay.

#### CCL17

CCL17 / TARC is a CC chemokine, released by dendritic cells, activated platelets and other cell types and attracts Th2 cells and regulatory T cells through ligation of its cognate chemokine receptor CCR4 [81, 82]. Besides this effect on T cells, CCL17 may also regulate other immune cells, such as mast cells and dendritic cell subsets. Several lines of *in vitro* and *in vivo* evidence support the notion that the immune response in ABPA is characterized by an overwhelming Th2 response. Accordingly, monitoring the magnitude of the Th2 response in airway fluids or blood in CF patients may represent a promising approach to detect a Th2 bias and the development of ABPA early in the course of disease, prior to the full clinical presentation of lung function loss and radiographic infiltration. Canonical Th2 markers include Th2-associated cytokines (IL-4, IL-5, IL-13, IL-25, TSLP), Th2-associated chemokines (CCL17, CCL22, and others) and Th2 cells itself. A novel Th2-like pro-allergic immune cell subsets includes the innate lymphoid cell subtype 'nuocytes', which could represent a substantial source of IL-13 at mucosal sites of allergic inflammation [83]. Inspired by these concepts, two studies investigated the potential usefulness of Th2-associated serum markers for the diagnosis and disease follow-up of ABPA in CF patients. The first study [38] involved mainly adult, clinically well-characterized, ABPA CF patients based on Nelson's criteria. Patients with CF colonized with *Aspergillus fumigatus* without ABPA, patients with CF sensitized to *Aspergillus fumigatus* but without ABPA, atopic patients with CF without ABPA, and atopic control subjects without CF were matched to the ABPA group by sex, age, disease severity, pathogens, and treatment characteristics. In these patients groups, we screened for a panel of Th2-associated mediators in serum fluids cross-sectionally and in CF ABPA patients both cross-sectionally and longitudinally. This study demonstrated that Th1- (IFN- $\gamma$ , IL-12) and Th2- (IL-4, IL-5 and IL-13) associated cytokines had a low detection rate, that means they were only detectable at low levels or had a very high range of detection (especially in ABPA patients), rendering them not useful for discriminating ABPA patients from patients sensitized with *Aspergillus fumigatus*. In contrast to cytokines, the Th2-associated chemokines CCL17 / TARC was readily detectable in serum fluids and was significantly increased in CF patients with diagnosed ABPA compared to CF patients colonized or sensitized to *Aspergillus fumigatus* but without clinical ABPA symptomatic. Serum levels of CCL17 / TARC showed a positive association to specific IgE levels to *Aspergillus fumigatus* and to specific IgE to the recombinant *Aspergillus fumigatus* allergen f4, suggesting, in line with animal studies, that the release of CCL17 related to an *Aspergillus fumigatus*-derived Th2 immune response. Patients undergoing corticosteroid treatment showed lower CCL17 / TARC serum levels. These data also underscore the notion, that immuno-

suppressive treatment is capable of dampening the high Th2 response in ABPA patients. Importantly, within the longitudinal course of ABPA flares, CCL17 / TARC levels increased in parallel to total serum IgE levels and reflected the clinical course of ABPA lung disease. This finding was not Th2 chemokine redundant, since CCL22 / MDC levels were less discriminative as compared to CCL17 / TARC, both cross-sectionally as well as longitudinally. Other chemokine serum levels, such as CCL2, CCL11 and CCL5 were higher in ABPA compared to *Aspergillus fumigatus* colonized patients, atopic patients and healthy controls, but did not discriminate ABPA patients from *Aspergillus fumigatus* sensitized patients. In a second independent study [84], the usefulness of CCL17 / TARC levels was compared between different putative ABPA biomarkers. This study included a younger, pediatric study cohort and involved a median longitudinal study duration of 40 months, with some patients followed-up until 80 months. In a nested matched case-control design substudy (n=10), CCL17 / TARC levels were able to discriminate between patients with clinical ABPA and without clinical ABPA when matched for total IgE levels, suggesting that CCL17 / TARC serum levels are not just reflecting IgE levels / Th2 response in general. Of note, two ABPA patients could not be included in this nested analysis due to very high serum IgE levels (3,710 and 5,263 IU·mL<sup>-1</sup>) without matching controls available. Longitudinally, this study also showed that serum CCL17 / TARC serum levels increased at ABPA flares, but were also increased earlier in the development of disease than IgE levels did. However, these observations were highly variable among individual ABPA patients. When viewed in combination, these studies indicate that the Th2-associated chemokines CCL17 / TARC was found to be increased in CF ABPA patients, but not in *Aspergillus fumigatus* colonized or sensitized patients. However, it has to be mentioned that the potential usefulness of CCL17 / TARC as biomarker for ABPA has several pitfalls: (i) CCL17 / TARC protein levels increase in serum at ABPA flares and were found to be increased in CF patients with a full blown clinically active ABPA, but were not commonly elevated in CF patients colonized with and sensitized to *Aspergillus fumigatus*. We observed that in individual CF patients, who developed later on full ABPA, CCL17 serum levels increased early in the course of disease, but not in others and we were unable to dissect the underlying reasons [84]; (ii) CCL17 levels are affected by age [85] and genetic polymorphism [86]. (iii) Preanalytical conditions are known to affect CCL17 quantifications and are dependent on storage duration, sample technique and platelet count (since platelets are a substantial source of CCL17). (iv) Corticosteroids lower CCL17 / TARC levels in serum, which has to be taken into account when interpreting ELISA results from treated patients. (v) The two studies on CCL17 / TARC in ABPA were supporting the concept of CCL17 as potential serum markers, but were nevertheless limited by patient numbers and follow-up time; (vi) the usefulness of quantifying CCL17 / TARC protein levels in ABPA sputum supernatants (as non-invasive ABPA biomarker method) remains elusive (despite studies on CCL17 / TARC levels in patients with asthma suggesting that CCL17 / TARC levels in sputum are also significantly increased compared to controls). When viewed in combination, prospective longitudinal multicenter studies are needed to evaluate the usefulness of CCL17 in serum as potential biomarker and corticosteroid response marker in the diagnosis and disease monitoring of ABPA in CF.

### Basophil Release Assay

Basophil activation tests are used to monitor specific allergen responses at single cell levels using flow cytometry and the upregulation of activation markers, such as interleukin (IL)-3 receptor CD123, the tetraspanin and marker for primary/azurophil granule release CD63 and the ecto-nucleotide pyrophosphatase/phosphodiesterase CD203c [87, 88]. Particularly, upregulation of CD203c has been used in patients with several atopic diseases, such as drug or wheat allergy [89, 90]. Gernez and coworkers now

utilized this technology to study its usefulness as marker for ABPA in CF patients [91]. Intriguingly, both baseline basophils as well as *Aspergillus fumigatus* extract-stimulated cells showed a significantly increased CD203c surface expression levels in CF patients colonized with *Aspergillus fumigatus* and the diagnosis of ABPA compared to CF patients colonized with *Aspergillus fumigatus*, but without ABPA or CF patients without *Aspergillus fumigatus* colonization. In contrast to CD203c, expression levels for CD63 and CD123 did not differ between the three patient groups, suggesting that CD203c expression levels after basophil activation might represent a promising and specific cellular immune biomarker for the diagnosis of ABPA in CF patients. The authors also observed that in patients treated with anti-IgE (omalizumab), the upregulation of CD203c expression was abolished, suggesting an IgE-mediated mechanism. The authors did not find statistically significant changes for CCL17 levels between their patient groups. However, CCL17 was analyzed in plasma, which may have affected the results.

### GENETIC FACTORS

Several studies implicated host genetic polymorphisms (SNPs) as disease modifiers for the susceptibility for and/or disease severity modulators of ABPA in CF or SAFS. These studies highlighted the potential role of distinct human leukocyte antigen (HLA) / MHC II alleles as well as polymorphisms in the IL-4 receptor alpha, the promoter region of the IL-10 gene, in Toll-like receptor (TLR) 9, mannan-binding lectin (MBL), Surfactant protein A2 (SP-A2) and mutations in the *CFTR* gene. For an in-depth discussion of this complex topic we refer to other recent excellent review articles [60, 92]. Given genetic risk factors for the development of ABPA would also imply a gradual spectrum of clinical presentations of ABPA or ABPA-like disease in CF, an issue requiring further attention in future studies.

### CURRENT AND FUTURE TREATMENT OPTIONS

The treatment of ABPA should control the acute ABPA episodes and should limit the development into chronic lung disease with irreversible loss of pulmonary function and tissue fibrosis [22, 60, 68, 93, 94]. As reviewed by Moss *et al.* [62] treatment of ABPA in CF is complicated by difficulty with diagnosis due to overlapping features of ABPA with CF itself, vulnerability to steroid toxicity, altered pharmacokinetics and drug-drug interactions. Currently high doses of corticosteroids are the standard treatment for ABPA, with a pivotal role for oral corticosteroids [95]. Mechanistically, steroid therapy is believed to rapidly clear the eosinophilic infiltrates and thereby dampen the pro-inflammatory cascade triggered by *Aspergillus fumigatus*. Initial therapy includes systemic corticosteroids, usually oral prednisone, with an initial dose of 0.5 – 2 mg / kg bw / day for two to four weeks followed by stepwise reduction over one to three 3 month. However, the duration of steroid therapy should be individualized in each patient based on the clinical course. The most commonly used strategy is two weeks of daily therapy with 0.5 mg / kg / day followed by alternate days for six to eight weeks, then tapered by 5 - 10mg every two weeks [22]. Importantly, some patients require continuous daily or alternate-day prednisone in order to prevent relapse [95, 96]. An alternative to this corticosteroids regimen is the use of monthly high-dose pulse intravenous methylprednisolone. Several steroid-dependent CF ABPA patients have been treated with 10–20 mg/kg daily for three days at a one to two month interval for up to four years with an observed reduction in steroid toxicity and improved clinical control of ABPA [97, 98]. However, adverse events (flushing, myalgia, malaise, restlessness, transient hyperglycemia and hypertension) occurred in some patients necessitating discontinuation of therapy.

Although oral steroids are effective and recommended first-line treatment for ABPA in CF, a large survey analyzing data from the Epidemiologic Registry of Cystic Fibrosis (ERCF) on 12,447 CF patients gathered from 224 CF centres in nine European countries,

found only 56% of CF-ABPA patients received oral steroids, whereas inhaled corticosteroids (ICS) were given to 75% despite a lack of published efficacy data [46], suggesting that concern for systemic steroid toxicity plays a substantial role in CF-ABPA treatment decisions [62]. An additional concern may be reports on acquisition of nontuberculous mycobacterial infection [47, 99]. However, in contrast to systemic intake of steroids, ICSs have failed to prevent the progression of lung damage in patients with ABPA [100, 101]. In order to guide therapy, total serum IgE is a critical marker to manage treatment as it is correlated with ABPA disease activity. Accordingly, total serum IgE levels should be measured every six to eight weeks after the initiation of corticosteroid treatment, then every eight weeks for at least one year [102]. Due to the fact that in many patients the total IgE serum levels do not decrease down to normal values, it is recommended to consider the lowest level of IgE during the follow up period as individual baseline parameter for each patient. In addition, repeated chest x-ray or CT scan of the lung is strongly recommended in order to monitor clearance of infiltrates. Pulmonary function tests are mandatory as well [103]. If mucus impaction persists and is associated with atelectasis, bronchoscopy should be performed to confirm the diagnosis and to attempt to remove the mucus plugs.

Apart from corticosteroids, an alternative strategy in the treatment of ABPA is to reduce or clear the lung of *Aspergillus fumigatus* colonization and diminish antigenic stimulation by employing anti-fungal agents. A number of antifungals have been used to treat ABPA in CF lung disease. Besides one recent double-blind randomized placebo-controlled pilot clinical trial involving 35 CF patients with *Aspergillus fumigatus* colonization [104] at present time, there are no randomized controlled trials (RCTs) to evaluate the use of antifungal therapies for the treatment of ABPA in people with CF [105]. The most highly accepted agent is itraconazole that belongs to a group of compounds, azoles, which can be taken orally and have been proven to exert activity against *Aspergillus fumigatus*. Itraconazole has been proven to have a significant therapeutic benefit in RCTs in patients with asthma and ABPA [95, 106] and in one single study of CF patients with *Aspergillus fumigatus* colonization [104]. The latter RCT showed that subjects receiving itraconazole for 24 weeks had a decrease in sputum eosinophils of 35% per week, but itraconazole had no statistically significant effects on lung function in the treated patients. In another study, there were fewer exacerbations requiring oral corticosteroids in those treated with itraconazole compared with the placebo group [106]. Further pooled analysis of studies confirmed that itraconazole could decrease the IgE levels but could not significantly improve the lung function [67]. The CFF Consensus Conference recommended use of itraconazole as a second-line treatment for ABPA in patients with slow or poor response to steroids, relapse after initial episode, steroid-dependence, and/or steroid toxicity. However, a recently published Cochrane review summarized that the currently available data is not sufficient to recommend the use of antifungal therapies for ABPA in people with CF [105]. Triazole antifungals have thus assumed a large role in the management of CF-ABPA, as, unlike steroids, they reduce the fungal burden and may act as steroid-sparing agents [67, 68]. However, it can be difficult to achieve efficacy with itraconazole in patients with CF due to poor bioavailability and the required acidic gastric pH for proper absorption. In order to ameliorate these problems, monitoring of blood levels is recommended [68]. Hope *et al.* [107] reviewed the role of monitoring triazoles currently licensed in the USA (fluconazole, itraconazole, voriconazole, and posaconazole). Itraconazole is metabolized to bioactive hydroxy-itraconazole and disposed of via cytochrome P450 (CYP)3A4-dependent hepatic metabolism. Through inhibition of CYP3A4, it increases exposure to methylprednisolone (but not prednisolone); similarly, concomitant use of itraconazole and inhaled budesonide or fluticasone can result in adrenal suppression [67, 68, 108]. Measurement of itraconazole by high performance liquid chromatography (HPLC) measures the native molecule and is

used by most reference laboratories, whereas bioassay includes the hydroxylated metabolite, resulting in reported itraconazole levels 2–10 times higher with bioassay than with HPLC. Itraconazole displays nonlinear pharmacokinetics and concentration dependent antifungal activity and toxicity. The recommended minimal steady-state (trough) level, based on *Aspergillus fumigatus* minimum inhibitory concentration and clinical studies in a variety of disease states, is 0.5mg/ml by HPLC. Toxicity includes fluid retention, gastrointestinal intolerance, elevated liver function tests, rash, headache, peripheral neuropathy, tremor, and sleep disturbance (all reported in  $\geq 4\%$  of patients) and is related to drug level [109]. The CFF Consensus Conference recommendation for itraconazole dosing is 5 mg/kg per day, with twice daily (b.i.d.) dosing if more than 200 mg/day [68]. Recently, pharmacokinetic modeling with pharmacokinetic data from 30 adults with CF employing a two compartment model with first-order metabolism to the hydroxy-metabolite and defining a target therapeutic range of 0.5–2mg/ml by HPLC (1.5–6mg/ml by bioassay) suggested an optimal dosing regimen of 500 mg b.i.d. for treatment of CF adults [110]. No clinical studies have reported results with this higher dosing regimen.

A few case reports have shown acceptable benefit from using voriconazole, which is a newer antifungal azole as well [111, 112]. Voriconazole, more expensive than itraconazole, has at least equal activity against *Aspergillus fumigatus* as itraconazole but a better oral bioavailability (96% in healthy persons), and encouraging results were reported in two small open trials in CF-ABPA [111, 113]. However, voriconazole has complex hepatic metabolism involving CYP2C19, CYP3A4, and CYP2C9, rendering it extremely unpredictable steady-state levels and drug–drug interactions. Voriconazole thus has a substantial greater interpatient variability than itraconazole (~100-fold vs. ~15-fold). A validated assay methodology using liquid chromatography–tandem mass spectrometry in various clinical settings has led to a recommended steady-state (trough) target range of 1–6 mg/ml. Whereas the recommended dose in adults of 200 mg b.i.d. generally produces steady-state levels of 2–3 mg/ml, higher doses of 4–13mg/kg may be required in children due to enhanced clearance [114]. Levels of more than 6 mg/ml are predictive of increased toxicity including hepatic, ophthalmologic, and photosensitivity reactions and rare but serious cardiac (torsades de pointes) and neurologic events [115]. The practical difficulty of achieving and maintaining therapeutic voriconazole levels in CF are illustrated in a study by Berge *et al.* [116] of lung transplant recipients. They found standard dosing on the one hand resulted in adequate levels in only 20% of patients and, on the other hand, neurologic or hepatic toxicity in 14 and 30% of the patients, respectively. Resistance to both itraconazole and voriconazole has been found in patients with ABPA without CF [117, 118]. So far there is only limited data about prevalence of azole-resistant *Aspergillus fumigatus* in adults with cystic fibrosis exposed to itraconazole [119]. Posaconazole, also available, has few drug interactions and is more active against *Aspergillus fumigatus* than either itraconazole or voriconazole. A recent review has reported that 70–80% of patients who tolerated either voriconazole or posaconazole have responded to this treatment [26]. Although useful, steroids and triazoles often either fail to adequately control ABPA or result in unacceptable toxicity.

Nebulized amphotericin B has been used to treat pulmonary fungal infection for over 50 years. Unfortunately, the literature on inhaled amphotericin is confused by a plethora of delivery systems and dose regimes, availability of multiple intravenous formulations (water-soluble lyophilized amphotericin deoxycholate and three commercially available lipid-based preparations), and the diversity of patients and diseases treated. Amphotericin B can be administered by jet nebulization to airways with good tolerability at doses capable of killing *Aspergillus fumigatus* in the epithelial lining fluid without the toxicity of parenteral administration [120]. One dose regimen reported able to do this is 20 mg amphotericin deoxycho-

late nebulized b.i.d. However, studies of CF-ABPA are limited to a few patients with varying formulations and regimes [120-124]. Far larger numbers are available for prophylaxis or treatment of *Aspergillus* infection in neutropenic patients and in lung transplant recipients.

Omalizumab, a humanized monoclonal antibody to IgE (anti-IgE) approved for use in patients older than 12 years in the USA and for patients older than 6 years in Europe with severe persistent allergic asthma, is being more widely used for CF-ABPA, but published results are sparse. Several case reports and small case series have suggested its potential in CF patients with ABPA [125-130], but in at least one instance, control was disappointingly partial and transitory [131]. A randomized controlled trial of omalizumab in CF-ABPA has been terminated (ClinicalTrials.gov identifier NCT00787917).

Apart from therapy with corticosteroids, antifungal drugs and anti-IgE there is evidence that supplementation of 1,25-OH-vitamin D3 may be effective to treat or prevent ABPA [132]. In the study by Kreindler *et al.* [132] heightened Th2 reactivity in the ABPA cohort correlated with lower mean serum vitamin D levels. Furthermore, *in vitro* addition of 1,25 OH-vitamin D3 substantially reduced Dendritic Cell (DC) expression of OX40 ligand and increased DC expression of tolerogenic TGF- $\beta$ . This treatment also resulted in increased Treg TGF- $\beta$  expression and reduced Th2 responses by CD4<sup>+</sup> T cells from patients with ABPA. However, clinical data about effects of Vitamin D supplementation in ABPA is lacking. One ongoing study investigating the role of Vitamin D in CF and ABPA is recruiting (ClinicalTrials.gov identifier NCT01222273).

## CONCLUDING REMARKS

Viewing the current microbiological, immunological and clinical data on ABPA in CF patients in combination, there is emerging evidence that there are several genetic and non-genetic heterogeneous layers of disease mechanisms behind the unifying term 'ABPA'. CFTR, non-CFTR gene variants, treatment approaches (mainly inhaled antibiotics), bacteria-fungus and fungus-fungus interactions, atopy and other factors are highly probable to modulate the predisposition, the clinical severity and the treatment response and have to be considered when investigating ABPA in CF and beyond SAFS. Besides ABPA, the relevance of *Aspergillus fumigatus* colonization in CF lung disease is increasingly appreciated, but remains discussed controversial. Further basic and clinical studies are required to better understand ABPA and ABPA-like disease conditions in CF, particularly therapeutic studies.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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