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# Immune responses to airborne fungi and non-invasive airway diseases

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## **Abstract**

Inhalation of fungal particles is a ubiquitous way of exposure to microorganisms during human life, however this exposure may promote or exacerbate respiratory diseases only in particular exposure conditions and human genetic background. Depending on the fungal species and form, fungal particles can induce symptoms in the lung by acting as irritants, aeroallergens or pathogens causing infection. Some thermophilic species, can even act in all these three ways (*e.g. Aspergillus, Penicillium*), mesophilic species being only involved in allergic and/or non-allergic airway diseases (*e.g. Cladosporium, Alternaria, Fusarium*). The goal of the present review is to present the current knowledge on the interaction between airborne fungal particles and the host immune system, to illustrate the differences of immune sensing of different fungal species and to emphasize the importance of conducting research on non-conventional mesophilic fungal species. Indeed, the diversity of fungal species we inhale and the complexity of their composition have a direct impact on fungal particle recognition and immune system decision to tolerate or respond to those particles, eventually leading to collateral damages promoting airway pathologies.

**Key words:** Mesophilic fungi, Immunity, Inflammation, Lung, Airway disease

Fungal particles are ubiquitous in outdoor as well as in built environment air. Their growth is greatly dependent on humidity, temperature and availability of nutritive substrates. Fungal species can be present in aerosols in different forms: hyphae fragments, spores or yeast. Their number vary from thousand in the outdoor air [1], to forty in the indoor air [2]. The species composition in the air varies depending on the season [3] and on the presence of particular conditions of fungal growth (water-damage, waste, grain etc.). However, allergic and non-allergic respiratory diseases have been associated only to particular aerosols compositions [4-6] or to the presence of one dominant fungal species in the inhaled air [7]. Noteworthy, it was recently proposed that this is not the exposure to a particular fungal species that favors the development of respiratory pathologies, but the low diversity of the fungal species present in the inhaled air [8]. Yet, a large number of thermophilic as well as mesophilic fungal species have been incriminated in airway pathologies. The factors that favor growth of fungal species have been identified outdoor [7] and in the built environment [9], such as the occupational positions most likely at risk (manipulation of plant or animal derived products). The mechanisms underlying the development of allergic and non-allergic pathologies induced or exacerbated by fungi have been mostly explored for thermophilic species, rarely for mesophilic species. The goal of this review is to make a state of the art on the early events of host responses which impact on the resistance (restricting fungal burden) or tolerance (limiting collateral damages caused by the immune response) to fungi and to illustrate to which airway pathologies the disruption of host-fungus relationship may lead. We will first describe the coordinated action of airway resident cells to sense the dynamic structure of the fungal cell wall, made of polysaccharides (> 80%) and proteins, as well as fungal derived cytoplasmic and secreted compounds. Second, we will present the mechanisms involving fungi in the development of airway allergic disorders including allergic asthma, allergic bronchopulmonary aspergillosis (ABPA) and mycosis (ABPM), allergic fungal rhinosinusitis (AFRS) and hypersensitivity pneumonitis (HP) and airway non-allergic disorders such as chronic bronchitis, organic dust toxic syndrome and chronic mycotoxicosis. Of note, we will not discuss about chronic obstructive pulmonary disease (COPD) since no convincing association between COPD and airborne fungi has been reported despite the fact that 15 to 20% of non-smokers have an occupational exposure etiology and that the exposure to organic dust has been incriminated for some of them [10-12]. Finally, we will briefly discuss the genetic variants favoring the development of non-invasive fungal airway pathologies.

## **1. Airway immune response to fungal compounds**

### **1.1. Processing of fungal particles in the airways**

Inhaled fungal particles deposit in different areas of the respiratory tract depending on their size, shape and aggregation with other substances. Large particles (over 5  $\mu\text{m}$ ) end in the nasopharyngeal region, whereas smaller particles may reach lung alveoli. Mucociliary clearance is the very first innate defense mechanism of the lung against the particles reaching the lung alveoli [13]. Cilia located along the airway walls provide a motive force for clearing microorganisms and toxic substances trapped in the mucus secreted by goblet cells. Moreover, mucus

contains an array of molecules with microbiocidal activity or that help neutralizing pathogens, among which  $\beta$ -defensins, lysozyme, lactoferrin, mucin, secretory IgA and surfactants.

The immune response to fungi is influenced by fungal species, fungus form (spore, hyphae or yeast), fungus-derived products (such as mycotoxins and allergens), co-inhalation of fungus with other substances and host genetic background. The respiratory tract is a complex structure made of multiple structural epithelial cell types broadly classified into three categories (basal, ciliated and secretory cells) and infiltrated by leukocytes [14]. Besides phagocytic cells such as alveolar macrophages and dendritic cells (DCs) that reside in the airways or sense the airway lumen, epithelial cells and possibly mesenchymal cells play an active role in the initial events driving pulmonary immune responses. The cytokines and chemokines released following fungal contact by resident airway cells stimulate the recruitment and instruct innate and adaptive cells, among which neutrophils, eosinophils, basophils, mast cells, T cells and B cells. Other cells such as the recently identified lineage-negative innate lymphoid cells (ILCs) of subgroup 2 (ILC2) are central to fungal-induced allergic inflammation due to the production of interleukin (IL)-5 and IL-13 at early stage of allergen recognition [15, 16].

DCs are permanently trafficking from lungs to draining lymph nodes, a path presumably activated by subtle maturation signals provided by the local flora at steady state and strongly amplified in inflammatory conditions during which the cytokines released by the stroma or epithelial cells instruct DCs [17, 18]. DCs are particularly well equipped to decipher the fine structure of fungi, and the extreme plasticity of their response shapes the development of naïve CD4<sup>+</sup> T cells into Th1, Th2 or Th17 cells that differentially affect antifungal responses [19]. For example, DCs exposed to *Eurotium amstelodami* or *Wallemia sebi*, two filamentous fungi involved in HP, induce a Th1 polarized pathogenic response [20], whereas DCs exposed to *Alternaria alternata*, a common allergenic fungi responsible of asthma, trigger a potent Th2 skewed immune response [21].

The differentiation of Th1, Th2 and Th17 cells is triggered by IL-12 and interferon (IFN)  $\gamma$ , IL-2 and IL-4 and IL-6, TGF $\beta$ , IL-21 and IL-23, respectively. Th1 responses are characterized by the production of IL-2 and IFN $\gamma$ , which promote protective immunity against fungal infection through activation of phagocytes, antigen presentation by antigen presenting cells and production of opsonizing antibodies. IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and amphiregulin are the main drivers of Th2 cell responses that inhibit Th1 cell responses and promote alternative activation of macrophages, production of immunoglobulin (Ig) E, eosinophilia and mast cell growth resulting in decreased fungal clearance and favoring the development of fungal allergic responses. Th17 cells, characterized by the production of IL-17A, IL-17F, IL-21, IL-22, granulocyte-macrophage colony-stimulating factor (GM-CSF) and CC-chemokine ligand (CCL) 20, have been identified more recently as an additional subpopulation of CD4<sup>+</sup> T cells involved in host defenses against extracellular microorganisms among which fungi [22, 23]. Th17 responses facilitate fungal clearance through the mobilization of neutrophils and the production of defensins. Th17 responses have also been implicated in the development of a number of autoimmune pathologies and in airway allergic diseases such as allergic rhinitis (AR) and chronic rhinosinusitis (CRS) [24].

The immune response to fungi has to be tightly regulated to prevent inflammation-mediated collateral damage and maintain lung immune homeostasis. Pulmonary stromal cells promote transforming growth factor  $\beta$  (TGF $\beta$ )-dependent generation of tolerogenic DCs that contribute to suppress T-cell proliferation through prostaglandin E2 (PGE2) and induce the generation of regulatory (Treg) cells [25]. Treg cells produce high amounts of IL-10 and TGF $\beta$  that help limiting immunopathology, which in return may facilitate fungal persistence.

## 1.2. Pathogen-associated molecular patterns and pattern-recognition receptors

The fungal cell wall is mostly composed of polysaccharides (80-90%) and proteins, mostly glycoproteins. Pathogen-associated molecular patterns (PAMPs) expressed by fungi are sensed by epithelial cells and sentinel phagocytic cells of the airways through pattern-recognition receptors (PRRs). The principal fungal PAMPs -  $\beta$ -glucans (especially  $\beta$ 1,3 and  $\beta$ 1,6 glucans), chitin ( $\beta$ 1,4-linked polymer of *N*-acetyl glucosamine) and mannans - are present in most aerosolized fungi (all Ascomycetes and Basidiomycetes). Other fungal PAMPs recognized by innate immune cells are  $\alpha$ -glucans, galactomannans, xylomannans and chitosan, a deacetylated derivative of chitin [26, 27], which may be expressed by particular fungal taxa. Whereas  $\alpha$ -glucans compose the gel-like polymer of Ascomycetes and Basidiomycetes, galactomannans are found in Ascomycetes (principally *Aspergillus*, but also *Penicillium* and few other species) and xylomannans in Basidiomycetes (*e.g.* *Cryptococcus*). Therefore, the composition of the cell wall varies significantly between fungal species [28].

The immune response to fungal PAMPs is modulated according to fungal PAMPs distribution at the surface of fungal particles, their size, shape and linkage to other components. Pathogenic fungi have developed strategies to avoid immune recognition, for example *Cryptococcus neoformans* that bears a capsule, and dormant conidia of *Aspergillus fumigatus* that are covered by an immunologically inert hydrophobic rodlet layer [29]. Moreover, the fungal cell wall is a dynamic structure uneven during cell cycle and morphological transition.  $\beta$ 1,3-glucans are exposed on conidia but masked on hyphae of *Candida albicans*. By masking immunogenic PAMPs, fungi circumvent inflammatory responses, favoring fungal adaptation and opportunism [19].

The main PRRs involved in the sensing of fungal PAMPs are C-type lectin receptors (CLRs, such as dectin-1, dectin-2, mincle, DC-SIGN, the mannose receptor, the mannose binding lectin and galectin-3), Toll-like receptors (TLRs, especially TLR2, TLR4 and TLR9) and Nod-like receptors (NLRs) [19, 30-32]. The cooperative recognition of fungal PAMPs by several PRRs is required for optimal anti-fungal responses [33-35]. Most of our knowledge about PRRs and pathways involved in host responses to fungi derives from studies using mostly the thermophilic species *C. albicans* and *A. fumigatus* [19, 26, 27, 30-32, 36] and can only be extrapolated to mesophilic species when these species are sharing similar cell wall characteristics and cytoplasmic composition.

$\beta$ 1,3 glucans are the major components of fungal cell wall. Immune cells sense  $\beta$ -glucans primarily through dectin-1 that is expressed at high levels by myeloid cells, yet with possibly different outcomes. For example,  $\beta$ -glucans trigger much higher TNF production by DCs than by macrophages [37]. TLR2, complement receptor 3 (CR3) and scavenger receptors (CD5, CD36 and SCARF1) have also been described to detect  $\beta$ -glucans. Ligand binding to

dectin-1 engages Syk-CARD9 and Raf-1 dependent signalling pathways that relay activation of the canonical (c-Rel) and non-canonical (RelB) nuclear factor (NF)- $\kappa$ B pathways, and stimulates endocytosis and phagocytosis, the respiratory burst, the production of cytokines and chemokines, the differentiation and maturation of DCs and the development of effector T and B cells responses [38-40]. The panel of cytokines produced following dectin-1 triggering drives antifungal Th1 and Th17 responses. Shaping the development of Th17 responses requires the interaction of dectin-1 with TLRs, which amplifies the production of IL-1 $\beta$ , IL-6 and IL-23 and down-regulates the production of IL-12 [41]. Interestingly, dectin-1 promotes pro-IL-1 $\beta$  processing via activation of both the canonical, caspase-1 dependent, NLRP3 (NLR pyrin domain containing 3) inflammasome and a noncanonical caspase-8 inflammasome [42, 43]. The density of  $\beta$ 1,3 glucans at the cell surface varies between fungal species and between fungal forms (yeast or hyphae). Mold-induced asthma in mice repeatedly exposed to *Aspergillus versicolor* and *Cladosporium cladosporioides* - fungal species with respectively high and low surface  $\beta$ -glucans - revealed that *A. versicolor* induces a dectin-1 dependent Th17 response with neutrophilia and low airway hyper-responsiveness (AHR) whereas *C. cladosporioides* induces a dectin-1 independent Th2 response with eosinophilia and strong AHR [44, 45]. These data suggest that fungi that expose low levels of  $\beta$ -glucans at their surface are more susceptible to induce allergic Th2 polarized immune responses. In agreement,  $\beta$ -glucans from *Wallemia sebi* and *Eurotium amstelodami*, fungal species associated to HP development, promote Th1 polarized responses by DCs [20]. Along with a primordial role for dectin-1 in antifungal defences, mutations in dectin-1 and CARD9 have been associated with fungal infections [46-48].

Chitin is the second-most abundant polysaccharide in fungal cell wall. Chitin is not surface-exposed, but chitin fragments are released and stimulate host cells following phagocytosis and digestion of fungal particles by lysozyme and chitinase/chitinase-like proteins (C/CLPs) that are constitutively expressed by epithelial lung cells and macrophages [49]. Moreover, chitin content and exposure at the cell surface may increase upon antifungal therapy with echinocandins, and therefore affect host immune responses [50, 51]. Experimental and clinical studies have associated dysregulated expression of C/CLPs with Th2 inflammation and asthma development [52]. Like-minded, intrapulmonary administration of chitin into mice induced a macrophage-dependent recruitment of IL-4 expressing eosinophils and basophils [53]. Interestingly, while both chitin and chitosan are phagocytosed by macrophages, only chitosan activates the NLRP3 inflammasome and IL-1 $\beta$  secretion [54]. How the host senses and responds to chitin remains to be fully elucidated. Chitin particles promote not only Th2 responses, but also Th1 as well as Th17 responses [52]. These paradoxical findings likely arise from the usage of chitin preparations differing in origin (commercially available chitin is usually extracted from crab/shrimp shells), purity and particle size. Whereas large chitin fragments are immunologically inert, chitin particles of 40-70  $\mu$ m stimulate tumor necrosis factor (TNF) through dectin-1 and TLR2 while chitin particles below 40  $\mu$ m induce TNF and IL-10 through dectin-1, the mannose receptor and TLR2 in macrophages [55]. Yet, a recent study using ultra-pure fungal-derived chitin of 1-10  $\mu$ m, a size likely to be relevant for host responses, showed that low concentrations of chitin stimulate IL-10 production while high concentrations of chitin induce predominantly TNF [56]. Intracellular delivery of chitin by

the mannose receptor was preceding chitin sensing by TLR9 and NOD2. Thus, one can postulate that protection against *Aspergillus*-driven acute allergic disease afforded by the administration of CpG (a TLR9 agonist) into mice might result from binding competition between CpG and chitin to TLR9 [56, 57]. Additional receptors expressed by myeloid cells possibly involved in chitin/chitosan recognition encompass the C-type lectins galectin-3 and RegIII $\gamma$ , FIBCD1 and NKR-P1 [49].

Mannans are  $\alpha$ -linked polymers of mannose covalently attached to fungal cell wall mannoproteins through ester linkage (O-linked) or amine bond (N-linked). Mannans are recognized by the mannose receptor (CD206), dectin-2, dectin-3, DC-SIGN (CD209), TLR2, TLR4 and possibly mincle [27, 32, 34, 36, 58]. While CLR recognizes N-branched mannans, TLR4 senses O-branched mannans [33]. TLR4 and TLR2 recognize glucuronoxylomannans from *C. neoformans* and phospholipomannan from *C. albicans*, respectively [59, 60]. The mannose receptor is predominantly expressed by macrophages and DCs as part of the endocytic pathway. The mannose receptor senses several fungi and mediates, in collaboration with the TLR2/dectin-1 pathway, the release of a number of cytokines among which IL-17 [61]. Dectin-2, expressed by myeloid cells, was the first PRR shown to induce proinflammatory cytokine expression in response to fungal hyphae [62, 63]. Indeed, dectin-2 senses  $\alpha$ -mannans and induces Th17 responses [64, 65]. Recently, dectin-2 was shown to form with dectin-3 a heterodimeric complex to recognize  $\alpha$ -mannans and mediate host defenses to *C. albicans* infection [58]. DC-SIGN expressed on DCs, macrophages and endothelial cells favors the uptake and phagocytosis of *C. albicans* and *A. fumigatus* conidia, which is inhibited by *Aspergillus*-derived galactomannans [66-68]. The sensing of *C. albicans* through DC-SIGN modulates co-signaling via TLRs and stimulates the production of IL-10, suggesting an important role of mannans in regulating DC response [67]. Mincle is predominantly expressed on activated macrophages and detects mannose derivatives or N-acetyl-glucosamine from *C. albicans* [69] as well as glycolipids from *Malassezia*, an opportunistic skin fungal pathogen [70, 71]. Interestingly, co-engagement of mincle and dectin-1 by *Fonsecaea monophora*, an etiologic agent of chromoblastomycosis, blocks IL-12 production and redirects dectin-1 mediated antifungal Th1 polarization to a Th2 response [72].

All these data indicate that fungi trigger multiple PRRs of the same or different families to modulate host immune response to their own benefit. Conversely, cross-regulation of cytokine production by immune cells through the engagement of multiple PRRs fine-tunes host defense mechanisms, allowing resistance and tolerance to fungi.

### 1.3. Fungal allergens

Fungi express multiple kinds of allergens - cytoplasmic and structural components released upon cell (auto) lysis and secreted metabolic products. In contrast to fungal cell wall components, allergens are mostly fungal species or family specific. Fungal allergens elicit hypersensitivity reactions not only of type I (IgE-mediated), but also of type II, type III (IgG-mediated) and type IV (delayed type hypersensitivity) that may act together to mediate pathogenesis. Yet, fungi are poorly studied in the field of molecular allergology, and the role of fungal allergens in airway diseases is poorly characterized [73]. Fungal allergenic proteins, secreted mainly during spore germination

and mycelia growth, can be classified into five chief categories: proteases, glycosidases, components of protein synthesis/secretion, proteins of the stress response and proteins of gluconeogenesis. Fungal proteases have been characterized in some fungal species relevant for allergic respiratory diseases, such as *A. fumigatus* (Asp f5, f6, f11, f13, f15, f18) and *Trichophyton rubrum* (Tri r4, Tri t4). Fungal proteases play a particularly important role in allergen sensitization, and represent one of the initiator of lung allergic responses [7, 74]. Indeed, proteases initiate Th2-skewed lung allergenic responses by inducing thymic stromal lymphopoietin (TSLP) secretion by lung epithelial cells [75]. Proteases may have also an adjuvant effect on allergenic properties of other proteins [7]. Proteases facilitate antigen access to host cells by direct receptor-specific interaction involving protease-activated receptor type-2 (PAR-2) with epithelial cells or by damaging epithelial cells allowing access to underlying cells. As an example, *A. alternata*, *A. fumigatus* and *Cladosporium herbarum* proteases not only stimulate cytokine secretion, but also promote epithelial cell barrier disruption [76, 77]. *A. alternata* extracts also activate eosinophils in a PAR-2-dependent manner, favoring allergen sensitization and Th2-biased inflammatory responses [78, 79].

#### 1.4. Fungal metabolites

##### 1.4.1. Non-volatiles low molecular weight metabolites: mycotoxins

Mycotoxins are secondary metabolites produced by a large number of Ascomycetes at particular stages of fungal growth and in particular environments. They are rarely specific of one fungal species and they are most frequently produced by close related fungal species. However, within the same species, some strains are mycotoxins producers and others not. As they are present within fungal particles, mycotoxins can be inhaled at the same time as fungal particles. Mycotoxins produced by *Aspergillus*, *Penicillium*, *Stachybotrys*, *Trichoderma* and *Chaetomium* species have been detected indoor [80], whereas those produced by *Aspergillus*, *Fusarium* or *Penicillium* species have been found in the aerosols of occupational places where plant derived material is manipulated. Only the mycotoxins produced by *Aspergillus*, *Fusarium* and *Penicillium* species (**Table 1**) have been classified as carcinogenic in humans and animals [81]. In addition to their toxic effects, mycotoxins may also display important immunomodulatory and immunotoxic effects depending on toxin concentration, toxin co-administration and the ratio between each toxin. Aflatoxin and ochratoxin suppress cell-mediated immunity [82]. Gliotoxin, patulin and citrinin impair IFN $\gamma$  production [83] and decrease intracellular glutathione in DCs, which is responsible for decreased IL-12 secretion and skewing toward Th2 immune responses [84]. Trichothecene mycotoxins (T-2 toxin, nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, fusarenon-X) have strong immunotoxicity as these toxins target mitotic tissues with important immune functions such as the bone marrow, intestinal mucosa, lymph nodes and thymus. While trichothecene mycotoxins are usually considered immunosuppressive, one of them, deoxynivalenol, may either stimulate or suppress immune functions depending on the dose and exposure conditions [85]. The estrogenic mycotoxin zearalenone (ZEA) and its metabolites bind the estrogenic receptor to inhibit immune functions such as cytokine production (TNF, IL-1 $\beta$ , IL-6, IL-8 and IFN $\gamma$ ) and T cell proliferation [86, 87]. Fumonisin B1 have animal species-specific effects, thus the immunosuppressive activities attributed to this mycotoxin in pigs are not necessarily extrapolated to humans [82, 88].

#### 1.4.2. Volatiles low molecular weight metabolites: microbial volatile organic components

All molds produce volatile organic components (VOCs). More than 200 different microbial VOCs (MVOCs) have been characterized in fungi, mostly alcohols, aldehydes, amines, chlorinated hydrocarbons, ketones, terpenes, aromatic and sulphuric compounds [89, 90]. MVOCs are often responsible of strong/unpleasant odors. Fungi have unique MVOC profiles depending on their development stage, size and substrate for development. For example, 1,3-dimethoxybenzene and 2,4-pentandione are produced by *A. versicolor* and *A. fumigatus* but not *Penicillium expansum*, *Penicillium chrysogenum*, *Aspergillus niger* and *C. cladosporoides* growing on wall paper [91]. Chronic exposure to MVOCs may lead to short as well as long term adverse health effects. In the airways of sensitive people, MVOCs induce irritation and inflammation [89, 90]. In agreement, two prevailing MVOCs,  $\alpha$ -pinene and 1-octen-3-ol, stimulate cytokine and nitric oxide production by immune cells (neutrophils and alveolar macrophages; hemocytes in *Drosophila*) [92]. Of note, some fungi produce both mycotoxins and MVOCs (*Aspergillus* and *Penicillium* spp.; **Table 1**), which may further affect the sensitive host. Yet, clearly much more work is required to fully appreciate the immunomodulatory properties of mycotoxins and MVOCs and their impact in human airway diseases.

## 2. Allergic responses after fungal particle exposure

### 2.1. Allergic asthma

Asthma is a multifactorial chronic and persistent pulmonary inflammation with airway obstruction, breathlessness, mucus hyper-secretion and airway hyper-responsiveness, clinically ranging from intermittent to severe forms [93, 94]. Inflammation leads to bronchoconstriction, vasodilatation and hyperplasia of bronchial smooth muscles. The prevalence of mold allergy to *Alternaria*, *Aspergillus*, *Candida*, *Cladosporium* and *Penicillium* is much higher in atopics and asthmatics when compared to the general population [73]. Moreover, numerous epidemiological studies have associated chronic exposure to fungi with asthma symptoms in children and asthma exacerbation in adults [4, 6, 80, 95, 96]. The most frequent fungal species associated with allergic asthma are *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Epicoccum*, *Helminthosporium* and *Penicillium* [97]. Bronchial provocation tests with spores in non-occupational mild asthmatic adult patients suggest that a minimal dose of  $9 \cdot 10^4$  spores of *A. alternata* and  $6 \cdot 10^4$  spores of *Penicillium* spp. is required to induce clinical symptoms [98]. To distinguish fungal sensitization from fungal involvement in asthma development and/or exacerbation, allergen-specific IgE serum levels should be systematically tittered for a large panel of fungal allergens, together with a systemic analysis of the dominant fungal species to which asthmatic subjects are exposed.

Etiologic agents associated with asthma of fungal origin are heterogeneous, and a role for fungal chitin and proteases as triggered of asthmatic reactions has been proposed [7]. As discussed earlier, fungi with low or unavailable cell surface  $\beta$ -glucans favor Th2 polarized immune response related to an asthmatic phenotype. In addition, mycotoxins such as gliotoxin and patulin impact on DCs to stimulate Th2 responses and trigger asthma-

like symptoms in a model of acute asthma in mice [84]. The underlying mechanisms involved in allergic airway diseases are not fully understood, but our knowledge has improved markedly over the last years. Allergens activate airway epithelial cells and DCs to produce IL-1, IL-25, IL-33, TSLP, GM-CSF, eicosanoids and danger signals that are involved in the expansion and activation of type 2 innate lymphoid cells (ILC2) and activate DCs migration to lymph nodes where they instruct Th2 cell differentiation with the help of IL-4 secreting basophils [99, 100]. Lung ILC2 are a major source of IL-5, IL-9 and IL-13 that, with other cytokines from the local milieu, induce eosinophilia, mast cell mobilization and IgE and mucus production. Of note, ILC2 have just been reported to be increased in the blood of asthmatic patients [101], but not yet in asthmatic lung tissues contrary to IL-25, IL-33 and TSLP. Asthma has long been considered as a Th2-mediated pathology, but recently Th17 cells have been pointed out as important effector cells in asthma, especially severe steroid-resistant asthma characterized by IL-17 production and neutrophilia [102]. Th17 differentiation is dictated by DCs secreting IL-1 $\beta$  and IL-23, and associated with the expression of IL-17 and IL-22, a member of the IL-10 family that can mediate both pro-inflammatory and anti-inflammatory effects [103]. Overall, the phenotypic heterogeneity of asthma might be related to T cell response polarization. Yet, the underlying mechanisms responsible for dominant Th2, Th17 or Th2/Th17 mixed responses during allergic asthma are not well understood.

## **2.2. Allergic bronchopulmonary aspergillosis (ABPA) and allergic bronchopulmonary mycosis (ABPM)**

ABPA refers to an exaggerated immune response to fungal colonization of the lower airways by *A. fumigatus* whereas ABPM refers to allergic bronchopulmonary diseases due to species other than *Aspergillus*. The principal etiological agents of ABPM reported worldwide are *C. albicans*, *Bipolaris* spp., *Schizophyllum commune* and *Curvularia* spp. [104]. ABPM/ABPA prevalence in the general population is unknown. ABPA prevalence in patients having mucociliary clearance impairment such as asthmatic and cystic fibrosis patients is around 1-2% and 2-15%, respectively [105, 106]. The prevalence of ABPM in asthmatic and cystic fibrosis patients is unknown, yet ABPM occurs more frequently than ABPA in patients without asthma [104]. The exposure level of spores triggering ABPA/ABPM is not clearly defined. Due to impaired mucociliary clearance, fungal particles from thermophilic species might remain in the lower respiratory tract long enough to germinate and to breach the epithelial barrier. This may lead to pro-inflammatory cytokine release, activation of antigen presentation cells and skewed Th2 responses with mast cell proliferation and eosinophilia [104, 107]. ABPA/ABPM are characterized by severe asthma initially associated with type I IgE-mediated hypersensitivity, subsequently with type III IgG-mediated hypersensitivity [104]. Type IV hypersensitivity is also present in ABPA. Fungal specific IgE, IgA and IgG can be detected in bronchoalveolar lavage and serum of patients [104]. The diagnosis of ABPA/ABPM is not trivial, and the identification of genetic risk factors should improve the recognition of high-risk patients (**Table 2**). It seems also important to enlarge the panel of fungi tested for ABPM diagnosis.

## **2.3. Allergic fungal rhinosinusitis (AFRS)**

Although the definition of AFRS is controversial, AFRS can be described as a subset of chronic rhinosinusitis (CRS) in immunocompetent patients due to fungal colonization of a sinus with impaired mucociliary clearance [108-110]. AFRS patients generally suffer from a unilateral chronic sinus infection with fungal allergy, nasal airway obstruction and congestion, purulent rhinorrhoea, postnasal drainage and headache [109]. Eosinophilic mucus, fungal detection in sinus, presence of nasal polyps and type I IgE-mediated hypersensitivity are characteristics of AFRS patients [111]. Symptoms associated with AFRS are related to luminal degranulation of mucosal eosinophils and release of cytotoxic proteins (Charcot-Leyden crystals) responsible of tissue damages [112]. *Aspergillus* species were expected to be a common causative agent of AFRS, however *Bipolaris spicifera*, *Curvularia lunata*, *A. alternata*, *Cladosporium* spp. and *Trichoderma longibrachiatum* are commonly incriminated in AFRS [109, 113-115]. Unexplained cases of CRS should be systematically screened for AFRS diagnostic since allergic fungal sinusitis represent 6 to 9 % of the CRS requiring surgery [116].

The prevalence of AFRS is higher in warm geographical regions with a high humidity rate (from 0 to 23% in USA) [117]. AFRS prevalence ranges from 5 to 10% in CRS patients, and AFRS patients can represent more than 87% of the patients with fungal rhinosinusitis (FRS) [118]. Controversy on the classification of CRS and difficulties of differential diagnosis between CRS subcategories may explain the absence of a fair description of the immunological cascade underlying AFRS pathogenesis. AFRS is characterized by mixed type I, III and IV hypersensitivity reactions, and T cell-derived IL-5 and IL-13 that activate eosinophils to release toxic factors for the epithelium in the sinus cavity. Interestingly enough, CRS was the first human pathology in which the accumulation of ILC2 in inflamed tissues was clearly shown [119]. Although allergic rhinitis is classically viewed as a Th2 skewed pathology, recent studies have shown the up-regulation of Th17 specific parameters in local mucosa and peripheral blood of patients, which correlated with eosinophilia and symptom severity [24].

#### 2.4. Hypersensitivity pneumonitis (HP)

HP, also called extrinsic allergic alveolitis, is a generic term defining a non IgE-mediated bronchial and alveolar inflammation caused by an exaggerated immunological response to a variety of organic particles. The most common antigens implicated in HP are bird proteins and bacteria; however fungi are also frequently incriminated in HP development in particular working populations and in the general population in Japan [120]. Acute HP patients suffer from fever, chills, dyspnea, dry cough, headache, malaise, interstitial mononuclear cell infiltration, non-necrotizing poorly formed granulomas, cellular bronchiolitis with spontaneous resolution after cessation of antigen exposure. Chronic HP patients also have chronic interstitial inflammation and alveolar destruction associated with dense fibrosis in 41% of the cases [3, 98]. The prevalence of HP in the general population is low (around 0.9 cases per 100'000/year in the United Kingdom) but much more elevated in populations chronically exposed to high amounts of fungal particles such as farmer's population [3, 121, 122]. Provocation tests suggest that repeated exposure to  $10^7$  to  $4.10^9$  spores/m<sup>3</sup> is required to set off fungal-related HP [98].

HP subtypes are classically defined based on the nature of inhaled antigens, the most common being the bird fancier's lung and the farmer's lung. The panel of fungi involved in HP is large (for example *Aspergillus*, *Alternaria*, *Aureobasidium*, *Cladosporium*, *Cryptococcus*, *Epicoccum*, *Eurotium amstelodami*, *Fusarium*, *Lichtheimia*, *Paecilomyces*, *Penicillium*, *Trichosporon* and *Wallemia*), reflecting the population and geographical area of interest [3, 123].

HP is a mix of type III and type IV allergic reaction. The immune response is characterized by neutrophilic and lymphocytic infiltration in the lower respiratory tract with macrophage and T cell-derived cytokines. In acute and subacute clinical forms, cell-mediated immunopathogenesis has been associated with Th1 responses [124]. However, Th2 mechanisms have been described in chronic HP with a fibrotic phenotype [125]. In addition, mouse models of *Stachybotrys chartarum*-induced HP suggest that Th17 responses are also involved in HP pathogenesis [126].

### 3. Non-allergic disease to fungal compounds

#### 3.1. Chronic bronchitis

Chronic bronchitis refers to a recurrent, often irreversible, inflammation of the bronchial mucous membrane with hypersecretion of mucus and hyperplasia of goblet cells and submucosal glands. Its prevalence in the general population is around 2.5% but can vary from 5 to 40% among workers depending on the level of exposure to antigens [98]. Chronic bronchitis occurs after long-term exposure to large concentrations of fungal spores ( $2 \cdot 10^9$  to  $14 \cdot 10^9$  spores/m<sup>3</sup> over 1 hour) resulting in irreversible damages to the lung. Predominant clinical symptoms are a persistent wet cough and breathing complications [98]. *A. fumigatus* and *Penicillium frequentans* (present on cork) are associated with chronic bronchitis [127, 128]. Our understanding of the pathophysiology of chronic bronchitis is rudimentary.

#### 3.2. Organic dust toxic syndrome (ODTS)

ODTS is also known as toxic alveolitis, pulmonary mycotoxicosis, silo unloader's (filler's) syndrome, atypical farmer's lung or grain fever. ODTS is an acute, non-allergic sickness characterized by flu-like symptoms associated with a local acute toxic effect due to exposure to very high amounts of organic dust [9]. In most cases, symptoms (cough, fever, breathlessness) develop 4 to 8 hours after exposure. Symptoms last generally less than 24 hours but may persist 5-7 days [9]. The prevalence of ODTS is around 36% among workers exposed to organic dusts; it is unknown in the general population [129]. Due to the increased protection of agricultural workers, ODTS cases are expected to decrease in the future. ODTS is related to inhalation of mycotoxins during mold contaminated grain handling (coffee, pepper, wheat) or animal (*e.g.* poultry) related activities. The principal fungal species incriminated are *A. fumigatus*, *Aspergillus nidulans* and *Penicillium* spp. [130]. The exact role of mycotoxins in ODTS is not clearly defined, yet adverse events are more related to chronic exposure to toxicogenic fungi than non-toxicogenic fungi [131]. Organic dust inhalation results in massive neutrophil recruitment to the lungs, following chemotactic

signals provided by lung epithelial cells and macrophages. Pyrogenic cytokines are likely contributing to fever and other inflammatory parameters of ODS [132].

### 3.3. Chronic mycotoxicosis and other pathologies associated with fungal metabolites

Water-damage building syndrome, also called “mold-related illness”, has been associated to the exposure to molds in water-damaged building. Main symptoms are fatigue, headache with dermatological, gastrointestinal as well as reproductive, rheumatologic and immune related symptoms [133]. A wide range of molds is found in water-damage buildings, among which *Penicillium*, *Aspergillus*, *Chaetomium*, *Acremonium*, *Ulocladium*, *Cladosporium*, *Mucor*, *Trichoderma*, *Alternaria*, *Sporothrix*, *Rhodotorula*, *Rhizopus*, *Stachybotrys*, *Calcarisporium*, *Scopulariopsis* and *Fusarium* spp. to cite the most representative ones [134].

Living and/or working in a water-damage building has been associated with the presence of toxicogenic fungi and of their mycotoxins (especially aflatoxin, ochratoxin and trichothecenes) in nasal wash and with an increased concentration of mycotoxins in urine [135, 136]. Mycotoxin toxicity may be aggravated by the formation of fungal biofilm in sinus [137]. Mycotoxins have also been shown to accumulate in lungs, spleen and lymph nodes [135], as well as in placenta, umbilical cord blood and breast milk in symptomatic pregnant women, which might be responsible for side effects on fetus or new-born [138]. Despite the established deleterious role of mycotoxins in animal models, epidemiological evidences struggle to establish a link between mycotoxins and respiratory health effects. Intra-tracheal instillation in rats of toxicogenic *S. chartarum*, a mold suspected to be the etiological agent of pulmonary hemorrhages in children due to its presence in the building and in air samples, induces lung inflammation related to the release of IL-1 $\beta$  and TNF by  $\beta$ -glucan-activated alveolar macrophages [139].

Since MVOCs accumulate in the air of water-damage buildings, they are suspected to participate to health adverse events. Exposure to MVOCs has been linked to symptoms such as headaches, nasal irritation, dizziness, fatigue, nausea and swelling/inflammation of the airways [140-142]. MVOCs such as 3-methylfuran were also associated with other respiratory adverse events summarized in the guidelines for indoor air quality [143].

## 4. Genetic variants as risk factors of fungal non-invasive related pathologies

Host genetic factors impact on host tolerance or resistance to microorganisms, as it is the case for its susceptibility to infection by opportunistic or non-invasive fungal infections. Both primary immune-deficiencies affecting a single gene at the individual level (monogenic inheritance) and genetic polymorphisms affecting several genes at the population level (polygenic inheritance) have been extensively studied in relation with invasive *Aspergillus* and *Candida* infections. These studies have implicated polymorphisms in numerous genes encoding for proteins involved in the whole immunological cascade activated upon fungal contact, such as PRRs, cytokines, chemokines and their receptors and signaling molecules [144, 145]. The most reliable associations, supported by functional effects, concern polymorphisms affecting pathways triggered by dectin-1 and pentraxin-3 (PTX3), a PRR involved in host defenses against *A. fumigatus* [146]. Unfortunately, numerous associations may not be robust due to several

limitations in study design [145]. This is also true for gene association studies concerning the pathologies discussed here, which moreover do not specifically incriminate fungi as the etiological agents of the studied patients apart from ABPA and AFRS. This being said, polymorphisms of human leukocyte antigen (*HLA*) genes have been associated with the development of allergic airway disorders (asthma, ABPA/ABPM, AFRS; **Table 2**). Additionally, mutations in *IL4RA* have been associated with *Alternaria*-sensitive moderate-severe asthma in children [158], whereas mutations in *TLR9*, *CFTR*, *SPA2*, *IL4RA* and *IL10* genes have been reported as risk factors for ABPA [147-150]. No other unambiguous or validated genetic association with allergic or non-allergic fungal airway disease has been reported.

## 5. Conclusion

Although it is well recognized that ubiquitous fungi are involved in the pathogenesis of a broad panel of allergic and non-allergic airway diseases, many issues should deserve more attention, for example the molecular characterization of fungal allergens in mesophilic fungal species, the screening for fungal allergy in relation with the identification of fungal species present in patient environment and the determination of the immunomodulatory or immunotoxic effects of mycotoxins and/or MVOCs delivered by these environmental fungal species. Moreover, the mechanisms controlling host responses to fungal particles and genetic susceptibility to respiratory pathologies induced by fungi were studied primarily in the context of fungal infections such as those promoted by *C. albicans* and *A. fumigatus*, while mesophilic species associated to the development of allergic or non-allergic airway diseases receive a much more modest attention. In depth characterization of the genetic, molecular and cellular actors involved in host-mesophilic fungus interactions, of the impact of lung microbial ecosystem on lung immunity and of the pathophysiological characteristics of patients with non-invasive airway diseases is also warranted as it would help to understand how different fungal species (and microorganisms at large) induce similar pathologies and, conversely, how the same fungal species can cause different pathologies. On the long term, answering these questions would pave the path for designing new therapeutic strategies with the aim to improve the outcome of patients suffering from life threatening airway diseases.

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## Conflict of interest

All the authors declare no conflict of interest.

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**Table 1. Fungal families producing mycotoxins and microbial volatile organic components (MVOCs).**

<b>Fungal family</b>	<b>Mycotoxin</b>	<b>MVOCs</b>	<b>Reference</b>
<i>Aspergillus</i>	Aflatoxin, citrinin, gliotoxin, ochratoxin, patulin	Yes	[151]
<i>Fusarium</i>	Deoxynivalenol [DON], 3-acetyl deoxynivalenol [3-ADON], fumonisin B1 [FB-1], fusarenon-X, nivalenol [NIV], T-2 toxin, zearalenone [ZEA]		[152-154]
<i>Penicillium</i>	Citrinin, gliotoxin, ochratoxin, patulin	Yes	[83, 84, 155]

1 **Table 2. Main characteristics of allergic and non-allergic airway diseases associated with an exposure to airborne fungi**

2

Allergic / non allergic	Pathology	Hypersensitivity reaction	Ig response	Cellular response	Principal fungus	Genetic risk factors
Allergic	Asthma	Type I	IgE (atopic asthma); non-IgE (nonatopic asthma)	Th2 with eosinophilia [156]; Th17 with neutrophilia [102, 157]	<i>Alternaria, Aspergillus, Aureobasidium, Cladosporium, Epicoccum, Helminthosporium and Penicillium</i> [97]	IL4RA and HLA-DQ in children of mixed ethnicity with <i>Alternaria</i> –sensitive asthma [158]
	ABPA/ABPM	Type I, III, IV	IgE and/or IgG, IgA	Th2, eosinophilia, T cells [104, 107]	<i>A. fumigatus, C. albicans, Bipolaris</i> spp., <i>Shyzyphyllum commune, Curvularia</i> spp. [104]	HLA-DR, HLA-DQ, IL4RA, IL10 and TLR9 in mixed populations from USA, France and Germany and UK; SPA2 and CFTR in Caucasians [107, 147-150]
	AFRS	Type I	IgE	Th2 with eosinophilia [159] (Th17) [24]	<i>Aspergillus</i> spp., <i>Bipolaris spicifera, Curvularia lunata, A. alternata, Cladosporium</i> spp., <i>Trichoderma longibrachiatum</i> [109, 113-115]	HLA-DQ in Hispanic and White Americans [160]
	HP	Type III, IV	IgG	Th1 (acute, subacute), Th2 (chronic) and Th17 (mouse model), neutrophilia, lymphocytosis [124, 125, 126, 161]	<i>Aspergillus, Alternaria, Aureobasidium, Cladosporium, Cryptococcus, Epicoccum, Eurotium amstelodami, Fusarium, Lichtheimia corymbifera, Paecilomyces, Penicillium, Trichosporon, Wallebia sebi</i> [162, 163]	<sup>1</sup>
Non allergic	Chronic bronchitis			Neutrophilia [164]	<i>A. fumigatus, P. frequentans</i> [127, 128]	<sup>1</sup>
	ODTS			Neutrophilia, cytokines [132, 165]	<i>A. fumigatus, Penicillium</i> spp., <i>A. nidulans</i> [130]	<sup>1</sup>
	Chronic mycotoxicosis			Cytokines [139]	<i>Penicillium, Aspergillus, Chaetomium, Acremonium, Ulocladiums, Cladosporium, Mucor, Trichoderma, Alternaria, Sporothrix, Rhodotorula, Rhizopus, Stachybotrys, Calcarisporium, Scopulariopsis and Fusarium</i> spp. [131, 134]	<sup>1</sup>

3 <sup>1</sup> No unambiguous association reported