Published in final edited form as:

Curr Opin Microbiol. 2017 December ; 40: 8–13. doi:10.1016/j.mib.2017.10.007.

Lung Epithelium: Barrier Immunity to Inhaled Fungi and Driver of Fungal-associated Allergic Asthma

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Abstract

Fungi are ubiquitous in the environment. The epithelium that lines our airways is the first point of contact with the frequent encounter of inhaled fungi. Consequently, the lung epithelium has evolved behaviors that instruct the earliest immune events to resist fungal penetration. Although the epithelium efficiently assists in immunity to invasive fungi, it also can be inappropriately triggered, to the detriment of the host, by normally innocuous fungi or fungal components. Thus, there is a tipping point of protective immunity against fungal pathogens versus inflammatory disease caused by an exuberant immune response to harmless fungal antigens. This review will discuss several aspects of barrier immunity to pulmonary fungal infection, as well as situations where fungal exposure leads to allergic asthma.

Keywords

Fungi; Epithelial cells; Immunity; Inflammation; Asthma

Introduction

For terrestrial vertebrates, the lungs are a primary interface with the external environment. The delicate and moist structures needed for efficient gas exchange between the blood and air also, unfortunately, present a suitable environment for fungal pathogens to invade and cause disease. This vulnerability is partially circumvented by the cavernous, cobbled architecture of the lungs (Figure 1). Particulates must navigate their way through ever winding and constricting passages of the trachea, bronchi, and bronchioles before reaching the fragile site of gas exchange in the terminal alveolar air space. Mucous and cilia lining the airways impose further physical constraints by capturing and reversing the trajectory of inhaled pathogens. Additionally, a heterogeneous assortment of epithelial cell subsets, each

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with unique functions, are distributed along the airways. Club cells, ciliated columnar cells, basal cells, and pulmonary neuroendocrine cells decorate the proximal airways, whereas type-1 and type-2 alveolar cells populate the distal epithelium. The asymmetric polarization of these epithelial cell subsets also augments their sophisticated behaviors. The apical surfaces of the epithelium expel antimicrobial peptides, mucous, and surfactants into the airway lumen. Conversely, the basolateral surfaces secrete chemotactic factors toward the lung parenchyma, thereby recruiting long ranging leukocytes and initiating the earliest events of immunity. The broad importance of the epithelium as a barrier to microbial invasion is widely recognized. However, the dynamic involvement of epithelial cells and their potent functions in fungal pathogen resistance are only beginning to be understood.

Humans inhale several liters of air every minute, and with each breath, we aspirate numerous fungal yeasts and spores [1]. Ensuing invasive disease is largely determined by the quality and quantity of inhaled fungi, as well as host intrinsic factors like immune status. Primary fungal pathogens (e.g. *Blastomyces*, *Coccidioides*, *Cryptococcus gattii*, and *Histoplasma*) cause symptomatic disease in otherwise healthy individuals, indicating that exposure is a major determinant of mycosis (Figure 2) [2]. Infections with other fungi (e.g. *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Pneumocystis*) commonly arise in people with weakened immune systems [2]. This suggests that opportunistic fungal pathogens are likely less virulent, yet more prevalent in the environment than primary fungal pathogens (Figure 2). In both cases, the earliest events of frontline defense after fungal exposure occur at the epithelial surfaces. Investigations into the evolutionary rivalry between lung epithelial cells and fungi could spur paradigm-shifting treatments that prevent or cure invasive fungal disease.

Asthma is a lifelong illness noted by periodic episodes of respiratory distress. According to the World Health Organization, asthma affects an estimated 235 million people and is the most common chronic disease in children [3]. The initial sensitization and subsequent recurrence of asthmatic events is often triggered by inhalation of environmental allergens [4]. Therefore, allergic asthma can be operationally defined as an inappropriate response to a normally innocuous extrinsic stimulus. A significant proportion of cases of allergic airway disease are associated with fungal exposure [5–8]. Household molds, like *Aspergillus* and *Penicillium*, as well as the outdoor fungus, *Alternaria*, account for a majority of these fungal-associated allergies [8]. Inhalation of intact fungi and fungal components triggers allergic responses at the respiratory mucosa. Unlike invasive fungal infections, most evidence to date implicates epithelial cells as having a detrimental impact on lung health in this allergic setting. Thus, identifying ways to turn off specific signals instigated by fungal allergens in epithelial cells would offer valuable therapeutic benefit to the many individuals stricken with allergic asthma.

Several excellent reviews have been written on the topics of lung epithelial cell development and maintenance [9], pulmonary barrier immunity to bacterial and viral pathogens [10, 11], and epithelium-dependent allergic responses to model allergens, such as ovalbumin and house dust mite extracts [12]. This review will discuss recent advances in our understanding of how epithelial cells recognize and respond to the threat of fungal invasion and promote antifungal immunity. We will also delve into how epithelial cells drive allergic asthma by

mishandling the exposure to normally innocuous fungi and fungal products. To fully appreciate the inherent complexity of the epithelium and its interaction with a broad repertoire of immune cells, this review will focus mainly on *in vivo* studies published within the past several years.

Epithelial cells and invasive fungal infection

Only a handful of reports in the literature address the role of the epithelium in combating invasive fungal infection *in vivo* (Figure 3). The challenge of applying reductionist science in a highly complex system has likely stymied this field. However, the availability of conditional genetics to manipulate gene expression in cell subsets, in addition to intravital imaging of the interaction of lung epithelial cells and fungi, will facilitate *in vivo* studies of epithelial cells and antifungal immunity. In the paragraphs below, we discuss several seminal papers and the resulting insights that have paved the way forward.

Wright and colleagues were among the earliest to investigate epithelial cell-dependent immunity to invasive fungal infection. Initially, the group showed that physical contact with *Pneumocystis* evoked NF κ B translocation to the nucleus in type-2 alveolar cell explants [13]. The group also noted that type-2 alveolar cells required MyD88 and interleukin (IL) receptor 1, signaling molecules upstream of NF κ B, for the production of chemokine ligand 2 in similar *ex vivo* assays with *Pneumocystis* [14]. This work established a foundation to formally test the requirement of lung epithelial cell-dependent NF κ B signaling in the orchestration of antifungal immunity [15]. These researchers generated mice with conditional deletion of inhibitor of nuclear factor kappa-B kinase subunit beta (IKK2) in cells that express surfactant protein C. IKK2 is a positive regulator of NF κ B and surfactant protein C is a lung-specific protein. Therefore, NF κ B signaling is abrogated in pulmonary epithelial cells of these mice (IKK2^{LEC}). Similar to immune-competent humans, wild type mice efficiently clear *Pneumocystis* from the lungs. However, IKK2^{LEC} mice infected with *Pneumocystis* exhibited impaired Th17 cell and B cell responses, delayed clearance of fungal burden, and sustained perivascular fibrosis [15]. These data collectively highlight the importance of epithelial cells in regulating immunity to lung *Pneumocystis* infection.

Another recent advance in understanding barrier immunity to fungal infection came from studies of invasive aspergillosis. Jhingran et al. [16] utilized a murine model of inhaled *Aspergillus* spores to discern the spatial and temporal response by lung stromal and resident myeloid populations. Bone marrow chimeras revealed that IL-1R and MyD88 signaling were required within the stromal compartment - presumably epithelium - for efficient production of CXCL1 and neutrophil recruitment within the first 10 hours of infection. By 36 hours post-infection, however, the neutrophils that accumulated in the lungs required intrinsic CARD9 to produce CXCL2. Finally, genetic disruptions in both CARD9 and MyD88 resulted in lethal invasive disease. These data corroborate some of the findings mentioned above regarding IL-1R/MyD88 signaling in the epithelium. In addition, this paper nicely demonstrates that the rapid response by lung epithelial cells promotes rapid recruitment of neutrophils and early immunity against inhaled *Aspergillus* spores.

How do inhaled fungi combat a hostile host response? A couple of papers have examined mechanisms deployed by fungi to counteract barrier immunity to promote tissue invasion. Geunes-Boyer et al. investigated the impact of surfactant protein D (SPD) expressed by lung epithelial cells on *Cryptococcus neoformans* infection [17]. Due to the opsonic properties of SPD and its role in protecting against bacterial infection, one might predict that SPD-deficiency would confer susceptibility to pulmonary fungal infection. On the contrary, mice with global deficiency or club cell-specific deletion of SPD fared much better than similarly infected, wild type controls. These authors showed that *Cryptococcus* bears the host's SPD on its surface to protect against oxidative damage, thus offering an explanation for the unexpected finding in that study. Another recent paper elucidated the function of a pH sensitive transcription factor, PacC, produced by *Aspergillus*. As revealed by *in vitro* studies and *in vivo* studies in lymphopenic mice, *Aspergillus* attaches to and enters epithelial cells in a manner that requires the Dectin-1 receptor and actin-mediated events. PacC regulates fungal cell wall characteristics, which facilitate this process. Upon cell entry, PacC further modulates the epithelial cell environment by altering fungal production of secreted products, including proteases. Regardless of whether fungal virulence is a consequence of evolution or accident, the relatively unexplored frontier of the lung epithelial interface with fungi is an untapped, medically important area of research that will deepen our understanding of lung health and disease in response to inhaled fungi.

Epithelial cells promote allergic disease

Allergic (also known as “type-2”) responses did not evolve to cause disease. Rather, these responses are known to assist in beneficial wound healing, and aid in the expulsion of invertebrate parasites. Thus, current paradigms assign fungal-associated allergic responses to two general pathways. First, fungal proteases are unequivocally important products of fungi that are directly linked to allergic asthma in humans. It has been posited and empirically substantiated that proteases coopt host processes involved in wound healing. Second, chitin is a polysaccharide found in the exoskeletons of invertebrates and cell walls of all fungi. This glycan polymer is not produced by mammals; consequently, chitin could be a means for the host to detect invading chitinous organisms (e.g. helminth parasites) to promote type-2 responses [18]. Unlike barrier immunity to fungal pathogens, there is a comparatively larger body of literature on the involvement of epithelial cells and type-2 responses to fungal allergens. Below, we discuss several aspects of epithelial cell-dependent allergic responses to fungal components (Figure 3).

Many fungi rely on secreted proteases to degrade organic material in their environment. One could logically predict that inhalation of saprophytic fungi or aerosolized proteases into the delicate structure of the airways could irritate the lung. IL-33 is an important signal that alerts the host of tissue injury [19]. Although IL-33 is normally sequestered in the nucleus, it is released into the interstitium upon damage to the cell. Leukocytes bearing IL-33R respond to this alarm and propagate a type-2 immune response. Sub-lethal infection of mice with *Cryptococcus neoformans* leads to allergic inflammation that is dependent on IL-33R [20]. Interestingly, IL-33 expression is restricted to type-2 alveolar cells in *Cryptococcus* infected lungs [21]. Thus, the signal of epithelial damage via IL-33 is the major determinant of allergic responses to *Cryptococcus* infection. The tight junctions between epithelial cells that

maintain barrier integrity are also vulnerable to protease degradation. A *Penicillium* protease (Pen c 13) induces forceful allergic inflammation in a mouse model [22]. Proteome analyses revealed cytoskeletal abnormalities associated with Pen c 13 activity in the lungs, and the protease was also responsible for degrading junctional proteins, such as zona occludens-1, e-cadherin, and occluding [22]. While the evidence of fungal proteolysis of junctional proteins is intriguing, the manner in which the epithelium recognizes this injury and signals an allergic response is poorly understood.

Several lines of evidence implicate fungal proteases in meddling with wound healing to drive allergic airway disease. Coagulation is the earliest wound-healing event, and it involves a cascade of proteases that ultimately generates a fibrin clot. A recent model, proposed by David Corry and colleagues [23], involves the degradation of fibrinogen (the molecule cleaved by thrombin to form fibrin polymers) by an *Aspergillus* protease. The fibrinogen cleavage products (FCP) were presumed to be bound by TLR4 on epithelial cells in this model, leading to mucin production and IL-13R expression. This seminal study also raised a number of questions. FCP aspiration alone did not direct a type-2 lymphocyte responses *in vivo*. Another shortcoming in this model is that TLR4-deficiency (in knockout mice studied) is known to alter the gut microbiota [24], which in turn influences pulmonary allergic inflammation [25]. Thus, further characterization of FCP and structural evidence to support a direct TLR4-FCP interaction would be necessary to clarify these possible confounds. Coagulation and the complement cascade are also intimately linked [26], and there are several reports of the involvement of complement in driving fungal-associated allergy [27–29]. Proteases contained within *Aspergillus* culture filtrates elicit C3aR-dependent allergic inflammation and airway hyper-reactivity [28]. Moreover, C3aR is expressed by epithelial cells [29], and C3aR is required for epithelial cell production of mucin in response to *Aspergillus* proteases [27]. Therefore, further consideration also should be given to the confounding effect that thrombin contaminants in the FCP could have on C3a detection by epithelial cells in the FCP-TLR4 model.

Although inhaled chitin was first recognized to induce allergic responses by the Locksley group in landmark work in 2007 [30], several years passed before chitin recognition was directly linked to the epithelium. Roy et al. [31] found that M2 alveolar macrophage polarization only occurred *in vivo* in the presence of chitin, indicating a requirement of an accessory signal. The authors went on to demonstrate that epithelial cells respond to chitin by producing CCL2, and CCL2 production correlated with the recruitment of eosinophils to the lungs [31]. Additionally, IL-25, IL-33 and thymic stromal lymphopoietin are known to emanate from lung epithelial cells. Mice with deletions in one or two of the genes that encode receptors for these proteins have significantly reduced eosinophil and alternative macrophage activation in response to chitin [32]. Moreover, the response to chitin is fully abrogated in the triple knockout, thus each of these epithelial cytokines have a partially redundant role in controlling allergic inflammation [32]. Chitin availability in the airway lumen is also regulated by a chitinolytic enzyme acidic mammalian chitinase, which is constitutively secreted by epithelial cells [33]. Thus, epithelial cells are now widely believed to be a major sensor and responder to chitin exposure in the lungs.

Concluding remarks

Several complex biological systems involving lung stroma and immune cells intersect shortly after fungi are inhaled into the lungs. The epithelial lining of the airways senses the presence of fungi and responds by summoning immune cells into action. Upon arrival, a network of diverse immune cells enacts their effective antimicrobial functions. If fungi attach to the epithelial cell surface, penetrate the lung parenchyma, and avoid immune surveillance, then invasive disease ensues. All three biological systems must be considered simultaneously if one is to learn about the natural process of fungal infection. Likewise, investigation of intact systems is necessary to unravel the means by which epithelial cells instruct exuberant allergic inflammation to benign fungi and fungal products. The papers highlighted in this review are recent examples of *in vivo* studies that establish a solid foundation for future investigations. Future work can unravel some of the remaining questions such as the means by which the epithelium recognizes inhaled fungi, whether and how these cells directly act on fungal spores or yeast, differences among subsets in how they interact with fungi, and fungal strategies for evading lung epithelial cell responses.

Acknowledgments

This work was supported by NIH grants AI035681 and AI040996 (BSK) and by postdoctoral fellowship grants from the Hartwell Foundation and the NIH (T32 HL007899) to DLW. We thank Robert Gordon (Pediatrics) for assistance with graphic illustrations.

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Highlights

- The lung epithelium orchestrates immune responses to inhaled fungi or their constituents
- Lung epithelium represents a “tipping point” shaping protective or pathological responses
- Studies of lung epithelial cell interactions with inhaled fungi represent a research frontier

Figure 1. Epithelial Architecture of the Lung

The lung is comprised of three zones. The trachea is lined with ciliated epithelial cells and club cells. The small conducting airways, including the bronchi and bronchioles, are a heterogeneous mix of club cells, ciliated cells, and goblet cells. Basal cells underlie these luminal cells, and pulmonary neuroendocrine cells tend to cluster at bronchiolar branch points. Finally, the terminal airways are occupied by alveolar cells. Type-1 alveolar cells are the primary sites of gas exchange, and as a result, they make up an overwhelming majority of the lung surface area. Type-2 alveolar cells produce surfactants that provide the surface tension to stabilize these delicate alveoli.

Prevalence in
the environment
i.e. frequency of
exposure in humans

Intrinsic Fungal
Virulence & Disease

Figure 2. Reciprocal Relationship of Fungal Virulence and Exposure

Invasive pulmonary mycosis is rare in immune competent individuals, yet quite common in people with immune deficiency. Interestingly, the fungi that cause disease in otherwise healthy people are usually different than the fungi that infect people with compromised immune systems. This suggests that highly virulent fungi are sparse in the environment, whereas less virulent fungal pathogens are more prevalent.

Figure 3. Epithelial Response to Fungal Exposure

Schematic of (A) barrier immunity to invasive fungal pathogen and (B) epithelial response to fungal allergens. AAM, alternatively activated macrophage; CLR, C-type lectin receptor.