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REVIEW ARTICLE

Cellular and functional heterogeneity of the airway epithelium

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The airway epithelium protects us from environmental insults, which we encounter with every breath. Not only does it passively filter large particles, it also senses potential danger and alerts other cells, including immune and nervous cells. Together, these tissues orchestrate the most appropriate response, balancing the need to eliminate the danger with the risk of damage to the host. Each cell subset within the airway epithelium plays its part, and when impaired, may contribute to the development of respiratory disease. Here we highlight recent advances regarding the cellular and functional heterogeneity along the airway epithelium and discuss how we can use this knowledge to design more effective, targeted therapeutics.

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INTRODUCTION

Respiration, be it through lungs, gills or skin, is a defining feature of life. In humans, the respiratory system is often divided into the proximal conducting airways, including the nasal cavity, trachea and bronchi, and the distal respiratory airways, including the respiratory bronchioles and alveoli.[1] Though the primary responsibility of the respiratory system is to carry out efficient gas exchange between inhaled air and the bloodstream, it also plays a pivotal role in maintaining respiratory homeostasis, and when dysregulated, can contribute to disease (Fig. 1 and Table 1). Constantly exposed to the environment, the lungs, which total over 70 m^2,[2] encounter countless pathogens, toxins, allergens, and other foreign particles, necessitating continual immunological surveillance. While innate and adaptive immune cells are fundamental for protection, the respiratory epithelium also plays an indispensable role in host defense. Recent research using single-cell RNA sequencing (scRNA-seq) has uncovered enormous cellular heterogeneity within the airways.[3–9] New subsets (e.g., pulmonary ionocytes) or differentiation states (e.g., deuterosomal cells, mucous ciliated cells) have been characterized, and their functions explored. Deuterosomal cells (precursors of multiciliated cells), for example, were shown to exclusively express several Notch transcriptional inhibitors, explaining shutdown of this pathway at the end of multiciliogenesis.[10] Mucous ciliated cells (a transitional state of ciliated cells), expressing high levels of IL-4/13-inducible genes, were highly enriched in asthmatic patients, and preceded mucous cell hyperplasia.[5] Ionocytes and PNECs, both expressing high levels of ion channels POU2F3 and FOXI1, were shown to be important for normal epithelial electrophysiology, as air–liquid interface cultures deficient in both those proteins displayed hyperpolarization and lower conductance.[7] Apart from cell-type-specific transcriptional signatures, location-specific differences between identical cell types were found. Multiciliated cells, for example, expressed higher levels of ACE2 (a receptor for SARS-CoV2) in the nose than in the tracheobronchial compartment[7,8] All these data contribute to our better understanding of the dynamics of differentiation and interactions between cellular populations within the airways. Here we seek to characterize the role that each subset plays in health and disease, and explore the immunological contributions of airway epithelium.

THE OLD AND THE NEW: CELLS OF THE AIRWAY EPITHELIUM AND THEIR CONTRIBUTIONS TO HEALTH AND DISEASE

Basal cells

Basal cells are cuboidal in shape, secured to the basement membrane,[11] and identifiable through expression of cytokeratins 5 and 14, as well as transformation-related protein 63 (Trp-63).[12] These cells comprise approximately 6–31% of AECs in humans, depending on their location across the proximal-distal axis.[13] Basal cells are key modulators of respiratory homeostasis and, crucially, epithelial regeneration following injury.[12] To that end, basal cells are the principal stem cells of the airway, with the ability to self-renew post-injury and differentiate into most other important cell types including goblet, club and ciliated cells, tuft cells, PNECs, and pulmonary ionocytes (Fig. 2).[3,14–16] In fact, multiple studies have suggested that at least two transcriptionally distinct sub-populations of basal cells are present in both human[9,11] and murine[17] airways, with one study differentiating between the two groups by classifying them as either basal cells that can serve as stem cell progenitors, or those committed to luminal differentiation.[18]

Inhalation of cigarette smoke may predispose an individual to respiratory illness. Such is the case in COPD, a condition for which tobacco smoking is the main causative factor.[19] Notably, one of the first respiratory abnormalities observable in both heavy smokers and COPD patients is basal cell hyperplasia, highlighting a key role for these cells in the development of this disease.[20] Chronic exposure to tobacco smoke can induce genetic changes in basal cells, cause a shift toward a stressed state, and cause a loss of regenerative capacity.[7,11] The levels of IL-1β and IL-33 are elevated in smoker basal cells,[21,22] further implicating basal cell aberrations in COPD. Alarmingly, airway basal cells of smokers were described to have hundreds of differentially expressed genes compared to non-smokers, most of which were upregulated, including genes relevant to transcription, development, signal

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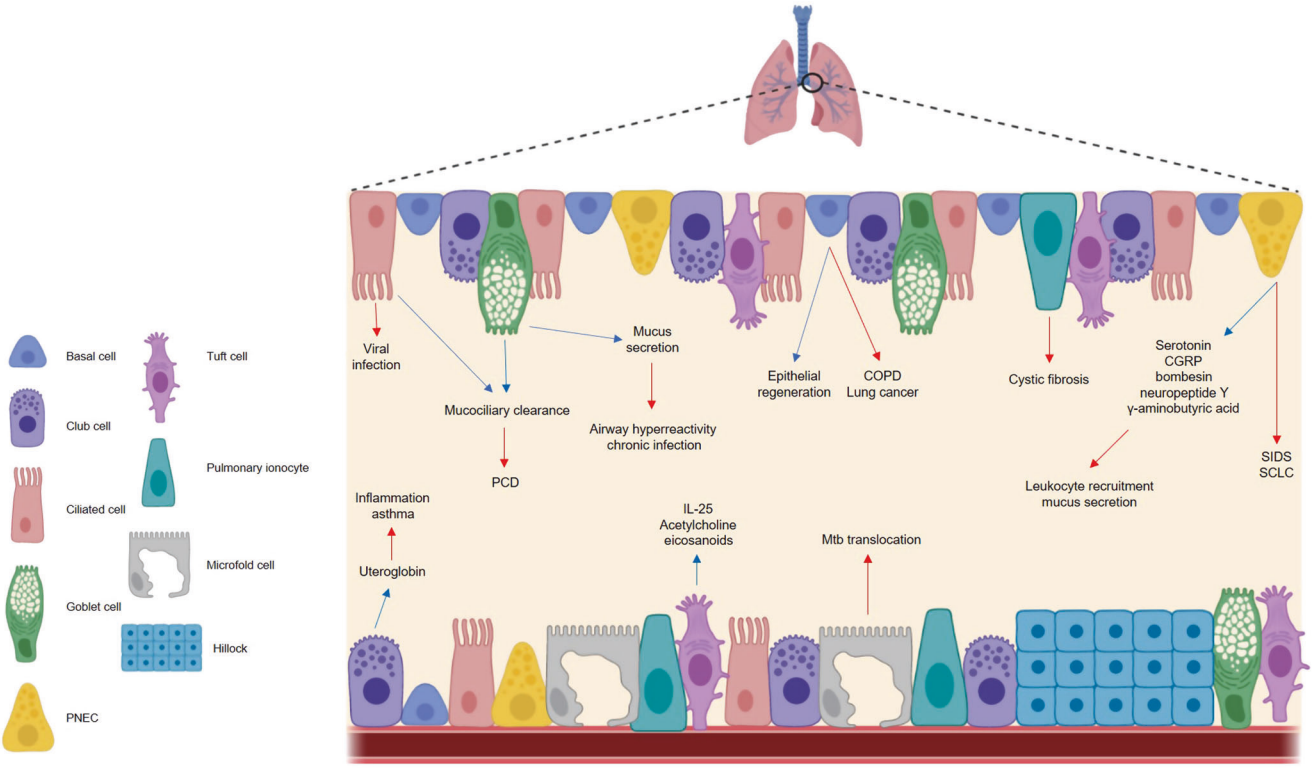

Fig. 1 The respiratory epithelium is important in maintaining respiratory homeostasis but can become implicated in disease. The cells of the airway epithelium each play a functionally distinct role in health and disease. In a healthy state (blue arrows), basal cells are the principal stem cells of the airway, facilitating epithelial regeneration. Club cells secrete the anti-inflammatory protein uteroglobin, and ciliated cells ensure effective mucociliary clearance in conjunction with goblet cells, the chief mucus producing cells of the airways. Pulmonary neuroendocrine cells secrete a range of neuropeptides, while tuft cells are thought to secrete IL-25, acetylcholine and eicosanoids, although the exact role of these molecules in a respiratory context is unclear. In a diseased state (red arrows), cells of the respiratory epithelium contribute to different illnesses. Basal cells have been linked to COPD and lung cancer, while uteroglobin deficiencies are seen in asthma sufferers. Ciliated cells are the target of viral infection and impaired cilia functionality can cause issues with mucociliary clearance e.g. PCD. Aberrations in mucus production can cause respiratory complications including chronic infection. Neuropeptides induce mucus secretion and leukocyte recruitment and contribute to the pathogenesis of SIDS and SCLC, and microfold cells facilitate Mtb translocation. The mechanisms by which pulmonary ionocytes contribute to cystic fibrosis are largely unknown, and the impact of tuft cells on respiratory disease are poorly characterized. PNEC pulmonary neuroendocrine cell, PCD primary ciliary dyskinesias, CGRP calcitonin gene-related peptide; SIDS sudden infant death syndrome, SCLC small cell lung carcinoma, Mtb mycobacterium tuberculosis, COPD chronic obstructive pulmonary disease.

transduction, metabolism, and apoptosis,[23] as well as interferon and inflammatory chemokine signaling, tissue repair, and mucus production.[7] Another disease where smoking is a contributing factor, lung cancer, can also have basal cell origins. A type of human lung adenocarcinoma classified as 'basal cell-high' was found to overexpress basal cell genes, a phenomenon associated with poorer prognosis and shorter survival time than 'basal cell-low' adenocarcinomas, linking these cells to a particularly aggressive form of lung cancer.[24] Together, this evidence suggests that smoking dysregulates basal cell populations in the respiratory tract and that this contributes to the onset of COPD and lung carcinoma.

Club cells

Previously known as Clara cells, these dome-shaped cells dominate the airway epithelium in the respiratory bronchioles.[25] Requiring the transforming growth factor-β receptor Alk5 (activin receptor-like kinase 5) for differentiation,[26] club cells, like basal cells, can act as stem cells and aid in epithelial repair.[27] Club cells can give rise to both ciliated and goblet cells (Fig. 2), and remarkably, have the ability to dedifferentiate into basal cells if required (e.g., upon basal cell injury/loss).[2,28] Club cells also function as secretory cells, commonly characterized by expression of Scgb1a1, a gene encoding for uteroglobin, the most abundant protein in the airway lining fluid.[29–31]

Club cells, along with their secreted products, are important in maintaining homeostasis, and their dysregulation contributes to many respiratory conditions, including asthma, idiopathic pulmonary fibrosis, cystic fibrosis (CF), pulmonary fibrosis, COPD, and acute respiratory distress syndrome.[32–34] For example, uteroglobin, otherwise known as Clara cell protein (CC10, CC16) or Clara Cell Secretory Protein, has been described as having general anti-inflammatory and immunosuppressive capabilities,[33,35] possibly through inhibition of neutrophil and monocyte chemotaxis and phagocytosis as well as inhibition of interferon-γ (IFN-γ), tumor necrosis factor-alpha (TNF-α) and IL-1 production by leukocytes[36–40] In fact, humans with uteroglobin deficiencies are more prone to an inflammatory state,[41] and intratracheal administration of uteroglobin in preterm infants with respiratory distress syndrome led to a reduction in leukocyte and neutrophil numbers in tracheal aspirate samples.[42] Uteroglobin also prevented the formation of immune complexes between IgA and fibronectin, and nephropathy, in mice,[43] although these findings were not recapitulated in humans.[44] Recent research highlights that sepsis, a condition that can be described as a systemic inflammatory response, is exacerbated in the absence of uteroglobin, and administration of this protein protected newborn rats with sepsis by blocking mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) signaling pathways.[45] Expectedly, smoking also affects club cells. Serum uteroglobin concentrations are inversely associated

Table 1. The proposed contributions of AECs to various respiratory and non-respiratory infections and diseases.

Disease	Cell	Proposed contributions to disease	Reference
COPD	Basal	Hyperplasia; smoking-induced loss of regenerative capacity and gene upregulation; increased cytokine secretion	11,20–23
	Ciliated	Reduced numbers of ciliated cells	63
	Goblet	Mucus hyperproduction; elevated SPDEF and Foxa3 expression	56
Asthma/Allergic asthma	Club	Decreased CC16 concentrations due to reduced Scgb1a1 expression	49
	Ciliated	Cdhr3 overexpression increases asthma susceptibility	59,60
	Goblet	Increased cell numbers; upregulation of proinflammatory and remodeling genes; elevated SPDEF and Foxa3 expression; MUC5AC overproduction	5,66,68,71
	PNEC	Bombesin-induced mast cell recruitment; CGRP-induced mucus secretion and ILC2 activation; γaminobutyric acid-induced goblet cell hyperplasia.	86,99,103–105
	Tuft	Express TAS2 receptors thought to attenuate allergic asthma symptoms	132–134
Cystic fibrosis	Pulmonary ionocyte	Possibly via mutation in CFTR, affecting chloride ion transport and leading to fluid accumulation in the airways, although the exact role of CFTR in ionocytes has not been described	3,4
Lung cancer	Basal	Overexpression of basal cell genes	24
	Club	Decreased serum CC16 concentrations	46
	PNEC	Source of lethal SCLC	82,111,112
	Tuft	POU2F3 expression characterizes a variant form of SCLC	147
Rhinovirus	Ciliated	High expression of Cdhr3 facilitates rhinovirus entry	58
PCD	Ciliated	Impaired cilia formation/function impedes mucociliary clearance	56
	Goblet	Aberrant mucus production	56,72
SIDS	PNEC	Hyperplasia and hypertrophy	109
Helminth infection	Tuft	Secrete IL-25 to maintain an intestinal IL-13 producing ILC2 population that stimulates tuft cell proliferation	113–115
Tuberculosis	Microfold	Facilitate Mtb binding and translocation via the B1 scavenger receptor	159,160

with lung cancer mortality in smokers,[46] and ablation of club cells in murine airways prevents lung tumor formation, indicating that these cells are a potential source of respiratory malignancies.[47] CC16 has even been suggested as a useful biomarker for the early detection of silicosis, a condition in which its levels are substantially decreased.[48] More recently, it has been shown that uteroglobin levels are reduced in asthma patients, and mouse models linked this reduction to increased airway hyperresponsiveness.[49,50] Evidently, uteroglobin abnormalities are associated with a wide array of respiratory conditions. Yet still, research has begun to uncover broader, previously unappreciated roles for club cells beyond uteroglobin secretion, including that in shaping antiprotease activity, barrier function, viral responses, host defense, and xenobiotic metabolism.[51] Future research will aid in further clarifying our understanding of these cells.

Ciliated cells

Columnar ciliated cells are found throughout the airways, with at least two transcriptionally distinct subsets having been identified across the proximal-distal axis.[9] These cells play a pivotal role in airway homeostasis by trapping and expelling microorganisms, mucus and other debris, achieved through the rhythmic beating of hair-like cilia, a process commonly termed mucociliary clearance (MCC).[52] Ciliated cells are descendants of club cells or goblet cells,[10] and their differentiation is governed by a complex transcriptional network involving Notch signaling,[53–55] MYB proto-oncogene activation and geminin coiled-coil containing protein 1 (GMNC), the 'master regulator' of ciliated cell fate.[56] Also expressed is forkhead box protein J1 (Foxj1), a transcription factor necessary for cilia formation.[30,57] ScRNA-seq of human airway epithelium from nasal swabs as well as in vitro 3D cultures of human AECs identified a transitional state preceding differentiation of ciliated cells, termed "deuterosomal cells". At this stage, Notch signaling appears to be switched off via induction of the Notch inhibitors HES6, DYRK1A, CIR1, and SAP30.[10]

In a diseased setting, ciliated cells play a central role in susceptibility to viral infection. Of all airway epithelial cells, ciliated cells express particularly high levels of cadherin related family member 3 (Cdhr3), a receptor required for rhinovirus C entry into the cell.[58] Moreover, Cdhr3 is considered an asthma susceptibility gene (especially in young children),[59,60] which may explain the strong association observed between infantile rhinovirus infection and subsequent asthma development.[61] The importance of cilia in health is highlighted by the observation that mutations in over 35 genes related to cilia formation are associated with chronic lung disorders characterized by impaired MCC, collectively referred to as primary ciliary dyskinesias (PCD). Notably, cigarette smoke shortens cilia, leading to impediment of MCC,[62] and a significant reduction in ciliated cell numbers are found in COPD patients.[63] Overall, it is clear that ciliated cells play an important role in respiratory health, yet can become malfunctional in disease.

Goblet cells

Goblet cells, named for their goblet-like appearance, are the chief mucus producing cells of the airways, which together with ciliated cells facilitate effective MCC. Mucus contains an assortment of products, namely electrolytes, metabolites, fluids, antimicrobial products, and mucins, further subdivided into cell-associated mucins, secreted mucins, and gel-forming mucins (principal examples being MUC5AC and MUC5B).[56] Like ciliated cells, goblet cells are derived from club cells through activation of alternative transcriptional pathways, primarily those regulated by Sam-pointed domain-containing Ets-like factor (SPDEF; Fig. 2).[56] SPDEF controls goblet cell transcriptional programs, including forkhead box A3 (Foxa3), and together these two transcription factors regulate goblet cell differentiation and mucus production.[64]

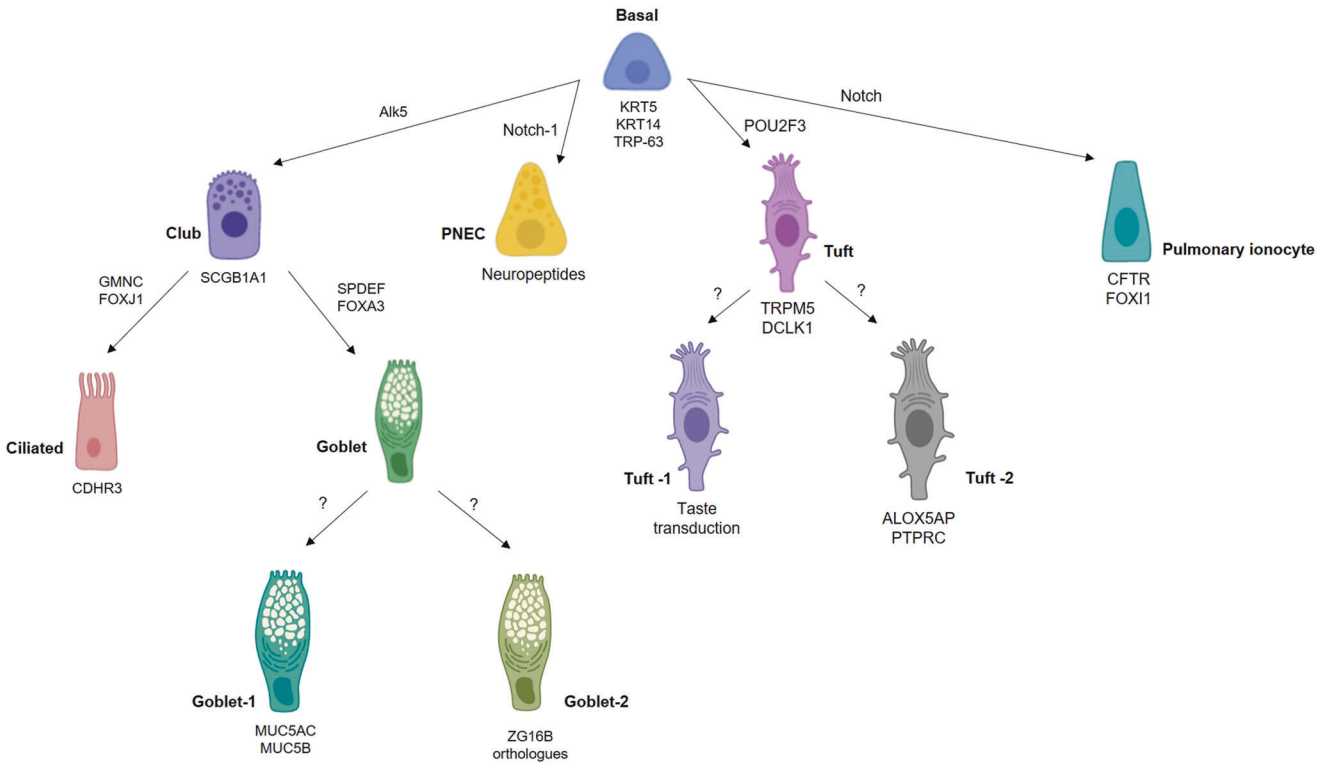

Fig. 2 Different transcriptional networks govern airway epithelial cell fate. Basal cells are the principal stem cells of the airways, differentiating into most other epithelial cell types. Different cell subsets can be characterized by expression of particular genes, for example, pulmonary ionocytes can be classified as expressing FOXI1 and CFTR, the gene mutated in cystic fibrosis. The transcriptional programs governing certain cell fates remains unclear. ZG16B, zymogen granule protein 16.

Overexpression of SPDEF is sufficient to induce goblet cell hyperplasia,[65] while its deletion causes an absence of goblet cells within the tracheal and laryngeal submucosal glands.[66] Recently, the goblet cell lineage was divided into two functionally different subsets, classified as goblet-1 cells, primarily producing mucus, and goblet-2 cells, which secrete orthologues of zymogen granule protein 16, a protein capable of binding to and aggregating gram-positive bacteria.[3,67]

Increased mucus production by goblet cells is a common feature of asthma, COPD, PCD, and CF.[5,56] SPDEF and Foxa3 levels are elevated in patients suffering from asthma, COPD and CF, as well as in chronic smokers.[56,66,68] Overexpression of SPDEF resulted in elevated levels of various inflammatory markers at baseline (e.g., IL-13, IL-25, IL-33, CCL17, CCL20, CCL24, and granulocyte-macrophage colony-stimulating factor (GM-CSF), likely contributing to enhanced recruitment of eosinophils, type 2 innate lymphoid cells and T cells in response to HDM.[64] In line with this, SPDEF$^{-/-}$ mice displayed a marked reduction in goblet cell differentiation, eosinophilic infiltration and T_H2 inflammatory markers in a mouse model of asthma.[64] This suggests that SPDEF expression facilitates the allergic response to HDM. Somewhat paradoxically, SPDEF may also dampen immune responses to respiratory bacterial or viral infection by binding to the TLR adapters MyD88 and TRIF, and inhibiting their signaling.[69] As a consequence, neutrophils are not efficiently recruited to the airways of mice overexpressing SPDEF, leading to the impaired clearance of an invading pathogen.[70] Dysregulated mucus production may have detrimental consequences for respiratory health. For example, overproduction of MUC5AC can cause allergic airway hyperreactivity,[71] while the absence of MUC5B may result in impaired MCC characterized by persistent inflammation, chronic bacterial infection and atypical breathing patterns.[72] Overall, these data clearly demonstrate that goblet cell anomalies are closely tied to pulmonary disease.

Pulmonary neuroendocrine cells

PNECs serve as key communicators between the immune and nervous systems. First discovered in the 1950s,[73–75] PNECs can best be described as very rare, innervated epithelial-resident cells that sense airway activity and secrete neuropeptides to stimulate immune responses.[76] These cells are estimated to comprise ~0.5% of all epithelial cells in human airways,[77] and 0.01% of all cells in the lung.[9] PNECs, whose origins can be traced back to the gills of fish,[78] often exist as solitary cells, but can form clusters termed neuroepithelial bodies.[79,80] They have recently been suggested to arise from tuft-like cells.[7] Similarly to basal and club cells, epithelial damage can trigger a subset of PNECs to reprogram and adopt other cell fates, thereby acting as stem cells and facilitating tissue repair.[81,82] Interestingly, PNECs are considered to play a substantial role in fetal lung development,[83] which may explain why they are one of the first cell types to appear in the murine respiratory epithelium,[84] identifiable between embryonic days 13–16.[74] Finally, it has been proposed that a subset of PNECs may have some degree of chemosensory function, given they express olfactory receptors.[85]

PNECs primarily modulate immunity through secretion of various chemicals including serotonin, calcitonin gene-related peptide (CGRP) and bombesin-related peptides, which interact with both structural and immune cells.[80,86] Serotonin induces angiogenesis,[87] activates fibroblasts,[88–90] and reduces alveolar epithelial fluid transport by acting on type II alveolar epithelial cells.[91] CGRP inhibits production of nitric oxide in endothelial cells[92] and promotes their proliferation.[93] It also induces proliferation and chemotaxis of fibroblasts[94] and affects the functioning of alveolar type II cells by inducing their proliferation[95] and inhibiting hypoxia-induced DNA damage and apoptosis.[84] Finally, bombesin or bombesin-like peptides induce proliferation and migration of fibroblasts[94] and induce angiogenesis[96] and proliferation/antigen presentation of bronchial epithelial cells.[97,98]

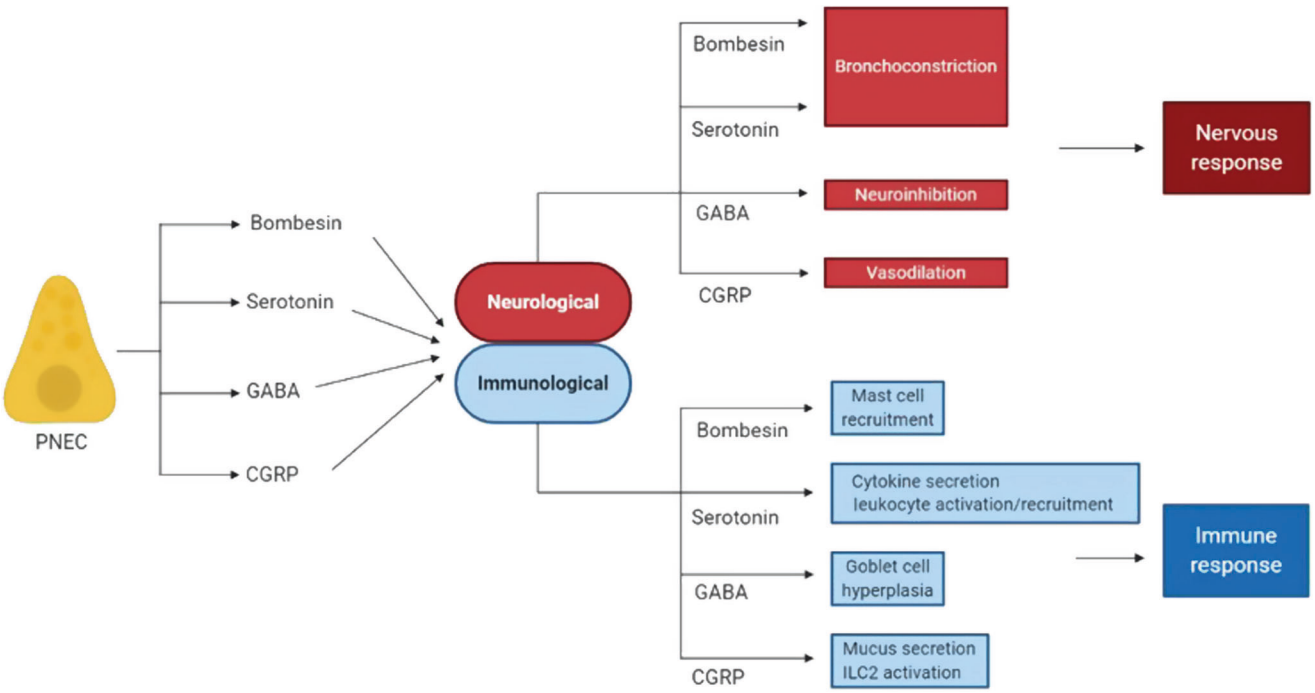

Fig. 3 Pulmonary neuroendocrine cells (PNECs) secrete neuropeptides that have both neurological and immunological effects. PNECs secrete bombesin/bombesin-related peptide, serotonin, GABA and CGRP, which in a neurological context can cause bronchoconstriction, neuroinhibition and vasodilation, respectively. These same effector molecules also stimulate immune responses. For example, bombesin recruits mast cells, serotonin influences cytokine secretion and leukocyte activity, GABA causes goblet cell hyperplasia, and CGRP elicits mucus secretion and ILC2 activation. CGRP calcitonin gene-related peptide, GABA gamma-aminobutyric acid, ILC2 innate lymphoid cell 2.

Elimination of PNECs was also shown to decrease expression of neuropeptide Y in the lungs, although a specific source was not identified.[80,86,99] Bombesin and serotonin influence neurons and can cause bronchoconstriction,[100,101] while CGRP is a potent vasodilator.[102] In addition to their neurological effects, these neuropeptides also have immunological functions, for example, bombesin-related peptides recruit mast cells to the lungs,[103] CGRP can induce mucus secretion in the airways from both glands and goblet cells,[86,104,105] and serotonin has been shown to modulate cytokine secretion in macrophages, suppress TNF-α and IL-1β production, and affect T-cell activation and neutrophil recruitment[106] (Fig. 3). In fact, serotonin is suspected to interact with almost all immune cells.[106] These observations are in line with recent findings that PNECs augment allergic responses by secreting γ-aminobutyric acid (otherwise known as an inhibitory neurotransmitter in the central nervous system) and CGRP. γ-aminobutyric acid and CGRP stimulated goblet cell hyperplasia and activated ILC2s, respectively, and their administration into PNEC-deficient mice rescued blunted type 2 responses observed in sham-treated animals.[99]

Looking retrospectively, it is possible that multiple studies may have investigated PNECs and their secreted products without classifying these cells as PNECs. For instance, Der p 2 administration to mice was shown to cause upregulation of nerve growth factor (NGF) within the bronchial epithelium and bronchoalveolar lavage fluid, as well as mucus gland hyperplasia and inflammatory cell infiltration.[107] Moreover, AECs were classified as a major source of NGF and brain-derived neurotrophic factor during allergic airway inflammation, thereby promoting eosinophil survival.[108] These studies did not, however, discriminate between different subsets of AECs. Thus, it is conceivable that the observed neuronal functions in these studies are attributable to PNECs.

Post-mortem examination of children who perished from sudden infant death syndrome (SIDS) revealed both PNEC hyperplasia and hypertrophy in the lungs of these patients.[109] SIDS is a devastating condition whose exact etiology remains unknown, but increased research into the role of PNECs may help clarify the pathophysiological mechanisms behind this disease. Dysregulation of PNECs, which often manifests as an increase in PNEC numbers, can also be detected in other pulmonary diseases, namely CF and bronchopulmonary dysplasia,[80] COPD,[85] and even in a rare case of emphysema.[110] It is unclear, however, whether PNEC dysregulation causes, or is a consequence of, disease. PNECs are also implicated in cancer, where they are a source of small cell lung carcinoma (SCLC), a potentially lethal malignancy affecting over 200,000 people annually.[82,111,112] Distinct from their immunomodulatory role, PNECs can also sense hypoxic conditions, respond to changes in carbon dioxide levels, and become activated upon exposure to nicotine.[75] Though much remains to be discovered about their functionality, PNECs are a prime example of a cell that comprises a tiny fraction of the total heterogeneity observed in the lungs, yet has meaningful consequences for health and disease. Finally, PNECs constitute an example of how the intricately intertwined immune and nervous systems work conjointly to facilitate respiratory homeostasis.

Tuft cells

To categorize tuft cells as 'newly discovered' would be misleading, but these cells attracted little immunological interest until 2016, when three independent reports identifying tuft cells as critical components of the gastrointestinal immune response against helminth infection were published.[113–115] These basal-derived cells were first discovered in 1956 in the murine gastrointestinal tract and rat trachea,[116–118] and are recognizable through their distinct microvillous apical tuft. Tuft cells are not limited to these sites, however. Instead, they have been found in the airways,[3] stomach,[119] intestines,[120] olfactory epithelium,[121] auditory tube,[122] conjunctiva,[123] bile duct,[124] gall bladder,[125,126] urethra,[127,128] and even the thymus,[129] where they have been proposed to present

antigens to developing thymocytes.[130] Somewhat confusingly, tuft cells are often referred to by a variety of different names depending on their anatomical location, including brush cells, solitary chemosensory cells, and microvillous cells.[131] Irrespective of this, they can be characterized by several common markers, including reliance on POU domain class 2 homeobox 3 (POU2F3) for their differentiation,[115] expression of transient receptor potential cation channel subfamily M member 5 (Trpm5),[120] and secretion of a specific set of effector molecules, namely IL-25, acetylcholine and eicosanoids.[131]

In keeping with this diverse group of secreted products, tuft cells are believed to have chemosensory, neuronal and immunological functions.[120] Tracheal tuft cells express a variety of taste receptors and G-protein-coupled receptors,[3] and share signaling pathways with taste bud cells.[131] For example, tuft cells express type 2 taste receptors, also known as bitter taste receptors,[132,133] which have been associated with allergic asthma.[134] Curiously, tuft cells share POU2F3 dependency with umami, bitter, and sweet taste cells,[135] suggesting that these cells are closely related.

In the human gut, tuft cells have been shown to reside in close proximity to nerve fibers.[136] Analogous observations have been made in the auditory canal and conjunctiva,[122,123,137] thereby designating tuft cells as potential mediators of immunological-neuronal communication. Similar co-localization in the airways is yet to be described.

As stated, tuft cells came to the fore in 2016 when they were revealed to be critical components of the immune response against helminths (for example, *Nippostrongylus brasiliensis*), and it is in this gastrointestinal context that these cells are best understood. Combined, these studies propose a model whereby tuft cell-derived IL-25 promotes the maintenance of a local ILC2 population. ILC2s, in turn, secrete IL-13 and induce tuft cell differentiation and proliferation via IL-4Rα,[113–115] in a sequence of events now termed the ILC2-epithelial or ILC2-tuft cell circuit. This immune response leads to eosinophil recruitment and goblet cell hyperplasia, both of which are important for the expulsion of helminths.

Tuft cells have been noted in the airways of rodents,[138] cows, and bulls.[139] Recent research has revealed the existence of multiple subsets of murine airway-resident tuft cells, termed immature tuft, tuft-1 and tuft-2 cells.[3] Tuft-1 cells were found to express genes related to taste signaling, whereas tuft-2 cells expressed genes associated with leukotriene synthesis and immunomodulation (*Alox5ap* and *Ptprc)*, suggesting the former has a chemosensory role while the latter serves a more immunological purpose.[3] Independent studies have made similar observations in the murine intestines, where an immunological role for tuft-2 cells is also suggested, but, in contrast to the above, tuft-1 cells are described as expressing genes associated with neuronal development.[140] It is not inconceivable, however, that tuft cell function may vary across different tissues.

Evidence implicating tuft cells in respiratory disease is limited. It is known that tuft cells express TAS2 receptors,[133] which have been shown to attenuate allergic airway inflammation.[134] It has also been demonstrated that tuft cells emerge ectopically in the lung after influenza virus infection, where they may contribute to dysplastic remodeling.[141] More broadly, the overexpressed tuft cell marker doublecortin-like kinase 1 (DCLK1) has been implicated in multiple malignancies including pancreatic,[142] colonic/colorectal[143–145] and gastric cancers,[146] while POU2F3 expression is associated with a form of SCLC.[147] Most recently, tuft-like cells were observed in the human trachea and were suggested to serve as progenitors for PNECs and ionocytes.[7] Despite this, tuft cells are still shrouded in mystery, and much remains to be discovered regarding their function as both epithelial cells and immune influencers. What is clear, however, is that these enigmatic cells are diverse and multifaceted, with important roles in respiratory and gastrointestinal health.

Pulmonary ionocytes

Pulmonary ionocytes were largely unknown to immunologists until 2018, when two separate studies identified pulmonary ionocytes in the airways of both mice and humans.[3,4] Generated from basal cell precursors or from tuft-like cells,[7] pulmonary ionocytes are estimated to comprise less than 2% of human AECs, express both forkhead box I1 (*FOXI1*) and CF transmembrane conductance regulator (*CFTR*), and rely on Notch signaling for their differentiation.[3,4] Ionocytes are evolutionarily ancient; they have been observed in the skin of both *Xenopus* larvae and zebrafish.[148,149] Interestingly, zebrafish ionocyte development is also dependant on Notch signaling, suggesting a close relationship with human pulmonary ionocytes.[150]

Because pulmonary ionocytes highly express *CFTR*, the mutation of which contributes to CF, there is hope that targeting these cells could be an effective treatment for this disorder, characterized by chronic mucus accumulation in the airways and failure of MCC.[151] The *CFTR* gene was unearthed and named in 1989,[152] and is now understood to be an ATP-gated ion channel that allows passive diffusion of chloride and bicarbonate ions.[151,153,154] Pulmonary ionocytes, despite only making up 0.42% of murine AECs, express almost 55% of the total detected *Cftr* transcripts; for comparison, the far more abundant ciliated cells express only 1.5%.[3] CFTR expression in human bronchial tissue is driven by FOXI1, another prominent marker of pulmonary ionocytes. *Foxi1*-knockout mice display a marked reduction in *Cftr* expression and disrupted airway fluid/mucus physiology,[3] potentially indicating a causative link between pulmonary ionocyte dysregulation and the development of CF. As more information comes to light surrounding pulmonary ionocytes and their contributions to CF, it is hoped that novel therapies targeting these cells will provide a much-needed breakthrough in the management of this genetic disease, for which there is currently no cure.

Hillock cells

Very little is known about hillock cells. Not to be confused with axon hillocks, hillock cells were described by Montoro et al. as a variety of club cells descending from the basal cell lineage and expressing keratin 13.[3] These cells are found in contiguous groups of stratified epithelial cells, forming structures termed 'hillocks', where they have a particularly high turnover rate and express markers associated with squamous epithelial differentiation, cellular adhesion and immunomodulation.[3] This population was also found by Deprez et al. and characterized as Keratin 13 (KRT13)-positive cells with high cycling capacity.[8]

Beyond this, hillock cells remain a closed book. Future investigations may expand our knowledge of these cells.

Microfold cells

Like tuft cells, microfold cells (M cells) are best understood in a gastrointestinal context, where their main function is to initiate immune responses via endocytosis and transport of antigens from the gut lumen to lamina propria-residing antigen presenting cells.[155] Nevertheless, M cells can also be found at other mucosal sights, including the airways of humans and mice, where they are structurally and functionally similar to those found in the gut.[156,157] Recent findings demonstrate that a small population of respiratory M cells, like their gastrointestinal counterparts, utilize receptor activator of nuclear factor-κB (RANK)-RANK ligand (RANKL) signaling, and express glycoprotein 2 and tumor necrosis factor alpha-induced protein 2 (Tnfaip2).[158] Airway M cells are the target of Mtb, which hijacks these cells to facilitate its spread.[159] A study published earlier this year identified M cell-bound scavenger receptor B1, a receptor for the Mtb virulence factor EsxA (ESAT-6), as essential for Mtb binding and translocation.[160] M cells also have the ability to take up *Salmonella typhimurium* and *Streptococcus* antigens,[157] potentially evincing a broader role for M cells in bacterial infection.

Box 1 Epithelial barrier integrity in health and disease

Integrity of the respiratory epithelial barrier is dependent upon its ability to form a continuous impermeable membrane, held together by tight junctions and adhesion proteins such as E-cadherin, β-catenin, occludins, zonular occluden (ZO) −1, −2 and −3, and claudins.[226] Often, disruption of this barrier may cause, or be a consequence of, respiratory disease. In asthma, for example, the prototypical T_H2 cytokines IL-4 and IL-13, or the proteolytic enzymes found in allergens, can disrupt barrier integrity by reducing the expression of, or cleaving, tight junction proteins including E-cadherin, occludins, and ZO-1.[227] Smoking, a causative factor of many disorders, can also increase epithelial permeability and lead to a loss of barrier function.[228] In agreement with the latter, barrier integrity is disrupted in people with chronic obstructive pulmonary disease (COPD) through the loss of claudin and occludin expression.[229] Beyond allergic diseases, many infections (viral, bacterial or fungal) may also compromise barrier integrity of the airway epithelium. For instance, polyinosinic:polycytidylic acid (polyI:C), mimicking a virus-derived double-stranded RNA, has been shown to affect the respiratory epithelium by severely disrupting tight junctions.[230] Furthermore, rhinovirus infection can induce ZO-1 loss and a corresponding decrease in transepithelial resistance,[231] and influenza infection may lead to leakage of albumin into the airways, indicating a loss of barrier integrity.[232] Although the airway epithelium represents much more than a physical barrier, efficient barrier function still plays a fundamental protective role.

THE EMERGING IMMUNOLOGICAL FUNCTIONS OF THE AIRWAY EPITHELIUM

Historically, the respiratory epithelium was thought to function as a first line of defense by constituting a passive barrier against pathogen invasion.[161,162] Indeed, while it does serve this purpose (Box 1), the airway epithelium also engages in constant immunological activity, with each cell subtype actively contributing to the maintenance of respiratory health in the face of viral and bacterial attack, or allergen exposure (Fig. 4). In response to rhinovirus infection (and many other viral infections)[163–165] AECs secrete type I and type III interferons (IFNs), whose production is induced through interactions with dsRNA receptors MDA5, RIG-I, and TLR-3,[166,167] as well as JAK/STAT signaling pathways.[168,169] IFNs are crucial in host defense as they stimulate infected and surrounding cells to ihibit viral replication and recruit/activate immune cells. IFNs can also increase MHC class I expression, leading to improved recognition of virally infected cells by cytotoxic T cells (Fig.4).[170] Similarly, AECs are important in the response against bacterial infection, and triggering of AEC-bound pattern recognition receptors is an important upstream event that promotes bactericidal activity. AECs produce antimicrobial peptides including members of the cathelicidin and β-defensin families, as well as lactoferrin, lysozyme, and secretory leukocyte proteinase inhibitor (SLPI), which each mediate bacterial killing through different mechanisms.[171] Lysozyme can rapidly destroy Gram-positive bacteria by degrading glycosidic links in bacterial surface proteoglycan, while the iron chelator lactoferrin can kill bacteria by depriving them of iron and can also disrupt the integrity of the outer membrane of Gram-negative bacteria.[172] SLPI has bactericidal properties against variety of Gram-positive and Gram-negative bacteria, including *S. typhimurium, Escherichia coli, Staphylococcus aureus, Mycobacterium tuberculosis* (Mtb) and *Neisseria gonorrhoeae*, although exact mechanisms remain elusive.[173] Defensins can efficiently eliminate bacteria via membrane depolarization and premature activation of cell wall lytic enzymes,[174] while the antimicrobial effects of the cationic human cathelicidin LL-37 depend on their capacity to attract monocytes, neutrophils and $CD4^+$ T cells and activate mast cells.[172,175,176] These antimicrobial effectors behave synergistically to ensure the effective and timely clearance of bacterial infections (Fig. 4).

In addition to delivering immune response themselves, AECs have also been shown to communicate with other cells to orchestrate immune responses. This communication is primarily mediated by the release of many cytokines and chemokines. RSV infection elicits secretion of an array of AEC-derived proinflammatory molecules including IL-1α and IL-1β, IL-6, GM-CSF, TNF-α, IFN-γ and a variety of chemokines, which influence the activity of multiple immune cell populations.[177] AECs have also been found to release B-cell activating factor in response to RSV infection, suggesting a role in shaping B-cell responses in the lung.[178] Upon internalization of influenza virus, airway epithelial cells sense viral products via TLR-3, retinoic acid-inducible gene 1–like receptors (RLRs, including RIG-I and MDA5), and NOD (nucleotide-binding oligomerization domain-containing protein)-like receptors (NLRs, including NLRP3). Activation of TLR-3 by dsRNA, which is an intermediate state of RNA species during influenza virus replication, leads to secretion of proinflammatory cytokines such as IL-6, IL-8, and RANTES.[179,180] Activation of TLR7 and TLR8, known sensors of ssRNA, leads to secretion of IL-6, IL-8, and IFN-λ.[181] RNA species from influenza virus are also sensed by RIG-I and MDA5 in airway epithelial cells, leading to expression of type I IFNs.[180,182] NLRP3 is activated by both viral ssRNA and danger-associated molecular patterns induced upon influenza virus infection,[183] and its activation leads to enhanced secretion of IL-1β.[183,184] Finally, severe acute respiratory syndrome (SARS)-CoV2 interacts with ACE2 on airway epithelial cells and leads to secretion of various inflammatory mediators, including CCL20, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CXCL16, IL-1β, IL-6, and TNF-α,[185,186] Aside from viruses, bacteria are also potent triggers of airway epithelial cell responses. Nontypeable *Haemophilus influenzae* induced epithelial cell expression of IL-8, IL-6, TNF-α, IL-1α, IL-1β, and CCL2,[187] while *Pseudomonas aeruginosa* or *Staphylococcus aurerus* induced IL-8 secretion by AECs.[188] Other inflammatory mediators secreted by epithelial cells in response to bacteria or bacterial products include GM-CSF, G-CSF, mucin, and nitric oxide.[189] Lastly, airway epithelial cells orchestrate anti-fungal immunity. Type II alveolar cells directly sense *Pneumocystis*, and secrete CCL2, in a MyD88 and IL-1R-dependent manner.[190] Also, selective ablation of IKK2 (a positive regulator of NF-κB) in the lung epithelium impairs pulmonary Th17 and B-cell responses to *Pneumocystis* and delays its clearance.[191] The importance of IL-1R/MyD88 signaling in the stromal compartment of the lung in fighting off fungal infections was also demonstrated by Jhingran et al., who showed the involvement of these pathways in the production of CXCL1 and recruitment of neutrophils following *Aspergillus fumigatus* infection.[192] Finally, dsRNA from the conidia of *A. fumigatus* led to the induction of IFN-β signaling pathway in human bronchial epithelial cells.[193]

Apart from soluble mediators, AECs may interact with cells via cell–cell contact. This is exemplified by the interaction between AECs and alveolar macrophages via CD200-CD200R. Murine alveolar macrophages express CD200R, a type 1 transmembrane glycoprotein, at higher levels compared to their interstitial and splenic counterparts, and its ligand, CD200, is expressed on type II alveolar epithelial cells and bronchial epithelial cells.[194–196] CD200 interactions are primarily inhibitory, with CD200R engagement impeding activation of myeloid cells (including macrophages) and T lymphocytes through the inhibition of complex ERK, MAPK, and JUN N-terminal kinase signaling pathways.[195,197,198] Mice lacking CD200 have more alveolar macrophages in the airways, express higher levels of TNF-α, IFN-γ, IL-6, and CCL3, and have more severe immunopathology associated with influenza virus infection.[194] Co-infection with human rhinovirus and *H. influenzae* induces IL-17C release from epithelial cells and drives production of the neutrophil chemoattractant, CXCL1.[199]

Cross-talk between AEC activation and other cell types is also evident in the pathogenesis of asthma. This was first suggested in 2000,[200] building upon previous observations that the bronchial epithelium is highly abnormal in asthma sufferers. Later research pointed to the importance of toll-like receptor 4 (TLR-4) activation, either by the allergen itself (e.g., house dust mite (HDM))[201] or cleavage products of allergen proteases (e.g., fibrinogen cleavage products of fungal proteases).[202] TLR-4 activation in response to allergens leads to secretion of GM-CSF and alarmins IL-25, IL-33 and TSLP, which collectively drive the allergic response.[201,203–206] More specifically, IL-25 amplifies IL-4, IL-5, IL-13, and eotaxin

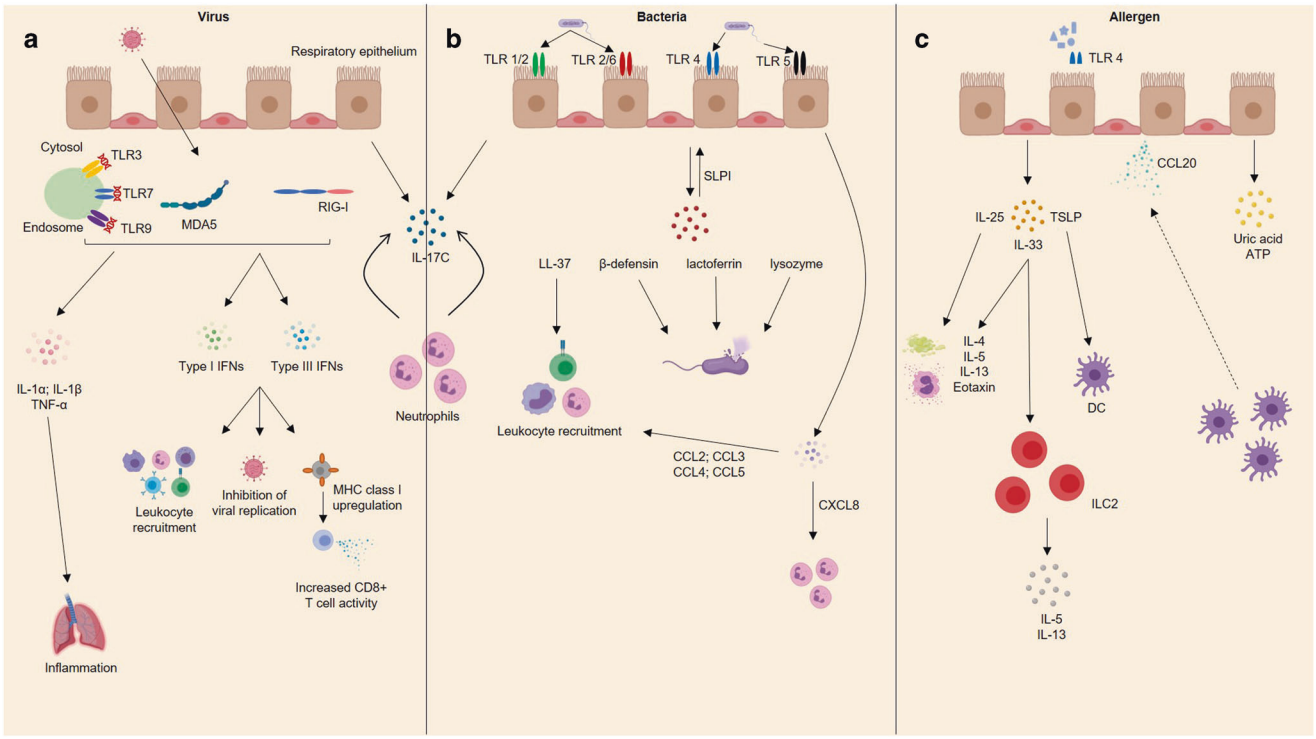

Fig. 4 The airway epithelium responds to viral, bacterial, and allergic attack. a Virus-associated molecular patterns are recognized by a variety of intracellular pattern recognition receptors including toll-like receptors and rig-like helicases e.g. MDA5 and RIG-I, which stimulates release of type 1 and 3 interferons. Collectively, interferons mediate antiviral immunity by inhibiting viral replication, recruiting immune cells (e.g., T cells, B cells, natural killer cells), and upregulating MHC class I expression, leading to increased $CD8^+$ T cell activity. Activated airway epithelial cells also secrete proinflammatory cytokines IL-1α, IL-1β, and TNF-α. **b** Bacteria can be recognized by multiple TLRs stimulating the release of antimicrobial peptides including human cathelicidin LL-37, defensins, lactoferrin, lysozyme and SLPI. LL-37 mediates recruitment of monocytes, neutrophils, and $CD4^+$ T cells, while defensins, lactoferrin, and lysozyme can destroy bacteria through different mechanisms. AECs also secrete SLPI, which acts in an autocrine/paracrine manner to protect the epithelium against serine proteases and proteolytic enzymes. In response to bacterial infection airway epithelial cells also release a plethora of chemokines, for example, the neutrophilic chemoattractant CXCL8, as well as CCL2, CCL3, CC4, and CCL5, which mediate recruitment of several leukocytes (e.g., monocytes/macrophages, T cells, DCs, neutrophils and natural killer cells). In response to viral and bacterial co-infection, the respiratory epithelium produces IL-17C, which is thought to stimulate neutrophil activity. **c** Allergens including HDM and fungi activate AEC-bound TLR-4, eliciting secretion of IL-25, IL-33, and TSLP which together drive the downstream allergic response by inducing T_H2 cytokine secretion, mucus production, eosinophilia, and DC and ILC2 activation. AECs also secrete CCL20, a potent dendritic cell chemoattractant, as well as uric acid and ATP. TLR toll-like receptor, MDA5 melanoma differentiation-associated protein 5, RIG-I retinoic acid-inducible gene I, IFN interferon, AECs airway epithelial cells, SLPI secretory leukocyte protease inhibitor, IL interleukin, TSLP thymic stromal lymphopoietin, DC dendritic cell, HDM house dust mite, IgE immunoglobulin E, TNF-α tumor necrosis factor-alpha.

secretion, mucus production, and eosinophilia.[207,208] IL-33 facilitates T_H2 cytokine responses and the subsequent recruitment of eosinophils,[209] and TSLP activates DCs.[210] AECs also produce CCL20, uric acid and ATP, recruiting and activating dendritic cells[211–214] and, in the case of ATP, driving migration and ROS production in eosinophils.[213]

In the context of allergic senitization, expression of several immune-related genes in DCs, including *Psmb9, Slamf1, and Fkbp5*, is partly controlled by MyD88 signaling in AECs.[215] Moreover, upon HDM inhalation, lung epithelial cells undergo fucosylation by fucosyltransferase 2 (Fut2), leading to accumulation of complement C3 at fucosylated sites. $Fut2^{-/-}$ mice, which lack fucosylation, exhibit attenuated allergic airway inflammation, significantly lower levels of C3a and a corresponding reduced accumulation of C3a receptor-expressing monocyte-derived DCs in the lung, indicating that epithelial glycosylation indirectly promotes allergic responses by enhancing DC recruitment via C3-related pathways.[216] AEC-derived cytokines are also needed to stimulate an ILC2 response within the mucosa. ILC2s, which lack conventional T-cell receptors, secrete IL-5, and IL-13 in response to AEC-derived IL-25 and IL-33,[217] while skin ILC2s contribute to atopic dermatitis by responding to TSLP.[218]

Apart from sending signals to other cell types, AECs also receive signals. Release of neutrophil extracellular traps (NETs) causes a significant increase in secretion of IL-6 and IL-8 by alveolar and bronchial epithelial cells, effects not observed when NETosis is inhibited.[219] Furthermore, neutrophil-derived antimicrobial peptides such as defensins and cathelicidin hCAP-18 can trigger AEC release of chemokines including neutrophil chemoattractants IL-8 and epithelial neutrophil-activating protein 78.[220]

UTILIZING OUR KNOWLEDGE OF LUNG HETEROGENEITY TO DESIGN IMPROVED THERAPEUTICS

It is now exceedingly clear that the airway epithelium comprises a diverse range of cells, both common and rare, newly discovered and long known. Each cell type is vital in respiratory health, and, when dysfunctional, can cause disease. Technological and experimental advances have allowed us to better grasp the functions and location of many relevant cells, but it is the next challenge to utilize this information for therapeutic purposes. Likely, a "one size fits all" approach will be insufficient, rather, each disease will require a specific, tailored treatment. First, drug delivery methods to the airways must be revisited. A common

method used to treat respiratory diseases is inhalation of particulate substances, but different parts of the respiratory system need to be targeted for different diseases. As such, drugs for targeting bronchial pulmonary ionocytes in CF might require accessory particles of a different size/diameter than for those targeting cells deep within the alveoli.[221] There are three key factors to consider for optimal drug delivery, namely airway geometry, airway flow, and drug particle characteristics.[222] The airways form a complex system of interconnected tubes with many branch points. Hence, it must be ensured that the drug can physically reach the region of interest. Consideration of particle characteristics is particularly important, with hydrophilicity, hydrophobicity, overall solubility, tissue retention, ionic charge and, as mentioned, size, all impacting on particle flow.[223] In addition, physical properties of the airways play a role, with mucus being a major obstacle to developing efficacious drugs as it affects particle absorption and behavior.[224] Careful analysis of these parameters will be pivotal in facilitating improved drug design and administration.

Combined with knowledge of these factors, an improved understanding of airway heterogeneity, and of the specific cell subtypes involved in disease, is already proving beneficial. Targeting club cell-derived uteroglobin has been suggested to be a potential option to treat various respiratory conditions due to its small size, stability at extreme temperatures and pH levels, and resistance to proteases.[41] Goblet cell metaplasia is a common feature of chronic lung disease, and recent research has identified the ability of geldanamycin, ordinarily an inhibitor of heat shock protein 90, to prevent and, remarkably, revert already established goblet cell metaplasia.[225] These are just a small number of examples of how a greater understanding of the airway epithelium allows the development of improved therapeutics. As discussed, research already implicates pulmonary ionocyte aberrations in CF, PNEC dysregulation in SIDS and M cells in Mtb infection. Appropriately targeting these cell types (with a preclinically promising drug formulated to reach a pulmonary region of interest) will be key to achieve clinical efficacy. Together, improved knowledge of the functional and anatomical heterogeneity of cells in the lungs has been, and will remain, crucial in allowing the discovery of more effective treatments for a variety of respiratory conditions.

PERSPECTIVES

Here we have described the airway epithelium, once thought to play only a passive barrier role, as an active contributor to respiratory health. The last two decades have seen an explosion of knowledge and a consequential increased appreciation for the diversity and functional heterogeneity of epithelial cells within the airways. It is now indubitable that alveolar epithelial, basal, club, ciliated, goblet, pulmonary neuroendocrine, tuft and M cells, and pulmonary ionocytes, are individually and collectively indispensable in proper respiratory function. It is the next challenge to map in detail communication networks formed by these cells and identify those of key importance for maintaining homeostasis. It will be equally important to explore the influence of distal body compartments on the respiratory epithelium. These include the gut microbiota and microbial metabolites, which might reach the lungs via the bloodstream and interact with cells of hematopoietic and non-hematopoietic origin. Combined with technological advances in drug delivery systems into the airways, these insights will be pivotal in translating fundamental research into novel treatments for respiratory diseases.

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ADDITIONAL INFORMATION

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