Airway epithelial cell cultures for studying obstructive lung disease effects of IL-13 and cigarette smoke

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CHAPTER 2

USE OF AIRWAY EPITHELIAL CELL CULTURE TO UNRAVEL THE PATHOGENESIS AND STUDY TREATMENT IN OBSTRUCTIVE AIRWAY DISEASES

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ABSTRACT

Asthma and chronic obstructive pulmonary disease (COPD) are considered as two distinct obstructive diseases. Both chronic diseases share a component of airway epithelial dysfunction. The airway epithelium is localized to deal with inhaled substances, and functions as a barrier preventing penetration of such substances into the body. In addition, the epithelium is involved in the regulation of both innate and adaptive immune responses following inhalation of particles, allergens and pathogens. Through triggering and inducing immune responses, airway epithelial cells contribute to the pathogenesis of both asthma and COPD. Various *in vitro* research models have been described to study airway epithelial cell dysfunction in asthma and COPD. However, various considerations and cautions have to be taken into account when designing such *in vitro* experiments. Epithelial features of asthma and COPD can be modelled by using a variety of disease-related invoking substances either alone or in combination, and by the use of primary cells isolated from patients. Differentiation is a hallmark of airway epithelial cells, and therefore models should include the ability of cells to differentiate, as can be achieved in air-liquid interface models. More recently developed *in vitro* models, including precision cut lung slices, lung-on-a-chip, organoids and human induced pluripotent stem cells derived cultures, provide novel state-of-the-art alternatives to the conventional *in vitro* models. Furthermore, advanced models in which cells are exposed to respiratory pathogens, aerosolized medications and inhaled toxic substances such as cigarette smoke and air pollution are increasingly used to model e.g. acute exacerbations. These exposure models are relevant to study how epithelial features of asthma and COPD are affected and provide a useful tool to study the effect of drugs used in treatment of asthma and COPD. These new developments are expected to contribute to a better understanding of the complex gene-environment interactions that contribute to development and progression of asthma and COPD.

INTRODUCTION

Asthma and chronic obstructive pulmonary disorder (COPD) are common disorders and affect 1 out of 12 people worldwide. Asthma and COPD are chronic inflammatory diseases characterized by airway obstruction which is reversible in asthma and often irreversible in COPD (1). Another important feature of COPD, and occasionally in severe asthma, is emphysema whereby the alveolar tissue is destroyed, resulting in impaired oxygen exchange (1-3). Since this review focuses on airway epithelial cells, studies investigating alveolar epithelial cells and their role in the development of emphysema are outside its scope. Inflammation of the airways is present in both asthma and COPD, but in asthma it affects mainly the conducting airways whereas in COPD it affects primarily the small airways, likely reflecting the distribution of inhaled provoking substances, such as allergens in asthma and cigarette smoke in COPD. Despite being different disease entities, both asthma and COPD share an important component of epithelial dysfunction (4, 5).

Approximately 20 to 35% of the world population smokes, with surprisingly similar smoking rates reported in patients with asthma (6-8). Cigarette smoking has been shown to worsen asthma symptoms, reduce responsiveness to corticosteroid treatment, accelerate lung function decline and increase exacerbation rates (9). In contrast, various characteristics typically assigned to asthma have also been found in patients with COPD, including reversibility of airway obstruction, atopy and T helper 2 (Th2)- mediated inflammation (1). Importantly, asthma and COPD share various dysfunctional features of the airway epithelium, in addition to several other disease features (4).

The epithelium of the conducting airways is a pseudostratified epithelial layer that comprises basal, ciliated and secretory cells. The epithelial barrier function in both asthma and COPD has been shown to be decreased, resulting from disrupted intercellular junctional proteins (10, 11). Other shared features of asthma and COPD include goblet cell metaplasia with increased mucus production, altered inflammatory responses, reduced antimicrobial peptide expression and activity, and altered basal function that may lead to defective repair responses following injury (5) (4, 10).

Epithelial dysfunction in both asthma and COPD implies an important role for these cells in the development and self-perpetuation of these diseases. Various research models have been applied to investigate the pathogenic mechanisms, diagnostic potential and

therapeutic targets of airway epithelial cells in chronic lung diseases. However, very few models have focused on the combined features of both asthma and COPD and how these may interact *in vitro*. In this review, we discuss recent advances and important considerations for *in vitro* models to study airway epithelial cell dysfunction in asthma and COPD.

ASSESSING EPITHELIAL FUNCTION IN VITRO

In contrast to patient studies and *in vivo* models, *in vitro* models allow us to deconstruct multi-layered mechanisms of disease pathogenesis and investigate the contribution of individual cellular components. Epithelial features of asthma and COPD can be investigated *in vitro* using patient derived primary cells, but can also be induced by known invoking substances involved in disease pathogenesis. Such substances can include complex mixtures such as cigarette smoke for COPD or allergen extracts for asthma, but also specific chemicals or proteins known to play a role in specific disease mechanisms can be used. Furthermore, the route of administration of invoking substances can vary. Using the culture media as the vehicle for the compound of interest is the most common approach, but for volatile compounds a more sophisticated technique may be required.

In vitro models can range from simple monolayers of epithelial cells to complex three-dimensional culture models involving multiple cell types. In a pseudostratified epithelium, all epithelial cells are attached to a basement membrane. Therefore, airway epithelial cells can be grown on a variety of different surfaces and careful selection of an appropriate support is warranted. Supports can range from uncoated tissue culture treated plastics to decellularized scaffolds of human tissue. Recent reviews provide an overview of various available supports and scaffolds and will not be revisited here (12-15).

Airway epithelial cells are available as continuous cell lines or as primary cells from various anatomical locations which vary in various characteristics including, but not restricted to apical-to-basal polarization, ciliary development, mucus production or barrier function. Primary epithelial cells can be obtained at a low passage from an increasing number of commercial sources, but can also be isolated from tissue by adequately equipped research laboratories if human samples are available. A major advantage of freshly isolated cells is also that they can be obtained from patients with disease and compared to cells derived from healthy persons. Primary cells can be grown as a submerged monolayer, but also as an

air-liquid interface culture with air exposure on the apical side and culture medium on the basolateral side of the membrane. In contrast, most tumour and immortalized cells lines are studied as submerged monoculture, which is partly explained by the fact that they do not differentiate into a pseudostratified epithelial layer at air-liquid interface. Airway epithelial cells can also be grown as organoids, in which cells are grouped and organized in a way similar to the organ they are representing (16, 17). Multiple structural, inflammatory and immune cell types can be included with the airway epithelial cells to create a more complex interacting system involving multiple cell types. Overall, various considerations have to be taken into account when modelling disease features *in vitro*.

Modelling epithelial changes of asthma and COPD in vitro

Various methods and techniques have been developed to recreate physiological relevant epithelial features of asthma and COPD *in vitro*. Reconstructing these disease features *in vitro* can be done by collecting airway epithelial cells from patients and culturing these cells using different techniques. Interestingly, when primary cells are isolated from asthma or COPD patients, several epithelial features observed *in vivo* are retained *in vitro*, including altered cytokine release, impaired immune responses and increased susceptibility to oxidative stress, suggesting that the epigenetic programming of the airway epithelial cells is retained after isolation (18-22). Nonetheless, it is important to consider that gene transcription, epigenetic programming and metabolism of the cells can be affected by the cell culture conditions. Airway epithelial cells can be collected by nasal or bronchial biopsy or brush, from resected lung tissue obtained during resection surgery, from resected lungs obtained during transplantation or from donor lungs not used for transplantation. However, in many research groups such studies are hampered by the fact that patient tissue is often difficult or expensive to obtain. Both primary airway epithelial cells or cell lines exposed to appropriate substances can be used to model certain features of disease, for example environmental exposures known to be involved in disease pathogenesis. Additionally, it is also important to consider exposure patterns and duration, as acute exposures may not reflect observations seen during chronic exposures.

Airway epithelial cells can be obtained *in vivo* through bronchoscopy or biopsies followed by morphology or expression analysis. Such analyses have been used by various groups to identify potential new therapeutic targets, but have helped in defining new phenotypes of asthma and COPD (23-27). Airway epithelial cells can be collected and cultured *in vitro*

followed by experimental exposures and other treatments and subsequent analysis. To this end, cigarette smoke and respiratory allergen exposures have been used to model COPD and asthma pathogenesis respectively (28). Alternatively, cytokines previously shown to be involved in disease pathogenesis have also been used to induce various signalling cascades that may lead to epithelial dysfunction. Th2 cytokines, including interleukin (IL)-4 or IL-13 are commonly used to model *in vitro* epithelial changes found in patients with asthma, whereas the pro-inflammatory cytokines TNFα and IL-1β have been used to model COPD (27, 29-31). Additionally, individual components of cigarette smoke or allergens can be used to induce epithelial dysfunction *in vitro* (32, 33).

Cigarette smoke is a complex mixture containing thousands of chemicals. Extracts of cigarette smoke have been made and used *in vitro* to study the effects cigarette smoke on airway epithelial cells. However, it is important to note that cigarette smoke consists of a volatile and a particulate fraction, with the particulate fraction being the minority fraction, contributing only to 4-9% of the total smoke weight (28). Cigarette smoke extract fails to capture the complete volatile fraction and consists mostly of the particulate fraction. Additionally, the particulate and the volatile fraction have been shown to have different properties (34). As an alternative to cigarette smoke extract, whole cigarette smoke can be used that contains both the particulate and volatile fraction of cigarette smoke, which resembles *in vivo* smoke exposure more closely (28). Various exposure designs, both commercial-available and self-made, have been developed to expose airway epithelial cells to whole cigarette smoke (35-39). Additionally, the availability of research grade cigarettes with defined chemical content allows for reproducible experiments between research groups. Moreover, cigarette smoke has been shown to contain harmful bacterial and fungal components that may affect epithelial responses following exposure (40). Cigarette smoke extract or whole cigarette smoke have both been used to expose airway epithelial cells *in vitro,* but also whole diesel exhaust or particles (28, 35, 41-47). Alternatively, individual components of cigarette smoke have also been used including nicotine, acrolein, formaldehydes or benzopyrene (32, 48-51). E-cigarettes, a recent commercially available alternative to cigarette smoking, has received a lot of attention regarding the safety and health risks and thus provide a new field to study the effects on airway epithelial cells (52). Whereas research focussing on the physiological effects of E-cigarette smoking remains limited, a recent publication provided important information regarding the use of E-cigarettes. The authors showed that electronic cigarette aerosols can induce nicotine-dependent gene expression changes in primary bronchial epithelial cells cultured at air-liquid interface, similar to whole cigarette smoke

induced changes. Moreover, they validated these *in vitro* findings in *in vivo* samples, overall suggesting that this *in vitro* model is relevant to study the *in vivo* effects of E-cigarette smoking (53).

Exposure of epithelial cells to inhaled allergens may provide important information on the pathogenesis on allergic airway disease such as asthma. The composition of allergen preparations used in such studies shows considerable variability, and a large variety of inhaled allergens exist, including house dust mite, pollen and fungi, which are most often applied as a crude extracts (54-57). Alternatively, individual components of allergen extracts have been used to investigate the effects on airway epithelial cells (58, 59). In addition to using extracts or individual components, it is important to consider the concentration applied and whether it reflects physiological concentrations encountered *in vivo*. Furthermore, extracts are prone to batch-to-batch variability and also extracts from commercial sources have been shown to vary in protein content (60, 61). Moreover, inhaled allergens can also contain numerous bacterial and fungal components due to close proximity of these compounds in the environment (62).

Comparing different sources of airway epithelial cells

In vitro airway epithelial cell cultures can be derived from cell lines or primary epithelial cells. Airway epithelial cell lines have acquired the ability to divide indefinitely either by nature occurring mutations such as tumours or through genetic transformation of primary tissue derived cells. These cells are generally easy to expand and cheap to culture and data obtained through cell lines are typically very reproducible. However, cell lines often fail to recapitulate the characteristics of an *in vivo* pseudostratified epithelium. On the other hand, primary airway epithelial cells have limited dividing capacity *in vitro*, additionally, these cells are expensive to culture and donor variability often hampers results. Despite these differences, primary airway epithelial cells retain the capacity to differentiate into a pseudostratified epithelial layer when cultured at air-liquid interface, thereby resembling more closely the *in vivo* epithelium morphologically and molecularly (63, 64).

To investigate the role of specific molecular targets in asthma or COPD, molecular techniques are available to genetically manipulate primary airway epithelial cells. However, primary cells are inherently difficult to manipulate genetically (65). Consequently, mouse tracheal epithelial cells from transgenic mice have been used as an alternative to human airway

epithelial cultures (57, 66-68). Moreover, disease models can be induced in transgenic mice followed by airway epithelial isolation. Nonetheless, transgenic animals are expensive and time consuming to establish and mouse tracheal epithelial cells are difficult to maintain in a proliferative state *in vitro*. As a result, to obtain an adequate amount of cells for experimental use, excessive animal numbers are needed for *in vitro* experiments which may hamper its applicability to study the role of airway epithelial cells in asthma and COPD.

Primary airway epithelial cells and various cell lines can be cultured either submerged or at air-liquid interface. The *in vivo* pseudostratified epithelium forms a physical and immunological barrier against inhaled particles and pathogens and consists of various epithelial cell types including club, goblet, ciliated and basal cells (69). Secretory epithelial cell types, club and goblet cells, maintain the airway surface liquid in which inhaled particles and pathogens are trapped followed by mucociliary clearance by ciliated cells (70). Upon damage of the epithelial layer, basal cells will proliferate followed by differentiation into specialized epithelial cell types (71, 72). Capturing these specific features of the airway epithelium *in vitro* is an important aspect of modelling asthma and COPD *in vitro*. To this end, airway epithelial cells have been cultured both submerged or at the air-liquid interface. Whereas submerged monolayers do not differentiate into a pseudostratified epithelial layer, they can be applied to investigate cell signalling pathways and basic cellular responses. Culturing airway epithelial cells at air-liquid interface allows mimicking *in vivo* exposures more closely by using e.g. aerosols (64). Primary airway epithelial cells cultured *in vitro* at air-liquid interface will differentiate into a pseudostratified epithelial layer consisting of club, goblet, ciliated and basal cells (73). Each of these cell types has its specific transcriptional program, thus it is important to verify the presence and composition of these cell types when culturing primary airway epithelial cells. Also, whereas *in vitro* cultured airway epithelial cells retain the ability to differentiate into a pseudostratified epithelial layer, it is important to consider that the transcriptional program can be also be affected by the *in vitro* culturing method including, but not limited to, the isolation procedure, culture medium containing antibiotics and the surface on which the cells are cultured. Additionally, primary airway epithelial cells can be cultured submerged to generate three dimensional spheroids which resemble a pseudostratified epithelium (74). Some epithelial cell lines also have the capability to be cultured at the air-liquid interface. However, whereas certain cell lines are able to develop the required robust barrier function that allows culture at the air-liquid interface, they will not differentiate into a functional pseudostratified epithelial layer. Both epithelial cell lines and primary airway epithelial cells, cultured either at ALI or submerged,

have been used to study the effects of whole cigarette smoke, cigarette smoke extract, allergens, chemicals or cytokines and are listed in table 1.

Table 1. Use of airway epithelial cell lines and primary airway epithelial cells under submerged or air-liquid interface culture conditions. ALI, air-liquid interface; PBEC, primary bronchial epithelial cells; SAEC, small airway epithelial cells.

Cell source	Cell type	Culture method	References
Immortalized cell lines	16HBE	ALI	(10, 75, 76)
		Submerged	(77-79)
	BEAS-2B	ALI	(80, 81)
		Submerged	(82-84)
	PBEC	ALI	(85)
		Submerged	(57, 86, 87)
	SAEC	Submerged	(88, 89)
Tumour cell lines	NCI-H292	ALI	(30, 90)
		Submerged	(91, 92)
	Calu-3	ALI	(54, 93-95)
		Submerged	(55, 95)
Primary cells isolated in research laboratories	PBEC	ALI	(29, 45, 96)
		Submerged	(78, 79, 97)
	SAEC	ALI	(98, 99)
		Submerged	(100-102)
Primary cells commercially available(*)	MucilAir	ALI	(103-105)
	EpiAirway	ALI	(52, 103, 106, 107)

(*) Multiple providers are available for primary bronchial or small airway epithelial cells. In addition, to Epithelix (providing MucilAir) and MatTek (EpiAirway), other major providers include Lonza, ATCC and ScienCell.

When using airway epithelial cell lines, it is important to consider that these may show marked differences in several important epithelial characteristics, including the capacity to form a physical barrier and their response to various exposures. Commonly used cell lines to resemble airway epithelial cells are 16HBE14o^{-} (16HBE), NCI-H292, Calu-3 and

BEAS-2B. 16HBE cells are transformed normal human bronchial epithelial cells that can form polarized monolayers with an intact barrier function, although conflicting reports exist on the presence of cilia and ciliary proteins in these cells (108-110). BEAS-2B cells, also transformed normal human bronchial epithelial cells, do not retain the ability to form an intact barrier function (111). Whereas BEAS-2B cells have limited differentiation capacity when cultured at air-liquid interface, they have been reported to develop cilia on the apical surface (111, 112). Calu-3 and NCI-H292 are both carcinoma-derived cell lines. Whereas Calu-3 cells are able to form a robust barrier function, NCI-H292 cells will only develop a robust barrier function when cultured on permeable supports (111, 113). Calu-3 cells have been reported to express ciliary proteins, although these were not expressed at the apical surface (111, 114). NCI-H292 cells have not been reported to express ciliary proteins (110). The adenocarcinoma cell line A549 is the most commonly used cell line to represent alveolar epithelial cells, from which it is also likely derived. A549 cells have several features of alveolar type II cells, but they lack the ability to form a strong barrier when cultured at the air-liquid interface, which is an essential feature of alveolar type II cells (115, 116). Because of their anatomical origin and features, A549 cells are not a suitable model to study airway epithelial cell function.

Primary airway epithelial cells can be isolated from human tissue or obtained at low passage from commercial sources. Primary cells have limited proliferation capacity and with increased passages, they suffer from senescence and diminished differentiation potential into a pseudostratified epithelial layer (73). However, recent advances have provided new techniques that allow extensive propagation of primary airway epithelial cells *in vitro*. Various studies have now shown that the combination of irradiated feeder cells, typically fibroblasts, with the RhoA kinase (ROCK) inhibitor Y-27632 enhances both the cell growth and life span of epithelial cells (117, 118). These so-called conditionally reprogrammed cells (CRC) are karyotype stable, and removal of the feeders and the ROCK inhibitor will allow cells to differentiate normally. Interestingly, human lung fibroblasts and mesenchymal stromal cells (MSC) were less efficient in supporting growth than mouse embryonic 3T3-J2 fibroblasts (117). A recent study showed that CRC technology can also be used to increase the availability of airway epithelial cells from patients with cystic fibrosis that retain their disease specific characteristics upon long-term culture (119). ROCK inhibition without the use of feeder cells has also been shown to induce basal cell proliferation without affecting their ability to differentiate (120). More recently, SMAD-signalling inhibition has also been shown to improve the proliferative capacity of primary airway epithelial cells

with subsequent air-liquid interface differentiation similar to low passage numbers (121). Whereas these approaches may increase the availability of primary airway epithelial cells, caution is needed. For instance, it is not clear whether disease-associated epithelial features of patient-derived epithelial cells are preserved using such cultures. Whereas the results with CF cultures generated using CRC technology are encouraging (119), this may be different in cultures from asthma and COPD patients since persistence of disease-specific features of such cells is more likely explained by epigenetic mechanisms than by genetic features. Additionally, genetic drift may affect the behaviour of these cells when high passage numbers are used. The same notes of caution are warranted when using airway epithelial cells that were generated using more recent immortalization techniques such as transduction overexpression of telomerase (hTERT) and inhibition of p16, that allow generation of cell lines that do form tight barriers and differentiate into mucociliary cell layers (122). As an alternative to primary airway epithelial cells, induced pluripotent stem cells (iPSC) have been shown to be able to differentiate into airway epithelial cells (123). Notably, iPSC can be derived from various sources (patients and controls) using minimally invasive or non-invasive techniques (e.g. skin, blood and urine). However, up to now, the generation of airway epithelial cells from multiple donors is expensive, time consuming and labour-intensive and therefore not yet readily applicable to a large number of laboratories.

Co-culture models

The major limitation of *in vitro* models is the capacity to model multifaceted interactions as seen *in vivo*. Using a single cell type does not capture the complex interplay between various cell types within the cellular environment of the human airways. To investigate the complex interactions of cells involved in asthma and COPD pathogenesis, various *in vitro* models were designed to include additional cell types. Co-culturing various cell types can be achieved by culturing epithelial cells with direct or indirect contact to other cells. Direct co-cultures allow for different cell types to make direct contact within the same culture environment, whereas in indirect co-cultures, the different cell types are separated without direct contact and cell-cell interactions occur through soluble factors. Co-culture models thus allow us to create a simplified and controllable *in vitro* system to mimic cell-cell interactions through either direct contact, soluble factors or both.

To establish a co-culture model, multiple factors have to be taken into account to warrant the quality of all cell types involved. Importantly, cell culture medium should be optimized

as growth of certain cell types may not be compatible with specific media formulations. Additionally, ratios of different cell types should reflect their *in vivo* physiologic relative abundance to ensure that results are not masked by irregular cell proportions. Both primary airway epithelial cells and cell lines have been used for co-culture models, grown as either monolayers or air-liquid interfaces. However, due to strict medium formulations for primary airway epithelial cells, cell lines are usually opted for as an alternative. Additionally, the accompanying cell types included in the co-culture models can originate from either cell lines or primary sources. Accompanying cell types can include structural cells (fibroblasts, airway smooth muscle cells, endothelial cells) or inflammatory and immune cells (macrophages, dendritic cells, B cells, T cells, neutrophils or eosinophils). Various co-culture models have been described using airway epithelial cells with various accompanying cell types although few have been specifically used to assess the role of epithelial cells in asthma or COPD. Even a tetra culture models has been reported, containing four cell lines including an alveolar type 2, macrophage, mast cell and endothelial cell line (124). An overview of recently used co-culture models is presented in table 2.

Table 2. Co-culture models using airway epithelial cells with accompanying cell types. ALI, air-liquid interface; BM, bone marrow, MDDC, monocyte-derived dendritic cell; MDM, monocyte-derived macrophage; iPSC, induced pluripotent stem cell; PBEC, primary bronchial epithelial cells; SAEC, small airway epithelial cells

Epithelial cells	Co-culture method	Accompanying cell types	References
PBEC 16HBE	Direct	MDDC	(125)
16HBE	Direct	MDDC MDM	(126)
16HBE	Direct	Fibroblast cell line (MRC-5) MDDC	(127)
ALI-PBEC	Direct	Fibroblast cell line (IMR-90)	(128)
BEAS-2B	Conditioned medium	Mesenchymal stem cells (iPSC or BM-derived)	(129)
16HBE PBEC	Indirect Conditioned medium	Primary fibroblasts Fibroblast cell line (MRC-5)	(130)
ALI-PBEC (MucilAir)	Indirect	Primary fibroblasts	(104)
ALI-PBEC	Indirect	B-cells	(131)
16HBE	Indirect	Fibroblast cell line (HFL-1)	(132)
BEAS-2B	Conditioned medium	Monocyte cell line (THP-1)	(133)
16HBE	Direct	MDDC MDM	(134, 135)
BEAS-2B ALI-PBEC	Conditioned medium	MDDC MDM	(136)
BEAS-2B	Direct or indirect	MDDC MDM	(136)
16HBE	Conditioned medium	Basophils	(58)
PBEC SAEC	Indirect	Microvascular endothelial cells	(42)
NCI-H292 ALI-PBEC	Conditioned medium	Mesenchymal stem cells	(30)
16HBE	Direct	Eosinophils Neutrophils	(137)
NCI-H292 PBEC	Direct	Umbilical vein endothelial cells	(138)
ALI-PBEC (EpiAirway)	Direct	Primary fibroblasts	(107, 139)

A novel approach is the development of a lung-on-a-chip which included alveolar and endothelial cells, but they also included a continuous flow of culture medium and mechanical stretch to mimic blood flow and breathing-induced stretch respectively (140). In this approach alveolar epithelial cells are cultured in an air-liquid interface, and additionally endothelial cells are grown on opposite sides of a porous membrane. Vacuum chambers on either side of the porous membrane were incorporated in the device to induce mechanical stretch. Despite some limitations including cell lines and the lack of other cell types, this novel model allowed for researchers to develop more sophisticated models that also allow human disease modelling (141). A more recent lung-on-a-chip model used air-liquid interface differentiated bronchial epithelial cells with microfluidics. Although this model did not include additional cell types, it did allow for kinetic analysis of epithelial responses following pollen exposure (142). Lung-on-a-chip models including multiple cell types will become useful tools for analysing the kinetics of epithelial responses following environmental exposures (143).

Precision cut lung slices

Precision cut lung slices (PCLS) are slices of lung tissue that are put into culture (144, 145). In contrast to *in vitro* co-culture models including airway epithelial cells, PCLS contain all the cell types present within a particular section of the lung in addition to retaining metabolic activity, tissue homeostasis and structural integrity, making PCLS particularly beneficial to study the pathophysiology and underlying mechanisms of asthma and COPD(146). Moreover, PCLS provide an important link between *in vitro* cell culture models and *in vivo* models of disease. Despite these advantages, lung tissue, particularly human lung tissue, is difficult to obtain and the quality of the lung tissue can vary a lot between donors. Due to limited availability of human lung tissue, animal lung tissue has been used as alternatives for PCLS with species including horses, sheep, mice, rats and guinea pigs. Moreover, PCLS have a limited, and likely cell-type specific variable life span *in vitro* with initial reports suggesting 72 h, although more recent reports suggest PCLS can be maintained up to 2 weeks while retaining metabolic activity, tissue homeostasis and structural integrity (147-149). Lung slices can vary in thickness (200 - 700 µm) which may affect gas diffusion and exposure efficiency. Moreover, the cutting edges of the slice will contain damaged cells, thus the thinner the slice, the higher the percentage of damaged cells per slice (150). PCLS can be cultured submerged, but also at air-liquid interface using porous membranes in cell culture inserts (151, 152).

So far no studies have reported the use of human PCLS from COPD or asthma patients. Additionally, the number of studies using human PCLS to investigate the effects of cigarette smoke, allergens or individual components remain low (153, 154). PCLS from animal models have been used more commonly, including *in vitro* exposed PCLS but also PCLS from disease models reflecting allergic airway disease or COPD pathogenesis (151, 155).

UTILIZING IN VITRO MODELS TO STUDY INFECTIONS AND EXACERBATIONS

Asthma and COPD patients are both at increased risk for acute exacerbations which can be triggered by viral or bacterial infection. Recurrent exacerbations are worrisome for patients and can lead to progressive worsening of the disease (1). Exacerbations involve complex interactions with multiple cell types, making *in vitro* models a respectable alternative to *in vivo* models to study cell-specific effects or cell-cell communication when using co-culture models. The airway epithelium is an important site for mounting an inflammatory response against inhaled bacteria and viruses. They can produce an array of inflammatory mediators, including cytokines and chemokines, thereby contributing to host defence and augmenting the inflammatory response by recruiting specialized inflammatory cells (5). Several concerns have to be taken into account when modelling infections *in vitro*. Epithelial cell types including goblet, ciliated and basal cells have been shown to have differential susceptibility to infection (156-158). Consequently, using cell lines or submerged monolayers of primary airway epithelial cells may not capture the full capabilities of the airway epithelium as they do not develop a pseudostratified epithelial layer. However, using submerged cultures of primary bronchial epithelial cells allows for studying basal cells specifically.

Airway epithelial cells from asthma or COPD patients cultured *in vitro* are more susceptible to viral infections compared to controls, suggesting that epithelial cells retain these features after isolation and that epigenetic mechanisms are involved (19, 159-161). This is in line with a report where active smoking has been shown to impair antiviral responses through epigenetic mechanisms (162). Additionally, cigarette smoke has been shown to increase epithelial susceptibility to infections although no similar evidence currently exists for inhaled allergens (94, 163-165). Also, no studies have currently investigated the effect of cigarette smoking in asthmatic airway epithelial cells nor the combined effect of cigarette smoke or air pollutants and inhaled allergens.

When studying inflammatory responses of airway epithelial cells following infection, the micro-organism studied can be applied alive or inactivated, but also lysates or specific microbial components can be used. Alternatively, conditioned medium can be used to study the effects of secreted components by these organisms (166). Using live fungi or bacteria in *in vitro* cultures can be quite challenging as epithelial cells alone may not be able to clear the infection, leading to overwhelming amounts of bacteria in the culture media with subsequent cell death of the airway epithelial cells. However, inactivated bacteria or bacterial lysates may not fully represent epithelial responses to a live infection (167). Live viral infection is often preferred over inactivated viral infection to allow for intracellular viral replication and subsequent activation of inflammatory mechanisms. The choice of microbial stimulus used is a major determinant of the epithelial response. Indeed, recent studies highlight the capacity of cells to sense microbial viability (in addition to e.g. discriminating pathogenic from commensal bacteria, colonizing versus infecting bacteria) to adapt their response based on the challenge encountered (168). Indeed, detection of bacterial death may be a sign of a successful immune response, requiring resolution of the immune response and initiation of a repair response.

Most studies to date, focus on epithelial exposure to a single microbial species. However, the epithelial surface of the airways contains a large variety of not only pathogenic, but also commensal bacteria, viruses and fungi that can affect the inflammatory response of airway epithelial cells against inhaled pathogens (169-171). This collection of commensal micro-organisms constitutes a major part of the microbiome, that has been shown to be altered in asthma and COPD compared to controls and likely attributes to disease pathogenesis (172, 173). Studying the effects of the microbiome on airway epithelial cells cultured *in vitro* is very challenging and thus far, research has focused on a selection of specific strains rather than the microbiome as a whole. Indeed, studying exposure to the complex mixtures of micro-organisms that constitute the microbiome is very challenging for various reasons. These include the fact that sampling techniques and *in vitro* culture conditions may result in selection of specific strains, thus altering the composition of the microbiome. Furthermore, also the absence of mucociliary clearance and non-epithelial components of the innate immune system in culture may affect the stability of the microbiome. Nonetheless, the microbiome has emerged as a critical player in lung homeostasis and disease development and will be an important research topic in the future.

EPITHELIAL CELL CULTURE: POTENTIAL ROLE IN DRUG SCREENING AND PERSONALIZED MEDICINE

Epithelial dysfunction is a common feature of both asthma and COPD (5). A better understanding of epithelial dysfunction will aid to identify new pathways and therapeutic strategies in asthma and COPD pathogenesis. Additionally, airway epithelial cells are the first cells to encounter not only inhaled toxic substances, but also inhaled pulmonary drugs. Consequently, airway epithelial cells cultures are a suitable model for drug screening and evaluation (Figure 1). Several considerations have to be taken into account when evaluating drugs *in vitro*. In a clinical setting, drugs can be delivered through various routes for e.g. inhalation, oral or injection. Accordingly, depending on the culture method of the airway epithelial cells, e.g. air-liquid interface, drugs can be applied apically, basolateral or a combination of both, representing different routes of application as seen *in vivo*. Moreover, drug metabolites encountered *in vivo*, may not be present when applying particular drugs *in vitro*. The importance of airway epithelial cell differentiation in metabolism of xenobiotics was recently demonstrated, highlighting the need to use differentiated cultures (174). Also, the dose used *in vitro* may not reflect clinically relevant concentrations, which may affect the observed results. Finally, especially when using e.g. aerosols, careful monitoring of drug deposition on the epithelial surface is important.

Despite these potential limitations and complicating factors, cultured airway epithelial cells are a representative and useful model to study the effects of inhaled pulmonary drugs. *In vitro* models using cultured airway epithelial cells have shown that muscarinic antagonists are able to reduce cigarette smoke and IL-13-induced mucus hypersecretion (175, 176). Inflammatory responses in cultured airway epithelial cells have been shown to be reduced by the corticosteroids, whereas oxidative-stress induced responses appear to be steroid resistant (177, 178). In addition to inhaled pulmonary drugs, also orally administered drugs, e.g. macrolides, have been studied *in vitro* using airway epithelial cells cultures (29, 179-182).

Airway epithelial cells line the conducting airways of the lung, providing a barrier against inhaled particles and pathogens. Being at the interface between environmental exposures and underlying tissue, makes airway epithelial cells ideal candidates as reporters of underlying tissue pathogenesis. Moreover, airway epithelial cells are reasonably accessible and bronchial brushings represent a relatively pure population of epithelial cells (183).

Consequently, airway epithelial cells derived from bronchial brushings have been applied in multiple transcriptomic studies to develop clinically relevant biomarker signatures, ultimately leading to biomarker-guided therapy. Also, gene expression profiles can be considered clinically at multiple time points during the course of treatment to study intermediate markers of therapeutic efficacy (24, 184).

Asthma and COPD are both heterogeneous chronic lung diseases with multiple clinical phenotypes existing within these diseases, including molecular phenotypes that show overlapping features of both asthma and COPD (23, 25, 27, 185). Additionally, differential therapeutic responses have been observed between these clinical phenotypes, indicating that patient-specific therapies are required (186). Biomarker guided therapy based on airway epithelial signatures has provided us with important information to delineate clinical phenotypes for tailored disease management. Furthermore, patient-specific airway epithelial cells allow for individualized drug screening, although current research is still limited. However, within cystic fibrosis, an autosomal recessive genetic disease caused by different classes of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, important progress was made towards patient-specific *in vitro* cultures to guide personalized treatment. Dekkers and colleagues developed a sphere-forming assay using patient-derived intestinal epithelial cells to study CFTR function. They demonstrated that forskolin-induced swelling of spheroids could be used to demonstrate patient-specific CFTR function by simple sphere swelling. Importantly, drug responses of the patient-specific spheroids could be positively correlated with clinical outcome data (187). This work highlighted the significant value of patient-specific *in vitro* cultures to guide personalised medicine, although current work using airway epithelial cells is still lacking.

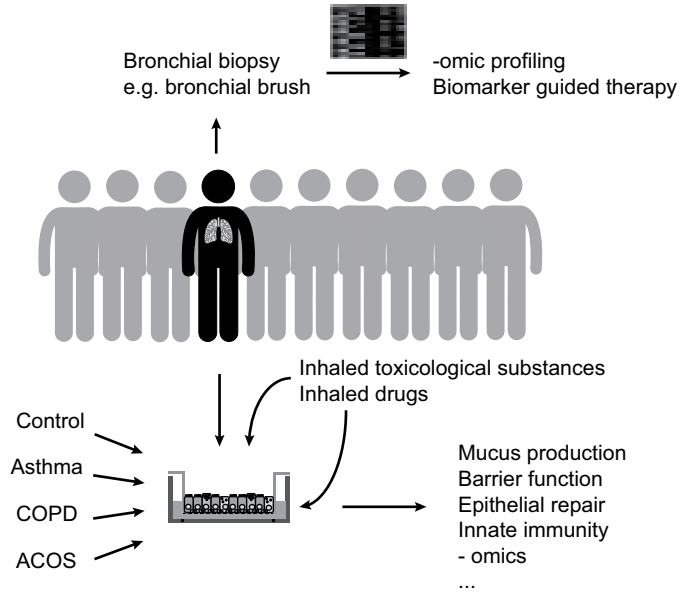

Figure 1. Epithelial cell cultures for drug screening and personalized medicine. ACOS, asthma – COPD overlapping syndrome; COPD, chronic obstructive pulmonary disease

ASTHMA AND COPD OVERLAP

Asthma and COPD are considered as distinct disease entities, however a hypothesis concerning a common pathophysiology has been described and named the "Dutch hypothesis" (1). In the Dutch hypothesis it was suggested that all obstructive airway diseases should be considered as different expressions of a single disease with shared genetic backgrounds. Environmental factors determined when and how the disease was clinically expressed (188). For both asthma and COPD it has become well recognized that within these diseases, several phenotypes exist that share overlapping features of both asthma and COPD. Airway hyperresponsiveness is typically attributed to asthma, although several reports indicate that airway hyperresponsiveness is a risk factor for the development of COPD and that the prevalence in COPD patients is up to 60% (2, 189, 190). Additionally, reversibility of airway obstruction and atopy can be present in COPD patients whereas these symptoms are typically recognized as features of asthma (191-194). Moreover, 20

to 35% of patients with asthma smoke, resulting in worsened asthma symptoms, reduced responsiveness to corticosteroid treatment, accelerated lung function decline and increase exacerbation rates (6-9).

In vitro models studying the shared epithelial features of asthma and COPD can be done by investigating the combined effects of COPD and asthma-related provoking substances. Cigarette smoke was shown to increase epithelial permeability for allergens with subsequent augmented histamine release from basophils (58). Moreover, cigarette smoke potentiated house dust mite-induced airway barrier function decrease and inflammatory cytokine release (195, 196). Alternatively, airway epithelial cells from asthma or COPD donors can be used in combination with COPD or asthma-related provoking substances respectively. Airway epithelial cells from asthma patients were shown to be more sensitive to diesel exhaust particles with increased pro-inflammatory cytokine release compared to control cells (20). Additionally, asthmatic airway epithelial cells are more susceptible to oxidative stress-induced apoptosis than control cells (18, 197). Nonetheless, *in vitro* studies investigating the shared epithelial features of asthma and COPD remain limited. In contrast, shared features of asthma and COPD have been more commonly studied in mouse models. Mouse models with share features of asthma and COPD focus mostly on the effect of cigarette smoke in allergic airway inflammation. Overall these models show conflicting results, with cigarette smoke either aggravating or attenuating inflammatory responses (198-202). These contradictory results are likely in part explained by the use of different models of allergic airway inflammation and different cigarette smoke exposure setups. Modern research allows us to use sophisticated transgenic animal models that enable us to investigate complex systemic interactions in asthma and COPD. However, these animal models do not fully reflect human anatomy, physiology and immunology. Despite these important differences, they can provide novel insights of complex interactions that we currently cannot model *in vitro*.

CONCLUSIONS AND FUTURE DIRECTIONS

Over the last decades we have gained increasing knowledge of airway epithelial cells and how they are involved in asthma and COPD pathogenesis. Airway epithelial cells form an important barrier against inhaled particles, allergens and pathogens and epithelial dysfunction is known to play an important role in asthma and COPD pathogenesis. Modelling these epithelial features *in vitro* is challenging and requires multiple considerations to be

made to mimic *in vivo* pathophysiology as close as possible. Currently there is no golden standard model to study the epithelial component in these diseases *in vitro*. Moreover, the large variety in epithelial cell sources, culture methods and exposure setups requires us to evaluate and reconsider our options with regard to ease-of-use, complexity and robustness of the *in vitro* model. Recent advances in *in vitro* models including lung-on-a-chip and precision cut lung slices, allow us to mimic the *in vivo* situation more closely. However, very few studies have incorporated these new models and techniques to study epithelial dysfunction in asthma and COPD. Overall, new research strategies should aim to include complex environmental interactions seen *in vivo* and combine these with physiologic relevant *in vitro* models to study epithelial dysfunction in asthma and COPD.

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