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Review Article

# Murine models of airway fungal exposure and allergic sensitization

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Inhalation of common indoor filamentous fungi has been associated with the induction or exacerbation of allergic respiratory disease. The understanding of fungal inhalation and allergic sensitization has significantly advanced with the use of small animal models, especially mouse models. Numerous studies have employed different animal exposure and sensitization techniques, each with inherent advantages and disadvantages that are addressed in this review. In addition, most studies involve exposure of animals to fungal spores or spore extracts while neglecting the influence of hyphal or subcellular fragment exposures. Recent literature examining the potential for hyphae and fungal fragments to induce or exacerbate allergy is discussed. Innate immune recognition of fungal elements and their contribution to lung allergic inflammation in animal models are also reviewed. Though physical properties of fungi play an important role following exposure, host immune development is also critical in airway inflammation and allergy. We discuss the importance of environmental factors that influence early immune development and subsequent susceptibility to allergy. Murine studies that examine the role of intestinal microflora and prenatal or early life environmental factors that promote allergic sensitization are also evaluated. Future studies will require animal models that accurately reflect natural fungal exposures and identify environmental factors that influence immune development and thus promote respiratory fungal allergy and disease.

**Keywords** mouse models, fungal allergy, spores, hyphae, inhalation

## Introduction

Non-dimorphic filamentous fungi are ubiquitous saprophytic, symbiotic, or parasitic heterotrophs that acquire extracellular nutrients for metabolic and physiological processes [1]. In the absence of critical nutrients and other environmental factors, fungi differentiate to produce a wide variety of reproductive structures that include asexual conidia, sexual spores, fragmented hyphae, and subcellular particulates [2]. In the environment, the mycoaerosol fraction contains various mycotoxins and allergens that represent a large burden to public health [1,3].

Inhalation of fungal spores, often considered the traditional route of exposure, has been associated with the induction or exacerbation of respiratory diseases such as asthma, allergic rhinitis, sinusitis, or hypersensitivity pneumonitis [4–7]. Spores derived from *Alternaria*, *Aspergillus*, *Penicillium*, and *Cladosporium* species are environmentally abundant, and predominantly correlated with exacerbations of allergic rhinitis and asthma [8–13]. With the exception of several preliminary studies, the contribution of other non-dimorphic filamentous fungi and

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fungal fragments has been widely ignored [2,14–17]. Some of these fragments release allergens and are reported to represent up to 56% of the total fungal particulate load [2]. Fungal fragmentation is particularly important in water damaged indoor environments where concentrations of fragments can be significantly higher than conidia [14,16]. Another important consideration is the role of fungal toxicity in respiratory disease. Although fungal allergen and mycotoxin exposure may pose a threat to immunocompetent individuals, immunocompromised patients (undergoing chemotherapy or infected with human immunodeficiency virus) are at greater risk. Immunocompromised patients exhibit decreased clearance of fungal spores from the lungs that potentiates widespread systemic fungal infection that may ultimately prove fatal [18].

Numerous studies have detailed the basic immune responses to fungal deposition in the lung and other peripheral tissues. In the lungs, alveolar macrophages are among the first cells to encounter inhaled fungi [19], and generally promote tolerance over immune activation [20]. Maintaining tolerance is an important function of surveillance in tissues such as the lungs that regularly encounter substantial amounts of innocuous antigens and microbes. Although large numbers of inhaled fungal spores are removed from the lungs prior to germination [21], conidia that escape phagocytosis may begin to germinate, and thus release fungal factors that initiate lung inflammation [22,23]. The early innate response to fungal deposition in the respiratory tract ultimately shapes the adaptive immune response. Lung dendritic cells (DCs) take up and transport fungal particles to bronchial lymph nodes where antigens are presented to naive T-cells. DCs also help to determine the level, direction, and priming of antigen-specific T-helper cells via co-stimulatory signals [24–26]. Dormant or non-viable *A. fumigatus* conidia uptake is associated with IFN-γ production and Th1 responses, while hyphae or swollen (germinating) conidia induce IL-4 production and eosinophil recruitment, a hallmark of allergic inflammation and Th2 responses [27]. Therefore, successful germination is likely to contribute to the development of fungal allergy, although the precise mechanism remains unclear and requires further investigation.

Although specific fungal structures and secreted factors play an important role in allergic sensitization, environmental factors are also critical. Results from epidemiological studies suggest that factors that influence early life immune development are critical for subsequent development of tolerance or allergic sensitization [28,29]. Early infections and non-invasive microbial exposures are generally protective against allergic sensitization. In contrast, decreased microbial exposure as a result of antibiotic use, dietary modification, or birth by caesarian section results in increased incidence of allergy [30]. Although the association is widely acknowledged, the mechanism by which early life microbial exposures or infections confer protection from fungal allergy remains poorly understood.

Studies of fungal inhalation, allergy, and infection have benefitted greatly from the use of small animal models. Mice, rats, rabbits, and guinea pigs are common species that are used to evaluate fungal pathology and lung immunity [1,31,32]. In addition to variability encountered between animal species, the route and method of lung exposure may also differ between individual studies. Some groups provide fungal exposure via inhalation of fungal spores or extracts in suspension through the nares, while others use involuntary aspiration or intratracheal instillation. Studies of fungal allergy involve chronic lung exposure to spores, extracts, or systemic sensitization to fungal allergens prior to respiratory challenge. Each type of animal species and method employed in these studies possess different strengths and weaknesses with regard to modeling fungal exposure and allergic sensitization in humans. The purpose of this review is to provide a summary and evaluation of animal studies of fungal inhalation and allergy, focusing on murine models of airway allergy to non-dimorphic filamentous fungi commonly found in indoor environments, the implications of the findings, as well as the potential of these models to answer future questions in the field of allergy and fungal disease.

## Animal models of fungal inhalation

Animal models are used to examine immune mechanisms associated with fungal clearance, infection, tolerance, and allergy. Several different methods of exposure have been employed in non-sensitized animals including rats, mice, rabbits, and guinea pigs (Table 1) [1]. *Aspergillus*, *Penicillium*, and *Stachybotrys* are examples of fungal genera commonly used in exposure models, especially as these fungi are associated with indoor fungal contamination arising from water-damaged building materials [33,34]. The most common methods used to expose mice to fungi include involuntary aspiration and intranasal instillation.

### *Aspiration*

Involuntary (pharyngeal) aspiration involves the delivery of either an aliquot of fungal spores or an extract suspended in saline, onto the vocal folds or base of the tongue of an anesthetized animal [35–37]. Nasal breathing is then blocked with a gloved finger tip, and mice are allowed to inhale the suspension. Using this method, the fungal suspension is delivered throughout the entire lung. In contrast, intranasal instillation delivers fungal suspensions into the lung through the nares of anesthetized animals

**Table 1** Methods of fungal respiratory exposure.

| Method of Exposure | Brief Description | Advantages | Disadvantages | References |
|---|---|---|---|---|
| Intranasal instillation | Suspension of fungi or extracts into the nares of an anesthetized animal | (a) Homogenous suspension,<br>(b) Ability to do repeat exposures,<br>(c) Reproducible | (a) Not representative of natural exposure,<br>(b) Suspension may dilute or alter surface antigen | [35,58,64,78,79,83] |
| Involuntary aspiration | Suspension of fungi or extracts into the pharyngeal folds of an anesthetized animal | (a) Homogenous suspension,<br>(b) Ability to do repeat exposures,<br>(c) Reproducible | (a) Not representative of natural exposure,<br>(b) Suspension may dilute or alter surface antigen | [35,37,54,59] |
| Intratracheal instillation | Suspension of fungi or extracts into the surgically exposed trachea of an anesthetized animal | (a) Homogenous suspension,<br>(b) Ag delivered directly to lung,<br>(c) Reproducible | (a) Single exposure only<br>(b) Not representative of natural exposure,<br>(c) Suspension may dilute or alter surface antigen | [21,35,39,52] |
| Aerosol exposure chamber | Whole body or nose only apparatuses; Fungal aerosols are dispersed into chamber and voluntarily inhaled | (a) Large number of animals exposed simultaneously,<br>(b) Animal exposed by normal breathing | (a) True lung exposure concentration unknown,<br>(b) Time consuming,<br>(c) Less reproducibility | [35,39–41,53,56] |

[35,38]. Both involuntary aspiration and intranasal instillation allow for a homogenous suspension of fungi to be administered to the animal. However, these methods are not reflective of natural fungal exposures (inhalation of dry aerosols) since extracellular and intracellular antigens may be directly released into the suspension. This may not only alter the concentration of soluble antigens, but may also alter the physical and metabolic properties of fungal particles.

### *Aerosol chambers*

Aerosol chambers are used to monitor the response of an animal to prolonged fungal exposures [35,39,40]. Experimental aerosols can be generated by dry particle aerosolization from growing fungal cultures or by nebulization using a suspension of fungal particles. During dry particle aerosolization, air flows across growing fungal cultures and spores, hyphae, and subcellular fragments are suspended and the aerosol is directed into an air flow controlled exposure chamber [39]. For nebulization, fungal particles are collected into a physiological buffer and then aerosolized into the chamber using a nebulizer. Animals are exposed by either a whole body or nose-only exposure system. Whole body exposures with fungal aerosols introduce the possibility that fungal antigens may be ingested by the animal during grooming [41]. Although chamber exposures allow for accurate monitoring and maintenance of airborne particle concentrations inside the chamber, the actual amount and distribution of fungal particles deposited inside the respiratory tract of the animals cannot be controlled. These attributes depend on the breathing patterns of the animal and vary due to aggregate formation, electrostatic interactions with the chamber wall, and the degree of mucociliary clearance of fungal particles from mucosal surfaces within the nares and trachea of the animal [1,39].

*Aspergillus fumigatus*. *A. fumigatus* is the most widely studied fungal species used in animal models. *A. fumigatus* is a ubiquitous, saprophytic, thermotolerant fungus that grows in moist environments, and may colonize the respiratory tract or inhabit the gastrointestinal tract of immunocompromised individuals [41–43]. It is commonly associated with invasive aspergillosis or allergic bronchopulmonary aspergillosis (ABPA) in immunocompromised patients, and is associated with allergic sensitization and exacerbations of asthma in immunocompetent individuals [5,44]. Rabbits exposed to a single dose of *A. fumigatus* spores using an exposure chamber developed an inflammatory response characterized by the recruitment of macrophages, lymphocytes, and a mild thickening of the alveolar walls [41]. Elevated levels of macrophages and lymphocytes were shown to persist for up to 8 days, and were visible in granulomas. Macrophages phagocytose approximately 19% of *A. fumigatus* spores within 4 h of exposure [21]. However, spores surviving beyond this interval may potentially germinate in the lungs. This ability to persist and germinate within macrophages is a unique characteristic of *A. fumigatus* [21]. *A. fumigatus* produces virulence factors that contain cellular adhesive properties [45] and inhibit epithelial cilia beat frequency [46], macrophage phagocytosis [46–48], and production of

reactive oxygen species [46,49]. These factors may promote spore germination and further colonization within the respiratory tract and bronchioles. Furthermore, germinated *A. fumigatus* spores express greater amounts of allergen compared to ungerminated spores [50,51]. These studies lead to the hypothesis that viable fungal elements, after germination in the human respiratory tract, may release greater concentrations of allergen than previously thought. It is unknown if the unique secretory products released from this fungus are potentially interacting with cytokines or chemokines to induce or hinder this observed effect, or if some other fungal-specific mechanism is responsible.

*Aspergillus terreus.* An early line of respiratory defense against fungal exposures involves the recruitment of alveolar macrophages. Intratracheal instillation studies using *A. terreus*, a ubiquitous thermotolerant human pathogen, showed that macrophages phagocytose 42% of spores immediately following instillation. Within 30 min, 78% of intratracheally instilled *A. terreus* spores were removed [52]. Using a single dose, granulomas were observed in some animals within 7 days of treatment. Despite the presence of lesions, no serum antibody response to *A. terreus* was detected [52]. In another study, using an exposure chamber, rabbits also failed to produce an anti-fungal antibody response to *A. terreus* [39].

*Aspergillus versicolor.* Aerosols of the non-thermotolerant fungus *A. versicolor*; a common indoor contaminant and aeroallergen, provoke respiratory irritation in mice after aerosol chamber exposure [53]. In another study, a dose-dependent inflammatory response was observed following involuntary aspiration [54]. Exposure of mice to single doses ranging from $1 \times 10^5$ to $1 \times 10^8$ spores resulted in increased neutrophils in bronchoalveolar lavage fluid (BALF) within 24 h. Furthermore, proinflammatory cytokines such as TNFα and IL-6 were detected in the sera within 6 h of exposure and remained elevated for 3 days at the highest dose. Albumin, total protein, and lactate dehydrogenase, indicating vascular leakage and cytotoxicity, were significantly increased and remained elevated for 7 days. Within 3 days of exposure, alveolar macrophages and neutrophils were the primary cells in the lung [54].

*Penicillium spinulosum. P. spinulosum* is commonly associated with water damaged buildings. Studies have demonstrated increases in TNFα and IL-6 levels within 6 h of exposure in the sera of mice aspirated with $5 \times 10^6$ spores [55]. Lung inflammation was characterized by elevated levels of neutrophils paired with a decrease in alveolar macrophages in the BALF within 24 h post exposure. Albumin, total protein, and LDH levels were not significantly increased in the lungs compared to control animals. Therefore, *P. spinulosum* can also induce inflammation in the lungs of mice, although the magnitude is decreased compared to inflammation induced by *A. versicolor* [54,55].

*Stachybotrys chartarum.* *S. chartarum* is another fungus associated with moisture damaged buildings [34]. Aspirated *S. chartarum* extracts were capable of inducing an acute systemic inflammatory response in mice as well as increasing total IgE levels [37]. A single dose of this extract was capable of inducing an influx of neutrophils and lymphocytes to the lung. Furthermore, mice exposed to *S. chartarum* extracts four times over a four week period also showed increased macrophage, neutrophil, lymphocyte, and eosinophil levels in the lung, as well as increased total protein and LDH levels in the BALF [37]. IL-5, a cytokine critical for eosinophil production and activation, was also increased. The use of extracts or fragments instead of whole spores in exposure studies of *S. chartarum* may have increased relevance, since the large spore size makes inhalation of intact airborne spores less likely, and inhalation of smaller fungal fragments more likely [56,57]. Another study examined the response of mice exposed to spores of *S. chartarum* twice a week over a 3 week time period via intranasal instillation [58]. In this study, two strains of *S. chartarum* were used, a non-toxic strain producing low levels of mycotoxins and a highly toxic strain. The toxic strain produced various mycotoxins such as satratoxin G, satratoxin H, stachybotrylactone, and stachybotrylactam. The results of this study showed that spores of the toxic strain induced infiltration of neutrophils, macrophages, and lymphocytes to the bronchioles and alveoli of the lung. Exposures to non-toxic spores yielded results similar to those in the control group. Thus, mycotoxin producing strains of *S. chartarum* can induce pulmonary hemorrhage as well as severe inflammation and destruction of lung tissue, demonstrating the need for studies to characterize the effects of long term exposure to other toxin-producing fungi.

### *Factors affecting fungal exposure*

In addition to fungal species considerations, the strain of animals used in exposure studies, as well as the frequency and dose of exposure are also important factors. It is well known that genetic factors contribute to the ability of individuals to respond to potential pathogens, including fungi. Genetic variation is also observable in animal fungal exposure studies. Of three congenic mouse strains (BALB/c, C3H/HeJ, C56BL/6), BALB/c mice exhibited

the strongest inflammatory response to *S. chartarum* [59]. Furthermore, exposed animals may be overwhelmed by large doses, potentially leading to non-specific lung immune responses. Human exposure to fungal concentrations exceeding $1 \times 10^6$ spores $m^{-3}$ are possible in environments that are known to harbor large quantities of fungi (i.e., moist/water damaged buildings and agricultural industries) [1], though it is rare that humans routinely encounter doses commensurate with those used in many animal studies. Therefore, models noting that the degree of inflammation and immune responses is dose-dependent may have limited biological relevance to natural human fungal exposure.

These studies indicate that characteristics of respiratory inflammation in response to fungal exposure are species-specific, and may be influenced by physical characteristics such as optimal growth temperature and by mycotoxin production. In the future, animal exposure models that will determine the contribution of these factors to acute and chronic health effects of fungal inhalation need to be developed. In addition, other studies will need to examine dry aerosol fungal particles and the relevance of fungal fragments on the severity of disease.

## Models of fungal allergy

Allergies affect up to 30% of the population in industrialized nations, with up to 12% suffering from asthma [5,6]. Many factors have been attributed to the rapid increases in the incidence of allergy over the last 20 years including decreased exposure to environmental microbes during prenatal and postnatal immune development. Reduced exposure may lead to a loss of immune tolerance mechanisms resulting in allergy or autoimmunity in susceptible individuals [29]. Although genes play an important role in susceptibility, the triggers for development of sensitization are largely environmental. Aeroallergens may be characterized as seasonal outdoor allergens and include pollen produced by trees, herbaceous shrubs and grasses, or as perennial indoor allergens including animal dander or cockroach allergens [5]. However, fungi are found in both indoor and outdoor environments. Respiratory symptoms are present in up to 30% of atopic individuals who are sensitized and produce specific IgE to fungal allergens [5].

The most common experimental allergen used in animal models of allergic asthma is chicken ovalbumin (ova). In a typical protocol, mice are sensitized by i.p. injection with ova and the adjuvant, aluminum hydroxide (Alum) [60]. In the weeks following the initial sensitization, mice are boosted with an intranasal instillation or pharyngeal aspiration of ova. Finally, a challenge of ova is given, and animals are sacrificed at least 24 hours later. Blood is collected for measurement of IgG and IgE antibody titers. Alternatively, sensitization may be achieved solely by airway challenge of ovalbumin, but this method is less efficient and may require the presence of lipopolysaccharide (LPS) in the ova preparation [60]. The most common mouse strain used in allergic sensitization models is Balb/c, due to immune responses which generally favor Th2 over Th1 [61,62]. However, C57BL/6 and other strains have also been used.

### *Fungal extract sensitization*

Studies that examine the potential of fungi to induce or exacerbate respiratory allergic responses have adapted the ova model to study fungal allergy. *A. fumigatus*, the etiologic agent of ABPA, is the most widely studied species in evaluating fungal sensitization [44]. In many of these studies, fungal extracts are instilled into the nares or trachea of previously sensitized mice (Table 2). Sensitization is induced by repeated intranasal exposures or injection of extracts with or without adjuvant [63,64]. In sensitized mice, fungal extracts induce eosinophilic airway inflammation, goblet cell hyperplasia, and mucous accumulation in the lungs [44]. Severe allergic inflammation in response to fungal extracts is characterized by pulmonary epithelial cell metaplasia, collagen deposition, and fibrotic lesion formation. Studies using this approach have demonstrated the requirement for CD4 T-cells and the Th2 cytokines IL-4 and IL-5, but not B-cells or IgE production, in the induction and maintenance of allergic airway responses to crude extracts of *A. fumigatus* [63,65–67]. The central component of the complement pathway, C3, and the receptor for the complement factor C3a (C3aR), are also important for Th2 effector functions in this model, as deficient mice displayed decreased airway hyperresponsiveness, eosinophilia, and IL-4 producing cells [68,69]. These studies demonstrate that Th2-effector cells and cytokines are important for allergic airway responses to crude extracts of *A. fumigatus*, with the complement pathway also a contributing factor. Although repeated fungal exposures of sensitized mice are generally administered by intransal inoculation due to ease and consistency of delivery, allergic airway responses may be induced by other methods of exposure. For instance, sensitized mice exposed to an aerosolized mycelial extract of *S. chartarum* in an exposure chamber model exhibited airway inflammation, increased IgE production, and airway hyperresponsiveness when compared to mice exposed to ovalbumin (Table 2) [56].

### *Whole spore exposure/sensitization*

Airway responses to intact fungal spores have also been examined in fungal-sensitized mice (Table 2). Hogaboam *et al.*

**Table 2** Murine models of fungal allergy.

| Fungal Species | Mouse strain | Immunization | Boost/Challenge | Pathology | Ab response | Reference |
|---|---|---|---|---|---|---|
| *Aspergillus fumigatus* | CBA/J | i.p. & s.c. culture extract in IFA | Boost: i.n. extract (weeks 3–5), Challenge: i.t. $5 \times 10^6$ spores (week 6) | Eosinophilic inflammation, goblet cell hyperplasia, collagen deposition | Increased IgE | [70] |
| *Aspergillus fumigatus* | Balb/c | i.p. culture extract ± Alum 2/w for 4 weeks | i.n. extract coupled to polystyrene beads 2/w for 4 weeks | Eosinophilic inflammation | Increased IgE | [64] |
| *Aspergillus fumigatus* | C57BL/6 | none. mice ingested cefoperazone (d-4-0) + *C. albicans* (d0) | i.n. $10^7$ spores 2× (d2,9) | Eosinophilic inflammation | Increased IgE | [110] |
| *Stachybotrys chartarum* | Balb/c | n/a | i.a. extract 1/w for 4 weeks | Increased BALF lymphocytes, eosinophils and neutrophils | Increased IgE, IgA | [37] |
| *Stachybotrys chartarum* | Balb/c | i.p. mycelial extract 3× on days 0, 14, 21 | AEROSOL chamber extract 2/w for 3 wks | Mild neutrophilic, eosinophilic inflammation | Increased IgE, $IgG_1$ | [56] |
| *Penicillium chrysogenum* | C57BL/6 | n/a | i.n. $1 \times 10^4$ viable or non-viable spores 1/w for 6w | Non-viable spores: increased neutrophils, viable spores: increased eosinophils | Non-viable spores: $IgG_{2a,}$ viable spores $IgG_1$, IgE | [79] |
| *Penicillium chrysogenum* | Balb/c, C57BL/6 | $1 \times 10^2$ spores or protease extract in Alum 1/w for 6 weeks | i.n. $1 \times 10^2$ spores 1/w for 2w (weeks 7–8) | Eosinophilic & neutrophilic inflammation | Increased IgE, $IgG_1$ | [83] |
| *Alternaria alternata* | Balb/c | i.p. $2 \times 10^6$ spores in Alum | i.n. $2 \times 10^5$ spores 1/d for 3d (d13–15) | Increased lymphocytes, eosinophilic & neutrophilic inflammation | Increased IgE, $IgG_1$ | [78] |
| *Cladosporium herbarum* | Balb/c | i.p. $2 \times 10^6$ spores in Alum | i.n. $2 \times 10^5$ spores 1/d for 3d (d13–15) | Eosinophilic & neutrophilic inflammation | Increased IgE, $IgG_1$ | [78] |

i.a., involuntary aspiration;
i.n., intranasal instillation;
i.t., intratracheal instillation;
s.c., subcutaneous injection;
i.p., intraperitoneal injection;
IFA, Incomplete Fruend's Adjuvant.

used fungal extracts of *A. fumigatus* in Incomplete Freund's Adjuvant (IFA) to sensitize CBA/J mice, boosted intranasally with the same extracts, followed by an intratracheal challenge with conidia suspended in saline [70]. Mice exposed to spores under this protocol exhibited airway hyperresponsiveness, lung eosinophilia, and increased IgE production when compared to control animals. Further studies using this model examined responses to fungal spores in sensitized mice deficient in a wide array of chemokines and chemokine receptors. Mice lacking leukocyte chemokine receptor CCR1 exhibited decreased airway remodeling [71], while the absence of CCR2, the receptor for the monocyte chemokine CCL2, enhanced pulmonary allergy and eosinophilia in sensitized mice exposed to *A. fumigatus* [72]. Furthermore, sensitized mice lacking the mast-cell chemokine receptor CXCR2 [73], the T-cell/macrophage receptors CCR4 [74] or CCR5 [75], or the Th2 cell-expressing receptor CCR8 [76], exhibited decreased chronic fungal allergy/asthma in response to intranasal challenge of conidia. Mice lacking the ability to produce the eosinophil

and Th2-attracting chemokine CCL11 (Eotaxin) [77] exhibited decreased acute, but not chronic airway allergy in response to *A. fumigatus* challenge. Therefore, the expression of chemotactic factors and receptors expressed by Th2-cells, neutrophils, eosinophils, mast cells, and monocytes/macrophages may influence the induction and maintenance of airway hyperreactivity and lung allergic responses to challenge with *A. fumigatus* spores in sensitized mice.

Spores of *Alternaria alternata*, *Cladosporium herbarum*, and *Penicillium chrysogenum* are other aeroallergen sources [5] that induce allergic inflammation in the lungs of mice (Table 2) [78,79]. In contrast to *A. fumigatus* studies that sensitized mice by i.p. injections of spore extracts prior to whole spore challenge, whole fungal spores of *A. alternata* or *C. herbarum* emulsified in Alum were used [78]. Sensitized mice exhibited airway inflammation, hyperreactivity, and eosinophilia after intranasal challenge with conidia. In a subsequent study, unsensitized mice treated with spores required chronic exposure to develop airway inflammation comparable to sensitized mice [80]. Furthermore, repeated exposures of mice to non-viable conidia of *P. chrysogenum* induced Th1 responses compared to Th2-type responses to viable conidia [79]. The effect of fungal inhalation on allergic sensitization to non-fungal allergens has also been examined. In a recent study, an extract of *A. alternata* demonstrated potent Th2-adjuvant effects to the antigen ovalbumin in the airways of exposed mice [81]. The Th2 adjuvant activity of *A. alternata* was significantly more potent than that of *A. versicolor*, suggesting that the effect on allergic sensitization by fungal exposure is species-specific.

## Induction of fungal allergy: tolerance vs. sensitization

### *Fungal-specific host recognition*

Another critical area of investigation has focused on the physical characteristics of fungi and fungal proteins that promote allergy and asthma in susceptible individuals. Many fungal and non-fungal allergens exhibit protease activity. Allergens derived from *Aspergillus* [82] and *Penicillium* [83] species are among the fungal protease allergens isolated [84,85]. Extracts of *A. alternata* contain proteases that stimulate inflammatory cytokine production in epithelial cells *in vitro* [86]. Sensitization with protease extracts of *P. chrysogenum* significantly increased allergic airway inflammation in mice exposed to viable conidia [83]. The commonality of protease activity in allergens suggests that proteases may function as an adjuvant for the induction of sensitization to other allergens. This hypothesis was tested by Kheradmand *et al*., who reported that fungal proteases were sufficient for ova-induced airway hyperresponsiveness, eosinophilia, and IgE production in C57BL/6 mice [87]. The role for protease activity was further shown to be mediated through the protease activated receptor 2 (PAR2) [88], a surface receptor expressed on airway epithelium, alveolar macrophages, and dendritic cells. Proteases act on DCs to downregulate release of IL-12, an important cytokine in induction of Th1 responses [89]. Protease activity also initiates epithelial detachment [90] and production of the inflammatory cytokines IL-6 and IL-8 [91]. Proteases may enhance subsequent innate and adaptive allergic immune responses by increasing airway infiltration by allergic immune cells. Although the mechanism of protease-mediated allergic sensitization is not completely clear, a recent animal study demonstrated a requirement for basophils in the generation of Th2-responses to the protease allergen papain [92].

In addition to PAR-mediated effects, other microbial pattern receptors play a role in early immune recognition of fungi. Among these are toll-like receptors (TLRs) 2 and 4 and the C-type lectin receptor Dectin-1. TLR2 and TLR4 recognize fungal mannan and β-glucan, respectively. These TLRs are important for recognition of *A. fumigatus* by murine macrophages, and concomitantly enhance macrophage activation and cytokine production [93–95]. Dectin-1 also acts as a receptor for β-glucan, a ubiquitous polysaccharide component of fungal spores and hyphae [96]. Dectin-1 is highly expressed by cells of the myeloid lineage [97]. Dectin-1-expressing macrophages and neutrophils are activated by β-glucan on the surface of germinating conidia to produce proinflammatory cytokines [22,98]. Mice deficient in Dectin-1 lack the ability for *in vivo* cytokine production in responses to and in subsequent removal of fungi [99,100]. Although activation of these pattern receptors by fungal ligands and subsequent activation of innate immune cells has been reported, much less is known about the influence of these signals on the development of adaptive allergic airway responses. In contrast to the Th2 promoting protease activation, the TLR signaling molecule MyD88 enhanced $CD4^+$ T cell priming and Th1 cytokine production during respiratory infection with *A. fumigatus* [101]. The role of Dectin-1 in $CD4^+$ T cell priming and differentiation in response to fungal infection remains unclear. However, recent evidence suggests a role in activation of Th17 cells, a T-helper cell lineage distinctly separate from Th1/Th2 phenotypes [102]. IL-17 produced by Th17 cells contributes to lung inflammation in a mouse model of pulmonary aspergillosis by stimulation of neutrophil-mediated killing of conidia [103]. The role of IL-17 in fungal allergic sensitization is not clear, although ova sensitized mice lacking IL-17 activity

exhibited decreased airway hyperreactivity and decreased influx of neutrophils, but not eosinophils [104,105]. Therefore, though PAR, Dectin-1, and TLRs 2 and 4 are involved in immune responses to fungi, only PAR-mediated effects have been demonstrated to promote allergic Th2 responses.

In addition to the variable immune responses to fungal molecular patterns, viable fungal particles also elicit contrasting responses when compared to non-viable or dormant particles. Large numbers of resting fungal spores introduced into the lungs of mice are phagocytosed by alveolar macrophages and result in little inflammation [1]. Swollen or germinating spores may be more immunologically reactive, as they rapidly release allergens during germination [50]. Therefore, although resting spores may become airborne in fungi-contaminated indoor environments, germinating spores may pose a greater threat to exposed individuals. Furthermore, common airborne fungal structures such as hyphae and subcellular fragments [2] may release greater quantities of fungal allergen than individual spores [13]. However, these particles are not efficiently removed by phagocytosis [106]. Exposure to fragments may require greater interactions with other inflammatory cells and thus exert greater influence on adaptive immune responses. Liquid suspensions containing *A. fumigatus* hyphae elicited Th2 responses in the draining lymph nodes of mice that received an intratracheal inoculation of fungi, compared to the Th1 responses induced by resting conidia [27]. Thus, while resting spores are taken up rapidly by alveolar macrophages and cause little or no inflammation in the lungs of exposed individuals, germinating spores and/or hyphae may play a larger role in allergic airway responses due to higher expression of allergens or other recognized fungal structures that skew airway immune responses toward an allergic Th2 phenotype.

### *Host-susceptibility*

Although the association between fungal exposure and allergy/asthma is widely accepted, the mechanism by which fungi induce allergy in some individuals but not in others remains less clear. Genetic susceptibility is an important component, although specific conditions of the environment during immune development are also critical [28]. Over the last two decades, the most popular explanation for increased allergic sensitization by environmental factors during development has been the 'hygiene hypothesis' [29,107]. The hygiene hypothesis suggests that infections and non-invasive microbial exposures confer protection from allergic disease. Subsequent epidemiological studies have provided a wealth of supporting evidence. Numerous studies have demonstrated decreased development of asthma in children raised on farms compared to similar children raised in non-agricultural households [29]. The timing of exposures appears critical in protection from allergy; exposures during the first year of life and even prenatal exposures may play a role [108]. Another interpretation of the hygiene hypothesis proposes that changes in gut microflora due to diet, antibiotic use, and birth by caesarean section lead to an increase in the incidence of airway allergic disease [30]. An absent intestinal microbiota alters immune development, as completely germ-free mice exhibit poorly formed secondary lymphoid tissue and antibody responses [109]. In addition, immunocompetent mice exposed to the antibiotic cefoperazone with subsequent oral inoculation of *Candida albicans* exhibited altered intestinal microflora and increased allergic airway responses to intranasal inoculation of *A. fumigatus* compared to control mice that did not receive antibiotic treatment [110,111]. Therefore, these results suggest that altered intestinal microflora may lead to fungal airway allergy.

Respiratory tolerance can be induced by dendritic cells through IL-10-mediated development of $CD4^+$ regulatory T cells ($T_{reg}$) [24]. In the lungs and draining lymph nodes of mice exposed to an intranasal suspension of *A. fumigatus* conidia, $T_{reg}$ cells acted early to suppress neutrophilic inflammation and also limited subsequent Th2 responses [112]. $T_{reg}$ suppression of airway allergy was dependent on the activity of indoleamine 2,3 dioxygenase (IDO). IDO is a tryptophan catabolizing enzyme highly expressed by DCs that, in addition to IL-10, mediates T-cell tolerance by induction of $T_{reg}$ cell development [113]. The two major subsets of regulatory T-cells are identified as natural and inducible $T_{reg}$ cells. Natural $T_{reg}$ cells develop in the thymus and are present before pathogen exposure, and inducible $T_{reg}$ cells are generated in the periphery and acquire regulatory function during immune responses [114]. Large numbers of $T_{reg}$ cells are found in gut-associated lymphoid tissue (GALT) [115]. It is likely that commensal microorganisms in the intestinal tract play an important role in the suppressive activity of $T_{reg}$ cells, as evidenced by the amelioration of inflammatory bowel disease by probiotic bacteria-mediated $T_{reg}$ induction [116]. In addition, $T_{reg}$ cells induced by intestinal helminth infection suppressed airway allergy in ova-sensitized mice [117]. Thus, induction of $T_{reg}$ cells in the GALT suppresses not only the local inflammation in the gut but also airway allergy. The effect of intestinal colonization or infection of mice with probiotic, parasitic, or pathogenic microorganisms on inflammatory responses to fungal exposure in the lungs remains unknown.

## Conclusion

Our understanding of the adverse health effects associated with fungal exposures in the home, workplace, or outdoors has been greatly enhanced through the use of animal models. Animal studies have examined acute and chronic exposures to spores and extracts, and have identified early lung inflammation and long term allergic airway responses. Although *A. fumigatus* serves as one model organism for fungal exposure, allergy, and infection, the potential for adverse respiratory effects to other species, including other common fungal conidia and fragments requires more detailed investigation. Future exposure assessment studies will likely identify other clinically important fungal species in indoor and outdoor environments. Small animal models will be required to confirm their relevance. Continued genome sequencing of medically important species will provide powerful tools for understanding the importance of fungal genetics in allergy, asthma, and infection using small animal models. Animal studies have aided in the characterization of the receptors involved in the immune recognition of fungi such as TLRs 2 and 4, and Dectin-1. These studies have characterized the role of multiple cytokines and chemokines in lung inflammation and allergy associated with fungal exposure. Future studies will be required to determine the mechanisms by which fungal allergens drive allergic airway responses, and the important genetic and environmental properties of both fungi and individuals exposed to fungi that increase the risk for allergic sensitization and disease.

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