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Published in:
3D Lung Models for Regenerating Lung Tissue

DOI:
10.1016/B978-0-323-90871-9.00006-1

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2022

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Hackett, T. L., Vasse, G. F., van der Does, A. M., Rae, B., Nawijn, M. C., & Heijink, I. H. (2022). The air–liquid interface model. In *3D Lung Models for Regenerating Lung Tissue* (pp. 51-72). Elsevier. https://doi.org/10.1016/B978-0-323-90871-9.00006-1

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CHAPTER

4

The air–liquid interface model

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Introduction

Lung structure and epithelium composition

Each minute, approximately 6 L of air are inhaled from the external environment through the branched airway structure of the lung for gas exchange and then are exhaled. Constituents of the inhaled environment, which contains allergens, gaseous pollutants, particulates, and pathogenic microorganisms, are deposited on the epithelium lining the airways. The cellular composition of the lung epithelium changes from proximal conducting airways to distal respiratory airways to meet the functional needs for mucociliary clearance, host defense, surface tension, and gas exchange. The main function of the conducting airway epithelium is to form a physical and chemical barrier, producing antioxidants, antiproteases, and mucus in which particles can be trapped and subsequently removed by beating cilia (mucociliary clearance). The airway epithelium also plays an important role in the innate immune system, expressing pattern recognition receptors that recognize molecular patterns from pathogenic microbes, parasites, fungi, and allergens as well as danger signals from damaged and necrotic cells to direct proinflammatory processes. Within the conducting airways, the upper airways (nasal cavity and pharynx) and lower airways (trachea, bronchi, and bronchioles) are lined with a pseudostratified columnar epithelium consisting mainly of basal progenitor cells, secretory club cells, goblet cells, and ciliated cells [1]. The last generation of conducting airways, the terminal bronchioles,

3D Lung Models for Regenerating Lung Tissue
DOI: https://doi.org/10.1016/B978-0-323-90871-9.00006-1

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consist mainly of a simple columnar epithelium of club cells with progenitor cell potential. The main function of the distal respiratory epithelium in the alveolar sacs is to provide the surface area for the exchange of oxygen and carbon dioxide to meet the metabolism requirements of the body. In the respiratory airways, which are composed of transitional bronchioles, respiratory bronchioles, and alveoli, the majority of the surface area is covered by alveolar type 1 (AT1) pneumocytes, flat-shaped epithelial cells that facilitate the transfer of oxygen and carbon dioxide to and from the bloodstream by close interaction with alveolar capillaries. Additionally, cuboidal-shaped AT2 cells are present; these maintain epithelial integrity by the production of surfactants, thereby reducing surface tension, maintaining structural integrity and binding to glycomolecules on pathogens to promote their clearance. AT2 cells also serve as progenitor cells for AT1 cells, contributing to the regeneration of alveolar tissue upon injury by reepithelialization.

In both animal models and humans, it has been shown that in the conducting airways, basal cells serve as progenitors, being able to differentiate into secretory club cells. Club cells are characterized by high expression of secretoglobin, family 1A member 1 (SCGB1A1) and can further differentiate into columnar mucus-producing goblet cells or mucus-clearing ciliated cells during homeostasis and repair processes [2–8]. Club cells have been shown to self-renew and generate ciliated cells after injury, repopulating damaged airway tissue, and also have the capacity to dedifferentiate into basal cells, as shown by lineage tracing studies in mice upon injury [9]. In mice the transition between respiratory bronchioles and alveoli, referred to as the bronchioalveolar duct junction, has also been shown to harbor a specific population of club cells that is responsible for the repair of terminal bronchioles [10]. In humans, basal cells from the conducting airways have been shown to include a stem or progenitor cell population, identified by the expression of breast cancer resistance protein, CK5, and p63, with the capacity to generate basal, club, mucus-producing, and ciliated epithelium at air–liquid interface (ALI) culture [8]. While some studies have shown that ciliated cells are terminally differentiated [11], others have reported that ciliated cells can undergo dynamic changes in cell shape and gene expression to redifferentiate into columnar cells upon injury. In the presence of type 2 cytokine interleukin 13 (IL-13), ciliated cells also undergo transdifferentiation into goblet cells [10], underscoring the plasticity of airway epithelial cells [7]. In addition, human basal epithelial cells have been reported to express progenitor markers, such as nerve growth factor receptor/CD271, integrin α6, cytokeratin (KTR5), KTR14, and transformation-related protein p63, and can generate differentiated cells when exposed to air or in organoid models, the latter of which is discussed elsewhere in this book (Chapter 5). Besides the main airway epithelial cell types, the human pseudostratified airway epithelium has been shown to contain a rare population of neuroendrocrine progenitor cells, which make up less than 1% of the epithelial layer [12], and other rare types, including brush/tuft cells and ionocytes [13].

Air–liquid interface models of the lung epithelium: what can we measure?

Many chronic lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD), and idiopathic pulmonary fibrosis, are known to involve complex changes in lung architecture and function. For valuable insights into airway epithelial responses in health and disease, in vitro models can be used to recapitulate the composition and three-dimensional (3D) structure of the pseudostratified human airway epithelial layer. As such, the ALI model was first described by Adler et al. in the early 1990s using tracheal epithelial cells from guinea pigs [14].

In this model, primary tracheal epithelial cells from guinea pigs were shown to maintain the morphologic characteristics of the differentiated epithelium in vivo. Cells were obtained by proteolytic treatment and grown on a collagen gel on top of a microporous membrane to recapitulate the basement membrane [14]. The cells were cultured in a biphasic manner with a serum-free medium comprising DME/F12, HEPES buffering, L-glutamine, insulin, transferrin, and fatty acids in the basolateral compartment and exposure to humidified 95% air/5% CO_2 in the apical compartment. In this ALI system the cells proliferated and differentiated to closely resemble epithelial morphology in situ [14]. A few years later, the first ALI culture study with human airway cells was published [15], which used airway epithelial cells derived from strips of postmortem tracheal tissue using protease treatment and from bronchial brushings [16,17]. Yamaya et al. subsequently cultured human airway epithelial cells on 0.45-μM pore inserts in serum-free DME/F12 containing the growth factors and hormones insulin, transferrin, hydrocortisone, triiodothyronine, epidermal growth factor, and endothelial cell growth supplement to retain the ultrastructure and ion transport properties of the original tissue and to form a tight barrier as assessed by transepithelial electrical resistance (TEER). To establish TEER, the epithelial cells were required to be cultured on an extracellular matrix (ECM), for instance, using a vitrogen coating.

The ALI model enables several measurements to assess the functional characteristics of the airway epithelium. First, the measurement of epithelial barrier function by TEER, which is useful over permeability with possible limitations due to the diffusion of the solute. Additionally, the mucus content in apical washes can be used as a readout for the presence or differentiation into goblet cells and/or activation of signaling programs that result in mucus secretion [18]. Ciliary beat frequency can be recorded by high-speed video microscopy as a measurement of cilia functioning [19]. Supernatants from the basolateral and apical compartments can be collected to study the secretion of cytokines and other proteins. ALI cultures can also be embedded for immunohistochemical, immunofluorescence, or electron microscopy analysis or can be harvested for DNA, RNA, or epigenetic profiling.

The measurement of barrier function using TEER has a strong advantage over the other models. It can be used to reflect the formation of a tight barrier, an important aspect of airway epithelial function, as well as epithelial damage upon exposure to insults. Epithelial barrier formation is thought to be a prerequisite for the polarization and differentiation of airway epithelial cells into a pseudostratified layer [20] (Fig. 4.1) and therefore is indispensable for recapitulation of the airway epithelium in situ or to study epithelial regenerative responses upon injury. Studies by our group confirmed that the increase in TEER measurement during ALI culture is prerequisite for successful culture with mucociliary differentiation, as depicted in Fig. 4.1, and correlates well with permeability measurements using labeled dextran [21]. TEER can be measured by using a conventional volt-ohmmeter, showing that epithelial resistance markedly increased in epithelial cells that were grown under ALI conditions, from values around $100-200\ \Omega \times cm^2$ at confluence under submerged conditions to levels above $1000\ \Omega \times cm^2$ upon mucociliary differentiation [22,23]. Epithelial barrier function is maintained by intercellular contact formation in so-called epithelial junctions. These intercellular junctions are mainly composed of tight junctions (TJs), which are located most apically, adherens junctions (AJs), and (hemi)desmosomes, which are located basolaterally. AJs regulate adhesion through homotypic interactions of the adhesion molecule E-cadherin on adjacent cells, providing the architecture that is required for TJs [24]. The major constituent of AJs is the transmembrane protein E-cadherin. E-cadherin is thought to be crucial for the formation of all other junctions, and its disruption

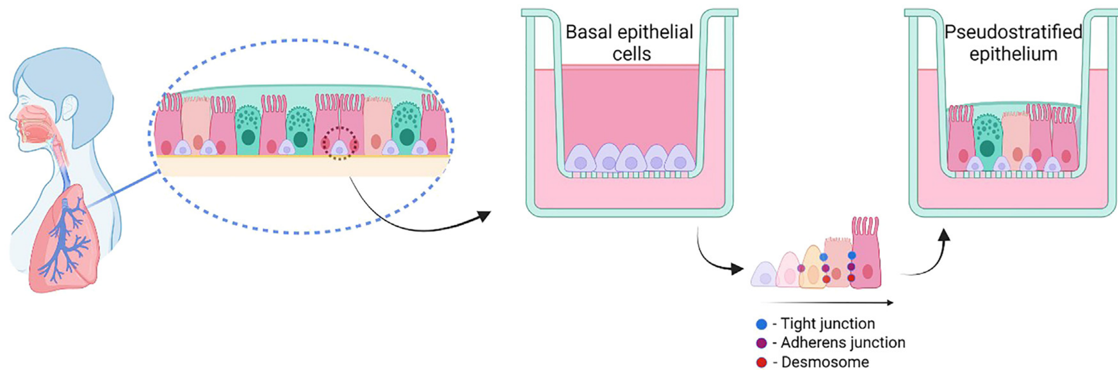

FIGURE 4.1 **The air−liquid interface (ALI) model representing the human airway epithelium.** Basal progenitor airway epithelial cells can be isolated from human lungs. Apical air exposure promotes isolated basal epithelial cells to develop tight barrier function in vitro by formation of tight junctions, adherens junctions, and desmosomes. Establishment of a tight barrier is thought to be a prerequisite for basal cells to polarize and differentiate. Over several weeks of air exposure, ALI-cultured basal cells will differentiate into pseudostratified epithelium consisting mainly of basal progenitor cells, secretory club cells, goblet cells, and ciliated cells and recapitulating the characteristics of the airway epithelium in vivo. Source: *Created with BioRender.com*.

results in delocalization of TJ proteins, as previously reviewed [24]. TJs are composed of the transmembrane proteins zona occludens-1 (ZO-1), occludin, claudins, and junction adhesion molecules and are the main regulators of epithelial permeability [24]. Desmosomes form adhesive bonds with the filament cytoskeleton between adjacent cells or between cells and the lamina propria by nonclassical cadherins [24]. Junctional expression of various of AJ and TJ proteins as well as desmosomes has been confirmed in ALI-cultured human bronchial epithelium [22,25,26]. Pell et al. have recently reported a robust and quantitative methodology to assess airway epithelial barrier integrity, using high-content imaging of junctional proteins in combination with TEER [27].

Under pathological conditions, disruption of epithelial junctions may lead to increased permeability and access of environmental factors such as pathogens to subepithelial layers, which may cause structural abnormalities and epithelial remodeling, that is, mucous hyperplasia and squamous cell metaplasia, as observed in patients with asthma and COPD [28−30]. Various insults, as will be described later in this chapter, can be used in ALI cultures to disrupt epithelial barrier function. In addition, injury of the epithelial layer can be mimicked in ALI cultures using a scratch wound model [31]. Upon this type of wounding, complete regeneration of the airway epithelium requires epithelial migration, proliferation, and reconstitution of functional intercellular junctions, followed by redifferentiation into a functionally intact epithelial layer.

For the (re-)differentiation into a mucociliary epithelium, not only are exposure to air and the presence of basement membrane ECM molecules required, but retinoic acid (RA) is also a crucial factor [32]. Without RA the epithelium became squamous, and mucin secretions drastically decreased [32]. For mucociliary differentiation upon air exposure, low concentrations of RA and seeding of the cells onto collagen plus fibronectin coating are currently most commonly used. Under these conditions, epithelial cultures established from protease digestion in bronchial tissue [25] as well as from bronchial brushes [23] have been shown to differentiate toward a mucociliary epithelium. This indicates

that cultures established from both enzymatic treatment and brushings of bronchial tissue contain cells with a basal epithelial phenotype that can (re)differentiate into columnar cell. Our group showed that the number and distribution of cells expressing the basal progenitor markers CK5/p63 in pseudostratified ALI cultures closely resembled that of airway epithelium in lung tissue and changes that in the composition of asthmatic airway epithelium are recapitulated by using this model, as will be described later in this chapter. In addition, Ghosh et al. performed RNA sequencing analysis to compare the transcriptome of fresh nasal brushings and ALI-cultured nasal epithelial cells and reported a strong correlation between the profiles with 96% of expressed genes showing similar expression, although specific pathways (including ECM organization/interactions, hemidesmosome assembly, keratinization, and tRNA-processing pathways) were affected by the culture conditions [33]. While the ALI system is suitable for investigating abnormal regenerative responses in lung disease, the successful differentiation of isolated airway epithelial cells into a pseudostratified layer is not always achieved. Of note, high variability exists not only between protocols and donors but also within donors in the ability of cells in ALI culture to consistently establish TEER levels and form a columnar epithelium [34]. In an attempt to overcome this, Gras et al. isolated epithelial cells from bronchial biopsies and demonstrated with the use of a logistic regression model that ALI culture success was independently associated with the presence of living ciliated cells within the initial biopsy [35].

For translation to the in vivo situation, the ECM forms an important component of in vitro models to study airway epithelial repair. In vivo, interaction with and remodeling of the ECM play a central role in epithelial cell migration, proliferation, and restoration of the barrier during wound repair. Epithelial release of growth factors such as transforming growth factor beta, which activate fibroblasts and myofibroblasts, promotes excessive deposition of ECM components, such as collagens and fibronectin, in the lamina reticularis just below the basement membrane [36]. ALI culture forms a solid basis for more complex models, including not only 3D ECM structures but also additional cell types such as stromal cells as well as endothelial cells and immune cells. For example, we have previously reported on coculture models of ALI-cultured primary epithelial cells with fibroblasts and T lymphocytes [37,38].

Unlike primary cells, cell lines are in general not suitable for studies of regeneration of mucociliary epithelial layers upon injury, not being able to differentiate into columnar epithelial cells. There are exceptions; for instance, Calu-3 cells produce mucus upon air exposure, reflecting differentiation into goblet cells, while the immortalized human bronchial epithelial cell line BCi-NS1.1 was shown to differentiate into a ciliated epithelium upon ALI culture [19,39]. The frequently used cell line 16HBE14o- is, to the best of our knowledge, not capable of mucociliary differentiation. However, these cells do form a tight barrier upon air exposure, with the establishment of TEER levels that are comparable to those in primary cells [22], and thus constitute a suitable model to study epithelial barrier function and its recovery upon injury. Finally, also human-induced pluripotent stem cells have been used to successfully establish ALI cultures expressing basal progenitor markers such as KRT5, P63, SCGB1A, goblet cell marker MUC5B, and ciliated cell marker FOXJ1 [40,41].

In the next section of this chapter, data from single-cell analysis will be described to provide comprehensive insight into the basal cell states and differentiated cell types that are present upon ALI culture in comparison to those that are present in fresh brushings and those that are present in the organoid model. The ALI model is a suitable model to study the effects

of inhaled drugs or the interaction of combined therapies. For example, pharmacodynamic and pharmacokinetic interactions between salmeterol and fluticasone particles have been studied upon their codeposition on ALI-cultured Calu-3 cells. Furthermore, ALI offers the ability to expose cells to gaseous phase particles, including cigarette smoke [42], mimicking the real-life situation of inhaled toxicants more closely than is possible with the use of cigarette smoke extract. The exposure of ALI-cultured epithelial cells to cigarette smoke as well as various other inhaled insults, including respiratory viruses such as respiratory syncytial virus (RSV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), will be discussed later in the chapter. Finally, we highlight the recapitulation of epithelial phenotype in ALI cultures of epithelial cells obtained from patients with asthma and COPD.

Single-cell RNA sequencing analysis of air–liquid interface–differentiated cell subtypes and states: how well do these represent the airway epithelium in vivo?

As was described in the chapter introduction, the airway epithelium is characterized by extensive cellular heterogeneity and plasticity [43]. ALI cultures are widely used as the in vitro model that most closely resembles the airway epithelial cell-type composition and cell-cell interactions that are observed in vivo. Immunohistochemical, RNA, and protein analyses show that the main epithelial cell types are all observed in well-differentiated ALI cultures, validating their use as a relevant model for airway epithelial function in vivo [44]. More recently, the application of single-cell RNA sequencing (scRNA-Seq) has facilitated the highly detailed characterization of cellular heterogeneity of the airway epithelium in nasal or bronchial brushes and biopsies as well as in ALI-cultured epithelial cells sourced from these locations. Initial scRNA-Seq studies involved ALI cultures of primary human nasal [45] and tracheal [46,47] epithelial cells. During the COVID-19 pandemic a number of studies have been performed employing ALI cultures as an optimal model to study the SARS-CoV-2 cell entry factor gene expression [48,49] as well as SARS-CoV-2 induced responses of the nasal and bronchial epithelium [50]. From these studies, it is clear that the ALI culture model represents an actively differentiating epithelium with relatively high proportions of (proliferating) basal cells. At baseline, five main epithelial cell types (with marker genes) can be distinguished: basal (KRT5 and TP63), suprabasal (KRT5 and KRT13 but lacking TP63), club (SCGBA1 but lacking MUC5AC/B), goblet (MUC5AC and/or MUC5B), and multiciliated cells (FOXJ1 and PIFO) as well as several rare cell types [13]. The first study to report on ALI-cultured upper airway epithelial cells also identified a discrete precursor to the multiciliated cell called the deuterosomal cell, identified by the expression of CDC20B [45]. Deuterosomal cells were subsequently also (reproducibly) observed in nasal and bronchial epithelial cells that had been freshly obtained from brushes or biopsies [45,46,51,52] or cultured at ALI [46]. A direct comparison between ALI cultures and freshly obtained nasal epithelial cells reveals that the relative proportion of basal to differentiated epithelial cells (goblet, multiciliated) strongly depends on duration after airlift and the culture media that are used [45]. For lower airway epithelial cells derived from tracheal brushings, the cell-type proportions stabilized around 14 days after airlift [46]. To directly compare molecular cell states (or transcriptional phenotypes), the individual epithelial cell subsets between ALI and nasal or bronchial brushes were analyzed directly ex vivo. Therefore we integrated data from bronchial biopsies obtained at the fourth- to sixth-generation airway by bronchoscopy in healthy control subjects with primary bronchial epithelial cells (sampled by bronchial brush at this same anatomical location in healthy control subjects) grown at ALI conditions and 3D spheroid culture conditions

according to an established bronchial epithelial organoid culture protocol and annotated cell types, using the labels from our initial description of bronchial epithelial cell states observed in bronchial biopsies from healthy controls and asthma patients [53]. From this analysis (Fig. 4.2) it becomes apparent that most epithelial cell subsets that are observed in vivo are also observed in ALI cultures, yet both ALI and spheroid cultured primary bronchial epithelial cells display low frequencies of unique cell states that are not observed in vivo, at least not under homeostatic conditions. Also, cell-type proportions differ in both culture models, with ciliated cells being present in lower numbers, especially in the 3D spheroid cultures, while basal cell subsets are increased in both spheroid cultures ("basal 2," which are suprabasal cells) and ALI cultures (activated basal cells) compared to brushes. The ALI culture media that were used here were also associated with lower frequencies of well-differentiated epithelial cells in nasal ALI cultures [45]. Therefore depending on the research question at hand, either 3D spheroid or ALI culture models can be applied, taking into account that resting, fully differentiated multiciliated cells will be present in lower numbers, while intermediate phenotypes will be overrepresented, making these culture models especially suited for the analysis of dynamic cell state transitions that are relevant to airway diseases such as airway epithelial (de)differentiation or repair processes. For ALI cultures, the selection of culture media will strongly determine the proportions of differentiated in basal epithelial cells and is therefore an important part of the experimental design of the study.

Recapitulation of epithelial phenotype in air–liquid interface cultures from asthma patients

Asthma affected an estimated 262 million people and caused over 461,000 deaths worldwide in 2019 [54]. The disease is characterized by chronic airway inflammation (which forms the target of all asthma therapeutics) and airway remodeling (for which there are no therapeutics) in response to inhaled allergens, smoke, and particulate matter. Airway remodeling has been documented for all stages of asthma severity and has been linked to reduced lung function, airways hyperresponsiveness, and greater use of asthma medications [55–58]. Features of airway remodeling include a damaged epithelium, increased mucus production, thickening of the basement membrane, increased deposition of the ECM within the lamina reticularis, increased numbers of myofibroblasts, and smooth muscle mass. Compelling evidence within the literature indicates that impairment of epithelial barrier function in asthma is a key player in airway inflammation and remodeling in the disease process [30].

Studies of the airway epithelium in situ have revealed structural changes in the epithelium of asthmatic patients, including disruption of TJs, detachment of ciliated cells, reduced expression of E-cadherin and other cell-cell adhesion molecules, and increased numbers of goblet cells and $KRT5^{+}/p63^{+}$ basal progenitor cells [22,59]. In line with these findings, functional studies of airway epithelial cells derived from children and adults with asthma cultured with ALI have replicated decreased expression of junctional molecules (e.g., E-cadherin, caveolin-1), increased permeability, increased numbers of basal cells as shown in Fig. 4.3, and a greater sensitivity to environmental insult compared with healthy controls. This has enabled the use of epithelial models to study the mechanisms of asthma in vitro.

Although the mechanisms contributing to the loss of airway epithelial barrier function in asthma have not been fully elucidated, a number of intrinsic and extrinsic factors have been shown to play a role. Several genome-wide association studies have found genes and

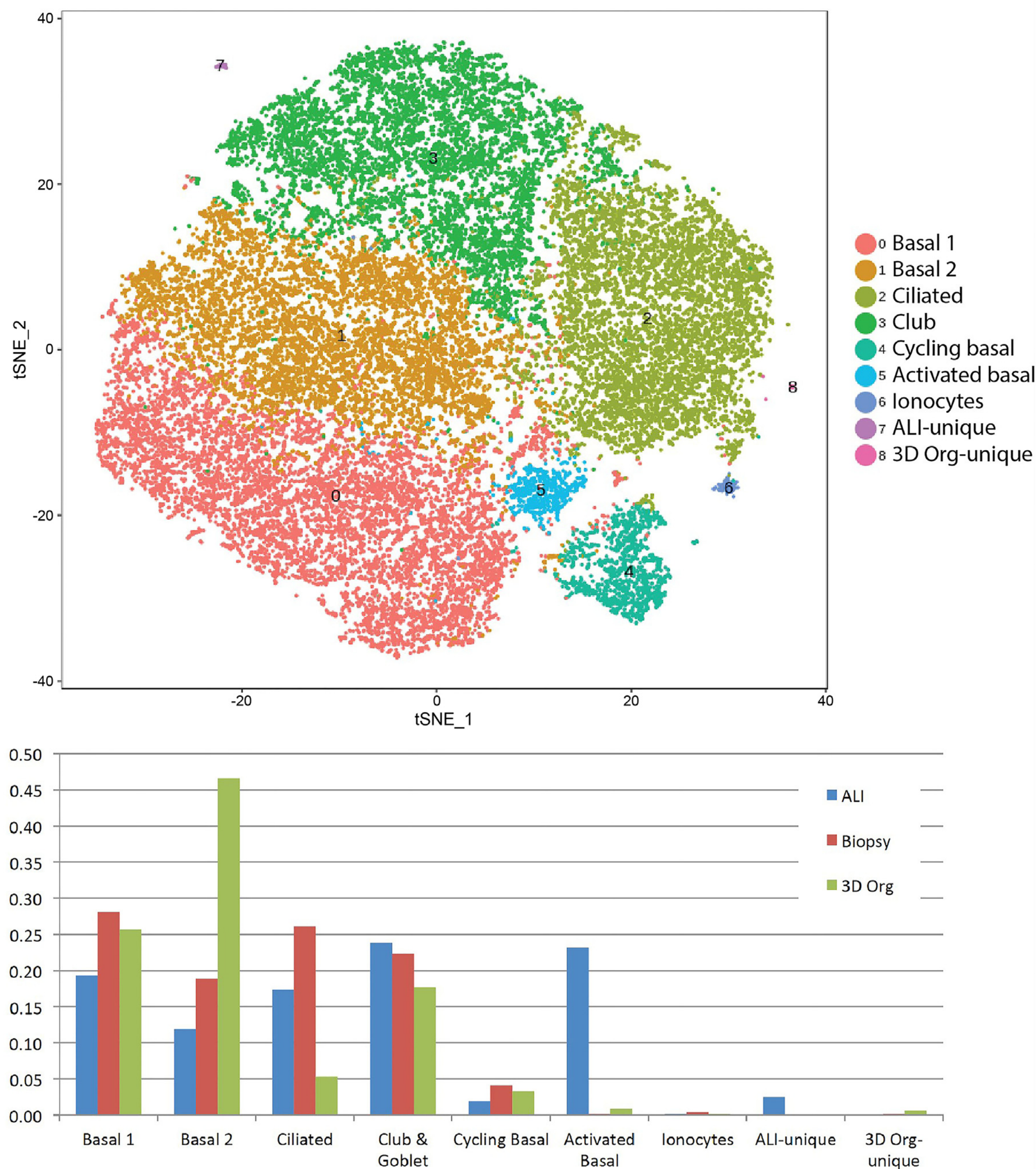

FIGURE 4.2 Analysis of primary bronchial epithelial cells from bronchial biopsies and in ALI and 3D spheroid cultures. Primary bronchial epithelial cells obtained by bronchial brushing of fourth- to sixth-generation airway of healthy control subjects were cultured under ALI conditions for 24–28 days or in 3D organoid cultures for 14 days and harvested for scRNA-Seq. Analysis was performed by merging the data with those of epithelial cells obtained in bronchial biopsies from healthy controls and clustering of the merged object. Cell-type annotation was performed, using the labels from Viera Braga et al. [53], and only small clusters exclusively containing cells from either ALI or 3D organoid cultures were observed. Relative proportions of the cells in the different clusters are shown in the bar graph, indicating that in 3D and ALI cultures, lower proportions of ciliated cells were observed compared to airway wall biopsies, while the number of activated basal cells (in ALI) or suprabasal (basal 2, in 3D organoids) is increased in the culture models.

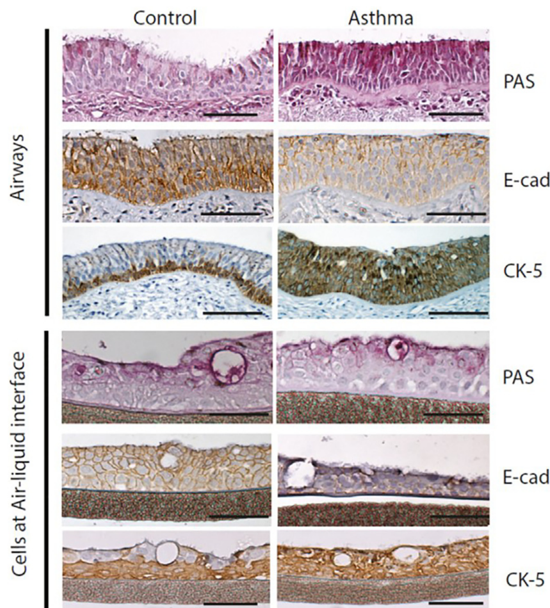

FIGURE 4.3 **Patient-matched airway sections and epithelial cells cultured at the air–liquid interface from a representative asthmatic patient and a nonasthmatic subject.** Sections were immunostained for periodic acid–Schiff (PAS) (stained in purple), E-cadherin(E-cad), and cytokeratin 5 (CK5). Scale bar = 100 μM. Source: *Adapted with permission of the American Thoracic Society. Copyright © 2022 American Thoracic Society. All rights reserved. Cite: Hackett, T.-L., Singhera, G. K., Shaheen, F., Hayden, P., Jackson, G. R., Hegele, R. G., et al. (2011). Intrinsic Phenotypic Differences of Asthmatic Epithelium and its Inflammatory Responses to Respiratory Syncytial Virus and Air Pollution. Am. J. Respir. Cell Mol. Biol. 45, 1090–1100. The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society. Readers are encouraged to read the entire article for the correct context at https://doi.org/10.1165/rcmb.2011-0031OC. The authors, editors, and The American Thoracic Society are not responsible for errors or omissions in adaptations.*

genetic loci associated with asthma that are expressed by the epithelium [60,61]. Asthma risk genes that are involved in cell adhesion and airway epithelial barrier function include PCDH1 (protocadherin1) [62], ORMDL3 (orosomucoid-like 3) [63–65], DPP10 (dipeptidyl peptidase 10) [66], and GPRA (G protein–coupled receptor for asthma susceptibility) [67]. CDHR3 (cadherin-related family member 3) has also been shown to be involved in cell adhesion and polarity and moreover is the receptor for rhinovirus (RV) C; thus variants of this gene can modulate the susceptibility to infection [68–70]. MUC5AC, MUC5B [71,72], and CLCA1 (calcium-activated chloride channel regulator 1) [73] variants are predicted to cause increased mucin production, and variants in TSLP and IL-33 lead to an increase in epithelial alarmins. This has been reflected in ALI cultures, in which higher levels of TSLP and IL-33 in basal supernatants of ALI-cultured epithelial cells have been shown from asthma patients compared to healthy controls [74]. These findings indicate that genetic defects or variations within the airway epithelium can cause, drive, or worsen asthma, most likely through interactions with the environment.

In terms of extrinsic factors, allergens, viral infections, and Th2 inflammation are strongly associated with the pathogenesis of asthma. Several in vitro studies using airway epithelial-ALI cultures have shown that protease-containing allergens, such as house dust mites, *Alternaria* fungi, and various pollen allergens [75,76], can directly via cleavage of epithelial TJ proteins or indirectly via epidermal growth factor receptor transactivation and protease-activated receptor 2 activation [77] disrupt the epithelial barrier, causing increased permeability and decreased TEER. Infections with respiratory viruses, such as RSV, have shown that infection disrupts epithelial TJs by activation of protein kinase D [78]. Studies focused on RV infection have shown that infection causes loss of TJs (ZO-1) in ALI-cultured airway epithelial cells from asthmatic children. This loss in barrier function is more pronounced and sustained in cells from children without asthma [79,80]. A reduction in occludin expression in a NADPH-oxidase-dependent manner has also been implicated in RV infection [81]. Recent coculture ALI models have shown that Th2 cytokine IL-13 released from ILC2 cells increases epithelial barrier permeability and reduces the expression of junctional proteins claudin-18, ZO-1, occludin, E-cadherin, and β-catenin [82,83].

Together, these studies demonstrate that intrinsic and extrinsic alterations in the airway epithelium can be modeled in vitro by the culture of airway epithelial cells at ALI using healthy versus asthma patient-derived epithelial cells.

Recapitulation of epithelial phenotype in air–liquid interface cultures from chronic obstructive pulmonary disease patients

According to the World Health Organization, COPD was the third leading cause of death worldwide in 2020, causing over 300 million deaths. COPD is a prevalent, disabling lung disease characterized by mucus hypersecretion (chronic bronchitis), small airway wall remodeling and reduction [84], and destruction of functional gas exchange units (emphysema). COPD is caused by the inhalation of noxious particles in combination with genetic and personal factors [85], such as age and lifestyle, which triggers predominantly neutrophilic inflammation and progressive lung tissue damage, leading to airflow limitation. Exposure to cigarette smoke is the main risk factor for COPD. Cigarette smoke consists of gaseous and particulate components and contains more than 7000 chemicals, including radicals and heavy metals. Inhaled smoke first encounters the airway epithelia, inflicting oxidative stress [86], damage, and immunogenic cell death [87], the initial pathological changes that are induced by smoking. This may eventually contribute to the development of COPD, leading to sustained recruitment of immune cells, tissue damage, and remodeling, including squamous metaplasia, mucus hypersecretion, and loss of ciliary beating in the airway epithelium. The loss of ciliary function is accompanied by affected cilia length and cilia recycling by a selective autophagy pathway, known as ciliophagy [88]. Cigarette smoking induces changes in the airway epithelium, leading to goblet cell hyperplasia [89–91], contributing to airway obstruction. In addition, oxidative stress induced by cigarette smoke disrupts the junctions between adjacent epithelial cells. In vivo models have shown that cigarette smoke induces the permeability of the airway mucosa [92]. We and others have previously demonstrated that in vitro, cigarette smoke transiently impairs epithelial barrier function, disrupting occludin and ZO-1 junctional expression [93,94]. Moreover, Milara et al. demonstrated that cigarette smoke extract reduces the expression of E-cadherin and ZO-1 in primary epithelial cells from COPD patients but not in those from control smokers [95]. In line with these findings, it has been reported that cigarette smoking reduces known apical junction genes in the airway epithelium, of which the majority is further reduced in lung tissue of COPD patients compared to that of smokers with a normal lung function [96]. Studies from our group confirmed the airway epithelial loss of E-cadherin expression and junctional ZO-1 expression in lung tissue from COPD patients who had formerly smoked [21,97].

To closely model the airway epithelium in COPD, the airway epithelial changes described above should be recapitulated in vitro, including loss of epithelial junctions, mucus hypersecretion, and ciliary impairments. RNA sequencing analysis upon intermittent whole cigarette smoke exposure during 28-day ALI culture of small airway epithelial cells from three non-COPD controls and three COPD patients (commercially obtained from lung tissue by enzymatic treatment) demonstrated deregulation of marker genes for basal and secretory cells (with higher expression of, e.g., CK5, CK13, P63, CD44 and MUC5AC and lower expression of SCGB1A and ciliated cell marker FOXJ1). A high degree of similarity was observed with signatures from a published dataset of bronchial epithelial brushings from smokers and nonsmokers [98]. These changes were accompanied by decreased epithelial barrier integrity (lower TEER and altered expression of several junctional proteins) in both control and COPD-derived cultures. We have demonstrated that the disrupted expression of ZO-1 and occludin of ex-smokers with severe

COPD is represented in ALI (re)differentiated airway epithelial cells [21]. Bronchial epithelial cells that were obtained by protease digestion from transplant recipient lungs of six former smokers with GOLD stage IV COPD were compared to cells from the lungs of eight non-COPD transplant donors. The disrupted expression of TJ proteins was accompanied by significantly lower barrier function upon submerged culturing, as measured by resistance using electrical cell-substrate impedance sensing. Similar findings were obtained by Gohy et al., who cultured primary bronchoepithelial cells from surgical lung tissue at the ALI [99]. Airway epithelial staining in lung tissue showed lower expression of ZO-1 in mild-to-moderate COPD compared to nonasymptomatic smokers, while E-cadherin expression was lower compared to that of nonsmokers. In vitro, ALI-differentiated bronchial epithelial cells from patients with mild-to-moderate COPD showed lower ZO-1 and E-cadherin expression than in cells from controls [99]. Similarly, Gohy et al. demonstrated that the epithelium in the large airways of COPD patients, when compared to the epithelia of smoking and nonsmoking controls, showed lower numbers of ciliated cells and lower expression of the ciliated cell marker β-tubulin IV. In addition, higher numbers of goblet cells and expression of one of the major glycoproteins of mucus, mucin (MUC)5AC, were seen in the COPD patients. Importantly, both features were recapitulated in the ALI-cultured epithelium from COPD patients [99]. Accordingly, our group has shown that COPD-derived ALI-cultured epithelial cells have higher levels of MUC5AC transcription factor SAM-pointed domain-containing Ets-like factor (SPDEF) than those from non-COPD controls [100]. Finally, COPD and non-COPD subject-derived ALI cultures have demonstrated differences in host defense responses between COPD patients and controls [101].

Together, these studies demonstrate that changes in the airway epithelial layer in COPD are well represented in vitro by the culture of airway epithelial cells at the ALI. Future studies should establish whether changes in communication with other cell types in coculture models can also be represented in ALI culture models and whether implementation of the altered ECM will provide a model that reflects the in vivo situation even more closely.

Use of air–liquid interface culture to study effects of environmental insults: respiratory virus

Infection of the lung epithelium with common viruses such as RV, RSV, influenza, and now SARS-Co-V2 are major causes of morbidity and mortality globally [102–104]. People with chronic respiratory diseases such as asthma and COPD are also more susceptible to viral infection and suffer more severe consequences of the infection, resulting in disease exacerbations [105,106]. Infection is typically initiated by exposure of the respiratory tract to viral particles through the inhalation of contaminated aerosols and droplets. The virus then binds to several surface receptors to endocytose, and once inside host epithelial cells, the virus uncoats and initiates the viral replication process. For example, RVs bind to intercellular adhesion molecule 1 (major group) or low-density lipoprotein receptors (minor group), and SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2), whereas influenza attaches to sialic acid residues. The epithelium senses viral nucleic acids upon their binding to pattern recognition receptors (PARs). In humans these include transmembrane toll-like receptors (TLRs) at the cell surface (TLR2 and TLR4) and in endosomes (TLR3, 7, 8, and 9) and cytoplasmic sensors, which include R-inducible gene (RIG)-I-like receptors, such as RIG-1 and melanoma differentiation-associated protein (MDA5), and nucleotide-binding oligomerization domain-like receptors (NLRs) such as NOD2 and NLRP3. The activation of these PARs results in the production of immune defense genes, including interferons

(IFNs), IFN-stimulated genes, and direct physical interactions with leukocytes that augment host defense mechanisms to kill the invading pathogens [107,108]. Subsequent humoral and dendritic cell–induced cell-mediated immunity results in the generation of B cells and $CD4^+$ T helper and $CD8^+$ cytotoxic that further contain the infections.

ALI cultures have been used to assess the infection of the upper (nasal) and lower (conducting and respiratory) airways with many human respiratory viruses [109,110]. Epithelial generation of IFNs is essential for effective antiviral responses and viral clearance. In terms of chronic diseases the airway epithelium from asthmatic patients compared with that of healthy individuals has been shown to have impaired IFN production [111] that is correlated with exacerbation severity [112–116]. Recently, the lung epithelial-ALI model has been used extensively to model SARS-CoV-2 infection in the lung [117–119] and for virus isolation [120]. Notably, differentiation of epithelial cells in ALI culture results in a drastic increase in the expression and the polar presentation of the viral receptor ACE2 [103,121] on the apical membrane [109,110]. The model has also been used to demonstrate that SARS-Co-V-2 predominantly targets nasal epithelial cells [48,122], ciliated cells, but also goblet cells, expressing MUC5AC [109,123]. Long-term modeling (51 days) of SARS-CoV-2 infection leads to airway epithelial damage, disruption of TJs, and loss of cilia [124]. The ALI culture system has also been used to study the effect of therapeutic strategies for COVID-19, which included the known antiviral cytokine IFNB1 and remdesivir and hydroxychloroquine, which are investigational drugs for COVID-19 treatment [125].

Together, these studies demonstrate that airway epithelial cells cultured at the ALI provide an important model to understand the detrimental effects of respiratory infections, reflecting viral responses in vivo, which cannot always be modeled by using animal models.

Use of air–liquid interface culture to study effects of environmental insults: cigarette smoke

Over the years, studies using ALI cultures of human epithelial cells have demonstrated their relevance for increasing our understanding of the harmful effects of cigarette smoke on the human lung epithelium. In particular, ALI models showed their importance as they allow differentiation of airway epithelial basal cells into a pseudostratified epithelium, as described earlier. Expression of genes related to xenobiotic metabolism, which is highly relevant for compounds derived from cigarette smoke exposure, were shown to be dependent on the differentiation status of the epithelium [126]. Several genes related to xenobiotic metabolism are relatively low in expression in undifferentiated submerged cultures, whereas upon differentiation at the ALI they increase [126]. This implies that ALI-epithelial differentiated cultures are more relevant for studying the effects of cigarette smoke exposure compared to undifferentiated cultures. To mimic cigarette smoking, studies mainly report the use of either water-soluble extract or condensate from cigarette smoke [127,128], whole cigarette smoke [129,130], or, more recently, e-liquids and vapors [131–133]. Whereas exposure to liquid (extracts) is straightforward, it covers the apical air-exposed side of the epithelial layer with a volume of fluid that exceeds the volume of the epithelial lining fluid. Furthermore, it comprises a selection of (water-soluble) mediators compared to whole cigarette smoke and does not allow studying the contribution of short-lived oxidants. On the other hand, many important compounds are still represented in the soluble fraction, including lipophilic compounds such as toxic aldehydes [86], that can pass the cell membrane. Use of smoke extract has provided valuable insights into effects on, for example, mitochondrial reactive oxygen species production, goblet cell differentiation, barrier dysfunction, and WNT

signaling [86,128,134,135]. Whole cigarette smoke is also applied to ALI cultures, which is especially relevant, as it allows the gaseous and particulate phase, as in smoking individuals, to be fused over the cells.

The use of many different exposure systems throughout the literature to expose ALI cultures to whole cigarette smoke contributes heavily to the complexity of data interpretation. Exposure systems are either commercial or custom made, resulting in differences between exposure duration, particle deposition, and intensity of the exposure [136,137]. Despite these differences, studies comparing transcriptional responses of cigarette smoke–exposed ALI cultures with those obtained from human studies showed overlap in for example xenobiotic metabolism pathways and antioxidant responses [138]. Additionally, established gene signatures derived from a variety of human cigarette smoke studies showed overlap with sets of genes that were differentially expressed in exposed ALI cultures [139]. Whole cigarette smoke is often applied to ALI cultures as a single, rather short exposure to study acute effects of cigarette smoke on, for example, inflammatory responses and/or barrier function [140,141]. Repeated exposures to cigarette smoke have been used to study more chronic effects, and for this, whole cigarette smoke is applied during repetitive exposures, often over a time course of days to weeks. This approach has allowed for the assessment of the effects of cigarette smoke on epithelial differentiation and remodeling [140,142,143]. In addition, consequences for functional responses of the epithelium including mucus secretion [144], barrier function [145], inflammation [146], ciliary functioning [147], and host defense [140,148] were established. With these repetitive applications, exposure to cigarette smoke seems to promote features that reflect COPD-related epithelial remodeling, such as increased numbers of goblet cells [142], whereas other researchers have reported impairment of differentiation of the epithelium in ALI culture [143] and expansion of a basal cell subset characterized by KRT6A expression that is associated with squamous differentiation [141]. These outcomes are likely explained by differences in cigarette smoke exposure and duration and whether smoke was applied during or after differentiation of the epithelial culture.

Primary epithelial-ALI cultures increasingly include additional cell types, such as fibroblasts [149] or immune cells [150], to assess disturbed crosstalk in the context of exposure to cigarette smoke. Here, the cocultured cells often have a supportive function by dampening stress responses or promoting wound repair [149,150]. Besides modeling airway epithelial responses, the ALI model could also be relevant in modeling responses of the alveolar epithelium to whole cigarette smoke, although only a few studies have addressed this particular setup. Isolation and culture of primary alveolar type II cells have only recently become more straightforward [151,152]; therefore studies mainly include the use of cell lines combined with cigarette smoke extract exposure.

Overall, the ALI cell culture model is highly suitable for studies addressing lung epithelial cell responses to airborne inhaled toxicants such as cigarette smoke. The variability between protocols for obtaining cigarette smoke extract or whole cigarette smoke does complicate the interpretation of results (e.g., the intensity of cigarette smoke exposure), and it will be important to further standardize these for future initiatives to refine and replace animal experimentation, for example, for drug-screening studies and studies of inhalation toxicology.

Use of air–liquid interface culture to study effects of environmental insults: air pollution

While the presence of cigarette smoke is perceptible upon inhalation, many other air pollutants are inhaled unnoticeably, both indoors and outdoors. Ambient air can be polluted by gaseous

substances, heavy metals, particulate matter, and organic substances [153]. Associations between air pollution and lung health are gaining more and more attention, especially in light of the SARS-CoV-2 pandemic. However, causal relationships are difficult to establish [154−157]. Exposure of ALI cultures to air pollution can give valuable insights into the possible mechanisms by which pollutants could affect the integrity and function of the lung epithelium.

Only a few studies have investigated the effect of local ambient air on cell behavior in ALI cultures, revealing clear changes in inflammatory gene expression when compared to clean air and even seasonal effects were found [158,159]. However, to investigate which pollutants are responsible for the observed harmful effects of ambient air, single components or mixtures from a specific source (such as a diesel engine) can be tested in ALI cultures in a dose-dependent manner. Exposure of epithelial cells in ALI culture to the exhaust of a diesel engine has been associated with increased expression or secretion of inflammatory and oxidative stress markers [160−162], matching the findings of controlled human exposure studies [163,164]. Furthermore, diesel exhaust has been shown to decrease the epithelial barrier function upon prolonged exposure, as demonstrated by TEER measurements [162]. Exposure of primary human bronchial epithelial cells to the exhaust of engines fueled by different ratios of diesel and biodiesel indicated clear variation in cell viability and the expression and secretion of antioxidants and inflammatory mediators, showing that biodiesel does not necessarily result in reduced adverse effects and emphasizing the sensitivity of this culture system [165].

Particulate matter is a complex mixture of particles of various sizes and compositions. The particulate matter that is found in diesel exhaust is thought to be the major contributor to the damaging effects, as exposure of ALI cultures to only diesel particulate matter has been shown to affect inflammation and oxidative stress in a way similar to that of whole diesel exhaust [166,167]. Particulate matter from other sources has also been linked to proinflammatory effects and reduced barrier integrity in ALI-cultured epithelial cells [25,168−172]. The diameter of particulate matter is believed to be an important variable in these responses, as smaller particles generally induce amplified responses in these culture systems [168,173].

In addition to particulate matter, a plethora of gaseous substances are present in polluted air that may affect lung health. Ozone (O_3), the main component of smog, has been shown to induce a proinflammatory response in ALI-cultured primary human bronchial epithelial cells, while its precursor NO_2 increased the expression of oxidative stress−associated genes [174] Volatile organic compounds, such as toluene and benzene, can also induce oxidative stress and inflammation in lung epithelial cells [175].

Although airborne particles and gaseous substances can be applied to ALI cultures in different doses to assess their effect, it is rather challenging to deposit the pollutants in a physiologically and environmentally relevant manner. The development of sophisticated exposure systems that facilitate prolonged and homogeneous exposure to air pollutants is essential to improve the translatability of these in vitro findings [176,177]. Furthermore, the use of fully differentiated human primary epithelial cells in ALI culture seems to be of great importance to optimally mimic the in vivo situation, as the response of these cells to air pollution is often dampened in comparison to the response of submerged primary epithelial cell cultures and immortalized cell lines [168,173,178−181]. Extracellular defense mechanisms in differentiated primary epithelial cells, such as mucociliary particle clearance, are likely to be responsible for this protective effect [168,182]. ALI cultures of primary human epithelial cells can also be applied to investigate disease-related sensitivity to air pollution, as

these cells preserve their healthy or diseased phenotype when cultured at the ALI. For example, bronchial epithelial cells from COPD patients in ALI culture are more sensitive to diesel exhaust and air pollution−derived particulate matter than non-COPD controls.

In conclusion, ALI cultures are suitable models to unravel the effects of air pollution on epithelial barrier function and integrity in health and disease. Although it is challenging to fully mimic in vivo exposure conditions in vitro, findings thus far do support the associations between air pollution and lung health.

Concluding remarks

The ALI culture model is highly useful for the study of airway epithelial responses in health and disease, particularly for investigating changes in barrier function, abnormal regenerative responses in lung disease, responses to inhaled gases and particulates, and innate immune responses. ALI culture allows epithelial cells to retain ion transport properties and ultrastructure of the original tissue and to form a tight barrier. Readouts in ALI include TEER measurement, quantitative analysis of mucus production, ciliary beat frequency measurement, analysis of soluble cytokines and other proteins, immunohistochemical analysis, and DNA/RNA isolation for (epi)genetic and transcriptomic profiles. In particular, the measurement of TEER is a strong advantage of the ALI model above other 3D models (e.g., organoids) as well as animal models, as it can be used to reflect the formation of a tight barrier, an important aspect of airway epithelial function, as well as epithelial damage upon exposure to insults. Intrinsic and extrinsic alterations in the epithelium of both asthma and COPD patients can be modeled well in ALI cultures. Furthermore, the ALI cell culture model is highly suitable for studies of the detrimental effects of lung epithelial cell responses to gas phase substances such as cigarette smoke or respiratory infections. Finally, although it is challenging to fully mimic in vivo exposure conditions in vitro, ALI cultures are suitable models to unravel the effects of air pollution on epithelial barrier function and integrity in health and disease.

Recent scRNA-seq data underscore that resting, fully differentiated multiciliated cells are present in lower numbers in ALI cultures compared to the human airway epithelium in vivo, while intermediate phenotypes are overrepresented. This makes ALI culture models especially well suited for the analysis of dynamic cell state transitions relevant to airway diseases, such as airway epithelial (de)differentiation or repair processes. Another limitation of the ALI culture is the variability between successful differentiation of isolated airway epithelial cells into a pseudostratified layer. Furthermore, there is variability between protocols for tissue culture media, such as coating with ECM and obtaining standardized cigarette smoke extract or whole cigarette smoke, which complicates the interpretation of results. In the future it will be important to standardize such methodologies to enable the use of the ALI model for drug-screening studies and replace animal experimentation. Future studies will also be important to establish if communication with other cell types in coculture models can also represent the in vivo environment more closely.

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