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

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AIRWAY EPITHELIAL CELL CULTURES FOR STUDYING OBSTRUCTIVE LUNG DISEASE:

EFFECTS OF IL-13 AND CIGARETTE SMOKE

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COLOPHON

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Voor mama en papa

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CHAPTER 1

GENERAL INTRODUCTION AND THESIS OUTLINE

INTRODUCTION

Asthma and chronic obstructive pulmonary disorder (COPD) are both chronic lung diseases characterized by chronic inflammation of the respiratory tract and airway obstruction which is often reversible in asthma, but mostly irreversible in COPD (1-3). Allergic asthma has classically been described as a T-helper 2 (Th2)-driven disease with predominantly eosinophilic infiltration, whereas COPD inflammation may involve CD8 cells, B cells, macrophages and neutrophils (3). Traditionally, these two diseases have been considered as two distinct disease entities, however, it has become evident over the last years that both asthma and COPD are very heterogeneous diseases (3-5). Disease heterogeneity in both asthma and COPD is characterized by a wide array of symptoms, variable exacerbation rates, inconsistent clinical findings, pathology abnormalities and diverse patient responses to therapeutics. The large degree of heterogeneity within asthma and COPD suggests that different underlying molecular pathways are contributing to disease pathogenesis. Elucidating these underlying molecular pathways may provide a basis to better delineate various phenotypes within asthma and COPD that respond to specific therapeutics.

Airway epithelial cell dysfunction is an important characteristic of both asthma and COPD, making it a likely candidate for molecular phenotyping to delineate clinical phenotypes (6-8). Because of their localization at the interface between environmental exposures and underlying tissue, airway epithelial cells are ideal candidates as reporters of both environmental exposures as wells as underlying tissue pathogenesis. Moreover, airway epithelial cells are reasonably accessible and bronchial brushings can be used to obtain a relatively pure population of epithelial cells. Collection of such bronchial brushings from patients and performing gene expression analysis has proven a successful method to delineate molecular pathways underlying specific disease phenotypes (8, 9). In addition, although considered to be invasive, repeated sampling may allow gene expression analysis at multiple time points during the course of treatment to study intermediate markers of therapeutic efficacy. Airway epithelial cells can be isolated from bronchial brushes or bronchial biopsies collected during bronchoscopy, or from lung tissues resected during surgery. These cells can subsequently be cultured *in vitro* to study molecular pathways underlying clinical phenotypes, but also to evaluate new potential targets for therapy. Moreover, airway epithelial cells cultured *in vitro* can be used to evaluate the effect of therapeutics on existing or induced molecular phenotypes representing clinically relevant molecular pathways.

Whereas animal models have contributed significantly to our knowledge of chronic lung diseases, they have been shown to have limited therapeutic predictive value and recreating phenotypes representing clinically encountered phenotypes may not be feasible (10, 11). Therefore, pre-clinical *in vitro* models using airway epithelial cells representing specific clinical phenotypes are essential to elucidate and unravel molecular pathways. These pre-clinical *in vitro* models will require extensive optimization and validation, but they could contribute tremendously towards elucidating molecular pathways and discovery of novel therapeutic targets. Also, using *in vitro* models representing particular features of asthma and COPD, for e.g. exacerbations, can contribute to our knowledge of understating disease progression. An important limitation of airway epithelial cells cultured *in vitro* is their limited potential for genetic manipulation as these cells are notoriously difficult to transfect. However, using airway epithelial cells isolated from transgenic animals can provide an alternative to human airway epithelial cells, circumventing the need to transfect airway epithelial cells.

THESIS OUTLINE

In the studies described in this thesis, *in vitro* models using airway epithelial cells were used to study molecular pathways involved in asthma and COPD and their disease heterogeneity. Optimized culture methods and a better understanding of these pathways could aid in the development of targeted treatments for these common lung diseases.

In **Chapter 2** a review and discussion is provided on epithelial features of asthma and COPD and how these can be modeled *in vitro*. Additionally, recent developments and the therapeutic potential of these *in vitro* models are discussed.

In **Chapter 3,** the effect of the macrolide antibiotic azithromycin on the interleukin (IL)-13-induced gene signature in primary human airway epithelial cells cultured *in vitro* is evaluated. This IL-13-induced gene signature has previously been described to represent the T helper 2 (Th2) gene signature encountered in a specific subset of asthma patients. An important environmental factor affecting asthma pathogenesis is cigarette smoking. Approximately 20 to 35% of the world population smokes and quite surprisingly, these smoking rates are reported similarly in asthma patients. Because cigarette smoking is an important environmental factor significantly influencing asthma pathogenesis, **Chapter 4** is focused on the effects of whole cigarette smoke exposure on the IL-13-induced gene signature in primary human airway epithelial cells cultured *in vitro*.

An important feature of both asthma and COPD are exacerbations, sudden worsening of disease, often requiring hospitalization. COPD patients have previously been shown to be more susceptible to exacerbations induced by infection. Therefore, in **Chapter 5** an *in vitro* model of exacerbations using UV-inactivated *Haemophilus influenzae* exposure or rhinovirus infection in primary airway epithelial cells derived from COPD patients or smoking controls was used to evaluate whether airway epithelial cells from COPD patients respond differently to infection compared to smoking controls.

It is difficult to obtain primary airway epithelial cells, and genetic manipulation of these cells is notoriously challenging. As an alternative, mouse tracheal epithelial cells can be isolated from transgenic animals to circumvent genetic editing of airway epithelial cells. However, these cells are difficult to maintain and expand in culture and numerous animals are needed to set up an *in vitro* experiment. Therefore, in **Chapter 6** an alternative and more efficient culture method for mouse tracheal epithelial cells is developed.

Finally, in **Chapter 7** the most important findings of the studies described in this thesis are discussed, followed by an outlook on the clinical implications and future perspectives based on the findings described in this thesis.

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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.

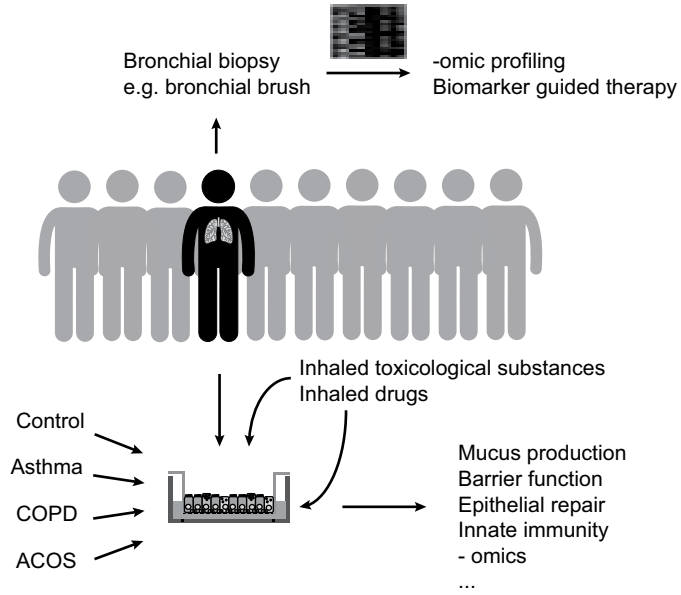

Figure 1. Epithelial cell cultures for drug screening and personalized medicine. ACOS, asthma – COPD overlapping syndrome; COPD, chronic obstructive pulmonary disease

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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CHAPTER 3

AZITHROMYCIN DIFFERENTIALLY AFFECTS THE IL-13-INDUCED EXPRESSION PROFILE IN HUMAN BRONCHIAL EPITHELIAL CELLS

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ABSTRACT

The T helper 2 (Th2) cytokine interleukin(IL)-13 is a central regulator in goblet cell metaplasia and induces the recently described Th2 gene signature consisting of periostin (POSTN), chloride channel regulator 1 (CLCA1) and serpin B2 (SERPINB2) in airway epithelial cells. This Th2 gene signature has been proposed as a biomarker to classify asthma into Th2-high and Th2-low phenotypes. Clinical studies have shown that the macrolide antibiotic azithromycin reduced clinical symptoms in neutrophilic asthma, but not in the classical Th2-mediated asthma despite the ability of azithromycin to reduce IL-13-induced mucus production. We therefore hypothesize that azithromycin differentially affects the IL-13-induced expression profile. To investigate this, we focus on IL-13-induced mucin and Th2-signature expression in human bronchial epithelial cells and how this combined expression profile is affected by azithromycin treatment. Primary bronchial epithelial cells were differentiated at air liquid interface in presence of IL-13 with or without azithromycin. Azithromycin inhibited IL-13-induced MUC5AC, which was accompanied by inhibition of IL-13-induced CLCA1 and SERPINB2 expression. In contrast, IL-13-induced expression of POSTN was further increased in cells treated with azithromycin. This indicates that azithromycin has a differential effect on the IL-13-induced Th2 gene signature. Furthermore, the ability of azithromycin to decrease IL-13-induced MUC5AC expression may be mediated by a reduction in CLCA1.

INTRODUCTION

Asthma is a syndrome characterized by airway hyperresponsiveness, chronic inflammation and mucus hypersecretion. Historically asthma has mainly been thought to be driven by a T helper 2 (Th2)-mediated immune response. However, it is now well recognized that asthma consists of multiple phenotypes with different pathophysiological pathways underlying airway inflammation, which may benefit from targeted treatment (1, 2). Different approaches have been taken to develop biomarkers to distinguish these phenotypes to guide clinical treatment. Recently various clinical trials have shown the potential of inhibitors of Th2 inflammation, including IL-13, to modulate clinical outcomes in asthma (3). IL-13 is one of the cytokines produced by Th2 CD4 T cells, and has been shown to have marked effects on the airway epithelium (4).

IL-13 induces goblet cell metaplasia in human bronchial epithelial cells *in vitro* and *in vivo*. The main mucins produced by goblet cells, MUC2, MUC5AC and MUC5B, are expressed in healthy human airways and their proportion can vary with health status. MUC2 and MUC5AC expression is increased in bronchial biopsies of Th2-high asthma patients compared to healthy controls. However, MUC5B expression is lower in Th2-high asthma patients compared to healthy controls (5). Whereas a variety of stimuli increase MUC5AC expression, the Th2 cytokine IL-13 appears a central trigger for its production in asthma. Different factors such as chloride channel regulator 1 (CLCA1), SAM pointed domain-containing ETS transcription factor (SPDEF) and forkheadbox A2 (FOXA2) have been implicated in the regulation of IL-13-induced MUC5AC expression (6-8).

In addition to increasing mucin gene expression, IL-13 is a central regulator in the epithelial gene expression of periostin (POSTN) and serpin B2 (SERPINB2), and expression of these genes together with CLCA1, has been used as a signature to classify asthma into Th2-high and Th2-low phenotypes (9). Periostin is of particular interest as a biomarker, as it is detectable in the circulation and therefore may be useful as a blood biomarker for IL-13-activated bronchial epithelial cells. Indeed, there is evidence suggesting that circulating periostin levels may be helpful to identify asthma patients that benefit from anti-IL-13 treatment (1, 10-13).

Several reports indicate that macrolide antibiotics have beneficial effects in the treatment of chronic inflammatory airway diseases such as cystic fibrosis, chronic obstructive pulmonary

disease (COPD) and asthma (14-16). These effects have partly been attributed to immune-modulatory actions, but the mechanisms involved are incompletely understood. Inhibitory effects of macrolides on goblet cell metaplasia and mucin expression induced by various stimuli may contribute to these clinical effects and also IL-13-induced MUC5AC expression has been shown to be inhibited by a macrolide antibiotic (17-23). However, whether macrolides also control the expression of the Th2 signature in epithelial cells is unknown.

Clinical studies have shown that the macrolide antibiotic azithromycin reduces clinical symptoms in neutrophilic asthma, but not in the classical Th2-mediated asthma (16). However, azithromycin has been shown to reduce IL-13-induced mucus expression in various studies. We therefore hypothesize that azithromycin differentially affects the IL-13-induced expression profile. To investigate this, we focus on IL-13-induced mucin and Th2-signature expression in human bronchial epithelial cells and how this combined expression profile is affected by azithromycin treatment.

MATERIAL AND METHODS

Bronchial epithelial cell culture and treatment

Human primary bronchial epithelial cells (PBEC) were isolated from macroscopically normal bronchial tissues obtained from lung cancer patients undergoing lobectomy at the Leiden University Medical Center (Leiden, The Netherlands). Cancer-free trimmed tissues were washed and incubated 2h at 37°C with 0.18% (w/v) proteinase type XIV (Sigma-Aldrich, St. Louis, MO, USA) in Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (Gibco, Bleiswijk, The Netherlands). Epithelial cells were gently scraped off the luminal surface, washed and subsequently cultured in serum-free keratinocyte medium (Gibco) supplemented with 0.2 ng/ml epidermal growth factor (Gibco), 25 µg/ml bovine pituitary extract (Gibco), 1 µM isoproterenol (Sigma-Aldrich), 100 U/mL Penicillin (Lonza, Verviers, Belgium) and 100 µg/ml Streptomycin (Lonza) on coated 6-well plates (coated at 37°C, 5% CO_2 for 2-24h with 30 µg/ml PureCol [Advanced BioMatrix, San Diego, CA, USA], 10 µg/ml Bovine serum albumin [Sigma-Aldrich] and 10 µg/ml fibronectin [isolated from human plasma] diluted in PBS). During the first week of culture following isolation of epithelial cells from lung tissue, 200 µg/ml of the anti-mycoplasm agent ciprofloxacin (Fresenius Kabi, Schelle, Belgium) was added to the medium. After reaching near-confluence, cells were trypsinized (0.03% [w/v] trypsin [Difco, Detroit, USA], 0.01% [w/v] EDTA [BDH, Poole, England], 0.1% glucose [BDH] in PBS) and stored in liquid nitrogen.

These PBEC were used for generation of mucociliary differentiated PBEC cultures by differentiation at the air-liquid interface (ALI) as described previously (24). Briefly, PBEC were cultured submerged on semipermeable transwell inserts with 0.4 µm pore size (Corning Costar, Cambridge, MA) that were coated with a mixture of collagen and fibronectin. Once full confluence was reached, apical medium was removed and PBEC were cultured at ALI during two weeks.

Study design

ALI-PBEC cultures were incubated in presence or absence of recombinant human IL-13 (Peprotech, Rocky Hill, NJ, USA) that was added to the basolateral compartment of the transwell insert during two weeks of ALI differentiation. Azithromycin (Sigma-Aldrich) was also added to the basolateral compartment during ALI differentiation; 0.04% (v/v) DMSO

(Merck, Darmstadt, Germany) was used as vehicle control for azithromycin. Medium with respective treatments was refreshed three times a week. After 14 days exposure, basal medium was collected and stored at -20°C until further use.

RNA isolation, reverse transcription (RT) and qPCR

Total RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Leiden, The Netherlands) and quantified using the Nanodrop ND-1000 UV-visible (UV-Vis) spectrophotometer (Nanodrop Technologies, Wilmington, DE). For cDNA synthesis, 1µg of total RNA was reverse transcribed using oligo(dT) primers and Moloney murine leukemia virus (M-MLV) polymerase (Promega) at 37°C. Primer sequences are listed in table 1. RPL13A and ATP5B were used as reference genes. All quantitative PCRs (qPCRs) were carried out in triplicate on a CFX-384 real-time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands) with the use of SensiFAST™ SYBR green (Bioline, Luckenwalde, Germany). Bio-Rad CFX manager 3.0 software (Bio-Rad) was used to calculate arbitrary gene expression by using the standard curve method.

Table 1. Primer sequences with gene names and NCBI gene ID used in present study.

Gene	Primer Sequence	NCBI Gene ID
POSTN	F: GAC CGT GTG CTT ACA CAA ATT G	10631
	R: AAG TGA CCG TCT CTT CCA AGG	
SERPINB2	F: TCC TGG GTC AAG ACT CAA ACC	5055
	R: CAT CCT GGT ATC CCC ATC TAC AG	
CLCA1	F: ATG GCT ATG AAG GCA TTG TCG	1179
	R: TGG CAC ATT GGG GTC GAT TG	
MUC5AC	F: CCT TCG ACG GAC AGA GCT AC	4586
	R: TCT CGG TGA CAA CAC GAA AG	
MUC2	F: GGA GAT CAC CAA TGA CTG CGA	4583
	R: GAA TCG TTG TGG TCA CCC TTG	
MUC5B	F: GGG CTT TGA CAA GAG AGT	727897
	R: AGG ATG GTC GTG TTG ATG CG	
FOXA2	F: ACT ACC CCG GCT ACG GTT C	3170
	R: AGG CCC GTT TTG TTC GTG A	
SPDEF	F: ATG AAA GAG CGG ACT TCA CCT	25803
	R: CTG GTC GAG GCA CAG TAG TG	
RPL13A	F: AAG GTG GTG GTC GTA CGC TGT G	23521
	R: CGG GAA GGG TTG GTG TTC ATC C	
ATP5B	F: TCA CCC AGG CTG GTT CAG A	506
	R: AGT GGC CAG GGT AGG CTG AT	

Mucin analysis

To determine levels of MUC5AC, MUC5B and MUC2 protein in ALI-PBEC, cell lysates were serially diluted in PBS and 50 µl was spotted on a methanol-preincubated polyvinylidene-difluoride (PVDF)-membrane using a Bio-Dot microfiltration apparatus (Bio-Rad). Non-specific binding sites on the membranes were blocked with PBS/5% (w/v) skim milk (Sigma-Aldrich) overnight at 4°C. Subsequently the membrane was incubated with mouse-anti-MUC5AC (1:100; 45M1; Thermo Fisher Scientific, Breda, The Netherlands), rabbit-anti MUC5B (1:500; H-300; Santa Cruz; Bio-Connect B.V., Huissen, The Netherlands) or rabbit-anti-MUC2 (1:100; H-300; Santa Cruz) in PBS/5% (w/v) skim milk for 1h at room

temperature. HRP-conjugated anti-mouse or anti-rabbit IgG (both 1:10000, Cell signaling Technologies) was used as a secondary antibody and detected using ECL Western Blotting substrate (Thermo Fisher Scientific). Densitometry was performed using Totallab image analysis software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

SDS-PAGE & Western blot

Protein lysates were diluted (2:3 [v/v]) in SDS sample buffer, containing 4% (w/v) SDS (Sigma-Aldrich), 20% (v/v) glycerol (Merck), 0.8% (w/v) DL-dithiothreitol (Sigma-Aldrich), 0.5 M Tris pH 6.8 and 0.003% (w/v) bromophenol blue (Sigma-Aldrich), heated for 5min at 100°C, and applied on a 4-15% SDS-PAGE gel (Mini-PROTEAN TGX , Bio-Rad). Next, proteins were blotted on a Trans-Blot Turbo Mini PDVF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Nonspecific binding sites were blocked in TBS/0.05% (v/v) Tween-20 containing 5% (w/v) skimmed milk. Membranes were probed with rabbit-anti-CLCA1 (1:2500; EPR12254-88; Abcam, Cambridge, United Kingdom), rabbit-anti-SERPINB2 (1:1000; ab47742; Abcam), rabbit-anti-POSTN (1:2500, ab14041, Abcam) or GAPDH (1:1000; 14C10; Cell Signaling Technologies, Leiden, The Netherlands) in 5% (w/v) BSA TBS/0.05% (v/v) Tween-20 overnight at 4°C. Afterwards, membranes were incubated with anti-rabbit IgG HRP-linked Antibody (1:10000, Cell Signaling Technologies) in blocking buffer for 1h and membranes were subsequently developed with enhanced chemiluminescence substrate (Thermo Fisher Scientific).

Statistical Analysis

Graphs were made and statistical analysis was performed in GraphPad PRISM 6.02 (GraphPad Software Inc., La Jolla, Ca). Data are shown as means ± SEM of cultures derived from several donors and differences were considered significant at p-values < 0.05. Differences were explored by one-way ANOVA with Dunnett's test.

RESULTS

Azithromycin lowers IL-13-induced MUC5AC and MUC2 expression, but does not significantly affect MUC5B expression

We first assessed the ability of IL-13 to induce MUC5AC expression in ALI-PBEC. The concentration of IL-13 was optimized in pilot experiments that showed inconsistent induction of MUC5AC at 1 ng/ml IL-13 whereas consistent induction of MUC5AC was observed for all donors at 5 ng/ml IL-13 (results not shown). We observed that IL-13 not only induced MUC5AC, but also increased MUC2 expression, whereas it lowered MUC5B expression (Fig. 1). Exposure to azithromycin caused a dose-dependent inhibition of IL-13-induced expression of MUC5AC and MUC2. These observations were confirmed at the protein level using dotblot analyses of protein lysates (Fig. 1).

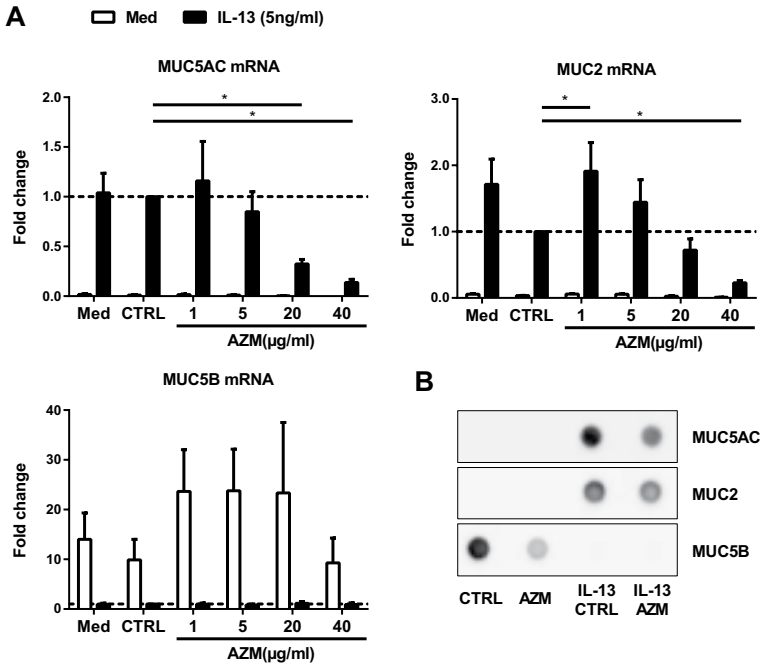

Figure 1. Modulation of mucin expression by azithromycin in IL-13-exposed ALI-PBEC cultures. ALI -PBEC were cultured for 14 days in the presence (black bars) or absence (white bars) of IL-13 (5 ng/ml) and various concentrations of azithromycin (AZM) or its solvent control (CTRL = DMSO; equivalent to the highest concentration of AZM). Mucin gene expression (A) was assessed by qRT-PCR and protein (B) by dotblot for which a representative example is shown. Results are expressed as mean ± SEM fold change compared to IL13 stimulated condition with CTRL (indicated by a horizontal dashed line) (n=4 different donors). *p<0.05.

Azithromycin lowers IL-13-induced SPDEF and CLCA1 expression

To investigate the mechanisms underlying the modulation of IL-13-induced MUC5AC expression by azithromycin, the effect on CLCA1, SPDEF and FOXA2 gene expression was investigated. IL-13 increased SPDEF and CLCA1 expression, while a non-significant decrease in FOXA2 was observed (Fig. 2). Whereas azithromycin caused an increase of FOXA2 expression in control-treated cells which did not reach statistical significance, azithromycin did not prevent the IL-13-induced decrease in FOXA2 expression. In contrast, azithromycin significantly reduced IL-13-induced CLCA1, whereas inhibition of SPDEF expression did not reach statistical significance (p = 0.11). CLCA1 gene expression data was confirmed by measurement of protein levels in basal medium and cell lysates (Fig. 2).

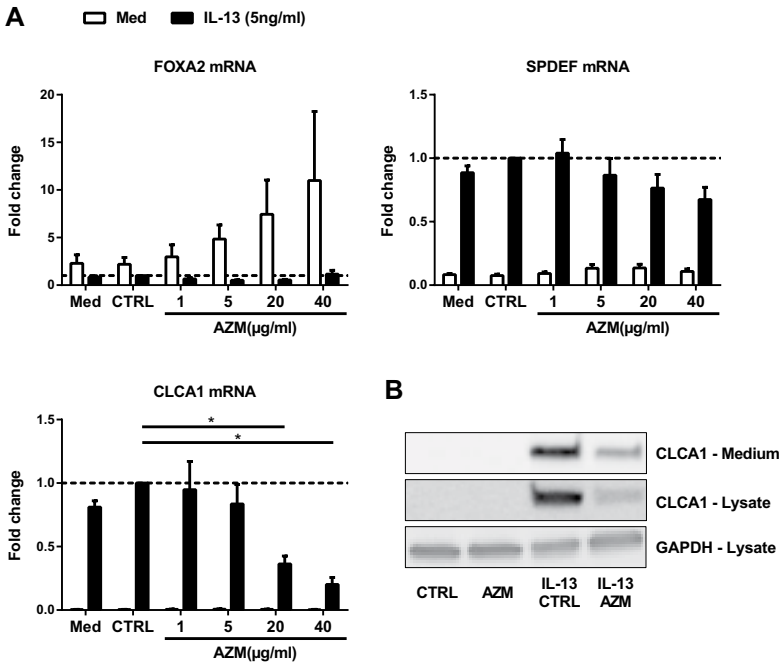

Figure 2. Modulation of SPDEF, CLCA1 and FOXA2 expression by azithromycin in IL-13-exposed ALI-PBEC cultures. ALI-PBEC were cultured for 14 days in the presence (black bars) or absence (white bars) of IL-13 (5 ng/ml) and various concentrations of azithromycin (AZM) or its solvent control (CTRL = DMSO; equivalent to the highest concentration of AZM). FOXA2, SPDEF and CLCA1 gene expression (A) was assessed by qRT-PCR and CLCA1 protein (B) by western blot for which a representative example is shown. Results are expressed as mean ± SEM fold change compared to IL-13 stimulated condition with CTRL (indicated by a horizontal dashed line) (n=4 different donors). *p<0.05.

Azithromycin differentially affects the IL-13-induced Th2 gene signature in human bronchial epithelial cells

In addition to CLCA1, POSTN and SERPINB2 are also increased in human airway epithelial cells following IL-13 exposure and have been suggested as biomarkers for Th2-mediated inflammation (5, 9). In the present study, IL-13 exposure significantly increased POSTN and SERPINB2 gene expression in the ALI-PBEC culture system (Fig. 3). Treatment of IL-13-exposed ALI-PBEC with various concentrations of azithromycin resulted in a significant reduction of SERPINB2 expression, similar to what was observed for CLCA1. In contrast, exposure to azithromycin led to a further dose-dependent increase in POSTN expression. Gene expression data was confirmed by measurement of protein levels in basal medium and cell lysate. In line with the gene expression data, azithromycin treatment resulted in lower protein levels of SERPINB2 in cell lysates. SERPINB2 could not be detected in the basal medium. Additionally, azithromycin further increased the IL-13-induced expression of POSTN protein in both cell lysates and basal medium (Fig. 3).

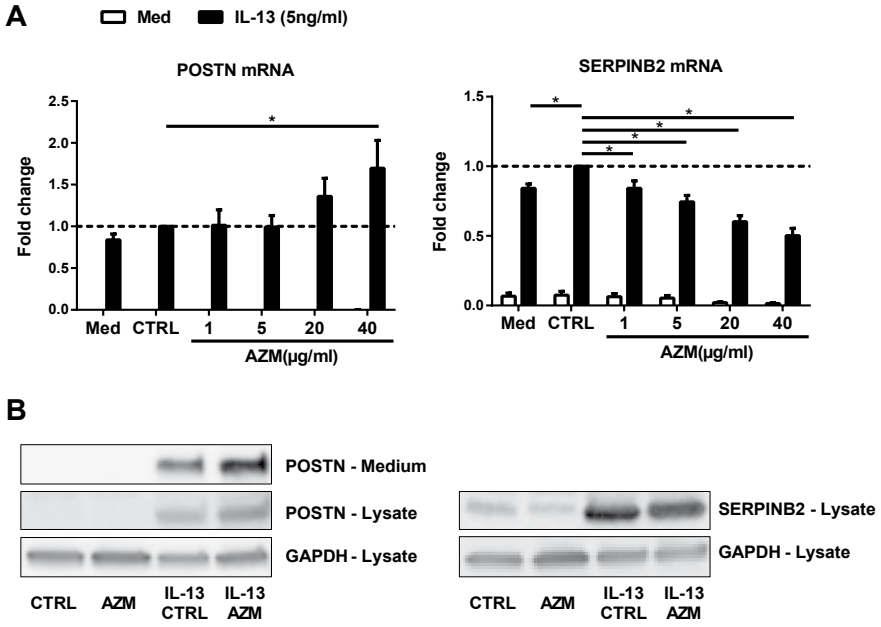

Figure 3. Modulation of the Th2 signature genes by azithromycin in IL-13-exposed ALI-PBEC cultures. ALI-PBEC were cultured for 14 days in the presence (black bars) or absence (white bars) of IL-13 (5 ng/ml) and various concentrations of azithromycin (AZM) or its solvent control (CTRL = DMSO; equivalent to the highest concentration of AZM). POSTN and SERPINB2 gene expression (A) was assessed by qRT-PCR and protein (B) by dot blot for which a representative example is shown. Results are expressed as mean ± SEM fold change compared to IL-13 stimulated condition with CTRL (indicated by a horizontal dashed line) (n=4 different donors). *p<0.05.

DISCUSSION

Results from the present study show that the macrolide azithromycin differentially affects the IL-13-induced expression profile in human bronchial epithelial cells. Azithromycin inhibits IL-13-induced MUC5AC expression, which is accompanied by inhibition of SPDEF, CLCA1 and SERPINB2 expression. In contrast, azithromycin further increases IL-13-induced POSTN expression. CLCA1, SERPINB2 and POSTN were recently described as a biomarker for Th2-high asthma. Our results indicate that azithromycin has a differential effect on this IL-13-induced Th2 gene signature. The modulatory effects of azithromycin on IL-13-induced changes in gene expression is summarized in table 2.

Table 2. Overview effects azithromycin on the IL-13-induced expression pattern. AZM, azithromycin; CTRL, control; IL-13, interleukin-13; ND, not detected

Gene	Effect of IL-13		Effect of AZM on IL-13-induced expression	
	mRNA	protein	mRNA	protein
SPDEF	↑	ND	-	ND
FOXA2	↓	ND	-	ND
MUC5AC	↑↑	↑↑	↓↓	↓
CLCA1	↑↑	↑↑	↓	↓
SERPINB2	↑	↑	↓	↓
POSTN	↑↑↑	↑↑↑	↑	↑

Our study confirms the potential of azithromycin to inhibit mucus production, as shown by its ability to inhibit expression of not only MUC5AC, but also MUC2. Azithromycin has been shown in previous studies to attenuate goblet cell metaplasia and airway inflammation in allergic airway inflammation models in mice (19). Additionally, clarithromycin is able to inhibit IL-13-induced goblet cell metaplasia in human bronchial epithelial cells (17). So far the pathophysiological mechanism is not well described.

The present results show that azithromycin attenuates IL-13-induced CLCA1 expression. Increased CLCA1 expression is found in airway epithelial cells of asthmatics and is linked to airway hyperresponsiveness and goblet cell metaplasia (25-27). A study by Alevy and colleagues indicated an important role for CLCA1 in IL-13-induced MUC5AC expression

(8). In this study, selective inhibition of CLCA1 and MAPK13 (activated by CLCA1) in IL-13-stimulated human airway epithelial cells significantly reduced MUC5AC expression. Furthermore, niflumic acid, a chloride channel inhibitor, has been shown to suppress IL-13-induced MUC5AC expression in human airway epithelial cells providing indirect evidence for a role of a role of one of these chloride channels, CLCA1 (28). Collectively, these data suggest that IL-13-induced CLCA1 expression mediates MUC5AC expression via MAPK13. Our results show that azithromycin attenuates IL-13-induced CLCA1 expression in addition to MUC5AC expression, and suggest that azithromycin blocks IL-13 induced goblet cell metaplasia by inhibiting CLCA1.

Two other molecules which have been shown to play an important role in IL-13-induced MUC5AC expression are SPDEF and FOXA2. SPDEF expression has been implicated in Th2-mediated goblet cell metaplasia both *in vivo* and *in vitro* (43, 44). Increased expression of spdef has been found in the respiratory epithelium of mice challenged with IL-13 (29). Additionally, epithelial overexpression of spdef resulted in goblet cell metaplasia whereas genetic deletion of spdef abrogated goblet cells in the respiratory epithelium after pulmonary allergen exposure (30). In human bronchial epithelial cells treatment with siRNA against SPDEF attenuated IL-13-induced MUC5AC expression (7). In the present study, IL-13 increased SPDEF gene expression, which was non-significantly inhibited by azithromycin. Therefore it can be suggested that azithromycin may reduce goblet cell metaplasia by lowering SPDEF expression although this effect remains to be investigated at the protein level. In contrast to the positive regulators of goblet cell metaplasia SPDEF and CLCA1, FOXA2 is an inhibitory regulator and IL-13 has been shown to reduce FOXA2 expression in human bronchial epithelial cells (6). FOXA2 expression is decreased in lung tissues from patients with a variety of pulmonary diseases (31). In mice, inhibition of Foxa2 causes spontaneous goblet cell metaplasia, whereas conditional expression of Foxa2 inhibits allergen-induced goblet cell differentiation (32). However, the present results show that azithromycin was unable to prevent the IL-13-induced inhibition of FOXA2, making this pathway unlikely to mediate the effects of azithromycin.

The airway epithelial Th2 gene signature can be induced *in vitro* by IL-13 as demonstrated by the ability of IL-13 to induce POSTN, CLCA1 and SERPINB2 expression in cultured airway epithelial cells (9). Our results in a two weeks exposure model confirm these findings. Importantly, our model also reflects mucin expression patterns found in airway epithelial brushings of Th2-high asthma patients with higher MUC5AC and MUC2 expression and

lower MUC5B expression compared to healthy controls (5). We observed that azithromycin lowered IL-13-induced MUC5AC and MUC2 expression, but did not significantly alter MUC5B expression. The observation that MUC5B expression is maintained in presence of azithromycin is important in view of a recent publication suggesting an important role for MUC5B in airway defense (33).

Azithromycin also inhibited IL-13-induced expression of SERPINB2, another member of the Th2 signature. SERPINB2 is a serine protease inhibitor that can inhibit plasminogen activators thereby preventing plasmin activation. Plasmin is able to degrade the extracellular matrix, either directly by removing glycoproteins or by activating metalloproteinases (34). Therefore, by inhibiting SERPINB2, more plasmin becomes available for extracellular matrix turnover and reducing airway remodeling.

The biological consequences of the increased levels of periostin are so far not clear. POSTN is an extracellular matrix protein and integrin ligand known to be produced by airway epithelial cells and fibroblasts. The functional role of POSTN in asthma remains controversial. Initial studies using POSTN-deficient mice suggested a protective role whereby POSTN was able to prevent allergen-induced goblet cell metaplasia in mouse tracheal epithelial cells (35). Additionally, POSTN has been shown to inhibit allergen-induced IgE production and airway hyperresponsiveness (36). However, a more recent study, also performed in POSTN-deficient mice, suggested that POSTN is required for airway hyperresponsiveness (37). Nonetheless, at present the development of serum POSTN as a biomarker is clinically more important and treatment responses to anti-IL-13 treatments have been linked to baseline levels of POSTN (1, 13). Therefore it is important to identify factors which influence the expression of POSTN and could also affect serum levels in this patient group.

Macrolides are increasingly used for chronic treatment of lung diseases and also in asthma macrolides have been used for prevention and treatment of exacerbations (16, 38-41). Interestingly, the long-term effects of macrolides seem to be dependent on asthma phenotype. Patients with predominantly neutrophilic airway inflammation show beneficial responses to macrolide treatment, whereas macrolides are not effective in patients with eosinophilic airway inflammation (16, 42). Overall, our results indicate that macrolide treatment differentially affects the Th2 signature that has been described to identify Th2-high asthma, which is usually associated with eosinophilic inflammation.

The concentrations of azithromycin used in the present study are likely relevant for understanding the clinical effects of azithromycin treatment. The concentrations used in the present study ranged between 1 and 40 μg/ml, which is within the range reported in the lungs of patients treated with this macrolide: 2.18 μg/ml in the epithelial lining fluid to 3.89 ± 1.2 μg/ml in bronchial mucosal tissue (43). Importantly, concentrations of azithromycin in tissues can be up to 100-fold higher than those in plasma, which range around 0.15 and 0.05 μg/ml (44-46). Additionally, phagocytes accumulate azithromycin which results in 200-fold higher intracellular than extracellular concentrations (47, 48). Since azithromycin accumulation differs in various cell types (47-49), it is likely that both passive and active transport are important for azithromycin accumulation. Overall this suggests that clinically relevant concentrations of azithromycin have been used in this study.

This study has some limitations. We used bronchial epithelial cells derived from macroscopically normal resected tissue obtained during surgery for lung cancer, indicating that cells from smokers were used. Additionally, bronchial epithelial cells from asthmatics were not available to further evaluate the effect of azithromycin on the Th2 signature. We have tried to further elucidate the role of FOXA2 in the effects of azithromycin using siRNA-mediated knockdown of FOXA2 as described previously by Ramachandran and colleagues for the CFTR gene (50). However, whereas transient suppression of FOXA2 gene expression was achieved, we were unable to maintain low levels of FOXA2 during two weeks differentiation. Future studies using alternative approaches using e.g. CRISPR/Cas9 technology are needed, but were outside the scope of the present study.

Our results indicate that azithromycin differentially affects the gene signature that has been described for Th2-high asthma. The observation that azithromycin increases IL-13-induced POSTN expression while decreasing SERPINB2 and CLCA1, is of potential clinical significance because especially POSTN is considered as an emerging, possibly important biomarker for Th2 inflammation in asthma. This study indicates that macrolide treatment may differentially affect biomarkers derived from this gene signature, and that data on modulation of Th2 inflammation based on such biomarkers should be interpreted with caution. Our study further extends previous findings on the ability of macrolides to inhibit goblet cell metaplasia, by demonstrating that azithromycin blocks MUC5AC and MUC2, possibly by inhibiting CLCA1. These observations on macrolide-induced suppression of goblet cell metaplasia secondary to Th2 inflammation, provide a mechanistic basis for the observed beneficial effects of macrolides in asthma and COPD. Clinical results that show that

azithromycin is not effective in Th2-mediated asthma. Azithromycin inhibits expression of several IL-13-induced genes with the exception of POSTN. Therefore, further investigations should focus on the role of periostin in asthma. Additionally, critical analysis is needed for the effect of various treatments on biomarker expression profiles.

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CHAPTER 4

CIGARETTE SMOKE DIFFERENTIALLY AFFECTS IL-13-INDUCED GENE EXPRESSION IN HUMAN AIRWAY EPITHELIAL CELLS

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ABSTRACT

Allergic airways inflammation in asthma is characterized by an airway epithelial gene signature composed of *POSTN*, *CLCA1* and *SERPINB2*. This Th2 gene signature is proposed as a tool to classify patients with asthma into Th2-high and Th2-low phenotypes. However, many asthmatics smoke and the effects of cigarette smoke exposure on the epithelial Th2 gene signature are largely unknown. Therefore, we investigated the combined effect of IL-13 and whole cigarette smoke (CS) on the Th2 gene signature and the mucin-related genes *MUC5AC* and *SPDEF* in air-liquid interface differentiated human bronchial (ALI-PBEC) and tracheal epithelial cells (ALI-PTEC). Cultures were exposed to IL-13 for 14 days followed by 5 days of IL-13 with CS exposure. Alternatively, cultures were exposed once daily to CS for 14 days, followed by 5 days CS with IL-13. *POSTN*, *SERPINB2* and *CLCA1* expression was measured 24h after the last exposure to CS and IL-13. In both models *POSTN*, *SERPINB2* and *CLCA1* expression was increased by IL-13. CS markedly affected the IL-13-induced Th2 gene signature as indicated by a reduced *POSTN, CLCA1* and *MUC5AC* expression in both models. In contrast, IL-13-induced *SERPINB2* expression remained unaffected by CS, whereas *SPDEF* expression was additively increased. Importantly, cessation of CS exposure failed to restore IL-13-induced *POSTN* and *CLCA1* expression. We show for the first time that CS differentially affects the IL-13-induced gene signature for Th2-high asthma. These findings provide novel insights in the interaction between Th2 inflammation and cigarette smoke that is important for asthma pathogenesis and biomarker-guided therapy in asthma.

INTRODUCTION

Asthma is a syndrome characterized by airway hyperresponsiveness, chronic inflammation and mucus hypersecretion. Historically asthma, and particularly allergic asthma, has been considered to be mainly driven by a T helper 2 (Th2)-mediated immune response. However, it is now well recognized that asthma is a heterogeneous disease with different pathophysiological pathways underlying airway inflammation (1). Molecular phenotyping of diseased airway tissue has the potential to unravel the multiple phenotypes of asthma. Furthermore, it allows the identification of biomarkers associated with specific disease patterns to select patients for personalized targeted therapies.

Approximately 50% of asthmatic patients have Th2-mediated disease (1, 2). A Th2-high subtype of asthma has been described and is associated with increased bronchial epithelial expression of periostin (*POSTN*), serpin B2 (*SERPINB2*) and chloride channel regulator 1 (*CLCA1*), and predicts a beneficial therapeutic response to corticosteroids (2, 3). Recently various clinical trials have shown the potential of inhibitors of Th2 inflammation, including monoclonal antibodies against interleukin (IL)-13, to modulate clinical outcomes in asthma (4). IL-13 is produced by Th2 cells and has been shown to have marked effects on airway epithelial cells (3, 5). IL-13 is an important mediator for the induction of goblet cell metaplasia in Th2-mediated asthma and is a central regulator in the epithelial expression of *POSTN*, *SERPINB2* and *CLCA1* (3). Periostin, the protein encoded by the *POSTN* gene, is of particular interest as a biomarker, as it is detectable in the circulation and may be useful as a blood biomarker for IL-13-activated bronchial epithelial cells. Indeed, there is evidence suggesting that circulating periostin levels may help in the identification of asthma patients that benefit from anti-IL-13 treatment (6-8).

Asthma has a genetic predisposition, but is it recognized that environmental factors are very important in the pathogenesis. An important environmental factor influencing asthma pathogenesis is cigarette smoking. Approximately 20 to 35% of the world population smokes, with surprisingly similar smoking rates reported in asthmatic patients (9-11). Cigarette smoking has been shown to worsen asthma symptoms, reduce responsiveness to corticosteroid treatment, accelerate lung function decline and increase exacerbation rates (12). Additionally, smoking is strongly predictive for the development of new onset asthma in atopic adults (13). As a history of current or former smoking is present in approximately 20 to 30% of the asthmatic population (9, 10), cigarette smoking could be considered as one

of the most important environmental factors influencing asthma pathogenesis.

We have previously shown that the IL-13-induced epithelial Th2-gene signature can be differentially affected by azithromycin treatment, suggesting that IL-13 induces its gene expression pattern through various pathways (14). Furthermore, a suppressive effect of cigarette smoke on *POSTN* and *SERPINB2* gene expression has previously been suggested based on a study focussing on the presence of a Th2-gene signature in patients with chronic obstructive pulmonary disease (COPD) (15). Surprisingly little is known about the effect of cigarette smoking on IL-13-activated airway epithelial cells and the IL-13-induced gene expression pattern described for Th2-high asthma. Therefore, we have investigated, for the first time, the combined effect of whole cigarette smoke exposure and IL-13 on primary human airway epithelial cells cultured at the air-liquid interface, thus providing novel insights in the interaction between Th2 inflammation and cigarette smoke that is relevant for asthma pathogenesis and biomarker-guided therapy in asthma.

MATERIAL AND METHODS

Cell culture

Human primary bronchial epithelial cells (PBEC) were isolated from macroscopically normal bronchial tissues obtained from lung cancer patients undergoing lobectomy at the Leiden University Medical Center (Leiden, The Netherlands). Primary tracheal epithelial cells were isolated from residual tracheal and main stem bronchial tissue from lung transplant donors post mortem at the University Medical Center Groningen (Groningen, the Netherlands). Use of lung tissue that became available for research within the framework of patient care was in line with the "Human Tissue and Medical Research: Code of conduct for responsible use" (2011) (www.federa.org), that describes the no-objection system for coded anonymous further use of such tissue. Therefore, individual written or verbal consent is not applicable. Details on isolation of PBEC (14) and PTEC (16) were described previously. During 14 days of differentiation, cell culture medium was replaced every two days.

Cultured PBEC and PTEC were used for generation of mucociliary differentiated cultures by differentiation at the air-liquid interface (ALI) as described previously (14). Briefly, PBEC and PTEC at passage 2 were cultured submerged on semipermeable transwell inserts with 0.4 µm pore size (Corning Costar, Cambridge, MA, USA) that were coated with a mixture of bovine serum albumin, collagen type 1 and fibronectin. Once full confluence was reached, apical medium was removed and PBEC or PTEC were used for subsequent experimental exposures.

Experimental design

Two experimental models were used to investigate the effects of whole cigarette smoke exposure on the IL-13-induced expression pattern (Figure 1). In exposure model A, ALI-PBEC or ALI-PTEC were grown to confluence and cultured for 14 days at the ALI in the presence of 1 or 2.5 ng/ml IL-13 which was added in the basolateral compartment of the transwell insert, followed by an additional 5 days once daily whole cigarette smoke or air exposure in the presence or absence of continued treatment with 1 or 2.5 ng/ml recombinant human IL-13 (Peprotech, Rocky Hill, CT, USA) which was added in the basolateral compartment of the transwell insert. In model B, ALI-PBEC or ALI-PTEC were grown to confluence, and next cultured at the ALI and exposed once daily to whole cigarette smoke or air for 14 days,

followed by an additional 5 days once daily whole cigarette smoke or air exposure in the presence or absence of continued treatment with 10 ng/ml IL-13 that was added to the basolateral compartment of the transwell insert. For both models, ALI-PBEC or ALI-PTEC were rinsed apically with 200 µl PBS, 4 h prior to whole cigarette smoke or air exposure. During 14 days of exposure, cell culture medium was replaced every two days. During the last 5 days of exposure, medium was refreshed daily, directly after whole cigarette smoke or air exposure. 24 h after the last whole cigarette smoke or air exposure, cells were lysed for RNA or protein extraction, and basal medium was collected and stored at -20°C until further use.

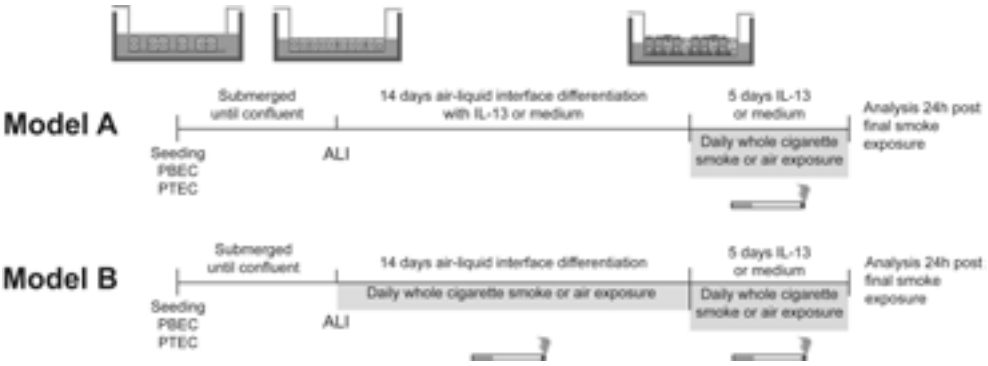

Figure 1. Exposure setup to investigate the effect of whole cigarette smoke exposure on the IL-13-induced expression pattern in human bronchial and tracheal epithelial cells. ALI, air-liquid interface; IL-13, interleukin-13; PBEC, primary bronchial epithelial cells; PTEC, primary tracheal epithelial cells.

Whole cigarette smoke exposure

ALI-PBEC or ALI-PTEC were exposed to cigarette smoke generated from 3R4F reference cigarettes (University of Kentucky, Lexington, KY, USA) in a whole cigarette smoke exposure model adapted from Beisswenger et al. (17) as described previously (18). In brief, ALI-cultures were placed into modified hypoxic chambers (Billups Rothenberg, Del Mar, CA, USA), localized inside an incubator at 37°C and 5% CO_2. Whole cigarette smoke derived from one cigarette, or air as negative control, was permeated inside the respective exposure chamber using a continuous flow of 1 l/min for a period of 4–5 min. After exposure, residual smoke inside the exposure chamber was removed by flushing the chambers with air derived from the incubator for a period of 10 min. After smoke or air exposure, cell culture medium

was refreshed and cells were incubated at 37°C and 5% CO_2.

RNA isolation, reverse transcription (RT) and qPCR

Total RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Leiden, The Netherlands) and quantified using the Nanodrop ND-1000 UV-visible spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). For cDNA synthesis, 1 µg of total RNA was reverse transcribed using oligo(dT) primers and Moloney murine leukemia virus (M-MLV) polymerase (Promega) at 37°C. Primer sequences are listed in table 1. RPL13A and ATP5B were used as reference genes following selection by the Genorm method (19). All quantitative PCRs (qPCRs) were carried out in triplicate on a CFX-384 real-time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands) with the use of SYBR green (Bio-Rad). Bio-Rad CFX manager 3.1 software (Bio-Rad) was used to calculate arbitrary gene expression by using the standard curve method.

Table 1. Primer sequences with gene names and NCBI gene ID used in present study.

Gene	Primer Sequence	NCBI Gene ID
POSTN	F: GAC CGT GTG CTT ACA CAA ATT G R: AAG TGA CCG TCT CTT CCA AGG	10631
SERPINB2	F: TCC TGG GTC AAG ACT CAA ACC R: CAT CCT GGT ATC CCC ATC TAC AG	5055
CLCA1	F: ATG GCT ATG AAG GCA TTG TCG R: TGG CAC ATT GGG GTC GAT TG	1179
MUC5AC	F: CCT TCG ACG GAC AGA GCT AC R: TCT CGG TGA CAA CAC GAA AG	4586
SPDEF	F: ATG AAA GAG CGG ACT TCA CCT R: CTG GTC GAG GCA CAG TAG TG	25803
RPL13A	F: AAG GTG GTG GTC GTA CGC TGT G R: CGG GAA GGG TTG GTG TTC ATC C	23521
ATP5B	F: TCA CCC AGG CTG GTT CAG A R: AGT GGC CAG GGT AGG CTG AT	506

Periostin and mucin 5AC ELISA

Periostin protein expression was measured in medium collected from the basolateral compartment of the transwell 24 h after the last whole cigarette smoke or air exposure. Periostin ELISA was performed according to the manufacturer's instruction (R&D Systems Europe Ltd, Abingdon, United Kingdom). For Mucin 5AC protein expression, cells were lysed in RIPA buffer 24 h after the last whole cigarette smoke or air exposure according to the manufacturer's instruction (Thermo Fisher Scientific, Breda, The Netherlands). Lysate was diluted in bicarbonate coating buffer without azide and incubated in a NUNC maxisorp ELISA plate (Thermo Fisher Scientific) at 37°C until dry. Plates were washed and non-specific binding sites were blocked with PBS/2% (w/v) BSA (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 2 h at room temperature, followed by 2h incubation with mouse anti-MUC5AC (1:200; 45M1; Thermo Fisher Scientific) in PBS/0.05% Tween-20 (v/v) (Sigma-Aldrich) at room temperature. Next plates were washed with PBS/0.05% Tween-20 and incubated for 1 h with goat anti-mouse HRP (1:2000, Dako Denmark A/S, Glostrup, Denmark) at room temperature. Plates were developed using tetramethylbenzidine-hydrogen peroxidase solution and the reaction was stopped with 2.5 M H_2SO_4. Absorbance was measured at 450 nm using a Microplate reader (iMark; Bio-Rad Laboratories, Hercules, CA, USA) and Microplate Manager Software (version 6.3, Bio-Rad).

SDS-PAGE & Western blot

Protein RIPA lysates were diluted (1:1 [v/v]) in sodium dodecyl sulfate (SDS) sample buffer, containing 4% (w/v) SDS (Sigma-Aldrich), 20% (v/v) glycerol (Merck), 0.8% (w/v) DL-dithiothreitol (Sigma-Aldrich), 0.5 M Tris pH 6.8 and 0.003% (w/v) bromophenol blue (Sigma-Aldrich), heated for 5 min at 100°C, and applied on a 4-15% SDS-PAGE gel (Mini-PROTEAN TGX , Bio-Rad). Next, proteins were blotted on a Trans-Blot Turbo Mini PDVF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Nonspecific binding sites were blocked in Tris-buffered saline (TBS)/0.05% (v/v) Tween-20 containing 5% (w/v) skimmed milk. Membranes were probed with rabbit-anti-CLCA1 (1:1000; EPR12254-88; Abcam, Cambridge, United Kingdom), rabbit-anti-SERPINB2 (1:1000; ab47742; Abcam), rabbit-anti-POSTN (1:1000, ab14041, Abcam) or GAPDH (1:1000; 14C10; Cell Signaling Technologies, Leiden, The Netherlands) in 5% (w/v) BSA TBS/0.05% (v/v) Tween-20 overnight at 4°C. Next, membranes were incubated with anti-rabbit IgG HRP-linked antibody (1:10000, Cell Signaling Technologies) in blocking buffer for 1h and binding was revealed using enhanced

chemiluminescence substrate (Thermo Fisher Scientific).

Statistical Analysis

Graphs were made and statistical analysis was performed in GraphPad PRISM 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences were explored by one-way ANOVA with Dunnett's test. Data are shown as means ± SEM of cultures derived from several donors and differences were considered significant at p-values < 0.05.

RESULTS

An established IL-13-induced gene expression pattern is differentially affected by whole cigarette smoke exposure

First we investigated the effect of whole cigarette smoke (CS) exposure on an established IL-13-induced Th2 gene expression pattern in ALI-PBEC. To this end, ALI-PBEC were differentiated for 14 days in the presence of IL-13 to establish this Th2 gene signature, followed by 5 days exposure to CS in the presence or absence of IL-13 (Figure 1, Model A). IL-13-differentiated ALI-PBEC showed indeed significantly increased expression of the Th2-signature genes *POSTN*, *SERPINB2* and *CLCA1*, in addition to the goblet cell metaplasia-related genes *MUC5AC* and *SAM pointed domain containing ETS transcription factor* (*SPDEF*) (Figure 2A). IL-13-differentiated ALI-PBEC exposed to CS in the presence of IL-13 had significantly reduced *POSTN* expression compared to the IL-13-differentiated ALI-PBEC exposed to air in the presence of IL-13. In contrast, *MUC5AC* and *SERPINB2* expression remained unaffected, whereas *SPDEF* expression was additively increased in CS-exposed IL-13-differentiated ALI-PBEC compared to air exposure (Figure 2A). Cessation of IL-13 exposure during the last 5 days reduced IL-13-induced gene expression back to baseline expression levels, suggesting that the effect of IL-13 does not persist (Figure 2A). Cigarette smoke exposure has previously been shown alter methylation patterns in airway epithelial cells (20). As mucin 5AC and periostin are both important biomarker proteins, we validated our gene expression findings for *MUC5AC* and *POSTN* using ELISA (Figure 2B). Mucin 5AC protein expression appeared to be lowered upon IL-13 exposure cessation; however, 5 days seems insufficient time to return protein levels to baseline expression levels. Additionally, periostin protein could only be detected in ALI-PBEC that were continuously exposed to IL-13 without the presence of CS (Figure 2B).

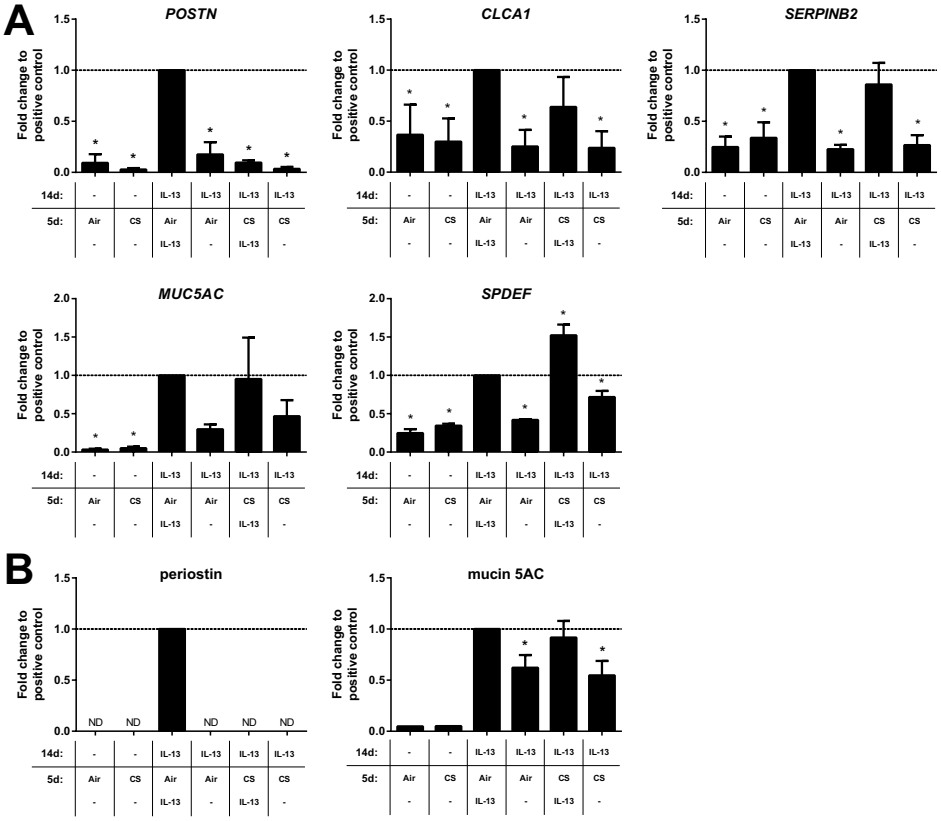

Figure 2. Effect of whole cigarette smoke exposure on IL-13-induced gene expression in ALI-PBEC. ALI-PBEC were differentiated with IL-13 (1 ng/ml) for 14 days followed by an additional 5 days with IL-13 (1 ng/ml) in the presence of air or CS exposure. MUC5AC, POSTN, SERPINB2, SPDEF and CLCA1 gene expression (A) was assessed by qRT-PCR; periostin and mucin 5AC protein (B) was assessed by ELISA. Results are expressed as mean ± SEM fold change compared to IL-13-exposed ALI-PBEC exposed to air (indicated by a horizontal dashed line) with n = 4 independent donors. *$p < 0.05$. ALI, air-liquid interface; CS, whole cigarette smoke exposure; IL-13, interleukin-13; PBEC, primary bronchial epithelial cells.

To investigate whether the effect of CS on IL-13-induced *POSTN* expression resulted from CS-induced DNA methylation effects, we daily treated CS-exposed IL-13-differentiated ALI-PBEC with the demethylating compound 5-azacytidine (5 or 25 µM) during the last 5 days of CS exposure. However, 5-azacytidine treatment during CS exposure was unable to restore *POSTN* expression levels (Figure 3).

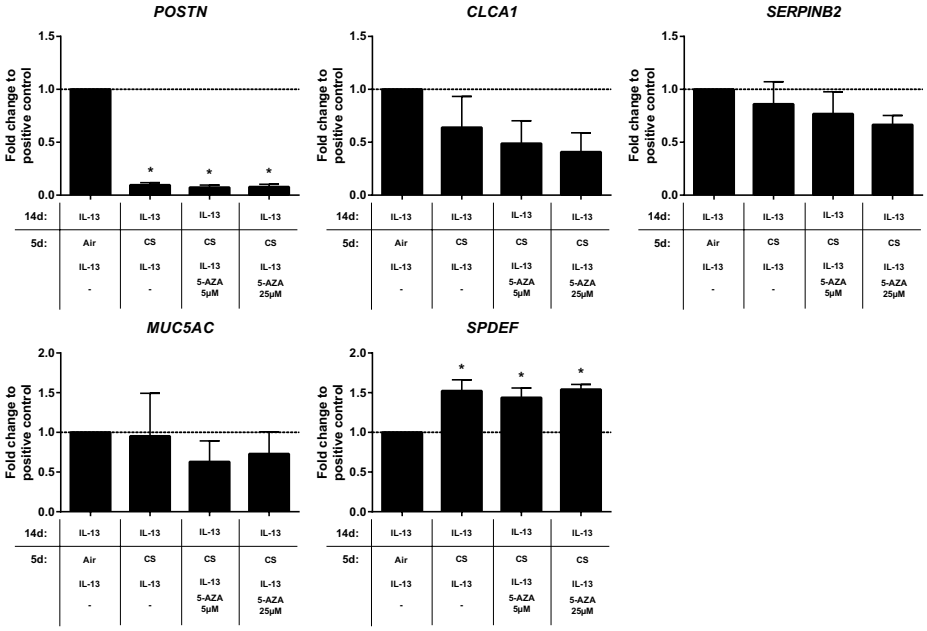

Figure 3: Whole cigarette smoke exposure does not affect the IL-13-induced Th2 gene signature through promoter methylation. ALI-PBEC were differentiated with IL-13 (1 ng/ml) for 14 days followed by an additional 5 days with IL-13 (1 ng/ml) in the presence of air or whole cigarette smoke exposure with or without 5-azacytidine. MUC5AC, POSTN, SERPINB2, SPDEF and CLCA1 gene expression (A) was assessed by qRT-PCR; POSTN and MUC5AC protein (B) was assessed by ELISA. Results are expressed as mean ± SEM fold change compared to IL-13-exposed ALI-PBEC exposed to air (indicated by a horizontal dashed line) with n = 4 independent donors. *p < 0.05. ALI, air-liquid interface; 5-AZA, 5-azacytidine; CS, whole cigarette smoke exposure; IL-13, interleukin-13; PBEC, primary bronchial epithelial cells.

Whole cigarette smoke exposure differentially affects IL-13-induced responsiveness

Our results showed a noticeably differential effect of CS exposure on an established IL-13-induced gene expression pattern in ALI-PBEC. Next we investigated whether chronic CS exposure in ALI-PBEC affected the ability of IL-13 to promote the expression of these genes. To this end, we differentiated ALI-PBEC with daily CS exposure or air as a control for 14 days, followed by another 5 days of daily CS (or air) exposure in the absence or presence of IL-13 (10 ng/ml, added in the basal chamber) as depicted in Figure 1, Model B. Pilot results indicated that short-term IL-13 exposure of ALI-PBEC differentiated in the presence of CS exposure induced STAT6 phosphorylation, an important downstream mediator of IL-13-induced changes, to the same extent as control differentiated ALI-PBEC, suggesting that CS-differentiated ALI-PBEC are still able to respond to IL-13 (results not shown). Control ALI-PBEC exposed for 5 days to IL-13 displayed an increased expression of the Th2-signature genes *POSTN*, *SERPINB2* and *CLCA1*, in addition to an increase in the goblet cell metaplasia related genes *MUC5AC* and *SPDEF* (Figure 4A). In contrast, CS-differentiated ALI-PBEC exposed to IL-13 had significantly reduced *POSTN* and *CLCA1* expression and a trend for reduced *MUC5AC* expression (p=0.094), whereas *SERPINB2* and *SPDEF* expression remained unaffected compared to IL-13-exposed control ALI-PBEC (Figure 4A). These data indicate that upon CS exposure, IL-13 is unable to promote the Th2-signature gene expression to a similar extend as it does in air-exposed controls.

We next investigated whether CS-induced reduction of *POSTN*, *CLCA1* and *MUC5AC* expression would return to IL-13-stimulated control levels upon cessation of CS exposure. After 14 days of daily CS exposure, CS-differentiated ALI-PBEC were exposed for 5 additional days to air in the presence or absence of IL-13. Results showed that upon CS cessation, both *POSTN* and *CLCA1* gene expression remained diminished in the presence of IL-13, however *MUC5AC* expression was fully restored to the level of IL-13-incubated air-exposed control ALI-PBEC (Figure 4A). The findings for mucin 5AC and periostin were confirmed on protein level using ELISA (Figure 4B). Overall, these results further support that CS exposure significantly affects IL-13-induced gene expression patterns in ALI-PBEC.

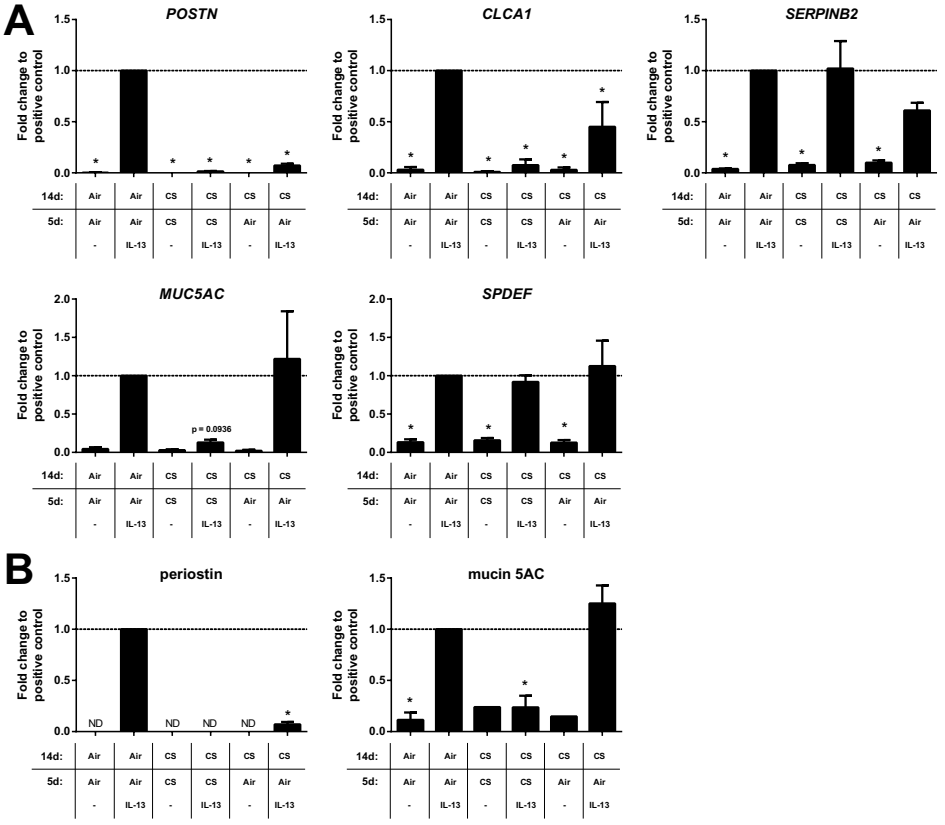

Figure 4. Effect of IL-13 on ALI-PBEC differentiated in the presence of air or whole cigarette smoke exposure. ALI-PBEC were exposed to once daily air or CS exposure during differentiation for 14 days, followed by an additional 5 days with IL-13 (10 ng/ml) in the presence of air or CS exposure. MUC5AC, POSTN, SERPINB2, SPDEF and CLCA1 gene expression (A) was assessed by qRT-PCR; periostin and mucin 5AC protein (B) was assessed by ELISA. Results are expressed as mean ± SEM fold change compared to IL-13-exposed ALI-PBEC exposed to air (indicated by a horizontal dashed line) with n = 4 independent donors. *p < 0.05. ALI, air-liquid interface; CS, whole cigarette smoke exposure; IL-13, interleukin-13; PBEC, primary bronchial epithelial cells.

Regional differences in the lung do not affect responses to cigarette smoke and IL-13

Primary tracheal epithelial cells (PTEC) are more easily accessible for biomarker studies due to their anatomic location compared to bronchial epithelial cells. Furthermore, tracheal epithelial cells have previously been shown to have a similar biological response to CS exposure compared to the small airway epithelium (21). To investigate whether ALI-PTEC show similar responses to ALI-PBEC following combined IL-13 and CS exposure, we exposed ALI-PTEC according to Model A and B (Figure 1). ALI-PTEC responded similar compared to ALI-PBEC, with a few exceptions (Figure 5 and 6). In addition to reduced *POSTN* expression by CS-exposed IL-13-differentiated ALI-PBEC, *MUC5AC* was also significantly reduced in ALI-PTEC compared to air-exposed controls (Figure 5A and 6A), suggesting a slightly stronger disturbance of the Th2-gene signature by CS in ALI-PTEC compared to the ALI-PBEC. Gene expression data for mucin 5AC and periostin was confirmed at the protein level in ALI-PTEC using ELISA (Figure 5B and 6B). Taken together, our results suggest that CS differentially affects the IL-13-induced expression in ALI-PTEC. Moreover, ALI-PTEC can be used as an alternative model for ALI-PBEC to study the effects of IL-13 and CS exposure with regard to the IL-13-induced Th2 gene signature.

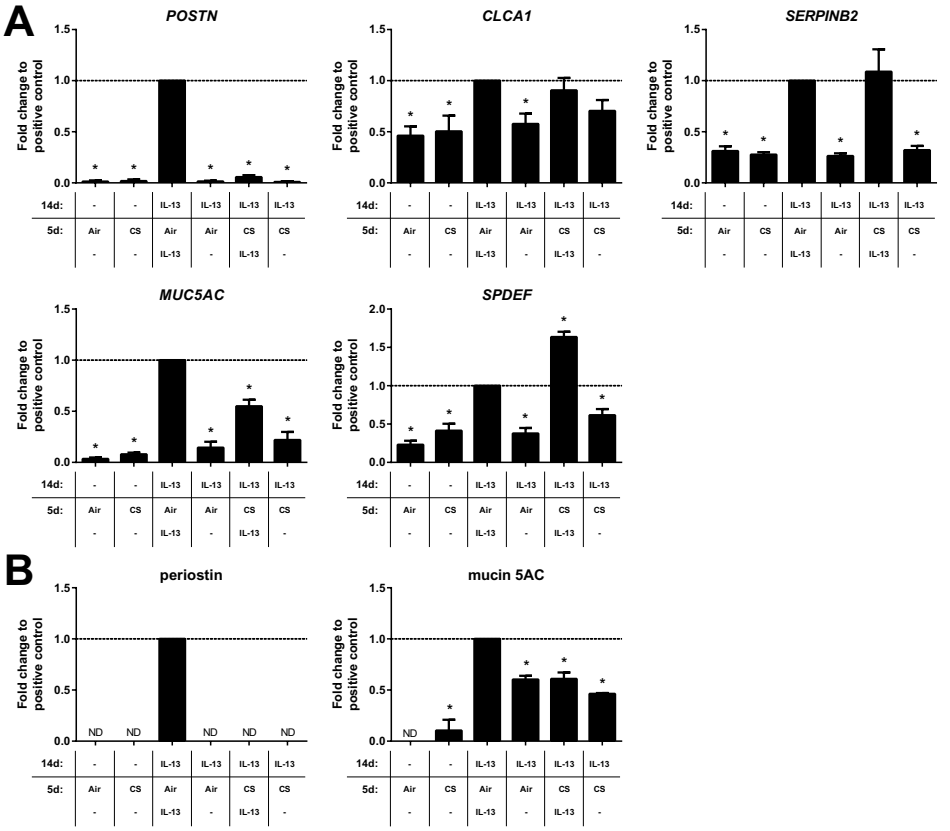

Figure 5. Effect of whole cigarette smoke exposure on IL-13-induced gene expression in ALI-PTEC. ALI-PTEC were differentiated with IL-13 (1 ng/ml) for 14 days followed by an additional 5 days with IL-13 (1 ng/ml) in the presence of air or CS. MUC5AC, POSTN, SERPINB2, SPDEF and CLCA1 gene expression (A) was assessed by qRT-PCR; periostin and mucin 5AC protein (B) was assessed by ELISA. Results are expressed as mean ± SEM fold change compared to IL-13-exposed ALI-PTEC exposed to air (indicated by a horizontal dashed line) with n = 4 independent donors. *p < 0.05. ALI, air-liquid interface; CS, whole cigarette smoke exposure; IL-13, interleukin-13; PTEC, primary tracheal epithelial cells.

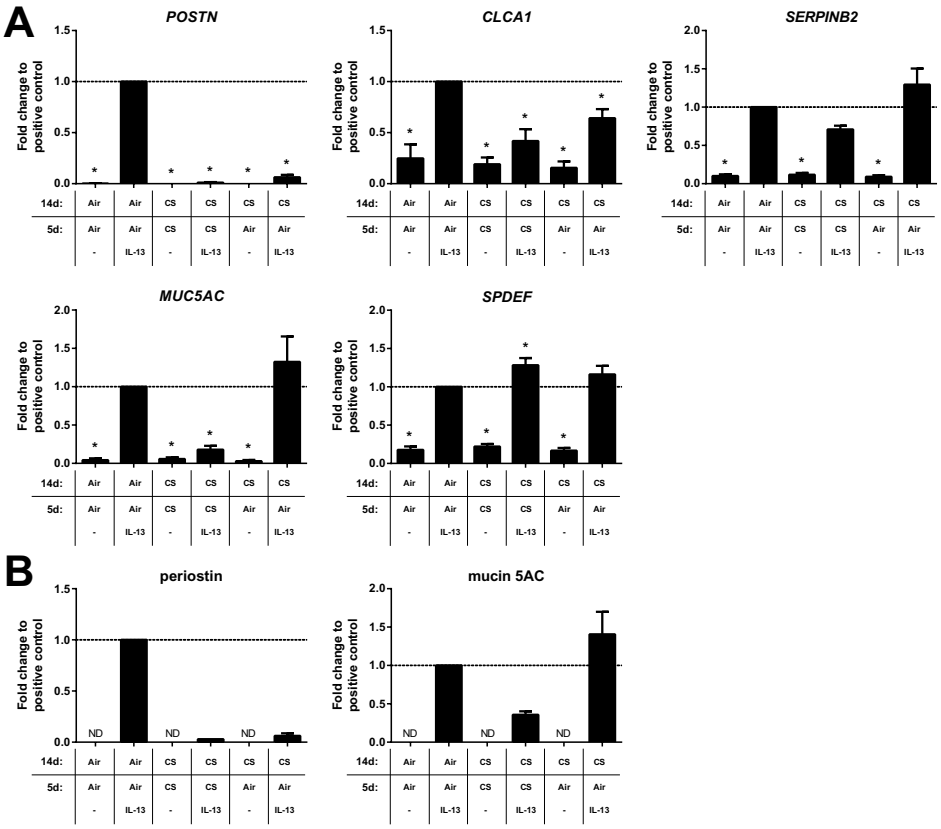

Figure 6. Effect of IL-13 on ALI-PTEC differentiated in the presence of whole cigarette smoke exposure. ALI-PTEC were exposed to daily air or CS exposure during differentiation for 14 days, followed by an additional 5 days with IL-13 (10 ng/ml) in the presence of air or CS. MUC5AC, POSTN, SERPINB2, SPDEF and CLCA1 gene expression (A) was assessed by qRT-PCR; periostin and mucin 5AC protein (B) was assessed by ELISA. Results are expressed as mean ± SEM fold change compared to IL-13-exposed ALI-PTEC exposed to air (indicated by a horizontal dashed line) with n = 4 independent donors. *$p < 0.05$. ALI, air-liquid interface; CS, whole cigarette smoke exposure; IL, interleukin; ND, not detected; PTEC, primary tracheal epithelial cells.

DISCUSSION

The present results show that chronic whole cigarette smoke (CS) exposure differentially affects the IL-13-induced gene expression pattern in primary bronchial and tracheal epithelial cells cultured at the air-liquid interface. Whereas IL-13-induced *POSTN* expression was lowered upon subsequent CS exposure, *MUC5AC*, *CLCA1* and *SERPINB2* expression remained unaffected and *SPDEF* expression was further increased. Conversely, IL-13-responsiveness of primary airway epithelial cells was also severely affected by CS. Differentiation of airway epithelial cells in presence of CS followed by IL-13 exposure resulted in reduced expression of *POSTN*, *CLCA1* and *MUC5AC*, whereas *SERPINB2* and *SPDEF* expression remained unaffected. Cessation of CS exposure in the presence of IL-13 was insufficient to restore *POSTN* and *CLCA1* expression, while *MUC5AC* expression was fully restored. Together these data suggest that CS affects, even upon cessation, the Th2-gene signature that has been suggested to distinguish Th2-high and Th2-low patients.

The clinical effects of smoking in asthmatic patients have been well described. However, little is known about the effect of cigarette smoking on the molecular phenotype that has been suggested for Th2-high asthma. Several mouse models of allergic airway inflammation have reported a suppressive effect of cigarette smoke on Th2-mediated inflammation, including goblet cell metaplasia (22-26). A suppressive effect of cigarette smoke on *POSTN* and *SERPINB2* expression could be expected based on observations on the presence of a Th2-gene signature in a subset of smoking and non-smoking COPD patients (15). Among the genes that comprise the Th2-gene signature, periostin is of particular interest as it can be detected in serum, thus serving as an easy accessible biomarker to distinguish Th2-high from Th2-low asthma patients. Furthermore, high serum periostin levels have been shown to predict therapy response to anti-IL-13 treatment (6, 7, 27). In our exposure models, we noticed a marked decrease in periostin mRNA and protein levels upon CS exposure. These data are also in line with a recently published study showing that serum periostin levels are lower in smoking asthmatics compared to nonsmoking controls (28). Indeed the current study shows the effect of smoking at a cellular level, in part explaining the results observed in the aforementioned patient study. In addition, the present study also shows that CS exposure cessation failed to restore IL-13-induced *POSTN* expression levels, indicating persistence of the effect of CS exposure on IL-13 responsiveness. Several studies have indicated a persistent effect on gene expression profiles in former smokers even several years after smoke cessation, suggesting the involvement of smoking-induced epigenetic mechanisms

(20, 29, 30). A long-lasting effect after smoking cessation in asthmatics on corticosteroid responsiveness has also been suggested by the observation of an attenuated response to corticosteroid treatment in former smokers with asthma (31). To investigate whether DNA methylation was involved in the observed effects of CS exposure on periostin expression, we used the demethylating agent 5-aza during CS exposure. However, treatment with 5-aza failed to prevent the CS-induced modulation of *POSTN* and *CLCA1* expression, suggesting that DNA methylation is not pivotal in the persistence of decreased expression after CS exposure. However, we cannot formally exclude that possibility that another demethylating agent would have prevented this CS-induced modulation of gene expression.

Both CS and IL-13 have been linked to goblet cell metaplasia in airway epithelial cells *in vitro* and *in vivo*. In Th2-mediated asthma, goblet cell metaplasia and associated MUC5AC overexpression is mainly attributed to the presence of IL-13. IL-13 induces SPDEF and CLCA1 expression, both essential genes involved in the development of IL-13-induced MUC5AC expression (32-34). Goblet cell metaplasia is increased in smokers, and several studies using cigarette smoke have shown the induction of MUC5AC (29, 35-39). Most studies have focused on the effects of cigarette smoke extract rather than whole cigarette smoke to induce goblet cell metaplasia. We observed an increase in *SPDEF* expression, a gene previously shown to be important in the development of goblet cell metaplasia (40, 41). In contrast, the present data showed no increase in *MUC5AC* expression in airway epithelial cells exposed to CS. Together these data suggest that smoking induces a first “hit” for the development of goblet cell metaplasia in smokers, but that an extra stimulus from e.g. underlying tissue inflammation may be required for the development of mucus hypersecretion following goblet cell metaplasia. In our experimental setup, exposure to CS reduced IL-13-induced epithelial markers of goblet cell metaplasia. This reduced *MUC5AC* and *CLCA1* expression in CS-differentiated airway epithelial cells exposed to IL-13 may be explained by the presence of heme oxygenase 1. Cigarette smoke has previously been shown to induce heme oxygenase 1 expression in our whole cigarette exposure model setup (42). Heme oxygenase 1 has been shown to inhibit IL-13-induced-MUC5AC expression and more recently, this process was shown to be associated with reduced CLCA1 expression in human bronchial epithelial cells (43, 44).

The relevance of our findings is further enhanced by the use of primary epithelial cells derived from multiple donors and from multiple anatomical locations instead of the use of cell lines. In addition, we used cells that were differentiated at the air-liquid interface

to allow mucociliary differentiation, instead of cell lines that do not differentiate and display other abnormalities. Finally, we used freshly prepared mainstream whole cigarette smoke containing both the gaseous and particulate components instead of the widely used aqueous extracts of CS. Whereas quantification of the exposure to whole smoke is difficult, in our view it does provide a more accurate representation of cigarette smoke exposure (gaseous and particulate constituents) compared to aqueous extracts. Bronchial epithelial cells were derived from macroscopically normal resected tissue obtained during surgery for lung cancer from patients that were largely (ex)-smokers. Furthermore, epithelial cells from asthmatics were not available to further evaluate the effect of CS on a clinically established Th2 signature. We considered the possibility that cigarette smoke also likely modulates epithelial gene expression induced by cytokines other than IL-13 that are known to be increased in asthma, such as IL-17. However, we focussed on IL-13 because it has been well described that the gene expression pattern observed in airway epithelial cells from patients with allergic asthma is well reflected by IL-13 treatment of cultured primary airway epithelial cells (2, 3).

In conclusion, our results indicate that CS differentially affects the IL-13-induced expression profile including the recently described epithelial 3-gene signature for Th2-high asthma. The observation that CS markedly reduces IL-13-induced *CLCA1* and *POSTN* expression, which does not recover after CS cessation, is an important finding for biomarker-guided therapy in asthma since especially periostin is considered as an emerging biomarker for Th2 inflammation. Possibly, periostin may not be a good biomarker for Th2 inflammation in asthmatics that smoke. The observation that CS is able to reduce *MUC5AC* expression but not *SPDEF* expression, remains to be further elucidated. Collectively, our results provide novel insight in the interaction between Th2 inflammation and cigarette smoke that is relevant for asthma pathogenesis and biomarker-guided therapy in asthma.

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CHAPTER 5

WHOLE CIGARETTE SMOKE EXPOSURE DIFFERENTIALLY ALTERS AIRWAY EPITHELIAL RESPONSES TO NON-TYPEABLE HAEMOPHILUS INFLUENZAE AND RHINOVIRUS

In progress

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ABSTRACT

Increased susceptibility to bacterial and viral respiratory tract infections is a characteristic of smokers with and without chronic obstructive pulmonary disease (COPD), and these infections contribute to acute exacerbations and disease progression in COPD. Cigarette smoke exposure is considered the main risk factor for the development of COPD. To better understand how mainstream whole cigarette smoke exposure (CS) modulates epithelial responses to viral and bacterial exposure, we use air-liquid interface (ALI) cultures of primary human bronchial epithelial cells (PBEC) of control and COPD patients. These cultures were exposed to CS followed by addition of UV-inactivated non-typeable *Haemophilus influenza* (NTHi) or human rhinovirus 16 (RV16). Exposure of ALI-PBEC to CS was found to inhibit the NTHi-induced expression of the antimicrobial peptide hBD-2 (*DEFB4)* in ALI-PBEC of both COPD patients and controls. In contrast, NTHi-induced expression of *GADD34*, a marker of endoplasmic reticulum stress and of the integrated stress response, was synergistically induced by cigarette smoke, which appeared to be more pronounced in ALI-PBEC from COPD patients compared to controls. CS also reduced RV16-induced expression of antiviral genes in ALI-PBEC of COPD patients, but not in controls with the exception viperin. In conclusion, our results indicate that CS differentially affects viral and bacterial infection responses in airway epithelial cells. The reduced antiviral response of airway epithelial cells from COPD patients, but not from controls, suggests that epigenetic mechanisms are involved in the acquisition of reduced antiviral protection of epithelial cells in COPD as these effects are preserved in culture.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease that is characterized by progressive airflow limitation that is not fully reversible. Both airway disease and parenchymal destruction contribute to airflow limitation in COPD, and the relative contribution of these varies amongst patients. The main risk factor for the development of COPD in Westernized societies is cigarette smoking (1). Despite being the main risk factor, only 25 to 30% of smokers develop COPD, suggesting that other factors contribute, such as genetic background and respiratory infections (2, 3). Furthermore, acute exacerbations that are frequently accompanied by respiratory infections further enhance airflow limitation and accelerate disease progression.

Acute exacerbations in COPD patients are defined by a sustained worsening of the patient's condition, which is acute in onset and necessitates a change in regular medication (4). Both bacterial and viral respiratory tract infections contribute to acute exacerbations in COPD. Non-typeable *Haemophilus influenzae* (NTHi) colonizes the lower respiratory tract of approximately 30% of COPD patients and is involved in both chronic airway infections and acute exacerbations (5, 6). In addition, also human rhinovirus (RV) contributes to COPD exacerbations. RV causes self-limiting infections in healthy individuals, but RV infections are associated with the majority of virus-related exacerbations in patients with COPD (7, 8).

Airway epithelial cells are actively involved in the protection of the lung against inhaled particles and pathogens. They line the surface of the conducting airway as a pseudostratified epithelial layer and function as a physical and immunological barrier. Cigarette smoke (CS) has been shown to alter various epithelial functions including a reduction in antimicrobial peptide expression, activation of the integrated stress response and increased inflammation (9). We and others have shown that CS impairs antibacterial defenses in *in vitro* cultures of human airway epithelial cells, and that *in vitro* cultured airway epithelial cells from patients with COPD display impaired antibacterial activity and impaired production of antimicrobial peptides (10). Additionally, previous *in vitro* studies on cigarette smoke extract (CSE) treatments showed a reduced antiviral epithelial response to RV16 (11).

To better understand how CS exposure modulates epithelial responses to viral and bacterial infection, we use air-liquid interface (ALI) cultures of primary human bronchial epithelial cells (PBEC) of COPD patients and controls in this study, and exposed these to CS followed by

exposure to RV16 or UV-inactivated NTHi. Additionally, since airway epithelial dysfunction has been described for COPD patients, we also compared epithelial responses of ALI-PBEC from control patients to ALI-PBEC of COPD patients.

MATERIALS AND METHODS

Subjects and cell culture conditions

Human primary bronchial epithelial cells (PBEC) were isolated from macroscopically normal bronchial tissues obtained from lung cancer patients undergoing lobectomy at the Leiden University Medical Center (Leiden, The Netherlands). Details on isolation and culture of PBEC were described previously (12). Clinical information on the control (CTRL) and COPD patients is presented in table 1.

Cultured PBEC were used to generate mucociliary differentiated cultures by differentiation at the air-liquid interface (ALI) as described previously (12). Briefly, PBEC at passage 2 were cultured submerged on semipermeable Transwell inserts with 0.4 µm pore size (Corning Costar, Cambridge, USA) that were coated with a mixture of collagen, fibronectin and bovine serum albumin. Once full confluence was reached, apical medium was removed followed by two weeks of ALI differentiation.

Table 1. Characteristics of control and COPD patients from whom primary bronchial epithelial cells were obtained. Data is represented as mean ± SD. (*) $p < 0.01$. BD, bronchodilator; BMI, body mass index; CTRL, control; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity.

Characteristics	CTRL (n = 8)	COPD (n = 8)
Age (Yr)	67.1 ± 11.38	70.4 ± 6.2
Gender (Male/Female)	4/4	7/1
BMI	24.2 ± 2.8	25.7 ± 1.4
FEV_1 (% pred)	100.5 ± 21.2(*)	65.7 ± 8.9
Pre-BD FEV_1 (L)	2.8 ± 0.6(*)	2.0 ± 0.3
FVC (% pred)	103.6 ± 16.5	95.9 ± 20.6
FEV_1/FVC (%)	80.0 ± 8.1(*)	54.7 ± 7.7

Whole cigarette smoke (CS) exposure

The apical surface of the cultures was washed with 100 µl and basal culture medium was replaced with infection medium 24 h prior to CS exposure. Infection medium is defined as PBEC culture medium without hydrocortisone, bovine pituitary extract, epidermal growth factor and bovine serum albumin. ALI-PBEC were exposed to CS generated from 3R4F reference cigarettes (University of Kentucky, Lexington, USA) in a CS exposure model adapted from Beisswenger et al. (13, 14). In brief, ALI-cultures were placed into modified hypoxic chambers (Billups Rothenberg, Del Mar, USA), localized inside an incubator at 37°C and 5% CO_2. Whole CS derived from one cigarette, or air as negative control, was guided through the respective exposure chamber using a continuous flow of 1 l/min for a period of 4–5 min, and CS was distributed within the exposure chamber using a small ventilator. After exposure, residual CS inside the exposure chamber was removed by flushing the chambers with air derived from the incubator for a period of 10 min. Bacterial or viral infection was performed directly after air or CS exposure.

Exposure to UV-inactivated non-typeable Haemophilus influenzae

A log-phase culture of non-typeable *Haemophilus influenzae* (NTHi) strain D1 (15) was obtained from a single colony as previously described (14). Bacterial cells in log-phase growth were washed and diluted in PBS to a concentration of $1*10^9$ colony forming unit (CFU)/ml. Bacteria were killed by exposure to UV-light for 2 h. ALI-PBEC were exposed to NTHi, by adding 100 µl of 10^9 CFU/ml of UV-NTHi to the apical surface followed by 3 h incubation at 37 °C with 5 % CO_2. For additional controls, ALI-PBEC were exposed for 3 h to TNF-α (20 ng/ml; Peprotech, Rocky Hill, USA) or tunicamycin (Sigma-Aldrich, St. Louis, USA) added to the basal media. Basal media and apical fluid (100 µl PBS ± NTHi) were collected directly after incubation, and stored at -80°C. Cells were lysed using 200 µl of lysis buffer added directly to the cells and stored at -20 °C until RNA extraction.

RV16 infection

Human rhinovirus type 16 (RV16, VR-283) and H1-HeLa cells (CRL-1958) were purchased from the American Type Culture Collection (ATCC, Rockville, USA). RV16 was propagated in H1-HeLa cells as described previously (16, 17). Prior to CS or air exposure, the cellular content of one insert per donor was trypsinized and counted to obtain the number of cells

per inserts to calculate a multiplicity of infection (MOI) of 1. RV16 was diluted to achieve a MOI1 by adding the virus in 100 µl of infection media to the apical surface of ALI-PBEC. After 1 h incubation at room temperature with intermittent swirling every 5 min, the apical liquid was removed and washed 3 times with infection medium to remove residual viral particles, followed by 24 h incubation at 37 °C with 5 % CO_2. Basal media and apical fluid (100 µl PBS ± RV16) were collected directly after incubation and stored at -80°C. Cells were lysed using 200 µl of lysis buffer added directly to the cells and stored at -20 °C until RNA extraction.

RNA isolation, reverse transcription (RT) and qPCR

Total RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Leiden, The Netherlands) according to the manufacturer's instructions and quantified using the Nanodrop ND-1000 UV-visible spectrophotometer (Nanodrop Technologies, USA). For cDNA synthesis, 1 µg of total RNA was reverse transcribed using oligo-(dT) primers and Moloney murine leukemia virus (M-MLV) polymerase (Promega) at 37°C. Primer sequences are listed in table 2. RPL13A and ATP5B were used as reference genes following selection by the Genorm method (18). All quantitative PCRs (qPCRs) were carried out in triplicate on a CFX-384 real-time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands) with the use of SensiFAST™ SYBR green (Bioline, Luckenwalde, Germany). Bio-Rad CFX manager 3.1 software (Bio-Rad) was used to calculate arbitrary gene expression by using the standard curve method.

Table 2. Primer sequences with gene names and NCBI gene ID used in present study.

Gene	Primer Sequence	NCBI Gene ID
DEFB4	F: ATC AGC CAT GAG GGT CTT G R: GCA GCA TTT TGT TCC AGG	1673
GADD34	F: ATG TAT GGT GAG CGA GAG GC R: GCA GTG TCC TTA TCA GAA GGC	23645
HMOX1	F: AAG ACT GCG TTC CTG CTC AAC R: AAA GCC CTA CAG CAA CTG TCG	3162
RV16	F: ACC CTC AAT ACA TAC GCC AAC T R: TTC CAA GCC ATC CAT TCC A	L24917
MDA5	F: TCG AAT GGG TAT TCC ACA GAC G R: GTG GCG ACT GTC CTC TGA A	64135
RIG1	F: CTG GAC CCT ACC TAC ATC CTG R: GGC ATC CAA AAA GCC ACG G	5920
IFNB	F: ATG ACC AAC AAG TGT CTC CTC C R: GGA ATC CAA GCA AGT TGT AGC TC	3456
ISG15	F: ATC CTG GTG AGG AAT AAC AAG GG R: GTC AGC CAG AAC AGG TCG TC	9636
Viperin	F: GTCA GCC AGA ACA GGT CGT C R: AGT GCT TTG ATC TGT TCC GTC	91543
RPL13A	F: AAG GTG GTG GTC GTA CGC TGT G R: CGG GAA GGG TTG GTG TTC ATC C	23521
ATP5B	F: TCA CCC AGG CTG GTT CAG A R: AGT GGC CAG GGT AGG CTG AT	506

$TCID_{50}$ calculation

Viral titers were assessed on the apical washes of RV16-infected ALI-PBEC using confluent layers of H1-HeLa in 96-well plates. A 10-fold serial dilution of the apical washes was made in DMEM containing 2% FBS (Bodinco, Alkmaar, The Netherlands), 2% (vol/vol) 1 M HEPES (Lonza) and 1% (wt/vol) $NaHCO_3$ (Gibco). The 50% tissue culture infection dose was evaluated by assessing the cytopathic effect after 5 days of incubation at 37 °C with 5 % CO_2 and expressed as $TCID_{50}$/ml.

CXCL8 and CXCL10 ELISA

CXCL8 and CXCL10 was measured in the basal media and assessed using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Minneapolis, USA).

Statistics

Within group (control and COPD) results were evaluated using one-way ANOVA for repeated measures with Bonferroni as post-hoc test with GraphPad Prism 6. Differences between control and COPD patients were compared with a non-parametric t-test for independent samples (Mann-Whitney). Differences were considered statistically significant at $*p<0.05$, $**p<0.01$ and $***p<0.001$.

RESULTS

Innate immune responses, integrated stress response and oxidative stress responses in airway epithelial cells of control and COPD patients cultured at air-liquid interface

To evaluate difference in the cellular response of ALI-PBEC derived from COPD patients and controls, we exposed control and COPD derived ALI-PBEC to TNF-α (20 ng/ml) or tunicamycin (20 ng/ml) for 3 h, or to whole cigarette smoke (CS) exposure (4-5 min exposure to one cigarette, followed by 10 min removal of smoke), followed by 3 h incubation. We measured gene expression patterns of *DEFB4*, *GADD34* and *HMOX1* to evaluate the innate immune response, integrated stress response and oxidative stress response respectively. Both ALI-PBEC of control and COPD patients showed increased expression of *DEFB4* by TNF-α, *GADD34* by tunicamycin and *HMOX1* by CS. Furthermore, ALI-PBEC from COPD patients showed a significantly higher induction of *DEFB4* expression following TNF-alpha exposure compared to ALI-PBEC from controls (Fig. 1). Together these results suggest that both ALI-PBEC of control and COPD patients are able to elicit a physiologic response to various stimuli, but this response may vary in ALI-PBEC from COPD patients compared to controls.

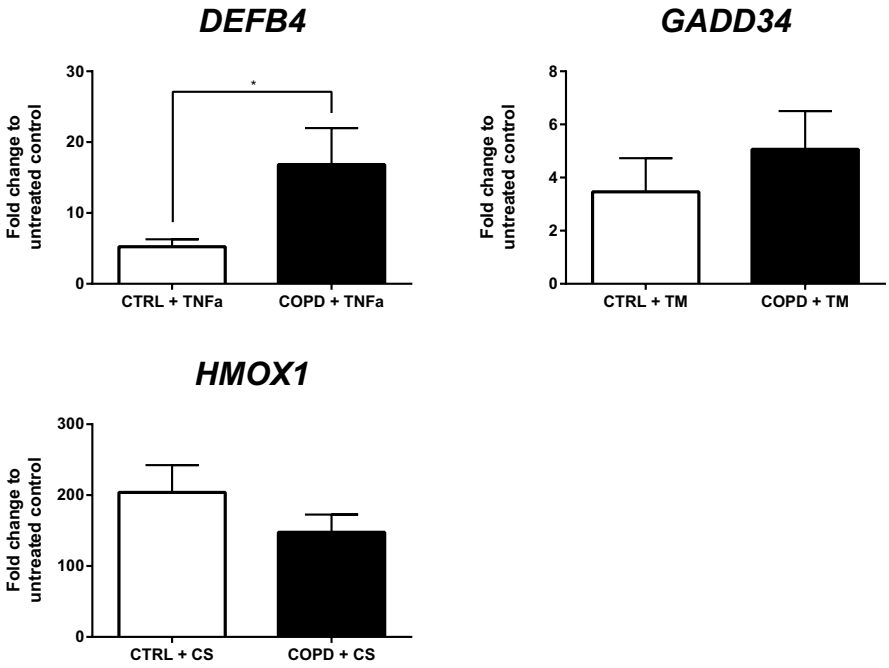

Figure 1. Analysis of antimicrobial peptide expression (innate immunity; *DEFB4*), activation of the integrated stress response (*GADD34*) and oxidative stress response (*HMOX1*) in ALI-PBEC from COPD patients and controls (CTRL). COPD and CTRL ALI-PBEC were exposed to CS, TNF-α (20 ng/ml) or tunicamycin (20 ng/ml) for 3 h. *DEFB4*, *GADD34* and *HMOX1* gene expression was assessed by qRT-PCR. Results are expressed as mean ± SEM fold change compared to incubator control with n = 8 independent donors. *p < 0.05. ALI, air-liquid interface; CS, whole cigarette smoke exposure; CTRL, control; PBEC, primary bronchial epithelial cells; TM, tunicamycin.

Differential airway epithelial responses to bacterial infection following cigarette exposure in ALI-PBEC of control and COPD patients

After evaluating whether ALI-PBEC from both control and COPD patients were able to elicit a physiologic response to various stimuli, we next evaluated whether whole cigarette smoke exposure (CS) alters epithelial responses to bacterial exposure, and whether this response differed between COPD patients and controls. Analysis of *DEFB4A* (innate immune response), *GADD34* (Integrated stress response) and *HMOX1* (oxidative stress response) expression was assessed on ALI-PBEC from control and COPD donors. These ALI-PBECs were exposed to air or CS, and next incubated for 3 h with or without UV-inactivated NTHi (10^9 CFU/ml) apically. CS exposure significantly inhibited the NTHi-induced *DEFB4A* expression in both control and COPD ALI-PBEC. Additionally, unexpectedly NTHi-induced *DEFB4* expression was higher in ALI-PBEC from COPD patients compared to controls ($p = 0.0353$). Together these data suggest that patient status can affect the intensity of the response to CS exposure followed by bacterial infection. *GADD34* expression showed a non-significant increase after CS exposure in both control and COPD ALI-PBEC. However, *GADD34* expression was significantly increased in CS treated ALI-PBEC following NTHi exposure, but not in air-exposed control and COPD ALI-PBEC. Furthermore, combined CS and NTHi exposure appears to synergistically increase *GADD34* expression in both control and COPD ALI-PBEC, suggesting that CS exposure exacerbates the NTHi-induced integrated stress response. CS alone significantly induced *HMOX1* mRNA in both donor groups, whereas NTHi exposure alone did not. However, HMOX1 induction by CS exposure appears to be lower in COPD ALI-PBEC compared to controls. No significant differences were observed in *HMOX1* expression between COPD and controls, suggesting that control and COPD ALI-PBEC have a similar capacity to induce an oxidative stress response following CS exposure which is unaffected by NTHi exposure (Fig. 2).

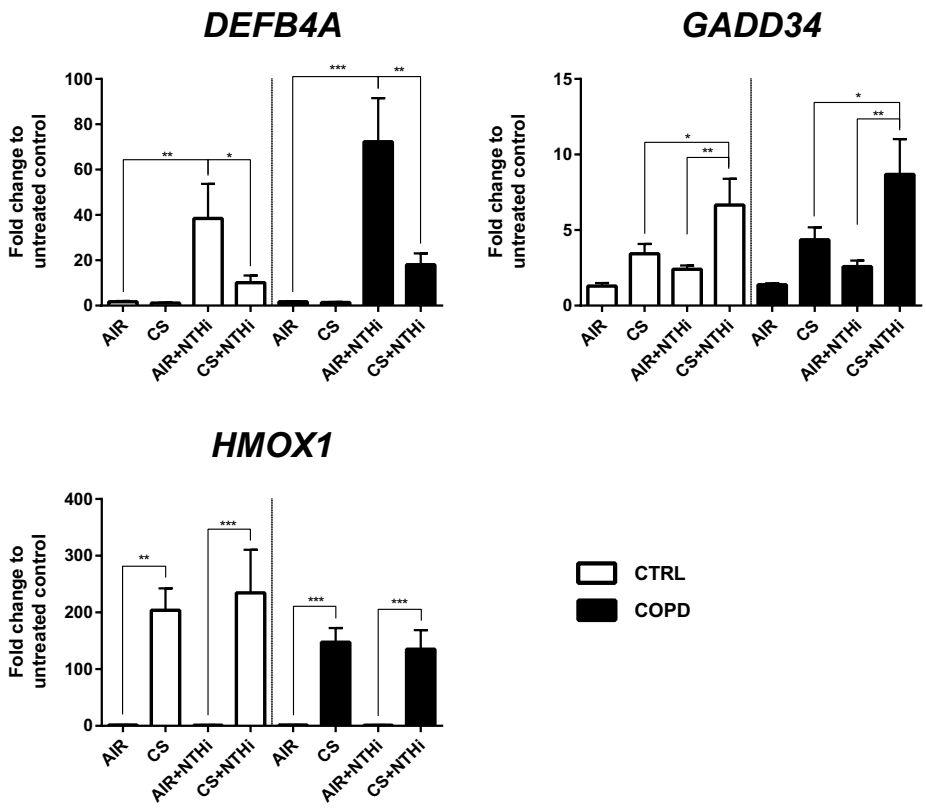

Figure 2. Whole cigarette smoke exposure differentially alters responses of COPD ALI-PBEC to NTHi. ALI-PBEC from COPD and CTRL donors were exposed to air or CS, followed by 3 h exposure to UV-inactivated NTHi (10^9 CFU/ml). *DEFB4, GADD34* and *HMOX1* gene expression was assessed by qRT-PCR. Results are expressed as mean ± SEM fold change compared to untreated control with n = 8 independent donors. *p < 0.05. ALI, air-liquid interface; CS, whole cigarette smoke exposure; CTRL, control; NTHi, non-typeable *Haemophilus influenzae*, PBEC, primary bronchial epithelial cells.

Differential airway epithelial responses to viral infection following cigarette exposure in ALI-PBEC of control and COPD patients

ALI-PBEC cultures from COPD patients show altered responses to bacterial infection following whole cigarette smoke exposure (CS). Next we evaluated if whole cigarette smoke exposure also alters the viral response in ALI-PBEC of COPD patients and whether these responses are different compared to ALI-PBEC from control patients. ALI-PBEC from 8 control and 8 COPD patients were exposed to air or CS and then infected with RV16 at a MOI of 1 for 1 h followed by 24 h incubation. Analysis of total viral RNA of RV16 showed a higher viral RNA load after CS exposure compared to air-exposed controls; this effect however did not reach statistical significance (Fig. 3A). Furthermore, shedding of infectious particles, indicated by the $TCID_{50}$, showed non-significant differences between COPD and control cultures, and prior exposure to CS had opposite effects on cultures derived from both groups. The CS-induced increase in viral particle levels in cultures from COPD patients nearly reached statistical significance (Fig. 3B). This apparent difference between shedding of infectious RV16 particles and viral load assessment by qPCR requires further investigation.

RV16 exposure significantly induced expression of the cytoplasmic viral RNA sensors *RIG-1* and *MDA5* in COPD-derived ALI-PBEC cultures, but not in CTRL-derived ALI-PBEC. This effect was unaffected by CS exposure (Fig. 3C). RV16 exposure induced a non-significant increase in *IFN*β expression, which followed a similar trend as *RIG-I* and *MDA5* expression (Fig. 3C). The expression of interferon-stimulated genes *Viperin* and *ISG15* was increased by RV16. This effect was unaffected by CS exposure (Fig. 3C). Taken together, these results suggest that CS exposure mainly affects RV16-induced responses in COPD-derived ALI-PBEC.

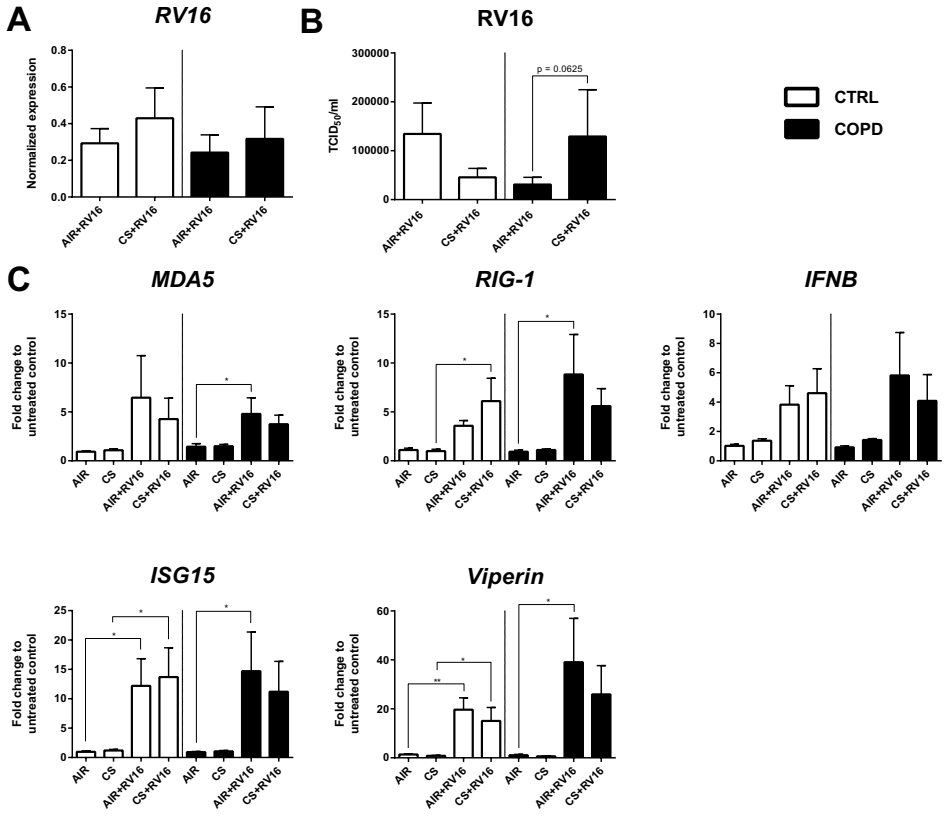

Figure 3. Whole cigarette smoke exposure differentially alters the response of COPD ALI-PBEC to RV16. ALI-PBEC from COPD and CTRL donors were exposed to air or CS, followed by 1 h infection with RV16 (MOI1) and 24 h incubation. (A) RV16 gene expression was assessed by qRT-PCR. Results are expressed as mean ± SEM normalized expression compared to incubator control with n = 8-9 independent donors. (B) Viral titration was performed on the apical wash and reported as $TCID_{50}$/ml. (C) *IFNß*, *Viperin*, *RIG-I*, *MDA5* and *ISG15* gene expression was assessed by qRT-PCR. Results are expressed as mean ± SEM fold change compared to incubator control with n = 8 independent donors.*p < 0.05, **p < 0.01. ALI, air-liquid interface; CS, whole cigarette smoke exposure; CTRL, control; MOI, multiplicity of infection; PBEC, primary bronchial epithelial cells; RV16, rhinovirus 16; $TCID_{50}$, 50% tissue culture infective dose.

Whole cigarette smoke exposure differentially alters cytokine secretion of ALI-PBEC following RV16 exposure

Whole cigarette smoke (CS) exposure mainly affected RV16-induced responses in COPD-derived ALI-PBEC compared to ALI-PBEC from controls. To investigate whether this effect was also observed when investigating cytokine protein release, ALI-PBEC from 8 control and 8 COPD donors were exposed TNF-α for 24 h or to air or CS followed by infection with 1 MOI of RV16 for 1 h followed by 24 h incubation. CXCL8 and CXCL10 release following TNF-α stimulation was similar in ALI-PBEC from control and COPD patients (Fig. 4A). CXCL8 was increased by CS exposure, but remained unaffected by RV16 exposure in ALI-PBEC of both control and COPD patients. Baseline secretion of CXCL8 appeared to be lower in ALI-PBEC of COPD patients compared to controls, but this effect was not significant (Fig. 4B). CXCL10 was detected only in RV-16 exposed ALI-PBEC. CS exposure appeared to lower RV16-induced CXCL10 secretion in both control and COPD ALI-PBEC, but this did not reach statistical significance (Fig. 4B). Overall, CXCL8 and CXCL10 secretion did not appear to be differentially regulated in ALI-PBEC of COPD patients compared to controls.

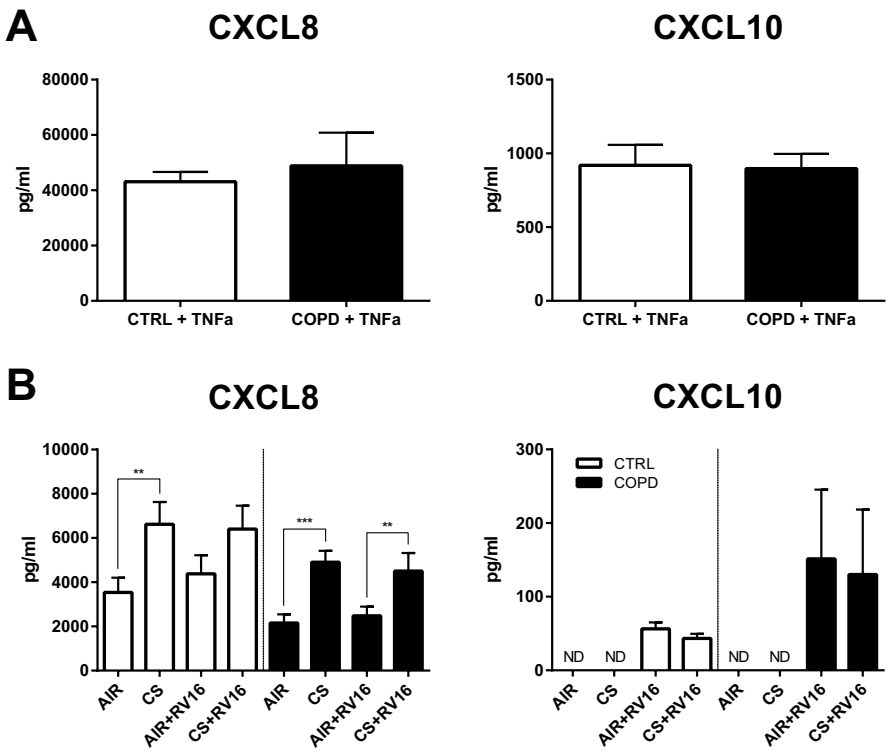

Figure 4. Effect of RV16 infection and whole cigarette smoke exposure on secretion of CXCL8/IL-8 and CXCL10/IP10 protein secretion of ALI-PBEC from COPD patients and controls. (A) ALI-PBEC from COPD and control donors were exposed to TNF-α for 24 h or (B) air or CS, followed by 1 h infection with RV16 (MOI1) followed by 24 h incubation. Protein quantification of CXCL8 and CXCL10 was performed by ELISA. Statistical significance is indicated as *p<0.05, **p<0.01 and ***p<0.001. ALI, air-liquid interface; CS, whole cigarette smoke; CTRL, control; MOI, multiplicity of infection; ND, none detected; PBEC, primary bronchial epithelial cells; RV16, rhinovirus 16.

DISCUSSION

In this study we aimed to compare responses to bacterial exposure and rhinovirus infection between primary bronchial epithelial cells (PBEC) from COPD patients cultured at air-liquid interface (ALI). Cigarette smoke (CS) exposure reduced NTHi-induced expression of the antimicrobial peptide *DEFB4* and decreased the viral titer in the supernatant of rhinovirus (RV) infected cells. Moreover, ALI-PBEC cultures from COPD patients and controls showed no significant differences in their responses to NTHi exposure or RV16 infection.

Cigarette smoking is considered the main risk factor for the development of COPD. Cigarette smoking has been shown to affect various epithelial cell functions through a range of different mechanisms. Cigarette smoke causes oxidative stress and we previously showed the cigarette smoke-mediated induction of *HMOX1* in cells from control donors cultured at the air-liquid interface (19). Here we observed a lower induction of *HMOX1* in COPD compared to control donors, which was not statistically significant. Cigarette smoke can also activate toll-like receptors through endotoxin contamination present in tobacco (20, 21). Furthermore, PBEC from active smokers have impaired antiviral responses (22). In a previous *in vitro* study from our group, NTHi stimulation of COPD patients resulted in a lower antimicrobial activity and a lower expression of *DEFB4A* compared to control patients at 24 h after treatment with NTHi (10). In the same study, exposure to cigarette smoke impaired the NTHi-induced *DEFB4A* expression at different time points and hBD2 release at 24 h after treatment with bacteria. In line with these findings, our results indicate that cigarette smoke dampens the innate immune response to NTHi, as suggested by the reduced *DEFB4* expression at 3 h after bacteria-treatment. In contrast, we observed a higher expression of *DEFB4A* in COPD compared to control patients after NTHi stimulation and this difference was significantly higher after TNFα treatment. Difference between ours and previous findings on the NTHi-induced expression of *DEFB4A* in control and COPD patients might be explained by the different time of incubation after stimulation. *DEFB4* expression has previously been shown to be lower in patients with smoking history during acute pneumonia (23), which is in line with our results *in vitro* using whole cigarette smoke exposure. Cigarette smoke synergistically increased the integrated stress response induced by bacterial stimulation in both ALI-PBEC of control and COPD patients. Since we only investigated expression of *GADD34*, we cannot rule out that its expression was part of the unfolded protein response to endoplasmic reticulum stress, instead of the integrated stress response. Interestingly, in a model of whole exposure to diesel exhaust followed by NTHi treatment, ALI-PBEC of

control and COPD patients showed similar responses to those induced by cigarette smoke (24). Diesel exhaust also reduced the NTHi-induced expression of *DEFB4A* but this inhibition was only significant in cultures from COPD patients. Both diesel exhaust and cigarette smoke increased the NTHi-induced expression of *GADD34* (24). Cigarette smoke has been shown to increase adhesion of *Haemophilus influenzae* to airway epithelial cells, which may help to explain the synergistic effect on the integrated stress response following bacterial infection (25). Overall, these data suggest that cigarette smoke may alter epithelial responses to bacterial infection.

Bronchial epithelial cells are the primary site for RV infection, indicating an important role for bronchial epithelial cells and their innate immune response against inhaled respiratory viruses (26). After entering the cell, viral RNA is recognized by cytoplasmic RIG-I-like receptors that recognize viral double-stranded RNA which subsequently trigger antiviral innate immune responses and induce type I interferon production (27). Important RIG-I-like receptors are RIG-1 and MDA5, which induce type I and type III interferons following viral infection. Interferons can then activate the expression of multiple interferon-responsive genes, including *CXCL10*, *ISG15* and *Viperin* (28, 29). Our results showed no obvious effects of CS exposure on RV16-indyced expression of *MDA5*, *RIG1* and their downstream effectors *IFNß*, *ISG15* and *Viperin*. It has been shown that PBEC from COPD patients have impaired induction of type I IFN, resulting from reduced protein kinase R (PKR) and decreased PKR-mediated stress granules, leading to increased viral replication after viral infection (30). Viperin, an interferon-responsive gene, can be induced by human rhinoviruses and has been shown to inhibit viral replication by inhibiting viral proteins that are required for viral assembly and maturation (29, 31). Whole cigarette smoke exposure did not inhibit *Viperin* expression 24 h after infection. Results by Proud and colleagues showed that *Viperin* expression is continuously increased even 48 h after infection, suggesting that we may have to investigate Viperin expression at later time points. Taken together, our results are not in line with current reported findings that show increased susceptibility to viral infections in COPD patients (7, 32-34). Nonetheless, we did observe a decreased viral titer following CS exposure in RV infected cells. Our *in vitro* smoke exposure setup may require further adaptations to mimic *in vivo* exposure more closely. Alternatively, our antiviral data is mostly limited to gene expression data that may not reflect actual protein expression.

Total viral RNA of RV16 showed a non-significant increase after CS exposure compared to air-exposed control ALI-PBEC. Primary bronchial epithelial cells of COPD patients have previously

been shown to have increased expression of antiviral and pro-inflammatory genes following human rhinovirus compared to healthy controls (35, 36). Various factors may contribute to the observed differences in the results. Various human rhinovirus serotypes exist and have been used for research, which may elicit different cellular responses. Additionally, our controls may have a smoking background whereas the aforementioned studies used non-smoking controls. Furthermore, Baines and colleagues used submerged cultures of primary bronchial epithelial cells, which do not form a pseudostratified epithelial layer. Submerged epithelial layers consist mainly of dividing basal cells, whereas a pseudostratified epithelial layer consists of multiple epithelial subtypes, which resembles the *in vivo* epithelium more closely (37). Moreover, basal cells have been shown to be more susceptible to RV infection (38). Overall, our data suggests that the reduced antiviral response in airway epithelial cells of COPD patients, but not in controls, may result from epigenetic mechanisms as these effects are preserved in culture.

The airway epithelium of COPD patients is exposed to persistent airway inflammation with increased numbers of inflammatory cells, despite smoke cessation in many patients (39). Various reports have indicated differences in epithelial characteristics between control and COPD patients. Airway epithelial cells of COPD patients have been shown to release more CXCL8 compared to smoking controls (40). This cytokine plays an important role in neutrophil recruitment to the airways. Additionally, rhinovirus induced-CXCL10 production in airway epithelial cells was shown to be reduced by cigarette smoke exposure (41-43). Our results do not recapitulate these findings for increased CXCL8 release in airway epithelial cells of COPD patients and reduced CXCL10 expression following RV16 infection after smoke exposure. This may be explained by differences in culture methods and the use of whole cigarette smoke exposure rather than an aqueous extract of cigarette smoke. Furthermore, since most of our donors were (ex-)smokers, the observed effects may be attributed to the effect of cigarette smoking rather than patient status.

Strengths of our study are the use of primary epithelial cells derived from multiple donors with and without COPD. Additionally, we used ALI differentiated cells to allow mucociliary differentiation, rather than using cell lines that do not differentiate and display other abnormalities. Finally, we used freshly prepared mainstream whole cigarette smoke containing both the gaseous and particulate components instead of the widely used aqueous extracts of cigarette smoke. Aqueous extracts of cigarette smoke fail to capture the complexity of whole cigarette smoke and also lack the presence of volatile compounds.

Nevertheless, this study also has some limitations. Bronchial epithelial cells were derived from macroscopically normal resected tissue obtained during surgery for lung cancer from patients that were largely (ex)-smokers. Furthermore, epithelial cells from healthy non-smoking controls were not available to further evaluate the effect of whole cigarette smoke on bacterial and viral infection in ALI-PBEC.

In conclusion, our results indicate that cigarette smoke differentially affects bacterial exposure responses in airway epithelial cells, but these effects do not appear to differ in airway epithelial cells from COPD patients compared to controls. The observation that cigarette smoke synergistically increases the integrated stress response by *Haemophilus influenzae* implies this pathway as a possible therapeutic target in COPD patients. Our study however could not confirm existing reports on reduced antiviral responses in airway epithelial cells from COPD patients. Collectively, our results provide novel insight in the interaction between cigarette smoke exposure and subsequent bacterial or viral infection that is relevant for COPD pathogenesis.

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CHAPTER 6

A NOVEL METHOD FOR EXPANSION AND DIFFERENTIATION OF MOUSE TRACHEAL EPITHELIAL CELLS IN CULTURE

Major revisions in Scientific Reports

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ABSTRACT

Air-liquid interface (ALI) cultures of mouse tracheal epithelial cells (MTEC) are a well-established model to study airway epithelial cells, but current methods require large numbers of animals which is unwanted in view of the 3R principle and introduces variation. Moreover, stringent breeding schemes are frequently needed to generate sufficient numbers of genetically modified animals. Current protocols do not incorporate expansion of MTEC, and therefore we developed a protocol to expand MTEC while maintaining their differentiation capacity. MTEC were isolated and expanded using the ROCK inhibitor Y-27632 in presence or absence of the γ-secretase inhibitor DAPT, a Notch pathway inhibitor. Whereas MTEC proliferated without DAPT, growth rate and cell morphology improved in presence of DAPT. ALI-induced differentiation of expanded MTEC resulted in an altered capacity of basal cells to differentiate into ciliated cells, whereas IL-13-induced goblet cell differentiation remained unaffected. Ciliated cell differentiation improved by prolonging the ALI differentiation or by adding DAPT, suggesting that basal cells retain their ability to differentiate. This technique using expansion of MTEC and subsequent ALI differentiation drastically reduces animal numbers and costs for *in vitro* experiments, and will reduce biological variation. Additionally, we provide novel insights in the dynamics of basal cell populations *in vitro*.

INTRODUCTION

Airway epithelial cells play a pivotal role in protecting the lung by acting both as a mechanical and an immunological barrier. The epithelial cells of the upper respiratory tract form a pseudostratified epithelial layer consisting of several epithelial cell types that constitute an efficient host defense system that employs a variety of mechanisms, including its ability to clear inhaled particles and pathogens from the lung using mucociliary clearance. Efficient mucociliary clearance depends on a proper balance between basal, ciliated and secretory cells. The relative distribution of these cell types in the epithelial layer varies depending on the anatomic location within the conducting airways. Altered composition of these epithelial cell types has been implied in several chronic lung diseases, including asthma and chronic obstructive pulmonary disorder (COPD) (1-3).

Air-liquid interface (ALI) cultures of human primary airway epithelial cells (AEC) are a well-established *in vitro* model to investigate the role of airway epithelial cells in chronic lung diseases (4, 5). Primary AEC are isolated from bronchial biopsies, brushes or resected lung tissue, and can either be cultured directly onto transwell inserts or the cells can first be expanded *in vitro* for subsequent experimental use. AEC freshly isolated from lung tissue consist of multiple cell types, but during *in vitro* culture under submerged conditions the main population that will expand is the basal cells, the epithelial progenitor population (6, 7). Following *in vitro* expansion, primary AEC can be cultured on transwell inserts to establish ALI cultures. To this end, once the cultures have reached full confluence the apical medium is removed to induce an ALI that allows AEC to differentiate into a pseudostratified epithelial layer containing basal, ciliated and secretory cells (8). Culturing primary airway epithelial cells at ALI provides a platform to investigate not only fully differentiated epithelial layers, but also the mechanisms of differentiation following airway epithelium damage and the dynamic processes of repair after injury (5). Importantly, ALI cultures allow us to study the effect of airborne exposures on airway epithelial cells, e.g. whole cigarette smoke exposure (9).

In addition to primary human AEC, various research groups are using cultures of mouse tracheal epithelial cells (MTEC) (7). These offers the opportunity to closely link *in vitro* and *in vivo* experiments, and make use of the large variety of transgenic mouse lines available. However, it is difficult to maintain MTEC in a proliferative state after isolation, and therefore MTEC are cultured directly onto transwell inserts without prior *in vitro* expansion. As a result,

large animal numbers are needed to obtain adequate cell numbers for *in vitro* experiments. Therefore, novel methods are required to subculture MTEC in order to achieve a drastic reduction in animal numbers needed for experiments.

Expanding the progenitor cell population is essential to subculture MTEC. Basal epithelial cells are considered as the progenitor cell type for the maintenance of a pseudostratified airway epithelium of the upper respiratory tract (6). The mechanisms that control progenitor cell renewal and differentiation to maintain the airway epithelium are still being uncovered, mostly owing to the complex cell-cell interactions and subsequent signaling involved in the decision making towards a specific cell fate. Notch signaling has been implied in the regulation of basal cell self-renewal and differentiation towards the specialized cell types of the epithelial layer. Importantly, inhibition of Notch signaling has been shown to allow expansion of the basal cell population (10-12).

To investigate the possibility of expanding MTEC while retaining the ability to differentiate, we have developed an alternative culture method that will lead to a drastic reduction in animal numbers needed for *in vitro* experiments. Moreover, subculturing MTEC would allow for increased numbers of *in vitro* experiments without using additional difficult-to-breed transgenic mice. To this end, we have used a combination of Notch signaling inhibition together with adaptation of existing cell culture methods to explore the possibility of subculturing MTEC and subsequent ALI differentiation. Additionally, we also investigated the effect of passaging MTEC on the basal cell type population as these cells are essential for subsequent differentiation into a pseudostratified epithelial layer consisting of multiple epithelial cell types.

METHODS

Culture media and supplements

A detailed overview of all culture media and supplements can be found in supplemental table 1. “Ham´s F12” is defined as Ham´s F12 (Gibco, Bleiswijk, The Netherlands) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza, Verviers, Belgium). “MTEC basic” medium is defined as DMEM/F12 (Gibco) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 0.03% (w/v) $NaHCO_3$ (Gibco). “KSFM expansion medium” is defined as KSFM (Gibco) supplemented with 1% penicillin-streptomycin, 1 µM isoproterenol (Sigma-Aldrich, St. Louis, MO, USA), 0.03 mg/ml bovine pituitary extract (Gibco), 25 ng/ml murine epidermal growth factor (Peprotech, Rocky Hill, NJ, USA) and 10 µM Y-27632 hydrochloride (Cayman Chemical, Ann Arbor, MI, USA). “MTEC proliferation medium” is defined as MTEC basic supplemented with 5% (v/v) Fetal Bovine Serum (FBS), 1.5 mM L-glutamine (Lonza), 1x (v/v) Insulin-Transferrin-Selenium (Gibco), 0.1 µg/ml cholera toxin (Sigma-Aldrich), 25 ng/ml murine epidermal growth factor, 0.03 mg/ml bovine pituitary extract, 0.05 µM retinoic acid (Sigma-Aldrich) and 10 µM Y-27632 hydrochloride. “MTEC differentiation medium” is defined as MTEC basic supplemented with 0.1% (w/v) bovine serum albumin (BSA) (Gibco), 1.5 mM L-glutamine, 1% (v/v) Insulin-Transferrin-Selenium, 25 ng/ml cholera toxin, 5 ng/ml murine epidermal growth factor, 0.03 mg/ml bovine pituitary extract and 0.05 µM retinoic acid. Retinoic acid and Y-27632 hydrochloride stocks were prepared and stored at -80 °C and -20 °C respectively, and were supplemented fresh for cell culture usage and used within the same day. Culture media containing retinoic acid were protected from light. An overview of the culture media and supplements are given in table 1.

Mouse tracheal epithelial cells (MTEC) isolation and culture

All animal experimental protocols were approved by the animal welfare committee of the veterinary authorities of the Leiden University Medical Center or the Erasmus Medical Center. Wild-type C57Bl/6 mice were used for isolating murine tracheas. MTEC were isolated as described previously (14). After isolation, MTEC were resuspended in MTEC basic containing 10% FBS for direct culture on transwell inserts, or in KSFM for further passage and expansion of MTEC.

In experiments in which MTEC were grown on transwell inserts directly after isolation

without passaging of the cells in KSFM expansion medium, the cells were first deprived of fibroblasts by incubating the cells on Primaria plates (Corning Costar, Cambridge, MA, USA) for 5 h at 37°C with 5% CO_2. Non-adherent cells were collected and centrifuged at 390xg for 10 min at room temperature. Cells were resuspended in MTEC proliferation medium, plated at 8 x 10^4 cells/cm^2 on transwell inserts (0.4 µm pore size; Corning Costar) and cultured submerged until confluent at 37°C with 5% CO_2. MTEC proliferation medium was refreshed every two to three days.

For MTEC subculture experiments, trachea-derived cells were collected in KSFM expansion medium and plated in T75 flasks at 5 x 10^3 cells/cm^2. The adherence step using Primaria plates to remove fibroblasts was omitted when using KSFM, since this medium is formulated by the provider to prevent fibroblast growth. KSFM expansion medium was supplemented with DAPT (Sigma-Aldrich), a Notch signaling inhibitor, according to the experimental design described in the results section. At 80 – 90% confluence, MTEC were passaged using 0.25% (w/v) trypsin (Gibco) and 2.7 mM EDTA (Sigma-Aldrich) in Cell Dissociation Solution Non-enzymatic 1x (Sigma-Aldrich). MTEC were plated at 5 x 10^3 cells/cm^2 in KSFM. Passage 1 or passage 2 MTEC were used for further experiments. For culturing onto transwell inserts, cells were dissociated with Cell Dissociation Solution (Sigma-Aldrich) supplemented with 0.25% trypsin and 2.7 mM EDTA at 37°C for 15 min, centrifuged at 390 g for 10 min at room temperature and resuspended in MTEC proliferation medium. MTEC were plated on transwell inserts at 8 x 10^4 cells/cm^2 and cultured in MTEC proliferation medium until confluent at 37°C with 5% CO_2. MTEC proliferation medium was refreshed every two to three days. A schematic overview of the expansion method followed is given in figure 1.

After MTEC cultured on transwell inserts had grown to full confluence, apical medium was removed to achieve air-liquid interface (ALI) culture conditions. After the start of culture at ALI, the apical surface was washed with warm PBS and the basal medium was refreshed using MTEC differentiation medium every two to three days.

For experimental exposures, ALI-MTEC were exposed to either 5 ng/ml murine IL-13 (Peprotech) or 5 µM DAPT, added in the basolateral chamber of the transwell insert for the indicated duration.

Allergic airway inflammation in mice

To induce allergic airway inflammation in mice, 8 to 12 weeks old female C57BL/6 mice were sensitized by intranasal instillation of 1 µg *Dermatophagoides pteronyssinus* extract (house dust mite extract (HDM)) in 50 µl PBS, followed by 5 daily intranasal challenges with 10 µg HDM in 50 µl PBS or PBS starting at 7 days after sensitization. Mice were sacrificed 2 days after the last challenge. Trachea were removed and fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) and stained for confocal analysis as described below.

Immunofluorescence of ALI-MTEC culture

MTEC on inserts were rinsed with PBS followed by fixation with 4% (w/v) paraformaldehyde (PFA) for 10 min at room temperature. After fixation cells were washed 3 times with PBS/0.2% (v/v) Triton-X100 (Sigma-Aldrich) to remove residual mucus from the apical surface. Non-specific binding sites were blocked with 5% (v/v) donkey serum (EMD Millipore, Billerica, MA, USA), 1% (w/v) BSA and 0.2% (v/v) Triton-X100 in PBS for 30 min at room temperature, followed by incubation with primary antibodies in blocking buffer at 4°C overnight (Supplemental table 1). The next day, MTEC were washed three times with PBS/0.02% Triton X-100 and incubated for 2 h at room temperature with Alexa Fluor labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) in blocking buffer (Supplemental table 2). After three washing steps with PBS/0.02% (v/v) Triton-X100 and one washing step with PBS, the cells were mounted with Vectashield hardset containing DAPI (Vector Laboratories, Burlingame, CA, USA). Images were acquired using a Leica SP5 confocal microscope. Cell counting was performed using ImageJ and 10-15 areas from each insert were analyzed for each replicate.

Immunofluorescence of tracheal sections

Mouse tracheas were fixed with 4% PFA overnight at 4°C, washed with PBS and embedded in paraffin for sectioning. Tracheas were sectioned (5 µm) and deparaffinized, rehydrated and subjected to antigen retrieval in Tris-EDTA (pH 9.0) at 600W for 15 min. After blocking with PBS plus 5% donkey serum and 0.05% Tween-20, sections were incubated with primary antibodies in blocking buffer overnight at 4°C (Supplemental table 2). The next day, sections were washed with PBS/0.05% Tween (3x, 5 min). Alexa Fluor labeled secondary antibodies were used in a 1:500 dilution in blocking buffer and sections were incubated for 2 h at room

temperature. After incubation, sections were washed (3x, 5 min) with PBS/0.05% Tween-20 followed by one final wash with PBS only. Sections were mounted with Vectashield hardset containing DAPI (Vector Laboratories) and confocal images were obtained using a Leica SP5 confocal microscope.

Trans-epithelial electrical resistance (TEER)

TEER values were measured with an STX2 electrode (World Precision Instruments, Berlin, Germany) connected to a Millicell ERS voltohmmeter (World Precision Instruments). Prior to measurement, 700 µl prewarmed PBS was added apically to the ALI-MTEC and incubated at room temperature for 10 min. Resistance measurements were corrected for the surface of the transwell inserts and expressed as Ohm * cm^2.

Western blot analysis

Cells were washed twice with PBS, scraped of the insert in PBS and pelleted. Cells were lysed in Carin lysis buffer containing 20mM Tris-HCl pH 8.0, 137 mM NaCl, 10 mM EDTA, 10% glycerol and 1% NP-40 and incubated on ice for 15 min. Complete protease inhibitors (Roche) was freshly added each time to the lysis buffer. Samples were centrifuged for 5 min at 18,711 g in an Eppendorf5417R at 4°C. Pellets were discarded and supernatant was used for western blot analysis. Protein concentrations were determined by the Pierce® BCA Protein Assay Kit (Thermos scientific) and equal concentrations of protein were eluted in 4x SDS sample buffer and 50mM 1,4-dithiothreitol (DTT, sigma). Samples were boiled and loaded on a 12% SDS-polyacrylamide gel and blotted onto a PVDF membrane (Immobilon®-P transfer membrane, Millipore). The blots were blocked for 1h in PBS containing 0.05% Tween-20 and 3% BSA at room temperature, and probed overnight with primary antibodies at 4°C. Next day, membranes were washed three times with PBS containing 0.05% Tween-20 and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO) at a dilution of 1:10,000. Signal was detected with Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare). Blots were developed using the Amersham Typhoon imaging system (GE Healthcare).

Statistical analysis

Data are represented as means with standard error of mean of measurements. Statistical differences between samples were assessed with a one way or two way analysis of variance (ANOVA) or unpaired t-test. Differences at P-values below 0.05 are considered significant (* $p<0.05$). All statistical analyses were performed using Graphpad PRISM version 5.02.

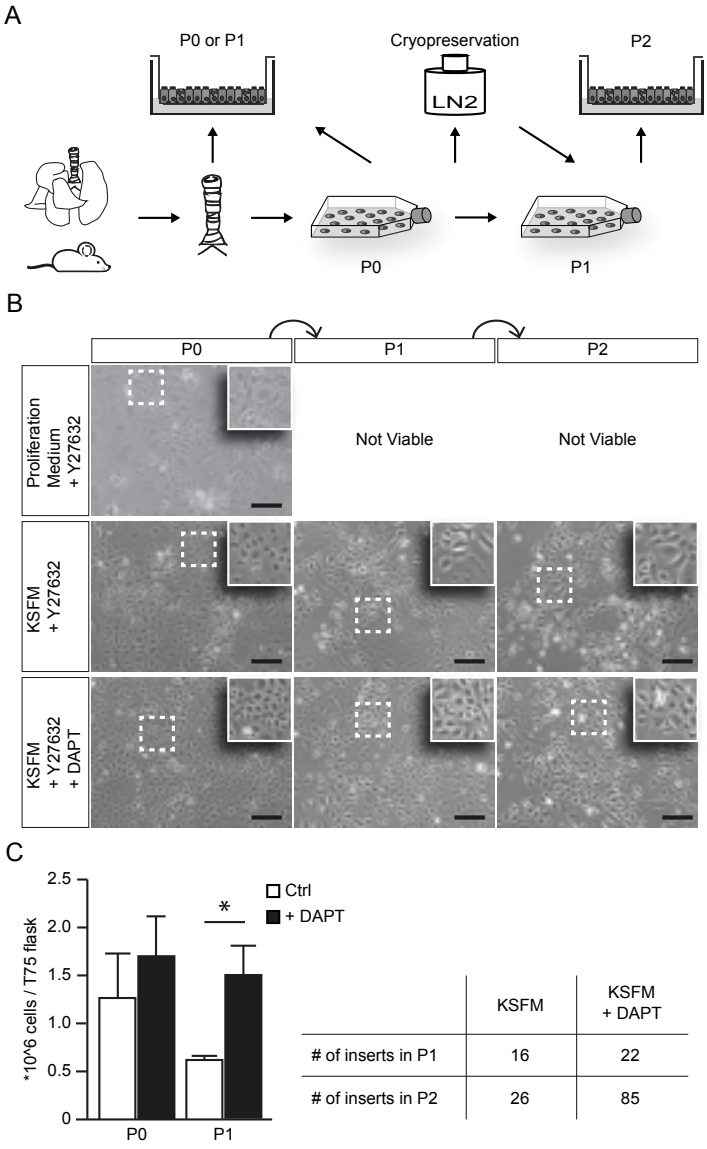
RESULTS

Serum free medium and the inhibition of Notch signaling enables basal cell expansion

Although the current methods of ALI culture represent the pseudostratified airway epithelium *in vitro*, a limitation of this technique is the relatively low number of cells obtained from a trachea. We first validated the existing culturing method of MTEC whereby isolated MTEC are cultured directly onto transwell inserts (Figure S1). This culture method allowed us to plate approximately 4 to 5 transwell inserts (50,000 cells per insert) using two mice (±100,000 cells per trachea). Improving the expansion of MTEC will allow for multiple experiments to be performed with fewer mice. Ideally, following isolation from the trachea, cells can be expanded in culture for at least two passages to generate sufficient cells for multiple experiments (Fig. 1a). In previous studies, MTEC are isolated and plated on inserts in proliferation medium and upon reaching confluency, cultured at ALI in differentiation medium (Table S1) (13, 14). Following expansion in proliferation medium, MTEC showed a limited proliferation capacity, with the presence of swollen cells with enlarged vacuoles (Fig. 1b). We were unable to successfully passage MTEC grown in proliferation medium. Keratinocyte Serum Free medium supplemented with epithelial growth factor (EGF), Bovine pituitary extract (BPE) and isoproterenol (KSFM) allows for expansion of human airway epithelial cells *in vitro* (15, 16). Additionally, adding a selective inhibitor of Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor, Y-27632, to the culture medium has been shown to increase proliferation of airway epithelial cells (17). Growing MTEC in KSFM with Y-27632 indeed resulted in improved cellular expansion, survival and morphology (Fig. 1b). However, despite improved cell morphology and survival, the expansion rate was very slow and still a large number of enlarged, hyperplastic cells were present. Notch signaling has been shown to play a pivotal role in preserving the basal cell population and in subsequent differentiation into specialized cell types. Furthermore, inhibition of Notch signaling has been shown to lead to an increased number of basal cells in mouse ALI cultures (10-12, 18, 19). To evaluate whether inhibition of Notch signaling promotes the expansion of MTEC after isolation, we added DAPT, a γ-secretase inhibitor and indirect Notch signaling inhibitor, to the KSFM medium containing Y-27632. Growing MTEC in the presence of DAPT and Y-27632 in KSFM medium resulted in a marked increase in proliferation rate with a slightly better morphology compared to cells grown in absence of DAPT; these differences were already apparent after 5 days of culture of P0 cells, but even more after passaging whereby

an increased number of transwell inserts could be obtained when DAPT was present during expansion (Fig. 1c). KSFM medium containing Y-27632 and DAPT will be further referred to as KSFM expansion medium.

Notably, expanding MTEC from two mice in KSFM expansion medium resulted in 42.5 million cells, sufficient for 85 transwell inserts, which is in stark contrast to the 200,000 cells required for 4-5 transwell inserts that are obtained if MTEC were isolated and cultured directly onto the transwell inserts. Also, no contaminating fibroblasts were observed during MTEC expansion. Taken together, we conclude that MTEC expansion is more efficient in the presence of the Notch signaling inhibitor DAPT than without.



	KSFM	KSFM + DAPT
# of inserts in P1	16	22
# of inserts in P2	26	85

Figure 1. Expanding MTEC in KSFM expansion medium with Notch signaling inhibitor. (A) MTEC are expanded to passage 1 in KSFM expansion medium followed by passaging and culturing on transwell inserts. MTEC are grown to full confluence in MTEC proliferation medium, followed by air-liquid interface differentiation in MTEC differentiation medium. (B) Serial expansion of MTEC in different medium conditions. Epithelial cells are cultured in proliferation medium with ROCK inhibitor (Y27632), in KSFM medium with ROCK inhibitor or KSFM medium with ROCK inhibitor (Y27632) and γ-secretase inhibitor (DAPT). Phase-contrast images of the MTEC at various passages showing the morphology of the cells. Scale bar, 100µm. (C) The graph represents the number of cells obtained after passaging when cultured in KSFM with ROCK inhibitor alone or in the presence of DAPT (mean ± SEM). * $p < 0.05$ by unpaired t-test (n=5). The table shows the number of 12-well inserts that can be obtained after each passage.

Subcultured MTEC retain the ability to differentiate and develop a pseudostratified epithelial layer

Differentiation into a pseudostratified epithelial layer is essential when using MTEC cultures to mimic *in vivo* airway epithelium. To evaluate whether MTEC that were expanded in KSFM expansion medium retain the ability to form a pseudostratified epithelial layer *in vitro*, MTEC were isolated, cultured submerged in KSFM expansion medium. Subsequently, MTEC were cultured on inserts in proliferation medium until confluent, followed by differentiation at ALI in differentiation medium (Fig. 2a). Polarization of the pseudostratified epithelial layer in an apical and basal side was evaluated using keratin 8 (KRT8) and keratin 5 (KRT5), which mark the luminal and basal cell layer respectively. Cellular differentiation was analyzed by staining for specialized cell types, including ciliated cells using forkhead box 1 (FOXJ1) and beta-tubulin IV (TUBB4B), club cells using secretoglobin, family 1A, member 1 (SCGB1A1) and basal cells using transformation-related protein 63 (TRP63). Additionally, zona occludens 1 (ZO-1)-specific staining indicated epithelial tight junction formation, an important feature of epithelial cells to maintain a tight barrier function. After 8 days of differentiation at ALI we observed stratification of the epithelial cell layer, indicated by KRT5 expression in the basal cell layer and KRT8 expression in the luminal cell layer, similar to ALI-MTEC cultures of P0 (Fig. 2a, Fig. S1). Although most of the cells were single positive for either KRT5 or KRT8, some cells were KRT5+KRT8 double positive, indicating the presence of basal luminal precursors which are cells transitioning from a basal to a luminal cell phenotype (20). Expanded MTEC developed tight junctions when cultured at ALI. Additionally, ciliated and secretory cells were detected after 8 days of differentiation at ALI (Fig. 2b). Epithelial polarization of expanded MTEC cultured at ALI was similar to the *in vivo* tracheal epithelium with TRP63 positive basal cells located at the basolateral side and the FOXJ1 positive ciliated cells at the apical side (Fig. 2b XZ plane, Fig. S1). Taken together, these data show that expanded MTEC retain the ability to develop a pseudostratified epithelial layer, mimicking the *in vivo* pseudostratified epithelium. We next compared the epithelial differentiation potential between P0, P1 and P2 MTEC by assessing ciliated cells and barrier development after 8 days ALI differentiation. Whereas the P2 ALI-MTEC were able to develop ciliated cells, the percentage of FOXJ1 positive cells was lower compared to passages P0 and P1 after 8 days of ALI differentiation (Fig. 2c, Fig. S2). Barrier function was assessed using trans-epithelial resistance measurement, whereby the reduction in FOXJ1 positive cells was accompanied by a significant increase in barrier development between P0 and P2 ALI MTEC (Fig. 2c and d). In addition, we investigated whether the passaged MTEC retained the ability

to develop ciliated cells in P2 ALI MTEC. Previously, it was shown that the presence of DAPT during differentiation increases the development of ciliated cells (11, 21). To this end, we differentiated expanded MTEC in the presence of DAPT during ALI and found an increased number of FOXJ1 positive ciliated cells compared to MTEC that were differentiated at ALI without DAPT (Fig. 2e). Moreover, increasing the duration of ALI differentiation also increased the number of FOXJ1 positive cells (Fig. 2e). Overall, expanded MTEC cultured at ALI retain their ability to develop a pseudostratified epithelial layer. The differentiation of expanded MTEC appeared to be altered as indicated by a decrease in ciliated cells compared to P0. However, addition of DAPT during differentiation and increasing ALI duration increases the number of ciliated cells comparable to the numbers as observed in P0.

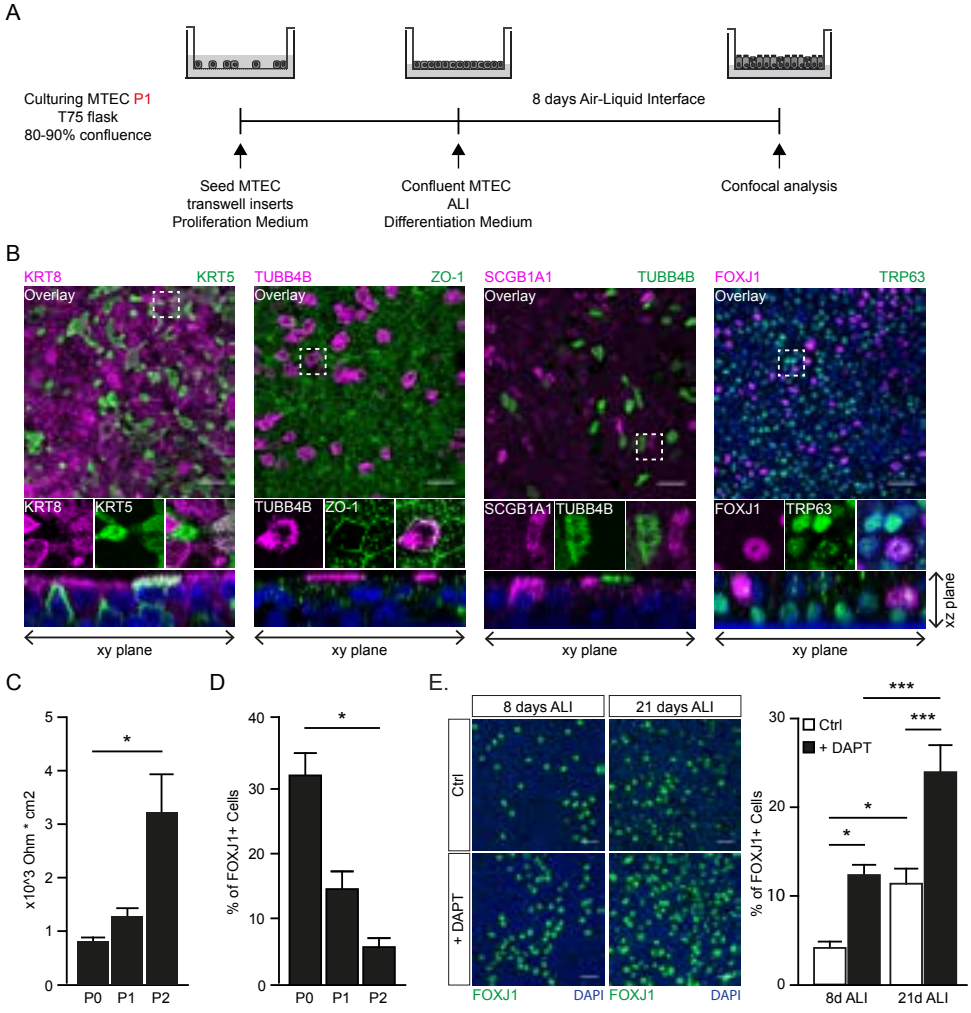


Figure 2. Expansion of MTEC *in vitro* leads to decreased ciliary cell differentiation. (A) Schematic representation of culture protocol. (B) Co-staining of different epithelial markers on MTEC passage 2 after 8 days of air liquid interface (ALI) culture. From left to right, inserts were stained with basal cell marker KRT5 and luminal marker KRT8, cilia cell TUBB4B with tight junction protein ZO-1, secretory club cell marker SCGB1A1 with cilia marker TUBB4B and the last panel shows TRP63 positive basal cells with ciliated cell marker FOXJ1. Nuclei are stained with DAPI (blue). Scale bar, 30 µm. (C) Resistance measurements of MTEC after different passages and 8 days of ALI (mean ± SEM). * $p < 0.05$ by one-way ANOVA (n=3). (D) Quantification of the percentage of FOXJ1 positive ciliated cells after 8 days of ALI comparing MTEC in passage (P) 0, 1 and 2 (mean ± SEM). * $p < 0.05$ by one-way ANOVA (n=3). (E) Representative images and quantification of ciliated cells (FOXJ1) after 8 days or 21 days ALI and with or without the presence of DAPT during differentiation (mean ± SEM). * $p < 0.05$, *** $P < 0.001$ by two-way ANOVA (n=6). Scale bar, 30 µm.

MTEC differentiation can be modulated using IL-13 to induce goblet cell metaplasia

Expanded MTEC cultured at ALI retained the ability to develop a pseudostratified epithelial layer. Next, we evaluated whether MTEC differentiation could be modulated using IL-13, which is important in the development of goblet cell metaplasia in mouse models of allergic airway inflammation (22, 23). We stained trachea of mice that were sensitized and exposed to house dust mite (HDM) allergen to induce allergic airways inflammation for the presence of goblet cells, and found these to be increased compared to placebo-treated mice (Fig. 3a). Previously, IL-13 has been shown to induce goblet cell metaplasia *in vitro* using ALI cultures of airway epithelial cells (15, 24-26). We exposed non-expanded (P0) and expanded (P1, P2) MTEC with DAPT to IL-13 during ALI culture and compared IL-13-induced MUC5AC expression patterns. Goblet cells are scarce in MTEC after 8 days of differentiation (Fig. 3c). However, we found that IL-13-exposed ALI MTEC cultures displayed increased numbers of goblet cells and there was no difference in the increase in goblet cells between P0 and P2 (Fig. 3c, Fig. S3). Furthermore, IL-13 exposure resulted in a reduced number of ciliated cells in both ALI cultures with freshly isolated MTEC or with expanded MTEC (Fig. 3e). Finally, IL-13 decreases epithelial barrier function *in vitro* (27, 28), which is in line with our results showing a similar reduction in barrier function following IL-13 exposure in expanded MTEC and isolated MTEC cultured directly onto inserts (Fig. 3d). Taken together our results indicate that expanding MTEC *in vitro* does not affect their ability to induce a physiologic response to IL-13 exposure, suggesting that this model could be an attractive alternative to evaluate new therapeutic compounds and their effects on airway epithelial cells *in vitro*.

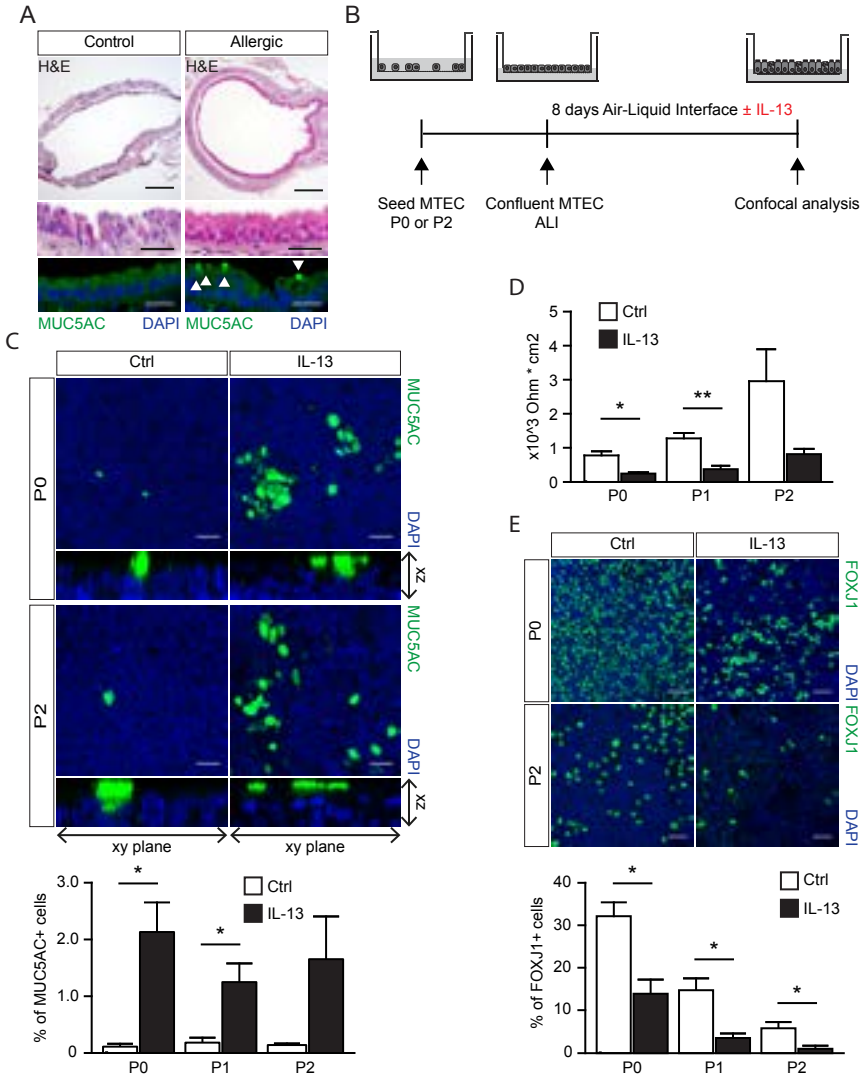


Figure 3. IL-13 exposure during MTEC differentiation induces goblet cells. *(A)* Hematoxylin and eosin on tracheal sections of control or house dust mite (HDM) treated mice. Immunofluorescence staining with Mucin 5AC (MUC5AC) shows the presence of goblet cells. MUC5AC expressing cells are indicated by the white arrows. Scale bar, 200 µm and 30 µm. *(B)* A schematic representation of the culture protocol. *(C)* Immunofluorescence staining of MUC5AC of MTEC passage 0 and 2 after 8 days of ALI culture with or without IL-13. Scale bar, 30 µM. Graph shows the percentage of MUC5AC expressing goblet cells in culture (mean ± SEM). * $p < 0.05$ by Student two-tailed t-test (n=3). *(D)* Resistance measurements after 8 days of ALI culture with or without IL-13 (5 ng/ml) treatment (mean ± SEM). * $p < 0.05$ by Student two-tailed t-test (n=3). Scale bar: 30 µm. *(E)* Immunofluorescence staining of FOXJ1 positive ciliated cells in a passage 0 (P0) and 2 (P2) after 8 days of ALI culture with or without IL-13 (5 ng/ml). Scale bar, 30 µm. Graph show the percentage of FOXJ1 expressing ciliated cells in culture (mean ± SEM). * $p < 0.05$ by Student two-tailed t-test (n=3).

Intrinsic changes of the basal cell population result in decreased differentiation potential

Expanding MTEC before ALI differentiation resulted in reduced numbers of ciliated cells, suggesting an altered differentiation potential of the basal cell population. The basal cell population consists of various subtypes with different functionality that can be identified by the presence of different basal cell markers (5, 7). To evaluate whether the altered differentiation potential could be explained by a change in basal cell composition at baseline, we first examined the TRP63 positive basal cell population at day 0 of ALI differentiation for P0, P1 and P2 (Fig. 4a). No significant differences were detected in the percentage of TRP63 positive basal cells between P0, P1 and P2 (Fig. 4b). This indicates that a difference in the number of TRP63 positive basal cells is not the cause of an altered differentiation potential, suggesting that the identity of the TRP63 positive basal cell is changed. We therefore next evaluated the expression patterns of other previously published basal cell markers. KRT8 and KRT5 were already used to show stratification of the MTEC cultured at ALI (Fig. 2). As indicated, KRT5+KRT8 double positive cells mark a population of basal luminal precursor cells. Another marker used to distinguish basal cells is P75-nerve growth factor receptor (NGFR), which is enriched in murine tracheal basal cells (6). We observed no overt differences in KRT5, KRT8 and NGFR positive basal cells in P2 compared with P0 (Fig. 4c and d).

A subpopulation of keratin 14 (KRT14) positive basal cells is reported to be present in a small subset of basal cells, but is highly increased after injury and serves as a progenitor for ciliated and secretory cells (29, 30). We investigated the presence of KRT14 positive basal cells in P0 and P2 MTEC on inserts at day 0 of ALI. The KRT14 positive basal cell fraction showed a marked increase in P2 compared to P0 (Fig. 4d and e). Overall, our data suggests that Notch signaling is involved in the maintenance of the basal cell population and that expanding MTEC during Notch signaling inhibition increases the KRT14 positive basal cell fraction without affecting the other investigated basal cell populations.

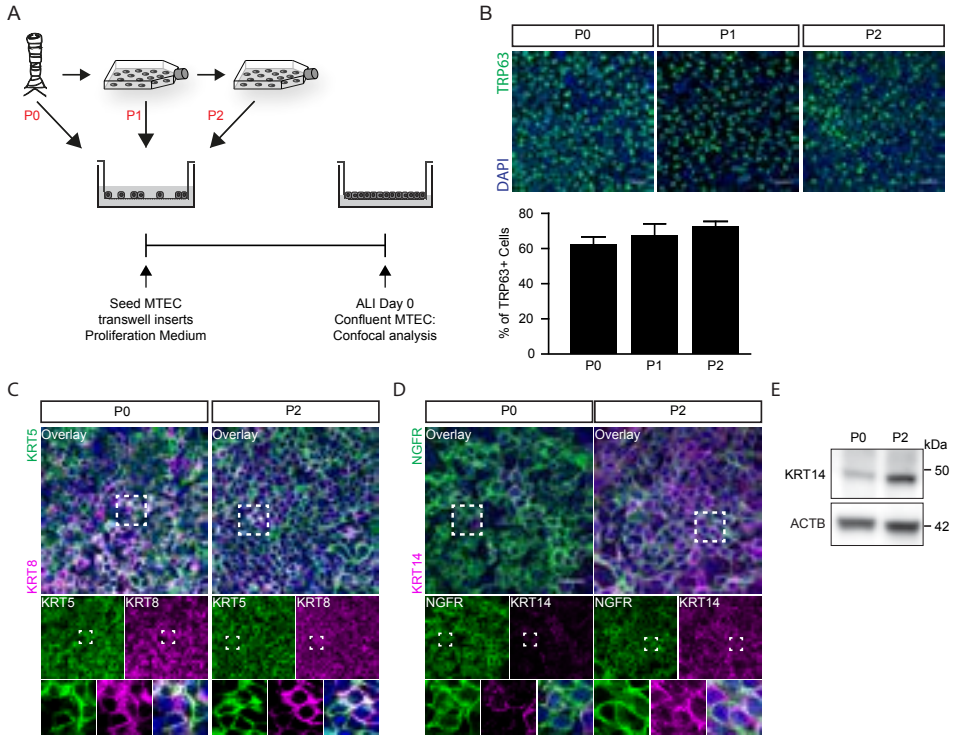


Figure 4. The population of TRP63 positive basal cells change during expansion. (A) Schematic representation of culture protocol. (B). Representative images of the number of TRP63 positive basal cells at ALI day 0. Scale bar, 30 µM. Graph shows the percentage of TRP63 positive basal cells at day 0 of ALI culture in a passage 0 (P0), passage 1 (P1) and passage 2 (P2) (mean ± SEM). One-way ANOVA (n=3). (C) Immunofluorescence of basal cell marker KRT5, and luminal cell maker KRT8 at day 0 of ALI. The overlay picture and the one-channel pictures are shown. The boxes indicate the area of which an enlarged image is presented. Scale bar: 30 µm. (D) Immunofluorescence of basal cell marker, P75-nerve growth factor (NGFR) and basal cell maker keratin 14 (KRT14) at day 0 of ALI. Scale bar: 30 µm. (E) Western blot analysis of KRT14 at day 0 of ALI in a passage (P) 0 and 2. Beta-actin (ACTB) is used as loading control. Scale bar: 30 µm.

DISCUSSION

Using a combination of Notch signaling and Rho-associated kinase (ROCK) inhibition and specialized media, we were able to subculture MTEC while preserving the mucociliary differentiation potential when cultured at ALI. This protocol provides a much more efficient way to culture MTEC, increasing the number of experiments that can be performed with two mouse tracheas, with approximately 42.5 million cells after passaging, which is in stark contrast to 200 000 cells if MTEC are plated on transwell inserts directly after isolation. Our culture method will contribute to a reduction in the number of animals used, in line with the 3R principle, and also has the potential to decrease technical and biological variation between experiments. Furthermore, a reduction in the number of animals needed is relevant when MTEC are isolated from difficult-to-breed mouse strains. To increase the efficient use of mouse trachea for MTEC culture, we have used KSFM medium to prevent outgrowth of fibroblasts (a common problem in MTEC cultures) supplemented with the RhoA-kinase inhibitor Y-27632 and the γ-secretase inhibitor and indirect Notch signaling inhibitor DAPT. Additionally, our results indicate that expanding MTEC *in vitro* results in altered stemness of the basal cell population which is accompanied by a shift of a KRT14 negative basal cell population observed at P0 towards a KRT14 positive basal cell population at P2. Further investigation is required to evaluate the contribution of the KRT14 positive basal cell population towards the altered stemness of the basal cell fraction.

MTEC have previously been shown to have the ability to grow in submerged culture. However, the ability of the MTEC to differentiate at ALI was not explored (31). More recently, SMAD signaling inhibition was shown to promote airway basal stem cell expansion *in vitro* from multiple species with subsequent ALI differentiation (32). Although this study contributed significantly to the *in vitro* research field, the authors did not elaborate on cell culture techniques and growth media used. We attempted to use the SMAD signaling inhibitors described by Mou and colleagues in combination with KSFM medium, but observed that MTEC could not be expanded under these growth conditions (results not shown). In the present study, we have used KSFM to expand MTEC *in vitro* prior to culturing on transwell inserts. KSFM has previously been used successfully to expand primary human bronchial epithelial cells (15, 16). In addition, KSFM has the advantage of preventing outgrowth of fibroblasts, which is likely attributed to the low calcium levels, thus impairing fibroblast migration and proliferation (33-35). An important advantage of the use of KSFM is therefore that it circumvents the need of culturing the isolated cell suspension for 4 to 5 hours on

culture dishes to remove fibroblasts by adherence. This is important, since maintaining epithelial cells for several hours in suspension prevents them from adhering to a matrix, which may result in anoikis (36). Our results indicate that MTEC do proliferate in KSFM, but the morphology, survival and expansion rates drastically improved when a ROCK inhibitor in combination with a Notch signaling inhibitor was used. Following expansion of MTEC in KSFM, we allowed MTEC to grow and differentiate on transwell inserts as previously described (14, 17, 37).

Expanding the progenitor basal cell population is essential for subculturing MTEC *in vitro*. ROCK inhibition is frequently used in embryonic stem cell cultures, induced pluripotent stem cells and some tissue-specific stem cell populations. More specifically, the ROCK inhibitor Y27632 has previously been shown to improve proliferation rates of human and mouse tracheal epithelial cells without affecting subsequent ALI differentiation (17). Feeder layers or conditional medium from feeder layers has proven effective in expanding airway epithelial cells (38). However, maintaining feeder layers adds significantly to the workload and exact mechanisms through which the feeder layers improve proliferation remain unknown. Therefore, using a defined culture medium without the need of feeder layers is preferred and is likely to result in increased consistence of the culture system.

A mechanism that has been suggested to underlie the ability of ROCK inhibitors to improve cell proliferation is the inhibition of downstream Notch signaling (39, 40). Whereas Y27632 improved MTEC proliferation rates in our experiments, cell morphology and survival suggested the presence of cell contact-induced impairment of cell proliferation. Cell-cell communication and related inhibition of proliferation has previously been attributed to Notch signaling (41). Notch signaling has previously been shown to be involved in basal epithelial cell proliferation and differentiation both *in vitro* and *in vivo* (10, 11, 18, 21, 41, 42). We therefore used DAPT, a γ-secretase inhibitor and indirect Notch signaling inhibitor, in combination with Y27632 in KSFM to expand MTEC *in vitro*. Our data shows that inhibiting Notch signaling is an effective way to expand the basal cell population.

Differentiation of the basal cell population into a pseudostratified epithelial layer containing secretory and ciliated cells is an important feature of the airway epithelium. We have shown that MTEC expanded to passage 2 retain the ability to differentiate into a pseudostratified epithelial layer. To this end, we used KRT5 and KRT8 staining to discriminate between basal and luminal precursor populations. Some cells show the presence of both KRT5 and

KRT8 which may mark basal cells differentiating towards a luminal precursor cell (11, 20). Furthermore, the pseudostratified epithelium of the trachea contains various cell types including basal, ciliated and secretory cells. Using FOXJ1 and TUBB4B in addition to SCGB1A1 staining, we showed the presence of ciliated and club cells in expanded MTEC after ALI differentiation. However, the expanded MTEC had reduced numbers of ciliated cells present after 8 days of ALI, despite the unchanged KRT8 positive luminal fraction in P2 compared to P0. This suggests that the cells may require more time or an additional trigger for efficient differentiation into ciliated cells. This hypothesis is strengthened by the observed increase in ciliated cells by extending the duration of ALI. This was further increased by adding the Notch inhibitor DAPT during ALI, which is in line with previous studies showing that inhibition of Notch signaling may drive epithelial cells towards ciliated cells (21, 43). Collectively, these findings indicate that after expansion in culture, basal cells retain the ability to develop a pseudostratified epithelial layer following ALI differentiation, but with an altered differentiation capacity, likely resulting from altered stemness of the basal cell population. Additional triggers generated by extended culture and Notch inhibition, are required to achieve more efficient mucociliary differentiation.

The ability to adapt to a changing environment to various stimuli is an important feature of airway epithelial cells. Furthermore, appropriate *in vitro* epithelial culture models should recapitulate the response to disease-specific stimuli observed in patients and/or animal models of disease. To this end, we used IL-13 exposure and assessment of subsequent goblet cell development of expanded MTEC cultured at ALI to mimic the *in vivo* allergic airways inflammation. IL-13 is a T helper 2 cytokine known to induce goblet cell metaplasia *in vivo* and *in vitro* (22, 23, 25, 44, 45). IL-13 exposure during differentiation induced MUC5AC positive goblet cells in both expanded MTEC and in freshly isolated MTEC that were cultured directly onto inserts. The presence of IL-13 during differentiation at the ALI resulted in an increased number of goblet cells, likely resulting from basal cells differentiating towards goblet cells rather than ciliated cells. Alternatively, the increased number of goblet cells may be resulting from IL-13 induced trans-differentiation of ciliated cells towards goblet cells, resulting in fewer ciliated cells (26, 45, 46). Moreover, IL-13 induced a reduction in barrier function in both expanded MTEC and isolated MTEC that had been cultured directly onto transwell inserts, which is in line with previous publications showing that IL-13 reduces barrier function *in vitro* (27, 28). Overall, expanded MTEC showed a robust response following IL-13 exposure including decreased barrier function and the development of goblet cells, indicating that expanding MTEC *in vitro* using a Notch signaling inhibitor does

not affect their ability to respond to IL-13. The observation that goblet cell differentiation induced by IL-13 exposure is similar in P0 and P2, suggests that the basal cells retain the ability to differentiate into goblet cells.

Basal cells are the main progenitor cells of the conducting airways. They have the proficiency to self-renew and differentiate into various cell types found in a pseudostratified epithelial layer including ciliated and secretory cells. Various cellular markers have been described to distinguish basal cells in the airway epithelium. Furthermore, basal cells can be separated in various subsets depending on the combination of cellular markers present (5, 7). KRT5, TRP63 and NGFR are commonly used markers to delineate basal cell populations. In contrast, only a small fraction of basal cells is positive for KRT14 at baseline. Our results indicated that expansion significantly induced a KRT14 positive basal cell population in P2 compared to P0, whereas the other investigated basal cell populations remained unaltered between passages. Together these data suggest that expanding basal cells *in vitro* can alter the composition of the basal cell population, which may explain why we see a reduced number of ciliated cells in expanded MTEC compared to isolated MTEC cultured directly onto inserts. Whether this alteration of the basal cell population results from the KRT14 negative basal cell population turning KRT14 positive or that the KRT14 positive basal cell population has an increased capacity to expand *in vitro* compared to the KRT14 negative basal cell population remains to be further investigated. However, KRT14 positive basal cells are still able to differentiate in ciliated cells and goblet cells when exposed to additional triggers, respectively DAPT and IL-13, in a similar way as KRT14 negative basal cells. More research is needed to investigate the role of KRT14 positive basal cells and their ability to differentiate.

In conclusion, we developed a clearly defined culture method that allows ALI differentiation of MTEC into a pseudostratified epithelial layer following *in vitro* expansion. Expanding MTEC *in vitro* did result in decreased differentiation of basal cells to ciliated cells, likely resulting in part from an altered composition of the basal cell population as indicated by an increase in KRT14 positive basal cells. Despite the altered basal cell population, the Th2 cytokine IL-13 was still able to redirect epithelial differentiation towards a phenotype observed in allergic airways inflammation. Furthermore, this culture method may be useful to study repair following injury combined with *in vitro* lineage tracing if MTEC from transgenic animals are used. Also, this new culture method will contribute to a reduction in animals needed for experimentation, in line with the principles of the 3R's (Replacement, Reduction and

Refinement).

SUPPLEMENTAL DATA

Supplemental Table S1. Overview of culture media and supplements

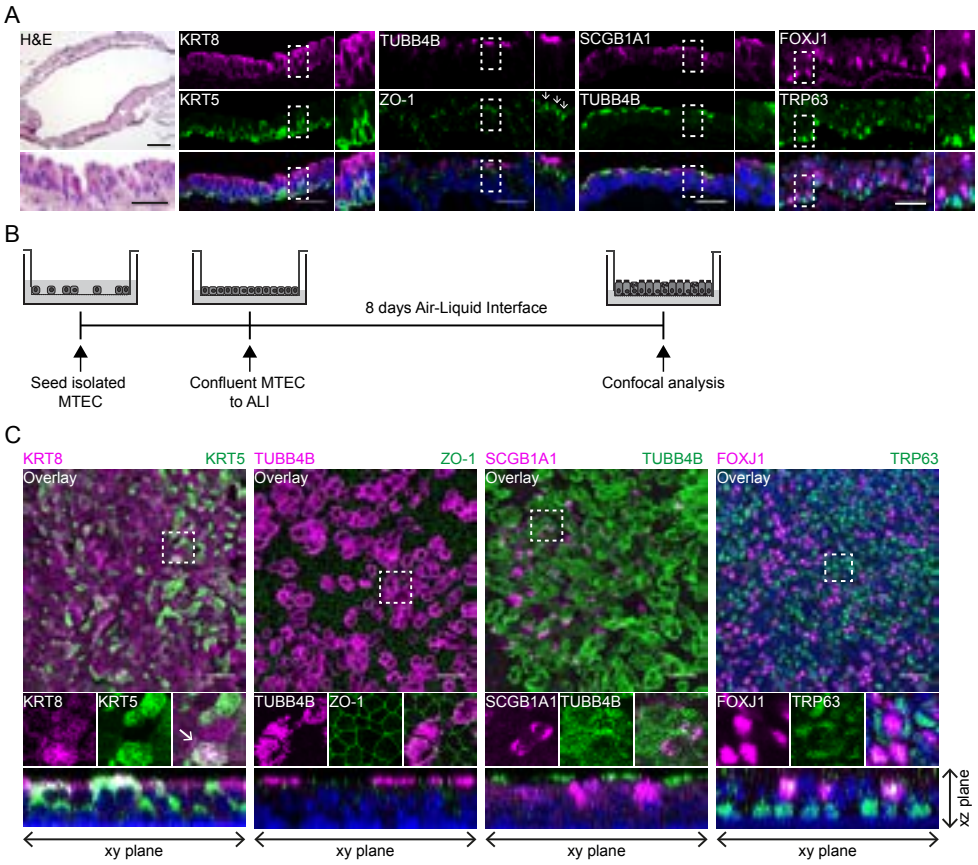
Medium	Component	Provider (catalogue number)	Final concentration
KSFM expansion medium	KSFM	Gibco (17005034)	
	Pen/Strep	Lonza (DE17-602e)	1 % (v/v)
	Murine EGF	Peprotech (315-09)	0.025 µg/ml
	Bovine Pituitary Extract	Gibco (13028014)	0.03 mg/ml
	Isoproterenol	Sigma (I-6504)	1 µM
	Y-27632 (add fresh)	Cayman Chemical (10005583)	10 µM
	DAPT (add fresh)	Sigma (D5942)	5 µM
MTEC Basic	DMEM/F12	Gibco (1133032)	
	Pen/Strep	Lonza (DE17-602e)	1 % (v/v)
	$NaHCO_3$	Gibco (25080094)	0.03 % (v/v)
MTEC proliferation medium	MTEC basic		
	L-Glutamine	Gibco (25030081)	1.5 mM
	Fetal Calf Serum	HyClone (SH30071.03)	5 %
	ITS-G	Gibco (41400045)	1x
	Cholera Toxin	Sigma (C8052-0.5mg)	0.1 µg/ml
	Murine EGF	Peprotech (315-09)	0.025 µg/ml
	Bovine Pituitary Extract	Gibco (13028014)	0.03 mg/ml
	Y-27632 (add fresh)	Cayman Chemical (10005583)	10 µM
	Retinoic Acid	Sigma (R2625-50mg)	0.05 µM

MTEC differentiation medium	MTEC basic		
	L-Glutamine	Gibco (25030081)	1.5 mM
	Bovine Serum Albumin	Gibco (***15260037)***	0.1 % (v/v)
	ITS-G	Gibco (41400-045)	1x
	Cholera Toxin	Sigma (C8052-0.5mg)	0.025 µg/ml
	Murine EGF	Peprotech (315-09)	0.005 µg/ml
	Bovine Pituitary Extract	Gibco (13028-014)	0.03 mg/ml
	Retinoic Acid	Sigma (R2625-50mg)	0.05 µM

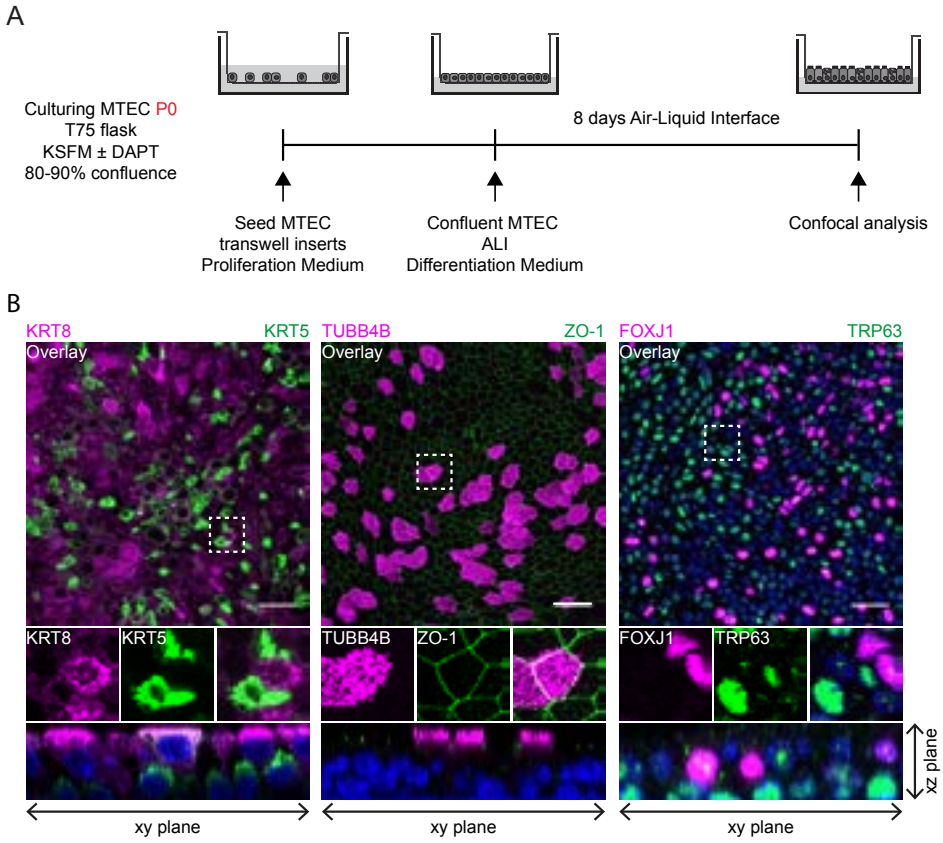
Supplemental table 2. Antibody overview and dilutions

Primary Antibody	Provider (Catalogue number)	Dilution
Beta-Tubulin IV	BioGenex, MU178-UC, Mouse monoclonal	1:100
SCGB1A1 (CCSP)	From Dr. Barry Strip, Goat polyclonal	1:5000
SCGB1A1 (CCSP, Uteroglobin)	Abcam, ab40873, Rabbit polyclonal	1:200
FOXJ1	eBioscience, 14-9965, Mouse monoclonal	1:300
KRT5	Biologend, Poly19055, Rabbit polyclonal	1:500
KRT8	DSHB, TROMA-I, Rat monoclonal	1:100
MUC5AC	Abcam, ab3649, Mouse monoclonal	1:500
TRP63	Santa Cruz, sc-8343, Rabbit polyclonal	1:50
Zona Occludens 1	Invitrogen, 61-7300, Rabbit polyclonal	1:100
KRT14	EMD Millipore, CBL197, Mouse monoclonal	1:100
NGFR	Promega, Anti-Human P75 pAb, Rabbit polyclonal	1:200
B-ACTIN	Cell Signaling, 4967, Rabbit polyclonal	1:1000
Secondary Antibody		
Alexa Fluor ®488, 594 Donkey anti Goat IgG	Jackson ImmunoResearch (111301), (112406)	1:500
Alexa Fluor ®488, 594 Donkey anti Mouse IgG	Jackson ImmunoResearch (112976), (110989)	1:500
Alexa Fluor ®488, 594 Donkey anti Rabbit IgG	Jackson ImmunoResearch (113254), (113078)	1:500

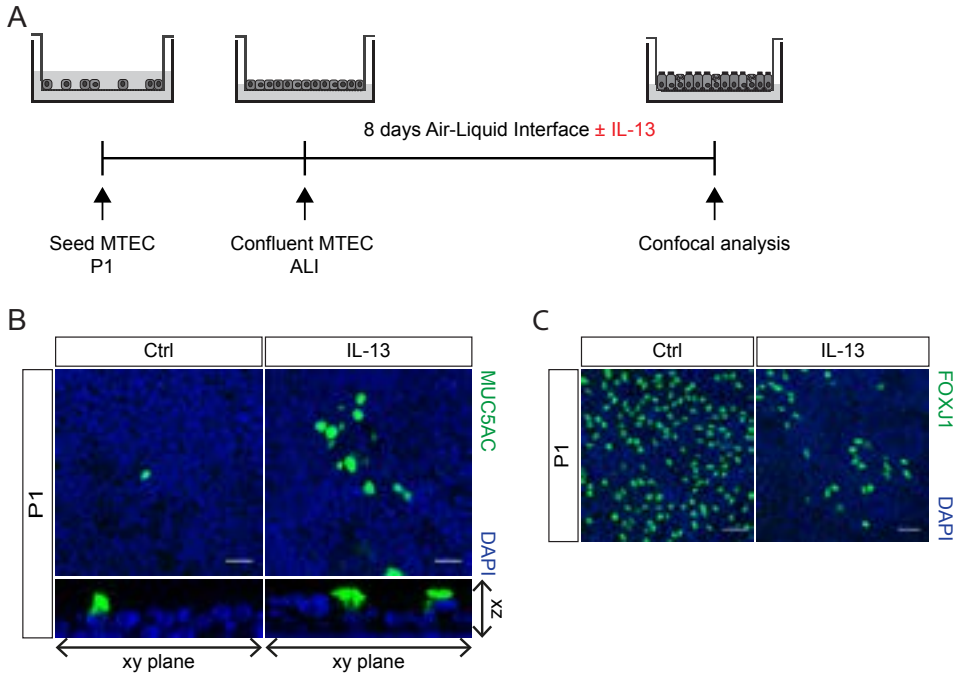
Alexa Fluor ®594 Donkey anti Rat IgG	Jackson ImmunoResearch (127426)	1:500
Anti-mouse HRP conjugated	DakoCytomation (P 0447)	1:10,000
Anti-rabbit HRP conjugated	DakoCytomation (P 0448)	1:10,000



Supplemental figure S1: MTEC ALI cultures recapitulate the *in vivo* **pseudostratified airway epithelium.** (A) Hematoxylin and eosin staining of a mouse tracheal sections and immunofluorescence co-staining on tracheal sections with basal cell marker KRT5 and luminal cell marker KRT8, cilia marker TUBB4B with tight junction protein ZO-1 (indicated by the arrows), secretory club cell marker SCGB1A1 with cilia marker TUBB4B and the last panel shows TRP63 positive basal cells with ciliated cell marker FOXJ1. Nuclei are stained with DAPI (blue). Scale bar, 200 µm and 30 µm. (B) Schematic representation of ALI culture. (C) Co-staining of the different epithelial markers on MTEC ALI culture. The arrow marks a basal luminal precursor cell that is both positive for KRT5 and KRT8. Orthogonal view shows a stratified epithelium. Nuclei are stained with DAPI (blue). Scale bar, 30 µm.



Supplemental figure S2. Representative images of P1 on inserts. (A) Schematic representation of ALI culture. (B) Co-staining of different epithelial markers on MTEC passage 1 after 8 days of Air Liquid Interface (ALI) culture. From left to right, inserts were stained with KRT5 for the basal cell layer and differentiation marker KRT8, Ciliated cell marker FOXJ1 with tight junction protein ZO-1, secretory cell marker SCGB1A1 with cilia marker TUBB4B and the last panel shows TRP63 positive basal cells with ciliated cells. Nuclei are stained with DAPI (blue). Scale bar, 30 µM.



Supplemental figure S3. IL-13 treatment stimulates the formation of goblet cells *in vitro***.** (A) Schematic representation of culture protocol. (B) Staining of Mucin 5AC (MUC5AC) expressing goblet cells in MTEC (Passage 1, P1) after 8 days of ALI with or without IL-13 (5 ng/ml). Nuclei are stained with DAPI (blue). Scale bar: 30 µM. (C) Staining of FOXJ1 positive ciliated cells after 8 days of ALI with or without IL-13 (5 ng/ml) in P1. Nuclei are stained with DAPI (blue). Scale bar: 30 µM.

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CHAPTER 7

GENERAL DISCUSSION

INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are both heterogeneous diseases with various clinical phenotypes and diverse underlying molecular pathways (1, 2). Disease heterogeneity makes treatment of these chronic obstructive diseases challenging, particularly for patients that respond poorly to existing therapies. Consequently, identifying patients using biomarkers indicating towards a specific disease phenotype and subsequent personalized treatment, will become important in improving therapy outcomes. Easy accessible tissues and body fluids allow us to discover novel phenotype specific biomarkers to drive biomarker-guided personalized therapy. Additionally, unravelling the specific molecular pathways underlying the various observed phenotypes will contribute to the discovery of new targets for the development of novel therapeutics. Indeed, the airway epithelium is increasingly in the focus as an important compartment in the development of airway diseases. Cultures of primary cells isolated from human tissue are a particularly relevant tool to study such pathways, because they offer the possibility to closely mimic events occurring in the tissues of patients. Ideally, the cell type investigated is easy accessible, can be isolated from patients and subsequently be cultured *in vitro*, and can be genetically modified to investigate potential new therapeutic targets.

Airway epithelial cells are at the interface of inhaled particles and pathogens, and the underlying tissues. Being at this interface makes airway epithelial cells ideal candidates to report underlying tissue inflammation. Moreover, airway epithelial cells are relatively accessible, can be cultured *in vitro* and can be cryopreserved. Culturing airway epithelial cells *in vitro* to study molecular pathways requires careful consideration of the culture conditions and experimental setup. In **chapter 2** we provide an overview of *in vitro* methods and models available to study asthma and COPD.

AIRWAY EPITHELIAL CELLS AND IN VITRO MODELS: COMPLEXITY, REPRODUCIBILITY OR BOTH?

In vitro models of airway epithelial cells are commonly used to model asthma or COPD. Despite their common use, there is a large discrepancy among the isolation and culture methods described in literature. Both cell lines and primary cells of airway epithelial cells are used, cultured submerged or at air-liquid interface (ALI). Cells are exposed to substances diluted in culture medium or when cells are cultured at ALI, *in vitro* exposures are feasible

that are more representative of exposure of *in vivo* airway epithelium to inhaled substances. In such ALI cultures, cells can be exposed to e.g. aerosolized substances or complex mixtures of gases and particles to mimic the air route of exposure, or substances can be added in culture medium present in the basolateral compartment of the transwell insert. When modeling the *in vivo* epithelium *in vitro*, multiple factors need to be taken into account. *In vivo*, the airway epithelial cells are surrounded by different cell types, both immune cells and structural cells. Additionally, the *in vivo* airway epithelium is not a sterile environment. In contrast, the epithelium is continuously challenged with inhaled microbial species in addition to commensal bacteria, viruses and fungi.

When culturing and/or exposing airway epithelial cells to model asthma or COPD, it is always important to consider the research question asked. Nonetheless, when modeling the *in vivo* epithelium, care should be taken to capture the *in vivo* characteristics as thoroughly as possible to improve the translational potential of the *in vitro* model. Given the complex environment of airway epithelial cells *in vivo*, modeling those characteristics *in vitro* will be very challenging. **Chapter 2** provides a discussion of co-culture models that can be used to mimic interactions of airway epithelial cells and other cell types.

Fully capturing the characteristics of the *in vivo* epithelium is particularly challenging and will add a significant degree of complexity to the *in vitro* model through the various cellular and microbial interactions discussed. Indeed, increasing complexity of the *in vitro* model may result in considerable variation that may introduce differences between experiments. Such reduced reproducibility will result in conflicting data between and also within laboratories. However, oversimplified *in vitro* models may not capture the intricate details of the *in vivo* epithelium, overall resulting in data that cannot be translated to a clinical setting. Finding a balance between complexity and reproducibility should be carefully considered when designing culture models.

An important feature of the *in vivo* epithelium that has received a lot of recent attention and is currently lacking from *in vitro* airway epithelial culture models, is the microbiome. The microbiome comprises of multiple species of micro-organisms of which the composition and total load can vary with health status (3, 4). However, modeling the microbiome *in vitro* will be particular challenging as current sampling methods and microbial culturing methods require optimizing. Also, current *in vitro* culturing methods of airway epithelial cells include the use of antibiotics in the culture medium to prevent infection. *In vivo*, antibiotics are only

encountered during treatment of bacterial infections. In contrast, *in vitro* cultures will always have antibiotics present in the culture medium to prevent infection of susceptible cells. One of the explanations for this increases susceptibility to infection, is that the mucociliary system provided by airway epithelial cells is unable to remove particles in a similar fashion as in the lung, but also the lack of adaptive immunity may help to explain this limitation of the model. It remains however important to consider the effects of antibiotics on *in vitro* cultures of airway epithelial cells and how these may affect experimental readouts. This is illustrated by a recent study showing the impact of antibiotics on growth and differentiation of cultured keratinocytes (5). Using antibiotics in the culture medium will not be feasible in *in vitro* models incorporating the microbiome. Vice versa, controlling outgrowth of particular microbial species under *in vitro* conditions will be particularly challenging. Nonetheless, *in vitro* studies of the gut epithelium combined with complex microbial mixtures have demonstrated the feasibility of such experimental approaches and may provide us with clues to set up an *in vitro* system using airway epithelial cells and the lung microbiome (6, 7).

Another important aspect of the *in vivo* epithelium that has received particular attention to be represented in *in vitro* airway epithelial models is the lung physiology and its associated mechanical forces, including rhythmic breathing patterns, mucociliary clearance and air flow, combined with the 3D environment of the airway epithelial cells containing different cell types such as immune cells and structural cells. Very recently, considerable effort has been made towards including these important features in airway epithelial *in vitro* models (8, 9). Benam and colleagues developed a small airway-on-a-chip model that integrates fully differentiated, pseudostratified small airway epithelial cells, microvascular endothelial cells and immune cells, combined with cyclic mechanical stretching, microfluidics and air flow (9). They used the small airway-on-a-chip to identify COPD-specific biological responses and discovered novel molecular signatures that may serve as potential therapeutic targets or diagnostic biomarkers (8).

In contrast to the aforementioned described complex models, are the oversimplified *in vitro* models of airway epithelial cells that have been widely used, consisting of mostly airway epithelial cell lines that do not develop a fully differentiated, pseudostratified epithelial layer. Whereas transformed or tumor cell lines can easily be grown in large quantities and their use often does not require advanced expertise and expensive culture media, care should be taken in the interpretation of the results since many cellular pathways may be altered in such cell lines. Also, epithelial cell lines are typically cultured submerged, thereby

preventing the archetypal apical to basal polarity of the *in vivo* epithelium. Moreover, the *in vivo* airway epithelium consists of multiple epithelial cell types including basal, secretory and ciliated cells whereas cell lines do not develop these specialized cell types. Ideally, results obtained through cell lines should be validated using air-liquid interface (ALI) cultured primary airway epithelial cells, which have become widely available through commercial sources. Also, culturing methods for ALI cultured primary airway epithelial cells have been thoroughly documented and should not require excessive optimization to culture for *in vitro* validation experiments.

Using an appropriate *in vitro* model is important when modeling asthma or COPD *in vitro*. Airway epithelial cells can be isolated from patients or alternatively, airway epithelial cells can be exposed to disease related substances to model disease characteristics *in vitro*. Ideally, patient and healthy control derived airway epithelial cells are used when comparing disease characteristics, but these are difficult to obtain and commercially available patient-derived airway epithelial cells often lack critical clinically information. Therefore, modeling asthma or COPD is typically done with disease related substances. Multiple considerations that need to be taken into account when modeling asthma or COPD *in vitro* have been described in **chapter 2**. In **chapter 3** we used interleukin (IL)-13, a T helper 2 (Th2) cytokine, to model the Th2-high asthma phenotype *in vitro*. Modeling features of asthma *in vitro* is very challenging as the actual cause of asthma is still highly debatable and asthma comprises of multiple phenotypes with different underlying pathways (10). IL-13 has long been recognized to be a pivotal player in asthma pathogenesis, but it has become quite clear that only in a subset of asthma patients IL-13 can be considered as a key player. These patients have been described to have a “Th2-high” phenotype comprising of airway epithelial expression of serpin family B member 2 (SERPINB2), chloride channel accessory 1 (CLCA1) and periostin (POSTN), with POSTN also being detectable in serum (11). Moreover, this epithelial expression pattern can be used to classify asthma patients into Th2-high and Th2-low phenotypes, with Th2-high asthma patients being more susceptible to inhaled corticosteroid treatment (12). The ability to model this gene signature *in vitro* allows us to study factors that could modulate this gene signature.

In **chapter 4** we used ALI cultured primary airway epithelial cells whereby we used disease related substances to induce disease characteristics, representing an *in vitro* model for Th2-mediated inflammation combined with cigarette smoking. Our *in vitro* model showed robust responses that could be reproduced in multiple donors. Of important notice is the

discrepancy between our observed results for mucin 5AC (MUC5AC) and the effects of cigarette smoke exposure. Multiple groups have reported increased expression of MUC5AC by cigarette smoke (13, 14), whereas we did not observe this effect. The aforementioned studies however used cigarette smoke extract, an aqueous solution of cigarette smoke. Cigarette smoke extract does not give a good representation of whole cigarette smoke as discussed in **chapter 2**, but using whole cigarette smoke does come with some challenges for application *in vitro*. We used a custom designed model to expose ALI cultured airway epithelial cells to whole cigarette smoke *in vitro*. Quantifying the actual deposition of whole cigarette smoke on the ALI airway epithelial cells is currently not possible in this model. Despite being the more representable model to expose cells to cigarette smoke *in vitro* compared to aqueous extracts, it remains to be investigated whether our observed effects on MUC5AC represent the *in vivo* situation and that underlying tissue inflammation may indeed be required for the development of mucus hypersecretion in smokers. Alternatively, the exposure setup may require further optimization to recreate the results observed by other researchers using cell lines and cigarette smoke extract.

In **chapter 5** we described an *in vitro* model with viral or bacterial exposure following cigarette smoke exposure. This model could be used as an *in vitro* setup to study viral or bacterial exacerbations in COPD. We used an UV-inactivated lysate of *Haemophilus influenzae* to model bacterial exposure. Using an inactivated bacteria may not fully represent a live infection, but using live bacteria *in vitro* can be particularly challenging and complex as discussed previously for the microbiome. We did however use live human rhinovirus 16 (RV16) infection to model viral exposure as inactivated virus does not induce proper antiviral responses in airway epithelial cells (15-17).

Taken together, *in vitro* models are very heterogeneous and should reflect the *in vivo* epithelium as closely as possible. When designing an *in vitro* model of airway epithelial cells there is a factor of complexity to consider, but it should not go at the cost of reproducibility. There is a vast amount of *in vitro* models and culture methods available for airway epithelial cells ranging from cell lines to primary cells cultured submerged or at ALI. Additionally, modeling asthma or COPD can be achieved in various ways. Given the large variety in culture methods, it should be considered to work towards a more robust standardized culture model for airway epithelial cells. Ideally, the model has to be compatible with the research question asked, has to be representative of the *in vivo* epithelium and should be reproducible.

PREDICTING PATIENT OUTCOMES AND THERAPEUTIC RESPONSES USING AIRWAY EPITHELIAL CELL CULTURES: "SCIENCE"-FICTION?

Predicting patient outcomes and therapeutic responses in asthma and COPD patients currently largely relies on lung function analysis, clinical information (e.g. exacerbation frequency) and blood analysis (cell differentials and allergy tests), combined with questionnaires. This current gold standard however fails to adequately classify certain patients that may present with a specific phenotype, suggesting the importance of working towards personalized treatment plans for patients. Using patient-specific biomarker profiles, personalized treatment could become the new gold standard, eventually resulting in better patient care and improved cost effectiveness. Airway epithelial cells are the gatekeepers to the lung, making them ideal informers for underlying tissue inflammation. The importance of airway epithelial cells in asthma and COPD pathogenesis has been outlined previously (18, 19). Additionally, the potential of airway epithelial cells as a source for biomarkers to phenotype asthma patients has been shown previously (11, 12). Interestingly, narrowing and disappearance of small conducting airways has been suggested to occur before the onset of emphysema (20, 21). Given the importance of airway epithelial cells in the early pathogenesis of asthma and COPD, these cells are likely candidates for biomarker profiling in patients. Moreover, predicting patient outcomes at an early stage is important to prevent rapid worsening of disease.

Patient phenotyping using airway epithelial cells has become popular over the recent years with the development of more accurate techniques to evaluate genetic profiles. However, predicting patient disease outcomes using airway epithelial cells has not received a lot of attention until quite recently. A recent study identified genes related to emphysema expressed in airway epithelial cells and their expression was associated with lung function (22). Steiling and colleagues identified COPD-related airway epithelial expression pattern changes associated with COPD and continuous COPD-related measures of lung function (23). Several studies have focused on lung function decline using other measures to investigate airway epithelial disease. Bhatt and colleagues used computed tomography (CT) to evaluate small airway disease. They reported that CT-assessed small airway dysfunction was associated with lung function decline (24). Another group used the impulse oscillometry system to assess small airway disease and suggested that small airway disease progressively increased with both old and new GOLD classifications (25). Together these data suggest that airway epithelial cells may have the potential to predict patient outcomes. Current data

is mainly limited to lung function decline, but it may become important to investigate the potential of airway epithelial cells to predict e.g. exacerbation risks in patients.

Airway epithelial cells can be obtained using e.g. bronchoscopy, and therefore patient-specific evaluation using an appropriate airway epithelial *in vitro* model is feasible. Furthermore, repeated sampling during treatment allows for evaluation of patient responses and therapeutic efficacy. The Groningen and Leiden Universities study of Corticosteroids in Obstructive Lung Disease (GLUCOLD) study showed that gene expression profiles of bronchial biopsies taken from COPD patients before and after corticosteroid treatment reflected therapeutic responses in these patients (26). In another study, airway epithelial signatures of COPD reflected lung tissue expression changes and these signatures were similarly affected following inhaled fluticasone treatment (23). Woodruff and colleagues reported a biomarker of corticosteroid responsiveness using *in vitro* airway epithelial cultures. They also showed that corticosteroid treatment could alter the IL-13-induced Th2-high gene signature *in vitro* which reflected *in vivo* patient responses to inhaled corticosteroids (11, 12). Furthermore, because high serum levels of periostin have been shown to be indicative of positive anti-IL-13 treatment responses clinically (27, 28), it is important to evaluate whether factors influencing periostin expression would alter these treatment responses.

In **chapter 3** we evaluated the effect of the macrolide antibiotic azithromycin on the IL-13-induced Th2-high gene signature *in vitro*. Specifically we showed that azithromycin differentially modulates this gene signature in IL-13 treated cultures, as shown by inhibition of SERPINB2 and CLCA1 expression in the absence of inhibition of POSTN. These findings may have implications for the use of the gene product of the POSTN gene, periostin, as a biomarkers in patients with asthma. However, how this effect is translated clinically remains to be investigated.

In **chapter 4** we extended our knowledge of the Th2-high *in vitro* model and combined it with cigarette smoke, the main contributing environmental exposure to the development of COPD. 20 to 35% of the world population smokes, with surprisingly similar smoking rates reported in asthmatic patients (29-31). Cigarette smoking has been shown to affect asthma patient responsiveness to inhaled corticosteroids (32). We have shown that cigarette smoke differentially affected the IL-13-induced gene signature *in vitro,* whereby POSTN expression was drastically lowered by cigarette smoke exposure and was not recovered upon cigarette smoke cessation. As the presence of the Th2-high gene expression profile has been linked

to sensitivity to inhaled corticosteroid treatment (12), it will be interesting to see how this *in vitro* gene signature may predict *in vivo* patient outcomes. This however remains to be studied and was outside the possibilities of this thesis. Nonetheless, using this Th2 gene signature *in vitro* may allow us to perform preclinical studies to predict *in vivo* effectiveness of existing and new therapeutics.

Current *in vitro* models that accurately predict therapeutic outcomes in patients are lacking, and such models would require extensive validation which thus far has not occurred. In addition, repeated isolation of airway epithelial cells from patients can be challenging and likely more accessible biomarkers are warranted. Blood biomarkers are ideal as they are easily accessible. However, local responses in the lung may not be captured by the blood. Nonetheless, using blood as a window to the lung should be evaluated alongside epithelial signatures to work towards more convenient patient sample retrieval. Alternatively, other sources for biomarkers can be used such as exhaled nitric oxide levels (Fe_{NO}), sputum or even urine. Possibly these aforementioned sources of biomarkers, combined with biomarker profiles of airway epithelial cells, could result in strong, phenotype-specific profiles leading to personalized treatment. Overall, airway epithelial cells cultured *in vitro* have the potential to predict patient outcomes and treatment responses, but they will require extensive validation prior to be used as approved preclinical models.

MOUSE MODELS FOR CHRONIC LUNG DISEASE: REPURPOSING EXISTING MODELS TO IMPROVE PREDICTIVE VALUE?

In vitro cultures are limited in their ability to recapitulate complex interactions between various cell types and tissues observed *in vivo*. To overcome this problem, multiple animal models were developed that recreate certain disease features of chronic lung diseases, including asthma and COPD. *In vivo* mouse models have contributed substantially to our knowledge of underlying disease mechanisms in asthma and COPD, and are essential in the drug discovery process. Despite their widespread use in both academic and industrial research, however, very few compounds that show potential in mouse models of disease have been shown to be successful in clinical studies. The problem is not just the mouse models and understanding how to interpret the findings in these models. Most importantly, poor understanding of the underlying disease mechanisms in human disease hampers proper disease modeling in mice. Also, there is a current lack in funding towards respiratory research, limiting the capacity of research and drug development. Nonetheless, important

differences exist between human and mouse lungs that could attribute to the poor predictive value of current existing mouse models for asthma and COPD.

The basic design of the lung is very similar between mice and human. However, important differences exist in part due to the large differences in body size. The length of the bronchial tree and the total surface area vary greatly between mice and humans. Also, mice are quadrupeds which results in altered physical forces affecting the mouse lung compared to the human lung (e.g. emphysema is generally worse in the lower lobes of COPD patients). Submucosal glands are present in the human airways containing cartilage which reach from the trachea and the intra-lobar airways down to the small bronchioles, whereas in mice the submucosal glands are restricted to only the trachea (33). This particular difference may suggest why mice with the cystic fibrosis-related mutation do not develop the fibrotic lesions in the lung as observed in human cystic fibrosis (34). Additionally, the conducting airways in humans contain basal cells whereas this is not the case in mice, where basal cells are only found in the trachea and main-stem bronchi (33, 35). Instead, mouse airways are lined with a simple epithelium with ciliated and club cells. In these airways, the club cells are mainly responsible for epithelial turnover and repair following injury (36).

Despite some differences between human and mouse lungs, using mice to study asthma and COPD has multiple advantages. Mice are relatively small and inexpensive compared to other available mammalian species to study chronic lung diseases. They are also easy to breed with a short gestational period. There are numerous inbred strains available to work with and importantly, genetic modification and consequent transgenic strains are widespread available. Moreover, the technology to develop transgenic mice is constantly evolving, resulting in more efficient genetic manipulations. Additionally, a large variety of immunological reagents are available, aiding significantly in the analysis of results. Taken together, these combined advantages make mouse models very attractive to discover novel disease mechanisms and to find new therapeutic targets in asthma and COPD.

The predictive value of drugs currently reaching the market for cardiovascular and HIV/AIDS-related research is 6 to 14% respectively, whereas in respiratory research this is only 3% (37). Whereas the problem cannot be fully attributed to poor predictive value of mouse models, it does remain an important point for improvement. Mouse models of asthma are typically induced by either ovalbumin (OVA) combined with an adjuvant or by house dust mite (HDM) extract. Whereas other mouse models for asthma exist, the OVA and HDM are

the most commonly used, whereby a mouse is initially sensitized followed several weeks later by single or multiple challenges to induce features of allergic airway inflammation. An extensive overview and discussion of mouse models of allergic airway disease is available elsewhere (38-41). Mouse models of COPD are most commonly induced by cigarette smoke exposure over a duration of several months to induce features of COPD. Other mouse models of COPD are discussed elsewhere (42, 43). Despite the common use of these mouse models, even with the use of similar exposure compounds, discrepancy between laboratory outcomes exist. This is likely due to differences in e.g. exposure protocols, inbred strains of mice, delivery systems and intensity in exposure. Moreover, these mouse models typically only recapitulate certain features of disease. In allergic airway disease models, airway remodeling is typically limited to subepithelial fibrosis with limited bronchial smooth muscle hyperplasia or hyper-proliferation (41). In COPD models, even after several months of cigarette smoke exposure, disease features are typically mild, unlike the disease severity observed in COPD patients (following decades of smoking).

A major pitfall is that both asthma and COPD are very complex syndromes comprised of multiple clinical phenotypes with different underlying pathologies which we have just started to unravel. Mouse models can only reflect our current knowledge of human disease and can only model a particular phenotype of the disease. Therefore, it is plausible that we should strive towards understanding human disease better before trying to model it *in vivo*. Stratifying patients into clinical phenotypes has shown to improve therapeutic responses to novel treatments. Anti-IL-5 treatment has been shown to have beneficial effects in a subset of asthmatic patients with high numbers of sputum eosinophils (44, 45). Similarly, a subset of asthmatic patients with high serum levels of periostin, indicating a Th2-high phenotype, were found to be more responsive to anti-IL-13 treatment compared to the overall population of asthmatic patients (27, 28). Overall, stratifying patients into clinical phenotypes may improve predictive values of novel therapeutics. Also, understanding the underlying disease mechanisms and working towards patient specific biomarkers becomes particularly important, especially with an industry working towards personalized medicine.

Improved understanding of the mechanisms underlying asthma and COPD and the clinically observed phenotypes will allow us to better understand the existing mouse models of chronic lung disease. As multiple clinical phenotypes exist for both diseases, it is conceivable that the various mouse models of asthma and COPD could be classified to reflect specific phenotypes, rather than the disease as a whole. It is well accepted that the OVA mouse

model of allergic airway disease is mainly driven by a Th2 phenotype, whereas the HDM mouse model is a more mixed response of Th2 and Th17-mediated disease (46, 47). For mouse models of COPD, the distinction of different phenotypes is not as obvious. However, the commonly used elastase mouse model of COPD is likely more reflective of the genetic form of COPD whereby a mutation in the alpha-1 antitrypsin gene causes unrestricted activity of neutrophil-derived elastase (48). Nonetheless, whereas classifying mouse models into specific phenotypes may improve their predictive value, it should also be noted that the existing models could be improved to reflect human disease more closely.

Both asthma and COPD are complex syndromes influenced by both genetics, lifestyle and environment. Single stimuli to induce features of disease in mouse models likely do not reflect the complex interactions observed in human disease. Therefore, mouse models of asthma and COPD should perhaps also include lifestyle effects on these conditions, such as high fat diet or physical activity. Likewise, environmental factors such as air pollution could also be considered. Exacerbations have also been modeled in mouse models of both asthma and COPD (49-51), although differences in pathogenicity of microbes between mice and humans should be taken into consideration. Additionally, it is important to consider that mice are kept in a clean and controlled environment, which is in stark contrast to human lifestyle. Another point to consider is that typical readout parameters used in mice studies are inflammation and remodeling, whereas in the clinic, changes in lung function are typically used. Various groups have started to include lung function data of mice studies, but it should always be considered that mouse lung physiology does not always reflect human lung physiology.

Reassigning mouse models to better reflect human disease phenotypes could improve their predictive value. Whereas there are genomic differences between mice and humans, the underlying molecular pathways and disease mechanisms are likely to be similar, particularly if specific disease phenotypes are considered. Given the current standing of *in vitro* models, *in vivo* models and patient-based studies, more effort should be put into finding translational links in those disease mechanisms and underlying molecular pathways using e.g. bioinformatics or –omics data. Changes in molecular signatures or pathways associated with functional and physiological data could further enhance the predictive value of mouse models of asthma and COPD. Another important feature of mouse models of chronic lung disease is that mouse airway epithelial cells can be isolated at different time points to study disease progression and perhaps how certain phenotypes develop over time. The

pseudostratified mouse tracheal epithelium contains basal cells, which are lacking in the lower airways of mice. Therefore, mouse tracheal epithelial cells give the best representation of the human pseudostratified airway epithelium. A major setback in isolating these cells however, is that current techniques to isolate mouse tracheal epithelial cells are particularly inefficient.

In **chapter 6** we developed a new culturing technique to expand and differentiate mouse tracheal epithelial cells. Expanding mouse tracheal epithelial cells *in vitro* for subsequent ALI differentiation allows a much more efficient use of animals, which is particularly interesting when mouse tracheal epithelial cells are isolated from difficult-to-breed mouse strains. Also, our culture method can decrease technical and biological variation between experiments and will ultimately lead to a reduction in experimental animal use and related costs. Furthermore, human tissue can be difficult to obtain and genetic modification of primary human bronchial and tracheal epithelial cells has proven to be very difficult (52). Mouse tracheal epithelial cells isolated from disease models or transgenic animals can offer a good alternative for primary human airway epithelial cells. Furthermore, since studies in patients with COPD and asthma have shown that some epithelial features are maintained in culture, mouse tracheal epithelial culture models can help to provide insight into the (epigenetic) mechanisms that may explain the persistence of disease specific features of epithelial cells in culture when cells are no longer present in the tissue of origin.

Overall, repurposing and improving existing mouse models should improve their predictive value. However, more effort should be put into understanding clinical phenotypes underlying human disease and how mouse models may correlate with these phenotypes. Therefore, combining data from *in vitro* models using airway epithelial cells with data from *in vivo* models, focusing on specific phenotypes and translating these results towards the clinic may overall improve respiratory research towards asthma and COPD.

CONCLUSIONS

The studies presented in this thesis were aimed at developing and using *in vitro* models that could benefit research towards understanding asthma and COPD. We used an *in vitro* model representing a Th2-high gene signature and studied how this gene signature may be affected by external factors such as cigarette smoke or drugs. Using these *in vitro* airway epithelial cell models may help to predict clinical outcomes, although they will require

extensive validation. We also investigated the possibility of using primary human airway epithelial cells to model bacterial and viral exacerbations. Whereas this model is currently still under investigation, it could be particularly useful to study possible biomarkers of exacerbations and how these may be affected by external factors. Additionally, we also developed a new method to expand and differentiate mouse tracheal epithelial cells *in vitro*. Whereas these cells do not fully represent human cultures, they have the major advantage that these cells can be isolated during a time course to investigate disease progression. Additionally, transgenic animals can be used to study the influence of particular genes on disease mechanisms. Overall, studying airway epithelial cells may provide important clues for understanding disease pathogenesis, lead to identification of new treatment targets, and may provide important biomarkers. Using airway epithelial cells and their derived biomarkers could significantly improve our understanding in disease phenotypes of asthma and COPD. Additionally, with increasing knowledge of the disease phenotypes, we could better address the unmet need in treatment of asthma and COPD.

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ADDENDUM

NEDERLANDSTALIGE SAMENVATTING

DUTCH SUMMARY

Astma en COPD (Chronic Obstructive Pulmonary Disease of chronisch obstructieve longziekte) zijn beide chronische longaandoeningen die gekenmerkt worden door ontsteking en obstructie van de luchtwegen. Bij astma patiënten is deze obstructie vaak te behandelen, maar bij COPD patiënten is dit vaak onomkeerbaar. Allergisch astma, de meest voorkomende en best gekende vorm van astma, wordt omschreven als een T-helper 2 (Th2)-gedreven aandoening met voornamelijk eosinofiele infiltratie, terwijl bij ontsteking in COPD patiënten voornamelijk CD8 cellen, B cellen, macrofagen en neutrofielen betrokken zijn. Traditioneel worden deze twee aandoeningen, astma en COPD, omschreven als twee verschillende entiteiten. De laatste jaren is het echter duidelijk geworden dat deze aandoeningen heel erg heterogeen zijn. Deze heterogeniteit suggereert dat verschillende moleculaire mechanismen bijdragen aan de variëteit aan ziektebeelden die in de kliniek worden aangetroffen. Het ontrafelen van de onderliggende moleculaire mechanismen kan ons helpen om betere kennis te vergaren om vervolgens de verschillende vormen van astma en COPD beter te kunnen behandelen.

Het slecht functioneren van het luchtweg epitheel, de bekleding van de luchtwegen, is een belangrijk kenmerk van zowel astma als COPD. Het luchtweg epitheel is vervolgens een goede kandidaat voor het moleculair ontrafelen van de verschillende klinische ziektebeelden die we momenteel moeilijk kunnen behandelen. Het luchtweg epitheel vormt een barrière tussen de ingeademde lucht en het onderliggend weefsel, een ideale locatie om aan te kunnen geven wat de effecten zijn van omgevingsblootstellingen en wat er gaande is in het onderliggend weefsel. Daarnaast bevindt het luchtweg epitheel zich op een relatief toegankelijke locatie om een biopt te nemen. Het verzamelen en analyseren van deze biopten is niet alleen een bewezen efficiënte methode om cruciale informatie te verzamelen die nodig is voor het stellen van een diagnose, maar in wetenschappelijk onderzoek ook zeer geschikt om moleculaire mechanismen te ontrafelen die ten grondslag liggen aan specifieke ziektebeelden. Daarnaast kunnen biopten die op verschillende tijdspunten bij een patiënt zijn verzameld een beeld geven over therapeutische effectiviteit in patiënten. Ook kunnen epitheelcellen geïsoleerd worden van luchtweg biopten of van tijdens een operatie verwijderd longweefsel (longresectie materiaal). Vervolgens kunnen we deze cellen opkweken *in vitro* om moleculaire mechanismen te bestuderen. Daarnaast kunnen deze cellen *in vitro* gebruikt worden om therapeutische effecten te bestuderen op bestaande of geïnduceerde ziektebeelden in de epitheelcellen.

Diermodellen hebben een belangrijke bijdrage geleverd aan onze kennis over chronische

longaandoeningen, maar de huidige diermodellen zijn maar beperkt geschikt voor het voorspellen van therapeutische effecten. Hierdoor is het essentieel dat we van andere preklinische modellen gebruik maken om moleculaire mechanismen te ontrafelen. Deze preklinische modellen zullen uitgebreid geoptimaliseerd en gevalideerd moeten worden, maar ze kunnen enorm bijdragen aan het ontrafelen van de ziektebeelden en het ontdekken van nieuwe therapeutische middelen. Een nadeel van gekweekt luchtweg epitheel dat rechtstreeks is verkregen uit longweefsel, is dat we deze cellen moeilijk genetisch kunnen veranderen door b.v. bepaalde genen uit te schakelen. Dit probleem kunnen we echter omzeilen door gebruik te maken van luchtweg epitheel dat is geïsoleerd uit genetisch gemodificeerde muizen. De huidige technieken laten echter nog niet toe om deze cellen efficiënt te kweken.

De studies in dit proefschrift hebben gebruik gemaakt van *in vitro* modellen om moleculaire mechanismen te ontrafelen die betrokken zijn bij astma en COPD. Geoptimaliseerde kweek methoden en een betere kennis van de onderliggende ziekte mechanismen kunnen helpen in de ontwikkeling van gerichte behandelingen voor deze veel voorkomende chronische longaandoeningen.

In hoofdstuk 2 wordt een uitgebreid literatuur overzicht gegeven over de eigenschappen van luchtweg epitheel in astma en COPD en hoe deze nagebootst kunnen worden gebruik makend van *in vitro* onderzoek met celkweken. Het luchtweg epitheel bestaat uit verschillende types epitheelcellen die elk een eigen specifieke functie hebben, noodzakelijk voor het normaal functioneren van het luchtweg epitheel. Deze verschillende types epitheelcellen kunnen we nabootsen *in vitro* als we deze cellen opkweken op een speciaal membraan dat toelaat dat de cellen aan de bovenkant blootgesteld worden aan lucht, terwijl ze langs de onderkant nutriënten krijgen die zijn opgelost in kweek medium. Dit is in tegenstelling tot het grote merendeel van *in vitro* kweken waarbij de cellen op de bodem van een plastic plaat gekweekt worden, ondergedompeld in een laag kweek medium. Deze klassieke manier van kweken laat de epitheelcellen niet toe om de specifieke celtypes te ontwikkelen die we zien in normaal luchtweg epitheel. Veel eigenschappen van luchtweg epitheel van astma en COPD patiënten kunnen behouden blijven na isolatie van epitheelcellen uit patiënten en het vervolgens opkweken hiervan. Als alternatief kunnen we ook kenmerken van astma en COPD nabootsen door epitheelcellen *in vitro* bloot te stellen aan stoffen die vaak gerelateerd worden aan chronische longaandoeningen zoals interleukin-13 (IL-13) bij astma, en sigaretten rook bij COPD. Tegenwoordig zijn er ook meer complexe varianten van *in vitro*

kweekmodellen beschikbaar waarbij we de omgeving van de epitheelcellen zo veel mogelijk proberen na te bootsten zoals deze in de long is. Dit zijn bijvoorbeeld *in vitro* kweken waarbij meerdere celtypes zoals bijvoorbeeld fibroblasten, endotheelcellen, macrofagen of gladde spiercellen aanwezig zijn in combinatie met de epitheel cellen. Daarnaast bestaan *in vitro* modellen waarbij ook rekening wordt gehouden met de opmaak van de longen zodat we de long als orgaan beter kunnen nabootsen. Deze nieuwe ontwikkelingen kunnen ons helpen bij het beter begrijpen van de complexe omgevingsinteracties met het longweefsel die bijdragen aan de ontwikkeling en progressie van chronische longaandoeningen.

Zoals eerder aangegeven is allergisch astma de meest voorkomende en best gekende vorm van astma. Deze vorm van astma wordt omschreven als een T-helper 2 (Th2)-gedreven aandoening. IL-13 is een belangrijk Th2 cytokine dat een centrale rol speelt bij allergische astma. Een aantal jaar geleden is door middel van genetische analyse van verschillende patiënten met allergisch astma, een specifieke signatuur van genen ontdekt die kenmerkend is voor de aanwezigheid van allergisch astma in patiënten. Deze specifieke signatuur, ook wel Th2-signatuur genoemd, is aanwezig in luchtweg epitheel en kan nagebootst worden *in vitro* door de epitheelcellen bloot te stellen aan IL-13. In hoofdstuk 3 hebben we gebruik gemaakt van deze *in vitro* opstelling om te kijken of deze Th2-signatuur gevoelig is voor de behandeling van macrolide antibiotica. Zo is het aangetoond dat azitromycine, een macrolide antibioticum, niet effectief is allergisch astma, maar wel in een an dere vorm van astme, neutrofiel astma. Onze resultaten gaven aan dat azitromycine een differentieel effect had op de Th2-signatuur, waarbij de expressie van een aantal genen onderdrukt werden terwijl de expressie een ander gen extra werd opgedreven. De gevolgen van deze differentiële effecten moeten nog onderzocht worden, maar dit kan wel een mogelijke indicatie zijn waarom azitromycine effectief is in neutrofiele astma in tegenstelling tot allergische astma.

Een aantal astma patiënten rookt waardoor ze vaak ongevoelig worden voor de standaard behandeling inhalatie steroïden. Daarnaast kan sigarettenrook de symptomen van astma verergeren, zorgen voor een snellere afname van de longfunctie en verhoogt het de kans op longaanvallen (exacerbaties). Omdat we in hoofdstuk 3 hebben aangetoond dat de Th2-signatuur gevoelig kan zijn voor behandeling, hebben we vervolgens in hoofdstuk 4 gekeken hoe sigaretten rook de Th2-signatuur kan beïnvloeden. Hiervoor hebben we gebruik gemaakt van de *in vitro* kweken waarbij we de cellen blootstellen aan lucht, in combinatie met IL-13 en sigaretten rook die we door middel van een gespecialiseerde opstelling genereren om een zo relevant mogelijke blootstelling na te bootsen. Ook hier zien we dat sigarettenrook een

differentieel effect heeft op de IL-13-geinduceerde Th2-signatuur. Daarnaast zagen we ook dat de Th2-signatuur niet herstelt als we de sigarettenrook blootstelling stoppen voor een aantal dagen. Aangezien de Th2-signatuur een indicatie is voor de gevoeligheid van allergisch astma patiënten voor inhalatie steroïden, kan dit mogelijk helpen om te verklaren waarom rokende astma patiënten ongevoelig kunnen worden voor deze standaard behandeling.

Een acute longaanval in COPD patiënten wordt gekenmerkt door een aanhoudende verslechtering van de patiënt, die vaak tot een ziekenhuisopname leidt. Zowel bacteriële als virale infecties hebben een belangrijke bijdrage aan het induceren van deze longaanvallen. Het luchtweg epitheel vormt een mechanische barrière voor geïnhaleerde stofdeeltjes en microben, maar het is ook in staat om een immuun reactie uit te lokken die belangrijk is bij het uiteindelijk verwijderen van de schadelijke deeltjes en microben. Het is eerder aangetoond dat sigarettenrook de luchtwegen gevoeliger kan maken voor infecties. Om beter te begrijpen hoe sigarettenrook dit effect tot stand brengt hebben we in hoofdstuk 5 onderzocht hoe sigarettenrook een infectie van epitheelcellen *in vitro* beïnvloed. Daarnaast hebben we ook onderzocht of epitheelcellen geïsoleerd uit COPD patiënten anders reageren op gecombineerde blootstelling aan sigarettenrook en microben dan epitheel cellen uit patiënten zonder COPD. Onze resultaten gaven aan dat sigarettenrook een differentieel effect heeft op virale en bacteriële immuun reacties van de epitheelcellen. Daarnaast hebben epitheelcellen van COPD patiënten een verminderde antivirale reactie dan epitheelcellen van patiënten zonder COPD, wat suggereert dat tijdens de ontwikkeling van COPD epigenetische veranderingen optreden in het luchtweg epitheel. Een verminderde antivirale reactie kan er vervolgens toe leiden dat de patiënt meer vatbaar is voor virale infecties.

Humaan luchtweg epitheel is niet altijd beschikbaar voor onderzoek. Daarnaast staan epitheelcellen ervoor bekend dat ze moeilijk genetisch te veranderen zijn wat onderzoek, gebruik makend van deze cellen, vaak lastig maakt. Een alternatief hiervoor is het tracheaal epitheel van een muis dat grote overeenkomsten heeft met het luchtweg epitheel dat we aantreffen in humane longen. Indien we de epitheel cellen uit een trachea van een transgene muis isoleren, dan voorkomen we de noodzaak om de cellen *in vitro* genetisch the moeten manipuleren. Een belangrijk probleem hierbij is echter dat de huidige protocollen om deze cellen te isoleren heel erg inefficiënt zijn. Zo zijn tientallen muizen nodig voor een enkel "*in vitro*" experiment met muis tracheaal epitheel om voldoende cellen te hebben. In hoofdstuk 6 is er een nieuwe kweekmethode ontwikkeld waarmee je met slechts twee muizen meerder

experimenten kan uitvoeren. Door middel van aangepaste kweekmedia waren we in staat om de geïsoleerde cellen *in vitro* te expanderen, wat voorheen niet mogelijk was, alvorens deze te gebruiken voor experimenten.

Tot slot wordt er in hoofdstuk 7 een bredere context gegeven, gebaseerd op de voorgaande hoofdstukken. Deze discussie is opgedeeld in drie onderdelen. Als eerste is besproken hoe *in vitro* modellen opgesteld kunnen worden en waarmee er rekening gehouden moet worden. *In vitro* modellen kunnen een uiterst complexe opmaak hebben, maar de keerzijde hiervan is dat deze modellen zeer moeilijk te reproduceren zijn. Bijgevolg is het belangrijk om de gulden middenweg te vinden tussen complexiteit en reproduceerbaarheid om vervolgens de resultaten in een duidelijke verband te kunnen plaatsen. Vervolgens is er in de algemene discussie besproken of het al dan niet mogelijk is om patiënt resultaten en responsen op therapeutische middelen te voorspellen gebruik makend van *in vitro* kweken van luchtweg epitheel. Hoewel dit op termijn mogelijkheid zou kunnen bieden om patiënten beter te karakteriseren om vervolgens gepersonaliseerde medicatie te geven, is het noodzakelijk dat we astma en COPD eerst beter begrijpen. Tot slot wordt er in het laatste onderdeel dieper in gegaan op de huidige muis modellen voor chronische longaandoeningen, en hoe we deze anders kunnen indelen om ze een beter voorspellend vermogen te geven. De huidige muismodellen voor chronische longaandoeningen zoals astma en COPD hebben een slecht voorspellende waarde voor de effectiviteit van nieuwe therapeutische middelen. Hoewel muis en humane longen sterk van elkaar verschillen, is een belangrijk probleem dat we astma en COPD nog steeds niet helemaal begrijpen. Daarnaast zijn zowel astma als COPD heel heterogene aandoeningen die moeilijk te reproduceren zijn in muismodellen. Bijgevolg is het belangrijk dat we de bestaande muis modellen toespitsen aan specifieke ziektebeelden binnen astma en COPD in tegenstelling tot ze onder een gezamenlijk gedeelde noemer plaatsten.

De onderzoeken in dit proefschrift waren toegewijd aan het ontwikkelen en toepassen van *in vitro* modellen die kunnen bijdragen aan onze kennis omtrent astma en COPD. Er is gebruik gemaakt van een *in vitro* model waarin een epitheliale Th2-signatuur kan worden nagebootst, en waarmee kan worden onderzocht hoe deze signatuur beïnvloed kan worden door externe factoren zoals medicijnen en sigarettenrook. Hoewel deze modellen kunnen helpen bij het voorspellen van klinische responsen, moeten ze nog wel extensief gevalideerd worden. Daarnaast hebben we ook gekeken naar de mogelijkheid om acute exacerbaties *in vitro* te modelleren. Dit model zou uiteindelijk belangrijk kunnen zijn voor

de identificatie en het bestuderen van biomarkers voor exacerbaties, en hoe deze beïnvloed kunnen worden door externe factoren. Ook hebben we een *in vitro* model geoptimaliseerd dat toelaat om efficiënt muis tracheaal epitheel te kweken. Hoewel deze *in vitro* modellen geen volledig beeld geven van de humane modellen, hebben ze wel het voordeel dat deze uit transgene dieren geïsoleerd kunnen worden. Daarnaast draagt de ontwikkeling van dit muis kweekmodel bij aan een reductie in het aantal dierproeven vanwege de efficiënte wijze waarop epitheel kan worden geïsoleerd en gekweekt. Ook kunnen pilot experimenten in het muismodel onnodige proefdierexperimenten voorkomen. Tenslotte kan door een rechtstreekse vergelijking van het muis en het humane kweekmodel een betere vertaling worden gemaakt van proeven verkregen in muismodellen naar de patiënt.

Het bestuderen van luchtweg epitheel kan ons belangrijke aanwijzingen geven voor het beter begrijpen van ziekte mechanismen, onderliggend aan chronische longaandoeningen. Dit kan uiteindelijk leiden tot de ontwikkeling van nieuwe therapeutische middelen, maar ook van nieuwe biomarker profielen. Het gebruik van luchtweg epitheel en hun biomarkers kan significant bijdragen aan onze kennis over de verschillende vormen van astma en COPD.

ADDENDUM

CURRICULUM VITAE
PUBLICATIES

CURRICULUM VITAE

Tinne C.J. Mertens is geboren op 30 april 1986 te Turnhout in België. Na haar middelbare school opleiding aan het Sint-Victor te Turnhout heeft zij een jaar in Brazilië doorgebracht als uitwisselingsstudente. In 2009 behaalde zij haar bachelor in de biomedische wetenschappen gevolgd door haar master in 2011 aan Universiteit Hasselt in Diepenbeek. In 2012 begon zij met haar onderzoek naar in vitro modellen voor het bestuderen van astma en COPD en de effecten van IL-13 en sigaretten rook op luchtweg epitheel, onder begeleiding van Prof. dr. Pieter S. Hiemstra en Prof. dr. Christian Taube in het Leids Universitair Medisch Centrum. Het resultaat van dit onderzoek is gebundeld in dit proefschrift. In 2016 is zij begonnen aan haar postdoctoraal onderzoek aan de *University of Texas Health Science Center at Houston* in Houston, Texas, onder begeleiding van dr. Harry Karmouty-Quintana. Hierbij is haar onderzoek gericht op pulmonale hypertensie in patiënten met chronische long aandoeningen.

PUBLICATIES

Azithromycin differentially affects modulation of airway epithelial gene expression by IL-13. ***Tinne C.J. Mertens***, Pieter S. Hiemstra, Christian Taube. Pulm Pharmacol Ther, 2016 Aug; 39:14-20.

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