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Airway epithelial innate host defence in chronic obstructive pulmonary disease

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AIRWAY EPITHELIAL INNATE HOST DEFENCE IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

GIMANO DANANG AMATNGALIM

Colophon

Airway epithelial innate host defence in chronic obstructive pulmonary disease
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Thesis Leiden University Medical Center

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AIRWAY EPITHELIAL INNATE HOST DEFENCE IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

PROEFSCHRIFT

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de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,
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geboren te Leidschendam in 1987

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"*DON'T SMOKE. I DID. WISH I NEVER HAD.*

LIVE LONG AND PROSPER."

LEONARD NIMOY (SPOCK, STAR TREK)

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CHAPTER 1

General Introduction: Airway epithelial cell function and respiratory host defense in chronic obstructive pulmonary disease (COPD).

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GENERAL INTRODUCTION

Our lungs have a vital role in mediating the exchange of oxygen and carbon dioxide between the air we breathe in and the body. This function is under constant pressure as inhaled air contains numerous particles, gasses and micro-organisms that may cause injury and infection to the lungs. Removal and neutralization of potential harmful substances from inhaled air is mediated by the airway epithelium. This pseudo-stratified layer of cells covers the surface of the conducting airways and plays an important role in protecting the alveoli, where gas exchange takes place, from injury. The airway epithelium has a range of properties that contribute to lung defense, including constitutive host defense mechanisms and regulation of airway innate immunity. Moreover, epithelial cells display wound healing properties, which allow rapid recovery of airway tissues upon injury. Airway epithelial host defense functions are important to maintain proper gas exchange and lung homeostasis. Despite this protective function, extensive epithelial exposures to noxious particles and gasses may have detrimental outcomes. This is seen in chronic obstructive pulmonary disease (COPD), in which an impaired epithelial function and epithelial remodeling caused by smoking contributes to an accelerated decline in lung function. COPD is characterized by increased colonization and infections with opportunistic respiratory pathogens, which is caused in part by impaired epithelial host defense functions. However, the molecular and cellular mechanisms that are affected in the airway epithelium by smoking and that may lead to COPD are largely unclear.

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

COPD is a severe inflammatory lung disease, regarded as one of the most prevalent burdens in global health (1). The disease has been ranked retrospectively in the top 10 causes of mortality in high-income countries between 1990 and 2013 (2), and COPD is predicted as the 3th cause of death and leading lung disease worldwide by 2030 (3). COPD is characterized by a progressive and largely irreversible decline in lung function. This is associated with long-term airway exposures to cytotoxic particles and gasses resulting in an abnormal response to inhalation of these substances (3, 4). Airflow limitation in COPD is accompanied by persistent inflammation, airway remodeling and destruction of lung tissue, resulting in clinical symptoms such as dyspnea, chronic cough, and fatigue. Moreover, COPD patients may suffer from comorbidities, such as cardiovascular disease, that contribute to disease severity and mortality (5, 6). In addition to a progressive decline in lung function in stable COPD, acute worsening in lung function may occur during disease exacerbations. Microbial infections are in most cases the trigger of these exacerbations. Furthermore, it has been shown that exacerbations are also associated with gastro-esophageal reflux and heartburn and that patients with frequent exacerbations are more susceptible for recurrent exacerbations (7).

COPD is a heterogenic disease in which airflow limitation may result from several mechanisms in which different, or multiple regions, of the respiratory tract are affected (8). Chronic bronchitis and small airway disease are characterized by remodeling and obstruction of the large and small conducting airways respectively. In contrast, emphysema is characterized by destruction of the alveoli located in the peripheral lung tissue resulting in airflow limitation, air trapping and a loss of diffusion capacity. Despite this heterogeneity, COPD

is in general associated with the exposure of the lung to cytotoxic particles and gasses that promote persistent inflammatory responses and induce lung tissue remodeling and damage (3). Exposure to biomass, occupational dusts, and chemicals are all cytotoxic insults that are associated with COPD development and progression (9-11). However, smoking is regarded as the main risk factor that is associated with the disease in Westernized societies. COPD can be largely prevented by non-smoking and it has been shown that smoking cessation decreases symptoms and to some extent may normalize the decline in lung function in smokers and patients with mild disease severity (12). Regardless of the significance of smoking in COPD pathogenesis, not all smokers develop the disease. Therefore, it is assumed that smokers can be divided into a susceptible and non-susceptible group which are likely defined based on additional risk factors such as genetic predisposition, (micro)nutrient deficiencies, environmental and lifestyle factors, and early life abnormalities in lung function (13-16).

MICROBIAL COLONIZATION AND RESPIRATORY INFECTIONS IN COPD

Microbial colonization and infections are an important pathophysiological aspect in certain COPD patients. Based on traditional culture-based techniques, it was shown that clinically stable COPD patients were colonized with opportunistic respiratory pathogens, most notably non-typeable *Haemophilus influenzae* (17-20). Colonization with respiratory pathogens were furthermore associated with elevated levels of inflammatory markers in upper- and lower airways fluid samples(18, 19, 21, 22). This suggests a role of microbial colonization in airway inflammation in COPD patients. Recent understanding of the presence of complex lung microbial communities (the lung microbiome), has further supported a role for microbial colonization in COPD pathogenesis. Compared to healthy individuals, it has been shown in various studies (23, 24) that COPD patients have altered microbiomes in the upper and lower airways, which are characterized by a less divers microbial composition. In line with culture-based studies, respiratory pathogens such as *Haemophilus* spp. were observed more frequently in the airway microbiome of COPD patients (25, 26). In addition, the COPD airway microbiome is characterized by the absence of microbes that are common in healthy individuals (25, 27). These promising findings suggest that imbalances in the microbiome - or dysbiosis - is a hallmark of COPD. Besides colonization in stable COPD, acute bacterial or viral infections are associated with approximately 50% of disease exacerbations. In particular, acquisition of new bacterial strains is assumed to cause acute worsening of patient symptoms (28). Also recent studies suggest alterations in the airway micobiome during COPD exacerbations, which are characterized by an increase in airway pathogens (29, 30). Overall, these observational studies highlight the importance of a better understanding of the role microbial colonization and infections in COPD pathogenesis.

The underlying mechanism linking smoking with microbial colonization and infections in COPD can be explained by the vicious circle hypothesis (23). According to this hypothesis, smoking stimulates the development and progression of COPD by initiating a vicious circle of airway injury, microbial colonization/infections and inflammation (Figure 1). Cigarette smoke exposure of airway tissues induces damage, which promotes local inflammatory responses and impairs host defense. Microbes further amplify airway inflammatory responses, whereas chronic inflammation contributes to tissue damage and degenerative repair. The persistence

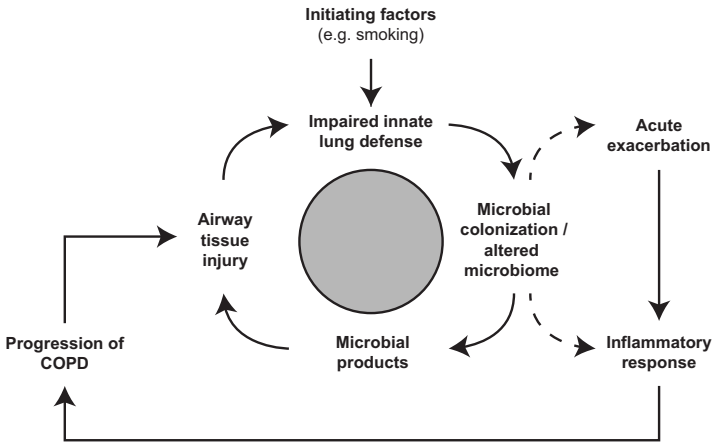

Figure 1. Vicious circle hypothesis of COPD. Model that explains the development and progression of COPD, focusing on a persistent cycle of microbial colonization and infections, inflammation and airway tissue injury. Adapted from: Mammen & Sethi, 2016 (23).

of this vicious circle due to repetitive smoking eventually modulates tissue repair and leads to remodeling of the airways, thereby causing progressive airflow obstruction. In line with this, endogenous lung tissue repair may be impaired in COPD as demonstrated by decreased nuclear β-catenin staining in emphysematous lung tissue (31).

THE AIRWAY EPITHELIUM

The airway epithelium is the first target of inhaled cigarette smoke. Furthermore, epithelial cells are the first defense lining of the respiratory tract that prevents microbial colonization and infections (32-34). Because the airway epithelium is also the first tissue to be exposed to inhaled toxicants such as those present in cigarette smoke, the airway epithelium has a central role in the vicious circle hypothesis, and alterations in host defense and epithelial remodeling may contribute to COPD development and progression.

The airway epithelium is a continuous layer that covers the surface of the respiratory tract and consists of cells that are connected by adhesion- and tight junctions (35-37). Two morphological and functional distinct types of epithelium are located respectively at the conductive airways and respiratory units in the lung peripheral tissue (Figure 2A). The conductive airways starts at the nasal cavity and ends at the small bronchioles in the lower airways. In these regions, the epithelium facilitates the moistening and warming of inhaled air before reaching the alveoli in the respiratory units where gas exchange takes place. The airway epithelium of the conductive airways furthermore has an active role in protecting the lungs against inhaled micro-organisms, which in a large extent is based on the morphology and composition of the epithelium.

In contrast to the simple columnar and cuboidal lining of the bronchioles and alveoli, the epithelium of the large conducting airways is characterized by a pseudostratified morphology (35, 38). Based on this morphology, epithelial cells can be divided into luminal cells (LCs), which are in direct contact with the environment, and basal cells (BCs) that are superimposed by LCs and located above the basement membrane (Figure 2B). The main cell types that make up the LC population are the ciliated cells and the secretory cells, which include the club cells and the mucus-producing goblet cells, and are discussed in the next paragraph. LCs and BCs have distinct functions in airway host defense, which depend on the degree of microbial threat and also whether the epithelial layer is intact or damaged. Based on this, airway epithelial host defenses can be categorized into 1) constitutive host defense mechanisms by LCs, 2) inducible

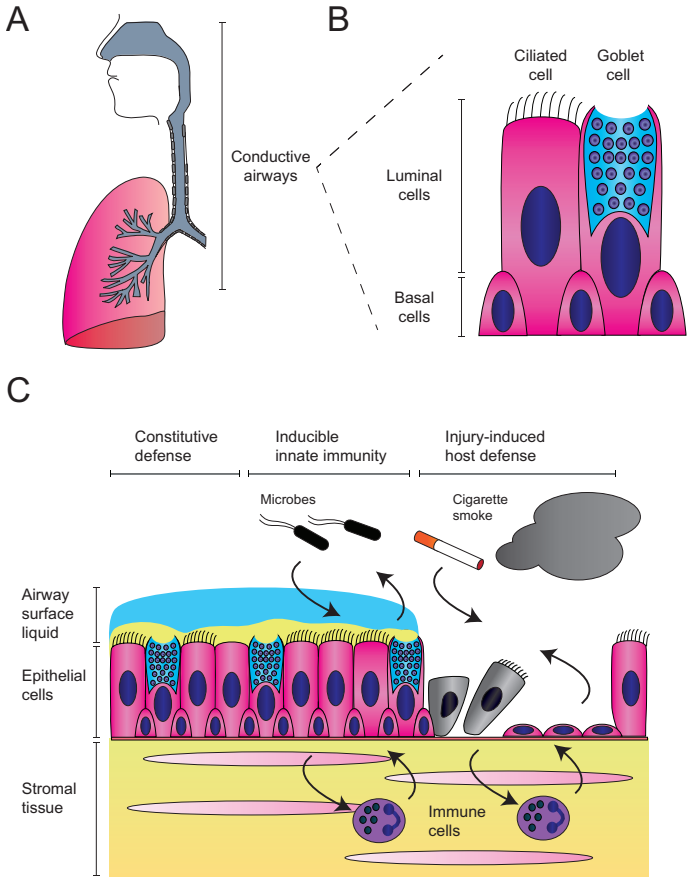

Figure 2. Schematic presentation of the airway epithelium. (A) The human respiratory tract, with the conductive airways highlighted in gray. (B) Composition of the pseudostratified airway epithelium, consisting of ciliated, secretory cells (i.e. goblet and club cells located in the upper and lower airways respectively), and basal cells. (C) Airway epithelial host defense mechanisms include: Constitutive host defense mechanisms, inducible innate immunity, activated for instance by microbes, and injury-induced host defense mechanisms, activated for instance by cigarette smoking. Both inducible innate immunity and injury-induced host defense mechanisms, lead to the chemo-attraction and interaction with immune cells.

innate immunity, and 3) injury-induced wound repair and defense by airway BCs (Figure 2C). In addition, the airway epithelium plays a central role in instructing adaptive immunity by interacting with dendritic cells and innate lymphoid cells.

CONSTITUTIVE LUMINAL CELL HOST DEFENSES

Constitutive epithelial host defense mechanisms are defined as those functions mediated by intact airway epithelium at baseline, homeostatic conditions (Figure 3A). This includes the physical barrier functions of connected epithelial cells, but also active mechanisms mediated by LCs that are directly exposed to environmental insults. LCs comprise mature high columnar cells with specialized functions. Ciliated cells are an abundant luminal cell type and are characterized by their multi-ciliated structures at the apical surface (35, 38). Moreover, the luminal epithelium includes specialized secretory cells, i.e. goblet and club cells, which are distinctively located in respectively the large and small airways (39). The constitutive defense of LCs dependents on the interaction between ciliated and secretory cells in regulating the fluid lining located at the epithelial surface. This airway surface liquid (ASL) consists of a mixture of host defense proteins and peptides that are secreted by the airway epithelium and immune cells (40-43). This mixture provides a chemical shield against micro-organisms and is responsible for the relatively low levels of microbes in the respiratory tract of healthy individuals. Antimicrobial proteins and peptides (AMPs) present in this ASL prevent microbial colonization and infections by displaying direct microbial killing activity or by reducing the availability of important micronutrients (44, 45). Another host defense mechanism is mediated by secreted gel-forming mucins, present in the ASL as discontinuing floating strands or rafts (46, 47). These mucins can entrap micro-organisms and large particles and are subsequently removed via mucociliary clearance. During this process, mucus is propelled from the airways towards the throat by the continuous ciliary beating of ciliated cells (48). MUC5B and MUC5AC, the main mucins of the mucus gel, are mainly produced by the goblet cells of the surface epithelium and by the submucosal glands (47). Moreover, it has been reported that club cells are able to produce MUC5B in the lower airways (49). The luminal airway epithelium and mucin gel are separated by a second constitutive defense lining that is formed by host defense mucins, tethered to the surface of the epithelium and in complexes with the glycosaminoglycan keratin sulfate (50). These complexes are mainly located at epithelial cilia and are assumed to shape a periciliary brush which creates an additional barrier that prevents penetration of particles and micro-organisms (51). LCs furthermore regulate the physiological conditions of the ASL. This is mediated by active ion transport, for instance by the cystic fibrosis transmembrane conductance regulator (CFTR) protein or calcium-activated chloride channels such as anoctamin-1 (ANO-1/TMEM16A) (52, 53). Chloride secretion and reabsorption of sodium by the epithelial sodium channel (ENaC), have been shown to regulate ASL volume (54). This has important consequences for mucociliary clearance as it determines the hydration state of the mucus gel, as well as the height of the periciliary layer which is an important determinant of ciliary movement (55). Moreover, transport of bicarbonate regulates the pH of the ASL (56), which may affect the activity of pH-sensitive AMPs and mucus viscosity (57, 58).

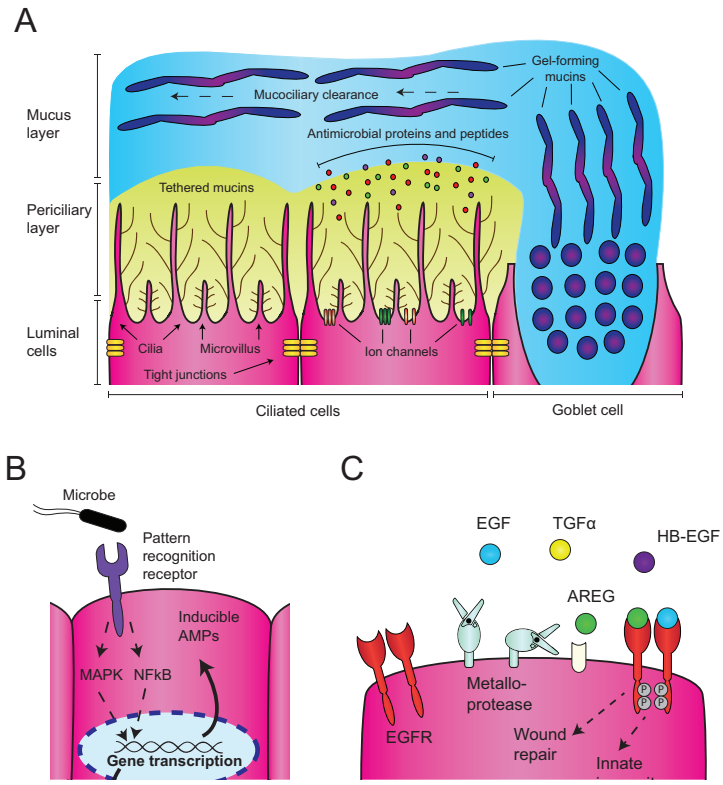

Figure 3. Airway epithelial host defense. (A) Constitutive host defense mechanisms of the luminal airway epithelium including barrier function, a cellular and tethered mucin barrier, defense via mucociliary clearance and secreted antimicrobial proteins and peptides, and regulation of airway surface liquid physiological properties though ion transport channels. (B) Inducible innate immunity can be activated upon recognition of microbes by epithelial pattern recognition receptors, which activate signaling pathways, i.e. MAPK and NFκB, which promote the expression of inducible AMPs and pro-inflammatory mediators. (C) Epithelial injury results in activation of EGFR located on basal cells, via various EGFR-ligands (i.e. EGF, TGFα, HB-EGF, AREG) produced in an autocrine manner, by luminal cells, stromal cells, or immune cells. The release of EGF-ligands is in part mediated via shedded by matrix-metalloproteases. EGFR activation subsequently promotes wound repair and innate immune responses.

INDUCIBLE INNATE IMMUNITY

Constitutive host defense mechanisms provided by LCs give baseline protection during relatively low microbial exposures. Evasion from host defense mechanisms or adaptation to the host micro-environment may allow microbial outgrowth, thereby overwhelming constitutive airway epithelial defense (59). Therefore, secondary host defense mechanisms are activated upon sensing of increased levels of microbes (Figure 3B) (60). This depends on recognition of microbes by host cell receptors, which is highly conserved between species. It was first observed in Drosophila that microbial recognition of the receptor toll, resulted in the expression of AMPs (61). Similar to Drosophila, human toll-like receptors (TLRs) are present at the surface of airway epithelial cells or located in membrane enclosed compartments (62). Moreover, other patter recognition receptors (PRRs), such as NOD-like receptors, MDA5

and RIG-1 are located in the cell cytosol (63). Ligation of PRRs leads to activation of cellular signaling transduction pathways such as MAPK and NFkB (64). This subsequently leads to expression of AMPs that are not produced at baseline conditions or only at very low levels. These "inducible" AMPs increase the antimicrobial activity of the ASL, counteracting the increased levels of microbes (65, 66). In addition to AMPs, activation of downstream signaling pathways leads to epithelial expression of pro-inflammatory cytokines and chemokines (67). These factors increase the attraction of immune cells to the site where increased microbial exposure is detected. Initially innate immune cells, such as dendritic cells, macrophages and neutrophils are directed to the epithelium, but in later stages also adaptive immune cells such as T- and B-lymphocytes are attracted. In addition to activation of the inducible innate immune system by microbes, airway epithelial are furthermore activated by the attracted innate and adaptive immune cells, which produce cytokines such as IL-1β and TNF-α (68). Moreover, airway epithelial cells display an autocrine mechanism, in which expression of the pro-inflammatory cytokine IL-17C leads to maintained innate immune defense mechanisms (69, 70). Moreover, the micronutrient vitamin D can also induce antibacterial responses, in part via expression of the antimicrobial peptide LL-37 (71). Taken together, inducible secondary host defense mechanisms are increased in the epithelium upon microbial exposure, during inflammation, during repair processes (discussed in the next paragraph) and upon exposure to vitamin D, and thereby provide protection upon outgrowth of microbes in the airways.

INJURY-INDUCED INNATE DEFENSE BY AIRWAY BASAL CELLS

The importance of maintaining an intact airway epithelium is emphasized by the low epithelial turn-over at steady-state levels (72, 73). However, exposure to cytotoxic particles and micro-organisms may cause epithelial injury, leading to shedding and cell death of LCs (38). Shedding of LCs provides defense by removal of infected cells (74). Moreover, epithelial death induced by injury or infection leads to the release of damage-associated molecular patterns, which can activate the innate immune system (75). Nevertheless, elimination of LCs compromises epithelial host defense. In this case, airway epithelial BCs play a role in providing airway protection (Figure 3C). BCs comprises approx. 30% of the airway epithelium in the large conductive airways, whereas its numbers decline at distal regions of the conductive airways (72). The cells are largely quiescent in intact epithelium. However, upon epithelial injury, BCs contribute to epithelial host defense by mediating recovery of the epithelial lining (76). Initially BCs spread and migrate on denuded basement membranes, followed by proliferation and differentiation towards mature LCs. A central role in the activation of epithelial repair involves activation of the epidermal growth factor receptor (EGFR) (77). This Erb family member is restricted to BCs and is activated by various ligands, including epidermal growth factor, amphiregulin and transforming growth factor-alpha (78-80). These ligands are produced and secreted by stromal cells or immune cells, however EGFR is also activated in an autocrine manner. This occurs for instance through release of EGF located at the surface of damaged luminal airway epithelial cells, but also via shedding of membrane-bound EGFR-ligands by matrix metalloproteases (77). In all cases, activation of EGFR leads to initiation of wound repair, particularly controlled by MAPK signaling transduction and downstream AP-1 family transcription factors. In addition, BCs contribute to airway innate immunity upon activation of PRRs (81). Moreover, EGFR activates innate immune responses

by promoting the expression of pro-inflammatory factors that lead to chemo-attraction of immune cells to the site of injury as well as epithelial expression of AMPs. High expression of integrins, and the cell type restricted expression of ICAM-1 allows homing of immune cells to BCs, which may provide protection against microbes at the site of injury (82, 83). Moreover, innate immune mediators produced by immune cells may increase wound repair or direct the differentiation of LCs (84, 85).

AIRWAY EPITHELIAL CELL CULTURES

Our understanding of airway epithelial cell biology large depends on basic research using cell culture models. These models furthermore are a helpful tool to understand epithelial cell responses to stimuli related to chronic inflammatory airway diseases or examine and compare cell cultures from diseased patients and control subjects (86).

Epithelial cells in conventional 2D submerged cultures are characterized by monolayers which lack differentiated luminal cells and display a basal cell phenotype (Figure 4A) (82). These undifferentiated airway epithelial cells have been used to study wound repair processes such as cell migration and proliferation (74). Moreover, studies examining the effect of pro-inflammatory stimuli have demonstrated innate immune activities of undifferentiated airway BCs (87, 88). Indeed, undifferentiated cells do not fully recapitulate the function of the differentiated epithelium because of the lack of specialized LCs. Therefore other cell culture approaches are required to study epithelial cell function.
The air-liquid interface (ALI) culture model is a well-established method to recapitulate the

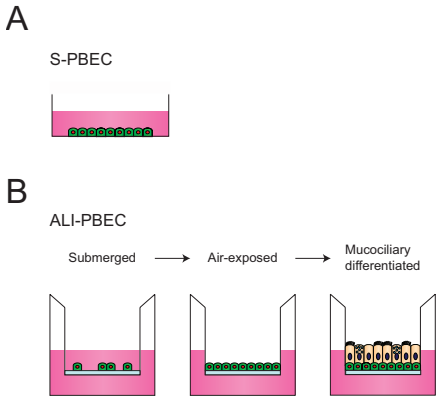

Figure 4. Airway epithelial cell cultures.
(A) Submerged cultured primary bronchial epithelial cells (S-PBEC) display an undifferentiated phenotype and resemble airway basal progenitor cells. (B) Culturing of undifferentiated cells on transwells at the air-liquid interface allows differentiation towards a mucociliary phenotype.

mucociliary phenotype of airway epithelial cells *in vitro* (Figure 4B) (89, 90). In this model, undifferentiated cells are seeded on semi-permeable transwell membrane supports, which are coated with an extracellular matrix substrate, i.e. collagen and/or fibronectin. The cells are initially cultured in submerged conditions to obtain confluent monolayers. Removal of the culture medium at the apical surface and further culturing under air-exposed conditions results in the development of tight junctions, which prevents leakage of basolateral medium to the apical compartment and results in the development of an epithelial barrier. Using

culture medium containing serum substitutes, or using a semi-defined culture medium including retinoic acid, air-exposed epithelial cells can differentiate in approx. 2-4 weeks towards a mature epithelium that includes ciliated and secretory cells (91). Differentiated ALI-cultures have been shown to display similar functional properties as the epithelium *in vivo*. This includes epithelial host defense mechanism, such as antimicrobial activity, mucin production, mucociliary transport, and ion transport (54, 92, 93). Moreover, epithelial cells stimulated with microbes or pro-inflammatory cytokines display innate immune properties, such as production of AMPs, cytokines and chemokines (70, 93).

EFFECT OF CIGARETTE SMOKE AND COPD DISEASE STATUS ON AIRWAY EPITHELIAL HOST DEFENSE

Both undifferentiated airway epithelial cells and differentiated ALI-cultures can be used to increase our understanding of how the epithelium is affected in COPD. This can be done for instance by studying the effect of cigarette smoke on cell cultures. In particular, aqueous solutions of cigarette smoke particles, i.e. extract or condensate, have been used to study this (94, 95). However, this approach primarily takes the effects of the soluble particulate phase of cigarette smoke into account and underestimates the effect of the vapor phase and especially that of short-lived oxidants (96). Therefore, instead of the conventional method of using an aqueous extract of cigarette smoke, we have set up a whole cigarette smoke exposure model (Figure 5) (97). In this model, epithelial cells are directly exposed to the particulate and vapor phase by leading smoke derived from a burning cigarette directly to the cells that are grown at the air-liquid interface. This allows the exposure of cells to airborne substances in a physiologically realistic fashion. Previous studies using a comparable exposure model have shown that cigarette smoke inhibits the antimicrobial activity of airway epithelial cells (98). These results suggest that further application of the whole cigarette smoke exposure model will give insight into how other airway epithelial cell host defense functions are affected by smoking.

Although smoking is regarded as the primary risk factor of COPD, not all smokers develop the disease (4). Therefore, it can be speculated that epithelial cells from COPD patients and non-COPD smokers display differences in host defense properties that may explain disease development. Recent studies have suggested that differences in airway epithelial activities persist in cell culture, such as an impaired airway epithelial barrier integrity, reduced wound repair and alterations in cell differentiation (99-102). Based on this, we hypothesize that persistent differences are present in other airway epithelial host defense properties of COPD patients and non-COPD controls.

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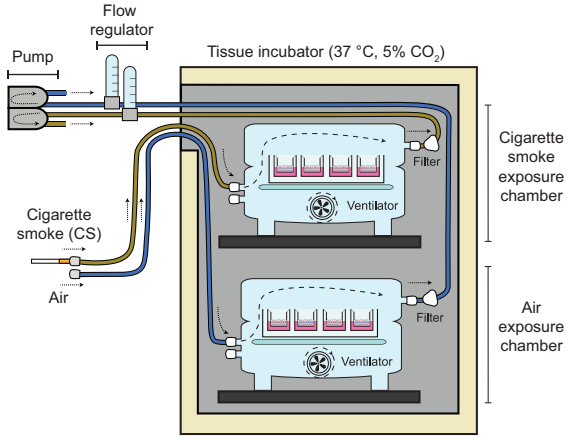

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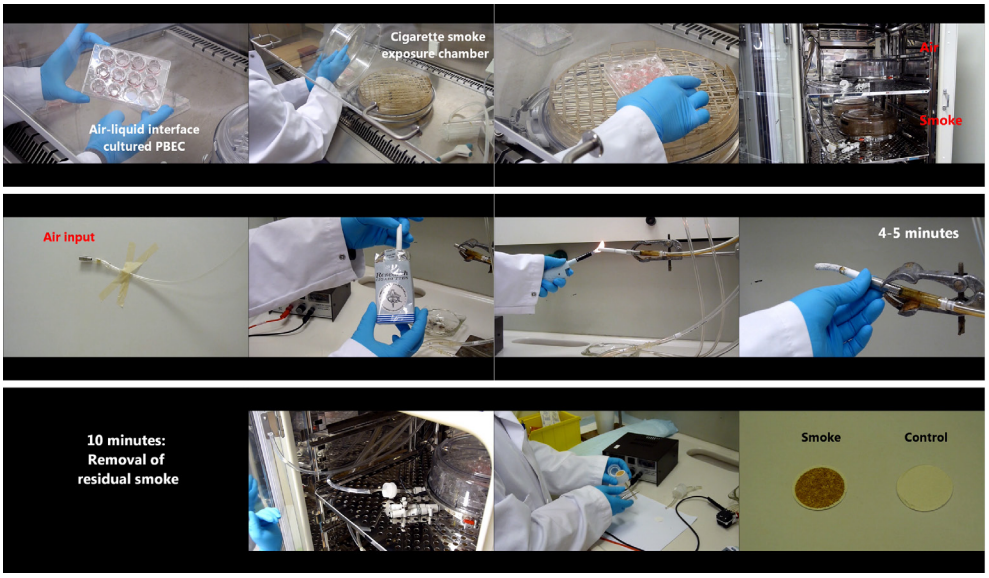

Figure 5. Whole cigarette smoke exposure model. (A) Illustration of the whole cigarette smoke exposure model setup. (B) In this model, epithelial cells are placed in modified hypoxic chambers in a tissue culture incubator at 37°C and 5% CO_2. During exposure, whole cigarette smoke (CS) derived from a Kentucky research cigarette is infused into the exposure chamber. This is mediated by a pump, which drives the flow of smoke using a continuous regulated flow. Simultaneously, cells are exposed in a separate chamber to room air, as negative control. The amount of exposed cigarette smoke is demonstrated by the deposition of particles on a filter located between the extracting pump and exposure chamber.

OUTLINE OF THE THESIS

In this thesis, studies are presented in which the impact of cigarette smoke exposure and COPD disease status on the innate host defense functions of the airway epithelium are explored. This was done by using cell culture experiments in which the effect of cigarette smoke was examined, or in which epithelial cultures of COPD patients and non-COPD (ex) smokers were compared. Antimicrobial proteins and peptides (AMPs) are a major contributor to airway epithelial host defense, and therefore a literature overview is given in **Chapter 2** on the potential role of AMPs in COPD pathogenesis. **Chapter 3** describes how microbial exposure and cigarette smoke induced injury increases expression of the antimicrobial protein ribonuclease 7 (RNase7), specifically in airway epithelial BCs. **Chapter 4** describes work in which the effect of cigarette smoke is studied on microbial-induced antibacterial activity of airway epithelial cells. Moreover, in this chapter the antibacterial activity, and expression of AMPs is studied in differentiated airway epithelial cells from COPD patients and non-COPD controls. In **Chapter 5** it is described how expression of constitutively expressed innate defense proteins is restricted to luminal airway epithelial cells, and how chronic cigarette smoke exposure impairs epithelial defense by affecting cell differentiation. In **Chapter 6** we examined the effects of cigarette smoke on wound repair and induction of RNase 7 by basal cells and how smoke-induced oxidative stress differentially affects host defense properties of the epithelium. **Chapter 7** describes the influence of cigarette smoke-induced oxidative stress on regulation of the cytoprotective cellular mechanism known as the integrated stress response. **Chapter 8** discusses work in which the effect of cigarette smoke was examined on COPD and non-COPD airway epithelial shedding of the IL-6 receptor and amphiregulin by the matrix metalloprotease ADAM17. **Chapter 9** describes the influence of COPD related risk factors on the expression of the host defence protein WFDC12, which is dynamically regulated in an epithelial cell differentiation dependent manner. In **Chapter 10**, discussion on the therapeutic potential of targeting AMPs in infectious and non-infectious lung diseases is presented. Finally, **Chapter 11** provides a summary and discussion of the studies presented in this thesis.

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CHAPTER 2

Antimicrobial Peptides in Chronic Obstructive Pulmonary Disease.

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a frequent, chronic lung disease associated with significant morbidity and mortality. Respiratory infections play a central role in the disease, not only during exacerbations but also in the stable phase of the disease. These infections contribute to the development and progression of the disease, and many patients are colonized by respiratory pathogens. The pathogens are present in the lung, despite the presence of large numbers of neutrophils, especially during acute states of inflammation. These neutrophils may release antimicrobial peptides (AMPs) that may not only serve to kill these pathogens but also contribute to tissue injury and inflammation. In addition, smoke affects many elements of the host immune system, including the expression of epithelial AMPs. Furthermore, the activity of AMPs may be decreased in the purulent airway secretions often present in COPD patients. Possibly vitamin D treatment may contribute to restoring local AMP deficiency and thereby to reducing exacerbations in COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a frequent respiratory tract disorder characterized by persistent airflow limitation (1). It is mainly caused by extensive exposure and an abnormal response toward harmful environmental substances and gases, most prominently, the exposure to cigarette smoke. Only ~20 % of the smoking population develops the disease, indicating the additional importance of genetics and other environmental predispositions. The progressive decrease in lung function in COPD is caused by airflow obstruction resulting from a variety of structural changes in the lung, including destruction of the alveoli and alveolar attachments (emphysema), mucus hypersecretion, and subsequent airway plugging especially in small airways, and changes in the airway wall. These structural changes are accompanied by a chronic inflammatory process in which a variety of cell types play a role. Small airway pathology is characterized by increased accumulation of neutrophils, macrophages, and T-cells during disease progression and the occurrence of B-cell lymphoid follicles in severe disease stages (2, 3). Neutrophils are regarded as one of the driving forces of emphysema, causing excessive tissue damage by an imbalance between neutrophil-derived proteases and protease inhibitors. This imbalance is also observed in genetically predisposed patients with alpha-1 antitrypsin deficiency development (4), a condition associated with liver disease and early onset emphysema.

RESPIRATORY INFECTIONS IN COPD

Chronic inflammation in stable COPD is frequently accompanied by bacterial and/or viral infections (5, 6). These infections contribute to persistence of airway inflammation and are thought to contribute to the etiology, pathogenesis, and clinical course of COPD. During acute exacerbations of COPD, a sudden decrease in airflow and an accompanying increase in airway inflammation is associated with the acquisition of new bacterial strains, most notable non-typeable *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis*, and *Streptococcus pneumoniae* (7). Interactions between bacterial and viral pathogens may contribute to the intensity of exacerbations. This is illustrated by a study from Wilkinson *et al.*, who showed that simultaneous detection of bacterial (mostly NTHi) and viral (mostly rhinovirus) pathogens during an exacerbation is associated with an increased bacterial load, inflammation, and symptoms and with decreased lung function (8). It is now clear that infections not only contribute to increased inflammation during exacerbations but also in the stable phase of the disease. This is illustrated by a study by Bresser *et al.*, showing that NTHi colonization may contribute to airway inflammation and airflow obstruction in COPD (9). Various mechanisms may explain the observed bacterial persistence in COPD, including antigenic variation, acquisition of new strains, tissue invasion, and biofilm formation. The observation that COPD patients are frequently unable to eradicate bacteria from their airways despite the presence of antibacterial antibodies and the abundant presence of neutrophils suggests that tissue invasion and biofilm formation are important processes. Adherence to airway epithelial cells is an important process in bacterial persistence. NTHi may penetrate between epithelial cells (10) and even into epithelial cells (11), which may protect them from host defense systems in the lung as well as from antibiotics. This protection from host immunity and antibiotics is also achieved by formation of biofilms, in which bacterial adhesion is the first

essential step (12). Biofilms are microbial communities that adhere to a surface and in which the microorganisms are embedded in a self-produced matrix. Importantly, microorganisms present in biofilms are not always readily detected by conventional culture techniques. Therefore, molecular techniques including microbiome sequencing are important to gain insight into the lung microbiome in COPD. Several recent studies have used such techniques and provided important insight in the microbial population composition in COPD. Hilty *et al.* demonstrated in bronchial brushings and bronchoalveolar lavage (BAL) specimens of COPD patients an increase in *Proteobacteria*, mainly *Haemophilus*, *Moraxella*, and *Neisseria* species, and a decrease in the phylum *Bacteroidetes* compared to healthy controls (13). Erb-Downward *et al.* furthermore showed in severe COPD that the diversity of the microbiome is reduced (14). These initial findings show some differences with other studies which may have been caused by differences in study design, sampling techniques, and small sample sizes (15). Nevertheless, the overall conclusions are the same, showing that the lung microbiome in COPD differs from that in healthy subjects and smokers with normal lung function. The COPD patient lung microbiome also differs from the microbiome in patients with cystic fibrosis. Further microbiome studies in COPD are needed to explore a wide range of topics, including changes in the composition of the microbiome over time, its relation to disease severity and response to therapy, the influence of antibiotic therapy on the microbiome compositions, its role in development and progression of the disease, as well as regional heterogeneity.

ANTIMICROBIAL PEPTIDES IN COPD

The prominent role of respiratory infections despite intense inflammation in the COPD lung suggests that the pulmonary immune system does not function optimally in COPD. Antimicrobial peptides (AMPs) play a central role in host defense against infection in the lung (16), which is also supported by, e.g., *in vivo* mouse studies showing that gene deletion or overexpression of AMP genes affects pulmonary host defense against NTHi (17) and *Pseudomonas aeruginosa* (18). Based on this role of AMPs, several studies have investigated expression and activity of AMPs in COPD in search for an explanation for the increased susceptibility to infection and increased inflammation in COPD.

Neutrophil-derived antimicrobial peptides

The excessive number of neutrophils in the lung consequently leads to high detectable quantities of neutrophil-derived AMPs. A-defensins (human neutrophil peptides; HNP) are abundantly present in sputum samples of COPD patients and found elevated in more severe disease stages compared to mild-to-moderate COPD(19). Analysis of sputum and bronchoalveolar lavage fluid (BAL) from COPD patients using a proteomic approach specifically demonstrated an increase of HNP1 and HNP2 levels, while HNP3 levels were similar to those of healthy controls (20, 21). In addition, also in α-1 antitrypsin-deficient patients, higher levels of HNPs were observed (22, 23). Similar to HNPs, concentrations of the cathelicidin antimicrobial peptide LL-37 are elevated in induced sputum samples of mild-to-very severe COPD patients (24). Compared to nonsmokers, LL-37 was already increased to some extent in smokers with a normal lung function, an observation not noticed for HNPs (21). This suggests that cigarette smoking plays an important role in increasing LL-37 levels in the airway. Moreover, a study examining the relation of LL-37 with bacterial colonization during acute exacerbations

revealed that increased levels of LL-37 in sputum samples correlated with the acquisition of NTHi and *M. catarrhalis* (25). The usually protective role of neutrophils in host defense against pathogens may be dysfunctional in COPD. High levels of neutrophil-derived HNPs and LL-37 are suggested to contribute to the chronic inflammatory state (Figure 1) (26), and HNPs were found to increase bacterial adherence to epithelial cells (27). The combined release of AMPs with reactive oxygen species and other neutrophil granule–derived proteins, such as cathepsin G, elastase, and S100 proteins, causes extensive tissue damage that contributes

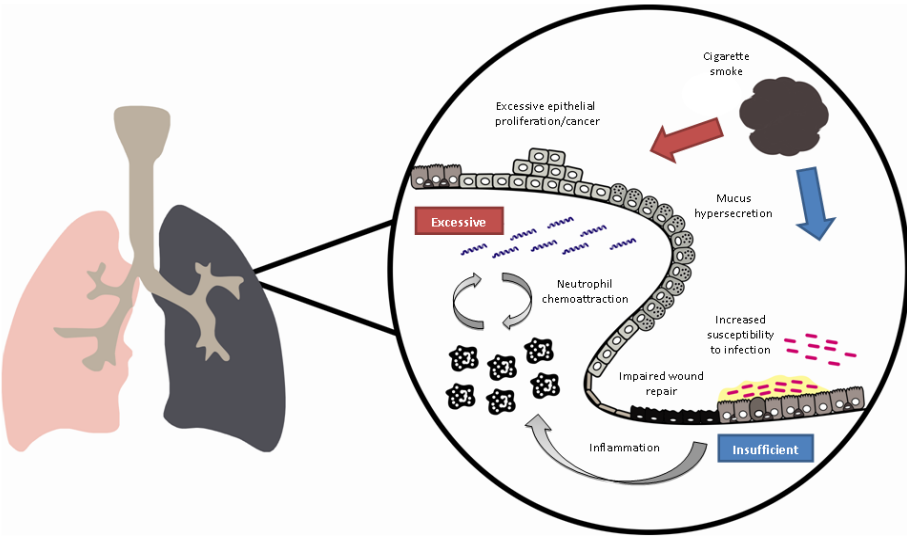

Figure 1. Dysregulation of antimicrobial peptide expression and activity by cigarette smoke. Cigarette smoke-induced chemoattraction of neutrophils causes excessive levels of neutrophil-derived AMPs in the lung, which may contribute to airway epithelial remodeling (including proliferation and mucus hypersecretion) and possibly carcinoma development, and further enhancement of neutrophilic inflammation. This mechanism may be increased by posttranslational modification of AMPs such as HNPs and LL-37, which may be increased in smokers and alter the activity of these peptides. Cigarette smoke furthermore not only impairs expression of AMPs by airway epithelial cells exposed to microbial stimuli but also decreases ciliary activity and increases mucus hypersecretion. These mechanisms contribute to an increased susceptibility to respiratory tract infections and may impair proper wound healing. Moreover, these respiratory infections lead to a further increase in airway inflammation.

to airway remodeling and the maintenance of inflammation (28). The release of neutrophil extracellular traps (NETs), consisting of complexes of DNA including high levels of LL-37 and HNPs, has been shown to contribute to autoimmune diseases via the development of autoantibodies (29). However, although various studies have provided evidence for an autoimmune response in COPD [e.g. (30), it is unclear whether autoimmunity contributes to COPD development and progression or may be a response to tissue injury that in itself is not pathogenic. In vitro experimental data support the increased cytotoxic activity and

immunomodulatory properties of HNPs and LL-37 at high concentrations. Exposure of airway epithelial cells to HNPs may result in cell death, whereas lower concentrations increase the secretion of pro-inflammatory cytokines, including the neutrophil chemoattractant IL-8 (31, 32). Furthermore, HNPs also increase the transcription and secretion of the mucin MUC5AC by an airway epithelial cell line (33, 34). This finding indicates a potential role in promoting mucus hypersecretion. A further role in decreased mucociliary clearance was suggested by the observation of an association between increased HNP levels and squamous metaplastic epithelium (35). This finding combined with the mitogenic activity of neutrophil defensins toward airway epithelial cells (33, 36) suggests a possible role in airway epithelial remodeling. HNPs can also contribute to the increased bacterial colonization in acute exacerbations as it has been demonstrated that the peptides increase the adhesion of NTHi, *M. catarrhalis*, and *S. pneumoniae* toward the airway epithelial surface (27, 37). Adhesion may be the first step in biofilm formation, or facilitate intra- or intercellular localization, mechanisms that may provide protection against the host immune system. Similar to HNPs, LL-37 can contribute to the increased neutrophilic infiltration in the lung by inducing the expression of IL-8 by airway epithelial cells and airway smooth muscle cells (38, 39) or via a direct chemotactic activity (40). Furthermore, it has been shown that LL-37 can drive macrophage differentiation toward a pro-inflammatory phenotype, thereby potentially increasing the inflammatory state in COPD (41). Similar to HNPs, it has been shown that LL-37 also increases airway epithelial cell proliferation (42), which may contribute to epithelial remodeling. The switch in function from antimicrobial effectors toward harmful mediators is not a unique property of HNPs and LL-37 at high concentrations. Several studies report that cigarette smoke-related posttranslational modifications of both AMPs may affect their function. The converting enzyme responsible for ADP-ribosylation of HNP1, arginine-specific ADP-ribosyltransferase 1, is increased in smoking individuals, and levels of ADP-ribosylated HNP1 are increased in BAL fluid from smokers 43. This may have important functional consequences, since ADP-ribosylation of HNP1 decreases the antimicrobial effect of the peptide, while increasing the cytotoxic and IL-8 inducing properties (43, 44). Recently, citrullination was described as a novel mechanism for posttranslational modification of LL-37 that may also be increased in smokers, and it was demonstrated that citrullination of LL-37 decreases antimicrobial activity and increases chemotactic activity (45). Citrullination of proteins is mediated by members of the peptidylarginine deiminase (PADI) family and expression of PADI2, and presence of citrullinated proteins was found to be increased in the lungs of smokers (45, 46). This points toward a potential mechanism by which modification of HNPs and LL-37 alters the activity of these peptides, which may contribute to the development and progression of COPD.

Epithelial expression of antimicrobial peptides

In contrast to HNPs and LL-37, levels of human β-defensin-2 (hBD-2) are decreased in induced sputum samples and BAL of COPD patients (47). Furthermore, Herr *et al.* found an association between decreased hBD-2 levels and cigarette smoking in patients hospitalized with an acute pneumonia (48). The cigarette smoke–mediated suppression of hBD-2 expression seems to be persistent, as 1 year smoking cessation did not result in an increase in hBD-2 sputum levels in asymptomatic smokers (49). In COPD patients, hBD-2 expression is predominantly decreased in the central airways, while in the distal airways the expression was increased compared to controls (50). A previous report showed that hBD-2

depletion in BAL supernatants increased the number of apoptotic neutrophils, suggesting a role of hBD-2 in protection of cells from apoptosis (51). In contrast to HNPs, hBD-2 does not induce IL-8 expression in airway epithelial cells (52), so it remains to be further investigated if hBD-2 contributes to inflammation in COPD. In vitro antibacterial assays demonstrate efficient killing activity of hBD-2 toward acute exacerbation-associated bacteria (53). This suggests that the observed suppression of hBD-2 expression in COPD in central airways contributes to the increased bacterial colonization during acute exacerbations. As the airway epithelium is regarded as the main cellular source of hBD-2 in the airways (54), it is hypothesized that airway epithelial cells display an impaired host defense activity (Figure. 1). *In vitro* experiments demonstrate that prior cigarette smoke exposure of air–liquid interface-cultured airway epithelial cells inhibits *P. aeruginosa*- and *M. catarrhalis*-induced expression of hBD-2 (48, 55). Furthermore, it was shown that apical surface fluid derived from these cells displayed decreased antimicrobial activity. Stimulation of epithelial cells with the cigarette smoke–derived compound acrolein similarly showed an inhibition of hBD-2 expression (56). In contrast to *in vitro* experiments, cigarette smoke exposure in murine models showed increased mouse β-defensin-2 expression in the lung (57), which is not in line with the findings in these *in vitro* studies, and observations in central airways and airway secretions in human studies (47, 48, 50). Recent studies have highlighted a role for vitamin D in the regulation of expression of antimicrobial peptides in epithelial cells and macrophages (58, 59). Furthermore, vitamin D deficiency is frequent in COPD and correlates with disease activity (60). This would suggest that vitamin D supplementation may be beneficial in COPD and may be a good strategy to prevent exacerbations that are so frequently associated with infections (61). A recent intervention study showed that high-dose vitamin D supplementation may indeed reduce exacerbations in those COPD patients with severe vitamin D deficiency (62).

Genetic and Epigenetic Mechanisms

Several studies have investigated the association between genetic and epigenetic differences of AMPs with COPD development. Both α- and β-defensin-coding genes are localized at highly polymorphic regions (63). Genetic association studies assessing the role of single nucleotide polymorphisms (SNPs) in the gene encoding human β-defensin-1 (hBD-1) with COPD development revealed population-dependent outcomes. The hBD-1 gene contains polymorphisms in both the promoter region and the two coding exons (64). In a Japanese population study, the nucleotide polymorphism in exon 2, resulting in a change of valine to isoleucine at position 38, was more frequent in COPD patients compared to controls (65). A study in a Chinese Han population describes an association between a polymorphism localized at exon 1 (44 C/G) with COPD susceptibility (66). In Caucasians, an association between SNPs in the hBD-1 gene and COPD could not be found (67, 68). In addition, also polymorphisms in HNP1/3 were not associated with COPD development in such populations (67). This indicates that the relation between polymorphisms and disease may differ between populations and that further studies on the functional consequences of these polymorphisms are needed. Janssens *et al.* examined the association of copy-number variations of the hBD-2 gene with COPD development (69). Using *in vitro* cultured epithelial cells, it was shown in this study that five and higher diploid copy numbers of the hBD-2 gene was significantly more often present in COPD patients compared to controls. Moreover, it was shown that epithelial cells with high diploid copy number displayed a higher expression of hBD-2 induced by TNF-α and furthermore have a higher bacterial killing activity. These results are

in contrast with earlier mentioned observations of a decrease in hBD-2 levels in BAL and induced sputum and inhibition of hBD-2 expression by airway epithelial cells after cigarette smoke exposure. Therefore, the additional effects of environmental factors on the induced expression of hBD-2 in COPD patients with high copy numbers of the hBD-2 gene should be taken into consideration. Andresen *et al.* investigated the role of epigenetics in hBD-1 expression in COPD (70). Using airway epithelium and cells derived from BAL, it was shown that mRNA levels of hBD-1 were higher in cells of COPD patients with mild to very severe disease compared to cells of healthy controls. The difference in mRNA levels was not due to a difference in DNA methylation of the hBD-1 gene promoter, but rather correlated with histone H3 lysine 4 methylation. These studies highlight that copy-number variations and epigenetic mechanisms may contribute to the control of expression levels of antimicrobial peptides in COPD.

Activity of Antimicrobial Peptides in COPD

During airway inflammation and infection, the local environment in which AMPs are active undergoes dramatic changes. Increased production of mucus as a result of smoking, inflammation, and infection may impact on local host defense against infection. Whereas the mucus layer that is positioned on top of the periciliary layer (Figure. 2) normally acts to trap and remove inhaled particles and pathogens, decreased mucociliary clearance in COPD

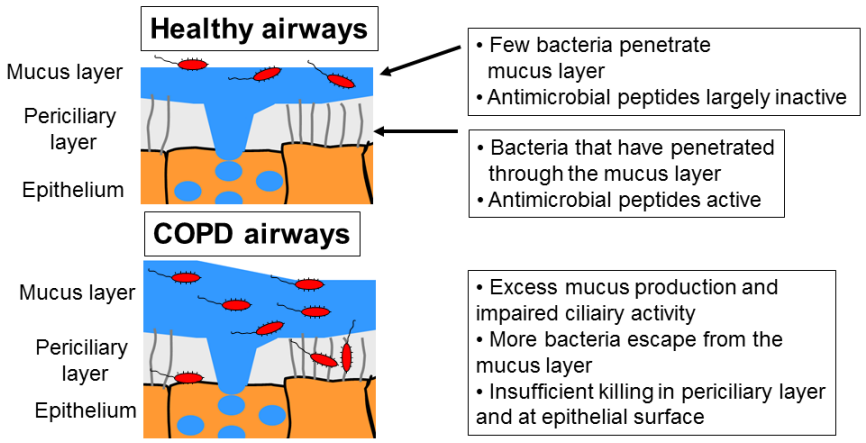

Figure 2. Mucus, antimicrobial peptides, and microbial pathogens. In COPD, mucus hypersecretion and decreased mucociliary clearance may allow bacteria to escape from the mucus layer in numbers that are too large for efficient killing in the periciliary layer and epithelial surface

prevents this process. Mucus itself does display antimicrobial activity because of its barrier and biochemical properties, but microorganisms have developed escape mechanisms such as flagella-mediated motility and enzymatic degradation of mucus (71). Mucins are large, heavily glycosylated glycoproteins that are essential components of mucus. Mucins have been shown to restrict the antimicrobial activity of LL-37 (72, 73), and therefore, these peptides may not contribute optimally to antimicrobial activity of mucus. In healthy airways, this is not

a problem, since mucus is removed. Furthermore, the bacteria that escape from the mucus layer are likely to be killed by antimicrobial peptides present in the periciliary layer (Figure. 2). However, because of excess mucus production and impaired ciliary activity, in COPD more bacteria may penetrate the mucus layer and reach the epithelial surfaces. However, also other factors in the inflamed airways of COPD patients may have a negative impact on the antimicrobial activity of AMPs. It has been shown that a wide range of microbial and host proteases are able to degrade and inactivate AMPs (74-76). In addition, products such as F-actin and DNA (77) that are released by dead cells, glycosaminoglycans (78, 79), and bacterial polysaccharides (80) may inhibit antimicrobial activity of AMPs. Finally, bacteria have evolved mechanisms to escape the antimicrobial activity of peptides. One such mechanism is the development of biofilms that provide protection against the action of the host immune system. Interestingly, bacteria in biofilms may not be fully protected against the action of antimicrobial peptides, since, e.g., LL-37 (81) and lactoferrin (82) have been shown to prevent biofilm formation and to act on bacteria present in biofilms.

CONCLUDING REMARKS

Recent studies point to a clear role of respiratory infection and AMPs in COPD. Microbiome analysis using unbiased molecular biological methods is still in its infancy but is pointing toward an altered microbiome also in stable COPD. Local excessive release of AMPs by, e.g., neutrophils may contribute to inflammation and possibly autoimmunity, whereas a local deficiency as a result of epithelial smoke exposure or inactivation of AMPs may contribute to respiratory infections. Biofilm formation impairs host defense against infection, but some AMPs may contribute to the fight against biofilm formation. Whether enhancement of local AMP production by, e.g., vitamin D treatment or administration of novel drugs based on the structure of endogenous AMPs holds a future in COPD treatment requires additional studies.

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CHAPTER 3

Basal cells contribute to innate immunity of the airway epithelium through production of the antimicrobial protein RNase 7.

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ABSTRACT

Basal cells play a critical role in the response of the airway epithelium to injury and are recently recognized to also contribute to epithelial immunity. Antimicrobial proteins and peptides are essential effector molecules in this airway epithelial innate immunity. However, little is known about the specific role of basal cells in antimicrobial protein and peptide production and about the regulation of the ubiquitous antimicrobial protein RNase 7. In this study, we report that basal cells are the principal cell type producing RNase 7 in cultured primary bronchial epithelial cells (PBEC). Exposure of submerged cultured PBEC (primarily consisting of basal cells) to the respiratory pathogen nontypeable *Haemophilus influenzae* resulted in a marked increase in expression of RNase 7, although this was not observed in differentiated air-liquid interface cultured PBEC. However, transient epithelial injury in air liquid interface–cultured PBEC induced by cigarette smoke exposure led to epidermal growth factor receptor–mediated expression of RNase 7 in remaining basal cells. The selective induction of RNase 7 in basal cells by cigarette smoke was demonstrated using confocal microscopy and by examining isolated luminal and basal cell fractions. Taken together, these findings demonstrate a phenotype-specific innate immune activity of airway epithelial basal cells, which serves as a second line of airway epithelial defence that is induced by airway epithelial injury

INTRODUCTION

Mucosal epithelial cells are an essential component of the host defense barrier against microbial pathogens. In the lung, a pseudostratified layer of airway epithelial cells provides this host defense via a passive barrier function but also active innate immune defense mechanisms (1, 2). These mechanisms are primarily executed by airway epithelial luminal cells (LCs), mainly composed of ciliated and secretory cells, which facilitate for instance mucociliary clearance, defense mediated by periciliary tethered mucins, production of innate immune mediators upon pathogen recognition, and Ig transcytosis toward the airway surface liquid (3-6). In contrast to LCs, it has been largely unexplored whether airway epithelial basal cells (BCs) have an innate immune function. BCs are characterized as epithelial progenitor cells and defined based on the expression of p63 and cytokeratin 5 (KRT5) (7-9). BCs display a low turnover at normal homeostatic conditions but rapidly expand and subsequently differentiate toward other epithelial phenotypes upon injury to restore the airway epithelial barrier. The basolateral localized BCs are shielded from direct microbial contact by the overlying LCs in intact airway epithelium. However, airway epithelial injury caused by disruption of the epithelial barrier because of the loss of cell–cell contact or shedding of LCs after extensive injury may increase microbial exposure. This may enhance the vulnerability of BCs and underlying tissues to respiratory infections. Recently, Byer *et al.* (10) demonstrated selective expression of the pro-inflammatory mediator IL-33 by a subpopulation of BCs in patients with chronic obstructive pulmonary disease. On the basis of this observation, we speculate that BCs might display other unique innate immune responses, which act as a second line of airway epithelial host defense in addition to the activity of LCs. Antimicrobial proteins and peptides (AMPs) are important innate immune mediators involved in airway epithelial host defense (11). AMPs display direct antimicrobial activity against bacteria, viruses, and fungi but also exhibit immunomodulatory and wound healing properties (12, 13). The antimicrobial RNase 7 is a secreted RNase A family member displaying antimicrobial activity toward a range of pathogens, including the respiratory pathogen *Pseudomonas aeruginosa* (14-16). Previous studies exploring the regulation of RNase 7 expression in skin, urinary tract, and ocular surface epithelial cells, demonstrated increased RNase 7 expression upon stimulation with microbes, microbial compounds, and pro-inflammatory cytokines (17-19). Moreover, mechanical injury and UV radiation induce expression of RNase 7 in keratinocytes (20, 21), demonstrating that epithelial injury is a potent inducer of RNase 7 expression. Although RNase 7 expression has been detected in cultured airway epithelial cells (14), it is currently unknown how RNase 7 expression is regulated in these cells. In this paper, we provide evidence that BCs are the principal cell producing RNase 7 in cultured human primary bronchial epithelial cells (PBEC) upon exposure to the respiratory pathogen nontypeable *Haemophilus influenzae* (NTHi) and epithelial barrier disruption caused by cigarette smoke exposure. Submerged cultured undifferentiated PBEC (S-PBEC), primarily consisting of BCs, but not mucociliary differentiated air-liquid interface–cultured PBEC (ALI-PBEC), expressed RNase 7 upon stimulation with UV-inactivated NTHi. Transient epithelial injury induced by whole cigarette smoke coincides with epidermal growth factor receptor (EGFR)–dependent expression of RNase 7 in ALI-PBEC, and this expression is specifically observed in BCs. These findings demonstrate a novel innate immune defense function of BCs, which act as a second line of airway epithelial defense induced upon injury.

MATERIALS AND METHODS

Bronchial epithelial cell culture

Human PBEC were isolated from tumour-free resected lung tissue from anonymous donors by enzymatic digestion as reported previously (22). Mucociliary-differentiated ALI-PBEC were cultured as previously described (23), using semipermeable Transwell membranes with a 0.4-mm pore size (Corning Costar, Cambridge, MA). Transwells were coated with a mixture of 30 µg/ml PureCol (Advanced BioMatrix, San Diego, CA), 10 µg/ml BSA (Sigma-Aldrich, St. Louis, MO), and 10 µg/ml fibronectin (isolated from human plasma) diluted in PBS, at 37°C, 5% CO_2 for 2–24 h. PBEC (passage 2) were seeded on coated Transwells at a density of 40,000 cells and cultured in submerged condition using a 1:1 mixture of DMEM (Life Technologies, Bleiswijk, the Netherlands) and bronchial epithelial growth medium (Lonza, Verviers, Belgium) (B/D medium) with supplementation of BEGM BulletKit singlequots (0.4% [w/v] bovine pituitary extract, 1 mM hydrocortisone, 0.5 ng/ml human hEGF, 0.5 µg/ml epinephrine, 10 µg/ml transferrin, 5 µg/ml insulin, T3, and 0.1 ng/ml retinoic acid) (Lonza), 1 mM HEPES (Lonza), 1 mg/ml BSA (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza), and additional supplementation of 15 ng/ml retinoic acid (Sigma-Aldrich) to induce mucociliary differentiation. After reaching confluence (~ 4–5 d), cells were cultured air exposed for an additional period of 2 wk before use. Undifferentiated S-PBEC were cultured in 12-well tissue culture plates in B/D medium including supplements as earlier described but without additional retinoic acid supplementation. Upon reaching 80–90% confluence, S-PBEC were used for further experiments.

NTHi

NTHi strain D1 (24) was cultured on chocolate agar plates (bioMérieux, Craponne, France) at 5% CO_2 and 37°C overnight. A selected colony was resuspended in 10 ml Tryptone soya broth + X- and V-factor (Mediaproducts BV, Groningen, the Netherlands) and incubated at 37°C while shaking overnight. Next, 1 ml of the overnight broth containing NTHi was transferred into new 10 ml Tryptone soya broth + X- and V-factor and incubated for 3 h at 37°C while shaking. Afterward, the bacteria containing broth was centrifuged for 10 min at 3000 rpm, and the pellet was washed with PBS. The bacterial pellet was resuspended in PBS after an additional washing step, and the concentration was adjusted to an OD_{600} 1 (1 x 10^9 CFU/ml). The NTHi was subsequently inactivated by UV exposure for 2 h.

Whole cigarette smoke exposure and other stimuli

ALI-PBEC were exposed to cigarette smoke using 3R4F reference cigarettes (University of Kentucky, Lexington, KY) in a whole smoke exposure model adapted from Beisswenger *et al.* (25) as depicted in Figure 2A. ALI-PBEC were placed into modified hypoxic chambers (Billups Rothenberg, Del Mar, CA), further referred to as exposure chambers, localized inside an incubator at 37°C and 5% CO_2. Next mainstream smoke derived from one cigarette or air as negative control was infused inside respectively a "Smoke" and "Air" exposure chamber by a mechanical pump using a continuous flow of 1 l/minute for a period of 4–5 min. A ventilator inside the exposure chambers equally distributed and mixed the infused air/smoke with humidified warm air derived from the incubator to prevent inflammatory responses by PBEC as reported previously (25). After exposure, residual smoke inside the exposure chamber was

removed for a period of 10 min by flushing the chambers with air derived from the incubator. The amount of smoke infused inside the exposure chamber was measured by determining the weight difference of a filter placed between the pump and exposure chamber before and after exposure. Approximately 2 mg cigarette smoke–derived particles were deposited on the filter as determined by measuring the filter of different exposures. After smoke or air exposure, the culture medium of ALI-PBEC was refreshed, and cells were incubated at 37°C and 5% CO_2 for the indicated period of times. UV-inactivated NTHi or Pam3CSK4 (InvivoGen, San Diego, CA) was administered in a volume of 100 µl in PBS at the apical surface of ALI-PBEC or added to the culture medium when stimulating S-PBEC. To examine cell signalling pathways, the following chemical inhibitors were applied: AG1478, anti-EGFR neutralizing Ab, and U0125 (all Calbiochem, Darmstadt, Germany). Inhibitors were added into the basal culture medium and pre-incubated for 1 h prior to smoke exposure. Stimulation of ALI-PBEC with rTGF-α (PeproTech, Rocky Hill, NJ) was done by adding this growth factor to the culture medium. In confocal imaging and FACS experiments, 5 µg/ml brefeldin A (Sigma-Aldrich) was added in the culture medium to prevent protein secretion.

Cell cytotoxicity and viability assays

Lactate dehydrogenase (LDH) release in the apical surface liquid, collected by washing the epithelial cultures apical with 100 µl PBS, and culture medium was assessed using a cytotoxicity detection kit (Roche, Basel, Switzerland), according to the manufacturer's protocol. One hundred microliters of 0.1% (v/v) Triton X-100 in PBS was applied on the apical surface as positive control. The transepithelial electrical resistance (TEER) was measured using the MilliCell-ERS (Millipore, Bedford, MA). Cell permeability was determined by the FITC–dextran permeability assay. In this assay, 1 mg/ml FITC–dextran (4 kDa; Sigma-Aldrich) diluted in PBS was added to the apical side and incubated for 2 h. Next, 100 µl of the basal culture medium was transferred into solid black 96-well plates (Costar-Corning), and the fluorescence was measured with an excitation/emission wavelength of 490/521 nm using a Wallac Victor 2 Microtiter Plate Reader (PerkinElmer, Waltham, MA).

RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Venlo, the Netherlands) following the manufacturer's protocol, and RNA quantities were measured using the Nanodrop ND-1000 UV-visible (UV-Vis) spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA synthesis was performed by RT-PCR of 1 µg RNA reverse mixed with oligo (deoxythymidine) primers (Qiagen) and Moloney murine leukemia virus polymerase (Promega, Leiden, The Netherlands) at 37°C. Quantitative PCR (qPCR) was performed using IQ Sybr green supermix (Bio-Rad) and a CFX-384 real-time PCR detection system (Bio-Rad). Reactions were performed using the primers indicated in Table 1. The housekeeping genes RPL13A and ATP5B were selected using the "Genorm method" (Genorm; Primer Design, Southampton, U.K.). 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

Gene	Forward primer	Reverse primer
RNASE7	5'-CCAAGGGCATGACCTCATCAC-3'	5'-ACCGTTTTGTGTGCTTGTTAATG-3'
IL8	5'-CAGCCTTCCTGATTTCTG-3'	5'-CACTTCTCCACAACCCTCTGC-3'
IL6	5'-CAGAGCTGTGCAGATGAGTACA-3'	5'-GATGAGTTGTCATGTCCTGCAG-3'
DEFB4	5'-ATCAGCCATGAGGGTCTTG-3'	5'-GCAGCATTTTGTTCCAGG-3'
CCL20	5'-GCAAGCAACTTTGACTGCTG-3'	5'-TGGGCTATGTCCAATTCCAT-3'
LCN2	5'-CCTCAGACCTGATCCCAGC-3'	5'-CAGGACGGAGGTGACATTGTA-3'
FOXJ1	5'-GGAGGGGACGTAAATCCCTA-3'	5'-TTGGTCCCAGTAGTTCCAGC -3'
MUC5AC	5'-ATTTTTTCCCCACTCCTGATG-3'	5'-AAGACAACCCACTCCCAACC-3'
TP63	5'-CCACCTGGACGTATTCCACTG-3'	5'-TCGAATCAAATGACTAGGAGGGG-3'
KRT5	5'-CCAAGGTTGATGCACTGATGG-3'	5'-TGTCAGACATGCGTCTGC-3'
RPL13A	5'-AAGGTGGTGGTCGTACGCTGTG-3'	5'- CGGGAAGGGTTGGTGTTCATCC-3'
ATP5B	5'-TCACCCAGGCTGGTTCAGA-3'	5'-AGTGGCCAGGGTAGGCTGAT-3'

Sequences of primers used for qPCR.

ELISA

Protein secretion by ALI-PBEC was determined in the apical surface liquid and in the basal medium. The secretion of IL-8 was determined by ELISA following the manufacturer's protocol (Sanquin, Amsterdam, The Netherlands). RNase 7 secretion was assessed by ELISA as described previously (16). The OD values were measured with a microplate reader (Bio-Rad).

Isolation of luminal and basal airway epithelial cells

The basal and luminal fraction of ALI-PBEC were isolated according to a method adapted from Jakiela *et al.* (26). Cultured ALI-PBEC were washed twice with calcium-free PBS. Next, calcium-free MEM (Life Technologies) was applied to the apical side (700 µl) and the basolateral compartment to break down the integrity of the intercellular junctions. This was monitored by TEER measurements, which reached background levels after ~15–20 min. Next, the apical medium was substituted by 700 µl Trypsin Versene (Lonza) and incubated for 7–10 min until luminal cells started to detach. The detached luminal fraction was collected, and the apical surface was subsequently washed twice gently with 700 µl DMEM supplemented with 5% heat-inactivated FCS (Life Technologies). The cells that were collected by this washing step were added to the luminal fraction, which was then centrifuged for 7 min at 1200 rpm. After removal of the supernatant, mRNA was isolated from the cell pellet (luminal fraction) and the epithelial cells that remained attached to the inserts (basal fraction).

Immunofluorescence staining and confocal imaging

For immunofluorescence staining, ALI-PBEC were fixed in 4% paraformaldehyde, permeabilized in 0.3% (v/v) Triton X-100 in PBS, and blocked in blocking solution consisting of 5% (w/v) BSA and 0.3% (v/v) Triton X in PBS. All incubation steps were 30 min at room temperature and contained washing steps with PBS in-between. Membranes containing ALI-PBEC were detached from the Transwell using a razorblade and transferred to 24-well tissues

culture plates for Ab staining. The primary Abs (mouse mAb against RNase 7 and rabbit mAb against p63 [Abcam, Cambridge, U.K.]) were diluted in blocking solution and added to the cells for 1 h at room temperature. The secondary Abs goat anti-mouse Alexa Fluor 488, goat anti-rabbit Alexa Fluor 568 (both Invitrogen), and DAPI, diluted in blocking buffer, were added to the cells for 30 min at room temperature in the dark. Next, the PBEC-containing membranes were placed on coverslips and mounted in Vectashield Hard Set Mounting Medium (Vector Labs, Burlingame, CA). Confocal images were taken using a Leica TCS SP5 confocal inverted microscope (Leica Microsystems, Wetzlar, Germany) and processed using the Leica Application Suite Advanced Fluorescence software (Leica Microsystems).

Flow cytometry

ALI-PBEC were detached from the Transwell by trypsinization using soft trypsin, consisting of 0.3 mg/ml trypsin 250 (BD Biosciences, San Jose, CA), 0.1 mg/ml EDTA, 1 mg/ml glucose (both BDH Chemicals, Poole, U.K.), 100 U/ml penicillin, and 100 μg/ml streptomycin in PBS (pH 7.45). Trypsinization was stopped using 1.1 mg/ml soy bean trypsin inhibitor (Sigma-Aldrich) in keratinocyte serum-free medium (Life Technologies). Cells were washed once in cold PBS and stained with live/dead stain aqua (Invitrogen). Then, cells were washed in PBS, permeabilized using 0.5% (v/v) saponin (Sigma-Aldrich) in PBS for 5 min on ice, and fixed with 3.9% (v/v) formaldehyde (Merck) in PBS for 15 min at room temperature. Next, primary Abs (mouse mAb against RNase 7 [Abcam, Cambridge, U.K.] and rabbit polyclonal Ab against KRT5 [Santa Cruz Biotechnology, Santa Cruz, CA]) were added, followed by secondary Ab treatment (goat anti-mouse Alexa Fluor 488 and goat-anti-rabbit Alexa Fluor 647 [both Invitrogen]), both diluted in 0.5% saponin supplemented with human FcR binding inhibitor (eBioscience, San Diego, CA). Both primary and secondary Abs were incubated for 30 min at room temperature. Cells were measured on a FACSCanto II (BD Biosciences), and analysis was performed using FlowJo (version 7.6.5) software (Tree Star, Ashland, OR). Gates were placed according to unstained and single stains with secondary Abs (Supplementary Figure 1).

SDS-PAGE and Western blot

SDS-PAGE gel electrophoresis and Western blotting was performed as described previously (27). In short, PBEC were washed with ice-cold washing buffer consisting of 5 mM Tris (pH 6.4), 100 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, and cell lysates were prepared in lysis buffer consisting of 0.5% (v/v) Triton X-100, 0.1 M Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ 1 mM Na_3VO_4, and protease inhibitor mixture (Roche) and subsequently suspended in SDS-PAGE sample buffer consisting of 4% (w/v) SDS, 10% (v/v) 2-ME, 20% (v/v) glycerol, 0.5 M Tris (pH 6.8), and 0.003% (w/v) Bromophenol blue. SDS-gel electrophoresis was conducted using 10–12.5% glycine-based gels, and afterward, separated proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked with blocking buffer consisting of 5% (w/v) skim milk (Sigma-Aldrich) in PBS/0.05% (v/v) Tween 20 and incubated with primary Abs (rabbit polyclonal Ab ERK1/2, p-ERK1/2, p-EGFR, and mouse mAb b-actin [all Cell Signaling Technology, Leiden, The Netherlands], mouse mAb EGFR [BD Biosciences], and mouse mAb RNase 7 [Abcam]) in 5% (w/v) BSA TBS/0.05% (v/v) Tween 20 overnight at 4°C. Afterward, membranes were incubated with secondary HRP-labelled Ab (Sigma-Aldrich) in blocking buffer for 1 h, and membranes were subsequently

developed with ECL substrate (ThermoScientific, Rockford, IL). Results were analysed by densitometry using Adobe Photoshop (Adobe Systems, San Jose, CA).

Statistical analysis

Graphs were made and statistical analysis was performed in GraphPad PRISM 6.0 (GraphPad Software, La Jolla, CA). Data are shown as means ± SEM. Differences were considered significantly different with $p<0.05$ explored by (un)paired Student t test or one/two-way ANOVA as appropriate.

RESULTS

NTHi increases expression of RNase 7 in undifferentiated S-PBEC but not in mucociliary differentiated ALI-PBEC

We first investigated whether the common respiratory pathogen NTHi induced RNase 7 expression in cultured PBEC. Therefore, undifferentiated S-PBEC were exposed to UV-inactivated NTHi for 6 and 24 h, and RNase 7 expression was assessed by qPCR. The results showed that NTHi induced RNase 7 mRNA expression by S-PBEC after 24 h but not 6 h of stimulation (Figure 1A). In contrast, mRNA expression of the well-defined bacterial-induced antimicrobial peptide human β-defensin-2 (hBD-2) was increased by NTHi both at 6 and 24 h. As shown by others (28), we observed that undifferentiated S-PBEC primarily consist of p63+ BCs (*data not shown*). Because mucociliary-differentiated ALI-PBEC are more comparable to the pseudostratified airway epithelium *in vivo* (29), we subsequently compared RNase 7 mRNA expression in ALI-PBEC and S-PBEC. Interestingly, ALI-PBEC did not display an increase in RNase 7 mRNA in response to various concentrations of NTHi after 24 h of stimulation, whereas dose-dependent induction was observed in S-PBEC (Figure 1B). In contrast, a dose-dependent NTHi-induced expression of hBD-2 was observed in both ALI- and S-PBEC. Further evaluation of the expression of other innate immune mediators demonstrated that NTHi induced mRNA expression of IL-8, IL-6, CCL20, and lipocalin 2 (LCN2) in both S-PBEC and ALI-PBEC (Figure 1C). To explore baseline RNase 7 expression during mucociliary differentiation, mRNA levels of RNase 7 were measured in S-PBEC and in ALI-PBEC at 1 and 2 wk after initial air exposure. Compared with S-PBEC, no difference in mRNA levels was observed in ALI-PBEC cultured 1 wk in air exposed conditions, whereas after 2 wk a significant decrease in RNase 7 mRNA was observed (Figure 1D). As expected, airway epithelial cell differentiation furthermore resulted in a decreased mRNA expression of the BC marker p63 and increased expression of ciliated and goblet cell markers, respectively, FOXJ1 and MUC5AC. These findings suggest, that in contrast to other examined innate immune mediators, RNase 7 is selectively produced by airway BCs but not by mucociliary-differentiated airway epithelial cells at baseline conditions and upon NTHi stimulation.

Cigarette smoke–induced transient epithelial injury results in induction of RNase 7 in ALI-PBEC

BCs display enhanced activity upon injury, and it has been demonstrated that injury induces RNase 7 expression in keratinocytes (7, 20, 21). Therefore, we hypothesized that injury of ALI-PBEC can reinitiate expression of RNase 7 by remaining undifferentiated BCs. Cigarette smoke is a well-known inhaled toxic mixture that induces airway epithelial injury (30, 31).

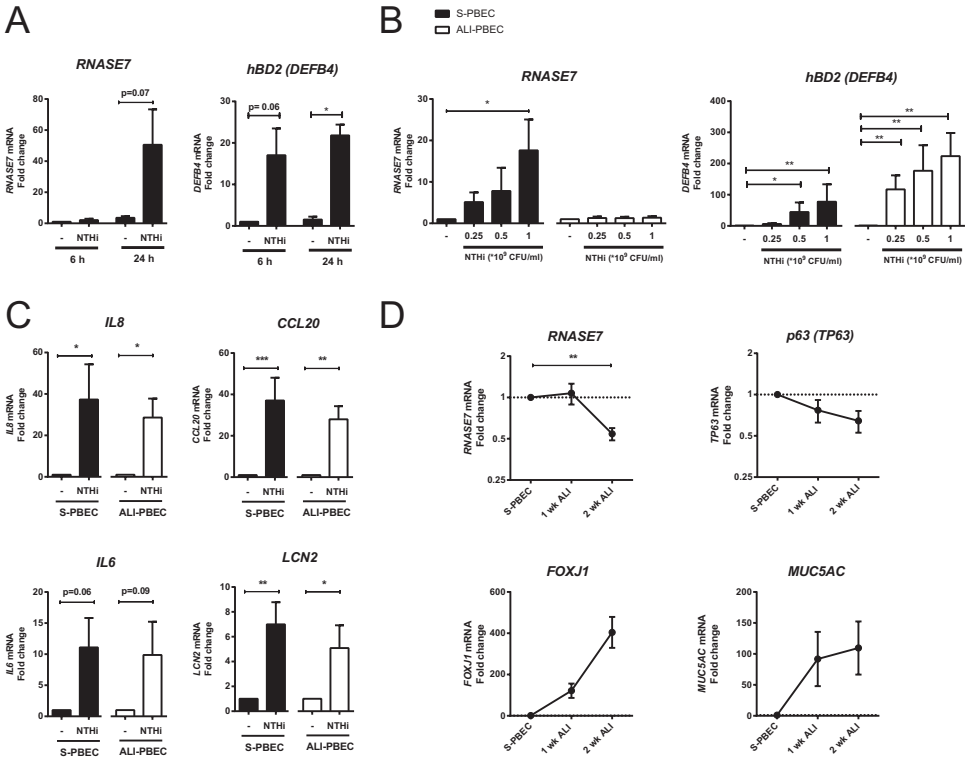

Figure 1. Differential expression of RNase 7 depending on the airway epithelial differentiation status. (A) S-PBEC were stimulated with 1*10^9 CFU/ml UV-inactivated NTHi for 6 and 24 h. mRNA expression of RNase 7 and hBD-2 was determined by qPCR. Data is shown as fold change compared to unstimulated cells (n=4 donors). (B) S-PBEC and ALI-PBEC were stimulated with 0.25, 0.5 and 1*10^9 CFU/ml UV-inactivated NTHi for 24 h. NTHi was stimulated on the apical surface of ALI-PBEC in 100 µl of PBS. mRNA expression of RNase7 and hBD-2 (DEFB4) was subsequently assessed. Data is shown as fold change compared to unstimulated cells (n=5 donors). (C) S-PBEC and ALI-PBEC were stimulated with 0.5*10^9 CFU/ml UV-inactivated NTHi for 24 h. mRNA expression of IL-8, IL-6, CCL20 and LCN2 was subsequently determined. Data is shown as fold change compared to unstimulated cells (n=5 donors). (D) Baseline mRNA expression of RNase 7, the basal cell marker p63 (TP63), ciliated cell marker FOXJ1, and goblet cell marker MUC5AC measured in undifferentiated S-PBEC, 1 wk (1 wk ALI) and 2 wk (2 wk ALI) differentiated ALI-PBEC, all cultured on transwells. Data is shown as fold change compared to undifferentiated cells (n=4 donors). All stimulations were performed in duplicate. * p<0.05, ** p<0.01, *** p< 0,0001.

We therefore examined cigarette smoke–induced injury in ALI-PBEC using a whole cigarette smoke exposure model (Figure 2A). First, the cytotoxic effect of smoke exposure was determined by a LDH release assay (Figure. 2B). No significant increase in LDH release was detected in the basal medium of smoke-exposed cells compared with air controls. However, a significant increase in LDH release was observed in the apical surface wash. Despite this

cytotoxic effect, no significant difference was observed in the TEER of air- and smoke exposed cultures after 24 h (Figure. 2C). In contrast, a significant decrease in TEER was observed in the first 2 h after smoke exposure, indicating transient injury. Further evaluation of the epithelial barrier function by FITC–dextran permeability also confirmed increased barrier disruption in the first 2 h after smoke exposure, whereas no difference in permeability was detected 24 h after exposure (Figure 2D). To examine whether transient epithelial damage induced by cigarette smoke affected the expression of RNase 7, mRNA expression levels were measured at different time periods after exposure. A time-dependent increase in RNase 7 mRNA expression was detected after smoke exposure, which peaked after 3 h and remained significantly different compared with air exposed cells after 6 h (Figure 3A). Similar to increased RNase 7 mRNA expression, protein expression was increased in lysates of ALI-PBEC at 6, 12, and 24 h after smoke exposure (Figure 3B,C). Moreover, increased RNase 7 secretion was measured in the apical surface liquid but not in the basolateral culture medium 24 h after exposure, indicating

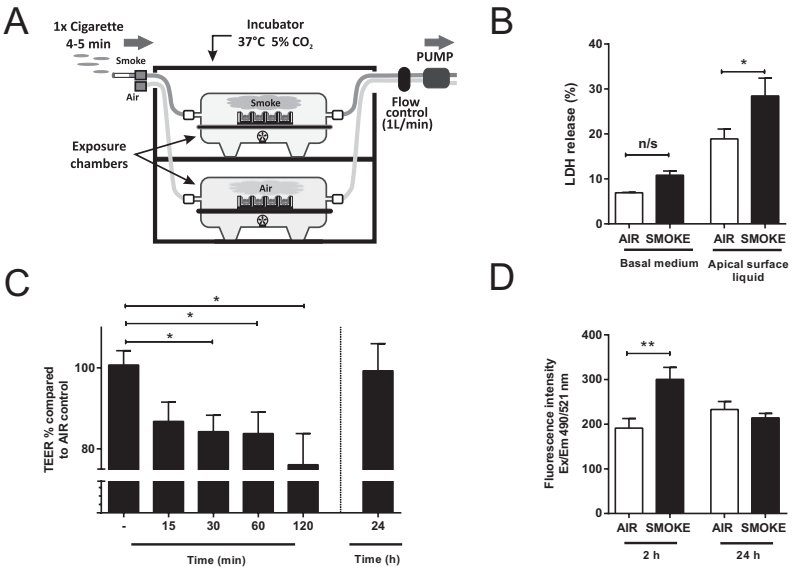

Figure 2. Attenuation of the airway epithelial barrier integrity by whole cigarette smoke exposure. (A) Schematic representation of the whole cigarette smoke exposure model (See materials and methods for details). (B) LDH release was measured in the basal medium 24 h after air/smoke exposure. Data is depicted as percentage cytotoxicity compared to the positive control Triton-X (n=5 donors). (C) Trans-epithelial electrical resistance (TEER) measurements 15-120 minutes and 24 h after air/smoke exposure. Data is shown as percentage compared to TEER measurements of air exposed cells (n=4-9 donors). (D) FITC-dextran was applied on the apical surface either directly or 24 h after air/smoke exposure, and incubated for 2 h. Afterwards the fluorescence was measured in basal medium samples. Data is shown as the measured fluorescence (n=4 donors). All exposures were performed in duplicate. * $p<0.05$, ** $p<0.01$.

polarized secretion of RNase 7 (Figure 3D). We further examined the expression of other mediators besides RNase 7 to delineate the innate immune response induced by cigarette smoke. In contrast to RNase 7, cigarette smoke did not increase mRNA expression of the AMPs hBD-2, hBD-3, LCN2, and Psoriasin/S100A7 (data not shown). However, smoke did induce mRNA expression of IL-8 and IL-6 (Figure 3E), in line with previous observations by others (25). Furthermore, cigarette smoke exposure increased mRNA expression of CCL20, which previously was reported to be induced in airway epithelial cells upon disruption of the airway epithelial barrier integrity (32). In line with mRNA expression, we also detected cigarette smoke–induced IL-8 protein secretion in the basal medium and apical surface liquid (Figure 3F). In summary, we demonstrated that transient injury induced by cigarette smoke exposure is paired with increased expression and polarized secretion of RNase 7 by ALI-PBEC. This response is part of a distinct innate immune response including the expression of IL-8, IL-6, and CCL20, but lacks induction of other AMPs that were investigated.

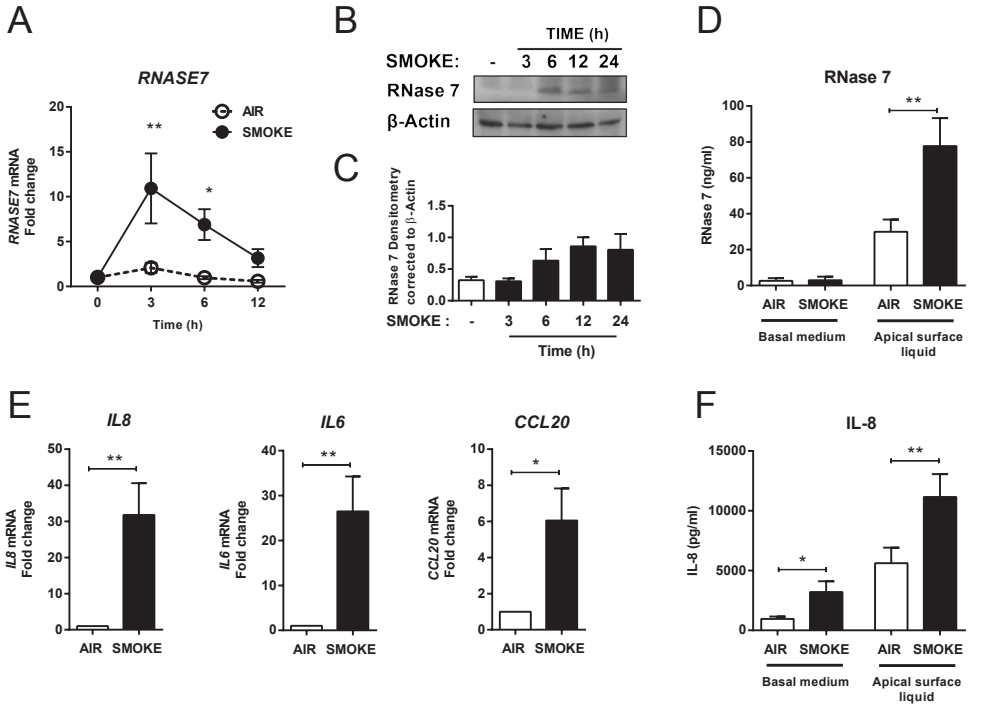

Figure 3. Production of RNase 7 and other innate immune mediators by ALI-PBEC in response to cigarette smoke. (A) ALI-PBEC were exposed to air or cigarette smoke and afterwards incubated for different time periods. mRNA expression levels of RNase 7 was determined by qPCR at 3, 6 and 12 h after air/smoke exposure. Data is shown as fold change compared to unexposed cells (n=6 donors). (B) RNase 7 protein detection in cell lysates of ALI-PBEC, 24 h after air/smoke exposure (data represents 3 independent donors). (C) RNase 7 protein expression was determined by ELISA in the basal medium and apical surface liquid 24 h after air/smoke exposure (n=5 donors). (D) qPCR of IL-8, IL-6 and CCL20, 3 h after air/smoke exposure. Data is shown as fold change compared to air exposed cells (n=6 donors). (E) IL-8 secretion was measured by ELISA in the basal medium and apical surface liquid 24 h after air/smoke exposure (n=6 donors). Air/smoke exposures were performed in duplicate. *p<0.05, ** p<0.01.

Smoke enhances RNase 7 expression specifically in BCs of ALI-PBEC

To determine whether cigarette smoke–induced RNase 7 was specifically expressed by BCs in ALI-PBEC, we analysed mRNA expression in the basal and luminal cell fraction of ALI-PBEC that were separated based on a previous described method (26). To validate the nature of the isolated fractions, we initially examined the mRNA expression of the BC markers p63 and KRT5. Both p63 and KRT5 mRNA levels were significantly higher in the basal fraction compared with the luminal fraction in smoke- and air- exposed cultures (Figure 4A). Interestingly, we observed a significant decrease in p63 and KRT5 mRNA levels in the basal fraction of smoke-exposed cultures. At later time points, these levels returned to baseline levels (*unpublished data*), suggesting that the effect of smoke exposure on these basal cell markers is only transient. In contrast, mRNA expression of RNase 7 was significantly increased 3 h after smoke exposure in the basal fraction, but not in the luminal fraction of ALI-PBEC, whereas smoke-induced mRNA expression of IL-8 was observed in both the basal and the luminal fraction. Examination of RNase 7 protein expression was subsequently determined by assessing co-localization of RNase 7 with p63 using immunofluorescence confocal imaging. Confocal imaging of the apical side of ALI-PBEC lacking $p63^+$ cells, demonstrated that RNase 7 expression could not be observed in both air- and smoke-exposed ALI-PBEC after 6 h (Figure 4B). However, RNase 7 co-localized with $p63^+$ cells at the basal side of ALI-PBEC, which was more abundant in cigarette smoke–exposed cultures than air controls. In these confocal studies, we did not observe a decrease in p63 protein levels or the number of $p63^+$ cells in smoke-exposed cultures compared with air controls, contrasting the observed decreased mRNA expression of p63. To quantify RNase 7 expression in BCs, we performed flow cytometric analysis examining RNase 7 expression in $KRT5^+$ cells in ALI-PBEC, 6 h after air/smoke exposure (Figure 4C). Cigarette smoke significantly increased RNase 7 in the $KRT5^+$ fraction compared with air exposed cultures (Figure 4D), whereas no effect was observed in the $KRT5^-$ fraction. Similar to p63 protein detection by confocal imaging, we did not observed a difference in the ratio of $KRT5^-$ and $KRT5^+$ cells upon smoke exposure (data not shown). Supporting our hypothesis, we observed that smoke exposure induced RNase 7 expression in ALI-PBEC selectively in BCs.

Bacterial stimulation does not further increase cigarette smoke-induced RNase 7 expression

We further evaluated the additional effect of bacterial stimulation on smoke-induced RNase 7 expression. After smoke exposure, ALI-PBEC were stimulated with either NTHi or the TLR2 ligand Pam3CSK4. Consistent with previous studies examining the effect of whole cigarette smoke on bacterial induced expression of IL-8 and hBD-2 (33), cigarette smoke inhibited NTHi- and Pam3CSK4-induced hBD-2 expression while increasing smoke-induced IL-8 mRNA expression (Figure 5). In line with the observation in Figure 1B, NTHi did not induce RNase 7 mRNA expression at 3, 12, and 24 h after exposure (Figure 5A,B). Moreover, NTHi did not further increase smoke-induced RNase 7 mRNA levels. Similar to NTHi, TLR2 activation with Pam3CSK4 did not affect RNase 7 mRNA expression after 3 h of stimulation (Figure 5C).

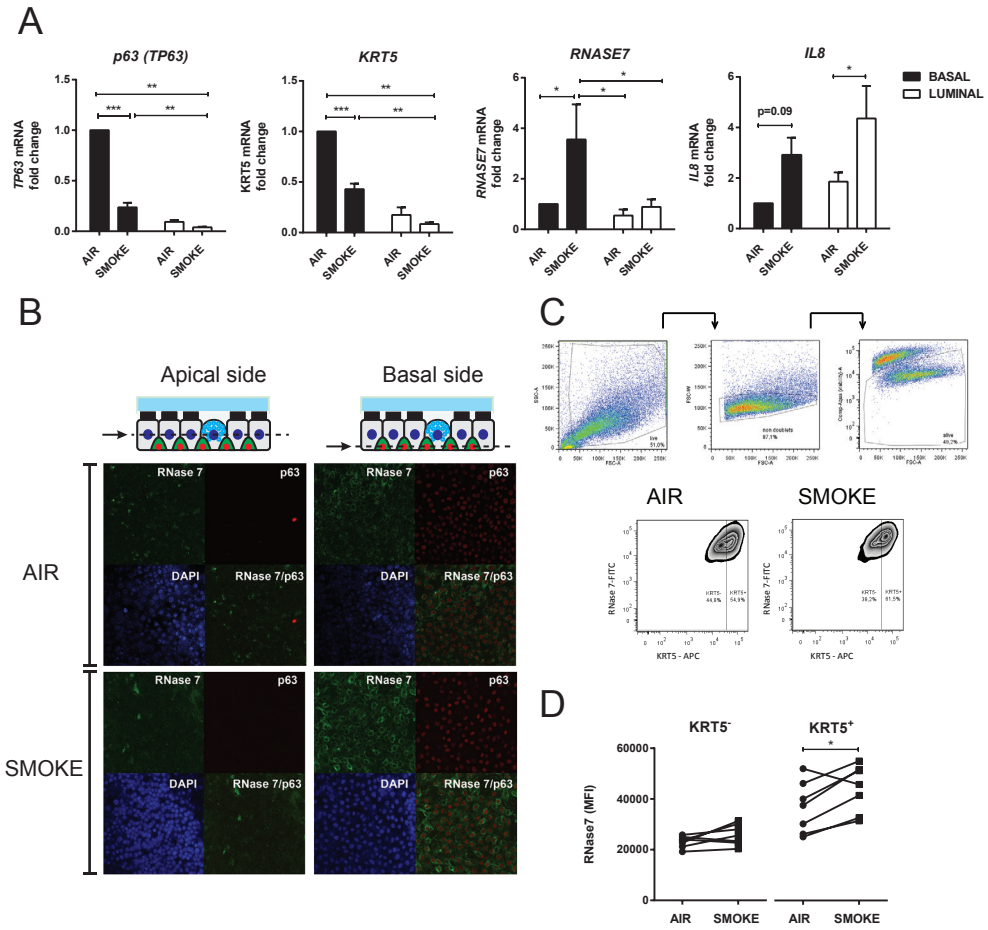

Figure 4. BC-specific expression of RNase 7 in ALI-PBEC after smoke exposure. (A) ALI-PBEC were exposed to air or smoke, and the basal and luminal cell fraction were isolated 3 h after exposure. mRNA expression of p63, KRT5, RNase 7 and IL-8 was determined by qPCR. Data is shown as fold change compared to the air exposed basal fraction (n=4 donors). For protein detection, ALI-PBEC were exposed to air or cigarette smoke, and after 3 h refreshed with culture medium containing 5 µg/ml Brefeldin A to prevent protein secretion. After an additional 3 h, RNase 7 protein localization was determined by confocal imaging and flow cytometry. (B) For confocal imaging, ALI-PBEC were immunostained for RNase 7 (Green) and p63 (Red) and DAPI (Blue). Confocal images were made of the apical site without p63+ cells and basolateral site containing p63+ cells (data represents 3 independent donors). (C) For flow cytometry, ALI-PBEC were detached from the transwell inserts by treatment with soft trypsin and cell suspensions were subsequently stained for RNase 7 and KRT5. Flow cytometric analysis was performed excluding doublets and dead cells by aqua staining. Data is shown as the mean fluorescence intensity (MFI) of the KRT5- and KRT5+ positive fraction (n=6 donors). Air/smoke exposures were performed in duplicate. *p<0.05, ** p<0.01, *** p< 0,0001.

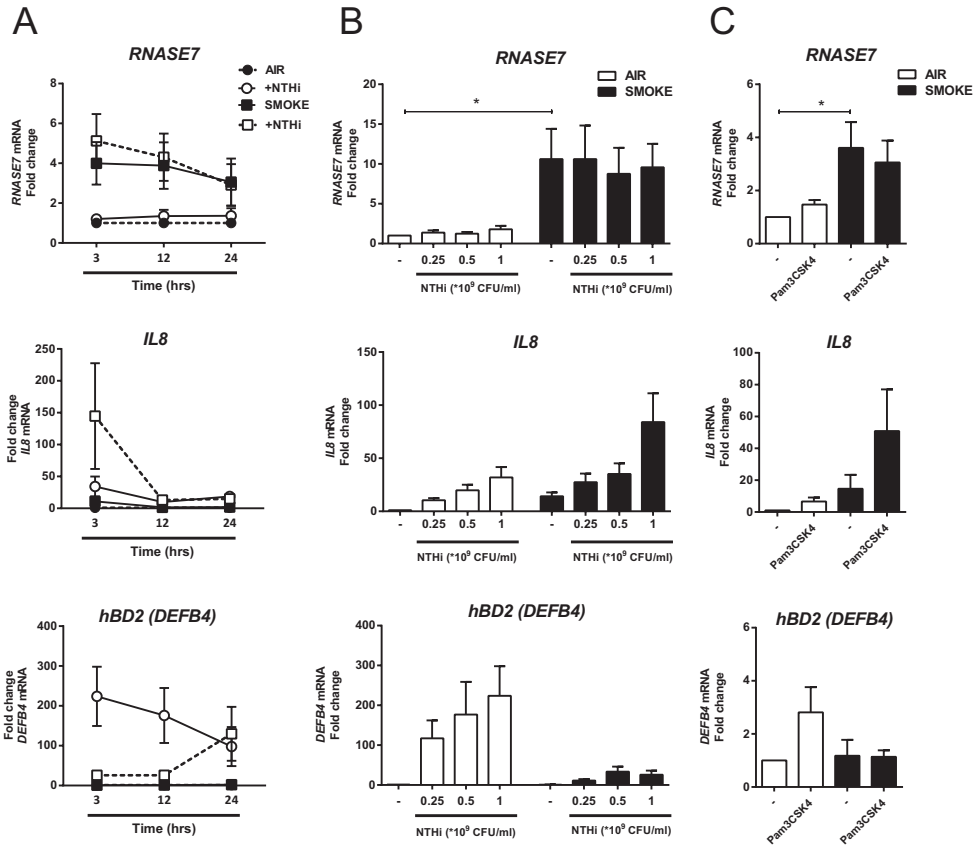

Figure 5. The effect of bacterial stimuli on smoke-induced RNase 7 expression by ALI-PBEC. After air/smoke exposure, (A) ALI-PBEC were stimulated apical with 100 µl of 10*10^7 CFU/ml NTHi for 3, 12 and 24 h (n=6 donors), (B) 100 µl of different concentrations of NTHi (0.25, 0.5 and 1 *10^9 CFU/ml) for 3 h (n=6 donors) or (C) Pam3CSK4 (1 µg/ml) for 4 h (n=5 donors). RNase 7, IL-8 and hBD-2 mRNA expression was assessed by qPCR. Data is shown as fold change compared to air exposed cells. All stimulations were performed in duplicate. *p<0.05.

Cigarette smoke–induced RNase 7 expression requires activation of the epidermal growth factor receptor

Airway epithelial BCs are characterized by selective expression of the EGFR, a receptor that is activated upon disruption of the epithelial barrier function by cigarette smoke (31, 34). Previous studies reported that cigarette smoke extract induced expression of IL-8 in airway epithelial cells, which required activation of EGFR and the downstream MAPK/ERK 1/2 (ERK1/2) (35, 36). Therefore, we studied whether EGFR and ERK1/2 signalling were required in cigarette smoke–induced expression of RNase 7. Our results clearly showed that similar to cigarette smoke extract, whole cigarette smoke exposure increased phosphorylation of EGFR and ERK1/2 in ALI-PBEC 15 min after exposure (Figure 6A,B). Inhibition of EGFR using a neutralizing Ab, the EGFR tyrosine kinase inhibitor AG1478, and the ERK1/2-activating kinases MEK1/2 inhibitor U0126, attenuated activation of ERK1/2, demonstrating that indeed smoke activates ERK1/2 in an EGFR dependent manner. Pharmacological inhibition

of EGFR with AG1478 and inhibition of the ERK1/2-activating kinases MEK1/2 with U0126 caused a significant decrease in smoke-induced mRNA expression of RNase 7 (Figure 6C). Similar to RNase 7, and in line with previous studies, mRNA expression of IL-8 and CCL20 was also impaired upon inhibition of EGFR and MEK/ERK1/2, while a trend was observed for IL-6 expression. To further support the conclusion that EGFR activation mediates RNase 7 expression, we examined the effect of direct receptor activation by stimulating ALI-PBEC with TGF-α. Indeed, in line with the effect of cigarette smoke, 3 h stimulation with TGF-α

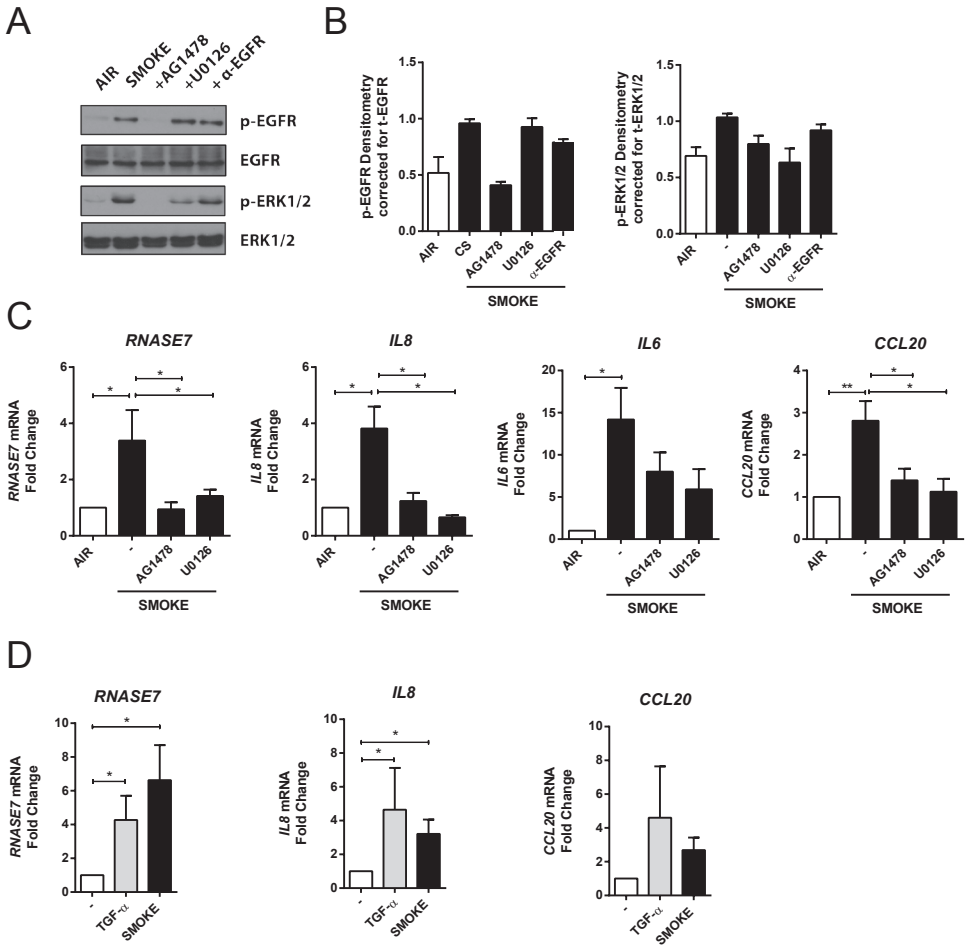

Figure 6. Requirement of EGFR and ERK1/2 in cigarette smoke induced RNase 7 expression by ALI-PBEC. (A) ALI-PBEC were pre-incubated for 1 h with AG1478 (EGFR inhibitor; 1 μM) or EGFR neutralizing antibody (2 μg/ml), prior to smoke exposure. Western blot analysis was performed of p-EGFR, p-ERK1/2 15 minutes after air/smoke exposure. Total EGFR and ERK1/2 were used as loading controls (data represents 3 independent donors). (B) PBEC were pre-incubated for 1 h with AG1478 (EGFR inhibitor; 1 μM) or U0125 (MEK1/2 inhibitor; 25 μM) prior to air/smoke exposure. RNase 7, IL-8, IL-6 and CCL20 mRNA expression was assessed 3 h after exposure by qPCR. Data is shown as fold change compared to air exposed cells (n=6). * $p<0.05$.

significantly enhanced RNase 7 mRNA expression and furthermore increased IL-8 expression; the increase in CCL20 did not reach statistical significance (Figure 6D). In conclusion, we observed that cigarette smoke–induced expression of RNase 7 requires activation of the BC-specific receptor EGFR and the downstream ERK1/2 signalling pathway.

DISCUSSION

In this study, we examined the expression of the antimicrobial RNase 7 in cultured airway epithelial cells. We demonstrated that undifferentiated S-PBEC, primarily consisting of BCs, but not mucociliary-differentiated ALI-PBEC, expressed RNase 7 upon stimulation with the respiratory pathogen NTHi. This was in marked contrast to other examined innate immune mediators that were induced by NTHi in both S-PBEC and ALI-PBEC. Furthermore, differentiated ALI-PBEC displayed a decreased baseline RNase 7 expression compared with S-PBEC after 2 wk of culturing in air-exposed conditions. Cigarette smoke exposure, which caused transient disruption of the epithelial barrier function, reinitiated RNase 7 expression in the remaining undifferentiated BCs in ALI-PBEC, which was demonstrated using confocal microscopy and isolation of luminal and basal cell fractions. Moreover, we showed that this induction required activation of EGFR and the downstream ERK1/2 signalling pathway and furthermore demonstrated that direct EGFR activation by TGF-α also induced RNase 7 expression. In contrast to the innate immune mediators IL-8 and hBD-2, bacterial co-stimulation did not further affect smoke-induced RNase 7 expression in ALI-PBEC. This data suggest that expression of RNase 7 is a unique innate host defence property of BCs that is induced in conditions of airway epithelial injury. To our knowledge, we demonstrate for the first time that BCs display a phenotype-distinct antimicrobial response by producing RNase 7. The epithelial phenotype–specific expression of AMPs has been well described in multiple mucosal tissues (37). For instance, intestinal Paneth cells selectively produce human α-defensin 5 and 6, which are not produced by enterocytes (38). In the lung, phenotype-specific expression of AMPs has been observed in submucosal gland serous cells, which produce lactoferrin and lysozyme (39, 40) and also in alveolar cells producing antimicrobial surfactant protein A and D (41). Phenotype-specific expression of RNase 7 has been reported in the skin and urinary tract, demonstrating specific expression by respectively suprabasal keratinocytes and α- and β-intercalated cells (16, 18). In contrast to these epithelial cells, phenotype-distinct expression of AMPs by airway epithelial BCs has not yet been reported. Similar to previous in vitro observations demonstrating BC specific expression of IL-33 and EGFR (10, 34), we observed decreased expression of RNase 7 after mucociliary differentiation of PBEC. Consistent with this observation, it was shown in a previous transcriptome analysis that RNase 7 transcripts were increased in BCs but not in differentiated airway epithelial cells (28). Another transcriptome analysis demonstrated enhanced mRNA expression of RNase 1, 4, and 6 in differentiated airway epithelial cells compared with undifferentiated cultures (42), suggesting that RNase 7 production by BCs is unique among other secreted RNases. Interestingly, we did not observe a difference in RNase 7 mRNA expression after 1 wk of mucociliary differentiation of ALI-PBEC. As the amount of BCs declines during the first week of differentiation, we reasoned that the attenuation of RNase 7 expression after 2 wk of differentiation was not caused by a decrease in the number of BCs in cultured ALI-PBEC but because of another mechanism. Because undifferentiated ALI-PBEC initially display a low epithelial barrier function that progresses during differentiation, we hypothesized that RNase

7 expression by BCs in ALI-PBEC is attenuated because of an enhanced airway epithelial barrier function. Our finding that cigarette smoke can reinitiate RNase 7 expression by BCs in ALI-PBEC supported this hypothesis. Previous data demonstrate that E-cadherin–mediated cell–cell contacts inhibit EGFR signalling in airway epithelial cells (43). Because we observed that cigarette smoke decreases epithelial barrier function, which has been shown to be related to interference with the function of E-cadherin (30), and because smoke-induced RNase 7 expression involved EGFR activation, we reason that smoke-induced barrier disruption contributes to RNase 7 expression. In skin host defence, it is well established that injury enhances EGFR-dependent innate immune responses by keratinocytes that provide protection against infections during wound repair (44, 45). Also, in airway epithelial cells, it has been shown that EGFR activation promotes innate immune responses that are depending on the MAPK/ERK1/2 signalling pathway, which also facilitates epithelial wound healing (36, 46). These studies primarily demonstrate EGFR-dependent expression of pro-inflammatory innate immune mediators such as IL-8, IL-6, and CCL20, which were also induced by cigarette smoke in our model. However, to our knowledge, this is the first study reporting EGFR-dependent AMP expression in airway epithelial cells. Although smoke-induced RNase 7 expression was specifically detected in BCs, we observed secretion of RNase 7 in the apical surface liquid. This may be the result of polarized secretion to the apical side, which can be mediated in part by trans-epithelial projections between the lateral intracellular spaces formed by BCs (47). These projections might explain the apical secretion of RNase 7. However, this requires further investigation. Besides decreased baseline RNase 7 expression in ALI-PBEC, we furthermore demonstrated that in contrast to S-PBEC, ALI-PBEC did not induce RNase 7 expression after stimulation with NTHi. Also, additional stimulation with NTHi or the TLR2 ligand Pam3CSK4 did not further enhance cigarette smoke–induced RNase 7 expression, whereas expression of IL-8 was further increased. It can be speculated that the inability of bacterial stimuli to induce RNase 7 expression in BCs of ALI-PBEC is caused by a lack of direct microbial contact of BCs because of apical application of the NTHi and Pam3CSK4. Similar to the pseudostratified airway epithelium *in vivo*, undifferentiated BCs in cultured ALI-PBEC are superimposed by differentiated LCs. In intact airway epithelium, this mechanism might protect BCs from direct microbial contact and subsequently prevents RNase 7 expression. Another explanation of the absence of bacterial induced RNase 7 expression in BCs of ALI-PBEC might be that pattern recognition receptors such as TLR2 are localized at the basolateral site of BCs because of cell polarization. Finally, we cannot formally exclude the possibility that, in contrast to UV-inactivated bacteria, life-NTHi may promote RNase 7 expression in basal cells *in vivo* (e.g., as a result of epithelial barrier disruption). In contrast to RNase 7, NTHi-induced hBD-2 in airway epithelial cells was independent of the epithelial differentiation status. Furthermore, cigarette smoke exposure impaired bacterial-induced hBD-2 (33). This finding is in line with a decreased antimicrobial defence of airway epithelial cells exposed to cigarette smoke, which provides an explanation for the increased susceptibility of smokers and chronic obstructive pulmonary disease patients toward respiratory infections (33). We argue that the decreased antimicrobial defence caused by smoking results from the attenuation of a large number of AMPs including hBD-2, which cannot be compensated by RNase 7 expression. To further explore the significance of RNase 7 in airway epithelial host defence upon barrier disruption, other environmental factors should be examined that affect the epithelial barrier function but do not attenuate the expression of other AMPs. On the basis of our observations, we propose a novel model of

airway epithelial host defence that includes a second line of defense mediated by airway BCs, which is induced upon injury. In the intact airway epithelium, LCs are the main cells that mediate host defence via, for example, production of AMPs such as hBD-2 and mucociliary clearance (Figure 7A). LCs furthermore prevent microbial contact of the basolateral localized BCs, thereby preventing RNase 7 expression. However, BCs do produce RNase 7 upon disruption of the epithelial barrier integrity by inhaled substances such as cigarette smoke (Figure 7B). Moreover, in damaged epithelium lacking LCs, microbial exposure of BCs induces innate immune responses including cell type–specific expression of RNase 7. This way BCs provide additional innate immune defence against microbial infections during the course of epithelial wound repair.

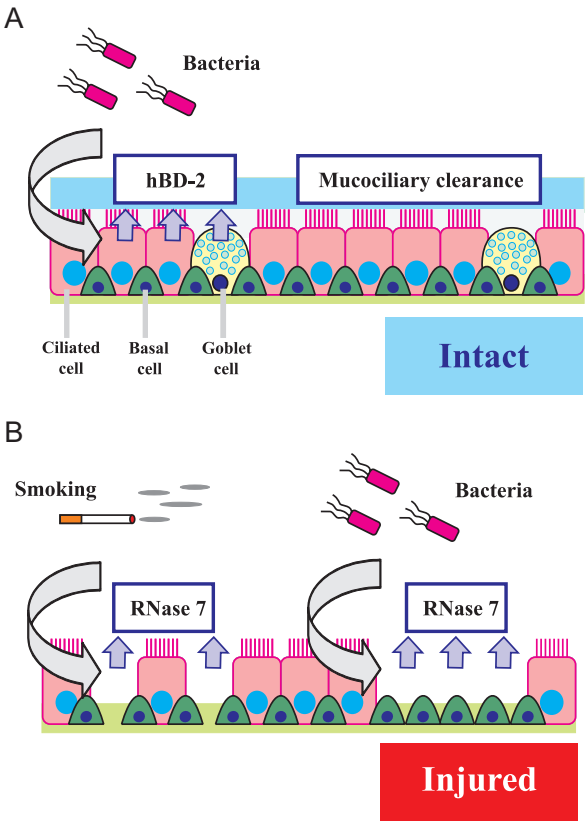

Figure 7. Proposed model. (A) Innate host defense of undamaged airway epithelium mediated by LCs containing ciliated and secretory/goblet cells, for instance providing protection against bacteria via production of hBD-2 and mucociliary clearance. (B) Second line of airway epithelial innate host defense upon injury mediated by BCs, which selectively producing RNase 7. Expression of RNase 7 is induced upon disruption of the epithelial barrier caused by cigarette smoking or upon bacterial exposure in regions lacking LCs.

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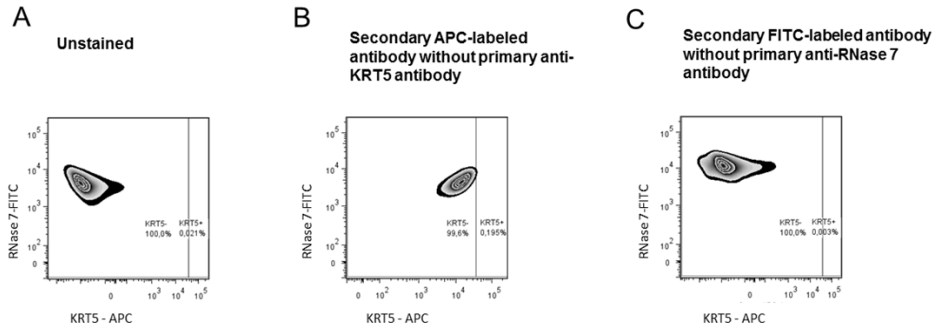
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Controls FACS analysis. (A) Unstained sample. (B) Staining with the secondary APC-labeled antibody in the absence of the KRT5-specific primary antibody. (C) Staining with the secondary FITC-labeled antibody in the absence of the RNase 7-specific antibody.

CHAPTER 4

Antibacterial defense of human airway epithelial cells from chronic obstructive pulmonary disease patients induced by acute exposure to nontypeable *Haemophilus influenzae*: modulation by cigarette smoke.

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ABSTRACT

Antimicrobial proteins and peptides (AMPs) are a central component of the antibacterial activity of airway epithelial cells. It has been proposed that a decrease in antibacterial lung defense contributes to an increased susceptibility to microbial infection of smokers and patients with chronic obstructive pulmonary disease (COPD). Whether reduced AMP expression in the epithelium contributes to this lower defense is however largely unknown. We investigated the bacterial killing activity and expression of AMPs by air-liquid interface cultured primary bronchial epithelial cells from COPD patients and non-COPD (ex)smokers that were stimulated with non-typeable *Haemophilus influenzae* (NTHi). In addition, the effect of cigarette smoke on AMP expression and activation of signaling pathways was determined. COPD cell cultures displayed reduced antibacterial activity, whereas smoke exposure suppressed NTHi-induced expression of AMPs and further increased IL-8 expression in COPD and non-COPD cultures. Moreover, smoke exposure impaired NTHi-induced activation of NF-κB, but not MAP-kinase signaling. Our findings demonstrate that the antibacterial activity of cultured airway epithelial cells induced by acute bacterial exposure is reduced in COPD and suppressed by cigarette smoke, whereas inflammatory responses persisted. These findings help to explain the imbalance between protective antibacterial and destructive inflammatory innate immune responses in COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a severe inflammatory lung disorder in which colonization and infections with opportunistic respiratory bacteria are a major disease hallmark (1). It is hypothesized that a vicious circle of attenuated antibacterial lung defense, enhanced bacterial colonization, and induction of lung inflammation and injury, contribute to the disease (2). However, the underlying mechanisms remain incompletely understood. Smoking is the main risk factor for COPD development, and is furthermore associated with an increased susceptibility to respiratory infections (3). Despite the strong association, only a subpopulation of approximately 15-20% of the smokers develops the disease. This suggests that there are differences between susceptible smokers and smokers with a normal lung function that may help to explain disease etiology. These differences may persist in cell culture (4, 5), for that reason, comparison of cell cultures from COPD patients and non-COPD smoker may reveal differences in host defense activities that may help to explain the increased microbial susceptibility.

Airway epithelial cells contribute to innate lung defense by displaying direct antibacterial activity, which is mediated in part by the production of antimicrobial proteins and peptides (AMPs) (6, 7). In addition to AMPs that are expressed at a steady-state basis, others are induced for example upon recognition of microbes or microbial structures by pattern recognition receptors. This response is highly similar amongst species, and murine models have shown that the inducible antibacterial activity of airway epithelial cells provides full protection against various pathogenic microbes without further involvement of immune cells (8). Impairment of this induced activity in COPD might therefore contribute to the increased susceptibility to infection with respiratory pathogens. Observational studies in lung tissue, airway secretions and tracheal washings have shown that the expression of several AMPs is reduced in smokers and patients with COPD, including the microbial-induced antimicrobial peptide human beta-defensin-2 (hBD-2) (9-12). Moreover, it has been demonstrated that cigarette smoke exposure attenuated the antibacterial activity and microbial-induced expression of hBD-2 in cultured airway epithelial cells (12-14). This suggests that the inducible antibacterial activity is affected in airway epithelial cells from smokers and COPD patients. However, it is unclear whether this activity differs between airway epithelial cells from non-COPD smokers and COPD patients. Interestingly, microbial-induced expression of the pro-inflammatory mediator IL-8 is not reduced by cigarette smoke (14, 15). Conflicting to this, cellular signal transduction pathways regulating the expression of AMPs and pro-inflammatory mediators largely overlap (16, 17), and it is unknown whether imbalances in these signaling pathways reflect the alterations in AMP and pro-inflammatory mediator expression.

To gain more insight in the role of the inducible antibacterial defense function of airway epithelial cells in COPD, we examined the antibacterial activity and expression of AMPs following acute bacterial exposure of cultured airway epithelial cells from mild-to-moderate COPD patients and (ex)smokers with a normal lung function (non-COPD). To understand the mechanism underling reduced AMP expression, we determined the effect of cigarette smoke on microbial-induced expression of AMPs, and the regulation of cellular signal transduction pathways.

MATERIALS AND METHODS

Primary bronchial epithelial cell cultures and stimuli

Primary bronchial epithelial cells (PBEC) were isolated from tumor-free resected lung tissue and cultured in an air liquid interface model (further referred to as ALI-PBEC) to obtain mucociliary-differentiated cultures, essentially as previously described (14, 18). PBEC cultures were used from a total of 28 patients, all undergoing lung resection surgery for lung cancer. Clinical history and lung function data were obtained from anonymized patients. Disease status was determined based on lung function according to the Global Initiative for Chronic Obstructive Lung Disease classification (19). The patients included 15 COPD and 13 non-COPD subjects (Tables 1 and 2 for indicated experiments). Both groups included

Table 1: Patient characteristics bacterial killing assay.

	COPD	non-COPD	p-value
N=	5	5	
Gender (Females/Males)	2/3	2/3	
Age, years	66±11	64±7	0.6
FEV_1 , % predicted	68±17	96±14	0.05
FEV_1/FVC %	58±7	78±4	< 0.01

Characteristics of COPD and non-COPD patients used the bacterial killing assay.

Table 2: Patient characteristics NTHi-induced AMP expression.

	COPD	non-COPD	p-value
N=	5	5	
Gender (Females/Males)	2/3	2/3	
Age, years	66±11	64±7	0.6
FEV_1 , % predicted	68±17	96±14	0.05
FEV_1/FVC %	58±7	78±4	< 0.01

Characteristics of COPD and non-COPD patients. Age and lung function are shown as means ± SEM. The mean differences in FEV_1 (% predicted) and FEV_1/FVC (%) were compared using the non-parametric Mann-Whitney test. Abbreviations: COPD = chronic obstructive pulmonary disease, FEV_1= Forced expiratory volume in one second, FVC = Forced vital capacity.

current smokers and ex-smokers. Non-typeable *Haemophilus influenzae* (NTHi) strain D1 (20) was cultured and UV-inactivated as described earlier (14). UV-inactivated NTHi was applied to the apical surface of ALI-PBEC in a volume of 100 µl; PBS was used for dilutions and as negative control. ALI-PBEC were exposed to mainstream cigarette smoke (CS) from 3R4F reference cigarettes (University of Kentucky, Lexington, KY, USA) using an exposure model previously described (14, 15). In brief, epithelial cultures were placed in air (control) or CS exposure chambers, localized in a tissue incubator at 37°C and 5% CO2. In these exposure chambers, ALI-PBEC were respectively exposed to air or CS from one cigarette (approximately 2 mg of CS particles), which was infused by a mechanical pump with a continuous flow of 1 liter/minute for a period of 4-5 minutes. Residual CS was removed by flushing the chamber

with air derived from the incubator for 10 minutes. After exposure cells were stimulated at the apical surface with UV-inactivated NTHi or PBS (negative control).

Bacterial killing assay

The bacterial killing assay was adapted from Pezzulo *et al.* (21). Instead of golden grids, bacteria were linked to glass coverslips. 6 mm round coverslips were rinsed in ethanol, dried at room temperature and subsequently silanized with 2% (v/v) 3-Aminopropyltriethoxysilane (APTES, $H_2N(CH_2)_3Si(OC_2H_5)_3$, Sigma-Aldrich) solution in acetone for 10 seconds. After rinsing in H2O for 10 minutes and drying at room temperature, coverslips were immersed in 1 mM 11-mercaptoundecanoic acid (MUA, $HS(CH_2)_{10}COOH$, Sigma-Aldrich) for 30 minutes at room temperature. Afterwards, coverslips were incubated in 0.1 M N-hydroxysuccinimide (NHS) and 0.1 M 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (EDC) (1:2 molar ratio) (both Sigma-Aldrich) for 30 minutes, and subsequently coated for 30 minutes with 10 μg/mL streptavidin (Sigma Aldrich) in PBS. Next, the coverslips were washed and immersed with 1 M glycine for 30 minutes. $1*10^8$ CFU/ml (OD_{600}) of log phase growth cultured NTHi was incubated in 0.88 mg/ml mg/mL N-hydroxysulfosuccinimide-biotin (sulfo-NHS-biotin, Thermo Scientific) for 30 minutes on ice. Next, biotin-labeled NTHi was linked to the surface of the streptavidin-coated glass coverslips in PBS for 30 minutes and subsequently washed two times in PBS and finally in 0.01M Phosphate buffer pH 7.4, to remove the unbound bacteria. Bacterial killing activity was determined of ALI-PBEC that were pre-stimulated with UV-inactivated NTHi or PBS (untreated control), which was applied at the apical surface. After 6 hours of incubation, cultures were washed with PBS and incubated for 48 hours. Afterwards, NTHi coated coverslips were placed on the apical surface of ALI-PBEC for 1 minute. Coverslips were subsequently stained for 30 seconds with SYTO 9 and propidium iodide (both Life Technologies, Darmstadt, Germany), to visualize live and dead bacteria respectively. Coverslips were mounted on microscopic slides and covered in Baclight mounting oil (Life Technologies). Digital images were made with a Zeiss Axio Scope A1 fluorescent microscope and Zeiss Axiocam mRc 5 camera (Carl Zeiss Microscopy, Göttingen, Germany). The number of live and dead bacterial was analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA). The bacterial killing activity was determined by calculating the percentage of dead bacteria.

Microarray gene expression analysis

Primary bronchial epithelial cells from 6 different donors were cultured in submerged conditions as previously described (14). Cells were left unstimulated or triggered with UV-killed NTHi. RNA from these cultures were then isolated at two timepoints: early (6 hour) and late (24 hour) and subsequently subject to whole genome profiling of gene expression by microarray (Affymetrix GeneTitan platform).

Quantitative real-time PCR (qPCR)

RNA was isolated from ALI-PBEC using the miRNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. RNA quantities were determined using the Nanodrop ND-1000 UV-visible (UV-Vis) spectrophotometer (Nanodrop Technologies, Wilmington, DE) and cDNA was synthesized by reverse transcription polymerase chain reaction (PCR) of 1 μg of RNA, using oligo(dT) primers (Qiagen) and Moloney murine leukemia virus

(M-MLV) polymerase (Promega, Leiden, The Netherlands). qPCR was done using IQ SYBR green supermix (Bio-Rad) and a CFX-384 real-time PCR detection system (Bio-Rad). qPCR reactions were performed using the primers shown in Table 3. The housekeeping genes RPL13A and ATP5B were selected using the "Genorm method" (Genorm; Primer Design, Southampton, United Kingdom). Bio-Rad CFX manager 3.0 software (Bio-Rad) was used to calculate arbitrary gene expression by using the standard curve method.

Table 3: qPCR primer sequences

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
DEFB4	ATCAGCCATGAGGGTCTTG	GCAGCATTTTGTTCCAGG
CCL20	GCAAGCAACTTTGACTGCTG	TGGGCTATGTCCAATTCCAT
LCN2	CCTCAGACCTGATCCCAGC	CAGGACGGAGGTGACATTGTA
S100A7	ACGTGATGACAAGATTGACAAGC	GCGAGGTAATTTGTGCCCTTT
TLR2	TCTCGCAGTTCCAAACATTCCAC	TTTATCGTCTTCCTGCTTCAAGCC
ATF3	CCTCTGCGCTGGAATCAGTC	TTCTTTCTCGTCGCCTCTTTTT
pri-mir-147b	TTCATGACTGTGGCGGCGGG	GGCGAGGGCTCGTCATTTGGT
A20	TCCTCAGGCTTTGTATTTGAGC	TGTGTATCGGTGCATGGTTTTA
DEFB3	AGCCTAGCAGCTATGAGGATC	CTTCGGCAGCATTTTGCGCCA
CAMP	TCATTGCCCAGGTCCTCAG	TCCCCATACACCGCTTCAC
SLPI	GAGATGTTGTCCTGACACTTGTG	AGGCTTCCTCCTTGTTGGGT
DEFB1	ATGAGAACTTCCTACCTTCTGCT	TCTGTAACAGGTGCCTTGAATTT
IL8	CAGCCTTCCTGATTTCTG	CACTTCTCCACAACCCTCTGC
IL6	CAGAGCTGTGCAGATGAGTACA	GATGAGTTGTCATGTCCTGCAG
NFKBIA	TGTGCTTCGAGTGACTGACC	TCACCCCACATCACTGAACG
ZC3H12A	GTACGTCTCCCAGGATTGCC	GGGACTGTAGCCCGTGTAAG
NFKBIZ	AGAGGCCCCTTTCAAGGTGT	TCCATCAGACAACGAATCGGG
FOS	CCTAACCGCCACGATGATGT	TCTGCGGGTGAGTGGTAGTA
JUN	TCCTGCCCAGTGTTGTTTGT	GACTTCTCAGTGGGCTGTCC
FOSL1	AACCGGAGGAAGGAACTGAC	CTGCAGCCCAGATTTCTCAT
RPL13A	AAGGTGGTGGTCGTACGCTGTG	CGGGAAGGGTTGGTGTTCATCC
ATP5B	TCACCCAGGCTGGTTCAGA	AGTGGCCAGGGTAGGCTGAT

ELISA

Secretion of innate immune mediators by ALI-PBEC was determined in the apical surface liquid, collected by washing the apical surface with 100 µl PBS for 15 minutes, and in the basal medium as indicated. The secretion of IL-8 (Sanquin, Amsterdam, The Netherlands) and CCL20 (R&D, Minneapolis, MN, USA) was determined by ELISA following the manufacturer's protocol. Reagent for hBD-2 detection was a generous gift of D. Proud (Calgary, Canada), and the hBD-2 ELISA was conducted as previously described (22). The optical density values were measured with a microplate reader (Bio-Rad).

Trans-epithelial electrical resistance

The epithelial barrier integrity of ALI-PBEC cultures was determined by measuring the trans-epithelial electrical resistance (TEER) using the MilliCell-ERS (Millipore, Bedford, MA, USA).

Western blot

ALI-PBEC were washed 3 times with cold PBS and cell lysis was done in lysis buffer consisting of 150 mM NaCl, 50 mM Tris HCl pH 7.4, 0.1% NP-40 (v/v) and EDTA-free protease inhibitor cocktail (Roche, Basel, Switserland) . Samples were subsequently mixed with sample buffer, consisting of 0.5 M Tris pH 6.8, 10% SDS (w/v), 20% Glycerol (v/v), 0.02% Bromophenolblue and 50 mM DTT. Nuclear fractions were isolated using the NE-PER Nuclear Protein Extraction Kit (Thermo Scientific, Rockford, IL) according to the manufactur's protocol. Protein samples were separated by SDS-PAGE gel electrophoresis on 10% glycine-based gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 5% (w/v) skim-milk (Sigma-Aldrich) in PBS/0.05% (v/v) Tween-20 and stained with primary antibodies in 5% (w/v) BSA PBS/0.05% (v/v) Tween-20 overnight at 4°C. Antibodies were used to detect p- IKKα/β, IKKβ, IκB-α, p- and t-ERK1/2, p- and t-p38, TBP (all Cell Signaling, Beverly, MA, USA), p50, p65 (both Santa Cruz), and β-actin (Leica), Next, membranes were stained with secondary HRP-labelled antibody (Sigma-Aldrich) in 5% (w/v) skim-milk (Sigma-Aldrich) in PBS/0.05% (v/v) Tween-20 for 1 hour. Afterwards, membranes were developed with enhanced chemiluminescence substrate (Thermo Scientific). Intensity of bands were quantified by densitometry using Image J software (National Institutes of Health, Bethesda, MD, USA)

Immunofluorescence confocal microscopy

ALI-PBEC were fixed in 4% paraformaldehyde for 30 minutes, permeabilized in 100% methanol for 10 minutes at 4°C, and blocked in blocking solution consisting of 5% (w/v) BSA, 0.3% (v/v) Triton X in PBS. In between each step, cells were washed 3 times with PBS. Afterwards, the PBEC-containing filters were cut from the Transwell using a razorblade and subsequently incubated overnight at 4°C with anti-mouse p50 or anti-rabbit p65 primary antibodies (both Santa Cruz Biotechnology, Santa Cruz, CA) diluted in blocking solution. Next, filters were washed 3 times in PBS and stained with goat anti-rabbit AlexaFluor 568 or goat anti-mouse AlexaFluor 488 (both Life Technologies) respectively, and DAPI as nuclear staining. Secondary antibodies and DAPI were diluted in blocking solution and incubated in the dark for 30 minutes at room temperature. Afterwards, the filters were washed three times with PBS and mounted on coverslips with Vectashield Hard Set Mounting Medium (Vector Lab, Burlingame, CA, USA). Images were made using a Leica TCS SP5 confocal inverted microscope (Leica Microsystems) and processed using the Leica Application Suite Advanced Fluorescence software (LAS AF; Leica Microsystems).

Data analysis

Statistical analysis was performed in GraphPad PRISM 6.0 (GraphPad Software Inc., La Jolla, Ca). Analysis of differences was conducted with a one/two-way repeated measurements ANOVA and Bonferroni post-hoc test and (un)paired Student's t-test as indicated. Differences were considered significant with p-values < 0.05.

RESULTS

Lower NTHi-induced antibacterial activity by COPD airway epithelial cells

We first determined the bacterial killing activity of cultured ALI-PBEC from COPD patients and non-COPD (ex)smokers. ALI-PBEC were first stimulated at the apical surface with UV-inactivated NTHi or PBS (negative control) to investigate microbial exposure-induced and baseline antibacterial activity. 6 hours after stimulation, the apical surface was washed and cells were incubated for an additional 42 hours prior to assessment of the antibacterial activity (Figure 1A). A killing assay was used that allows direct assessment of the antibacterial activity of the airway surface liquid (ASL) of cultured ALI-PBEC by placing NTHi-coated coverslips

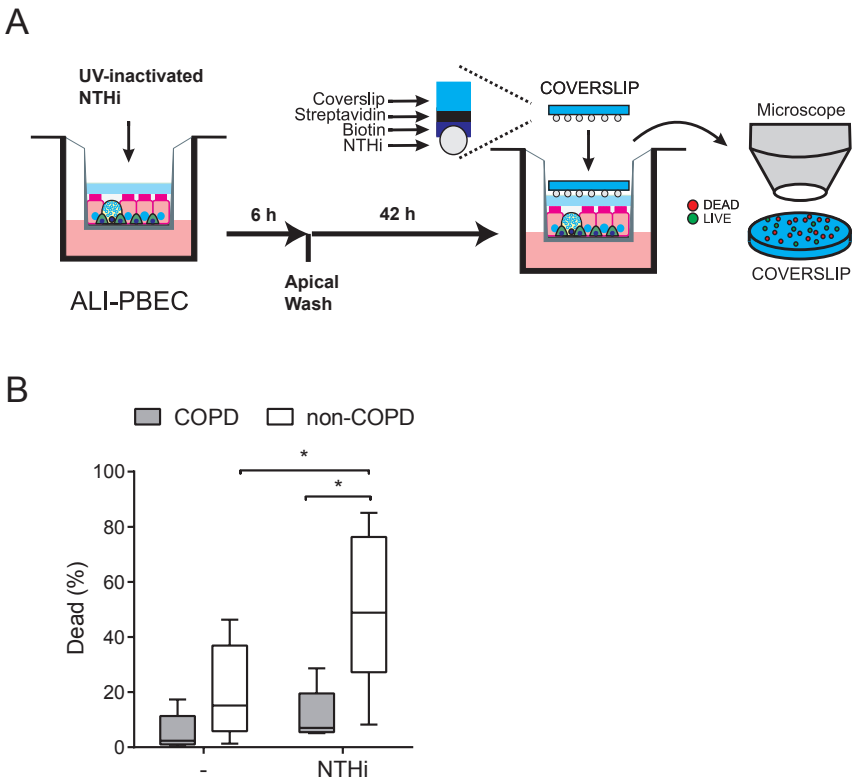

Figure 1. Impaired bacterial killing by COPD ALI-PBEC. (A) Schematic representation of the bacterial killing assay. ALI-PBEC cultures were stimulated with $0.5*10^9$ CFU/ml UV-inactivated NTHi or PBS as negative control for 6 h, washed at the apical surface, and incubated for 42 h. Next, streptavidin-coated glass coverslips linked to biotin-bound NTHi were placed on the apical surface of ALI-PBEC. Bacterial killing was determined by counting individual live and dead bacteria. (B) Bacterial killing was assessed of cultured ALI-PBEC from COPD patients (grey boxplots, n=5 patients) and non-COPD smokers (white boxplots, n=5 patients), either unstimulated or stimulated with $0.5*10^9$ CFU/ml UV-inactivated NTHi. Data is shown as % dead bacteria. The killing assay was performed in triplicates. COPD and non-COPD comparison results were depicted as boxplot with whiskers from minimum to maximum or bars (means ± SEM). Analysis of differences was conducted with a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$.

on the apical surface. Minimal antibacterial activity was seen in control-treated ALI-PBEC from both COPD and non-COPD donors (Figure 1B). Exposure to NTHi significantly increased antibacterial activity of non-COPD cultures. In contrast, this increase was not observed in COPD epithelia. These findings suggest that cultured airway epithelial cells from COPD patients have reduced microbial-induced antibacterial activity compared to cell cultures from non-COPD (ex)smokers.

NTHi-induced expression of hBD-2 and S100A7 is altered in COPD ALI-PBEC

To investigate the role of AMPs in the reduced antibacterial activity of COPD airway epithelial cells, we examined the expression of microbial-induced AMPs in COPD and non-COPD ALI-PBEC exposed to various concentrations of UV-inactivated NTHi for 24 hours. We first focused for this analysis on microbial-induced AMPs that were found to be highly expressed in submerged cultured and undifferentiated PBEC upon exposure to UV-inactivated NTHi, based on a micro-array gene expression analysis (Supplementary Table 1). These genes included *DEFB4*, encoding human β-defensin-2 (hBD-2), the antimicrobial chemokine *CCL20*, lipocalin 2 (*LCN2*), and *S100A7* (23-26). NTHi stimulation induced the expression of all examined genes in both COPD and non-COPD cultures (Figure 2A-D) However, COPD ALI-PBEC displayed lower NTHi-mediated expression of *DEFB4* and *S100A7* compared to non-COPD cultures (Figure 2A,B), whereas *LCN2* and *CCL20* did not reveal statistically significant differences (Figure 2C,D). We also did not observe differences in the expression of other AMPs that were not observed in the micro-array gene expression analysis (LL-37/CAMP, SLPI, β-defensin 1 and 3 [hBD-1 and hBD-3]) (Supplementary Figure 1). We further analyzed the NTHi-induced protein secretion of hBD-2 and CCL20 in apical washes and basal medium of COPD and non-COPD ALI-PBEC 24 hours after stimulation. In contrast to the mRNA expression, we did not detect a difference in hBD-2 peptide release at the apical surface and basal medium between COPD and non-COPD ALI-PBEC (Figure 2E,F). Moreover, no differences were observed in secretion of CCL20. To explain this discrepancy between hBD-2 mRNA expression and peptide levels, we examined whether the difference in mRNA expression was time-dependent. Indeed, in contrast to NTHi-induced mRNA expression at 24 hours, we did not detect differences between COPD and non-COPD at 3 and 12 hours of stimulation (Figure 3A-D). These findings suggest that the attenuated antibacterial defense of COPD airway epithelial cells observed 48 h after microbial stimulation was not accompanied by differences in the early effects of microbial stimuli on AMP expression, but possibly related to lower expression of hBD-2 and *S100A7* in COPD cultures only at later time points.

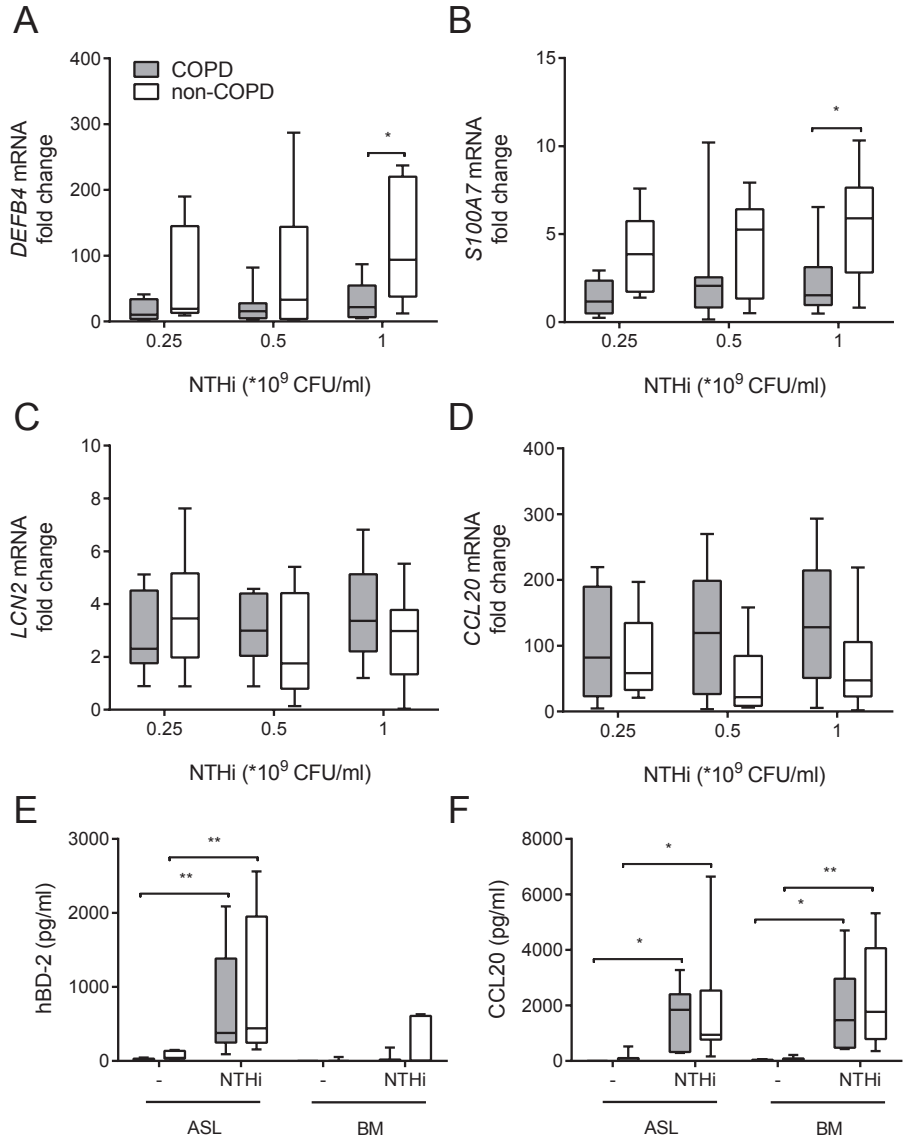

Figure 2. AMPs expression by COPD ALI-PBEC is lower compared to non-COPD. COPD (grey boxplots, n=12 patients) and non-COPD (white boxplots, n=8 patients) ALI-PBEC were stimulated with different concentrations of UV-inactivated NTHi for 24 hours. mRNA expression of the AMPs: (A) *DEFB4*/hBD-2, (B) *S100A7*, (C) *LCN2* and (D) *CCL20* was assessed by qPCR. Stimulations were performed in duplicates. Data is shown as fold change in mRNA compared to untreated cells. Assessment of (E) hBD-2 and (F) CCL20 protein secretion in the apical surface liquid (ASL) and basal medium (BM) of COPD (grey boxplots, n=9) and non-COPD (white boxplots, n=7-8) ALI-PBEC stimulated with 1*10^9 CFU/ml UV-inactivated NTHi for 24 hours. Stimulations were performed in duplicates. Results are shown as boxplot with whiskers from minimum to maximum or bars (means ± SEM). Analysis of differences was conducted with a two-way ANOVA and Bonferroni post-hoc test. * p<0.05, ** p<0.01, *** p<0.001.* p<0.05.

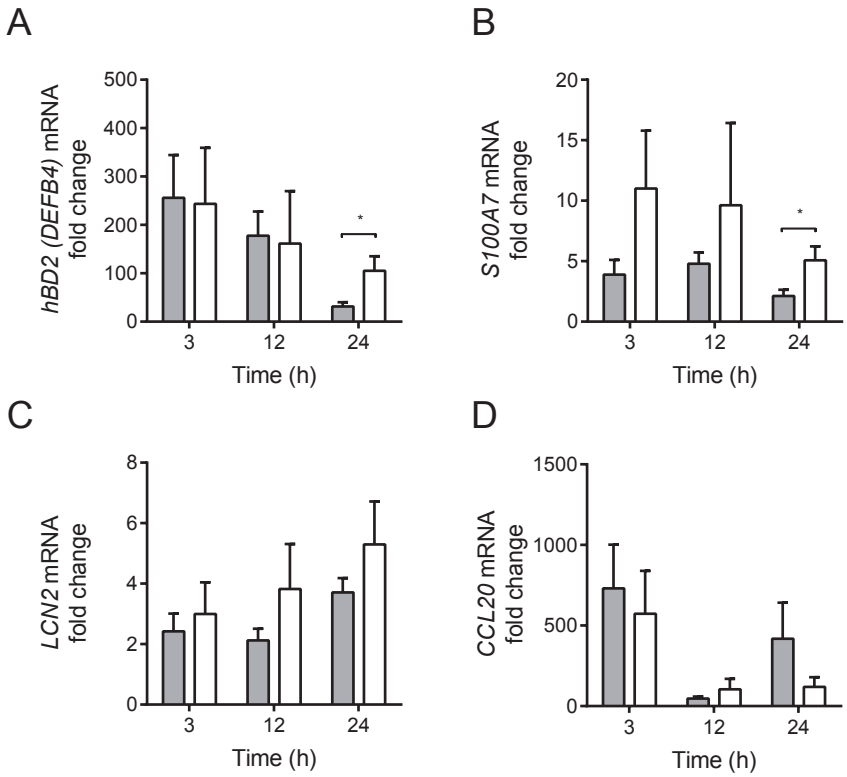

Figure 3. Differences in early- and late-induced transcriptional responses between COPD and non-COPD ALI-PBEC. Time course of NTHi-induced mRNA expression in COPD (grey bars, n=12) and non-COPD AL-PBEC (white bars, n=9). COPD and non-COPD ALI-PBEC were stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 3, 12 and 24 hours, afterwards mRNA expression of (A) *DEFB4*/hBD-2, (B) *S100A7*, (C) *LCN2* and (D) *CCL20* was examined by qPCR. Stimulations were performed in duplicates. Data is shown as fold change compared to unstimulated cells. All results are depicted as mean ± SEM. Analysis of differences was conducted with an unpaired t-test.* $p<0.05$.

Cell differentiation, barrier function and expression of regulatory genes do not differ between COPD and non-COPD ALI-PBEC

We further studied the cause of the impaired expression of AMPs in COPD airway epithelial cells. Previous research has demonstrated a reduced host defense function of airway epithelial cells from severe COPD patients due to impaired cell differentiation (5, 27). In addition, it was reported that COPD airway epithelial cells display a reduced barrier function (4). Therefore, we examined cell differentiation and the epithelial barrier integrity of COPD and non-COPD ALI-PBEC. In contrast to previous findings, we did not observed differences in mRNA expression levels of the club cell marker *SCGB1A1*, ciliated cell marker *FOXJ1*, goblet cell marker *MUC5AC*, and basal cell marker *TP63* (Figure 4A-D). Moreover, we did not observed differences in the epithelial barrier function between COPD and non-COPD ALI-PBEC, based on trans-epithelial electrical resistance (TEER) measurements (Figure 4E). COPD

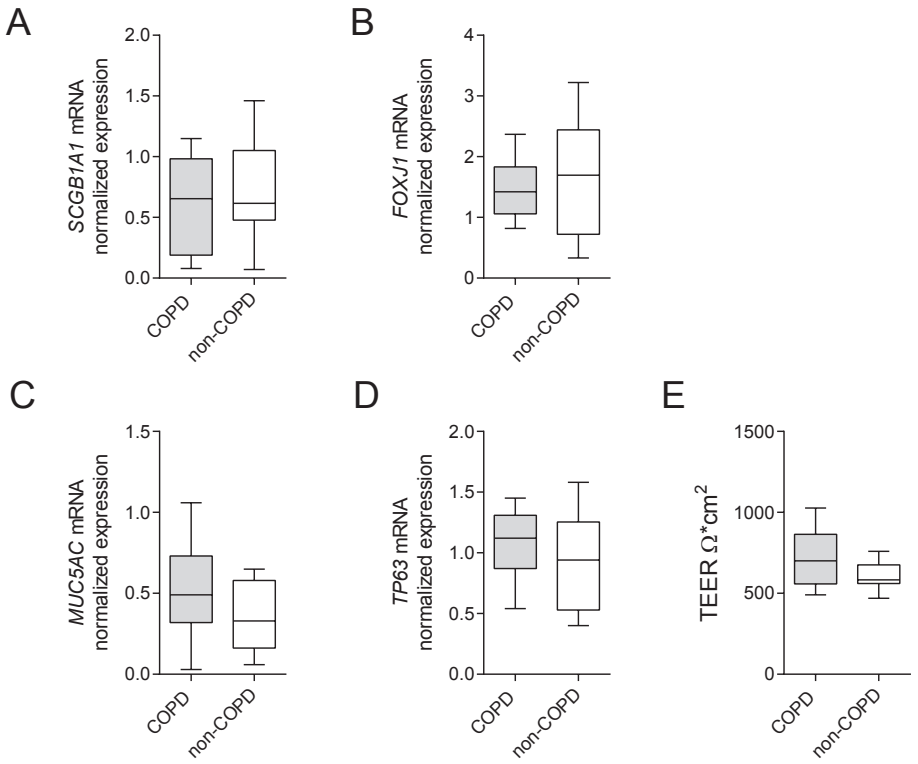

Figure 4. Expression of epithelial differentiation markers and barrier function in COPD and non-COPD ALI-PBEC. Baseline mRNA expression of the cell differentiation markers: (A) *SCGB1A1* (club cell), (B) *FOXJ1* (ciliated cell), (C) *MUC5AC* (goblet cell), and (D) TP63 (basal cell), was determined in differentiated ALI-PBEC from COPD (grey boxplots, n=11 patients) and non-COPD (white boxplots, n=8 patients) ALI-PBEC. Data is shown as normalized values. (E) The epithelial barrier integrity of COPD (grey boxplots, n=9 patients) and non-COPD (white boxplots, n=7 patients) ALI-PBEC was determined by measuring the trans-epithelial electrical resistance (TEER). TEER values are shown as Ω*cm2. ALL results are shown as boxplot with whiskers from minimum to maximum or bars (means ± SEM). Analysis of differences was conducted with a two-way ANOVA and Bonferroni post-hoc test.

ALI-PBEC furthermore did not display low TEER values as observed in a previous study (4). These findings suggest that the altered NTHi-induced antibacterial defense of COPD airway epithelial cells is not caused by impaired cell differentiation or a reduced barrier function. Another possible explanation for the persistence of this difference after prolonged culture could be the presence of epigenetic modifications in the epithelium from COPD patients. These modifications can lead to a variety of changes that may help to explain the observed differences. First, this differential expression might be caused by altered expression of pattern recognition receptors at later time points. It has been shown that NTHi increases expression of *TLR2* (28), and impairment of this induction in cells from COPD patients can influence late-induced innate immune responses. However, we did not observe differences in *TLR2* mRNA expression between COPD and non-COPD cultures (Supplementary Figure 2). Second, alterations in negative-feedback loop mechanism of the NF-κB signaling pathway may also result in impaired expression of AMPs, such as differential expression of micro-RNA's, like

mir-147b, the deubiquitinating enzyme/ ubiquitin ligase A20, or the transcription factor ATF3 (29-31). However, we furthermore did not observe differences in the expression of the primary mir-147b transcript, and expression of *A20* and *ATF3* mRNA (Supplementary Figure 2). This suggest that other mechanism underlie the altered microbial-induced expression of AMPs in COPD airway epithelial cultures.

Cigarette smoke differentially affects the induction of AMPs and pro-inflammatory mediators in ALI-PBEC

We next set out to investigate the effect of cigarette smoke (CS) exposure on the expression of AMPs and pro-inflammatory mediators. This was done using a whole CS exposure model in which ALI-PBEC are exposed to mainstream smoke derived from one cigarette or room air (AIR) as negative control. The exposure is done for a period of 15 minutes, after which the cells are stimulated with UV-inactivated NTHi for 3, 12, and 24 hours. After the indicated time points mRNA expression was measured by qPCR. Corresponding with earlier reports (12-14), CS exposure significantly reduced the NTHi-mediated expression of *DEFB4* at 3 and 12 hours after exposure (Figure 5A). This effect was no longer observed at 24 hours after exposure. CS also attenuated the NTHi-induced expression of *LCN2*, *S100A7* and *CCL20* after 3 hours (Figure 5B-D). In accordance with preceding studies (14, 15), we observed a further increase in IL-8 and IL-6 mRNA expression upon CS and NTHi co-stimulation (Figure 5E,F). We also found decreased NTHi-induced hBD-2 and CCL20 protein secretion in CS exposed cultures (Figure 5G,H), whereas IL-8 secretion was not attenuated and further enhanced (Figure 5I). Overall, this data demonstrates that CS exposure differentially modulates microbial-induced innate immunity, impairing induction of AMPs while increasing pro-inflammatory mediators.

Cigarette smoke impairs NTHi-induced expression of AMPs in both COPD and non-COPD ALI-PBEC

Next, we determined the effect of CS on COPD and non-COPD ALI-PBEC. As we observed an acute effect of CS exposure, which was primarily seen at 3 hours after exposure, we studied mRNA expression levels of AMPs at this particular time point. AMP expression was reduced by smoke in both COPD and non-COPD cultures, thus suggesting that attenuation of AMP expression in our CS exposure model occurs independent of disease status (Figure 6A-D). We furthermore did not observe a significant difference in IL-8 mRNA expression between CS and NTHi exposed COPD and non-COPD ALI-PBEC (Figure 6E). This suggests that modulation of the expression of AMPs and pro-inflammatory mediators by CS occurs in a similar extent in COPD and non-COPD ALI-PBEC cultures.

Cigarette smoke inhibits NTHi-mediated activation of NF-κB but not MAPK signaling in ALI-PBEC

We subsequently examined the underlying mechanism of the differential regulation of AMPs and pro-inflammatory mediators by CS. Microbial- and cytokine-mediated expression of AMPs and pro-inflammatory mediators are regulated in particular by the NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways (16, 17). Therefore, we examined the effect of CS exposure on NF-κB and MAPK signal transduction in ALI-PBEC. Epithelial cultures were exposed to CS or air as control and subsequently stimulated with UV-

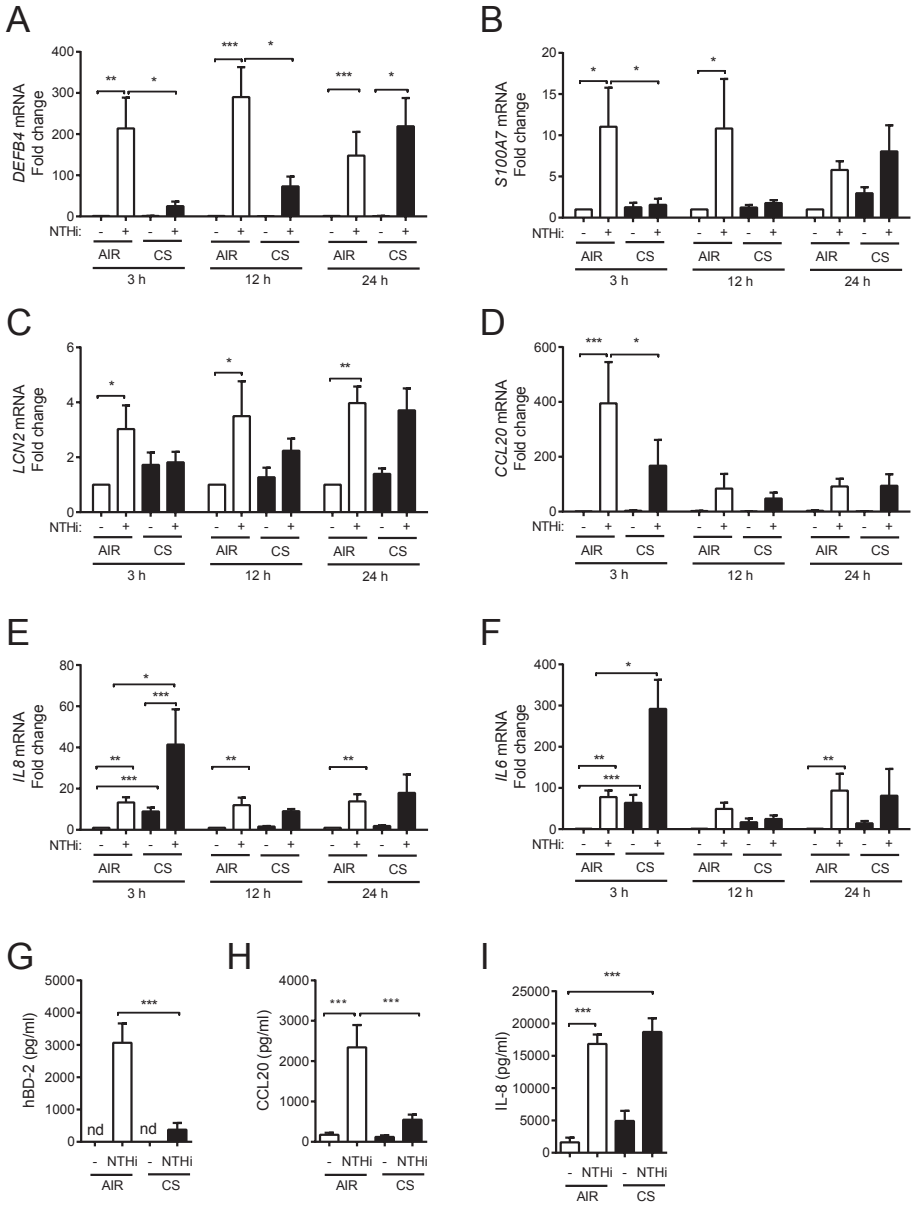

Figure 5. Cigarette smoke differentially modulates innate immune gene expression. ALI-PBEC (n=7) were exposure to AIR or CS and subsequently stimulated with 1*10^9 CFU/ml UV-inactivated NTHi for 3, 12, and 24 hours. mRNA expression of (A) *DEFB4*, (B) *S100A7*, (C) *LCN2*, (D) *CCL20*, (E) *IL8* and (F) *IL6* was measured by qPCR. Stimulations were performed in duplicates. Data is shown as fold change in mRNA compared to untreated cells. Assessment of (G) hBD-2 secretion in the apical surface liquid and (H) CCL20 and (I) IL-8 secretion in the basal medium of AIR/CS exposed ALI-PBEC (n=7) stimulated with 1*10^9 CFU/ml UV-inactivated NTHi. Stimulations were performed in duplicates. All results are shown as mean ± SEM. Analysis of differences was conducted with a one-way ANOVA and Bonferroni post-hoc test (A-F), and paired t-test (G-I). * p<0.05, ** p<0.01, *** p<0.001.

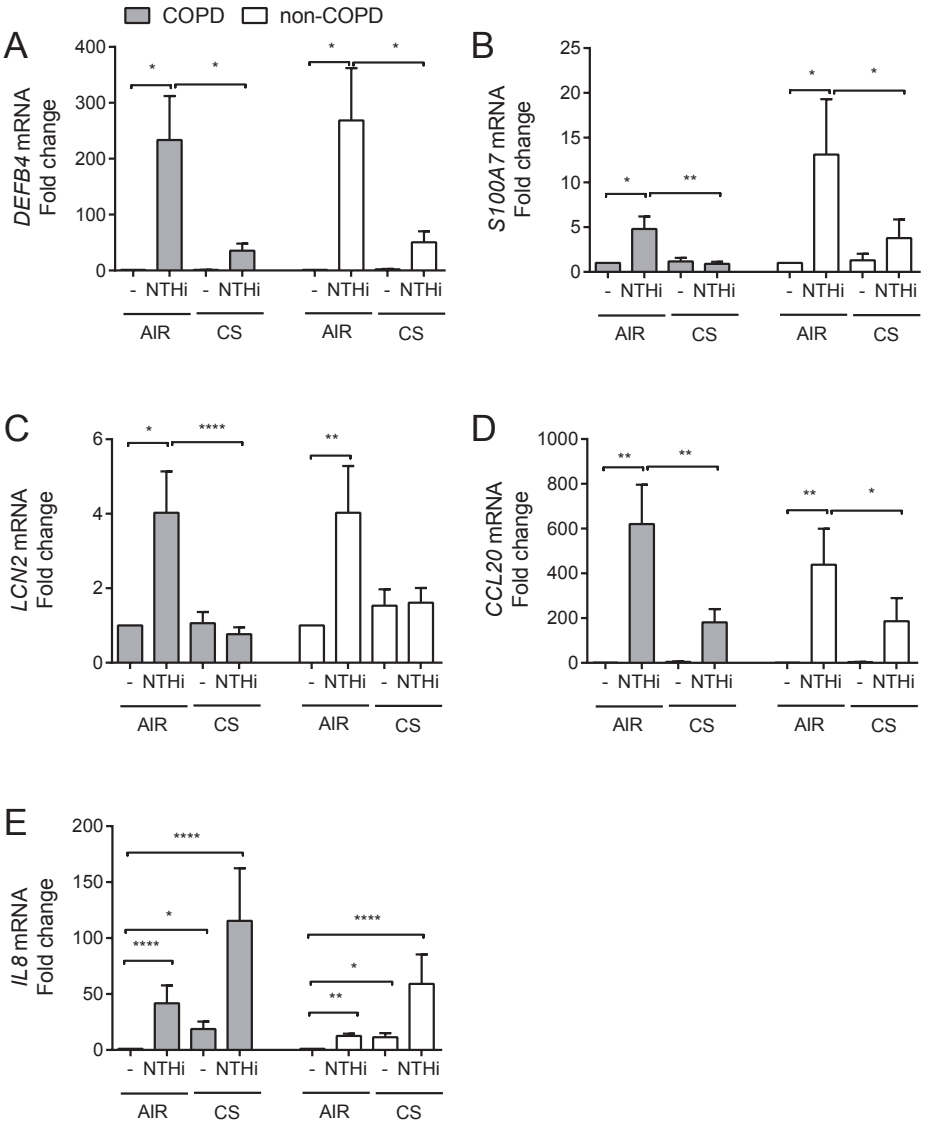

Figure 6. Suppression of AMPs by CS in both COPD and non-COPD ALI-PBEC. COPD (grey bars, n=12 patients) and non-COPD (white bars, n=8 patients) ALI-PBEC were exposure to AIR or CS and subsequently stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 3 hours. mRNA expression of (A) *DEFB4*, (B) *S100A7,* (C) *LCN2*, (D) *CCL20*, and (E) *IL8* was determined by qPCR. Stimulations were performed in duplicates. Data is shown as fold change in mRNA compared to untreated cells and depicted as mean ± SEM. Analysis of differences was conducted with a paired t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

inactivated NTHi at the apical surface for 30 minutes. Activation of NF-κB was determined by assessing phosphorylation of the upstream NF-κB signaling kinases IKKα/β and degradation of the NF-κB inhibitor IκB-α. MAPK signaling was evaluated by assessing phosphorylation of p38 and ERK1/2. NTHi stimulation of air-exposed cultures resulted in enhanced activation of NF-κB signal transduction based on phosphorylation of IKKα/β and degradation of the NFκB inhibitor IκB-α (Figure 7A-C). In contrast, CS exposure suppressed IKKα/β phosphorylation and IκB-α degradation. Both NTHi and CS induced phosphorylation of the MAPKs ERK1/2 and p38, and CS exposure did not attenuate the NTHi-mediated phosphorylation of ERK1/2 and p38 (Figure 7A,D,E). These findings demonstrate that CS alters NF-κB and MAPK signaling, which corresponds with attenuated expression of AMPs and enhanced expression of pro-inflammatory mediators.

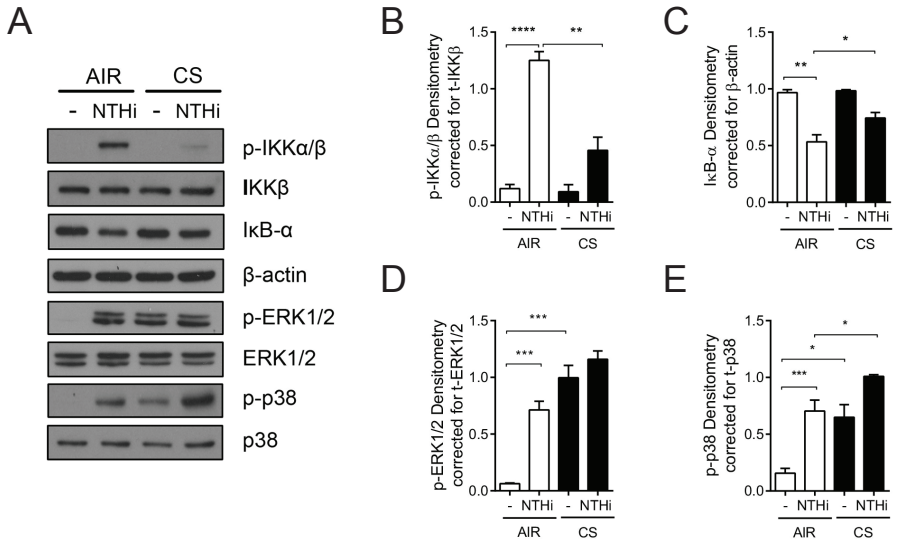

Figure 7. CS impairs NTHi-induced NF-κB but not MAPK signal transduction in ALI-PBEC. (A) ALI-PBEC (n=4-8) were exposed to AIR/CS and stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 30 minutes. NF-κB activation was assessed by measuring phosphorylation of IKKα/β, and degradation of IκB-α. MAPK signaling was assessed by determining phosphorylation of ERK1/2 and p38. (B) Analysis of data by densitometry. Results are shown as mean ± SEM. Analysis of differences was conducted with a paired t-test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Cigarette smoke suppresses NF-κB-mediated transcriptional activity

To further assess whether alterations in NF-κB signaling corresponds with reduced AMPs expression we investigated nuclear translocation of NF-κB and transcription of early induced target genes. In agreement with impaired IκB-α degradation, CS inhibited nuclear translocation of the p50 and p65 NF-κB subunit, as shown by immunofluorescence staining and analysis of isolated nuclear fractions (Figure 8A-C). To validate whether this reduced nuclear localization impaired NF-κB-mediated transcription, we examined the expression of early induced NF-κB-target genes, including the negative feedback regulators *NFKBIA* and *ZC3H12A* (32), and *NFKBIZ*, which represent an essential co-transcription factor required

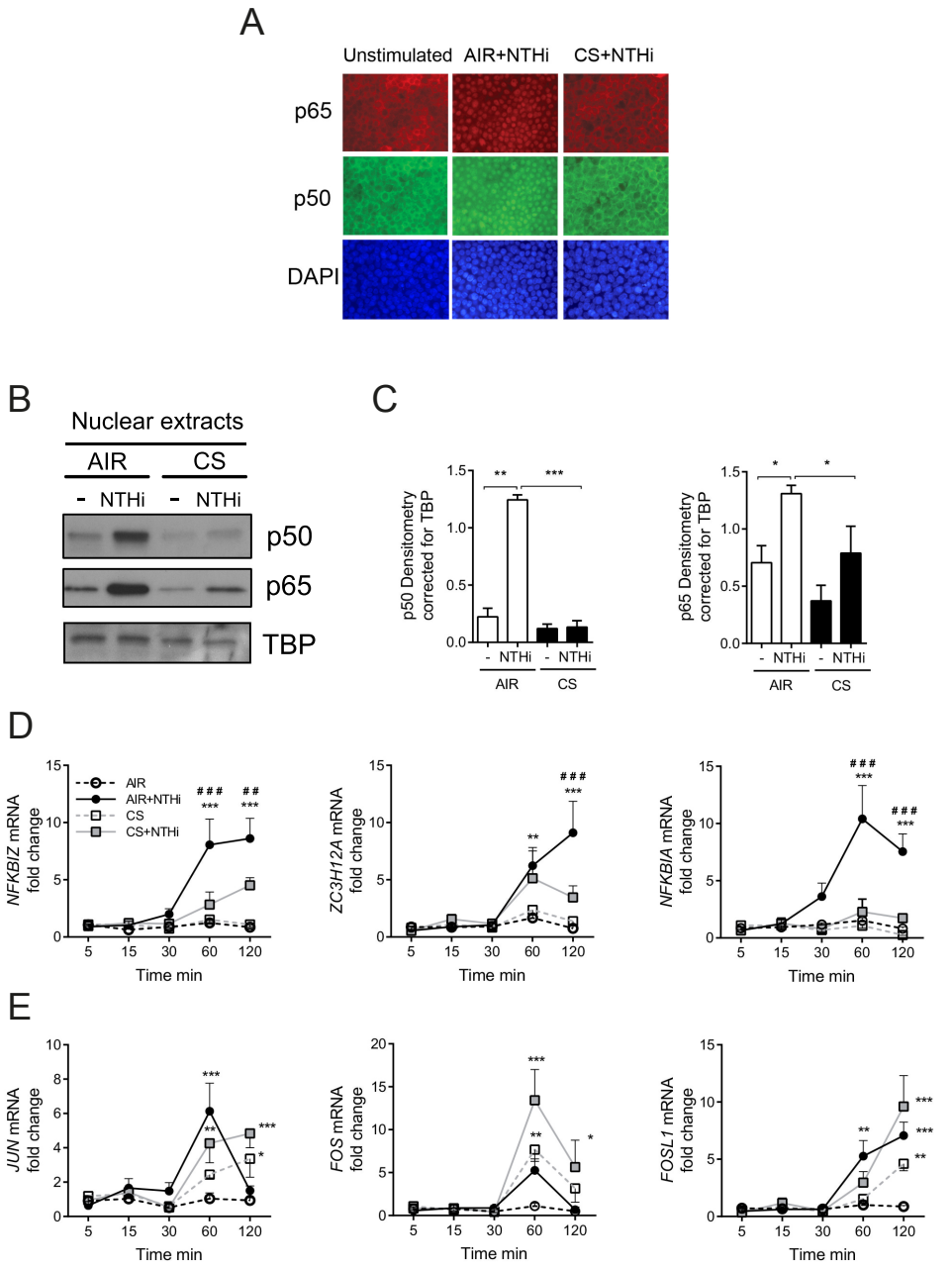

Figure 8. CS impairs NF-κB transcriptional activity by ALI-PBEC. ALI-PBEC were left untreated or exposed to AIR or CS and stimulated with UV-inactivated NTHi for 1 h. (A) Cellular localization of the NF-κB subunits p50 and p65 was determined by immunofluorescence microscopy. Data shown represent n=3 independent donors. (B) ALI-PBEC (n=4) were exposed to AIR or CS and stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 1 hour. Protein expression of p50 and p65 was measured in isolated nuclear extracts. (C) Data was quantified by densitometry. ALI-PBEC were unstimulated or exposed to AIR or CS and stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi

for 1 hour. ALI-PBEC (n=4) were exposure to AIR or CS and subsequently stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 5, 15, 30, 60 and 120 minutes. mRNA expression of (D) early induced NF-κB-target genes: *NFKBIA*, *ZC3H12A* and *NFKBIZ*, and (E) early induced MAPK-target genes *FOS*, *JUN* and *FOSL1*, was determined by qPCR. Data is shown as fold change in mRNA compared to air exposed cells. All results are shown as mean ± SEM. Analysis of differences was conducted with a paired t-test (C) and two-way ANOVA with Bonferroni post-hoc test (D,E). Significant different compared to air: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Significant different compared to CS+NTHi: ## p<0.01, ### p<0.001.

for AMP expression (33). Moreover, induction of MAPK-regulated early induced target genes and AP-1 transcription factors: *FOS*, *JUN* and *FOSL1* (34) was determined. CS exposure attenuated the NTHi-induced expression of *NFKBIZ* and *NFKBIA* at 60 and 120 minutes after stimulation, whereas induction of *ZC3H12A* was impaired at 120 minutes (Figure 8D). In contrast, CS exposure further increased the induction of FOS at 60 and 120 minutes, and *JUN* and *FOSL1* expression after 120 minutes of stimulation (Figure 8E). Overall, these observations demonstrate selective attenuation of NF-κB-mediated transcriptional activity by CS, which reflects impaired expression of AMPs.

DISCUSSION

In this study we provide evidence that the antibacterial defense of airway epithelial cells from COPD patients is decreased compared to non-COPD (ex)smokers. In addition, we show that the impairment of microbial-induced expression of AMPs by CS exposure may be related to inhibition of NF-κB activation. The persistence of pro-inflammatory responses to microbial stimulation in presence of CS may be explained by enhanced MAPK signaling. In contrast to the impaired antibacterial activity of cystic fibrosis airway epithelial cells at steady state conditions (35, 36), COPD cultures only display an attenuated antibacterial activity upon microbial-stimulation, suggesting that the induction of antibacterial factors is impaired. Indeed, a previous study reported lower hBD-2 expression in airway epithelial tissues from COPD patients compared to non-COPD (ex)smokers (10). In line with this study, we detected lowered NTHi-mediated expression of hBD-2/*DEFB4* mRNA expression in cultured COPD airway epithelia. In addition, we also observed lower NTHi-induced *S100A7* mRNA by COPD epithelial cultures. In contrast to hBD-2, *S100A7* displays antibacterial activity by impairing bacterial acquisition of zinc (26), which suggests that expression of AMPs with different modes of action is affected in COPD. In contrast to the differential regulation of *DEFB4* at 24 hours after NTHi stimulation, we did not observe differences in mRNA levels at earlier time points and protein secretion at 24 hours. This suggests that COPD and non-COPD airway epithelial cells differ in late-induced mRNA expression.

It has been shown that ALI-PBEC cultures from severe COPD patients display impaired epithelial differentiation and epithelial barrier integrity, which causes impaired host defense (4, 5, 27). However, we did not observed differences in the mRNA expression of cell differentiation markers and the epithelial barrier function. It is therefore unlikely that altered epithelial differentiation is the underlying cause for the impaired antibacterial activity of COPD cultures observed in this study. In contrast to the airway epithelial cell cultures from mild-to-moderate COPD patients used in this study, impaired epithelial differentiation

in severe COPD may have an additional impact on antibacterial host defense. Impaired differentiation may for example lead to a loss of CFTR expression, which in turn may result in impaired activity of AMPs in the airway surface liquid (37).

We further hypothesized that the differences in late-induced mRNA expression between COPD and non-COPD airway epithelial cells might be caused by the differential expression of second messengers, post-transcriptional and/or post-translational mechanisms influencing these late-induced responses. TLR-2, pri-mir-147b, A20, and ATF3, are known regulators of microbial-induced innate immune responses that might be affected in COPD cultures and thereby explain the impaired antibacterial defense. Despite of this, we did not observe differences in the NTHi-induced mRNA expression of these regulators.

Differences in the antibacterial activity between COPD and non-COPD cell cultures were determined with an assay previously used to assess the antibacterial activity of cystic fibrosis airway epithelial cell cultures (21, 36). Conventional killing assays rely on collection of apical washes or inoculation of bacteria to the apical surface that may result respectively in selective collection of antimicrobial substances and dilution or modulation of the physiological conditions of the apical surface liquid. This may have a major influence on determining the antibacterial activity of epithelial cells, and the assay used in the present study is not affected by these factors.

Although it is tempting to speculate that a reduced antibacterial activity of COPD airway epithelial cells is due to a reduced expression of *DEFB4* and *S100A7*, a clear link between these findings is missing. Indeed, other antibacterial defense mechanisms may also be affected in the epithelium of COPD patients, and contribute to the reduced antibacterial activity. In addition to various AMPs, also antimicrobial lipids and reactive oxygen species contribute to the bacterial killing activity of the airway epithelium (38, 39). Moreover, lower antimicrobial activity might also result from other alterations in the airway surface liquid, such as reduced pH, and the presence of mucins, F-actin and proteoglycans (21, 40-44). Therefore we cannot exclude that changes in the presence or activity of other antibacterial mediators and changes in the composition of the airway surface liquid also contributed to the reduced antibacterial activity of the COPD airway epithelial cells.

In line with previous studies demonstrating reduced antibacterial activity and inhibition of hBD-2 expression caused by cigarette smoke (12-14), we showed that the expression of *S100A7*, *LCN2* and *CCL20* was also inhibited in cigarette smoke-exposed COPD and non-COPD cultures. In contrast to AMPs, we showed increased NTHi-induced expression of IL-8 and IL-6 upon CS exposure. This further demonstrates that AMPs and pro-inflammatory mediators are differentially regulated in airway epithelial cells. The modulation of innate immune responses by CS was primarily observed 3 h after exposure, which is in line with the transient effect of the acute exposure (14).

Our study has several limitations. Our findings are limited to the acute effects of microbial- and cigarette smoke on airway epithelial innate immune responses, and chronic exposures of airway epithelial cells to both NTHi and CS may cause different effects. Moreover, differences in smoking status of both COPD and non-COPD subjects may have an influence on our

findings. Current and ex-smokers were both included in the COPD and non-COPD group, however we only have limited information about i.e. pack years smoked. Further research regarding the influence of smoking status is therefore needed.

To assess whether alterations in AMPs and pro-inflammatory mediator expression are reflected by changes in cellular signal transduction pathways, we examined the effect of CS on NF-κB and MAPK signaling. Whereas CS increased MAPK signaling, it reduced NTHi-induced NF-κB signal transduction. NTHi-induced activation of MAPK and NF-κB is mediated by the common upstream signaling kinase TAK1, which directly phosphorylates IKKα/β (45). Therefore, we speculate that CS causes selective suppression of NF-κB signaling by directly modulating IKKα/β phosphorylation, rather than affecting TAK1 or more upstream signaling components (Supplementary Figure 3). This appears in contrast to some studies that have reported increased NF-κB activation in COPD lung tissue (46-48). However, this is not observed in all studies and it has been suggested that NF-κB signaling may not contribute to COPD pathogenesis (49). Furthermore, our results are also in line with results of a study showing that long-term passive cigarette smoke exposure inhibits UVB-induced NF-κB signaling in skin (50), and with earlier reports showing impaired IKKα/β phosphorylation and kinase activity caused by post-translational modifications, mediated for instance by oxidative stress (51, 52). Previous studies demonstrated the importance of NF-κB in both microbial-induced hBD-2 and IL-8 by airway epithelial cells. However, our findings suggest a more fundamental role of NF-κB in the antibacterial defense, whereas induction of IL-8 and IL-6 persisted independent of NF-κB. Restoration of NF-κB signaling may therefore improve the airway epithelial antibacterial defense in smokers. However, further research is required to study this.

As also discussed in the previous section, the role of NF-κB in COPD remains a matter of debate since inflammation in COPD may occur independent of NF-κB (49). Based on our findings, it can be speculated that MAPK signaling has a more prominent role in CS-induced airway epithelial inflammatory responses than NF-κB. This is furthermore supported by findings from a recent study examining ozone-induced pro-inflammatory responses by differentiated airway epithelial cells, revealing MAPK-dependent and NF-κB-independent induction of inflammatory mediators (53).

Our study focused on airway epithelial responses to NTHi, because of the critical role of this microbe in COPD pathogenesis (54). However, often multiple respiratory pathogens are isolated from the airways of COPD patients and these micro-organisms may affect epithelial cell function in a different way than NTHi. In recent years there is an increased awareness of the role of the microbiome in COPD development and progression (55). Cigarette smoke-induced changes in airway epithelial defense may affect the composition of this microbiome, and its altered composition may contribute to COPD development and progression. We reported in a previous study that cigarette smoke exposure increased expression of the antimicrobial protein RNase 7 in ALI-PBEC cultures (14). This raises the possibility that, in addition to a selective down-regulation of microbial-induced AMPs, expression of other AMPs might be increased by smoke exposure. These and other changes in airway epithelial defense may contribute to changes in the microbiome. However, further research is needed to study the role of AMPs in regulating the airway microbiome in COPD.

In summary, our findings demonstrate that cultured airway epithelial cells from COPD patients have reduced antibacterial activity. Moreover, we observed an imbalance between the protective antibacterial defense and destructive inflammatory innate immune response by airway epithelial cells from COPD patients and in response to CS. This imbalance explains in part the enhanced bacterial burden and increased lung inflammation observed in COPD development and progression. Therefore, application of exogenous AMPs to compensate for the loss of AMPs might have therapeutic potential in COPD (56). Furthermore, the differential regulation of AMPs and pro-inflammatory mediators at the signal transduction level suggest that selective therapeutic targeting of airway inflammation, without affecting the beneficial antibacterial defense, might be possible.

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SUPPLEMENTARY TABLES

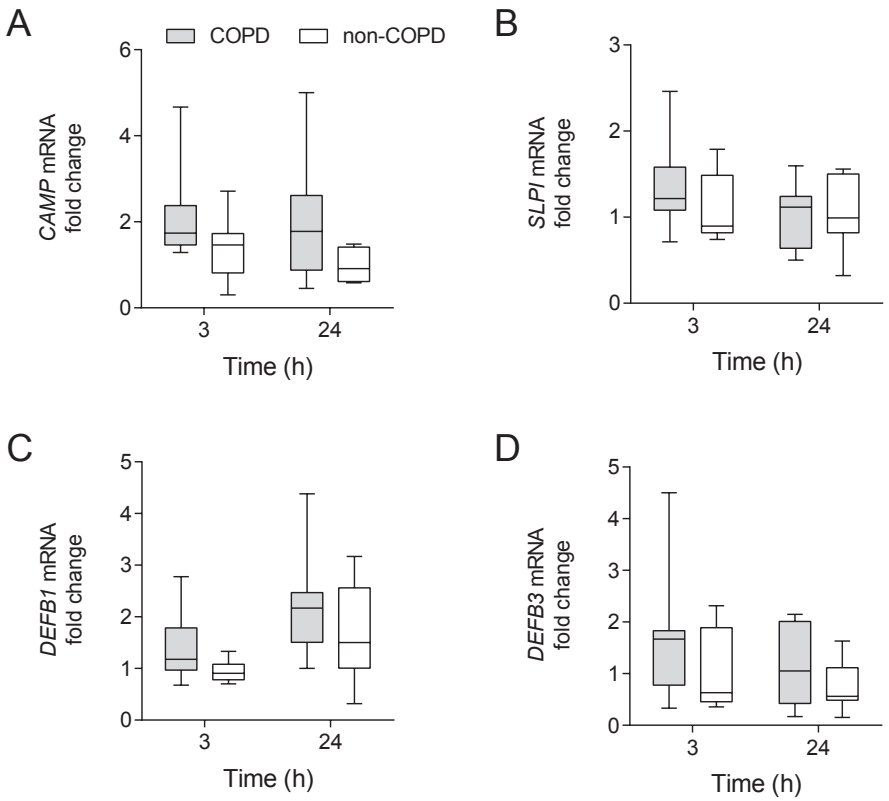
Supplementary Table 1: Top 25 most differentially expressed genes in NTHi stimulated PBEC

Gene Symbol	FC NTHi vs UNST (6 h)
DEFB4	*60.3*
CCL20	*33.0*
PLAT	21.3
BCL2A1	20.0
IL8	18.3
SPRR2C	16.2
LCE3D	15.9
CYP1A1	15.7
IL1R2	12.6
SPRR2G	12.1
C15orf48	11.9
MRGPX3	10.5
MMP9	8.5
TLR2	8.3
IL1F9	8.1
SOD2	7.5
CXCL3	7.0
IL13RA2	6.7
MMP1	6.3
LCN2	6.0
MMP10	5.9
ADH7	-5.3
S100A7	5.2
G0S2	5.0
APOBEC3A	5.0

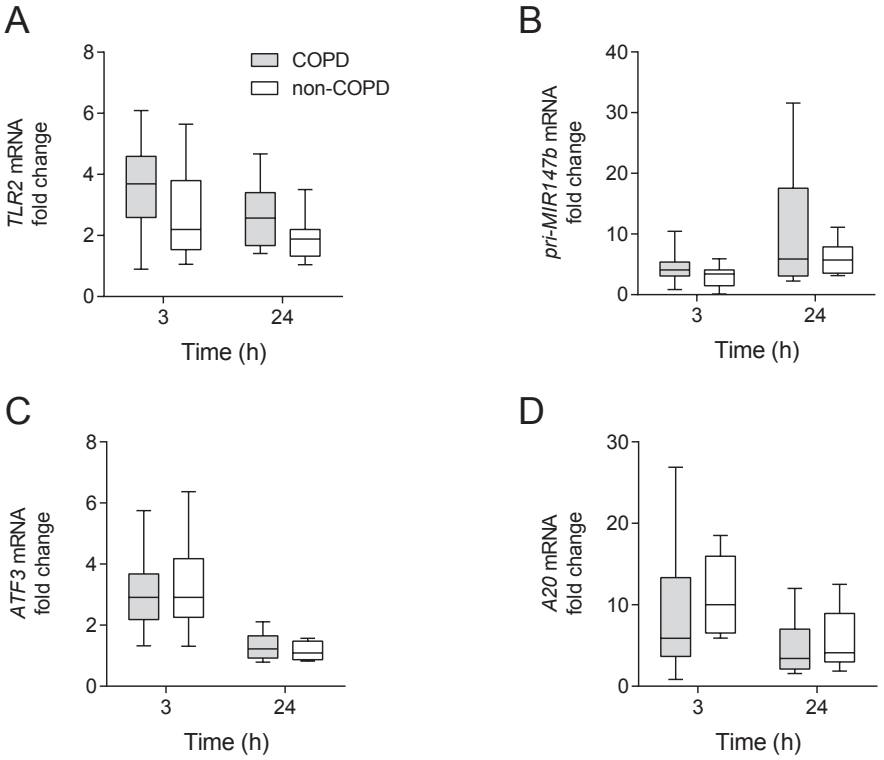
Gene Symbol	FC NTHi vs UNST (24 h)
DEFB4	110.6
IL1F9	0.3
SPRR2G	37.0
SPRR2C	31.6
IL8	17.2
RNASE7	16.1
IL1R2	14.7
CCL20	34
CYP4B1	-12.8
ADH7	-12.6
LCN2	11.9
IL1RL1	11.1
LCE3D	10.8
KRT15	-10.7
DAPL1	-10.7
IL13RA2	10.6
MRGPX3	10.0
XDH	9.9
WFDC12	9.8
PPIF	9.7
MMP1	9.6
MMP9	8.2
HMOX1	8.2
SLC16A9	-8.1
C15orf48	8.1

Gene expression analysis by microarray of unstimulated (UNST) and UV-inactivated NTHi stimulated PBEC. Fold change values are shown for the top 25 most differentially expressed genes at 6 h (left table) and 24 h (right table) after stimulation. Selected AMPs that are further analysed are highlighted in bold and grey boxes. PBEC from 6 independent donors were included in the analysis. The criteria for differential expression was an absolute fold change > 2 and $p < 0.05$, corrected for multiple testing.

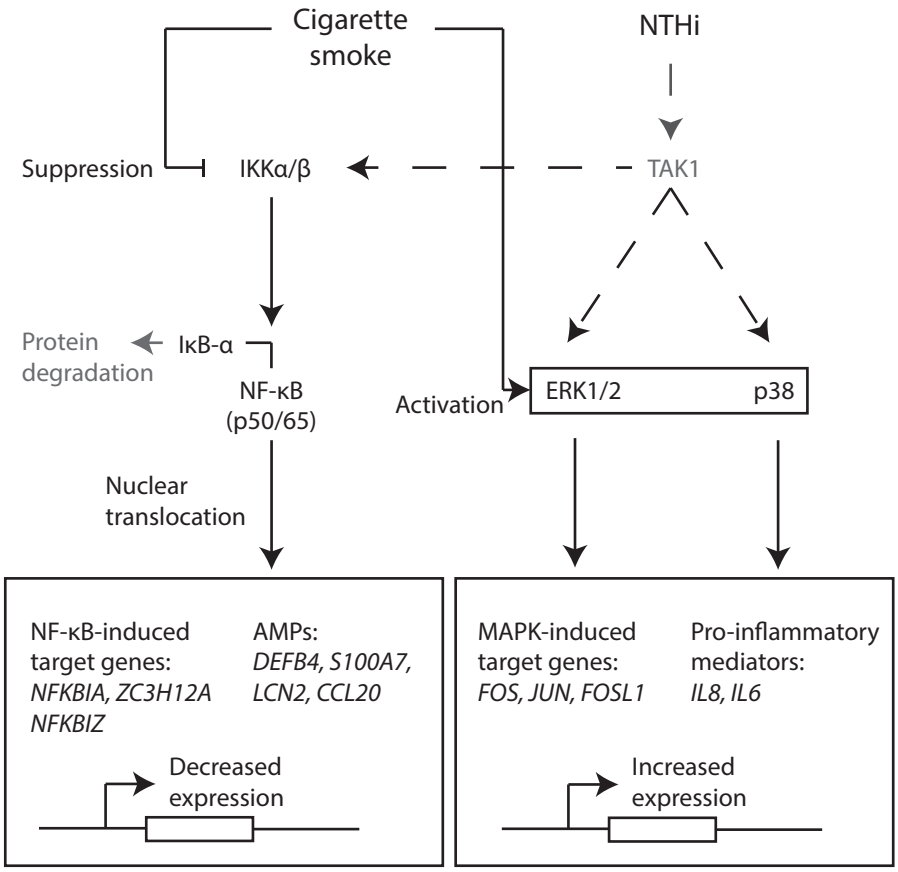
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Expression of LL-37/*CAMP, SLPI*, *DEFB1* and *DEFB3* in COPD and non-COPD ALI-PBEC. COPD (grey boxplots, n=11 patients) and non-COPD (white boxplots, n=8 patients) ALI-PBEC were stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 3 and 24 hours. mRNA expression of (A) LL-37/*CAMP,* (B) *SLPI,* (C) *DEFB1* and (D) *DEFB3* was assessed by qPCR. Stimulations were performed in duplicates. Data is shown as fold change in mRNA compared to untreated cells. Results are shown as boxplot with whiskers from minimum to maximum or bars (means ± SEM). Analysis of differences was conducted with a two-way ANOVA and Bonferroni *post-hoc* test.



Supplementary Figure 2. Expression of NTHi-induced innate immune regulators by COPD and non-COPD airway epithelial cells. COPD (grey boxplots, n=11 patients) and non-COPD (white boxplots, n=8 patients) ALI-PBEC were stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi for 3 and 24 hours. Afterwards, mRNA expression of (A) *TLR2*, (B) the primary microRNA 147b (pri-mir-147b) transcript, (C) *ATF3* and (D) *A20* was assessed by qPCR. Stimulations were performed in duplicates. Data is shown as fold change in mRNA compared to untreated cells. All results are shown as boxplot with whiskers from minimum to maximum or bars (means ± SEM). Analysis of differences was conducted with a two-way ANOVA and Bonferroni *post-hoc* test.



Supplementary Figure 3. Hypothetical model of the effect of CS on airway epithelial cell innate immunity. NTHi activates both the NF-κB and MAPK signal transduction through the kinase TAK1. Cigarette smoke suppresses IKKα/β phosphorylation, which results in impaired NF-κB transcriptional activity reflected by impaired expression of early-induced target genes (*NFKBIA*, *ZC3H12A*, and *NFKBIZ*) and AMPs. In contrast, CS further enhances activation of the MAP-kinases ERK1/2 and p38, promoting expression of AP-1 transcription factor (*FOS*, *JUN*, and *FOSL1*) and pro-inflammatory mediators.

CHAPTER 5

Aberrant epithelial differentiation by cigarette smoke dysregulates respiratory host defense.

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ABSTRACT

Research question: It is currently unknown how cigarette smoke-induced airway remodelling affects highly expressed respiratory epithelial defence proteins and thereby mucosal host defence.

Methods: Localization of a selected set of highly expressed respiratory epithelial host defence proteins was assessed in well-differentiated primary bronchial epithelial cell (PBEC) cultures. Next, PBEC were cultured at the air-liquid interface and during differentiation for 2-3 weeks daily exposed to whole cigarette smoke. Gene expression, protein levels and epithelial cell markers were subsequently assessed. In addition, functional activities and persistence of the cigarette smoke-induced effects upon cessation were determined.

Results: Expression of pIgR, SLPI, long and short PLUNC was restricted to luminal cells and exposure of differentiating PBEC to cigarette smoke resulted in a selective reduction of the expression of these luminal cell-restricted respiratory host defence proteins compared to controls. This reduced expression was a consequence of cigarette smoke-impaired end-stage differentiation of epithelial cells, and accompanied by a significant decreased trans-epithelial transport of IgA and bacterial killing.

Conclusions: These findings shed new light on the importance of airway epithelial cell differentiation in respiratory host defence and could provide an additional explanation for the increased susceptibility of smokers and patients with COPD to respiratory infections.

INTRODUCTION

Respiratory infections and microbial colonization are a major health burden in smokers, and contribute to exacerbations and to the development and progression of chronic obstructive pulmonary disease (COPD) (reviewed by Sethi(1)). The mechanisms underlying this increased susceptibility of smokers with or without COPD are incompletely understood, but can be attributed in part to epithelial injury and remodelling resulting in a disrupted mucociliary clearance(2). In addition to mucociliary clearance, the airway epithelium contributes to host defence with a wide variety of additional activities(3) that include secretion of antimicrobial peptides that act as endogenous antibiotics or modulate important antimicrobial immune responses via a variety of mechanisms(4). Furthermore, the epithelium produces cytokines and chemokines that initiate an immune response to act against microbial invaders. Finally, transport of polymeric IgA and IgM to the lumen by the polymeric immunoglobulin receptor (pIgR) contributes to adaptive immunity in the lung by inhibiting adherence and facilitating clearance of pathogens, a process called immune exclusion(5). Several of these respiratory host defence proteins (HDPs) in the airways are highly expressed during homeostasis by epithelial cells, suggesting their importance for airway epithelial barrier function. Highly expressed proteins and peptides include -but are not limited to- antimicrobial peptides such as human beta defensin-1 (hBD-1) and lipocalin 2 (LCN2), the secretory leukocyte protease inhibitor (SLPI), pIgR and the epithelial sodium channel regulators short and long palate, lung and nasal epithelium clone protein (s/lPLUNC or *BPIFA1/BPIFB1*)(6-8). Expression of other peptides involved in airway host defence such as Ribonuclease 7 (RNase 7), LL-37 and human beta defensin-2 (hBD-2) is low during homeostasis but can be induced by e.g. inflammatory mediators, microbial products and upon injury of epithelial cells, and thus contribute to clearance of the pathogen and the resulting inflammatory process(4). The pseudostratified airway epithelium is composed of several cell-types, including goblet, club and ciliated cells that reach out toward the lumen of the airways, while basal cells do not reach this lumen in the intact epithelial layer(9). Based on their distinct anatomical positioning, it is not surprising that these different cell-types also produce different types of mediators. For example, expression of pIgR is restricted to the luminal cells of the pseudostratified airway epithelium and is therefore largely regulated by airway epithelial cell differentiation(10), similar to e.g. mucin production by goblet cells. In contrast, expression of the antimicrobial protein RNase 7 is restricted to basal cells(11). Cigarette smoke is known to induce airway epithelial remodelling in smokers and patients with COPD, characterized by an increase in goblet cells and a reduction in presence of ciliated cells(2). As a result higher levels of mucus are produced by the epithelium, while mucus transport is impaired, thereby compromising mucociliary clearance activity of luminal airway epithelial cells. Currently it is unknown if the expression of proteins that are important for airway epithelial defence is polarized in the epithelium, and if so, how cigarette smoke-induced remodelling of the airway epithelium affects their expression. We hypothesized that cigarette smoke-induced alterations in epithelial cell differentiation result in a decreased expression of proteins that contribute to respiratory host defence (HDPs), which may render the host more susceptible to infection.

MATERIALS AND METHODS

Collection of cells and cell culture

Primary bronchial epithelial cells (PBEC) were obtained from tumour-free resected lung tissue at the Leiden University Medical Center, Leiden, the Netherlands. For this, bronchial epithelial cells were isolated from a bronchial ring by enzymatic digestion for 2 h 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 (Life Technologies Europe B.V., Bleiswijk, The Netherlands). Next, the obtained cell fraction was expanded in serum-free keratinocyte medium (KSFM, Life Technologies Europe B.V.) supplemented with 0.2 ng/ml epidermal growth factor (Gibco), 25 μg/ml bovine pituitary extract (Life Technologies Europe B.V.), 1 μM isoproterenol (Sigma-Aldrich), 100 U/mL Penicillin (Lonza, Verviers, Belgium), 100 μg/ml Streptomycin (Lonza) and 5 μg/ml Ciproxin. Upon reaching confluence, cells were trypsinized in 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 until further use. For our cultures, cells were thawed in KSFM medium supplemented with the above mentioned supplements until near confluence, seeded on semipermeable transwell inserts with 0.4 μm pore size (Corning Costar, Cambridge, USA) that were coated with a mixture of bovine serum albumin, collagen and fibronectin and cultured as described (11). PBEC were cultured at the air-liquid interface (ALI) for 13 to 19 days (Figure 1A). Apical washes were performed daily; medium was refreshed every other day.

Fractionation of the airway epithelial cultures

Luminal and basal cell-enriched fractions were obtained from 3-4 weeks differentiated ALI-PBEC cultures as described previously(11). The luminal cell fraction was spun down and either lysed in RNA lysis buffer or fixed with 1% paraformaldehyde (Millipore B.V., Amsterdam, the Netherlands) in PBS for 10 minutes on ice and washed afterwards in ice-cold PBS. The remaining basal epithelial cell fractions on the transwell inserts were also either lysed in RNA lysis buffer (Promega) or fixed with 1% paraformaldehyde (Millipore B.V.) in PBS for 10 minutes on ice and washed afterwards with ice-cold PBS. Next, cells were stained with antibodies described in Supplementary Table 2.

Chronic cigarette smoke exposure

When confluent, PBECs were air-exposed (day 0) by removal of medium from the apical side of the transwell insert and 4 h later exposed to freshly generated whole cigarette smoke (CS) using 3R4F reference cigarettes (University of Kentucky, Lexington, KY). CS exposure was repeated daily as described in(11), in the figure legends of Figure 1 and in Supplementary Figure 1 and illustrated in Figure 1B and Supplementary Figure 1. Briefly, cells were exposed in modified hypoxic chambers for 4-5 minutes to either cigarette smoke from 1 cigarette or to room air, after which smoke was removed by ventilation with air during 10 minutes. and cells were subsequently placed back in the incubator overnight. Approximately 18-20 h later, ALI-PBEC were washed apically with PBS and 4 h hereafter exposed to cigarette smoke. This cycle was repeated every day until day 13-19. Cells were harvested for analysis 18-20 h after the last cigarette smoke exposure.

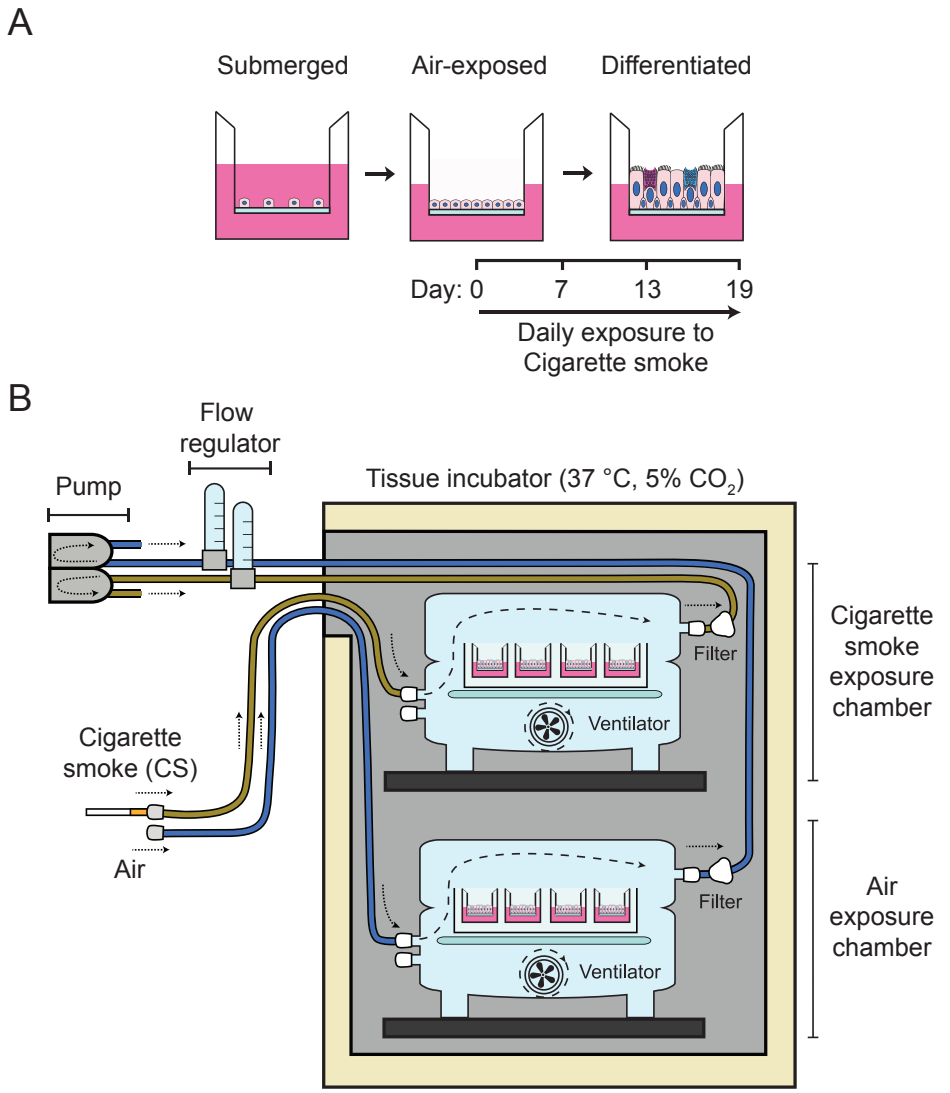


Figure 1. Cell culture set-up and cigarette smoke exposure of primary bronchial epithelial cells differentiated at the air-liquid interface (ALI-PBEC). (A) Primary bronchial epithelial cells (PBEC) were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cultures were air-exposed and cultured for additional 13-19 days to allow mucociliary differentiation. (B) Each day, starting at day 0, cultures were exposed to cigarette smoke (CS) by placing them in an exposure chamber that was infused with either cigarette smoke or with air for 4-5 min. Next, residual smoke in the chamber was removed for a period of 10 min. by infusing the chambers with air derived from the incubator. Approximately 4 h before each CS exposure the apical surface of the cultures was washed to remove mucus. Basal medium was changed every other day.

RNA isolation, cDNA synthesis and qPCR

Cells were lysed using lysis buffer from Promega, Leiden, the Netherlands. Next, RNA was extracted using the Maxwell tissue RNA extraction kit (Promega) and quantified using the Nanodrop ND-1000 Spectrophotometer (Nanodrop technologies, Wilmington, DE). cDNA synthesis was performed using oligo dT primers (Qiagen, Venlo, the Netherlands) and M-MLV Polymerase (Promega) in the presence of RNAsin (Promega). For qPCR analysis, diluted cDNA was mixed with primers (Supplementary Table 1) and iQ™ SYBR® Green Supermix (Bio-Rad, Veenendaal, the Netherlands). Reactions were performed in triplicate and results were corrected for the geometric mean of expression of 2-3 reference genes selected using the Genorm method. Expression values were determined by the relative gene expression of a standard curve as determined by CFX manager software (Bio-Rad).

Confocal microscopy

Following fixation with 1% PFA, cell culture inserts and/or cytospins containing luminal epithelial cells were treated with methanol for 10 min at 4 °C, washed with PBS and cells were permeabilized with 1% w/v BSA, 0.3% v/v Triton-X100 in PBS (PBT) for 30 min at 4 °C. After washing with PBS, cells were pre-treated with SFX-signal enhancer (Life Technologies Europe B.V.) followed by incubation with primary antibodies in PBT for 1 h at RT (supplementary Table 2). Next inserts were washed in PBS and incubated with an Alexa Fluor 488 or 568-labeled secondary antibody (Alexa Fluor 488 donkey-anti--mouse IgG; Alexa Fluor 568 donkey--anti-rabbit IgG, Life Technologies Europe B.V.) together with DAPI in PBT for 30 min at RT. Images were acquired using a TCS SP5 Confocal Laser Scanning Microscope (Leica Microsystems B.V., Eindhoven, The Netherlands) and LAS AF Lite software (Leica Microsystems B.V.).

Transcytosis experiment

Transcytosis capacity of the epithelial cultures was assessed in cultures exposed daily to whole cigarette smoke for 13 days, or air as a control. Dimeric IgA was added to the basal compartment of the cell cultures and 24 h thereafter, apical washes (PBS) were collected and stored at -20°C for further analysis. Apical washes were assessed for secretory (S-)IgA levels by sandwich ELISA(10).

Antibacterial activity assay

Direct antimicrobial activity was assessed in cultures of ALI-PBEC that were exposed daily to whole cigarette smoke or air controls for 13 days, followed by replacement with antibiotics-free cell culture medium for an additional 48 h period. *Moraxella catarrhalis* strain LUH2760 and *Klebsiella pneumoniae* strain LUH2754 were cultured in Tryptic Soy broth (TSB) while shaking overnight at 37^{0} C. Next, the overnight cultures were transferred into fresh TSB medium (1/50 dilution) and incubated for 4 h at 37°C -while shaking- to obtain mid log-phase-growing bacteria. Bacterial concentrations of log-phase cultures were determined by OD_{600} nm measurements, pre-diluted in PBS and final dilution was made in antibiotics-free cell culture medium. Twenty µl of bacterial suspension was added on the apical surface of the cells at a concentration of ~6x10^{5}/ml CFU/ml for *M. catarrhalis* and ~1x10^{4} CFU/ml for *K. pneumoniae* and incubated at 37°C, 5% CO^{2} for 2 h. Hereafter, membranes containing the cells with bacteria were dissected from the inserts and placed into tubes containing

sterile glass beads and 1% TSB in PBS. Next cells were disrupted by using a minilys personal homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) for 2 times 30 s and kept on ice in between. Serial dilutions of both bacterial suspensions were plated on Tryptic Soy Sheep blood (TSS) agar plates (Biomerieux, Zaltbommel, The Netherlands), and incubated overnight at 37°C to assess surviving bacteria by CFU determination.

ELISA and Trans-epithelial electrical resistance

CXCL8/IL-8 production by ALI-PBEC was determined in the basal medium by use of the CXCL8/IL-8 Duoset kit from R&D (MN, U.S.A.). hBD-1 was measured in the apical wash and in the basal medium using the hBD-1 kit from Peprotech (London, U.K.) and SLPI was measured as described(46). Epithelial barrier integrity of ALI-PBEC cultures was determined during cell differentiation by measuring the trans-epithelial electrical resistance (TEER) using the MilliCell-ERS (Millipore, Bedford, MA). TEER values were shown as Ω^*cm^2 and calculated as TEER = (measured value – background value) *surface transwell insert in cm^2.

Inhibition of differentiation by DAPT

At day 0, PBEC were air exposed by removal of the medium in the insert and culture medium of ALI-PBEC was refreshed with medium supplemented with either 5 μM DAPT (Notch signalling inhibitor, Sigma Aldrich, Zwijndrecht, The Netherlands), or solvent control. Every other day, basal medium was changed in a similar fashion up to day 13 when the cells were harvested.

Statistics

Statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, U.S.A.). Data are shown as mean ± SEM and significance was tested with use of a paired t-test or two-way ANOVA with a Bonferroni corrected post-hoc test. Differences were considered significant at $p< 0.05$.

RESULTS

Respiratory host defence proteins display a polarized distribution in airway epithelial cell cultures

In this study we have focussed on a set of proteins and peptides that are important for respiratory host defence. These host defence proteins (HDPs) were selected based on their constitutive and/or high expression by airway epithelial cells during homeostasis: i.e. SLPI, PLUNC (short/long), pIgR, hBD-1 and LCN2. We first investigated whether expression of these proteins was polarized in the airway epithelial cultures. To this end, we prepared luminal and basal epithelial cell-enriched fractions of well-differentiated primary bronchial epithelial cells (PBEC), cultured at the air-liquid interface (ALI, Figure 2A). We confirmed the successful enrichment of fractions for luminal and basal cells by determining the gene expression of the typical basal cell markers TP63 and KRT5 and luminal epithelial cell markers FOXJ1 (ciliated cells), SCGB1A1 (club cells), MUC5AC and MUC5B (both goblet cells) (Figure 2A), and by immunofluorescence staining for p63 (basal cells), CC16 (club cells) and acetylated α-tubulin (ciliated cells) (supplementary Figure 2). Further analysis of these fractions showed that the luminal cell-enriched fraction expressed significantly higher levels of BPIFA1 (sPLUNC),

BPIFB1 (lPLUNC) and SLPI (Figure 2A). In contrast, LCN2 and DEFB1 expression did not differ between the luminal and basal cell-enriched fraction (Figure 2A). The luminal cell-specific expression of SLPI and sPLUNC was further confirmed using confocal imaging in which the staining of both proteins did not co-localize with p63⁺ basal cells, but was highly present at the apical side of the PBEC culture and in the luminal cell-enriched fraction (Figure 2B and Supplementary Figure 2).

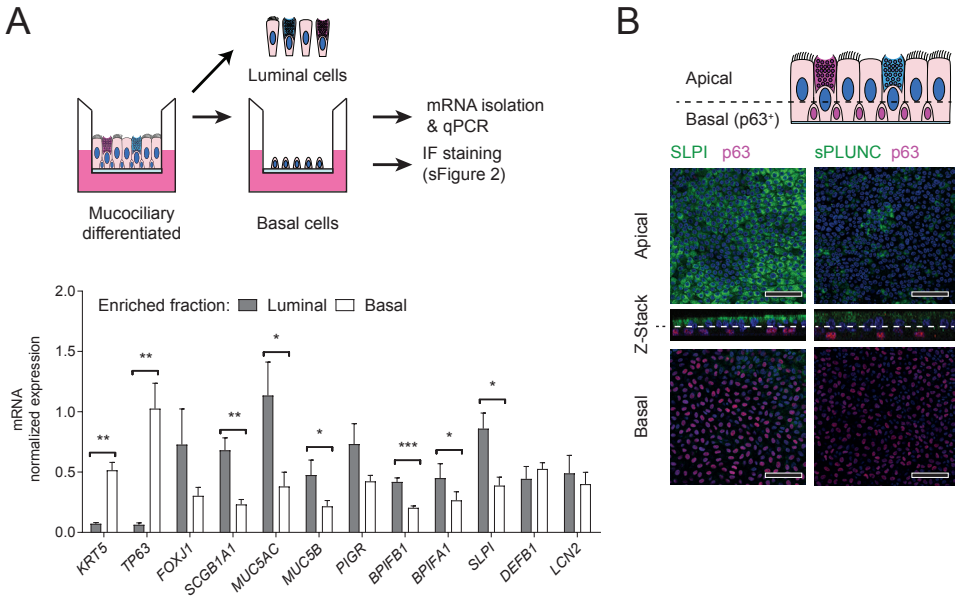


Figure 2. Respiratory host defence proteins display a polarized distribution in air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC). (A) PBEC were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cultures were air-exposed and cultured at the air-liquid interface. After 3-4 weeks of differentiation luminal and basal cell-enriched fractions were separated followed by RNA isolation, cDNA synthesis and qPCR analysis. Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin (*B2M*) and Ribosomal Protein L13a (RPL13A), n=5-7 different donors. Open bars are basal cell-enriched fractions; grey bars are luminal cell-enriched fractions. Statistical significance was tested using a paired t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (B) Confocal images to visualize polarized distribution of secretory leukocyte protease inhibitor (SLPI) and short palate, lung and nasal epithelium clone protein (sPLUNC) in differentiated ALI-PBEC cultures cells. After 3 weeks of differentiation, cells were fixed in 1% paraformaldehyde and stained using immunofluorescence with primary antibodies against p63 (basal cell marker, red) in combination with primary antibodies against SLPI and/or sPLUNC (both green) and DAPI for nuclear staining (blue). Z-stacks and images of the apical and basal side of stained cells were made by confocal imaging, scale bars equals 50 µm. Images shown are representative for results obtained with cells from 4 different donors.

Chronic cigarette smoke exposure of airway epithelial cell cultures reduces expression of respiratory host defence proteins

We next investigated if cigarette smoke-exposure affected expression of this set of respiratory HDPs. To this end, ALI-PBEC cultures were exposed on a daily basis during 2-3 weeks of differentiation to whole cigarette smoke (CS) (Figure 1 and Supplementary Figure 1). Gene expression analysis showed that DEFB1 (hBD-1) mRNA levels decreased during differentiation, but were not affected by CS exposure (Figure 3A). On the other hand, expression of SLPI, BPIFA1 (sPLUNC), BPIFB1 (lPLUNC) and PIGR strongly increased during differentiation, and this increase was significantly prevented by CS (Figure 3A). In contrast, gene expression of LCN2 (lipocalin 2) was increased by CS exposure during differentiation (Figure 3A). These findings were further confirmed by assessment of hBD-1 and SLPI protein levels in the apical wash and in basal medium from the ALI-PBEC cultures (Figure 3B). Indeed, hBD-1 levels reduced over the time of differentiation in the apical wash, but were not significantly affected by chronic CS-exposure, whereas SLPI levels were significantly lower in chronic CS-exposed cell cultures (Figure 3B). We next performed immunofluorescence staining of the airway epithelial cell cultures and found strongly reduced presence of SLPI-, sPLUNC- and pIgR-positive cells in chronic CS-exposed epithelium compared to air controls (Figure 3C). These results confirmed selective impairment of specific respiratory HDPs by chronic CS exposure during airway epithelial differentiation. To exclude that possible toxic effects of the chronic CS exposure affected the observations we made, we performed a selection of additional experiments. We assessed trans-epithelial electrical resistance (TEER) of ALI-PBEC exposed to CS or air as a control: results showed that chronic CS-exposed ALI-PBEC displayed a slight but non-significant decrease of TEER in CS-exposed cultures in the first week of exposure, and a similar TEER as the air-exposed controls in the second week of exposure up until day 19 (supplementary Figure 3A). LDH levels in chronic CS-exposed cell cultures were not increased, but rather reduced compared to air-exposed cultures (supplementary Figure. 3B). Indirect evidence for absence of marked cytotoxicity was the observation that chronic CS exposure significantly increased secretion of the neutrophil-attracting chemokine IL-8 at 13 days of differentiation in CS-exposed cells compared to air-exposed controls (supplementary Figure 3C). The cell size in chronic CS-exposed cell cultures seemed bigger in some donors, but not all, compared to air-exposed cultures, but no other morphological changes could be detected by microscopic inspection (an example illustrated in supplementary Figure. 3D). Together these data show that chronic CS-exposure-mediated loss of specific HDP expression by ALI-PBEC is unlikely to be a result of toxicity. This conclusion is further supported by measurements on the expression of a selection of inducible HDPs. We previously reported induction of *RNASE7* mRNA and protein in ALI-PBEC upon acute exposure to one cigarette (11), in line with these findings, chronic CS exposure also caused a progressive increase in *RNASE7* compared to air-exposed controls (supplementary Figure 3E). In addition also increased *CAMP* gene expression (LL-37-coding gene) was detected in chronic CS-exposed cultures (supplementary Figure 3E). In contrast, we did not observe a significant difference in the expression of *DEFB4* (human β-defensin 2) (supplementary Figure 3E).

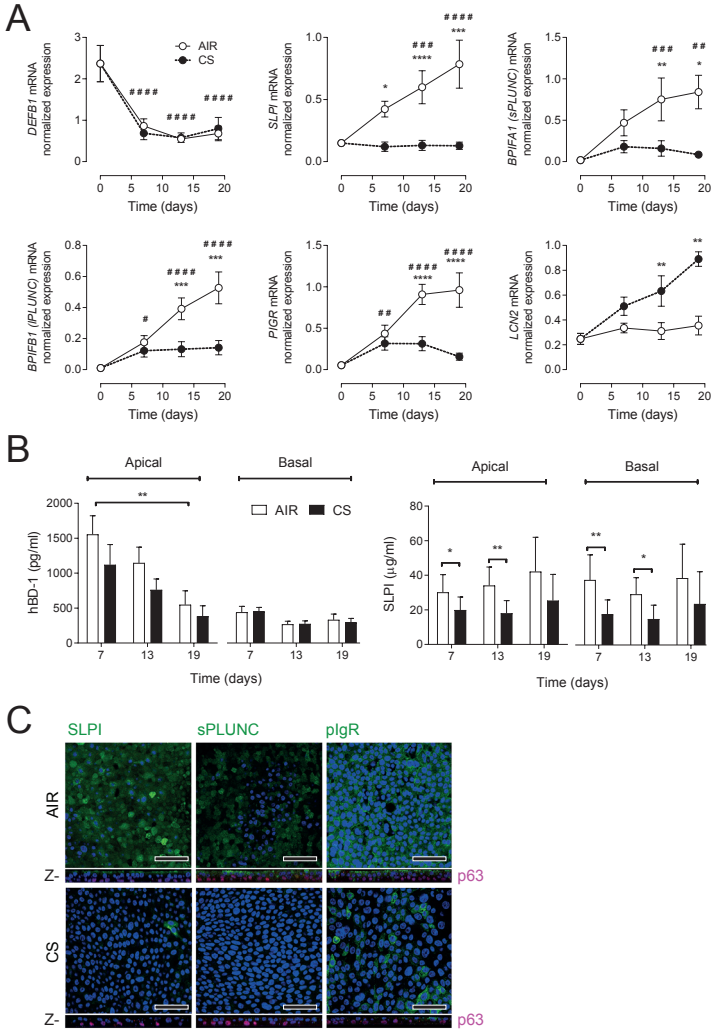


Figure 3. Chronic cigarette smoke exposure of air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) lowers the expression of luminal cell-restricted host defence proteins. (A) ALI-PBEC were daily exposed to whole cigarette smoke (CS) or air as a control (AIR) during differentiation for 13-19 consecutive days. Cells were lysed at several points during this course of time and RNA was isolated followed by cDNA synthesis to assess gene expression of *DEFB1* (human beta defensin-1), *SLPI* (secretory leukocyte protease inhibitor), *BPIF1A* (short palate, lung and nasal epithelium clone protein), *BPIF1B* (long palate, lung and nasal epithelium clone protein), *PIGR* (polymeric immunoglobulin receptor) and *LCN2* (lipocalin 2). Open circles: air-exposed controls, black circles: CS-exposed cell cultures; data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin (*B2M*) and Ribosomal Protein L13a (*RPL13A*); day 0, 7, 13 n=8 different donors and day 19 n=4 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ between AIR and CS. # $p<0.05$, ## $p<0.01$, ### $p<0.001$, #### $p<0.0001$ between AIR at day 7, 13 and 19 and unexposed cultures at day 0. (B) ELISA for hBD-1 and SLPI was performed on

the apical wash (Apical) and basal medium (Basal) of these cultures. Open bars: air controls (AIR), black bars: CS-exposed cell cultures (CS); day 7 and 13, n=8 different donors and day 19: n=4 different donors. Statistical differences on T=7 and T=13 (not T=19) was tested using a paired two-way ANOVA to compare AIR and CS. * p<0.05, ** p<0.01. (C) ALI-PBEC were differentiated for 2-3 weeks in which they were daily exposed to CS or air as a control (AIR). Subsequently the cells were fixed in 1% paraformaldehyde and stained using primary antibodies against SLPI, sPLUNC and pIgR (all green staining) in combination with DAPI to stain the nuclei (blue staining). Scale bars are equal to 50 μm. Images shown are representative for results obtained with cell cultures from 4 different donors.

Chronic cigarette smoke exposure reduces host defence of the airway epithelial cell cultures by decreasing apical release of secretory IgA and bacterial killing of Moraxella catarrhalis and Klebsiella pneumoniae

Next, we assessed if the strong reduction in SLPI, BPIFA1 (sPLUNC), BPIFB1 (lPLUNC) and PIGR expression levels in the CS-exposed airway epithelial cultures had functional consequences for host defence. We selected pIgR-mediated transfer of dimeric (d)IgA across the epithelium as a proof-of-principle for the consequences on immunomodulatory host defence functions and found this to be significantly reduced in chronic CS-exposed cultures (Figure 4A). We furthermore analysed bacterial killing by chronic CS-exposed cell cultures of the Gram-negative bacteria *Moraxella catarrhalis* and *Klebsiella pneumoniae,* pathogens that are found to be increased in the lungs of patients with stable or acute exacerbations of COPD (12). We observed significantly higher bacterial counts (indicating lower antibacterial activity) in chronic CS-exposed PBEC cultures when compared to air-exposed cultures for both pathogens (Figure 4B). These data indicate that various host defence mechanisms are

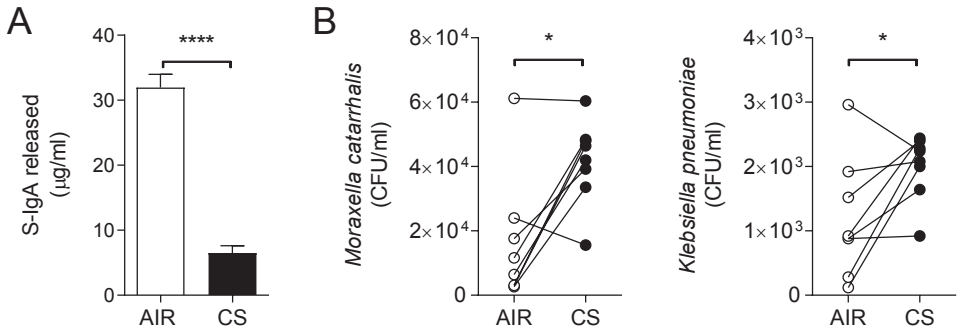


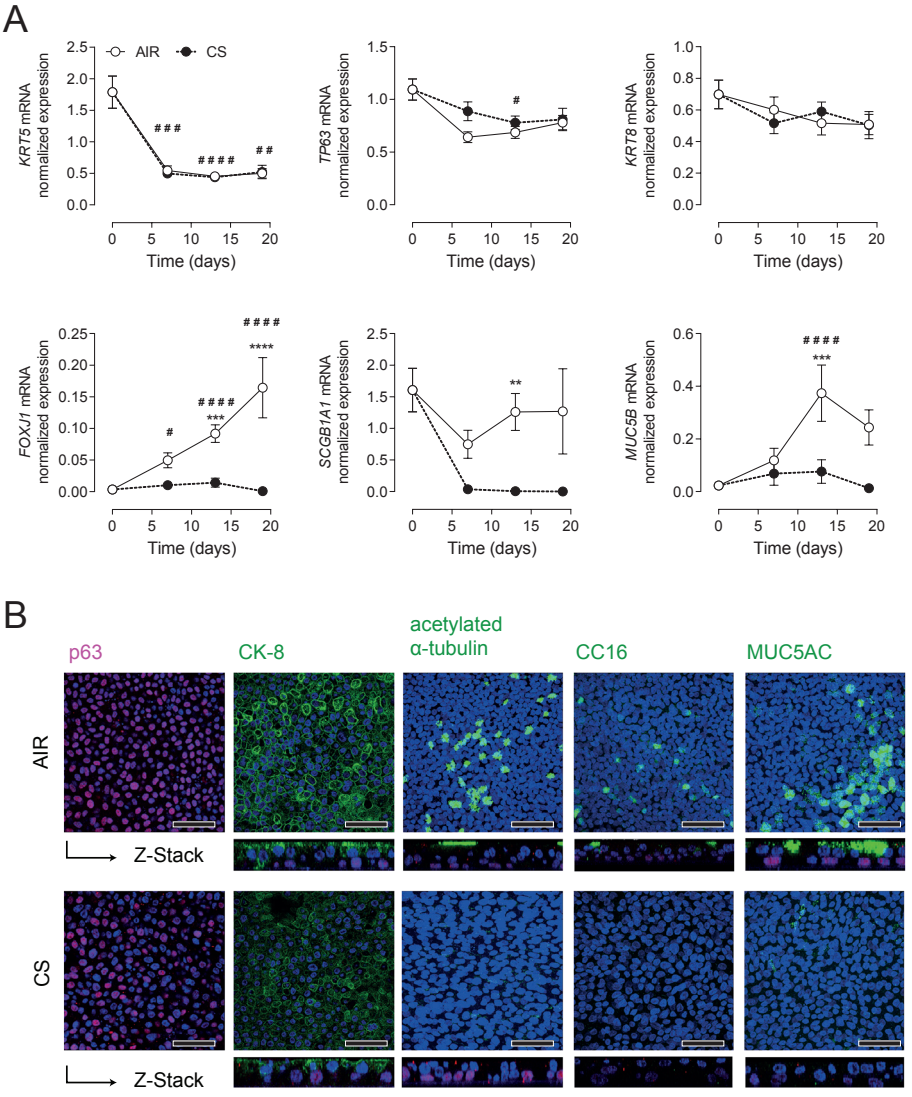
Figure 4. Chronic cigarette smoke exposure of air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) impairs host defence activities.

(A) ALI-PBEC were daily exposed to whole cigarette smoke (CS) or air as a control (AIR) during differentiation for 13 consecutive days. After 13 days of chronic CS exposure, dimeric (d)IgA transcytosis capacity of the epithelial cultures was assessed by determining secretory (S)-IgA levels in apical washes by ELISA (no S-IgA could be detected in the basal medium, as a control of the assay that does only recognize S-IgA and not d-IgA), n=10 different donors. Open bars: air-exposed cell cultures, black bars: CS-exposed cell cultures. (B) After 13 days of chronic CS exposure, ALI-PBEC were cultured for 48 h in antibiotics-free cell culture medium after which they were exposed for 2 h to *Moraxella catarrhalis* or *Klebsiella pneumoniae* at the apical surface of the ALI-PBEC. The surviving bacteria are depicted as colony forming units (CFU)/ml, n=8 different donors. Significance was determined using a paired t-test. *p<0.05, **** p<0.0001.

functionally impaired in CS-exposed epithelial cell cultures, which corresponds with impaired expression of respiratory defence proteins.

Cigarette smoke affects end-stage differentiation of airway epithelial cells

Next we assessed if chronic CS exposure affected differentiation of ALI-PBEC by measuring gene expression of epithelial cell-specific markers. Gene expression of the basal cell markers cytokeratin-5 (KRT5) and TP63, and of cytokeratin-8 (KRT8) that is expressed by intermediate/committed progenitor epithelial cells(13), was not affected by CS (Figure 5A).



In contrast, expression of the specialized luminal epithelial cell-specific genes FOXJ1 (ciliated cells), SCGB1A1 (club cells) and MUC5B (goblet cells) increased during differentiation, and this increase was significantly prevented by CS (Figure 5A). Confocal imaging confirmed the aberrant epithelial differentiation in CS-exposed cultures as cells positive for cilia marker acetylated α-tubulin, the club cell marker CC16, and the goblet cell marker MUC5AC were reduced in chronic CS-exposed cultures, while cytokeratin-8 (CK-8)$^+$ and p63$^+$ cells remained unchanged between air and CS-exposed cultures (Figure 5B).

Reversibility of cigarette smoke-induced effects on host defence protein expression

To assess the persistence of the CS-induced reduction in SLPI, BPIFA1 (sPLUNC), BPIFB1 (lPLUNC) and PIGR expression levels and of its effect on cellular composition, we allowed the cultures to recover from 13 days of CS exposure by culturing the cells for an additional 6 days without CS exposure. Chronic CS-exposed cultures were able to (partly) recover from the lack of end-stage differentiation, since all specialized luminal cell markers, except for SCGB1A1 (club cells), significantly increased in expression (Figure 6A). Furthermore, also SLPI, BPIFA1 (sPLUNC), BPIFB1 (lPLUNC) and PIGR showed enhanced expression compared to day 13 (Figure 6A). In addition, KRT5 (basal cell marker), and DEFB1 and LCN2 increased upon CS cessation, whereas TP63 and KRT8 were unaffected (Supplementary Figure 3A). This indicates that the inhibitory effects of cigarette smoke exposure on epithelial differentiation and expression of specific respiratory defence proteins are at least in part reversible. Furthermore, in an attempt to better mimic the in vivo situation and establish if the effects observed after chronic CS exposure also can be obtained when exposing an already partly differentiated epithelium to chronic CS, we performed a separate experiment. In this experiment, we first allowed the cultures to differentiate for 1 week, after which we started chronic CS exposure for an additional 12 days. Here we found similar effects of CS exposure on ALI-PBEC cultures regarding cell-type specific markers and HDP expression compared to CS exposure starting from day 0 (Figure 6B and Supplementary Figure 3B).

Figure 5. Chronic cigarette smoke exposure of air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) changes cellular composition. (A) ALI-PBEC were exposed during differentiation for 13-19 consecutive days to whole CS. Cells were lysed at several time-points and RNA was isolated followed by cDNA synthesis, to assess gene expression of basal cell markers cytokeratin-5 (*KRT5*) and *TP63,* of early progenitor cell marker cytokeratin-8 (*KRT8*) and of specialized cell markers *FOXJ1* (ciliated cells), *SCGB1A1* (club cells) and *MUC5B* (goblet cells). Open circles: air-exposed controls (AIR), black circles: CS-exposed cell cultures (CS); data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin (*B2M*) and Ribosomal Protein L13a (*RPL13A*); day 0, 7, 13 n=8 donors and day 19 n=4 donors. Significance was determined using a two-way ANOVA and Bonferroni post-hoc test. ** p<0.01, *** p<0.001, **** p<0.0001 between AIR and CS. # p<0.05, ## p<0.01, ### p<0.001, #### p<0.0001 between AIR at day 7, 13 and 19 and unexposed cultures at time 0. (B) ALI-PBEC were differentiated for 2-3 weeks and daily exposed to CS. Subsequently the cells were fixed in 1% paraformaldehyde and stained using primary antibodies against basal cells (p63), presented as a red staining, in combination with primary antibodies against cytokeratin-8 (CK-8), acetylated α-tubulin (ciliated cells), CC16 (club cells) and MUC5AC (goblet cells), which are presented as a green staining; DAPI was used to stain the nuclei (blue staining). Z-stacks and images of the apical and basal side of stained cells were obtained by confocal imaging. Scale bars equals 50 μm. Images shown are representative for results obtained with cells from 4 different donors (CK-8; n=3 different donors).

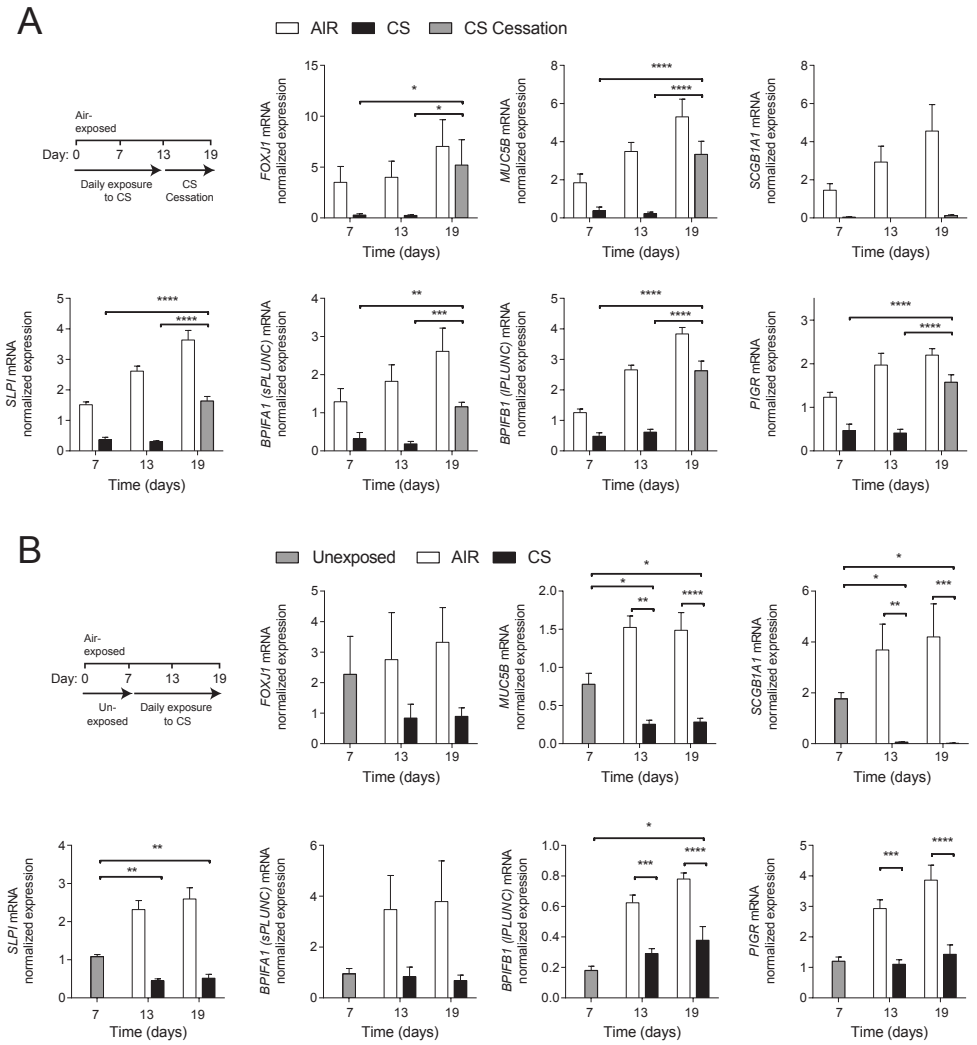


Figure 6. Cigarette smoke-induced impairment of host defence proteins and differentiation markers are partly persistent upon cigarette smoke cessation. (A) Air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) were exposed during differentiation for 13 consecutive days to whole cigarette smoke (CS) after which cultures were continued for another 6 days without CS exposure. Cells were lysed at several points during this course of time and RNA was isolated followed by cDNA synthesis, to assess gene expression of the cell specific markers: *FOXJ1* (ciliated cells), *MUC5B* (goblet cells) and *SCGB1A1* (club cells) and of respiratory defence proteins: *SLPI*, *BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR*. Open bars: air-exposed controls (AIR), black bars: CS-exposed cell cultures (CS), grey bars: CS-exposed cultures that were cultured for an additional week without CS exposure (CS cessation). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin (*B2M*) and Ribosomal Protein L13a (*RPL13A*). n=8 different donors. Statistical differences were evaluated only for the difference between cessation and previous CS expression using a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, ***

p<0.001, **** p<0.0001. (B) ALI-PBEC were air exposed at day 0 and cultured for 7 days under standard conditions. At day 7 cultures were exposed to CS for 12 consecutive days after which the cells were lysed and similar analysed as in (A). Grey bars (start point of culture at day 7): unexposed, open bars: air-exposed controls, black bars: CS-exposed cell cultures. Data are shown as target gene expression normalized for the geometric mean expression of the reference genes *ATP5B*, *B2M* and *RPL13A*; n=6 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

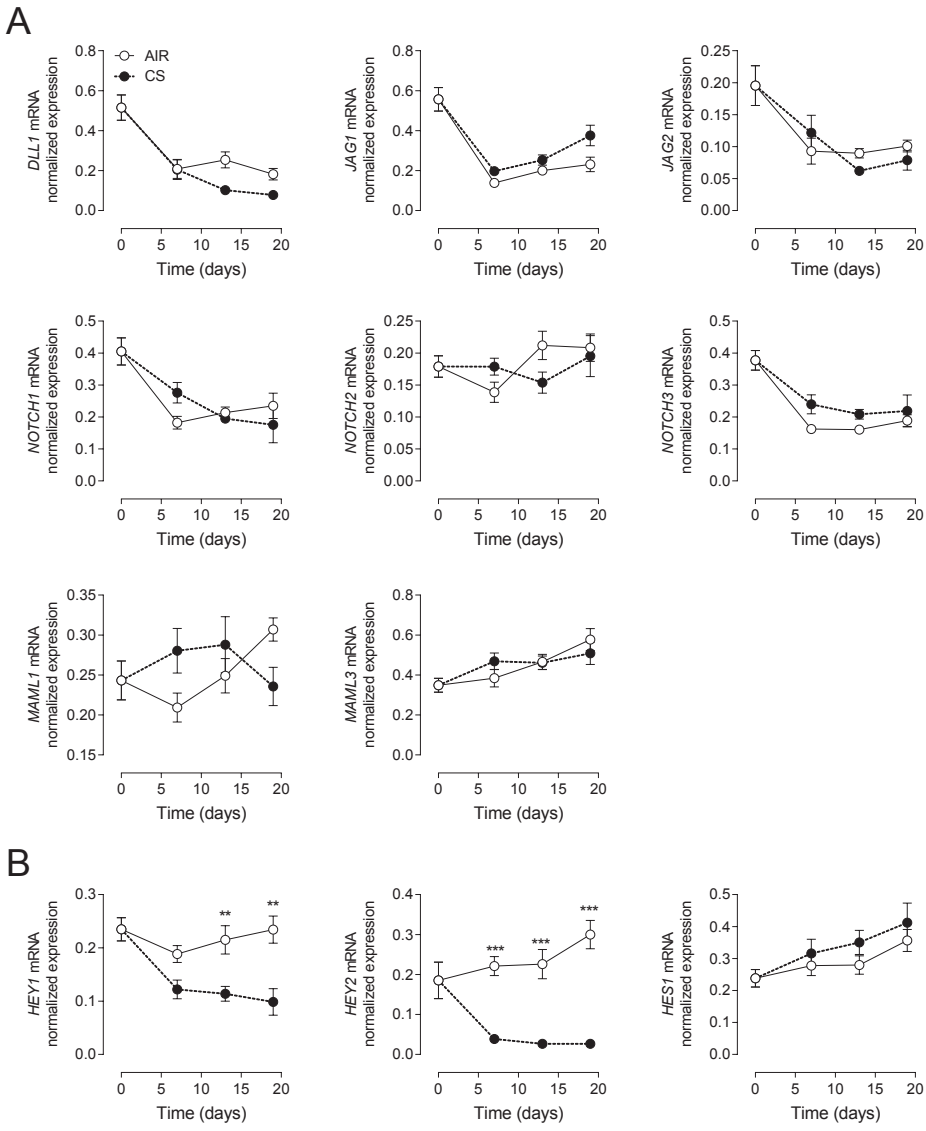


Figure 7. Chronic cigarette smoke exposure of air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) results in selective impairment of Notch signalling. (A) After 2 weeks of differentiation and daily cigarette smoke exposure, ALI-PBEC were lysed, RNA was isolated and cDNA synthesized. Subsequent qPCR analysis was performed on notch signalling ligands *DLL1*, *JAG1* and *JAG2*, on Notch receptors 1-3 and on the transcriptional co-activators

MAML1 and *MAML3*; data are shown as target gene expression normalized for the geometric mean expression of ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin (*B2M*) and Ribosomal Protein L13a (*RPL13A*). Open circles: air-exposed controls (AIR), black circles: CS-exposed cell cultures (CS); n=8 different donors. (B) Subsequent qPCR analysis was performed on Notch signalling target genes *HEY1*, *HEY2* and *HES1*. Data are shown as target gene expression normalized for the geometric mean expression of the reference genes *ATP5B*, *B2M* and *RPL13A*; n=8 different donors. Statistical significance was tested using a two-way ANOVA and Bonferroni post-hoc test . ** $p<0.01$, *** $p<0.001$ between AIR and CS.

Notch signalling inhibition impairs host defence protein expression during differentiation

Our results so far showed an impaired end-stage differentiation into specialized luminal epithelial cells in CS-exposed cultures resulting in reduced levels of SLPI, sPLUNC, lPLUNC and pIgR. Previous studies have shown that Notch signalling is involved in airway epithelial differentiation and that the airway epithelium of smokers displays reduced Notch signalling(14). Therefore, we next examined if Notch signalling was impaired in CS exposed cultures, and if Notch signalling inhibition modulates HDP expression during differentiation. First, we assessed gene expression of components of the Notch signalling cascade and found that chronic CS exposure did not influence gene expression of Notch ligands, receptors or transcriptional co-activators in these cultures (Figure 7A). However, the Notch signalling target genes, HEY1 and HEY2, were significantly reduced by chronic CS exposure, while HES1 was not (Figure 7B), indicating that chronic CS exposure selectively affects target genes of the Notch signalling pathway. To further investigate the importance of Notch signalling in the expression of host defence proteins, we examined the effect of the γ–secretase inhibitor DAPT, which acts as an inhibitor of Notch signalling (Figure 8A). After 15 days of PBEC differentiation in the presence of DAPT, we measured expression of HDPs. DEFB1 (hBD-1) and LCN2 were not affected by DAPT, while gene expression of SLPI, BPIFA1 (sPLUNC), BPIFB1 (lPLUNC) and PIGR were strongly reduced by DAPT incubation (Figure 8B). Furthermore, DAPT-exposed cultures showed a skewing of cell differentiation away from a secretory phenotype towards an increase in ciliated cells (Figure 8C) that was also confirmed by confocal imaging (Figure 8D).

DISCUSSION

Here we demonstrate that cigarette smoke negatively affects expression of the respiratory HDPs: pIgR, SLPI, lPLUNC and sPLUNC. Their expression was significantly reduced in epithelial cells daily exposed to CS during differentiation as a result of an impaired end-stage differentiation of specialized luminal cells. As a consequence, remodelling of the airway epithelium by cigarette smoke has a significant impact on respiratory host defence, underscored by the severely diminished IgA transport across the CS-exposed epithelium and impaired antibacterial defences against *M. catarrhalis* and *K. pneumoniae.* Our data suggest that increasing expression of specific respiratory HDPs (or preventing their decrease) could be of therapeutic interest to improve host defences in the lungs of COPD patients. Furthermore, this (selective) loss may also contribute to changes in lung microbiome composition, which is increasingly recognized as an important contributor to chronic inflammatory lung diseases(15, 16).

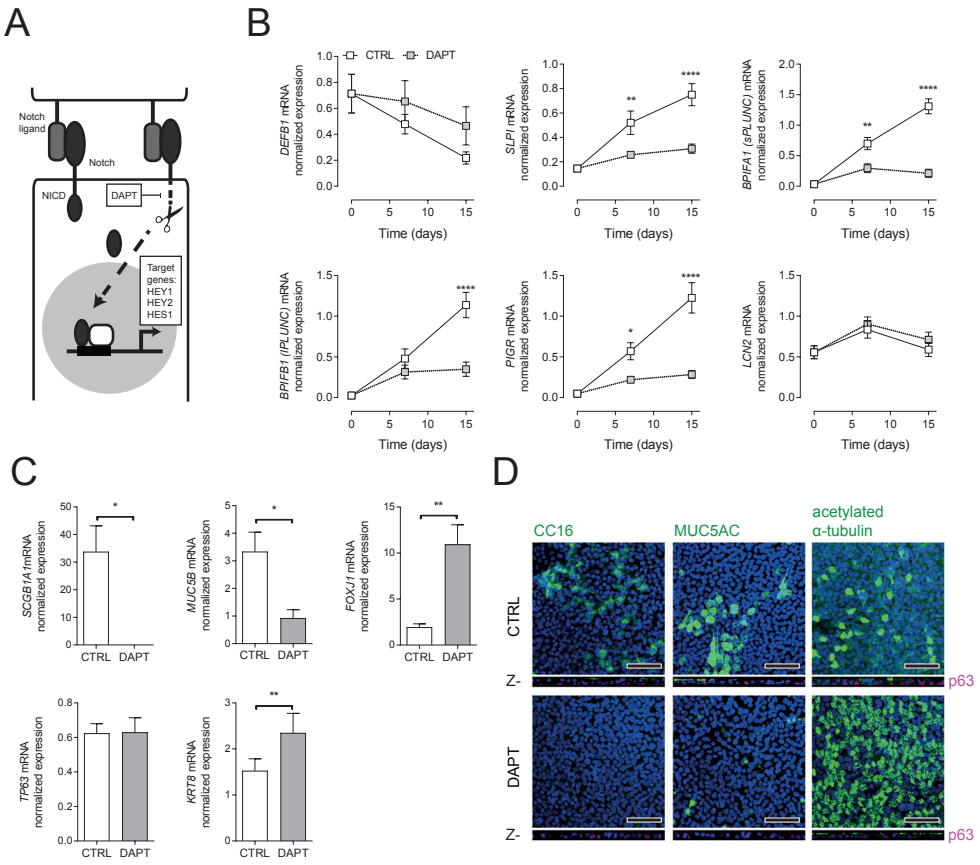


Figure 8. DAPT inhibits host defence protein expression in air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC). (A) Mechanism of action of the Notch inhibitor DAPT, a γ-secretase inhibitor that prevents proteolytic cleavage of the Notch intracellular domain (NCID). (B) PBEC were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cells were differentiated for an additional 15 days in the presence of 5 μM of the Notch signal transduction inhibitor DAPT in the basal medium or solvent as control. At day 0, 7, and 15 cells were lysed, RNA was isolated and cDNA synthesized. Subsequent qPCR analysis was performed to assess expression of respiratory defence proteins and epithelial cell-specific genes such as: *DEFB1* (human beta defensin-1), *SLPI* (secretory leukocyte protease inhibitor), *BPIFA1* (short palate, lung and nasal epithelium clone protein), *BPIFB1* (long palate, lung and nasal epithelium clone protein), polymeric immunoglobulin receptor (*PIGR*) and *LCN2* (lipocalin 2). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin (*B2M*) and Ribosomal Protein L13a (*RPL13A*); n=7 different donors. Statistical significance was tested using a two-way ANOVA and Bonferroni post-hoc test . ** p<0.01, *** p<0.001, **** p<0.0001 between CTRL and DAPT. (C) qPCR analysis was performed to assess mRNA expression of the epithelial cell markers *SCGB1A1* (club cells), *MUC5B* (goblet cells), *FOXJ1* (ciliated cells), *TP63* (basal cells), and cytokeratin-8 (*KRT8*) (intermediate cells) after 15 Days of differentiation with DAPT or solvent control, n=6 different donors. Statistical significance was tested using a paired t-test * p<0.05, ** p<0.01 between CTRL and DAPT (D) ALI-PBEC were differentiated for 15 Days with DAPT or solvent control. Subsequently the cells were fixed in 1% paraformaldehyde and stained using primary

antibodies against CC16 (club cells), MUC5AC (goblet cells), and acetylated α-tubulin (ciliated cells), which are presented as a green staining; DAPI was used to stain the nuclei (blue staining). Scale bars equals 50 μm. Images shown are representative for results obtained with cells from 3 different donors.

The cellular composition of the ALI-PBEC cultures changed drastically upon chronic CS-exposure. While presence of intermediate CK-8^{+} cells (or also called early, intermediate or committed progenitor epithelial cells(13, 17)) was not affected by chronic CS exposure, the specialized luminal cell markers were reduced in chronic CS-exposed cultures. These results indicate that specifically end-stage differentiation seems impaired by CS exposure. The effects of chronic CS exposure were also observed when the cells were first allowed to differentiate for one week in absence of CS. Furthermore, upon cessation of CS exposure, gene expression of most luminal cell markers showed a strong recovery. In contrast, SCGB1A1 mRNA expression remained absent after almost 1 week of recovery, suggesting an exceptional detrimental effect of CS on club cell differentiation or the regulation of SCGB1A1 gene expression. This is underscored by a recent study showing that expression of the club cell-protein CC16 (SCGB1A1) is reduced in COPD patients and in CS-exposed mice. Loss of CC16 was correlated with increased severity of the disease and CS-induced pulmonary inflammation was lower in mice overexpressing CC16(18).

Cytotoxicity is unlikely to have a major contribution to the CS-induced effects on the ALI-PBEC cultures since we detected no difference in trans-epithelial electrical resistance (TEER) as a measure of barrier function during the course of differentiation between CS and air-exposed controls. We previously observed transient decreases in TEER after acute single CS exposures, normalizing after 24 h (11). In our chronic CS set-up we measured TEER 18-20h after the previous CS exposure, which may explain why we did not observe significant differences in TEER between air and CS-exposed cultures. Previous studies have shown decreases in TEER by CS, but often use cigarette smoke extract (CSE) and not whole CS (19, 20). CSE has a different composition and concentration than whole CS used in our study. In addition, in some studies CSE was added to the basal medium, resulting in stimulation from the basal side of the transwells (19). This may also contribute to differences found in effects on TEER. Lastly, LDH release was not increased in our CS-exposed cultures, while cellular size appeared larger in the CS-exposed cultures for some donors. Finally, the CS-exposed cultures produced higher amounts of IL-8 and displayed increased mRNA expression of the inducible antimicrobial peptides RNase7 and LL-37 (but not hBD-2). These results are described in the online data supplement and in Supplementary Figure 4

Unexpectedly, we did not observe goblet cell hyperplasia in cultures that were exposed to CS, shown previously in smokers (21), guinea pigs (22), rats (23) and in cell lines (23, 24) or PBEC exposed to cigarette smoke extract (CSE)(19). However, Brekman *et al.* (25) also observed a decline in goblet cells markers in PBEC continuous exposed to CSE. Data are therefore conflicting and dependent on the type of cell culture used. Obviously, in our primary differentiated cultures, whole CS exposure alone is insufficient to induce goblet cell hyperplasia within 19 days. We strongly consider that the findings in patients are more likely explained by a secondary effect of the CS-induced inflammation (which is obviously incompletely represented in our in vitro model). For example, neutrophil recruitment as a consequence of the CS-induced inflammation and subsequent release of proteases and other

molecules, may help to explain goblet cell formation in patients. In addition, also the presence of other cell types besides neutrophils such as macrophages seem important for goblet cell hyperplasia. This has also been suggested in literature (26-29). Furthermore, several studies suggest that various other factors might have an important role in promoting goblet cell hyperplasia that are involved in COPD pathogenesis, including bacterial and viral infections (30, 31).

Whereas previous studies have shown that CS reduces the presence of ciliated cells (19, 25, 32), so far CS-induced airway epithelial remodelling was not yet linked to changes in levels of the highly expressed respiratory HDPs, despite the fact that changes in expression in these proteins have been reported in smokers or patients with COPD. Aarbiou *et al.* showed that expression of SLPI was significantly reduced in damaged bronchial epithelium of COPD patients compared to non-COPD individuals(33), and Gohy *et al.* showed reduced levels of pIgR in the lungs of patients with COPD, however not in smokers with a normal lung function compared to healthy controls(10). Finally, reduced levels of PLUNC were detected in bronchial brushes performed in current smokers compared to never smokers(34).
We observed impaired anti-bacterial activity of the CS-exposed airway epithelial cultures against *M. catarrhalis* and *K. pneumoniae*. We also evaluated direct antibacterial activity of the chronic CS-exposed cultures using a grid assay with live/dead staining of bacteria (35, 36) and via conventional plating methods against Pseudomonas aeruginosa and non-typeable Haemophilus influenzae,, but could not detect any differences (*data not shown*). These data suggest that the observed CS-induced impairment of antimicrobial activity may be pathogen-specific, since it is not observed with all pathogens studied. In addition to impaired antibacterial host defence activities, the loss of HDP expression by the airway epithelium can have further negative effects for the host. For example, loss of SLPI expression (highly expressed in normal lung tissue) can promote inflammation in the lungs of patients with COPD. SLPI acts as an inhibitor of detrimental proteases such as neutrophil elastase, acts as an inhibitor of NFκB activation and it modulates macrophage functions(37-39). sPLUNC is involved in regulation of the epithelial sodium channel (ENaC), thereby regulating airway surface liquid (ASL), and reduced levels could result in lowered ASL volume and impaired mucociliary clearance(40).

Since Notch signalling was previously reported to be impaired in COPD(14), we first analysed the effect of chronic CS exposure on components of the Notch signalling pathway and Notch target genes, and found that CS decreased the expression of selected Notch target genes. When we next used the Notch signalling inhibitor DAPT to inhibit airway epithelial cell differentiation, we found similar effects compared to CS exposure on expression of the selected set of respiratory HDPs. To our knowledge, this is the first study linking Notch signalling to expression of a range of host defence proteins that are increased upon differentiation. Whereas chronic CS exposure resulted in a reduction of all luminal-cell markers, DAPT-exposed cultures showed higher levels of ciliated cells when compared to control-treated cells, in line with previous studies(41, 42). These results suggest that expression of the luminal cell-restricted HDPs is confined to mature secretory epithelial cells, rather that the ciliated epithelium. Further studies using single cell RNA sequencing, may reveal whether the expression is restricted to a certain secretory cell phenotype. The partial similarity between the DAPT-incubated cultures and the CS-exposed cultures suggests involvement of altered

Notch signalling in the CS-induced effects. However, alterations in other signalling pathways might also be involved in the observed effects of CS, such as those involving EGFR(32), TGF-β(25) Wnt(43) and BMP(44). Most likely a more systems/-omics approach has the potential to elucidate in detail all effects of chronic CS exposure on Notch and other signalling pathways(45).

In summary, these findings shed new light on the role of dysregulated host defence in smokers and patients with COPD by highlighting the importance of airway epithelial cell differentiation in the expression of respiratory HDPs. Further investigations into how suppression of epithelial cell differentiation by cigarette smoke contributes to microbial colonization and infections of the airways are warranted in order to develop new therapeutics that restore airway epithelial host defence in patients with COPD.

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SUPPLEMENTARY TABLES

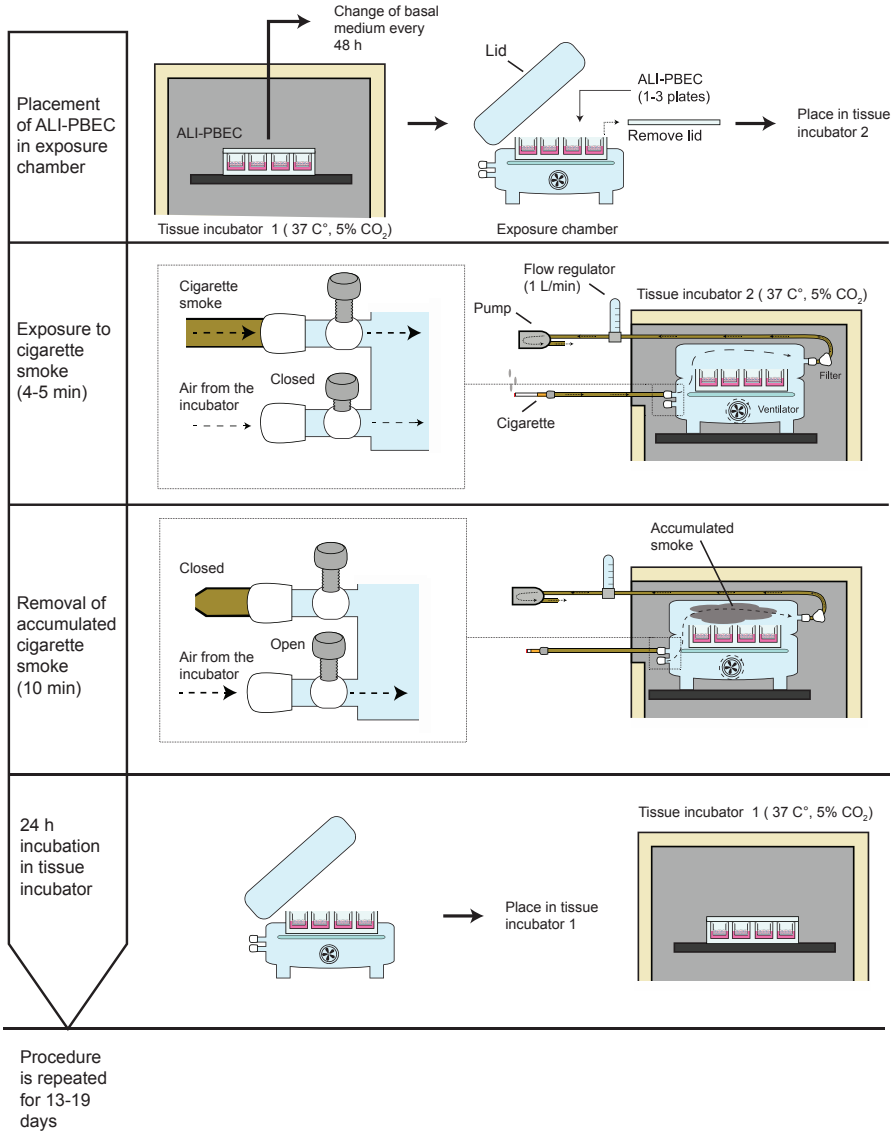
Supplementary Table 1. Primer sequences.

Gene	forward sequence (5' to 3')	reverse sequence (5' to 3')
ATP5B	TCACCCAGGCTGGTTCAGA	AGTGGCCAGGGTAGGCTGAT
RPL13A	AAGGTGGTGGTCGTACGCTGTG	CGGGAAGGGTTGGTGTTCATCC
B2M	GACCACTTACGTTCATTGACTCC	CAGGGTTTCATCATACAGCCAT
DEFB1	ATGAGAACTTCCTACCTTCTGCT	TCTGTAACAGGTGCCTTGAATTT
SLPI	CCA GGG AAG AAG AGA TGT TG	CCT CCA TAT GGC AGG AAT C
BPIFA1	CTTGGCCTTGTGCAGAGC	CAACAGACTTGCACCGACC
BPIFB1	CAGTGCCATGCGGGAAAAG	GCTGGAGGATGTTAGCTGTGA
PIGR	CTCTCTGGAGGACCACCGT	CAGCCGTGACATTCCCTG
LCN2	CCTCAGACCTGATCCCAGC	CAGGACGGAGGTGACATTGTA
KRT5	AGGAGTTGGACCAGTCAACAT	TGGAGTAGTAGCTTCCACTGC
TP63	CCACCTGGACGTATTCCACTG	TCGAATCAAATGACTAGGAGGGG
KRT8	TCCTCAGGCAGCTATATGAAGAG	GGTTGGCAATATCCTCGTACTGT
FOXJ1	GGAGGGGACGTAAATCCCTA	TTGGTCCCAGTAGTTCCAGC
SCGB1A1	ACATGAGGGAGGCAGGGGCTC	ACTCAAAGCATGGCAGCGGCA
MUC5B	GGGCTTTGACAAGAGAGT	AGGATGGTCGTGTTGATGCG
MUC5AC	CCTTCGACGGACAGAGCTAC	TCTCGGTGACAACACGAAAG
JAG2	TGGGACTGGGACAACGATAC	AGTGGCGCTGTAGTAGTTCTC
DLL1	GACGAACACTACTACGGAGAGG	AGCCAGGGTTGCACACTTT
NOTCH1	GAGGCGTGGCAGACTATGC	CTTGTACTCCGTCAGCGTGA
NOTCH2	CCTTCCACTGTGAGTGTCTGA	AGGTAGCATCATTCTGGCAGG
NOTCH3	CGTGGCTTCTTTCTACTGTGC	CGTTCACCGGATTTGTGTCAC
NOTCH4	GATGGGCTGGACACCTACAC	CACACGCAGTGAAAGCTACCA
HES1	CCTGTCATCCCCGTCTACAC	CACATGGAGTCCGCCGTAA
HEY1	ATCTGCTAAGCTAGAAAAAGCCG	GTGCGCGTCAAAGTAACCT
JAG1	GCCGAGGTCCTATACGTTGC	CCGAGTGAGAAGCCTTTTCAA
MAML1	CCCCAGTGAGTCATTTCCTCT	GAGGTTGCTTTGCGATATGGA
MAML3	CTTAGGACCTCCCTCTAGTCCA	GTTTTGGTTGTTAAAGGCTTGGG
RNASE7	CCAAGGGCATGACCTCATCAC	ACCGTTTTGTGTGCTTGTTAATG
DEFB4	ATCAGCCATGAGGGTCTTG	GCAGCATTTTGTTCCAGG
CAMP	TCATTGCCCAGGTCCTCAG	TCCCCATACACCGCTTCAC

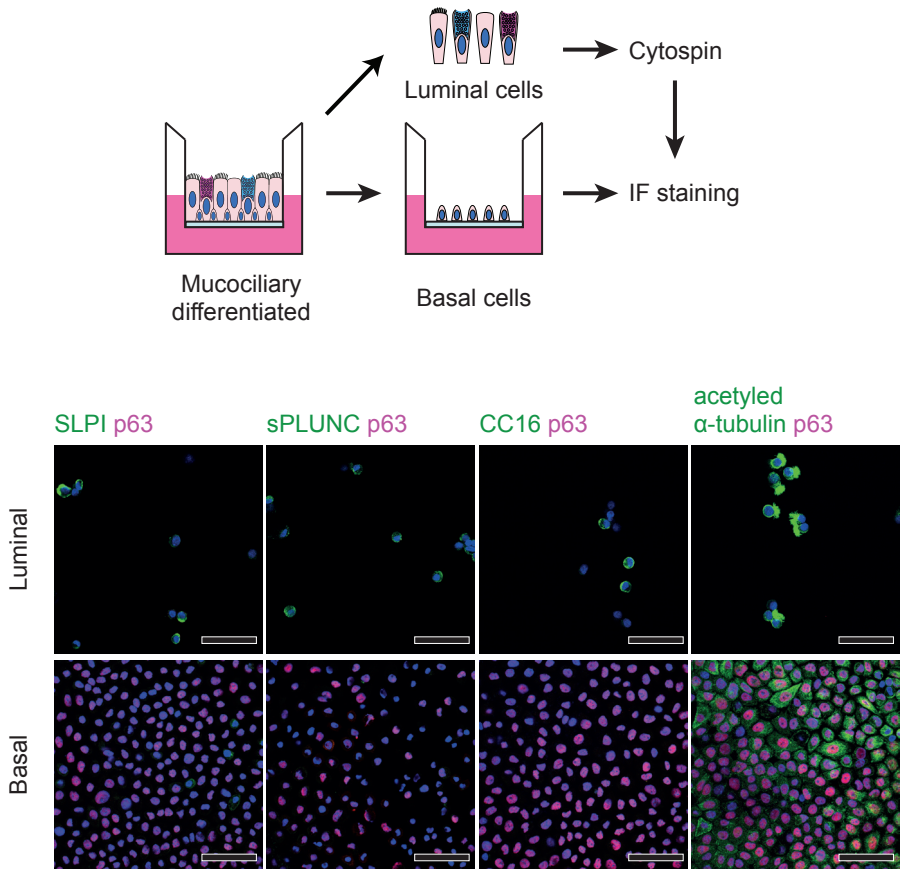
Supplementary Table 2. Antibodies used for confocal imaging

Antibody	**Supplier**	**Catalog#**	**species**	**Antibody dilution**
CK-8	Novus Biologicals	NBP2-34266	mouse	1/100
pIgR	R&D Systems	MAB27171	mouse	1/100
p63	Abcam	ab124762	rabbit	1/100
sPLUNC	Hycult Biotech	HM2314	mouse	1/100
SLPI	Hycult Biotech	HM2037	mouse	1/100
Mucin 5AC	Labvision Neomarkers	MS-145-P1	mouse	1/1000
CC16	Hycult Biotech	HM2178	mouse	1/50
Acetylated α-Tubulin	Sigma Aldrich	T6793	mouse	1/100

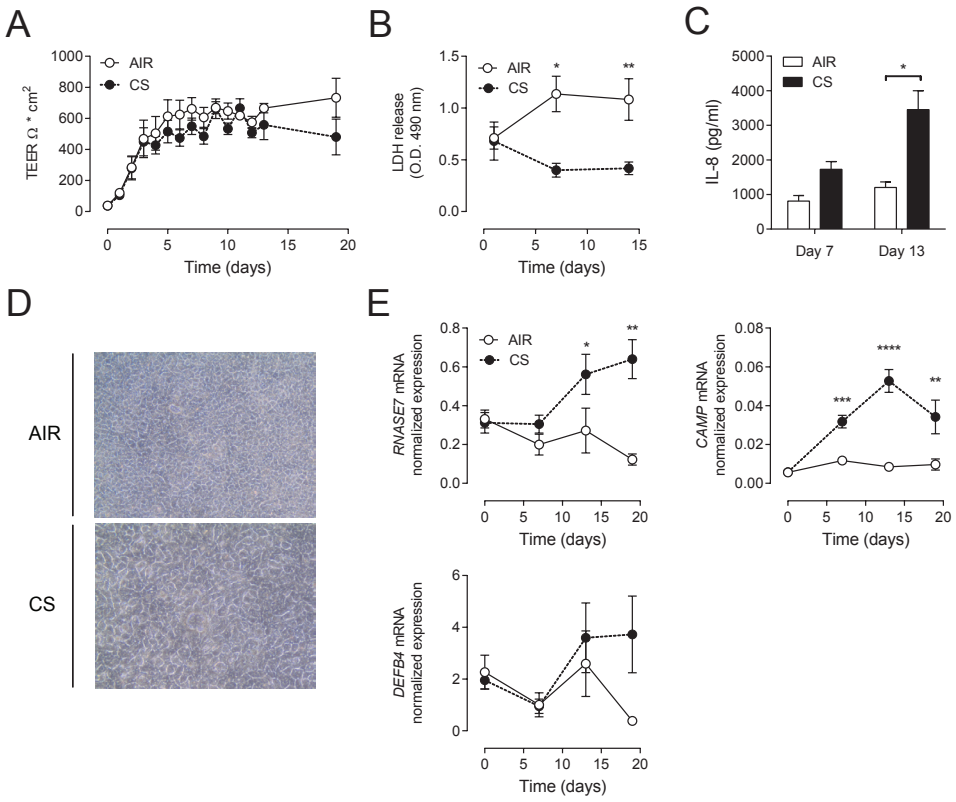
SUPPLEMENTARY FIGURES



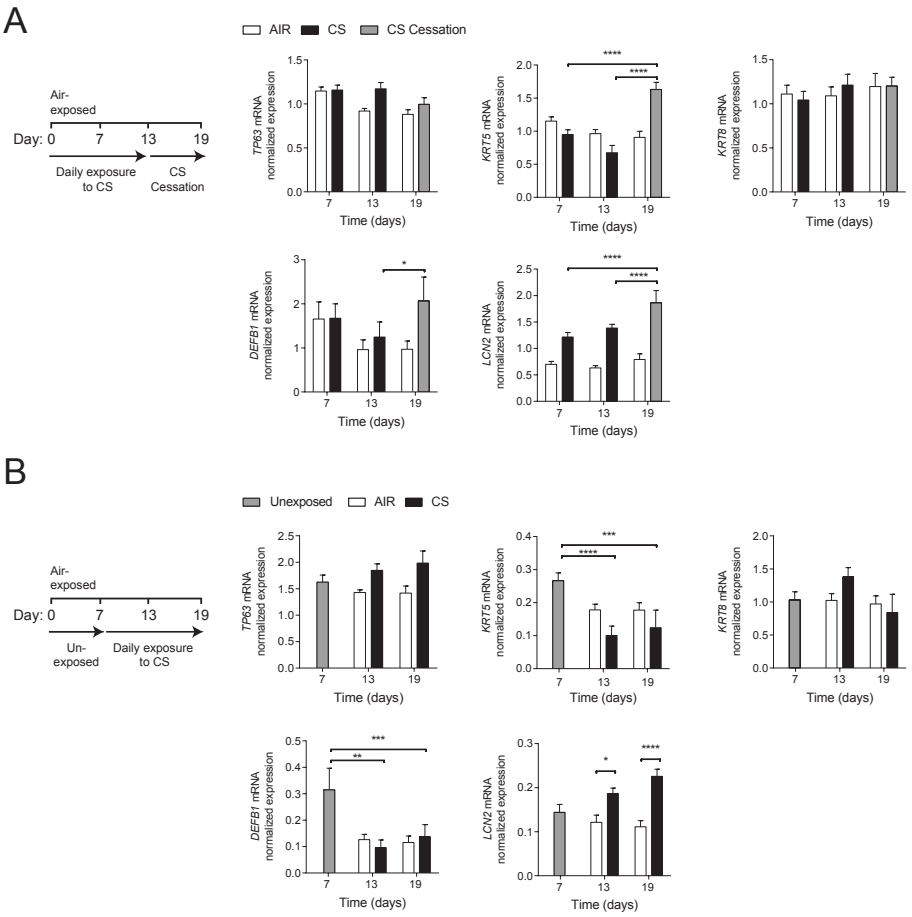
Supplementary Figure 1. Details of the cigarette smoke exposure design and procedure. Approximately 4 h before the cigarette smoke exposure, the apical surface of the cell cultures were washed with PBS and every other day the basal medium was replaced. Next, the cells were placed in the exposure chamber and the lid was removed. The closed exposure chamber was then infused with cigarette smoke from 1 cigarette for 4-5 min, or normal air in the control chamber. Hereafter, the tubing from the cigarette is clamped and vents on the exposure chamber connecting to the space in the incubator are opened and the air is refreshed with air from the incubator for an additional 10 min. The smoke-containing air is removed via separate tubing outside the incubator into a fume hood. After the exposure and refreshing, the chamber is opened, the lid placed back on the cells and the cells are placed back in a separate incubator for 20 h when the procedure is repeated.



Supplementary Figure 2. Expression of respiratory host defence proteins in the luminal cell fraction of air-liquid interface-differentiated primary bronchial epithelial cells (PBEC). PBEC were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cultures were air-exposed and cultured at the air-liquid interface (ALI). After 3 weeks of differentiation, luminal and basal cell fractions were separated. Cells were fixed in 1% paraformaldehyde and cytospins were prepared of the luminal cell enriched fraction. Luminal cell cytospins and the basal cell enriched fraction located on the transwell inserts were subsequently stained using immunofluorescence with primary antibodies against p63 (basal cell marker, red) in combination with primary antibodies against SLPI, sPLUNC, CC16 and acetylated α-tubulin (all green) and DAPI for nuclear staining (blue). Scale bars equal 50 μm. Images shown are representative for results obtained with cells from 3 different donors.



Supplementary Figure 3. Chronic cigarette smoke exposure of airway epithelial cell cultures does not lead to cell toxicity. Air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) were daily exposed to whole cigarette smoke (CS) or air as a control (AIR) during differentiation for 13-19 consecutive days. (A) Each day trans-epithelial electrical resistance (TEER) measurements were performed ~18 h after the previous CS exposure. Data are expressed as Ω*cm2. Open circles: air-exposed controls, black circles: CS-exposed cell cultures; n=8 different donors. Significance was determined using a two-way ANOVA and Bonferroni post-hoc test. (B) At several time-points during differentiation apical washes were collected and assessed for LDH content. Open circles: air-exposed controls, black circles: CS-exposed cell cultures; n=6 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * p<0.05, ** p<0.01 between AIR and CS. (C) At day 7 and Day 13 (~18 h after the last CS exposure), IL-8 protein levels were assessed by ELISA in the basal medium of the ALI-PBEC cultures. Open bars are air-exposed controls, grey bars are chronic CS-exposed cultures; n=8 different donors. Statistical differences were tested using a paired t-test. * p<0.05. (D) Illustrating phase contrast light microscopy images showing the increasing effects of 13 days of CS exposure (CS) or air as a control (AIR) on cell morphology in some donors. (E) At several time-points during differentiation, cells were lysed and RNA was isolated followed by cDNA synthesis, to assess gene expression of *RNASE7*, *CAMP* (LL-37) and *DEFB4* (human beta defensin-2). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B)*, β2-microglobulin (*B2M*) and Ribosomal Protein L13a (*RPL13A*), n=8 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 between AIR and CS.



Supplementary Figure 4. Persistence of cigarette smoke-induced changes in airway epithelial HDP expression and cellular composition. (A) Primary bronchial epithelial cells (PBEC) were cultured at the air-liquid interface (ALI) and exposed during differentiation for 13 consecutive days to whole CS after which cultures were continued for another 6 days without CS exposure. Cells were lysed at several points during this course of time and RNA was isolated followed by cDNA synthesis, to assess gene expression of the cell specific markers: *TP63*, *KRT5* (basal cells) and *KRT8* (intermediate cells), the HDPs: *DEFB1* (human beta-defensin 1) and *LCN2* (lipocalin 2). Open bars: air-exposed controls (AIR), black bars: CS-exposed cell cultures (CS), grey bars: CS-exposed cultures that were cultured for an additional week without CS exposure (CS cessation). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin (B2M) and Ribosomal Protein L13a (*RPL13A)*; n=8 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * p<0.05, **** p<0.0001. (B) ALI-PBEC were air exposed at day 0 and cultured for 7 days under standard conditions. At day 7 cultures were exposed to CS for 12 consecutive days after which the cells were lysed and similar analyzed as in (A). Grey bars: T=0 (day 7), open bars: air-exposed controls (AIR), black bars: CS-exposed cell cultures (CS). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes *ATP5B*, *B2M* and *RPL13A*. n=6 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

CHAPTER 6

Cigarette smoke modulates repair and innate immunity following injury to airway epithelial cells.

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ABSTRACT

Cigarette smoking is the main risk factor associated with chronic obstructive pulmonary disease (COPD), and contributes to COPD development and progression by causing epithelial injury and inflammation. Whereas it is known that cigarette smoke (CS) may affect the innate immune function of airway epithelial cells and epithelial repair, this has so far not been explored in an integrated design using mucociliary differentiated airway epithelial cells. In this study, we examined the effect of whole CS exposure on wound repair and the innate immune activity of mucociliary differentiated primary bronchial epithelial cells, upon injury induced by disruption of epithelial barrier integrity or by mechanical wounding. Upon mechanical injury CS caused a delayed recovery in the epithelial barrier integrity and wound closure. Furthermore CS enhanced innate immune responses, as demonstrated by increased expression of the antimicrobial protein RNase 7. These differential effects on epithelial repair and innate immunity were both mediated by CS-induced oxidative stress. Overall, our findings demonstrate modulation of wound repair and innate immune responses of injured airway epithelial cells that may contribute to COPD development and progression.

INTRODUCTION

Smoking has been shown to increase epithelial inflammation and injury, and has been suggested to disrupt the host defense function of the airway epithelium (1, 2). These effects may be highly relevant for our understanding of the development of smoking-induced lung diseases (3), including chronic obstructive pulmonary disease (COPD), an inflammatory lung disorder that is characterized by a progressive and irreversible obstruction of airflow (4). Changes in the airway epithelium resulting from exposure to smoke are early and key events in the development and progression of COPD (5, 6). Airway epithelial cells, which line the surface of the respiratory tract, normally function as the first host defense barrier against respiratory pathogens (2). However, extensive epithelial injury, for instance caused by cigarette smoking, respiratory pathogens and inflammation, may lead to disruption of the epithelial barrier integrity and cell death (7-9). Upon injury, a rapid wound repair process is initiated during which airway epithelial cells produce innate immune mediators to enhance host defenses at the wounded area (10). These repair responses are essential for restoration of the barrier function of the epithelium, and subsequent regeneration of a pseudostratified layer of epithelial cells. However, the repair process might be altered directly by CS exposure or indirectly by CS-induced inflammation, and this modulation of repair might contribute to COPD development and progression by promoting epithelial remodeling and persistent airway inflammation.

The direct effects of CS on wound repair of airway epithelial cells have been primarily studied by applying an aqueous extract of CS on undifferentiated submerged cultures of airway or alveolar epithelial cell lines or primary airway epithelial cells (7, 11, 12). However, to gain more insight in the effect of smoking on airway epithelial repair, further research is required using conditions that better reflect the local conditions in lungs of smokers. Air-liquid interface cultures of mucociliary differentiated primary bronchial epithelial (ALI-PBEC) represent a widely accepted model to investigate airway epithelial cell functioning in lung diseases (2, 5). These cultures are highly similar to the airway epithelium of the small and large conducting airways, and display a pseudostratified morphology including ciliated, secretory, intermediate columnar and basal cells (BCs) (13, 14). We have used exposure of ALI-PBEC cultures to whole CS to better mimic smoke exposure *in vivo* (9, 15). Using this model, we have previously shown epithelial injury and transient disruption of the epithelial barrier integrity upon acute exposure to whole CS (9). This response was accompanied by epidermal growth factor receptor (EGFR)-mediated expression of innate immune mediators, including expression of the neutrophil chemoattractant C-X-C Ligand 8 (CXCL8, or IL-8) and selective expression of the antimicrobial protein Ribonuclease 7 (RNase 7) by BCs present in ALI-PBEC cultures. These findings provided important evidence for a dual function of airway BCs in epithelial repair and innate immunity that requires further investigation (16). Especially, the influence of CS on the dual function of airway epithelial BCs in mediating wound repair and innate immunity is of interest in view of the development of smoking related diseases such as COPD.

In the present study we examined the effect of epithelial exposure to whole CS on repair and induction of innate immune responses by wounded ALI-PBEC cultures. Epithelial injury was induced by disrupting the epithelial barrier integrity, via disruption of cell junctions

or via mechanical wounding of epithelial layers. EGFR-induced innate immune responses were examined by determining the expression of the BC-specific mediator RNase 7 and the luminal airway epithelial cell- and BC-expressed chemokine IL-8. In addition, the role of BCs in wound repair after mechanical injury was determined by assessment of the number of BCs at the wound edge. Moreover, we studied the contribution of oxidative stress and EGFR signal transduction to wound repair and innate immune responses.

MATERIALS AND METHODS

ALI-PBEC and whole CS exposure model

Primary bronchial epithelial cells (PBEC) were isolated from macroscopically normal lung tissue obtained from patients undergoing resection surgery for lung cancer at the Leiden University Medical Center. Use of such 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. PBEC were cultured and differentiated at the air-liquid interface as previously described (9, 17). In short, cells at passage 2 were seeded at a density of 40.000 cells/0.9 cm^2 on 0.4 μm pore sized semi-permeable transwell membranes (Corning Costar, Cambridge, MA, USA) that were coated with a mixture of 30 μg/ml PureCol (Advanced BioMatrix, San Diego, CA, USA), 10 μg/ml bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) and 10 μg/ml fibronectin (isolated from human plasma) in PBS. Cells were cultured in Bronchial epithelial growth medium (BEGM) (Lonza, Verviers, Belgium) and Dulbecco's modified Eagle's medium (DMEM) (Gibco, Bleiswijk, The Netherlands) (1:1 mixture) containing 1 mM Hepes (Lonza) and supplemented with SingleQuot supplements and growth factors according to the manufacturer's instructions (bovine pituitary extract [BPE], hydrocortisone, human epidermal growth factor [hEGF], epinephrine, transferrin, insulin, T3 and retinoic acid; all from Lonza), and additional 15 ng/ml retinoic acid (Sigma-Aldrich), 1 mg/ml BSA (Sigma-Aldrich), 100 U/mL penicillin and 100 μg/ml streptomycin (Lonza). Cells were first cultured in submerged conditions until confluence, followed by air-exposed culturing during 2-3 weeks to allow mucociliary differentiation. Exposure of ALI-PBEC to whole cigarette smoke (CS) was done according to a previously described model (9). In this model, ALI-PBEC were placed in exposure chambers and exposed to air (control) or whole cigarette smoke (CS) derived from one cigarette (3R4F reference cigarettes, University of Kentucky, Lexington, KY, USA) during a period of 15 minutes. Following exposure, the culture medium was refreshed.

Calcium switch assay

The effect of disruption of the epithelial barrier integrity was examined using the calcium switch assay (18). In brief, ALI-PBEC were incubated for 15-30 minutes with 700 μL of calcium-free minimum essential medium (Gibco) that was added at the apical side and 1000 μL in the basolateral compartment to deprive cells of calcium to disrupt cell-cell contacts. Epithelial barrier disruption was determined by measuring the trans-epithelial electrical resistance (TEER) using MilliCell-ERS (Millipore, Bedford, MA, USA). After a complete

loss of the barrier integrity, the apical medium was removed and cells were exposed to air or CS. Following exposure, the basal medium was replaced with calcium-containing culture medium with growth factors to allow reformation of junctions.

Wound healing assay

Wound healing assays were performed according to a previously described protocol (7), adapted for use in ALI-PBEC. In brief, the apical side of ALI-PBEC cultures was washed with PBS, and cells were starved for growth factors overnight in starvation medium (supplemented BEGM:DMEM without BPE and hEGF). 500 µL PBS was added to the apical surface of ALI-PBEC to facilitate mechanical injury, which was induced by scraping the cell layer with a sterile Pasteur pipette with a soft tip, creating a wound with a diameter of 3 mm. After wounding, the apical surface of the cultures was washed with 200 µL PBS to remove cellular debris. In designated experiments, 10 mM of N-acetylcysteine (NAC) (Sigma Aldrich) was used to determine the role of oxidative stress. To investigate EGFR and ERK signaling, cells were incubated with AG1478 (EGFR tyrosine kinase inhibitor) or U0126 (MEK1/2 inhibitor) (both Calbiochem, Darmstadt, Germany). After wounding, ALI-PBEC cultures were exposed to whole CS, and culture medium was replaced by fresh starvation medium, including additional inhibitors as indicated. For live imaging experiments, images of wounded ALI-PBEC were acquired using a Leica DM16000 phase-contrast light microscope (Leica Microsystems, Wetzlar, Germany), collecting digital images of the wound every 15 minutes up to 48 hours. During this period, cells were placed inside a micro cell incubator at 37°C in a 7.5% CO_2 humidified atmosphere. The acquired images were used to create a time-lapse movie. For other wound healing experiments, digital images were collected on a digital camera connected to an inverted phase-contrast light microscope using Cell Sense Entry imaging software (both Olympus, Tokyo, Japan), at time 0, 6, 24 and 48 h after wounding. The surface of the wound area was measured using Photoshop CS6 (Adobe, San Jose, California, USA) in order to assess remaining wound size and wound closure rates.

Immunofluorescence confocal imaging

Immunofluorescence staining of wounded ALI-PBEC was conducted as previously described (9). Cells were stained with a monoclonal anti-rabbit p63 antibody (ab124762, Abcam, Cambridge, UK) (1:100) to detect BCs, and DAPI to stain all nuclei. Z-stack images of the wound edge were made using a Leica TCS SP5 confocal inverted microscope (Leica Microsystems, Wetzlar, Germany) and processed using the Leica Application Suite Advanced Fluorescence software (LAS AF; Leica Microsystems). Five random images of air and CS-exposed wounded ALI-PBEC were used from independent donors to determine the number of $p63^+$ cells. The percentage of $p63^+$ nuclei was determined at the leading wound edge and at a randomly selected unwounded area. Moreover, the average number of $p63^+$ cells at the wound edge was calculated per 400 µm, and the internuclear distance between a $p63^+$ cell at the leading wound edge and its first adjacent $p63^+$ cell was quantified in approximately 20-30 nuclei per image. Further explanation of this method is provided in Supplementary Figure 2.

Quantitative real-time PCR

RNA extraction, cDNA synthesis and quantitative real-time PCR (qPCR) was conducted as described previously (9). mRNA expression was examined for the genes described in Table

S1. Relative gene expression compared to reference genes *ATP5B* and *RPL13A* was calculated according to the standard curve method. Reference genes were selected using the "Genorm" software (19).

Western blot

Western blot analysis of EGFR and ERK1/2 phosphorylation was done as previously described (9). The following primary antibodies were used: rabbit monoclonal Ab EGFR #D38B1 (1:1000), rabbit polyclonal phospho-EGFR #2234 , rabbit polyclonal ERK1/2 #9102 (1:1000), and rabbit polyclonal phospho-ERK1/2 #9101 (all Cell Signalling, Leiden, The Netherlands). Protein bands were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

ELISA

Secretion of IL-8 (R&D, Minneapolis, MN, USA) was determined according to the manufacturer's protocol.

Statistics

Data were analyzed using GraphPad Prism 6.0 (GraphPad Inc., La Jolla, CA, USA). Statistical tests used for data analysis were 2-way ANOVA, with post-hoc Bonferroni correction for multiple analyses. Differences with a p-value < 0.05 were considered as statistically significant

RESULTS

Cigarette smoke delays barrier recovery and enhances innate immune responses by ALI-PBEC

We first used a calcium switch assay to determine the effect of CS on recovery of the airway epithelial barrier, and to explore the importance of the loss of barrier integrity for the induction of RNase 7 and IL-8. In this assay, calcium-depleted culture medium was applied at the apical surface and basal compartment of ALI-PBEC, resulting in a complete impairment of the airway epithelial barrier integrity as determined by measuring the trans- epithelial electrical resistance (TEER). Subsequently, cells were exposed to CS or air as negative control. The effect of CS on barrier recovery was determined at different time points, and induction of innate immune responses was assessed at 24 h. Both air- and CS-exposed ALI-PBEC displayed complete recovery of the airway epithelial barrier integrity 24 h after barrier disruption (Figure 1A). However, CS exposure caused a delay in this recovery at 6 h after exposure, which was significantly different compared to air-exposed cells. Assessment of RNase 7 mRNA expression demonstrated a significantly higher expression in CS-exposed ALI-PBEC incubated with calcium-depleted medium (Figure 1B). In control cultures, we did not detect CS-induced expression of RNase 7 at 24 h; in a previous study we also no longer detected CS-induced expression of RNase 7 at this time point (9). Similar to RNase 7, we observed enhanced secretion of IL-8 in the basal medium upon barrier disruption and CS exposure (Figure 1C). Overall, these findings suggest that CS delays restoration of epithelial barrier function following calcium deprivation, while further increasing innate immune responses.

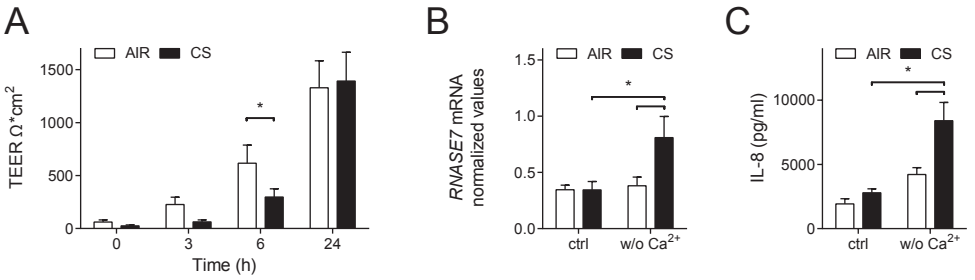

Figure 1. Effects of CS on airway epithelial barrier recovery and innate immunity. Barrier integrity in ALI-PBEC was disrupted using calcium depletion, and cells were subsequently exposed to air or CS. (A) The trans-epithelial electrical resistance (TEER) was subsequently measured at 0, 3, 6, and 24 h after exposure to assess loss and recovery of barrier integrity in air- and CS-exposed cultures. TEER values in ohm (Ω). n = 7 independent donors. (B) At 24 h, mRNA expression of *RNASE7* was assessed in ALI-PBEC that were incubated with calcium-depleted medium (w/o Ca2+) versus control medium (ctrl), and subsequently exposed to either air or CS and further incubated in calcium containing medium. Normalized mRNA expression compared to *RPL13A* and *ATP5B* is depicted in the graph. n = 4 independent donors. (C) Secretion of IL-8 in the basal culture medium was assessed by ELISA. n = 5 independent donors. Data are shown as mean; error bars represent SEM; experiments were conducted using duplicate exposures in all donors, * $p < 0.05$.

Cigarette smoke delays repair and further increases RNase 7 expression in ALI-PBEC

To further examine epithelial repair and induction of innate immune responses in wounded airway epithelial cells, we used a wound healing model in which ALI-PBEC cultures were mechanically injured by applying circular wounds. In this model, ALI-PBEC displayed intrinsic wound healing, and full wound closure was observed within approximately 48 h, as measured by live imaging (Supplementary Figure 1). Whole CS exposure directly following epithelial injury impaired wound healing of ALI-PBEC during the first 24 h after exposure (Figure 2A,B), with significantly decreased wound closure rates during the first 6 h after CS exposure, but not at later time intervals (Figure 2C). Live imaging experiments further demonstrated impaired wound repair at early time points, with recovery of wound closure rates approximately 6 h after CS exposure (Figure 2D). Next, we determined the effect of CS exposure following mechanical injury on mRNA expression of RNase 7 and protein secretion of IL-8. Comparison between intact and wounded cultures demonstrated significant higher CS-induced mRNA expression of RNase 7 in wounded ALI-PBEC at 6 h after exposure (Figure 2E). In contrast, we did not observe such an effect on IL-8 protein secretion (Figure 2F). Taken together, these findings further demonstrate impairment of epithelial repair upon CS exposure, which was accompanied by induction of RNase 7, but not of IL-8.

$p63^+$ cells at the wound edge are increased in CS exposed ALI-PBEC

Previously, we reported cell-type specific expression of RNase 7 by BCs in response to CS, whereas expression of IL-8 was observed in both luminal cells and BCs (9). The selective increase in RNase 7 expression in CS-exposed wounded cells suggests that CS in particular affects the activity of BCs in wounded ALI-PBEC. Therefore, we next examined the contribution of BCs to wound repair of ALI-PBEC. This was determined by assessing the

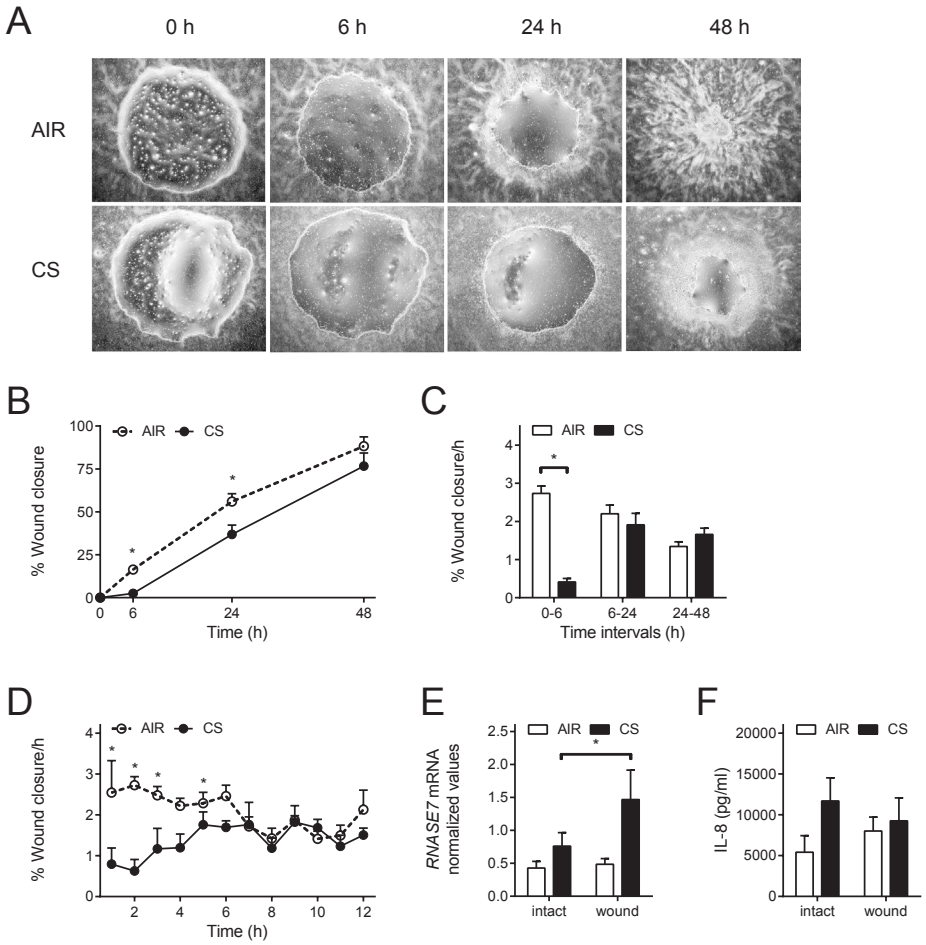

Figure 2. Effect of CS on airway epithelial wound healing and innate immunity. ALI-PBEC were mechanically injured and subsequently exposed to air (control) or whole cigarette smoke (CS). (A) Phase-contrast light microscopy images were made of air- and CS-exposed ALI-PBEC at 0, 6, 24 and 48 h after exposure. (B) Wound closure is shown in percentage in air- versus CS-exposed cells and (C) wound closure rate in percentage per hour at different time intervals was calculated. n = 8 independent donors. (D) Wound closure rates per hour in air- and CS-exposed ALI-PBEC up to 12 h after exposure were determined using live imaging. n = 7 independent donors. (E) *RNASE7* mRNA expression was determined in intact or wounded ALI-PBEC exposed to air or CS, at 6 h after exposure. Values shown represent normalized mRNA expression compared to *RPL13A* and *ATP5B*. n = 7 independent donors. (F) IL-8 secretion was determined in the basal culture medium. n = 9 independent donors. Data are shown as mean; error bars represent SEM; experiments were conducted in duplicate i, * $p < 0.05$.

number of cells at the wound edge that stained positive for the nuclear BC-marker p63 (Figure 3A and Supplementary Figure 2). The majority of cells (approximately 80%) directly located at the wound edge stained p63$^+$, which was a significantly higher proportion compared to intact areas of the same culture (appr. 35%) (Figure 3B). In CS-exposed cultures, p63$^+$ cells appeared to accumulate in higher numbers at the wound edge (Figure 3C). Moreover, we observed in CS-exposed cells significant smaller internuclear distances between p63$^+$ cells located at the leading wound edge and located directly adjacent to the wound edge (Figure 3D and Supplementary Figure 2 for explanation of the method). These observations suggest that CS impairs spreading and migration of BCs, a process that is important especially in the initial phase of wound repair. Collectively, these findings suggest that CS not only affects the innate immune function of BCs during repair, as shown by increased expression of RNase 7, but also affects the wound repair activity.

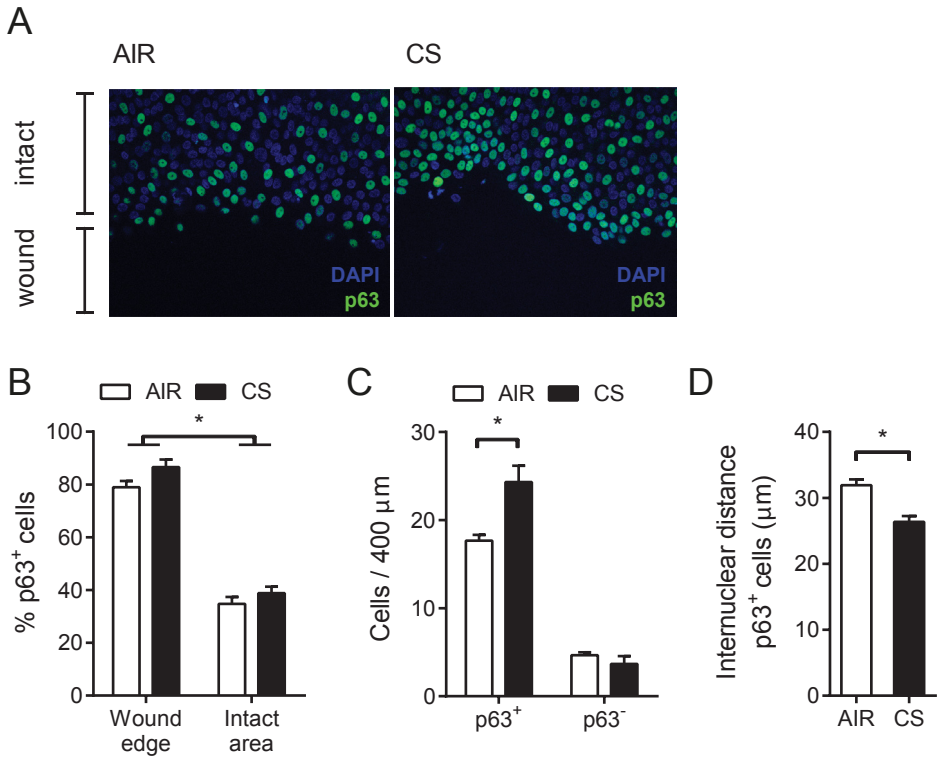

Figure 3. p63+ cells at the wound edge of ALI-PBEC. (A) Immunofluorescence staining for p63 (green) and nuclei (blue (DAPI)) of mechanically injured ALI-PBEC. (B) Percentage of p63$^+$ cells at the first line of cells directly at the wound edge or in intact areas, in air- versus CS-exposed cells. (C) Number of p63$^+$ cells and p63$^-$ cells at the wound edge per 400 µm length of wound edge, in air- versus CS-exposed cells. (D) Internuclear distance in µm between p63$^+$ cells located directly at the leading wound edge and the first adjacent p63$^+$ cell. All graphs: data are shown as mean; error bars represent SEM, experiments were conducted in duplicate, n = 3 independent donors, * $p < 0.05$.

Cigarette smoke differentially affects wound repair and innate immune responses through oxidative stress

To understand the mechanism of CS-mediated modulation of wound repair and innate immune responses we next examined the role of oxidative stress. The presence of oxidative stress upon CS exposure was demonstrated indirectly by showing CS-induced mRNA expression in wounded ALI-PBEC of heme oxygenase (decycling) 1 (*HMOX1*) and smoke and cancer-associated IncRNA-1 (*SCAL1*), both target genes of the oxidative stress-dependent Nrf2 pathway (20, 21). This induction by CS was blunted by treatment with the antioxidant N-acetylcysteine (NAC), suggesting involvement of oxidative stress (Figure 4A,B). Treatment with NAC in CS-exposed cultures partially restored wound repair (Figure 4C). In contrast, CS-induction of RNase 7 in wounded ALI-PBEC was completely inhibited by NAC (Figure 4D), and also CS-induced IL-8 mRNA expression was significantly inhibited (Figure 4E). These findings demonstrate a differential effect of CS-induced oxidative stress on wound repair and innate immune responses, which are suppressed and enhanced respectively.

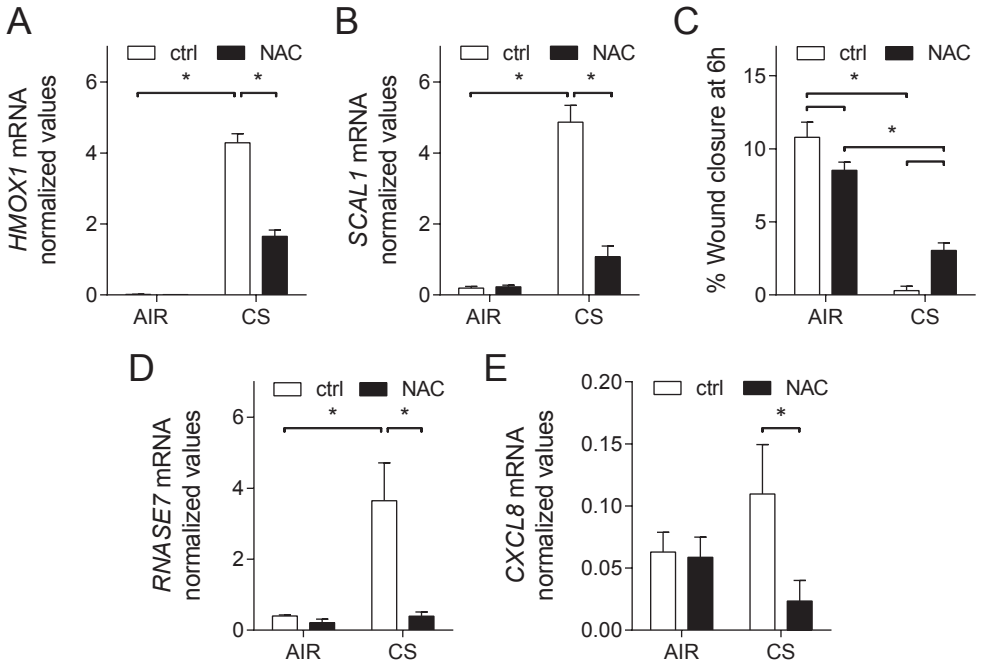

Figure 4. Role of CS-induced oxidative stress in modulating airway epithelial repair and innate immunity. Wounded ALI-PBEC were pre-incubated with NAC (10 mM) and subsequently exposed to air or CS. mRNA expression of the oxidative stress-induced genes (A) *HMOX1* and (B) *SCAL1* was determined 6 h after exposure. Values shown represent normalized mRNA expression compared to *RPL13A* and *ATP5B*. n = 3 independent donors, * $p < 0.05$. (C) Wound closure in presence or absence of NAC (10 mM) 6 h after wounding, in air- versus CS-exposed cells. Data are shown as percentage wound closure compared to t = 0 h. n = 7 independent donors. (D) mRNA expression of *RNASE7* and (E) *CXCL8* was assessed in wounded ALI-PBEC incubated with NAC (10 mM), at 6 h after exposure to air or CS. Data are shown as normalized mRNA expression compared to *RPL13A* and *ATP5B*. n = 3 independent donors. In all graphs data are shown as mean; error bars represent SEM; experiments were conducted in duplicate; * $p < 0.05$.

EGFR- and ERK1/2-dependent signaling are required for wound repair and innate immune responses

The epidermal growth factor receptor (EGFR) and downstream MAP-kinase/extracellular signal-regulated kinase (ERK)1/2 signaling pathway are important in both wound repair and induction of innate immune responses (10). Therefore, we examined the role of EGFR and ERK1/2 in the repair and induction of innate immune responses in wounded ALI-PBEC. First, the contribution to the intrinsic wound repair of ALI-PBEC was demonstrated by inhibitor experiments, showing impaired wound healing in the presence of AG1478 (EGFR tyrosine kinase inhibitor) and U0126 (Mitogen activated protein kinase/ERK kinase (MEK)1/2 inhibitor) (Figure 5A). As CS completely impaired wound healing at 6 h, we did not observe

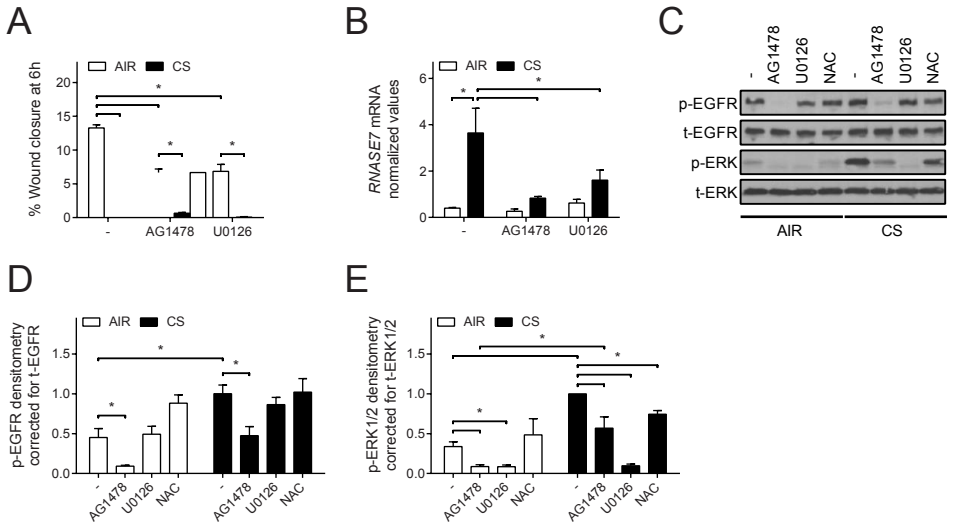

Figure 5. EGFR and ERK1/2 signaling in wounded ALI-PBEC. (A) Intrinsic wound healing of ALI-PBEC was determined in the presence of the EGFR tyrosine kinase inhibitor AG1478 (1 μM) or the MEK1/2 inhibitor U0126 (25 μM) at 6 h after exposure with either air or CS. Data are shown as percentage wound closure compared to t = 0 h. (B) mRNA expression of *RNASE7* was determined by qPCR. Data are shown as normalized mRNA expression compared to *RPL13A* and *ATP5B*. (C) Western blot analysis of EGFR and ERK1/2 phosphorylation of wounded ALI-PBEC exposed to air or CS in the presence of AG1478, U0126, and NAC, at 6 h after exposure. (D) Bands were quantified by densitometry for analysis of EGFR and (E) ERK1/2 phosphorylation and corrected for total-EGFR and total-ERK1/2, respectively. For all graphs data are shown as mean; error bars represent SEM; experiments were conducted in duplicate; n = 3 independent donors; * $p < 0.05$.

an additional effect of EGFR or MEK1/2 inhibition. In agreement with our previous study (9), CS-induced mRNA expression of RNase 7 in wounded ALI-PBEC cultures was significantly inhibited upon EGFR and ERK1/2 inhibition (Figure 5B). We subsequently determined EGFR and ERK1/2 phosphorylation in wounded ALI-PBEC. Phosphorylation of both proteins was observed in CS-induced wounded ALI-PBEC (Figure 5C-E). EGFR inhibition completely suppressed ERK1/2 phosphorylation in air-exposed cells, whereas in CS-exposed cells only a partial inhibition was observed. In contrast, inhibition of ERK1/2 phosphorylation did not

affect EGFR phosphorylation. The contribution of CS-induced oxidative stress to EGFR and ERK1/2 phosphorylation was determined by examining the effect of antioxidant treatment. CS-induced EGFR phosphorylation in wounded ALI-PBEC was not altered by NAC (Figure 5C-E), whereas NAC did decrease CS-induced ERK1/2 phosphorylation. This suggests an EGFR-independent activation of ERK1/2 mediated by oxidative stress. In summary, these findings indicate involvement of EGFR- and ERK1/2 signaling in intrinsic wound healing, and in CS-induced innate immune responses during repair. Moreover, CS increases ERK1/2 phosphorylation in both an EGFR-dependent and -independent pathway mediated in part by oxidative stress (Figure 6).

DISCUSSION

In this study we examined the effect of whole CS exposure on wound repair and innate immune responses of injured ALI-PBEC cultures. We observed a detrimental effect of acute CS exposure on the restoration of the epithelial barrier integrity and wound closure after mechanical wounding. In contrast, induction of innate immune responses, in particular expression of RNase 7, was further enhanced in CS-exposed injured ALI-PBEC. The impairment of epithelial repair in the mechanical injury model was accompanied by an accumulation of BCs at the wound edge. Moreover, oxidative stress contributed to the CS-induced attenuation of wound repair and the induction of innate immune responses. Both intrinsic wound repair and CS-induced innate immune responses required EGFR- and ERK1/2 mediated signal transduction.

Whole CS exposure of ALI-PBEC attenuated airway epithelial repair, in particular during the first 6 h following CS exposure. Such a transient effect of CS is in line with our previous study, in which CS was shown to transiently affect the epithelial barrier integrity followed by restoration within 24 h (9). Oxidative stress contributed to the observed effects of whole CS exposure on epithelial wound repair, as NAC partly reversed the observed induction of an anti-oxidant response and impairment of epithelial wound repair. This is in line with our previous findings in undifferentiated (submerged cultured) PBEC, using an aqueous extract of cigarette smoke (7), and suggest that findings made in these less physiological conditions remain relevant.

In contrast to the suppressive effect of CS on epithelial repair, CS-exposure resulted in increased innate immune responses in injured airway epithelial cell layers. Following CS exposure, increased IL-8 secretion was observed in the calcium switch model, whereas increased expression of RNase 7 was detected in the calcium switch as well as the mechanical wound model. Previously, we reported cell type-specific expression of RNase 7 by BCs present in ALI-PBEC cultures (9). The current observation further suggests that BCs are particularly affected upon injury of ALI-PBEC. Indeed, BCs are regarded as a heterogenic population including epithelial progenitor cell of the pseudostratified airway epithelium (22, 23), and it is assumed that BCs repopulate denuded wound areas through cell spreading and migration after injury (24). In agreement with this, the majority of cells at the leading wound edge of injured ALI-PBEC stained positive for the BC marker p63. In line with earlier reports (7, 25), this could not be explained by increased proliferation, as only limited numbers of proliferating

cells were observed at the wound edge as assessed by BrdU incorporation (*data not shown*). However, $p63^+$ cells displayed smaller internuclear distances upon CS exposure, suggesting that spreading and migration of BCs is impaired. Further studies are needed to determine whether cigarette smoke specifically targets subpopulations of BCs.

We speculate that the transient effect of CS in our model reflects the acute effects of smoking on the airway epithelium that is in a process of repair. Normally, BCs of the airway epithelium will close denuded wound areas through cell spreading before starting cell proliferation (26). However, primarily under the influence of oxidative stress caused by smoke exposure, the cells shift towards a different function, displaying reduced repair-promoting migratory activity but increased innate immune and cytoprotective anti-oxidant responses. The transient effect of CS on wound repair suggests recover of airway epithelial cells from mild damage that do not cause extensive cell injury and promote cell death. Indeed, CS exposure induced the expression of genes involved in the Nrf2-mediated antioxidant and survival response, which suggests that this response is involved in the restoration of wound repair following CS exposure. This mechanism may be impaired in COPD, since previous studies have reported attenuated Nrf2-dependent antioxidant responses in the bronchial tissues from COPD patients compared to non-COPD smokers (27, 28). Moreover, it has been shown that COPD airway epithelial cells display reduced wound repair and epithelial barrier properties (29, 30). Therefore, it can be speculated that an impaired oxidative stress response is related to epithelial dysfunction during COPD disease progression. It needs to be noted that our experimental design was adapted to mimic the effects acute cigarette smoke exposure, and not that of the repeated exposures that are typical form smoker's lungs. Further studies are needed to explore such effects during repeated CS exposure. Although the airway epithelial cells start to recover from the effects of CS in both the calcium switch and wound repair model at 6 h after exposure, induction of RNase 7 in BCs persisted at later time points. In particular, in the calcium switch assay enhanced expression of RNase 7 was observed at 24 h after smoke exposure, when the epithelial barrier integrity had recovered. This suggests that BCs display innate immune responses after the epithelium has recovered from injury. Epithelial injury results in activation of the EGFR signalling pathways in BCs, which is important for both wound closure and RNase 7 expression.

We propose that the reduced wound repair activity of the airway epithelium upon CS exposure increases the susceptibility of the epithelium and underlying tissues to microbial colonization and infections. The increased expression of the antimicrobial RNase 7 by BCs that occurs in parallel with impaired wound repair might be a compensatory mechanism to provide a last-resort antibacterial defense against invading microbes. We did not study the host defence activity of CS-exposed and injured airway epithelial cells using functional assays. Therefore, further research is required to determine the additional effects of adding live microbes in our wound healing model, and the putative modulating effect of increased RNase 7 expression. Antimicrobial proteins and peptides such as RNase 7 display immunomodulatory and wound repair enhancing properties (31, 32). These responses might contribute to the wound repair process but might also contribute to cell injury when these mediators are produced in high amounts and/or during prolonged periods. There is however currently no evidence for other activities of RNase 7, and therefore further research is required to demonstrate this. We used mechanical wounding of the epithelial layer by scraping, which is widely used in studies on

repair but is obviously a less physiological relevant model of injury than e.g. bacterial or viral infection or repeated smoke exposure. An important advantage of the model is, however, that it allows creation of a defined wound and quantification of its repair. Another advantage is that it allows an analysis of the interaction between microbial infection or smoke exposure and wound repair.

Previously, we reported the importance of EGFR signaling in induction of innate immune responses by CS, which was mediated by downstream ERK1/2 activation (9). Antioxidant treatment did not reduce EGFR signaling, but did decrease ERK1/2 phosphorylation, suggesting an EGFR-independent activation of ERK1/2 by oxidative stress (Figure 6). Thus, although EGFR-signal transduction is critical in airway epithelial wound repair and innate immunity (33), these findings demonstrate that other signaling transduction pathways contribute to repair and might be affected by CS exposure. Further research on CS effects on other repair pathways is required, and might also elucidate the differential regulation of epithelial wound repair and RNase 7 expression in mechanically wounded ALI-PBEC.

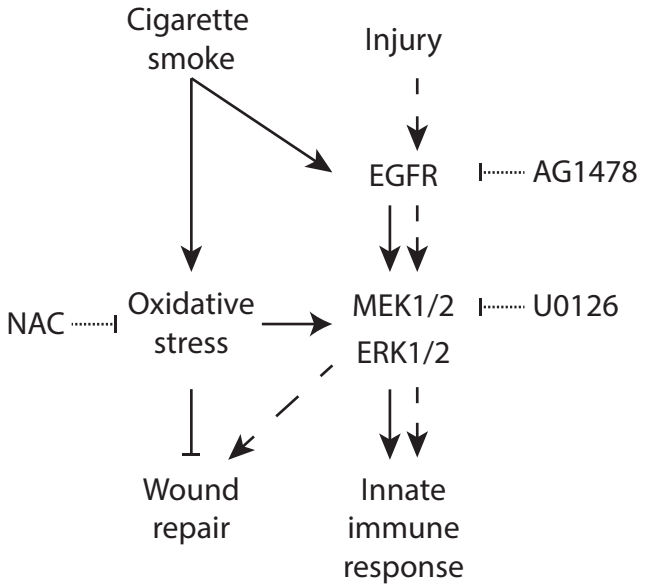

Figure 6. Proposed model. EGFR signaling is activated by CS and injury, and this leads to MEK1/2-mediated phosphorylation of downstream ERK1/2. CS furthermore directly causes phosphorylation and activation of ERK1/2 via oxidative stress, which is independent of EGFR signaling. EGFR/ERK1/2-mediated wound repair is suppressed by CS via oxidative stress. In contrast, activation of ERK1/2 due to a combined effect of CS-induced oxidative stress and injury, results in an enhanced innate immune response. Solid lines represent the effect of CS, dashed lines the effect of injury. NAC, AG1478 and U0126 were used to inhibit oxidative stress, EGFR phosphorylation, and ERK/12 phosphorylation respectively.

In summary, our findings demonstrate disturbances in the repair of injured airway epithelium and epithelial innate immunity upon cigarette smoke exposure. Oxidative stress caused by smoking is a key mechanism in modulating these responses, and in particular affects the activity of basal cells. These findings contribute to our understanding of how the repair and innate immune activity of wounded airway epithelial cells can be affected by cigarette smoking and might contribute to the development and progression of COPD.

ACKNOWLEDGEMENTS

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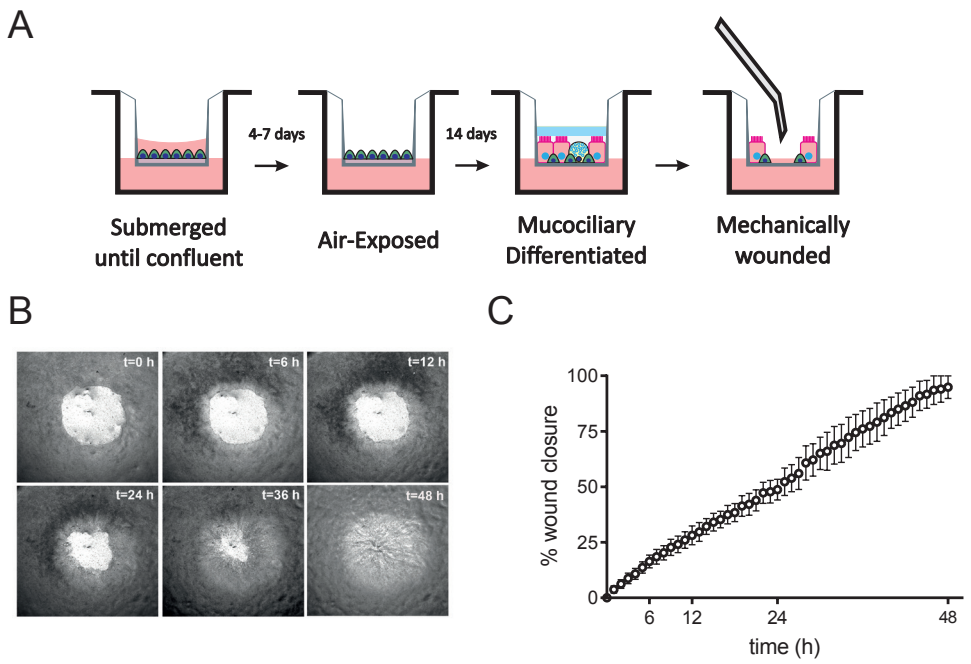
SUPPLEMENTARY TABLES

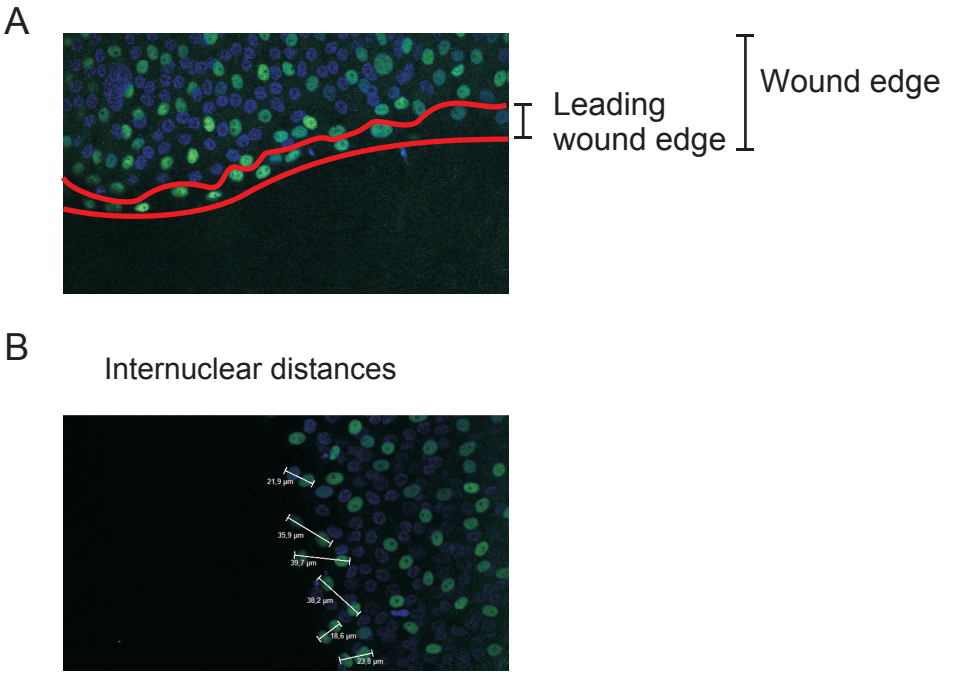
Supplementary Table 1: qPCR primer sequences.

Gene	Forward	Reverse
HMOX1	5'-AACCCTGAACAACGTAGTCTGCGA-3'	5'-ATGGTCAACAGCGTGGACACAAA-3'
SCAL1	5'-GGCATTTACCAGCTGAGGGA-3'	5'-TACCCCTACCTAGCACAGCA-3'
RNASE7	5'-CCAAGGGCATGACCTCATCAC-3'	5'-ACCGTTTTGTGTGCTTGTTAATG-3'
IL8	5'-CAGCCTTCCTGATTTCTG-3'	5'-CACTTCTCCACAACCCTCTGC-3'
RPL13A	5'-AAGGTGGTGGTCGTACGCTGTG-3'	5'-CGGGAAGGGTTGGTGTTCATCC-3'
ATP5B	5'-TCACCCAGGCTGGTTCAGA-3'	5'-AGTGGCCAGGGTAGGCTGAT-3'

SUPPLEMENTARY FIGURES



Supplementary Figure 1. Live imaging of intrinsic airway epithelial wound repair. (A) Primary bronchial epithelial cells (PBEC) were cultured and differentiated in an air-liquid interface (ALI) model, and subsequently mechanically wounded to assess wound repair. (B) The wound closure of ALI-PBEC was followed by live imaging at 0, 6, 12, 24, 36 and 48 h after wounding. (C) Wound closure was determined each hour, up to 48 h, by live imaging. Data are shown as the percentage wound closure compared to t = 0. Data are shown as mean; error bars represent SEM; experiments were conducted in duplicate. N = 3 independent donors.



Supplementary Figure 2. Quantification methods of p63+ cells in wounded ALI-PBEC. (A) Analysis of p63+ and p63- DAPI-stained nuclei at the leading wound edge. (B) Graphic example of how the internuclear distances were determined between p63+ cells located at the leading wound edge and p63+ cells that were perpendicular to the wound. p63+ cells at the leading wound edge were defined by the absence of other p63+ cells in the 45°-135° angle in its front perpendicularly to the wound edge. The most proximate p63+ cell that did not fulfill this definition was considered the reference cell to be selected for the measurement of the internuclear distance between adjacent p63+ cells. The distance between the outside edges of these two cells was regarded the internuclear distance. To prevent underestimation of distances in p63+ denser areas, each non-wound edge cell could be used only once for internuclear distance assessment, targeting overall at the lowest mean distance. The measurements were done in 5 randomly taken images of air- and CS-exposed ALI-PBEC. This analysis was performed in cultures derived from 3 independent donors.

CHAPTER 7

Effect of acute cigarette smoke exposure on airway epithelial activation of the integrated stress response.

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In preparation

ABSTRACT

Maintaining airway epithelial integrity is critical for lung defense. However, exposure to inhaled micro-organisms and toxicants constitutes a continuous threat to this defense. The integrated stress response (ISR) is a cellular mechanism that may contribute to the protection and recovery of airway epithelial cells upon injury. However, especially following chronic activation, the ISR may also mediate cell death. It has been proposed that the ISR contributes to the development and/or progression of the lung disorder chronic obstructive pulmonary disease (COPD). Moreover, it has been shown that prolonged exposure of cultured airway epithelial cells to cigarette smoke, which is the main risk factor of COPD, causes ISR-mediated cell death. This suggests a role of the ISR in smoke-induced effects on the airway epithelium that may contribute to COPD development. However, activation of the ISR has been investigated mainly in cell lines, while activation in primary airway epithelial cell cultures from COPD patients has not been studied. In the present study, we therefore further examined the influence of acute exposure to whole cigarette smoke or cigarette smoke extract on activation of the ISR in differentiated and undifferentiated airway epithelial cells respectively. We demonstrate that the ISR is activated in both smoke exposure models. Moreover, we observed higher expression of ISR-related target genes in whole smoke-exposed COPD cell cultures compared to controls, and provide data to propose a mechanism by which smoke-induced oxidative stress modulates the ISR. Taken together, our findings provide evidence for the involvement of activation of the ISR in the airway epithelial response to cigarette smoking and the importance of oxidative stress in modulating this response. More pronounced activation of the ISR in smoke-exposed epithelial cells from COPD patients, suggests involvement of the ISR in COPD pathogenesis.

INTRODUCTION

The airway epithelium is the first cellular defense lining of the respiratory tract and provides protection through its barrier function, mucociliary clearance, and innate immune defense mechanisms (1-4). Environmental stressors, including inhaled pathogens and cytotoxic particles and gases, may have detrimental effects on the airway epithelium, causing injury and promoting cell death (5). Adaptation of airway epithelial cells to stressors is therefore critical, and various intrinsic mechanisms provide protection from inhaled toxicants, including the detoxification by metabolic enzymes and antioxidants (6).

The main risk factor for the development and progression of the inflammatory lung disorder chronic obstructive pulmonary disease (COPD) in Westernized societies is cigarette smoking (7). Various studies have provided evidence that cigarette smoke exposure results in activation of the unfolded protein response (UPR) to endoplasmic reticulum (ER) stress (8-11). It was shown that cigarette smoke extract may selectively activate the PERK arm of the UPR in the bronchial epithelial cell line BEAS-2B and that this could be inhibited by anti-oxidants (12). PERK is an ER stress-sensing kinase that phosphorylates the eukaryotic translation initiation factor 2 (eIF2) subunit, eIF2α (13). Since eIF2α can also be phosphorylated by other stress-sensing kinases (GCN2, HRI and PKR), the pathway starting with phosphorylation of eIF2α is also referred to as the integrated stress response (ISR) (14). Phosphorylation and inactivation of eIF2α leads to the protection of the cell by inducing a broad inhibition of protein translation. This inhibition may provide protection against e.g. the accumulation of misfolded proteins in the ER. Phosphorylation of eIF2α also results in activation of gene expression through selective translation of the transcription factor ATF4, resulting in production of the transcription factor C/EBP homologous protein (CHOP), that regulates expression of a range of genes including growth arrest and DNA damage-inducible protein 34 (GADD34) (15). Interestingly, the gene expression signature present in bronchial tissue from COPD patients is enriched for genes regulated by ATF4 (16). By dephosphorylating eIF2α, GADD34 enables the recovery of protein synthesis and thus controls the duration of ISR. In contrast, during sustained stress, recovery of protein translation by GADD34 may promote cell death (15). GADD34 is therefore a critical regulator of cell viability, which depends on the resolution or persistence of cellular stress.

Although evidence suggests that the UPR and ISR can be activated by cigarette smoke (CS), many studies were performed in cell lines or undifferentiated primary cells using CS extract. Consequently, relatively little is known about activation of the UPR and ISR in differentiated primary airway epithelial cells by whole CS, or whether these responses are affected by the COPD status of the tissue donor. This is important, since we and others have shown that biochemical features of COPD may persist in epithelial cell cultures *ex vivo* (17). Moreover, the mechanistic relationship between CS and activation of the ISR remains obscure.

In a previous study we showed that short-term exposure of airway epithelial cells to whole CS had a mild cytotoxic effect, while cells displayed transient inflammatory responses and a temporary impairment in the epithelial barrier integrity (18). Activation of the ISR might contribute to this recovery of epithelial cells after acute CS exposure. Therefore, in the present study we examined ISR activation in airway epithelial cells during short-term exposures

to whole CS, and compared the activation of the ISR between cell cultures derived from individuals with COPD and non-COPD controls. We provide evidence that CS increases expression of GADD34 both via the classical ISR and via a non-ISR oxidative stress-dependent pathway. These findings suggest a new mechanism by which oxidative stress can modulate activation of the ISR.

MATERIALS AND METHODS

Cell culture

Primary bronchial epithelial cells (PBEC) were isolated from tumour-free resected lung tissue obtained during surgery for lung cancer, and cultured at the air-liquid interface (ALI-) or submerged conditions (S-). Culturing of ALI-PBEC and S-PBEC was performed as previously reported (18). In ALI-PBEC cultures, cells were seeded on 0.4 μm pore sized semi-permeable transwell membranes (Corning Costar, Cambridge, MA, USA) and first cultured in submerged conditions in a 1:1 mixture of bronchial epithelial growth medium (BEGM) (Lonza, Verviers, Belgium) and Dulbecco's modified Eagle's medium (DMEM) (Gibco, Bleiswijk, The Netherlands) containing 1 mM HEPES (Lonza), 100 U/mL penicillin and 100 μg/ml streptomycin (Lonza) (hereafter referred to as B/D culture medium), supplemented with singleQuot BEGM supplements (except Gentamycin) (Lonza), 15 ng/ml retinoic acid (Sigma-Aldrich) and 1 mg/ml BSA (Sigma-Aldrich). When monolayers were confluent, the apical medium was removed and cells were cultured in air-exposed conditions for at least 2 weeks to allow mucociliary differentiation. In western blot experiments, cells were cultured overnight and during the experiment with starvation medium, consisting of B/D medium without BSA and the SingleQuot supplements BPE and EGF. ALI-PBEC cultures were used from COPD patients and non-COPD (ex)smokers, for whom the disease status was determined based on pre-surgery lung function data according to the Global Initiative for Chronic Obstructive Lung Disease classification (7) (Table 1). S-PBEC were cultured in regular tissue culture plates with B/D culture medium as earlier described, but without the additional 15 ng/ml retinoic acid supplementation. Cells were used when approximately 80-90% confluent. In all experiments, S-PBEC were cultured overnight prior to the experiment and during the experiment with starvation medium, which was similar to ALI-PBEC starvation medium, but lacked the additional 15 ng/ml retinoic acid supplementation. eIF2α^{AA} and eIF2α^{SS} mouse embryonic fibroblasts (MEFs) were cultured as previously described (19).

Cigarette smoke exposure models and other stimuli

ALI-PBEC cultures were exposed to whole cigarette smoke (CS) using an exposure model previously described (18). In short, cells were exposed to whole smoke derived from one standard research cigarette (3R4F), after which cells were incubated for the indicated periods of time. S-PBEC and MEF cultures were treated with cigarette smoke condensate (CSC), which was prepared as previously reported (20). Cells were treated with CSC via a "pulse" method described in Figure 4 and corresponding text in the Results section, similar to the whole CS exposure protocol. In indicated experiments, cells were pre-treated for 1 hour and treated during the experiment with the anti-oxidant N-acetylcysteine (NAC) (Sigma). As indicated, in some experiments cells were treated with thapsigargin (Tg) or tunicamycin (Tm) (both Sigma) as positive controls for ER stress

Table 1: Characteristics of COPD and non-COPD patients.

	COPD	non-COPD	p-value
Number of donors	7	6	
Gender (females/males)	2/5	2/4	
Age, years	58±7	67±9	
FEV_1, % predicted	64±11	96±20	< 0.002
FEV_1/FVC %	53±8	76±4	< 0.0001

Patient characteristics: Age and lung function are shown as means ± SD. The mean differences in FEV_1 (% predicted) and FEV_1/FVC (%) were compared using the non-parametric Mann-Whitney test.

Western blot

Cell lysates were prepared in Harvest Buffer (Buffer H), consisting of 10 mM HEPES pH 7.9, 50 mM NaCl, 0.5 mM sucrose, 0.1 mM EDTA, 0.5% (v/v) Triton X-100, 1 mM DTT, Protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany), phosphatase inhibitors (10 mM tetrasodium pyrophosphate, 17.5 mM β-glycerophosphate, and 100 mM NaF), and 1 mM PMSF. Nuclear extraction was performed using the NE-PER Nuclear Protein Extraction Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. Cell lysates were diluted in 4x sample buffer consisting of 0.2 M Tris-HCl pH 6.8, 16% [v/v] glycerol, 4% [w/v] SDS, 4% [v/v] 2-mercaptoethanol and 0.003% [w/v] bromophenol blue. Samples were separated using a 10% SDS-PAGE gel and proteins were transferred onto nitrocellulose membrane. Primary antibodies were used against: eIF2α, phospho-eIF2α, ATF4, TBP (Cell Signaling Technology), GADD34 (ProteinTech) and puromycin (Millipore) (all 1:1000 diluted). After secondary antibody incubation, proteins detection was visualized using ECL (ThermoScientific) or the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences). Quantification of protein bands was done by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

qPCR

RNA was isolated with the Maxwell tissue RNA extraction kit (Promega) or Qiagen RNeasy mini kit (Qiagen). cDNA synthesis and qPCR was performed as previously described (see Table 2 for qPCR primer pairs) (18, 19). Relative gene expression was calculated according to the standard curve method. ATP5B and RPL13A were selected using the "Genorm method" (Genorm; Primer Design, Southampton, UK) as reference genes for PBEC. β-actin was used as reference gene for MEFs.

ELISA & LDH release assay

Protein secretion of IL-8/CXCL8 (R&D systems), and LDH release (Roche) was determined in the cell culture medium according to the manufacturer's protocol.

Statistics

Results were analyzed using GraphPad Prism 6.0 (GraphPad Inc). As indicated in the figures, statistical tests used for data analysis were (un)paired t-test or 2-way ANOVA, with post-hoc Bonferroni correction for multiple analyses. Differences with a p-value < 0.05 were considered statistically significant.

Table 2 qPCR primers

Gene	Forward primer (5' - 3')	Reverse primer (5' - 3')
Human		
GADD34	ATGTATGGTGAGCGAGAGGC	GCAGTGTCCTTATCAGAAGGC
CHOP	GCACCTCCCAGAGCCCTCACTCTCC	GTCTACTCCAAGCCTTCCCCCTGCG
Bip/GRP78	CGAGGAGGAGGACAAGAAGG	CACCTTGAACGGCAAGAACT
sXBP1	TGCTGAGTCCGCAGCAGGTG	GCTGGCAGGCTCTGGGGAAG
IL8	CAGCCTTCCTGATTTCTG	CACTTCTCCACAACCCTCTGC
RPL13A	AAGGTGGTGGTCGTACGCTGTG	CGGGAAGGGTTGGTGTTCATCC
ATP5B	TCACCCAGGCTGGTTCAGA	AGTGGCCAGGGTAGGCTGAT
PTGS2	TAAGTGCGATTGTACCCGGAC	TTTGTAGCCATAGTCAGCATTGT
DUSP5	TGTCGTCCTCACCTCGCTA	GGGCTCTCTCACTCTCAATCTTC
cFOS	CCTAACCGCCACGATGATGT	TCTGCGGGTGAGTGGTAGTA
Mouse		
GADD34	CCCGAGATTCCTCTAAAAGC	CCAGACAGCAAGGAAATGG
CHOP	GGAGCTGGAAGCCTGGTATGAG	GCAGGGTCAAGAGTAGTGAAGG
ACTB	TCCTGGCCTCACTGTCCA	GTCCGCCTAGAAGCACTTGC

RESULTS

Whole cigarette smoke exposure increases the ISR in ALI-PBEC

We first examined the effect of whole cigarette smoke (CS) on activation of the integrated stress response (ISR) in mucociliary differentiated primary bronchial epithelial cells (ALI-PBEC). Cell cultures were exposed to whole CS from a single cigarette (Figure 1A), which in previous studies was shown to cause an acute and transient disruption of the epithelial barrier, impairment of wound repair and induction of innate immune responses with minimal cytotoxic effects (17, 18, 21). An acute activation of the ISR upon CS exposure was detected based on the increased phosphorylation of eIF2α and nuclear accumulation of ATF4 within the first 2 hours after exposure (Figure 1B,C). In addition, a time-dependent expression of *GADD34* and *CHOP* mRNA was observed, which continued to increase up to 2 hours after exposure (Figure 1D). In previous studies it has been shown that smoke-induced activation of the ISR pathway is induced by the unfolded protein response (UPR) to endoplasmic reticulum (ER) stress (12). Therefore, we further examined activation of the UPR by determining the expression of the UPR target gene *BIP/GPR78* and splicing of *XBP1* mRNA. We did not observe an increased expression of *BIP* nor increased splicing of *XBP1* mRNA within the 2 h timeframe that was examined (Figure 1E). Similar results were obtained when expression levels of *BIP* and spliced *XBP1* were determined at 6 h after CS exposure, by which time the ER stress inducer tunicamycin was able to increase the expression of both markers in ALI-PBEC (Figure 1F). In contrast, both the levels of *CHOP* and *GADD34* mRNA expression were induced by both CS and tunicamyin at 6 hours.

The persistence of CS-induced ISR activation was subsequently determined at time points ranging from 0-24 h post-CS exposure (Figure 2A) by determining GADD34 protein

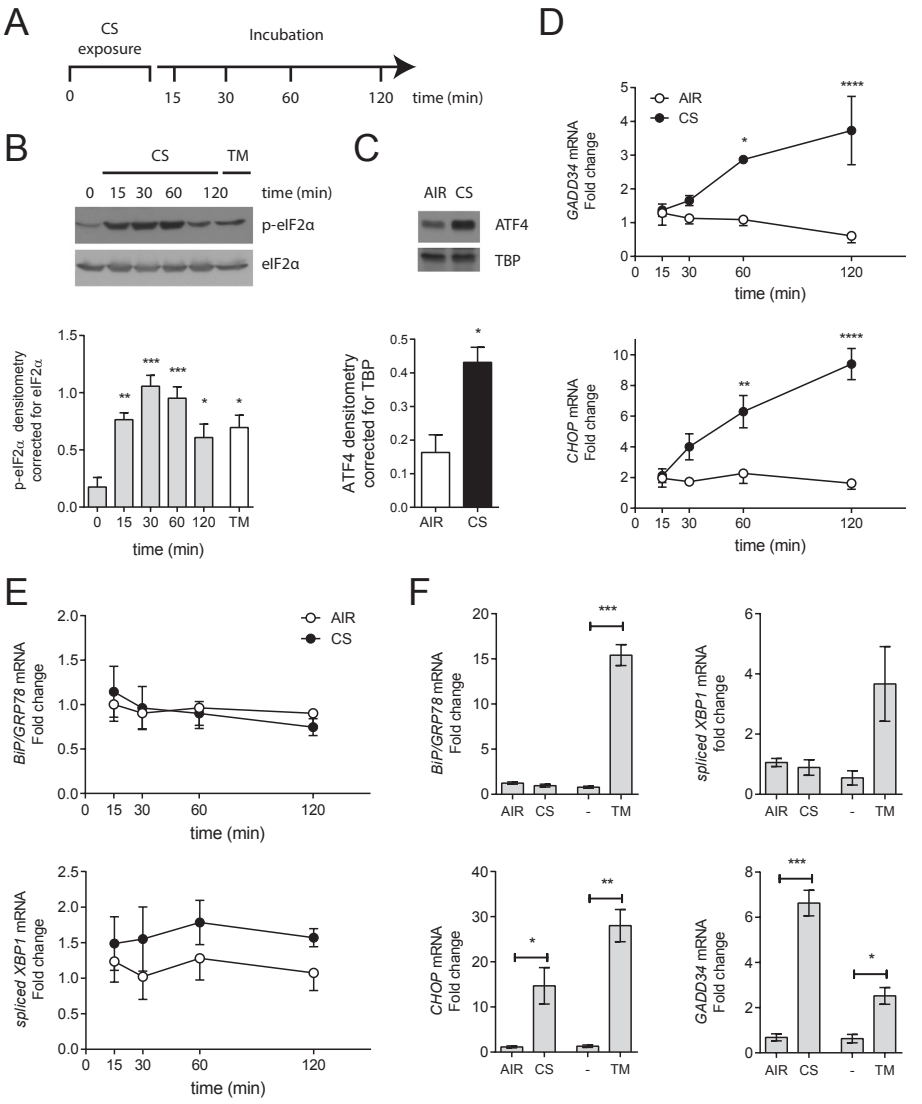

Figure 1. Effect of whole cigarette smoke (CS) exposure on ISR-activation in ALI-PBEC cultures. (A) Schematic of the whole CS exposure experiment, in which ALI-PBEC were exposed to CS from a single cigarette, followed by incubation for different time periods ranging from 15-120 min. (B) eIF2α phosphorylation was determined 15-120 min after whole CS exposure. The ER stress inducing agent tunicamycin (TM) was used as positive control. Results were quantified by densitometry using total eIF2α as loading control. (C) Nuclear localization of ATF4 was determined 2 h after CS exposure. Results were quantified by densitometry using TATA binding protein (TBP) as loading control. (D) Analysis of *GADD34* and *CHOP*, (E) BiP/*GRP78* and *sXBP1* mRNA expression 15-120 min after CS exposure. (F) mRNA expression of *GADD34*, *CHOP*, BiP/*GRP78* and *sXBP1* after Air/CS exposure or TM after 6 h incubation period. Results are shown as mean ± SD of at least 3 different donors. Analysis of differences was conducted using (B,D,E) a one-way ANOVA with a Bonferroni post-hoc test and (C,F) a paired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0005$.

expression, eIF2α phosphorylation, and puromycin incorporation as measurement of protein synthesis. Increased GADD34 protein expression was observed at 3 h and peaked at 6 h after CS exposure (Figure 2B,C). This was preceded by an earlier increase in CS-induced eIF2α phosphorylation (Figure 1B), which fell below baseline levels at 6-12 h before returning to baseline by 24 h (Figure 2B,C). CS exposure impaired protein synthesis, as reported by puromycin incorporation, and this inhibition was most prominent at 3 h after CS exposure but protein synthesis progressively recovered at 6, 12 and 24 h after exposure. Taken together, these findings suggest that acute exposure to whole CS causes a transient activation of the ISR.

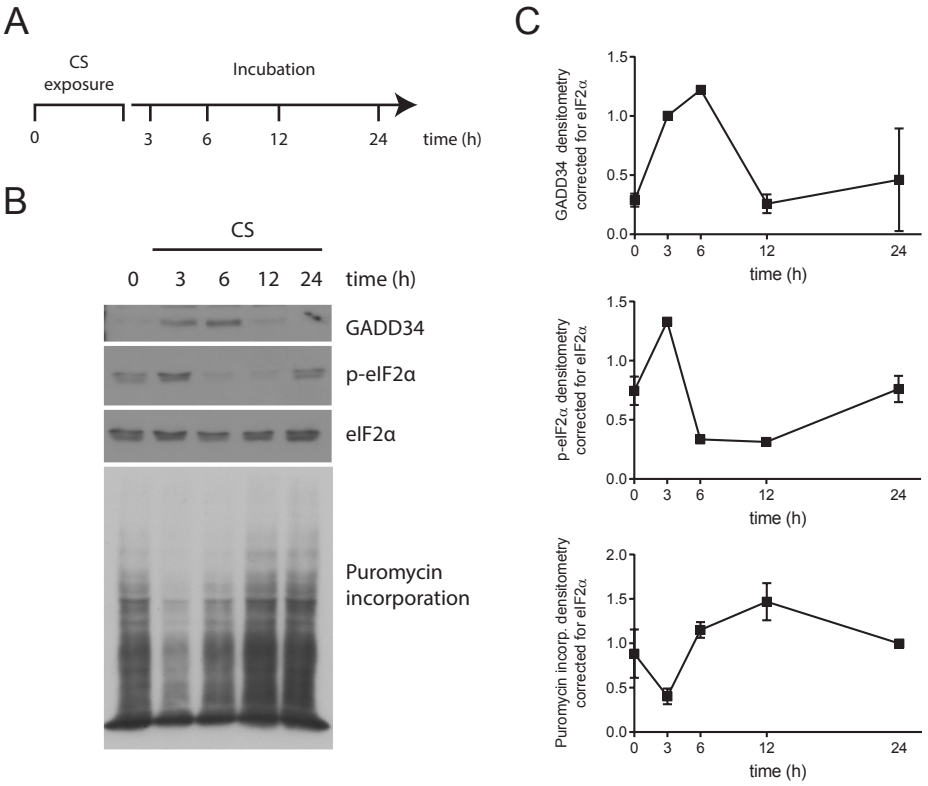

Figure 2. Effect of whole CS exposure on protein translation in ALI-PBEC cultures. (A) Schematic of the experiment, in which ALI-PBEC were exposed to CS from a single cigarette, followed by incubation for different time periods ranging from 0-24 h. (B) Analysis of GADD34 protein expression, eIF2α phosphorylation, and puromycin incorporation as marker of protein translation. (C) Results were quantified by densitometry using total eIF2α as loading control. Experiments were conducted in 2 independent donors showing similar results.

COPD airway epithelial cells display higher CS-induced expression of CHOP and GADD34

Next, we examined the induction of the ISR in ALI-PBEC from COPD patients and non-COPD (ex)smokers by comparing expression of *CHOP* and *GADD34* mRNA. ALI-PBEC were exposed to whole CS and mRNA expression levels were determined at 3, 12 and 24 h after exposure. In line with earlier observations, CS-induced *CHOP* and *GADD34* expression was observed early after exposure, whereas the expression decreased at later time points (Figure

3A). A comparison between COPD and non-COPD airway epithelial responses demonstrated a significantly higher expression of *GADD34* and *CHOP* in COPD airway epithelial cultures. In addition, and in line with this observation, we observed a negative correlation between CS-induced GADD34 and CHOP expression and FEV_1 (Figure 3B) and FEV_1/FVC (Figure 3C).

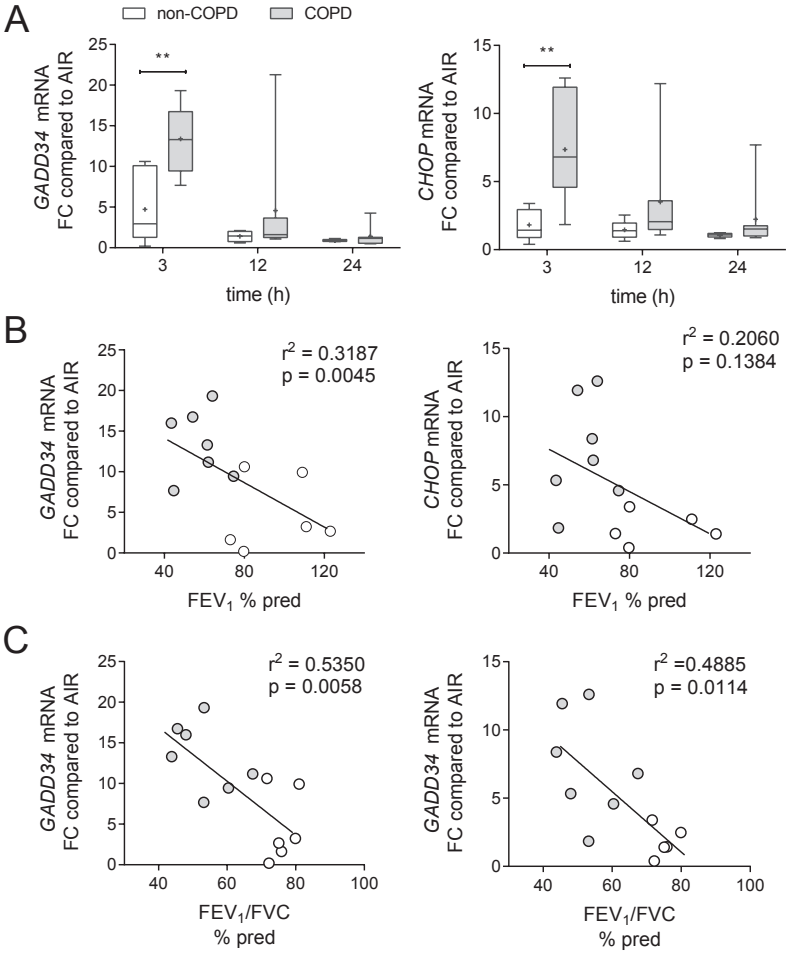

Figure 3. CS-induced expression of GADD34 and CHOP in COPD and non-COPD ALI-PBEC. (A) ALI-PBEC from COPD and non-COPD donors were exposed to whole CS and incubated for 3,12, and 24 h after which *GADD34* and *CHOP* mRNA expression was determined. (B) Correlation between fold change in mRNA expression compared to air controls of *GADD34* and *CHOP* mRNA, with FEV_1 (% pred), and (C) FEV_1/FVC (% pred). n=7 COPD and n=6 non-COPD donors were used in the analysis. Analysis of differences was conducted using a one-way ANOVA with a Bonferroni *post-hoc* test. ** $p < 0.01$. Correlations were assessed by determining the linear regression.

In addition to *GADD34* and *CHOP*, we also determined the expression of *PTGS2*, *DUSP5* and *cFOS,* which have previously been found to be enhanced in airway epithelial tissue from COPD patients and to be linked with ATF4-dependent gene transcription (16). However,

whereas expression of these genes was also transiently increased at 3 hours post-CS exposure, we did not observe differences in the expression of these genes between COPD patients and non-COPD controls (Supplementary Figure 1).

Cigarette smoke extract increases the ISR in submerged cultured PBEC

Although whole CS exposure of ALI-cultured PBEC may adequately mimic *in vivo* smoke exposure of epithelial cells in lung tissue, this model does not readily allow exploration of the mechanisms regulating ISR activation. Therefore we also determined the effect of short-term cigarette smoke exposure on ISR activation using cigarette smoke condensate (CSC), which can be used in conventional submerged cell cultures. First, we examined the effect of CSC on submerged cultured primary bronchial epithelial cells (S-PBEC), to determine whether activation of the ISR could be reproduced in this model. Similar to ALI-PBEC exposed to whole CS, S-PBEC were stimulated using a pulse exposure method in which cells were incubated with CSC for 15 minutes, after which the culture medium was refreshed and cells were incubated for different time periods (Figure 4A). This was done using CSC concentrations that in a previous study were shown to be cytotoxic to epithelial cells upon prolonged exposure (20). In contrast to prolonged exposures, pulse exposure only caused a mild cytotoxic effect based on the release of LDH in the culture medium (Figure 4B). However, CSC exposure of S-PBEC did cause a dose-dependent protein secretion of the neutrophil chemoattractant IL-8/CXCL8 (Figure 4C). This was accompanied by an early and dose-dependent mRNA expression of *IL8* (Figure 4D,E). These findings suggest that, similar to our studies using the whole CS exposure model (18), pulsed CSC stimulation also induces pro-inflammatory responses with minimal cytotoxic effects. We next determined ISR activation in S-PBEC stimulated with CSC by determining mRNA expression of *CHOP* and *GADD34*. Similar to *IL8*, *GADD34* and *CHOP* mRNA expression was rapidly but transiently induced, reaching maximal levels at 3 h after exposure (Figure 4D). Moreover, a dose dependent increase of *GADD34* and *CHOP* mRNA expression was observed at 3 h after exposure (Figure 4E). Interestingly, in contrast to our observations with differentiated ALI-PBEC, CSC also increased mRNA expression of *BIP* and spliced *XBP1*, peaking at 6 h after exposure (Figure 4F).

CSC –induced GADD34 expression occurs independent of the ISR

Next, to gain insight into the mechanism by which CS induced expression of *GADD34* and *CHOP*, we repeated the exposure to CSC experiments in MEFs, comparing wild type eIF2α^{SS} cells with MEFs lacking the ability to phosphorylate eIF2α owing to mutation of serine-51, eIF2α^{AA} (22). As expected, CSC induced expression of both *CHOP* and *GADD34* in eIF2α^{SS} MEFs, however, in eIF2α^{AA} MEFs CSC remained able to induce GADD34 expression, but the induction of CHOP was lost (Figure 5A). Indeed, in eIF2α^{AA} MEFs CSC induced GADD34 at a concentration below that required to induce GADD34 in wild type cells. In line with this observation, increased levels of GADD34 protein was also observed in both eIF2α^{AA} and eIF2α^{SS} MEFs following (Figure 5B), suggesting that an ISR-independent mechanism contributes to CSC-induced GADD34 expression.

ISR-deficient cells are known to be impaired in their response to oxidative stress (23). It is also known that altered redox plays an important role in the cytotoxic effects of cigarette smoke (20). We therefore determined whether the ISR-independent expression of GADD34 might

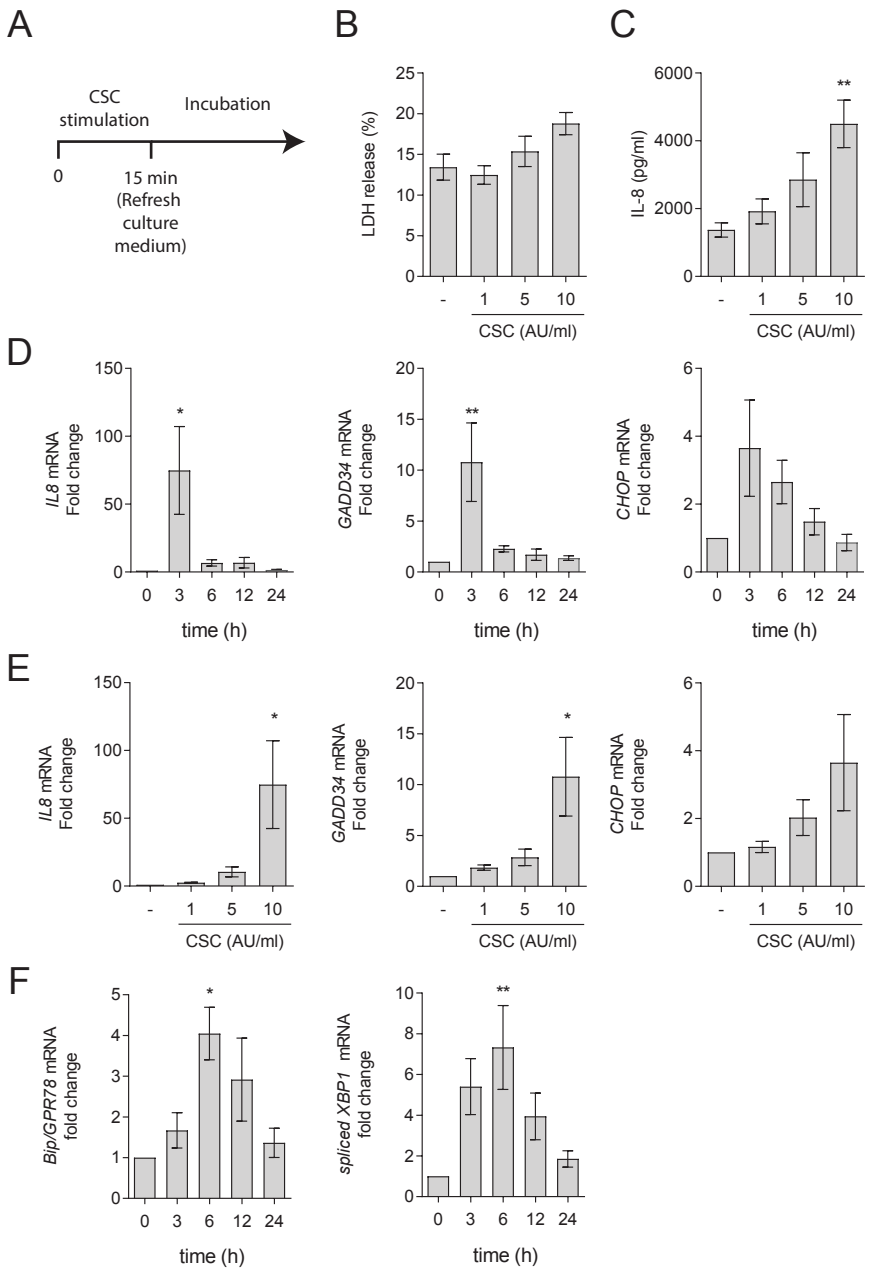

Figure 4. Effect of CSC on ISR activation in undifferentiated S-PBEC. (A) Schematic of experiments with CSC pulse exposure of S-PBEC cultures. (B) Assessment of the cytotoxic effect of 1-10 AU/ml CSC on S-PBEC after 24 h incubation. Percentage LDH release was used as measurement of cytotoxicity. (C) Assessment of IL-8 protein release in the cell culture supernatant of S-PBEC stimulated with 1, 5 and, 10 AU/ml CSC and incubated for 24 h. (D) Assessment of *IL8*, *GADD34* and *CHOP* mRNA expression at different time points (3-24 h) after 10 AU/ml CSC exposure of S-PBEC. mRNA expression is shown as fold chance compared to control-treated cells. (E) Assessment of

IL8, GADD34 and *CHOP* mRNA expression in S-PBEC after stimulation with 1, 5, and 10 AU/ml CSC exposure and 3 h incubation. mRNA expression is shown as fold chance compared to control-treated cells. (F) mRNA expression of BiP/*GRP78* and *sXBP1* at different time points (3-24 h) after 10 AU/ml CSC exposure of S-PBEC. mRNA expression is shown as fold chance compared to controls. Results are shown as mean ± SD using cells of at least 3 independent donors. Analysis of differences was conducted using a one-way ANOVA with a Bonferroni *post-hoc* test . * $p < 0.05$ and ** $p < 0.01$.

be mediated by oxidative stress. eIF2α^{AA} and eIF2α^{SS} MEFs were treated with the anti-oxidant N-acetylcysteine (NAC) and CSC in combination. CSC-induced expression of GADD34 was inhibited by NAC in both eIF2α^{AA} and eIF2α^{SS} MEFs (Figure 5C). In contrast, the ER stress activator thapsigargin induced GADD34 expression only in ISR-competent eIF2α^{SS} and this induction was insensitive to NAC (Figure 5C). This finding suggests that CSC can modulate the ISR via oxidative stress-induced GADD34 expression.

DISCUSSION

In the present study, we have used two complementary cigarette smoke exposure models to show that that short-term exposures of airway epithelial cells to cigarette smoke results in activation of the ISR. Whole cigarette smoke rapidly caused ISR activation in ALI-PBEC, which declined at later time points. Moreover, during the recovery period we observed that the kinetics of eIF2α dephosphorylation and recovery of protein synthesis corresponded with that of GADD34 protein expression. This suggests that the transient activation of the ISR by smoke is attenuated by GADD34.

We have previously shown that acute exposure to cigarette smoke caused a transient disruption of the epithelial barrier integrity in ALI-PBEC (18). Furthermore, we have shown that cigarette smoke transiently impaired epithelial wound repair by reducing cell migration (21). Interestingly, we found that recovery of epithelial barrier and wound repair functions were occurred 6 hours after exposure, which corresponds with the kinetics of GADD34 protein expression and the recovery of protein translation it mediates. It is therefore tempting to speculate that GADD34-dependent recovery of protein translation might be responsible, at least in part, for the recovery of ALI-PBEC cultures following smoke exposure. However, we currently lack conclusive evidence that proves that GADD34 expression is cytoprotective in this context.

We observed more pronounced expression of *CHOP* and *GADD34* mRNA in ALI-PBEC cultures from COPD patients when compared to non-COPD controls, and a correlation between gene expression and lung function parameters. This finding is in line with our earlier study that detected an ATF4 gene signature within the airway of individuals with COPD, although *GADD34* and *CHOP* were not part of the transcriptome data (16). Other genes, such as *PTGS2*, *DUSP5* and *cFOS*, that were found to be increased in COPD airways (16), were not different between COPD and non-COPD cultures exposed to CS in the current study. This is likely reflects the existence of additionally signaling pathways acting upon epithelial cells *in vivo* that do no persist *ex vivo*. More pronounced activation of ISR in COPD cultures may report and innate increased susceptibility to cigarette smoke of some individuals, although

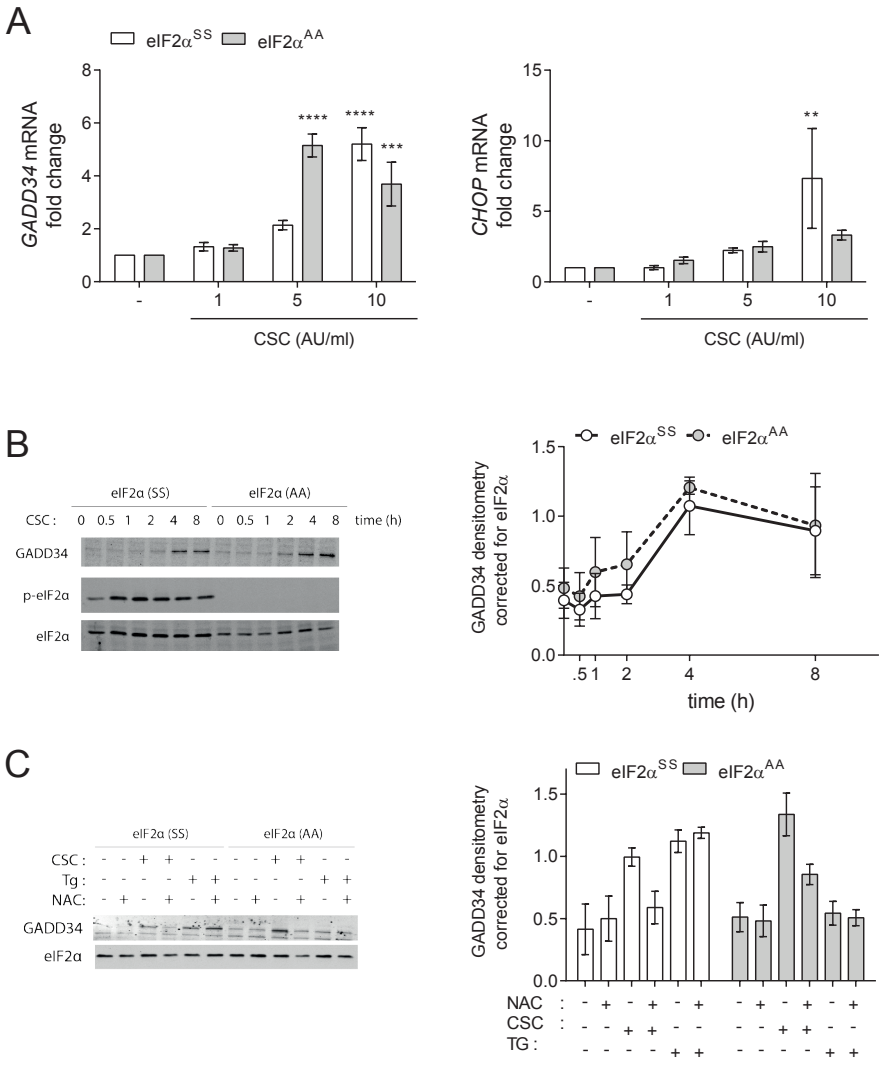

Figure 5. ISR-independent expression of GADD34 in cigarette smoke condensate (CSC) exposure of MEFs. (A) Assessment of GADD34 and CHOP mRNA expression in eIF2α^{AA} and eIF2α^{SS} MEFs stimulated with 1,5 or 10 AU/ml CSC after pulse stimulation and followed by 24 h incubation. Results are shown as fold change in mRNA compared to control-treated cells. (B) Assessment of GADD34 protein expression in eIF2α^{AA} and eIF2α^{SS} MEFs stimulated with 10 AU/ml CSC after pulse stimulation and followed by incubation at different time points. Results were quantified by densitometry using total eIF2α as loading control. (C) Assessment of the effect of N-acetyl cysteine (NAC) on CSC and thapsigargin (Tg)-induced expression of GADD34 in eIF2αAA and eIF2αSS MEFs after 8 h incubation. Results were quantified by densitometry using total eIF2α as loading control. Results are shown as mean ± SD of at least 3 different experiments. Analysis of differences was conducted using a one-way ANOVA with a Bonferroni *post-hoc* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0005$.

this might be considered out of keeping with a proposed protective role for the ISR during exposure to smoke. However, the chronicity stress has major effects both on the output of the ISR and on the pathological consequences of smoke, which may account for some of these apparent disparities (24). For example, it is plausible that ISR-independent expression of GADD34 caused by oxidative stress may enhance the recovery of protein synthesis that would otherwise by inhibited by CS. Alternatively, a cytoprotective effect of the ISR during acute cigarette smoke exposure might support the persistence of other airway epithelial cells functions that contribute to COPD pathogenesis. It has been shown, for instance, that CHOP can regulate airway epithelial expression of CXCL8/IL-8 in airway epithelial cells (25, 26). Moreover, the recovery of protein translation mediated by GADD34 has been shown to enable for the synthesis of innate immune mediators including IL-6, interferon-α and -β (27, 28). In addition, it has been shown that GADD34 knockout mice display reduced lung inflammation upon exposure to the cigarette smoke component acrolein (29). Overall, these studies suggest that both CHOP and GADD34 may contribute to inflammatory responses, and therefore increased expression in COPD airway epithelial cells may further enhance airway inflammation in response to cigarette smoke.

Similar to airway epithelial cells, CSC also increased expression of CHOP and GADD34 in MEFs. This is in line with the non-cell type specific activation of the ISR by cigarette smoke, which has been shown in other cell types besides airway epithelial cells (8, 30). When examining the role of ISR activation in this response, using cells expressing a non-phosphorylatable mutant of eIF2α, we demonstrated that GADD34 expression occurred independent of the ISR. This alternative pathway is dependent upon oxidative stress and can be inhibited by antioxidants. This effect could be reproduced in the airway epithelial cell line 16HBE (Supplementary Figure 2A), suggesting conservation of this mechanism in airway epithelial cells. Previous reports have shown that activation of the p38 MAPK pathway led to increased expression of GADD34 (31). Indeed, CSC-induced activation of p38 in airway epithelial cells was shown to be oxidative stress-dependent (20), and therefore it is possible that this signaling axis is involved in the ISR-independent expression of GADD34. In experiments using the airway epithelial cell line 16HBE, we observed reduced CS extract-induced GADD34 protein expression upon inhibition of p38 (Supplementary Figure 2B). However, further research is required to demonstrate this in primary airway epithelial cells. As oxidative stress has an important role in the induction of inflammatory responses in airway epithelial cells, it can be further speculated that the oxidative stress-mediated expression of GADD34 may contribute to this response. Moreover, persistent GADD34 expression induced by oxidative stress resulting from prolonged exposure to cigarette smoke may contribute to cell death by completely inhibiting activation of the ISR.

In summary, we have shown that ISR activation is part of the response of airway epithelial cells to cigarette smoke. This response is more pronounced in COPD airway epithelial cells and correlates with disease severity, suggesting a role of the ISR in COPD. Moreover, an oxidative stress-dependent and ISR-independent expression of GADD34 suggests the involvement of alternative mechanisms initiated by cigarette smoke exposure that may affect airway epithelial cell functions.

ACKNOWLEDGEMENTS

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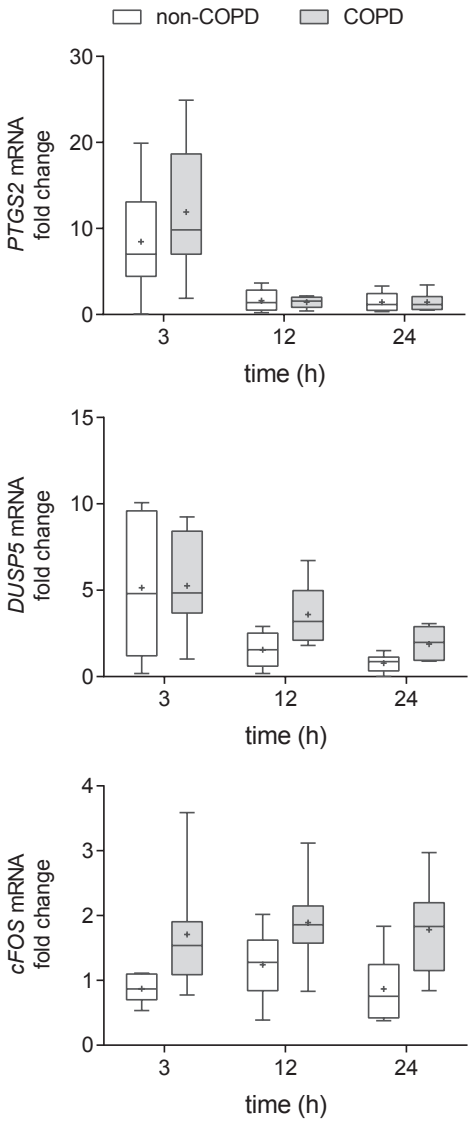
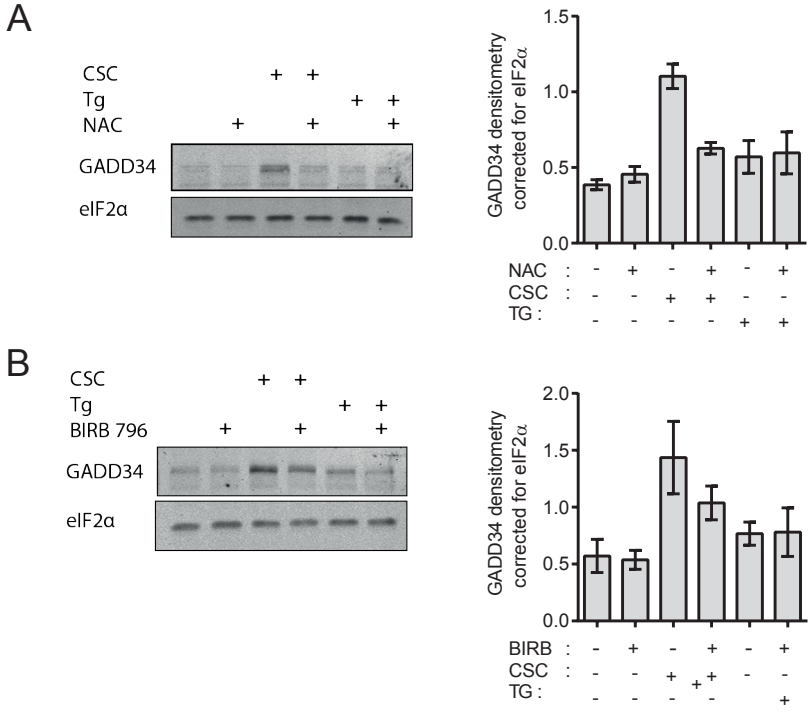
SUPPLEMENTARY FIGURES



Supplementary Figure 1. CS-induced expression of ATF4-related and COPD enhanced target genes in COPD and non-COPD ALI-PBEC. ALI-PBEC from COPD and non-COPD donors were exposed to whole CS and incubated for 3, 12, and 24 h after which *PTGS2*, *DUSP5* and *cFOS* mRNA expression was determined. n=7 COPD and n=6 non-COPD donors were used in the analysis. Analysis of differences was conducted using a one-way ANOVA with a Bonferroni *post-hoc* test.



Supplementary Figure 2. Effect of N-acetyl cysteine and p38 inhibition on cigarette smoke condensate-induced GADD34 expression in 16HBE. (A) Assessment of the effect of N-acetyl cysteine (NAC) on CSC and thapsigargin (Tg)-induced expression of GADD34 in the airway epithelial cell line 16HBE after 8 h incubation. (B) Assessment of the effect of the p38 inhibitor BIRB 796 on CSC and thapsigargin (Tg)-induced expression of GADD34 in the airway epithelial cell line 16HBE after 8 h incubation. Results were quantified by densitometry using total eIF2α as loading control. Results are shown as mean ± SD of 3 independent experiments.

CHAPTER 8

ADAM17 and EGFR regulate IL-6 receptor and amphiregulin mRNA expression and release in cigarette smoke-exposed primary bronchial epithelial cells from patients with chronic obstructive pulmonary disease (COPD).

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ABSTRACT

Aberrant activity of a disintegrin and metalloprotease 17 (ADAM17), also known as TACE, and epidermal growth factor receptor (EGFR) has been suggested to contribute to chronic obstructive pulmonary disease (COPD) development and progression. The aim of this study was to investigate the role of these proteins in activation of primary bronchial epithelial cells differentiated at the air–liquid interface (ALI-PBEC) by whole cigarette smoke (CS), comparing cells from COPD patients with non-COPD. CS exposure of ALI-PBEC enhanced ADAM17-mediated shedding of the IL-6 receptor (IL6R) and the EGFR agonist amphiregulin (AREG) toward the basolateral compartment, which was more pronounced in cells from COPD patients than in non-COPD controls. CS transiently increased IL6R and AREG mRNA in ALI-PBEC to a similar extent in cultures from both groups, suggesting that posttranslational events determine differential shedding between COPD and non-COPD cultures. We show for the first time by in situ proximity ligation (PLA) that CS strongly enhances interactions of phosphorylated ADAM17 with AREG and IL-6R in an intracellular compartment, suggesting that CS-induced intracellular trafficking events precede shedding to the extracellular compartment. Both EGFR and ADAM17 activity contribute to CS-induced IL-6R and AREG protein shedding and to mRNA expression, as demonstrated using selective inhibitors (AG1478 and TMI-2). Our data are consistent with an autocrine-positive feedback mechanism in which CS triggers shedding of EGFR agonists evoking EGFR activation, in ADAM17-dependent manner, and subsequently transduce paracrine signaling toward myeloid cells and connective tissue. Reducing ADAM17 and EGFR activity could therefore be a therapeutic approach for the tissue remodeling and inflammation observed in COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a progressive lung disorder characterized by irreversible airflow obstruction due to airway inflammation, infection, and tissue remodeling (1). Airway epithelial cells play a central role in the pathogenesis of COPD through a variety of mechanisms, including production of inflammatory mediators, antimicrobial peptides, and growth factors (2). Exposure of the lung tissue to triggers like cytotoxic particles and gasses, including cigarette smoke, microbes, and innate immune mediators (3-7) activate various matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) expressed by airway epithelial cells (8, 9). The activity of MMPs and ADAMs contributes not only to proteolytic degradation of lung tissue, but also to regulation and processing of numerous receptor activating proteins (10-13). Through these various activities, MMPs and ADAMs are implicated in a broad spectrum of processes ranging from inflammatory responses to airway epithelial repair. It has been proposed that their aberrant activity might lead to chronic inflammation and abnormal tissue remodeling in the lungs of COPD patients (9).

One of the ADAMs, a ubiquitously expressed Zn^{2+}-dependent disintegrin and metalloprotease 17 (ADAM17), formerly known as TNFα converting enzyme (TACE), is recognized as an important regulator of pulmonary inflammation, cell proliferation, and epithelial barrier function (5, 14). In bronchial epithelial cells, ADAM17 modulates these processes by cleaving membrane-bound cytokines (TNFα), several EGF receptor (EGFR) agonists (TGF-α, amphiregulin, epiregulin, HB-EGF), cytokine receptors (IL6R, TNF-R), growth factor receptors (NOTCH receptors), and adhesion proteins (L-selectin, ICAM-1, E-cadherin) (11-13, 15). Moreover, ADAM17 phosphorylation and activity is enhanced in airway epithelial cell lines and in undifferentiated primary cells upon exposure to cigarette smoke extract (5, 6, 16).

Our studies focus on two ADAM17 substrates implicated in COPD pathogenesis: the IL-6 cytokine receptor (IL6R) and the growth factor amphiregulin (AREG), one of the EGFR agonists produced by bronchial epithelial cells (10). Elevated levels of IL6R have been observed in peripheral blood leukocytes of COPD patients (17), and recently genetic variants of IL6R have been linked with COPD severity (18). However, the regulation of shedding of IL6R and AREG from COPD airway epithelium has not been studied. Upon shedding from epithelial cells, IL6R and AREG activate the shared interleukin receptor gp130 and EGFR, respectively, on epithelial cells (autocrine), as well as on underlying myofibroblasts and myeloid cells (paracrine) (19-22). Both IL6/IL6R/gp130 and AREG/EGFR/ERK pathways are involved in the resolution of lung inflammation and repair of injury, but also in progression of subepithelial fibrosis and collagen deposition (7). These signaling pathways involve the JAK kinase and/or MAP kinase pathway, which are druggable targets in COPD pathology (23). Excessive ligand-mediated EGFR activation results in epithelial hyperproliferation and increased production of the inducible mucin MUC5AC, processes observed in smokers with or without COPD (5, 6, 16, 21, 24-26). Moreover, EGFR activation results in subsequent transcriptional regulation of inflammatory mediators such as IL-8 (10), a chemokine that has been implicated in COPD development.

So far, studies on the effect of cigarette smoke on epithelial ADAMs activity has largely relied on the use of airway epithelial cell lines or undifferentiated primary cell cultures, stimulated with an aqueous extract of cigarette smoke. However, whole cigarette smoke exposure and primary differentiated airway cell cultures represent more relevant physiological conditions. Firstly, fresh whole cigarette smoke (CS) contains unstable active components and particulate matter that are largely absent from extracts. Furthermore, immortalized epithelial cells are poor models of bronchial epithelium in situ, since they are frequently karyotypically unstable and heterogeneous, do not show characteristic features of differentiation and inherently carry multiple mutations in pathways essential for growth, differentiation, cell-cell interaction, and polarization. Furthermore, submerged cultures of primary airway epithelial cells fail to differentiate. Finally, using cell lines does not allow a comparison of patient populations. Therefore, we examined the effect of whole CS exposure on shedding of the soluble interleukin-6 receptor (sIL6R) and the EGFR-ligand amphiregulin (AREG) by well-differentiated, air–liquid interface cultured human primary bronchial epithelial cells (ALI-PBEC).

This allowed us to compare CS-induced ADAM17-mediated protein shedding and mRNA expression of sIL6R and AREG in well-differentiated ALI-PBEC from COPD and non-COPD (ex)smokers. Moreover, we established in this model the involvement of both EGFR and ADAM17 not only in shedding of ADAM17 substrates, but also in the regulation of mRNA levels of ADAM17 substrates and IL-8. Finally, for the first time, we observed intracellular CS-induced phosphorylated ADAM17-substrate interaction via an in situ proximity ligation assay. Overall, our results provide novel insights into the activation of airway epithelial cells by cigarette smoke in COPD, and highlight a possible role of ADAMs and EGFR in COPD pathology.

MATERIALS AND METHODS

Air–liquid interface cell culture of human primary bronchial epithelial cells

Human airway epithelial cells were obtained from macroscopically normal, anonymous bronchial tissue obtained from lung cancer patients undergoing resection surgery for lung cancer at LUMC. This material was be used for research according to the "Code of Conduct for Responsible Use" (FEDERA code) based on the condition that the patient has no objection against such use. Primary bronchial epithelial cells (PBEC) were isolated from tumor-free lung resection material (27), and passage 2 expanded cells were cultured at the air–liquid interface (ALI) to achieve mucociliary differentiation as previously described (28). Briefly, 40,000 cells were seeded on 0.65 cm Transwell inserts (Corning Costar, Cambridge, MA) with a 0.4 μm pore size, which were coated with 30 μg/mL PureCol (Advanced BioMatrix, San Diego, CA), 10 μg/mL Bovine serum albumin (Sigma-Aldrich, St. Louis, MO), and 10 μg/mL Fibronectin (isolated from plasma). Cells were cultured in Bronchial epithelial growth medium (BEGM) (Lonza, Verviers, Belgium) and Dulbecco's modified Eagle's medium (DMEM) (Gibco, Bleiswijk, The Netherlands) (1:1 mixture) containing 1 mmol/L Hepes (Lonza) and supplemented with SingleQuot supplements and growth factors according to the manufacturer's instructions (bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, insulin, T3 and retinoic acid; all from Lonza),

additional 15 ng/mL retinoic acid (Sigma–Aldrich), 1 mg/mL BSA (Sigma–Aldrich), 100 U/mL penicillin, and 100 µg/mL streptomycin (Lonza). PBEC were initially cultured on inserts in submerged conditions until cell layers were confluent. Next, apical medium was removed and cells were cultured at air-exposed conditions for at least 2 weeks to allow mucociliary differentiation. Clinical history and lung function data were obtained from anonymized patients (Table 1), and COPD disease status was based on lung function data according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification (1). Donor cells were randomly allocated to experimental groups.

Table 1: COPD and non-COPD patient characteristics

	COPD	non-COPD	P-value
Number of donors	15	11	
Gender (females/males)	4/11	2/9	
Age, years	70 ± 8	66 ± 6	0.1667
FEV_1, % predicted	65 ± 16	81 ± 16	<0.01
FEV_1/FVC %	55 ± 9	79 ± 9	<0.0001

Characteristics of PBEC donors. Age in years, and lung function as FEV_1 (% predicted) and FEV_1/FVC are shown as means ± SD. The mean differences were compared using the nonparametric Mann–Whitney test. COPD, chronic obstructive pulmonary disease, FEV_1, Forced expiratory volume in one-second, FVC, forced vital capacity.

Cigarette smoke exposure

Air–liquid interface cultured human primary bronchial epithelial cells were exposed to whole cigarette smoke (CS) in an exposure model, adapted from (29) and previously described in more detail (28). In this model, ALI-PBEC cultures were placed in either a CS- or air (negative control) exposure chamber located in a tissue incubator at 37°C and 5% CO2. Smoke derived from one cigarette (3R4F reference cigarettes [University of Kentucky, Lexington, KY]) was infused into the exposure chamber by a mechanical pump with a constant flow of 1 L/min, and equally distributed by a ventilator inside the chamber. After infusion (approximately 4–5 min), residual smoke was removed by infusion of air from the tissue incubator for 10 min. Directly after CS exposure, the basal medium of the cell cultures was refreshed and cells were incubated for the indicated periods of time. Untreated cells used as controls were subjected to the same procedure omitting the smoke (AIR).

Inhibitors

TMI-2 (1 µmol/L; PF-5480090), a highly selective inhibitor of ADAM17 activity (30), was obtained from Wyeth inc. (Philadelphia, Pennsylvania) and the selective EGFR inhibitor AG1478 (1 µmol/L) was from Sigma Aldrich. Cells were preincubated for 1 h with inhibitors before CS exposure, and directly after CS exposure media were replaced and inhibitors were freshly added.

ELISA

ALI-PBEC conditioned culture media were collected from the basolateral side of the inserts (1 mL), and apical washes were obtained by washing the apical surface with 100 µL PBS at

different time points, dependent on the experiment. Collected samples were diluted 1:1 with BEGM media and analyzed for IL6R and AREG by human IL6R or AREG ELISA kit, R&D. Further steps were performed according to the manufacturer's protocol. Data were corrected for the dilution factor and insert size, and the amount of the shed IL6R and AREG was expressed as pg/mL per cm^2.

RNA isolation and quantitative real-time PCR

RNA was isolated using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, and cDNA was synthesized by reverse-transcription PCR using oligo(dT) primers (Qiagen) and Moloney murine leukemia virus (M-MLV) polymerase (Promega, Leiden, The Netherlands). mRNA expression was determined by quantitative real-time PCR as described previously (28) with primer pairs presented in Table 2. mRNA expression was quantified using the standard curve method (31), in which arbitrary expression levels were normalized to the housekeeping genes RPL13A and ATP5B. The housekeeping genes were selected based on stable expression using the "Genorm method" (32).

Table 2 Primer sequences

Gene	Forward primer	Reverse primer
IL8	5'-CAGCCTTCCTGATTTCTG-3'	5'-CACTTCTCCACAACCCTCTGC-3'
AREG	5'-GTGGTGCTGTCGCTCTTGATA-3'	5'- ACTCACAGGGGAAATCTCACT-3'
full-IL6R	5'-GCTGTGCTCTTGGTGAGGAAGTTT-3'	5'-CTGAGCTCAAACCGTAGTCTGTAGAAA-3'
spliced IL6R	5'-GCGACAAGCCTCCCAGGTT-3'	5'-CCGCAGCTTCCACGTCTTCTT-3'
RPL13A	5'-AAGGTGGTGGTCGTACGCTGTG-3'	5'- CGGGAAGGGTTGGTGTTCATCC-3'
ATP5B	5'-TCACCCAGGCTGGTTCAGA-3'	5'-AGTGGCCAGGGTAGGCTGAT-3'

Proximity ligation assay

Chronic obstructive pulmonary disease ALI-PBEC were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X100 in PBS twice for 15 min, blocked with 1% BSA and 0.15% glycine. Next, they were incubated overnight in 4°C with two different first antibodies simultaneously: against ADAM17 (rabbit polyclonal, C-terminal ADAM17, 25 µg/mL, ab78162, Abcam) or ADAM17P^{T735} (rabbit polyclonal, phospho-ADAM17 in position T735, 25 µg/mL, ab60996, Abcam) and IL6R (goat anti-human IL6R recognizing extracellular domain, 20 µg/mL, AF-227-NA, R&D) or AREG (polyclonal goat anti-human Areg, 25 µg/mL, AF-262, R&D). All washing steps were repeated three times with 0.5% Triton-X100 in PBS (Sigma-Aldrich). Further steps were performed according to the DuoLink manufacturer's protocol. Briefly, Proximity ligation assay (PLA) probes for anti-goat PLUS and anti-rabbit MINUS were incubated at 37°C for 1 h. Ligation and amplification steps were performed with Detection Red Reagent. Finally, inserts with ALI-PBEC were cut out and mounted on the slides with DAPI (Vectashield mounting medium for fluorescence with DAPI, H-1200). Z-stacks were acquired using confocal microscopy (Leica604).

PLA image analysis

The number of dots was counted in the whole Z-Stack with Image J software. The threshold values were adjusted with the Intermodes algorithm (the filter size set between 10 and 437 microns to exclude the small and large dots, which were in the range of 10% of the total dot count). The objects on the edges of the culture inserts were excluded from the analysis. The number of nuclei was counted in each Z-stacks by hand to express the number of dots per nucleus.

Statistical analysis

Data were analyzed with GraphPad Prism Software, using the appropriate statistical test, as indicated underneath each figure. Cells from various donors (number indicated by n in the legends of figures) were used for each experiment, samples were collected from two or three wells from a single donor, averaged and represented as a single dot in the figure. Statistical analysis was performed on the averaged data. Values are presented as mean with SEM values. Differences at values *P < 0.05 were considered to be statistically significant. ns > 0.05, *P < 0.05, **P < 0.01, ** P < 0.001, **** P < 0.0001.

RESULTS

CS induces shedding of IL6R and AREG by ALI-PBEC into basolateral medium, but not apical

We first examined the effect of cigarette smoke (CS) exposure on the release of sIL6R and AREG by ALI-PBEC at the apical surface and in the basal medium, which contains a maintenance level of EGF, associated with a basal level of EGFR activity. This was done, using a previously described whole CS exposure model (28), in which CS caused a transient disruption in the airway epithelial barrier integrity, accompanied by minor cytotoxic effects measured at the apical surface. Both sIL6R and AREG were barely detectible in the apical washes collected from ALI-PBEC of 17 COPD donors at different stages of disease, following exposure to either CS or air (Figure 1A,B). In contrast, sIL6R and AREG were markedly released into the basal medium in both conditions. CS significantly increased release of sIL6R into the basal medium at 12 h post exposure, while AREG levels were increased at 12 and 24 h after CS exposure. These results demonstrate that shedding of sIL6R and AREG by ALI-PBEC occurs mainly to the basolateral compartment, and is enhanced by CS exposure.

CS significantly induces shedding of IL6R and AREG in COPD ALI-PBEC but not in non-COPD ALI-PBEC

Next, we explored whether shedding of sIL6R and AREG differs between ALI-PBEC isolated from COPD patients and non-COPD (ex)-smokers upon CS and air exposure. Based on the previous result, the release was only determined in the basal medium 24 h after exposure. Shedding of sIL6R and AREG did not differ between COPD and non-COPD ALI-PBEC exposed to air (Figure 2A,B), indicating no differences at baseline conditions. In contrast, shedding of sIL6R (Figure 2A) and AREG (Figure 2B) was significantly higher after CS exposure only in COPD ALI-PBEC, and not in non-COPD ALI-PBEC. These data show that CS-induced release of sIL6R and AREG was more pronounced in airway epithelial cells from COPD in comparison to non-COPD donors.

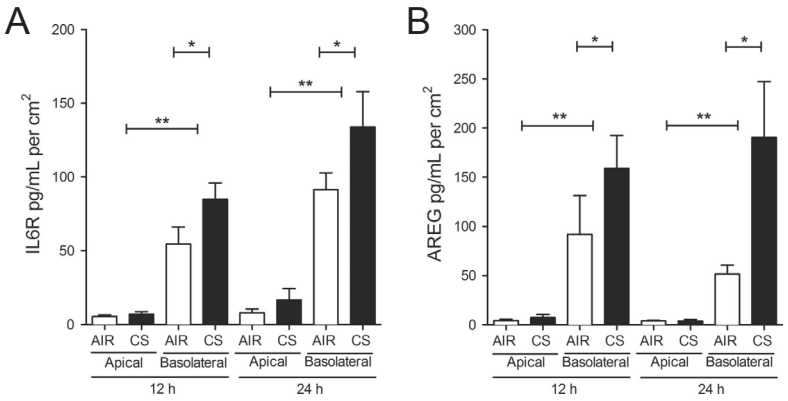

Figure 1. Cigarette smoke induces shedding of IL6R and AREG by ALI-PBEC into basolateral medium. IL6R (A) and AREG (B) were mainly shed to the basolateral compartment in ALI-PBEC (COPD donors at different stages, n = 12 for IL6R and n = 5 for AREG). Basolateral media (basolateral) were collected and apical PBS washes (apical) were performed 12 h and 24 h after CS or air exposure. Both IL6R and AREG were readily detectable in the basolateral compartment, and barely present in the apical washes. The response of cells from each donor was analyzed within one experiment using duplicate or triplicate inserts. Statistical analysis was performed by two-way ANOVA (Bonferroni) on the averaged data from each donor, comparing apical versus basolateral shedding at air and CS exposure, and basolateral shedding at air versus CS exposure.

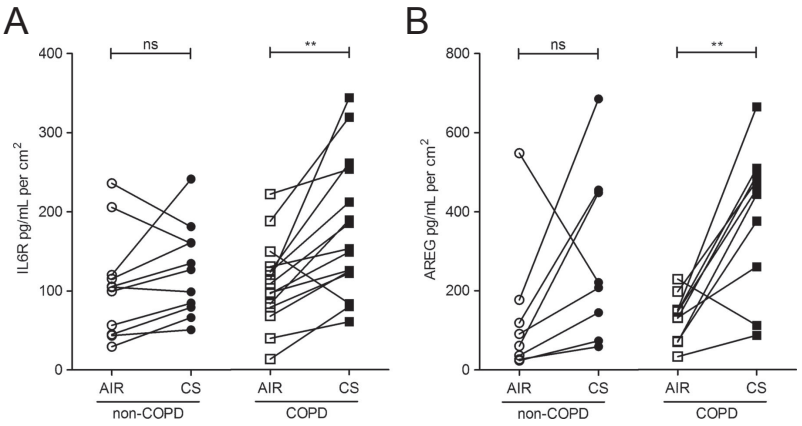

Figure 2. Cigarette smoke significantly induces shedding of IL6R and AREG into basolateral medium by COPD ALI-PBEC. Soluble forms of IL6R (A) and AREG (B) shed into the basolateral compartment were detected 24 h after CS or air exposure in ALI-PBEC derived from non-COPD and COPD donors (Table 1). (A) IL6R levels were significantly increased 24 h after CS treatment in COPD-ALI-PBEC (n = 15), but this increase was not significant in the non-COPD group (n = 11 donors). (B) Similarly, CS exposure significantly increased AREG levels in ALI-PBEC cells derived from COPD donors (n = 10), but not in non-COPD ALI-PBEC (n = 8 donors). Statistical analysis: paired t-test. n refers to the number of donors, duplicate or triplicate data were averaged per donor. Statistical analysis was performed on the averaged data from each donor.

CS-induced IL6R and AREG mRNA expression is lower in COPD ALI-PBEC compared to non-COPD cultures

We further determined mRNA expression of IL6R and AREG in CS and air exposed ALI-PBEC cultures from COPD and non-COPD patients. The soluble form of IL6R can be generated either by shedding of the membrane anchored form or by de novo synthesis of the alternatively spliced isoform that differs at the C-terminus (22). Therefore, we determined mRNA expression levels of both IL6R variants: the membrane-anchored (full-IL6R mRNA) and the alternatively spliced (spliced-IL6R mRNA) variant.

Time-course analysis revealed that CS increased full-IL6R mRNA 3 h after exposure, but not at later time points (Figure 3A). In contrast, baseline expression of spliced-IL6R mRNA did not differ from the expression after CS treatment (Figure 3B), suggesting that the increase in

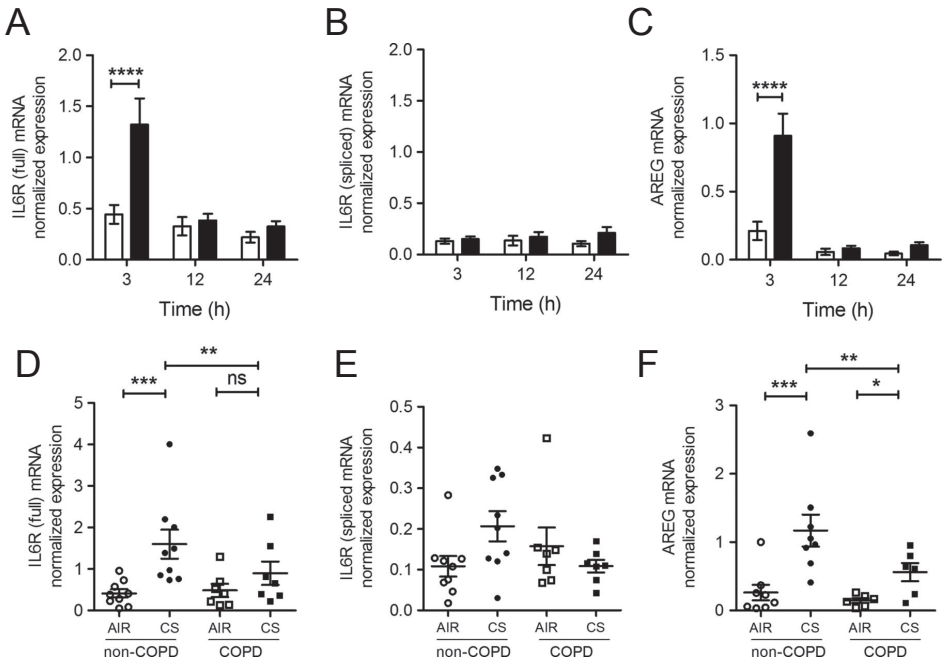

Figure 3. CS exposure transiently enhances IL6R and AREG mRNA expression in COPD and non-COPD ALI-PBEC. mRNA levels of the IL6R full-length variant (full-IL6R) (A), the IL6R splice variant (spliced-IL6R) (B) and AREG (C) were determined by qPCR 3, 12, and 24 h after CS (black bars) or air exposure (open bars) (n = 14 unspecified donors). A transient induction of full-IL6R (A) and AREG (C), but not spliced-IL6R (B) was observed at 3 h after CS exposure. In COPD (n = 7) and non-COPD (n = 8) ALI-PBEC, mRNA of full-IL6R (D), spliced-IL6R (E), and AREG (F) were determined 3 h after CS exposure. mRNA expression of full-IL6R and AREG, was lower on average but not statistically significant in COPD compared to non-COPD donors. Data were normalized for expression against two reference genes (ATP5B and RPL13A). n refers to the number of donors. The response of cells from each donor was analyzed within one experiment using duplicate inserts and data were averaged per donor. Statistical analysis was performed on the averaged data from each donor. Statistical analysis: Two-way ANOVA With Tukey's multiple comparison test.

sIL6R protein levels in culture supernatants did not result from alternative splicing. Similar to full-IL6R mRNA, CS significantly induced AREG mRNA expression 3 h after exposure, but not at later time points (Figure 3C). These findings suggest that the CS-induced increase in IL6R and AREG shedding is mediated at least in part via regulation of their mRNA expression levels.

Baseline expression of full-IL6R and AREG mRNA did not differ between COPD and non-COPD ALI-PBEC (Figure 3D,F). After CS exposure, full-IL6R and AREG mRNA were expressed at higher levels in both non-COPD and COPD ALI-PBEC. Interestingly, after CS induction, COPD cells expressed full-ILR and AREG at lower levels on average but this did not reach statistical significance (Figure 3D,F). Spliced-IL6R mRNA expression did not differ between investigated groups either after CS or air exposure (Figure 3E). These findings suggest that COPD patients may have impaired transcriptional or posttranscriptional responses to inflammatory and tissue regenerative triggers. The apparent contrast with the more pronounced shedding from COPD cells after CS challenge (Figure 2) suggests that posttranslational mechanisms determine shedding rate, rather than substrate mRNA levels.

ADAM17 is required for CS-induced release of IL6R and AREG in ALI-PBEC

To confirm the previously established involvement of ADAM17 in the shedding process of IL6R and AREG in our model, we used the selective ADAM17 inhibitor TMI-2 (Wyeth) (30). TMI-2 only partially decreased baseline IL6R release at all investigated time points (Figure 4A), plausibly because release of the product of spliced-IL6R mRNA, which cannot

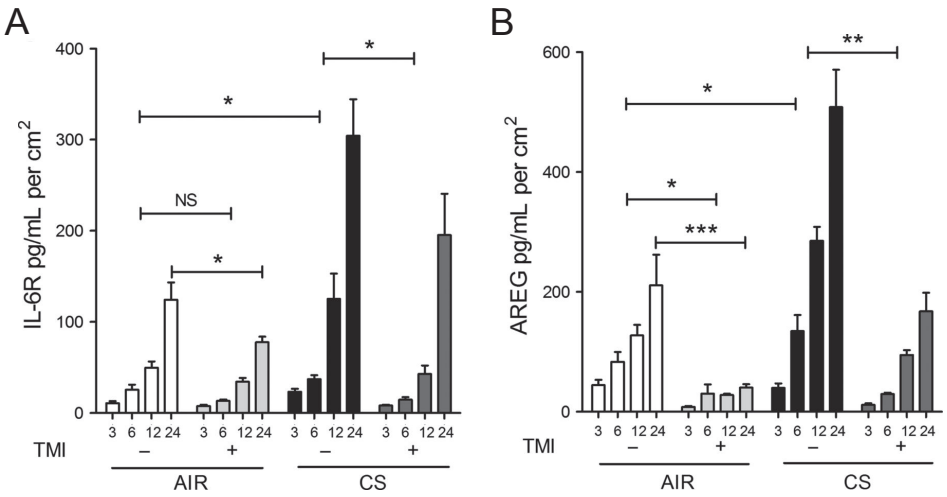

Figure 4. ADAM17 is involved in the release of soluble IL6R and AREG in ALI-PBEC. The selective ADAM17 inhibitor, TMI-2 (Zhang et al. 2004) decreases basal and CS-induced IL6R (A,B) and AREG (C,D) shedding in ALI-PBEC cells (n = 3 COPD donors) at 3, 6 (A,C), 12, and 24 h time points (B,D). n refers to the number of donors. The response of cells from each donor was analyzed within one experiment using duplicate or triplicate inserts and data were averaged per donor. Statistical analysis was performed on the averaged data from each donor, by two-way ANOVA (Bonferroni), confirming first the effect of CS on IL6R and AREG shedding at different time points, and second the effect of TMI-2 on shedding during air and CS exposure.

be distinguished from shed IL-6R with the available antibodies, is not sensitive to inhibitors of ADAMs (33). In contrast, TMI-2 significantly decreased baseline AREG shedding at all time points (Figure 4B). Importantly, CS-induced shedding of IL6R and AREG was significantly inhibited by TMI-2 at all timepoints after CS exposure, indicating that ADAM17 activity is involved in CS-induced ADAM17 substrate release (Figure 4).

ADAM17- and ADAM17P-substrate interactions are increased after CS exposure in an intracellular compartment of ALI-PBEC

Next, we explored the interactions of IL6R or AREG with ADAM17 3 h after CS treatment in ALI-PBEC with an in situ proximity ligation assay (PLA) (34), using antibodies against ADAM17 phosphorylated at Thr735 (ADAM17-P^{T735}) or total ADAM17. Protein IL6R/AREG-ADAM17 and IL6R/AREG-ADAM17-P^{T735} interactions were visualized as fluorescent red dots in x-y confocal sections (representative confocal pictures shown in Figure 5A,B).

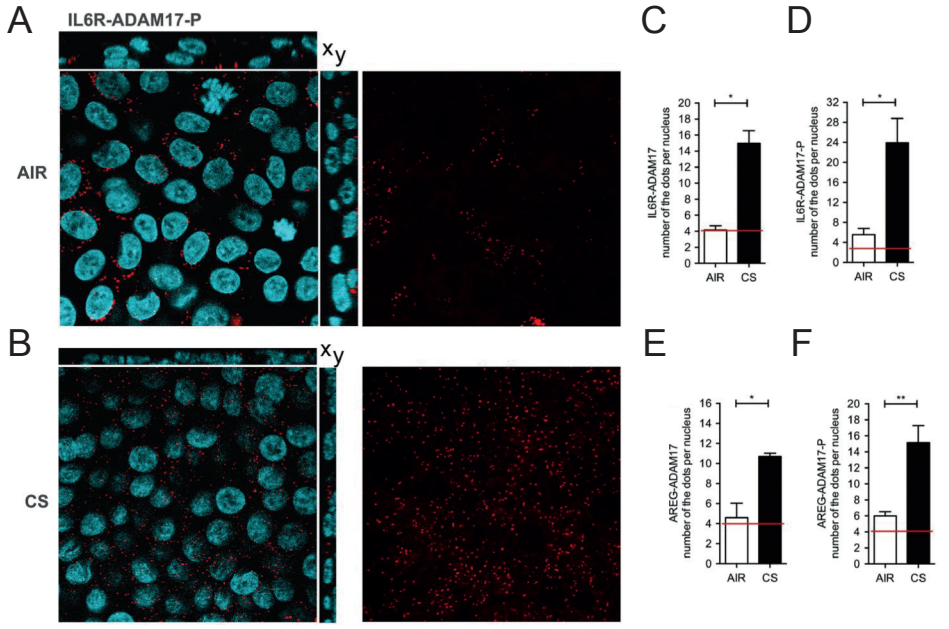

Figure 5. CS increases substrate-ADAM17 and substrate-ADAM17-P^{T735} interactions in pseudostratified COPD ALI-PBEC cells. The proximity ligation assay (PLA) signal in COPD- ALI-PBEC exposed to CS or air was performed for the following interactions: IL6R-ADAM17, IL6R-ADAM17P^{T735}, AREG-ADAM17, and AREG-ADAM17-P^{T735}. Relevant control data are shown in Figure S1. Here, we show a representative figure of IL6R-ADAM17P^{T735} 3 h after air (A) and CS exposure (B). Left panels show merged signals of nuclei (blue) and PLA (red) in the x–y sections of the confocal z-stack and right panel presents PLA signal in the apical region (red dots). The number of PLA interactions was counted for all interactions as described in the methods section and expressed per nucleus 3 h after CS or air exposure in the whole z-stack of the ALI-PBEC (C–F). The red lines on the graphs indicate the maximal dot count in the PLA assay controls, in which one of the antibodies for the interaction was omitted (background staining not shown). For each interaction, cells from one donor were analyzed. Different filters (n = 4) were used to show distinct interactions. Statistical analysis: unpaired t-test.

In air-exposed cells, PLA signals were largely confined to the basal region, as in the control incubations, and not significantly higher than background (data not shown), as indicated by red lines in Figure 5C–F (relevant control data are shown in Supplementary Figure 1). Interestingly, CS exposure significantly increased the total number of PLA signals for interactions of IL6R or AREG with ADAM17 (Figure 5C,E). We observed that CS strongly enhanced interactions of IL6R or AREG with ADAM17-P^{T735} (Figure 5D,F), which further extends previous findings showing that ADAM17 is phosphorylated after smoke extract (CSE) exposure in submerged immortalized NCI-H292 cells (5). CS-induced PLA signals of substrate-ADAM17 and substrate-ADAM17-P^{T735} were primarily detected in the apical region of the cells and were not confined to a lateral membrane pattern suggesting an intracellular vesicular localization of protein complexes in ALI-PBEC. These data for the first time demonstrate that CS exposure strongly increases the interaction of ADAM17 and ADAM17-P^{T735} with IL6R or AREG in an intracellular vesicular compartment of ALI-PBEC, suggesting a CS induced effect on protein trafficking.

EGFR is required for basal and induced AREG shedding in ALI-PBEC

ADAM17-dependent shedding of EGFR ligands such as AREG results in activation of EGFR through an autocrine feedback loop, which modulates basal EGFR activity (35). This mechanism was shown to be activated by CS extract in submerged cultured PBEC and in cell lines (16). In our experimental set-up, we have previously shown that CS enhances basal EGFR activity by increasing its phosphorylation (28). To illustrate the involvement of EGFR in CS-induced ADAM17-related shedding in ALI-PBEC, we assessed sIL6R and AREG shedding after starvation for growth factors, using medium devoid of EGF and bovine pituitary extract (BPE). Removing these factors from the medium substantially reduced baseline shedding of IL6R and AREG (Figure 6), when compared to standard culture conditions including EGF and BPE (Figure 4A,C). Both sIL6R and AREG release were significantly increased at 3 h after CS exposure. The selective ADAM17 inhibitor TMI-2 and the EGFR tyrosine kinase inhibitor (AG1478) added prior to CS exposure, partially inhibited sIL6R shedding, consistent with a substantial contribution of the ADAM-insensitive splice variant sIL-6R levels in the basal

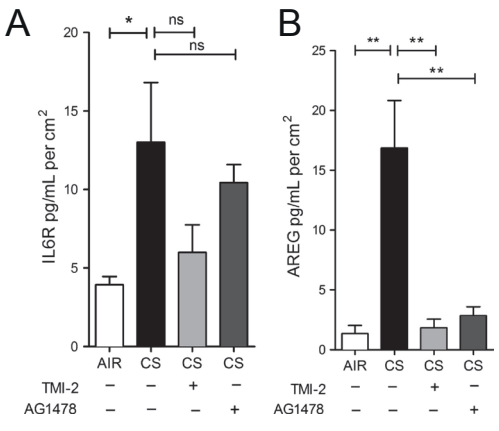

Figure 6. IL6R and AREG shedding depends on ADAM17 and EGFR activity in COPD-ALI-PBEC. COPD ALI-PBEC (n = 3 donors) were starved for growth factors for 48 h prior to CS or air exposure. Three hours after CS exposure, IL6R (A) and AREG (B) shedding were significantly increased compared to air. The ADAM17 inhibitor (1 µmol/L TMI-2) and the EGFR inhibitor (1 µmol/L AG1478) significantly reduced AREG, but IL6R shedding to a lesser extent. The response of cells from each donor was analyzed within one experiment using triplicate inserts and data were averaged per donor. Statistical analysis was performed on the averaged data from each donor by one way ANOVA (Tukey multiple comparison test), only relevant comparisons are shown, air versus CS-treated cells and the effect of inhibitors in CS-treated cells.

medium (Figure 6A). AG1478 strongly impaired AREG shedding, to a similar extent as TMI-2 (Figure 6B). These findings together demonstrate a critical role of EGFR activation in ADAM17-mediated basal and CS-induced shedding activity.

EGFR and ADAM17 are required for CS-induced IL6R and AREG mRNA expression

We previously observed that EGFR activation is involved in CS-induced expression of several genes in ALI-PBEC (28). The molecular mechanism by which CS activates EGFR are not known. Here, we explored the effect of ADAM17 and EGFR inhibition on CS-induced IL6R and AREG mRNA levels in ALI-PBEC. At 3 hours after CS exposure in the absence of EGF in the medium, both TMI-2 and AG1478 significantly impaired CS-induced expression of full-L6R mRNA (Figure 7A), but not the splice variant (Figure 7B). Both inhibitors strongly

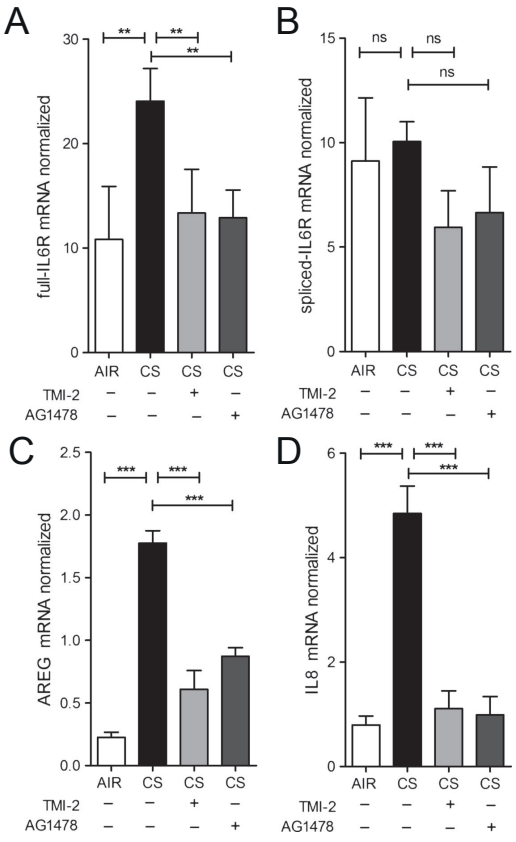

Figure 7. IL6R and AREG mRNA expression are regulated by ADAM17 and EGFR activity in ALI-PBEC. ALI-PBEC (n = 3 donors) were starved for growth factors for 48 h prior to CS exposure. At 3 h, CS-induced mRNA levels of full-length IL6R (A), AREG (C), and IL8 (D) were diminished upon ADAM17 (1 μmol/L TMI-2) and EGFR (1 μmol/L AG1478) inhibition, whereas that of the alternatively spliced form of IL6R was not affected (B). n refers to the number of donors. The response of cells from each donor was analyzed within one experiment using triplicate inserts and data were averaged per donor. Statistical analysis was performed on the averaged data from each donor, by one-way ANOVA (Tukey multiple comparison test), only relevant comparisons are shown, air versus CS-treated cells and the effect of inhibitors in CS-treated cells.

diminished CS-induced AREG mRNA levels (Figure 7C) as well as IL-8 mRNA expression (Figure 7D). Overall, these findings for the first time demonstrate that ADAM17, next to EGFR, is essential in the CS-induced mechanism regulating not only the mRNA of ADAM17 substrates (IL6R and AREG), but also IL-8 in ALI-PBEC.

DISCUSSION

Many studies have demonstrated that airway epithelial cells are activated by exposure to environmental triggers like cigarette smoke, which contributes to COPD pathology (28, 36-38). In contrast to most studies, we used fresh whole cigarette smoke instead of (aged) cigarette smoke extract, and ALI-differentiated PBEC from COPD and non-COPD donors instead of submerged cultures of non-differentiated primary cells or cell lines.

While the obvious advantage of this approach is that we can study well-differentiated primary cells from different patient populations, a limitation is that confirmation of data obtained with experimental pharmaceutics by, for example, gene editing or RNAi technology is not feasible in this context. Aside from efficiency issues and off-target effects in primary cells, knocking down EGFR or ADAM17 likely affects the growth and differentiation of primary bronchial epithelial cells, which essentially defeats our purpose. However, the two inhibitors that we apply here to inhibit EGFR (AG1478) and ADAM17 (TMI-2), respectively are widely used and are known to be highly selective.

Importantly, our data demonstrate for the first time that CS triggered increase of basal shedding of IL6R and AREG into the basal medium, in the presence of EGF in the growth medium providing basal EGFR activity, was more pronounced in ALI-PBEC derived from COPD patients compared to non-COPD controls. We further report the ability of CS to increase mRNA expression of these genes in an EGFR- and ADAM17-dependent way in ALI-PBEC cells, under these conditions, with a lower tendency to induction in the COPD group. These results extend previous studies showing dysregulated responses of COPD airway epithelial cells to cellular stress, and provide novel evidence for the mechanism of CS-induced and COPD-related proinflammatory and profibrotic responses (Figure 8).

The differential effect of CS on sIL6R and AREG release between COPD and non-COPD ALI-PBEC might be related to differences in epithelial barrier function as previously described (39). Using the current CS exposure system, we have previously shown that CS causes a transient decrease in epithelial barrier function (28). However, in contrast to Heijink et al., we did not observe differences between COPD and non-COPD cultures at baseline conditions and upon CS exposure (40), which may be explained by the fact that Heijink et al. focused on severe (GOLD stage IV) COPD. Another explanation might be differences in epithelial cell differentiation, as it has been shown that COPD epithelial cells display a more mesenchymal phenotype due to enhanced autocrine expression of TGF-β1 (41).

As previously shown, the EGFR-ADAM17 pathway is essential for IL-8 release from a bronchial epithelial cell line exposed to particulate air pollution (42) and implicated in CS extract-induced expression of the mucin MUC5AC (6). Further, autocrine production of EGFR ligands is involved in CS-induced IL-8 release from airway epithelial cells (10). Our studies extend these observations by showing the involvement of the ADAM17-EGFR pathway in the release of IL6R and AREG upon CS exposure of differentiated PBEC, both in the presence (Figure 4), and absence (Figure 6) of EGFR ligand (EGF) in the basal medium respectively. Notably, the basal shedding rates are considerably lower in cells pre-incubated in medium lacking EGF, resulting in a much larger ADAM17- and EGFR-dependent stimulation

effect of CS (compare Figures 4 and 6). Which of these extreme conditions of basal EGFR activation apply in normal and COPD lungs in situ, and to what extent autocrine feedback signaling through ADAM-dependent EGFR ligand shedding determines EGFR activity (Figure 8) remains to be established.

Additionally, EGFR and ADAM17 were both essential for CS-induced IL6R and AREG mRNA expression (Figure 7). These results provide novel insights into the mechanisms of airway epithelial cell activation by cigarette smoke in COPD, and highlight a role of ADAMs and EGFR in this process (Figure 8).

We further found that CS increases shedding of IL6R and AREG to the basal medium, but not to the apical side (Figure 1). This is in line with report in polarized Madin-Darby canine kidney cells (MDCK cells) showing that newly synthetized AREG is directly delivered to the basolateral surface with >95% efficiency (43). However, this is in contrast to the secretion of the innate immune mediators IL-8 and ribonuclease 7, which were also detected at the apical surface (28). A polarized ADAM17-mediated secretion toward underlying tissue may be relevant for lung tissue remodeling through autocrine, paracrine, extracrine (exosomal targeted receptor activation) pathways in COPD (7, 44). Further examination of this phenomenon in epithelial–mesenchymal co-culture systems is in progress.

Amphiregulin release and phosphorylation of ADAM17 after CS extract treatment in ALI-PBEC has been previously detected by ELISA or western Blotting (5). Our proximity ligation assay (PLA) data show for the first time that CS-induced shedding involves an intracellular interaction between phosphorylated ADAM17 and its substrates (Figures 5 and 8), whereas the majority of the literature suggests that shedding occurs mainly at the plasma membrane surface. This interaction likely takes place in intracellular membranes that sequester active phosphorylated ADAM17 and its transmembrane substrates upon activation. This process may relate to the transient change in barrier function upon CS treatment in our system and the subsequent activation of EGFR (28). Our observation is supported by other reports showing the presence ADAM17 or its substrates in a vesicular compartment in lysosomes (45), endosomes (46, 47), and exosomes negative for the ER marker calreticulin (48). Moreover, Gutwein et al. demonstrated that ADAM10-mediated L1 migration factor cleavage occurs in Golgi-derived vesicles in tumor cells (49). This was further supported by a recent paper suggesting that also ADAM10/ADAM17-mediated release of soluble FasL occurs from an intracellular vesicular pool of secretory lysosomes in stimulated T lymphocytes (45).

Moreover, after ligand binding, EGFR traffics in endosomes from the plasma membrane to an intracellular compartment to continue its signaling (50, 51). EGF-dependent MAPK signaling occurs from late endosomes and lysosomes (52). Interestingly, the MAPK/ERK pathway regulates trafficking of ADAM17 phosphorylated at Thr735 from the endoplasmic reticulum toward the plasma membrane (53, 54), which can be also activated through ligand binding to EGFR. Higginbotham et al. showed that AREG containing exosomes are rapidly internalized by recipient cells in an EGFR-dependent manner (48), enhancing invasion of LM2-4175 cells through Matrigel and wound healing. In our ALI-PBEC system, we observed a predominantly lateral localization of EGFR under basal culture conditions. After exposure to CS, we observed a more cytoplasmic localization, consistent with EGFR activation (Supplementary Figure 2).

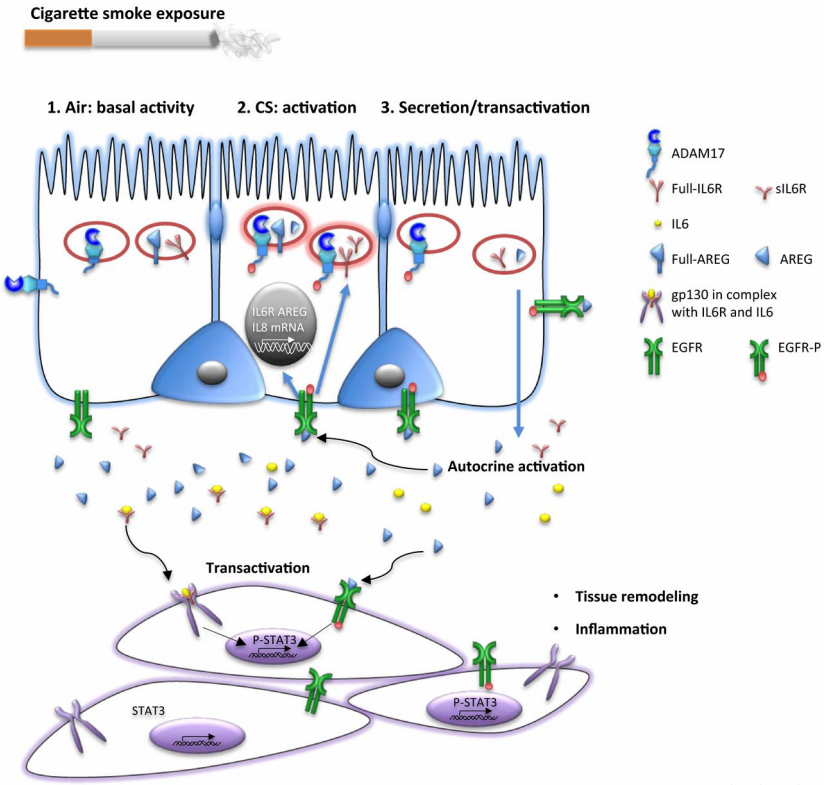

Figure 8. Cigarette smoke exposure activates EGFR-ADAM17 axis in airway epithelial cells. Under basal conditions, there is ADAM17 related AREG and IL6R shedding activity, depending on the level of EGFR activity (compare Figure 4 and 6). Cigarette smoke exposure (CS) initiates an interaction of the phosphorylated form of ADAM17 (ADAM17-P) with the full-length transmembrane forms of IL6R and AREG in an intracellular compartment of the airway epithelial cell (Figure. 5), resulting in proteolysis and subsequent secretion (shedding) of the soluble active domains of IL6R and AREG toward the basolateral compartment. This involves both ADAM17 and EGFR activity (Figures 4 and 6). CS exposure also affects IL6R and AREG gene expression or mRNA stabilization in airway epithelial cells through ADAM17 and EGFR activation. sAREG and sIL6R secreted towards the basolateral compartment may change the level of activity of EGFR and the interleukin receptor IL6st/gp130 on the airway epithelial cells (autocrine). This may contribute to the activity of the EGFR/ADAM17 axis (positive feedback), which is likely kept in check by inactivation of internalized EGFR. Paracrine activity of sAREG and sIL6R may further transactivate EGFR and the interleukin receptor IL6st/gp130 on the underlying myofibroblasts, and myeloid cells, activating downstream pathways, including STAT3, involved in inflammation, collagen deposition, and myofibroblast proliferation.

Therefore, in line with these and published observations, our findings suggest that in HBEC-ALI, CS triggers EGFR-mediated trafficking of ADAM17 and its substrates to a common subcellular compartment to allow proteolysis and subsequent secretion of soluble products (Figure 8). At this time, we cannot establish to what extent autocrine signaling through shed ADAM substrates determine this response, or whether alternative mechanisms such as transactivation by intracellular kinases or oxidation or the extracellular receptor domain plays

a role. Additional studies of triggered trafficking of EGFR, ADAM17-P, and its substrates in polarized airway cells are required to further establish this mechanism.

In addition to CS-enhanced release through ADAM17 enzymatic activity, we observed transiently enhanced mRNA expression of both AREG and full-IL6R in ALI-PBEC upon CS exposure (Figure 3). CS did not affect the level of the alternatively spliced form of IL6R, so we conclude that alternative splicing unlikely contributes to CS-enhanced release of IL6R. Previously, we observed upregulated IL-8 mRNA expression in ALI-PBEC exposed to CS as a result of enhanced EGFR phosphorylation and activation of the downstream MAPK/ERK1/2 signaling pathway (28). Here, we report that CS-induced IL6R and AREG mRNA expression was also reduced upon EGFR inhibition. In addition, we show here for the first time that inhibition of ADAM17 has the same effect on these mRNA levels (Figure 7). Therefore, our data suggest that CS enhances factors common for activation of IL6R, AREG, and IL-8 mRNA expression likely via an autocrine ADAM17-EGFR axis (Figure 8). The transcriptional and posttranscriptional regulation of these genes upon inhaled toxic substances has not been fully elucidated. Induced EGFR signaling is able to activate transcription of target genes. In addition, it has been shown that CS extract enhances HuR-mediated IL-8 mRNA stability in airway epithelial cells (55). Moreover, UV-exposure of keratinocytes enhances mRNA HuR-mediated stability of AREG, in an EGFR-dependent manner (56). These observations suggest that CS-induced activation of EGFR enhances sIL6R, AREG, and IL8 mRNA stability in ALI-PBEC. Furthermore, mRNA regulation may be altered in cultured airway epithelial cells from COPD patients (57). CS-treated COPD ALI-PBEC expressed lower AREG and IL6R mRNA levels on average compared to non-COPD controls, but this difference was not statistically different (Figure 3). Further studies on mRNA stability in this system are required to establish this. Nevertheless, this observation contrasts with the shedding data (Figure 2) and suggests that ADAM17-dependent AREG and sIL6R output is not primarily regulated on the mRNA level, but involves posttranslational regulation.

Our data support the relevance of the ADAM17/EGFR pathway in COPD development and progression. Selective inhibitors of ADAM17, EGFR, and other components of this signaling pathway such as JAK and MAPK potentially expand therapeutic possibilities. The development of ADAM inhibitors for clinical use has been studied intensively (58-60). In cellular and animal tumor models, positive results were recorded (61). An ADAM17 inhibitor, TAPI-0, reduced bleomycin-induced lung inflammation (62). The selective inhibitor TMI-2 used in this study, reduced LPS-induced inflammation in vivo (30). However, due to a lack of target specificity of available compounds, and side effects associated with the various other biological functions of ADAMs, chronic and systemic application of these compounds in humans is so far prohibited (63). Clearly, more advanced intervention tools are required. Our data offer new insights in the regulation of mRNA expression, secretion, and release of ADAM17 substrates in airway epithelial cells upon triggering, which in combination with state of the art molecular design and advanced organotypic cellular modeling of airways could allow development of more selective inhibitors, targeted to specific cells and subcellular domains.

In summary, this study provides evidence that ADAM17-mediated release and shedding of IL-6R and AREG is highly enhanced in airway epithelial cells in response to CS-induced injury. Next to ADAM17, we highlight the importance of EGFR in the regulation of IL6R and

AREG release and mRNA expression. Moreover, CS-induced ADAM17-mediated shedding of IL6R and AREG is especially high in COPD ALI-PBEC, suggesting that reducing ADAM17 activity in COPD might be a potential therapeutic approach.

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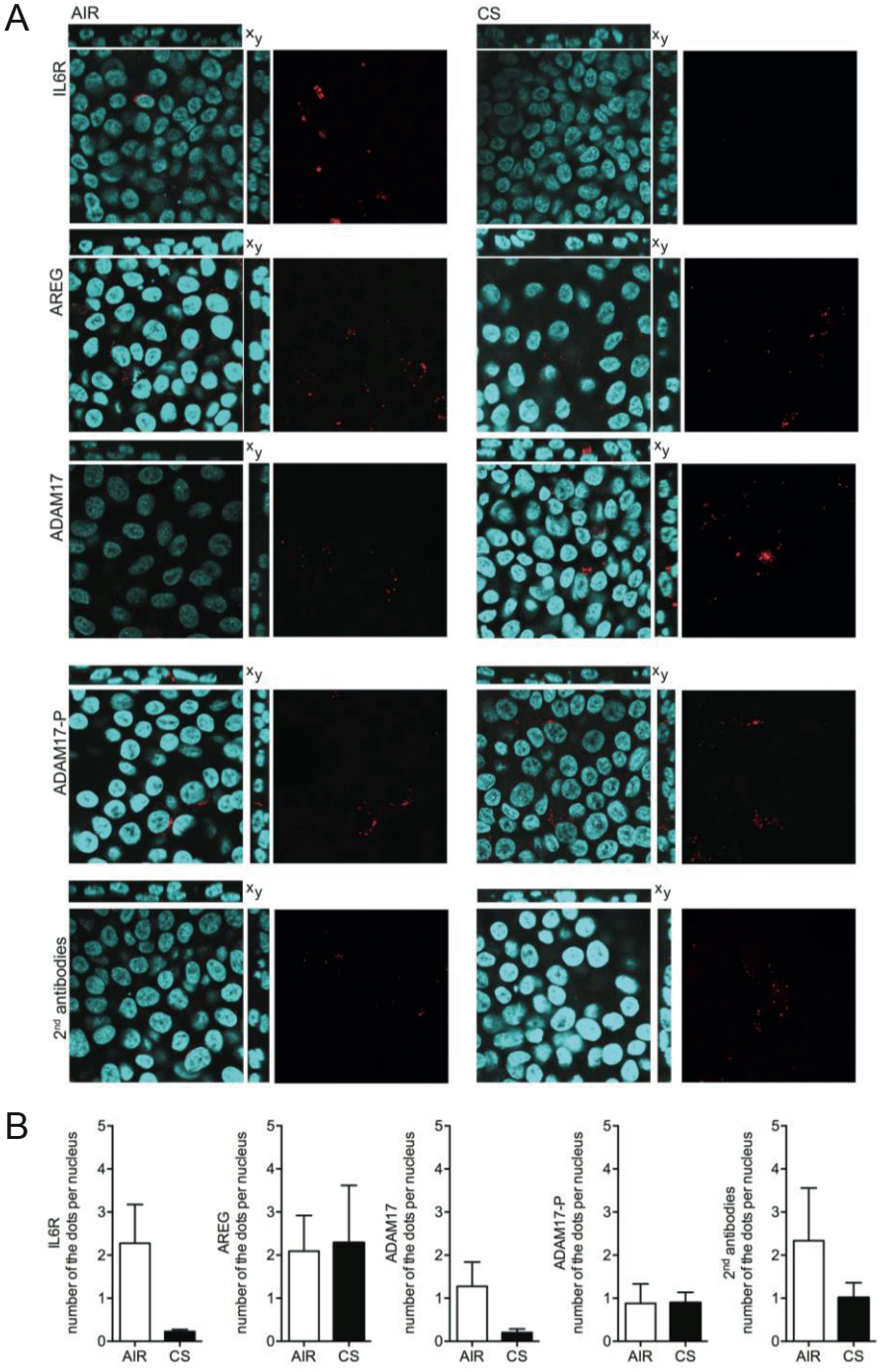
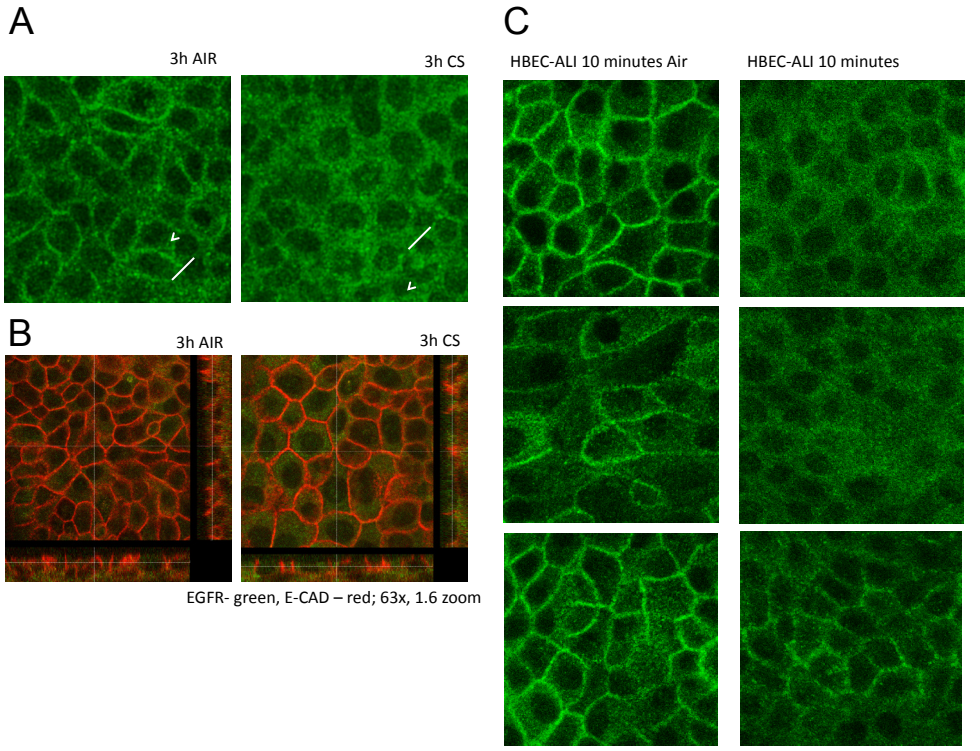
SUPPLEMENTARY FIGURES



Supplementary Figure 1. The proximity ligation assay (PLA) background signal. PLA recognizes two proteins that are in close proximity by using a combination of two specific antibodies and two complementary secondary antibodies carrying the ligation probes (Frederiksson et al., 2002). To check the specificity of the PLA signal, the filters with ALI-PBEC used for the assay of Figure 5 (COPD n=1) were incubated with only one of the first antibodies: IL6R, AREF, ADAM17, ADAM17PT735, or no first antibodies (2nd antibodies control). (A) The representative immunofluoresence pictures are shown. Left panel shows PLA signal of the apical region (red dots) and right panel presents merged signals of nuclei (blue) and PLA (red) in the x-uy sections of the confocal z-stack. (B) The number of dots detected by confocal microscopy in each condition is expressed per nucleus.



Supplementary Figure 2. Lateral EGFR in ALI-PBEC, internalized after CS treatment. ALI-PBEC cultured under basal conditions, including (EGF and BPE) were treated with air or CS as described in the methods section (A) Lateral EGFR immunofluoresence signal (green) becomes more diffuse three hours after CS treatment. (B) Lateral E-cadherin (red) illustrates the partial cytoplasmic localisation of EGFR. (C) This is confirmed in a seperate experiment, after 10 minutes exposure to air or CS, with three seperate filters each.

8

CHAPTER 9

Expression of the innate immunity mediator WFDC12 in airway epithelial cells: role of cell differentiation and expression in COPD.

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Submitted

ABSTRACT

Whey acidic protein four-disulfide-core 12 (WFDC12) is an anti-inflammatory mediator that is expressed during airway inflammation. Airway epithelial cells are centrally involved in regulating airway inflammation, however the expression of WFDC12 in these cells, and its role in the chronic inflammatory lung disease COPD, has not been investigated. Therefore, we examined the expression of WFDC12 in (un)differentiated primary bronchial epithelial cells (PBEC) that were exposed to inflammatory stimuli, and compared the expression in COPD and control cell cultures. Exposure to the COPD-related pathogen non-typeable *Haemophilus influenzae* increased mRNA of WFDC12 in undifferentiated PBEC, whereas it only affected WFDC12 secretion in differentiated PBEC. TNFα/IL-1β stimulation increased WFDC12 mRNA in differentiated PBEC, whereas supplemented and endogenously produced TGF-β1 suppressed the expression. Baseline WFDC12 mRNA levels were increased upon differentiation and were mainly observed in luminal cell of differentiated PBEC. This constitutive expression of WFDC12 was furthermore lower in cell cultures from COPD patients when compared to controls. Overall, these findings demonstrate a dynamic regulation of WFDC12 in airway epithelial cells during differentiation. Decreased production as observed in COPD epithelial cell cultures might favor the persistence of inflammation in this disease.

INTRODUCTION

The whey acidic protein four-disulfide-core (WFDC) domain-containing protein family comprises an evolutionary conserved group of proteins, including secretory leukocyte protease inhibitor (SLPI) and elafin (protease inhibitor 3; PI3) (1). These proteins are important regulators of innate immune responses in the lung that display a range of functions including antiprotease and antimicrobial activity, anti-inflammatory effects, and wound healing properties.

Recently, the expression and function of another, previously uncharacterized WFDC domain protein, WFDC12, was described (2). In contrast to the mouse orthologue (3), human WFDC12 did not display antibacterial properties. However, functional studies showed antiprotease activity against neutrophil-derived cathepsin G and reduced IL-8 and MCP-1 secretion *by in vitro* cultured monocytes stimulated with LPS (2). Moreover, LPS increased the expression of WFDC12 in the lungs *in vivo*, and increased expression levels were detected in secretions and tissues derived from the lungs of patients with acute respiratory distress syndrome. Together, these findings suggest a role of WFDC12 in regulating inflammatory responses in the airways.

In addition to immune cells, the airway epithelium plays a central role in regulating airway innate immunity and inflammation (4, 5). Airway epithelial cells are a major source of WFDC proteins in the lung and express several protein family members either constitutively or upon exposure to pro-inflammatory stimuli (6, 7). It remains however unknown if WFDC12 is also expressed by human airway epithelial cells, which mechanisms are involved in its expression, and furthermore whether its expression is affected in chronic inflammatory lung diseases.
Chronic obstructive pulmonary disease (COPD) is a smoking-related lung disorder that is characterized by irreversible, chronic and progressive airflow limitation, bacterial colonization and infection, a persistent inflammatory response in the lung, and remodeling of lung tissue (8-10). It is postulated that alterations in airway epithelial innate immunity contribute to the onset and progression of COPD (5), but the mechanisms that are affected remain incompletely understood. Indeed it has been shown in previous studies that airway epithelial cells in COPD display reduced anti-inflammatory activity, for instance due to reduced expression of club cell secretory protein-16 (11). In addition to this, reduced expression of other anti-inflammatory proteins, such as WFDC12, may also contribute to the reduced anti-inflammatory activity of airway epithelial cells in COPD.

In the present study we examined the expression of WFDC12 in primary bronchial epithelial cells (PBEC) in response to microbial and inflammatory stimuli related to COPD. To this end we examined the effect of the COPD-related respiratory pathogen non-typeable *Haemophilus influenzae* (NTHi), cigarette smoke, the pro-inflammatory cytokines TNFα/IL-1β and the growth factor TGF-β1 on WFDC12 expression. Moreover, expression of WFDC12 was compared between air-liquid interface cultured and mucociliary differentiated PBEC (ALI-PBEC) from COPD patients and non-COPD (ex)smokers.

MATERIALS AND METHODS

Cell culture and stimuli

Primary bronchial epithelial cells (PBEC) were isolated from macroscopically normal lung tissue obtained from patients undergoing resection surgery for lung cancer at the Leiden University Medical Center. Use of such 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. Cells were cultured as previously described (12, 13) either in transwells at the air-liquid interface (ALI) to allow mucociliary differentiation (ALI-PBEC), or cultured in undifferentiated submerged conditions in regular tissue culture plates (referred to as S-PBEC). In Figure 5 ALI-PBEC cultures were derived from COPD patients or non-COPD controls with a smoking history. The disease status of anonymized donors was determined based on lung function data according to the Global Initiative for Chronic Obstructive Lung Disease classification (14) (Table 1). PBEC were stimulated with UV-inactivated non-typeable *Haemophilus influenzae* (NTHi) and exposed to whole cigarette smoke (CS) essentially as previously described (13). The pro-inflammatory cytokines TNFα, IL-1β and the growth factor TGF-β1 (all at 20 ng/ml; PeproTech, Rocky Hill, NJ) were added to the basal culture medium. Neutralization of endogenous TGF-β1 was performed by adding 10 μg/ml anti-TGF-β1 neutralizing antibody to the culture medium, and equal amounts of a monoclonal mouse IgG1 was used as isotype control (both R&D, Minneapolis, MN, USA). In indicated experiments, basal and luminal cell-enriched fractions were isolated from ALI-PBEC cultures based on differential disassociation upon treatment with Ca^{2+} free medium, essentially as previously described (13, 15).

Table 1: Characteristics of COPD and non-COPD patients.

	COPD	**non-COPD**	**p-value**
N=	10	8	
Gender (Females/Males)	2/8	2/6	
Age, years	67±7	66±8	
FEV1 , % predicted	64±14	92±22	< 0.02
FEV1/FVC %	57±9	80±9	< 0.0001

Patient characteristics of airway epithelial cell donors. Age and lung function are shown as means ± SD. The mean differences in FEV_1 (% predicted) and FEV_1/FVC (%) were compared using the non-parametric Mann-Whitney test. Abbreviations: COPD = chronic obstructive pulmonary disease, FEV_1= Forced expiratory volume in one second, FVC = Forced vital capacity.

RNA isolation and qPCR

RNA isolation, cDNA synthesis and qPCR analysis was performed as previously described (13) using the primers shown in Table 2. mRNA expression levels were normalized against the reference genes RPL13A and ATP5B, which were selected using the "Genorm method" (Genorm; Primer Design, Southampton, United Kingdom) .

Table 2 Primer sequences

Gene	Forward primer	Reverse primer
WFDC12	5'-AGCCAGGATGGGAGGCCAAGT-3'	5'- CTCAGGGGCAGGTGCCAAGTG-3'
SLPI	5'-CAGGGAAGAAGAGATGTTG-3'	5'-CCTCCATATGGCAGGAATC-3'
PI3	5'-CCGCTGCTTGAAAGATACTG-3'	5'-GAATGGGAGGAAGAATGGAC-3'
IL8	5'-CAGCCTTCCTGATTTCTG-3'	5'-CACTTCTCCACAACCCTCTGC-3'
TGFB1	5'-CTAATGGTGGAAACCCACAACG-3'	5'- TATCGCCAGGAATTGTTGCTG-3'
TP63	5'-CCACCTGGACGTATTCCACTG-3'	5'-TCGAATCAAATGACTAGGAGGGG-3'
KRT5	5'-CCAAGGTTGATGCACTGATGG-3'	5'-TGTCAGACATGCGTCTGC-3'
FOXJ1	5'-GGAGGGGACGTAAATCCCTA-3'	5'-TTGGTCCCAGTAGTTCCAGC -3'
SCGB1A1	5'- ACATGAGGGAGGCAGGGGCTC-3'	5'- ACTCAAAGCATGGCAGCGGCA-3'
RPL13A	5'-AAGGTGGTGGTCGTACGCTGTG-3'	5'- CGGGAAGGGTTGGTGTTCATCC-3'
ATP5B	5'-TCACCCAGGCTGGTTCAGA-3'	5'-AGTGGCCAGGGTAGGCTGAT-3'

ELISA

The secretion of IL-8/CXCL8 (R&D, Minneapolis, MN, USA) was assessed following the manufacturer's protocol. Secretion of WFDC12 by ALI-PBEC was determined using a previously described ELISA (2). To this end, conditioned medium was collected from the basal compartment, and the apical surface samples were collected by washing the cell cultures with 100 µl PBS. Collected samples were directly incubated in a high-binding 96 well plate to allow optimal detection of WFDC12.

Data analysis

GraphPad PRISM 6.0 (GraphPad Software Inc., La Jolla, Ca) was used for statistical analysis. Analysis of differences was conducted by using a (un)paired Student's t-test, Mann-Witney U test, or one/two-way repeated measurements ANOVA and Bonferroni *post-hoc* test. Differences between groups were considered significant at p-values < 0.05.

RESULTS

NTHi increases WFDC12 protein secretion but not mRNA expression in ALI-PBEC

As monocytes express WFDC12 in response to the microbial component LPS (2), we first studied the effect of microbial stimuli on WFDC12 expression by airway epithelial cells. This was done by stimulating differentiated ALI-PBEC to the COPD-related respiratory pathogen non-typeable *Haemophilus influenzae* (NTHi). Various concentrations of UV-inactivated NTHi were added to the apical surface of ALI-PBEC cultures and after 3 h incubation mRNA expression of *WFDC12* was determined by qPCR. We did not observe an increase in *WFDC12* expression in NTHi stimulated cells (Figure 1A), and therefore assessed whether *WFDC12* expression was induced in a time-dependent manner by NTHi. This was done by exposing ALI-PBEC cultures to NTHi and determining mRNA expression levels after 3, 12, and 24 h of incubation. However, similar to the NTHi-dose response experiments, we did not observe a NTHi-induced expression of *WFDC12* at other time points (Figure 1B). We subsequently

determined whether the inability of ALI-PBEC to increase *WFDC12* gene expression after NTHi stimulation was accompanied by unchanged protein secretion levels. First, we compared the baseline WFDC12 protein release in the basal culture medium and apical surface washes from ALI-PBEC cultures, and observed that the protein was more abundantly secreted in the basal culture medium (Figure 1C). Next, we assessed WFDC12 secretion in

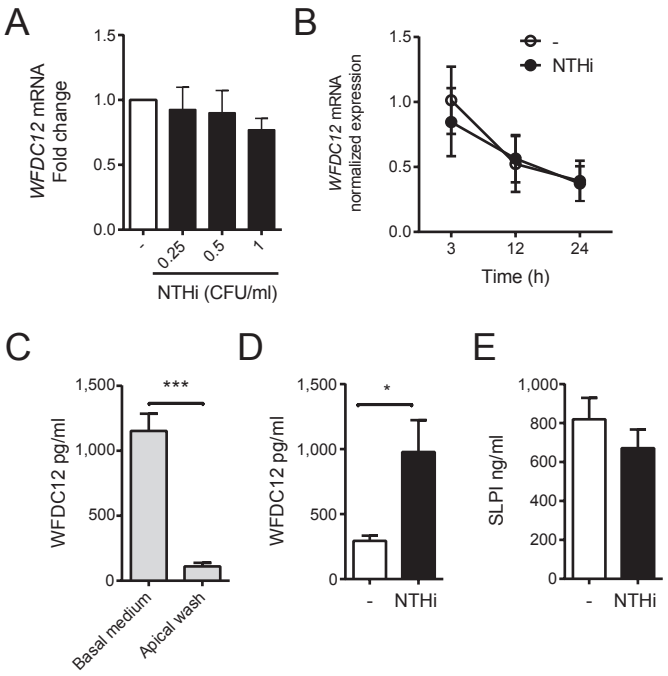

Figure 1. Effect of NTHi on WFDC12 mRNA expression and protein secretion in ALI-PBEC. ALI-PBEC were stimulated with 0.25, 0.5, and 1*10^9 CFU/ml UV-inactivated NTHi and incubated for 3 h (A) or stimulated with 1*10^9 CFU/ml UV-inactivated NTHi, for 3, 12 and 24 h incubation (B), followed by analysis of *WFDC12* expression by qPCR. mRNA expression is shown as normalized values corrected for the reference genes *RPL13A* and *ATP5B*. n=10 independent donors. (C) Baseline WFDC12 protein secretion was assessed in basal culture medium or washings of the apical surface of ALI-PBEC. n=9 independent donors. ALI-PBEC were stimulated with 1*10^9 CFU/ml UV-inactivated NTHi for 24 h. (D) WFDC12 and E) SLPI protein secretion was determined in the basal medium. n=4 independent donors. Results are shown as mean ± SEM. Analysis of differences was conducted with a (A,B) two-way ANOVA with a Bonferroni *post-hoc* test and (C-E) paired t-test. * $p < 0.05$, *** $p < 0.001$.

the basal medium of ALI-PBEC exposed at the apical surface with NTHi. In contrast to the unchanged *WFDC12* mRNA levels, NTHi increased protein secretion in ALI-PBEC cultures after 24 h stimulation (Figure 1D). SLPI secretion was not affected in NTHi-stimulated ALI-PBEC (Figure 1E), suggesting a distinct regulation of WFDC12 protein secretions in response to microbial stimuli. In summary, these findings suggest that NTHi increases the secretion, but not the mRNA expression, of WFDC12 in ALI-PBEC cultures.

NTHi induces mRNA expression of WFDC12 in undifferentiated airway epithelial cells

In a previous study, we observed NTHi-induced expression of the antimicrobial protein RNase 7, which was dependent on the differentiation status of the airway epithelial cells (13). Therefore, we further examined the induction of *WFDC12 i*n undifferentiated submerged PBEC (S-PBEC) and differentiated ALI-PBEC following exposure for 24 hours to UV-inactivated NTHi. Similar to RNase 7, *WFDC12* was induced in S-PBEC but not in ALI-PBEC cultures (Figure 2A). To compare this response with the expression of other WFDC-family members, we also examined *SLPI* and *PI3* mRNA expression levels. *SLPI* was not significantly

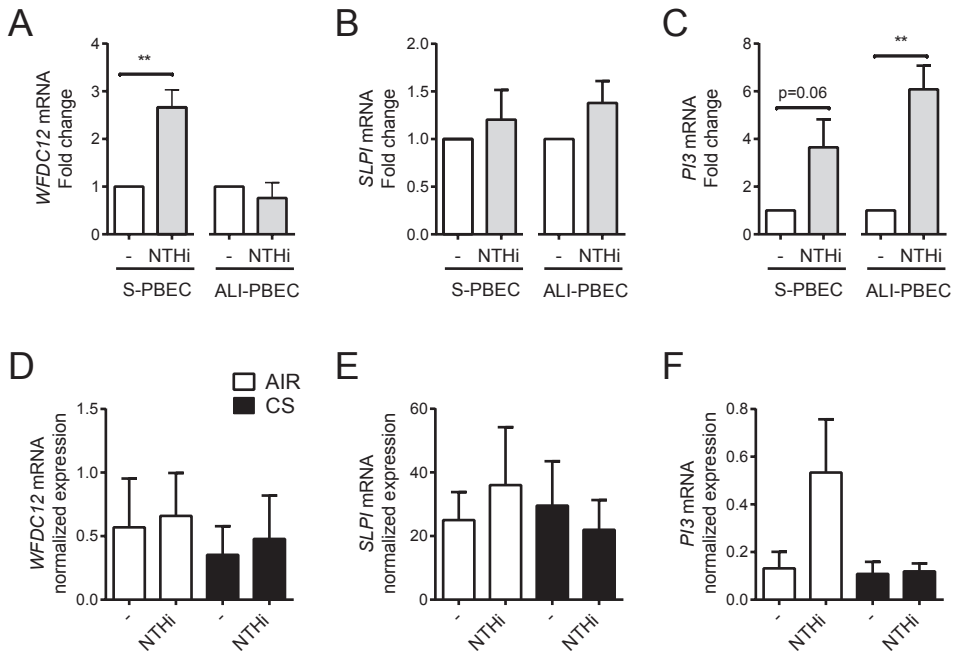

Figure 2. NTHi-induced expression of WFDC12 in S-PBEC and ALI-PBEC and effect of cigarette smoke. S-PBEC and ALI-PBEC were stimulated with $0.5*10^9$ CFU/ml UV-inactivated NTHi, and mRNA expression was determined of (A) *WFDC12*, (B) *SLPI*, and (C) elafin (*PI3*), at 24 h after stimulation. mRNA expression was corrected for the reference genes *RPL13A* and *ATP5B*, and shown as fold change compared to air exposed ALI-PBEC. n=4 independent donors. Results are shown as mean ± SEM. ALI-PBEC were exposed to air or CS and subsequently stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi. mRNA expression of (D) *WFDC12*, (E) *SLPI,* and (F) elafin (*PI3*) was determined by qPCR after 3 h incubation. mRNA expression is shown as normalized values corrected for the reference genes *RPL13A* and *ATP5B.* n=4 independent donors. Results are shown as mean ± SEM. Analysis of differences was conducted with a (A-C) paired t-test and (D-F)one-way ANOVA with a Bonferroni *post-hoc* test. ** $p < 0.01$.

induced in both S- and ALI-PBEC (Figure 2B). In contrast, *PI3* expression was induced by NTHi in both types of PBEC cultures, although its induction in submerged cultures did not reach statistical significance (p=0.06; Figure 2C). We have recently shown that cigarette smoke exposure, in contrast to NTHi, increases RNase 7 expression in ALI-PBEC (13, 16).

Therefore, we questioned if cigarette smoke (CS) exposure, and the combined effect of CS and NTHi, could affect the expression of WFDC12 in ALI-PBEC as well. Cell cultures were first exposed to air or CS during a period of 15 minutes, which in a previous report was shown to cause a transient impairment of the epithelial barrier integrity and induction of innate immune responses (13). Subsequently, cells were stimulated at the apical surface with UV-inactivated NTHi. 3 h after exposure the *WFDC12* expression was determined by qPCR. This post-exposure incubation time was used, because in previous studies we observed that the main effects of CS on mRNA expression of innate immune genes were observed at early time points due to a transient effect (13). Similar to earlier observations, NTHi did not affect *WFDC12* expression, but also CS exposure and the combined exposure of NTHi and CS did not influence the expression (Figure 2D). *SLPI* expression was also not affected by NTHi and CS (Figure 2E). Although not significant, *PI3* induced by NTHi was inhibited by CS (Figure 2F). In summary, these findings suggest that NTHi-induced *WFDC12* expression depends on cell differentiation, but in contrast to RNase 7, is not expressed upon cigarette smoke-induced injury. Moreover, the expression of *WFDC12* is distinct from *SLPI* and *PI3*.

TNFα/IL-1β and TGF-β1 modulate the expression of WFDC12 by ALI-PBEC

Secreted cytokines and growth factors produced by airway epithelial cells or underlying immune and stromal cells, can influence activity of the epithelium (4)(Whitsett and Alenghat, 2014). Therefore, we determined the effect of the pro-inflammatory cytokines TNFα and IL-1β on the expression of *WFDC12* by ALI-PBEC. Combined stimulation with TNFα and IL-1β significantly increased *WFDC12* expression in ALI-PBEC (Figure 3A). As positive control, TNFα and IL-1β also promoted mRNA expression of the pro-inflammatory chemokine IL-8/*CXCL8* (Figure 3B). Previous reports have shown that the growth factor TGF-β1 is increased in COPD (17, 18) and that TGF-β1 can inhibit the expression of SLPI in airway epithelial cells. Therefore, we explored the influence of TGF-β1 on WFDC12 production by determining the effect of recombinant TGF-β1 on ALI-PBEC. TGF-β1 stimulation attenuated the expression of *WFDC12* mRNA in ALI-PBEC (Figure 3C). However, we did not observe a significant suppression of WFDC12 protein secretion into the basal medium of ALI-PBEC (Figure 3D). Interestingly, mRNA expression and protein secretion of IL-8/CXCL8 was also increased by TGF-β1 in ALI-PBEC (Figure 3E,F). This suggests a differential effect of TGF-β1 on WFDC12 and IL-8/CXCL8 expression, which is in contrast to the effects of TNFα/IL-1β stimulation. To further examine whether endogenous TGF-β produced by airway epithelial cells caused suppression of WFDC12, the effect of a neutralizing antibody directed against TGF-β1 was investigated. In line with our findings following addition of TGF-β1, anti-TGF-β1 increased WFDC12 mRNA and protein expression, when compared to an isotype antibody control (Figure 3G,H). Overall, these findings suggest that the pro-inflammatory cytokines TNFα/IL-1β can increase *WFDC12* mRNA expression in airway epithelial cells, whereas exogenously added or endogenously produced TGF-β1 impairs the expression.

TGF-β1 and WFDC12 are differentially expressed in basal and luminal airway epithelial cells

Increased levels of TGF-β1 have been shown to impair the expression of the polymeric immunoglobulin receptor (pIgR) by affecting cell differentiation (18, 19). Therefore, we examined the influence of airway epithelial cell differentiation on the expression of *WFDC12* in ALI-PBEC cultures, and furthermore determined expression levels of TGF-β1 mRNA

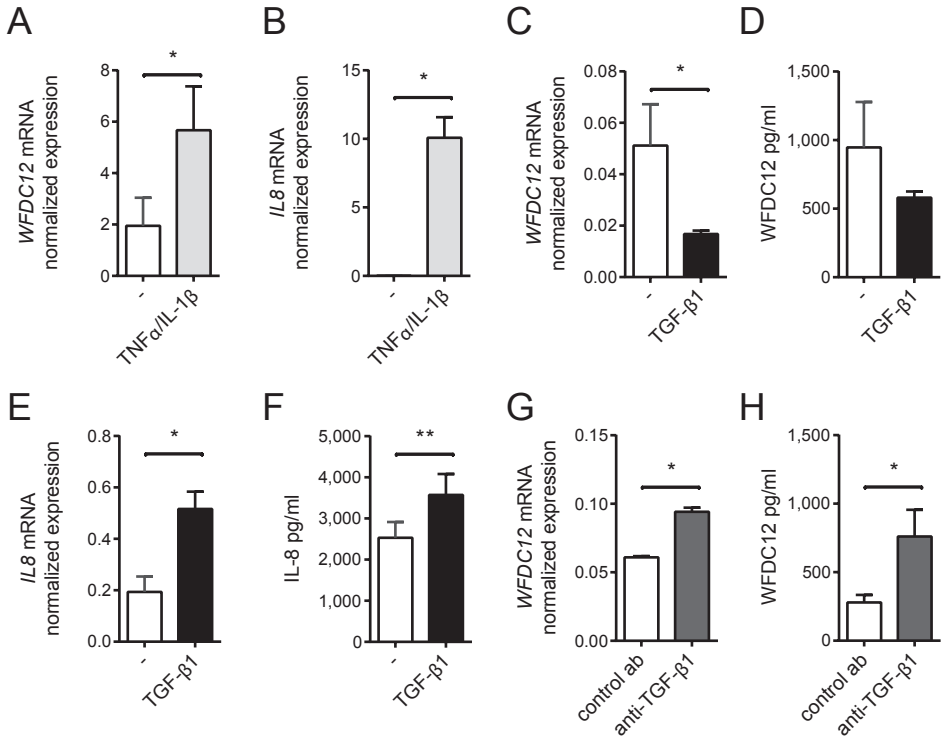

Figure 3. Effect of TNFα/IL-1β and TGF-β1 on the expression of WFDC12. (A) ALI-PBEC cultures were stimulated with TNFα/IL-1β (both 20 ng/ml) and after 24 h incubation expression of WFDC12 was determined. mRNA expression is shown as normalized values corrected for the reference genes *RPL13A* and *ATP5B*. n=4 independent donors. (B) IL-8/CXCL8 mRNA expression was also determined after TNFα/IL-1β (both 20 ng/ml) stimulation. (C) ALI-PBEC cultures were stimulated with TGF-β1 (20 ng/ml) for 24 h. After incubation *WFDC12* expression was determined. mRNA expression is shown as normalized values corrected for the reference genes *RPL13A* and *ATP5B*. n=4 independent donors. (D) Protein secretion of WFDC12 was assessed in the basal medium of unstimulated or TGF-β1 (20 ng/ml) - stimulated ALI-PBEC, 24 h after stimulation. n=3 independent donors. IL-8/CXCL8 (E) mRNA and (F) protein secretion was also determined after TGF-β1 (20 ng/ml) stimulation. (G) Differentiated ALI-PBEC were incubated for 24 h with an anti-TGF-β1 or isotype control antibody (both 10 μg/ml) that was added to the basal compartment. mRNA expression of WFDC12 was determined by qPCR. (H) Secretion of WFDC12 into the basal medium was assessed by ELISA. n=3-4 independent donors. Results are shown as mean ± SEM. Analysis of differences was conducted with a paired t-test. * $p < 0.05$, ** $p < 0.01$.

(*TGFB1*) during differentiation. First, baseline expression of *WFDC12* and *TGFB1* was determined in undifferentiated ALI-PBEC (i.e. confluent layers of PBEC grown in Transwell inserts), and cells that were differentiated at the ALI for 1 and 2 weeks. *WFDC12* expression levels were significantly increased upon 1 and 2 weeks of airway epithelial cell differentiation at the air-liquid interface (Figure 4A). In contrast, expression of *TGFB1* was significantly reduced after 1 and 2 weeks of cell differentiation (Figure 4B). These findings suggest an inverse relationship between *WFDC12* and *TGFB1* expression, which are respectively increased and decreased during differentiation. Since basal cells predominate in undifferentiated ALI-PBEC,

whereas differentiated cultures are composed of basal and luminal cells, we next isolated enriched luminal and basal cell fractions from fully differentiated ALI-PBEC cultures and determined mRNA expression levels in these fractions. We confirmed successful separation of both fractions by showing higher expression of the basal cell markers *TP63* and *KRT5* in the basal cell fraction, whereas *FOXJ1* (ciliated cells) and *SCGB1A1* (club cells) expression

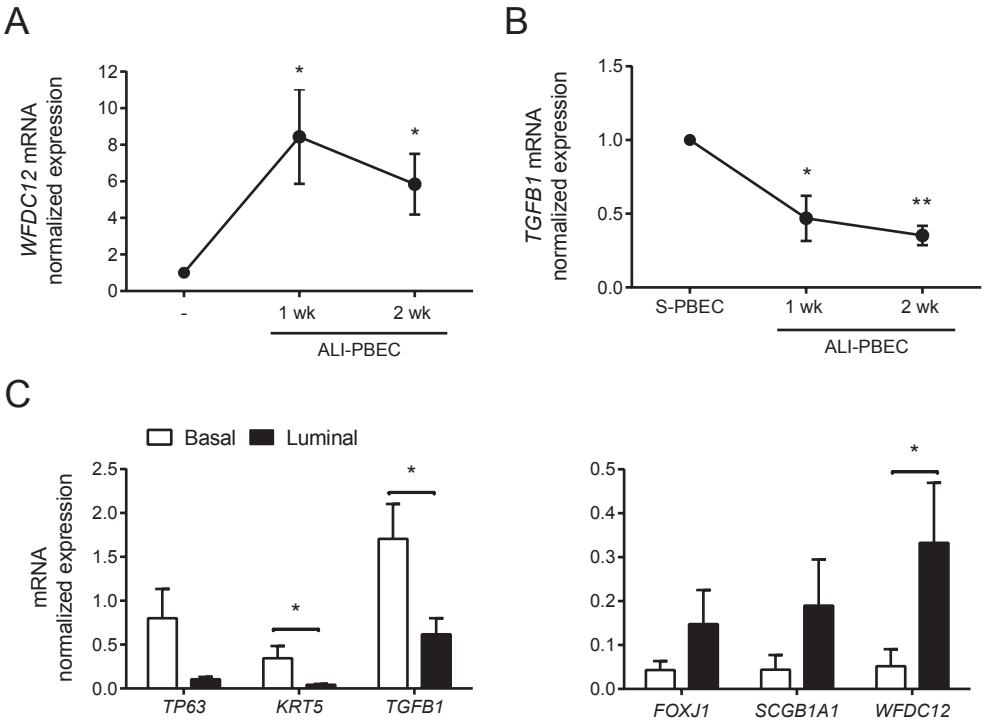

Figure 4. Airway epithelial cell differentiation regulates WFDC12 and TGF-β1 expression. Airway epithelial cell mRNA expression of (A) WFDC12 and (B) *TGFB1* was determined in undifferentiated PBEC (-) and ALI-PBEC differentiated for 1 or 2 weeks. n=6-9 independent donors. (C) Luminal and basal cell fractions were separated in differentiated ALI-PBEC and mRNA expression of *WFDC12* and *TGFB1* was determined. In addition, the expression of *TP63, KRT5* (both basal cell markers), *FOXJ1* (ciliated cell marker), and *SCGB1A1* (club cell marker) was determined. n=4 independent donors. Results are shown as mean ± SEM. Analysis of differences was conducted with (A,B) a one-way ANOVA with a Bonferroni *post-hoc* test (compared to (-) undifferentiated) and (C) a paired t-test.* $p < 0.05$.

was more abundant in the luminal cell fraction (Figure 4C). A significantly higher expression of *WFDC12* in the luminal cell enriched fraction was observed, whereas *TGFB1* was more abundant in the basal cell fraction. Overall, these findings suggest that constitutive expression of WFDC12 in airway epithelial cells is increased during differentiation by formation of luminal cells, while TGF-β1 is reduced during differentiation and mainly expressed by basal cells.

Lower expression of WFDC12 in COPD airway epithelial cells

In previous experiments we used epithelial cells that were not selected based on COPD disease status. However, as gene expression may be altered in COPD patients, we further examined the expression levels of *WFDC12* in cultured ALI-PBEC from COPD patients and non-COPD (ex)smokers. Compared to non-COPD cultures, COPD ALI-PBEC displayed a significantly lower expression of *WFDC12* (Figure 5A). Because of the large spread in mRNA expression between individual donors, we further assessed the correlation between lung function and *WFDC12* levels. A significant correlation was observed between WFDC12 and FEV_1 (% predicted) (Figure 5B) and FEV_1/FVC (% predicted) (Figure 5C). These findings suggest lower baseline expression of *WFDC12* in COPD cultures compared to non-COPD, which is correlated to lung function

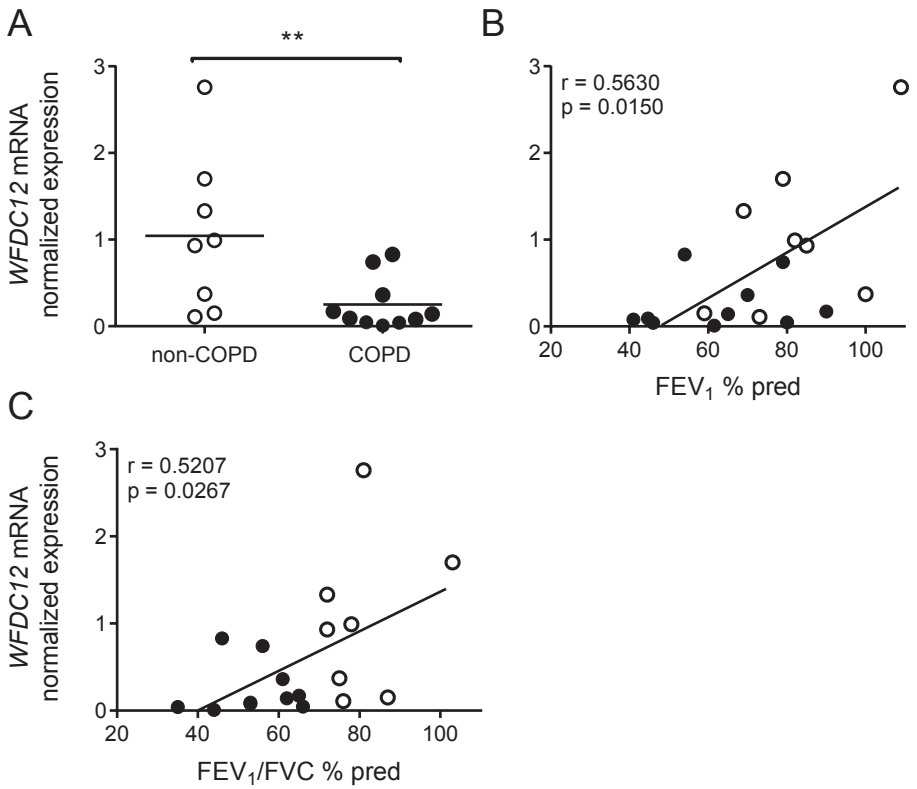

Figure 5. Expression of WFDC12 in COPD and non-COPD ALI-PBEC. (A) Expression of *WFDC12 i*n ALI-PBEC cultures from non-COPD (ex)smokers (open circles, n=8) and COPD patients (black circles, n=11). mRNA expression is shown as normalized values corrected for the reference genes *RPL13A* and *ATP5B*. Individual data points are shown with median and interquartile range. Analysis of differences was conducted with a Mann-Whitney test. ** $p < 0.01$. (B) Correlation between FEV_1 (% predicted) and *WFDC12* expression (r=0.5630, p=0.0150). (C) Correlation between FEV_1/FVC (% predicted) and *WFDC12* expression (r = 0.5207, p = 0.0267).

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DISCUSSION

We report a dynamic regulation of WFDC12 expression in airway epithelial cells, which increases during cell differentiation, and furthermore is distinct from other WFDC-family members. In contrast to the expression of other innate immune mediators (13), *WFDC12* mRNA expression by ALI-PBEC was not affected by stimulation with UV-inactivated NTHi or acute exposures to cigarette smoke. We cannot formally exclude that live bacteria or prolonged exposures to cigarette smoke could have affected WFDC12 expression. Interestingly, UV-inactivated only increased WFDC12 mRNA expression in undifferentiated PBEC, but did increase WFDC12 protein secretion in ALI-PBEC cultures suggesting release of stored WFDC12. In contrast to differentiated ALI-PBEC, *WFDC12* mRNA was further increased in undifferentiated S-PBEC exposed to UV-inactivated NTHi. This finding corresponds with an earlier study examining the expression of the antimicrobial protein RNase 7 (13), and further indicates that differentiated and undifferentiated airway epithelial cells display distinct innate immune properties.

The increase in *WFDC12* in S-PBEC upon stimulation with NTHi suggests that a pro-inflammatory environment may increase the expression of WFDC12 in basal cells. Such a mechanism may contribute to the regulation of airway epithelial innate immunity upon injury, when basal cells may be exposed to respiratory pathogens in the absence of a luminal cell layer (20, 21). Examination of the influence of pro-inflammatory cytokines demonstrated that, in contrast to NTHi, TNFα/IL-1β stimulation did increase *WFDC12* expression in ALI-PBEC. This is likely explained by the fact that these cytokines were added to the basal compartment of the culture and therefore were readily able to stimulate basal cells. In addition to TNFα/IL-1β, we have observed in preliminary studies that IL-4 and IL-17 could also increas *WFDC12* expression in undifferentiated S-PBEC (*unpublished data*). This suggests that a range of other cytokines can induce the expression of WFDC12.

We also observed a decrease in WFDC12 expression by TGF-β1, which is similar to earlier reports demonstrating that TGF-β1 also impaired expression of SLPI (22-24). It becomes more obvious that TGF-β and related growth factor family members play an important role in COPD pathogenesis (25). Besides decreasing the expression of WFDC12, TGF-β1 increased airway epithelial expression of the pro-inflammatory chemokine IL-8/CXCL8. This suggests that TGF-β1 causes imbalances in the expression of anti- and pro-inflammatory factors by the airway epithelium that may contribute to the persistence of airway inflammation. Interestingly, it was reported that TGF-β did not affect the expression of IL-8 in undifferentiated S-PBEC (26). This suggests that airway epithelial responses to TGF-β are also cell differentiation dependent.

Supporting our earlier assumption that differentiated ALI-PBEC express WFDC12 in sufficient quantities, we observed that *WFDC12* mRNA expression became more abundant during cell differentiation. We furthermore detected *WFDC12* mainly in luminal cells of differentiated ALI-PBEC cultures. Indeed, the luminal airway epithelium consists of different cell types, i.e. ciliated, goblet and club cells. Therefore, expression of WFDC12 may be more restricted to a certain luminal cell type, which requires further study. In line with previous studies (27, 28), *TGFB1* expression levels were decreased upon mucociliary differentiation,

and its expression was mainly observed in the basal cell fraction of ALI-PBEC. This suggests an inverse relationship between WFDC12 and TGF-β1 expression during cell differentiation, in which a reduction in TGF-β1 may allow an increase in WFDC12. The distinct expression of WFDC12 in luminal and basal cells of ALI-PBEC, might be explained by a cell differentiation dependent effect of TGF-β. Another explanation for the low expression of WFDC12 by basal cells is the quiescent state of the cells in intact ALI-PBEC cultures, whereas expression in injured conditions can be elevated by inflammatory stimuli.

Previous studies have revealed impaired defense mechanisms in cultured epithelial cells from patients with COPD. This includes a recent report from our group showing that COPD epithelial cell cultures display reduced antibacterial activity and expression of the antimicrobial mediators hBD-2 and S100A7 (29). In the present study we extend these findings by showing that airway epithelial cell expression of *WFDC12* is intrinsically lower in differentiated ALI-PBEC from COPD patients compared to non-COPD controls. Based on the previously described function of the protein as a protease inhibitor and anti-inflammatory mediator (2), it can be speculated that the attenuated expression of WFCD12 by airway epithelial cells in COPD contributes to enhanced airway tissue injury and inflammation. However, the protein may also have additional functions, such as affecting wound repair. It

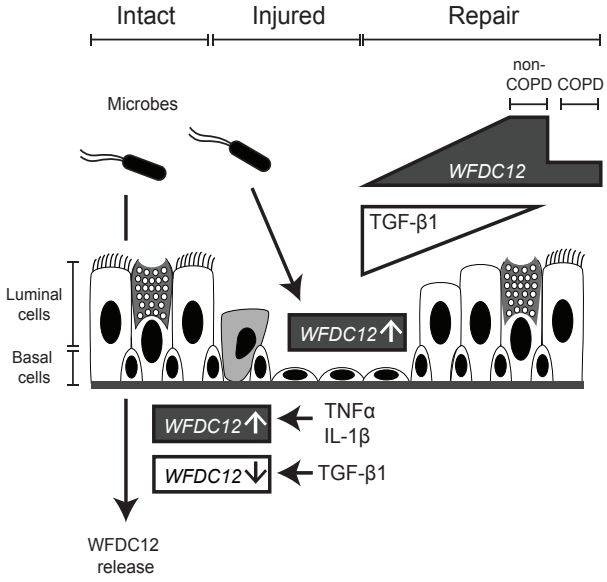

Figure 6. Proposed model for regulation of epithelial WFDC12 expression. Intact airway epithelium consists of luminal cells and basal cells. Release of stored WFDC12 protein by intact epithelium is increased upon exposure to microbes, whereas mRNA expression is increased by TNFα/IL-1β, and inhibited by TGF-β1. Injured epithelium consists of basal cells that restore the injured epithelial layer and that express *WFDC12* upon exposure to microbes. Moreover, basal cells express TGF-β1, which inhibit *WFDC12*. Upon regeneration of the airway epithelium, basal cell numbers decrease, resulting in a reduced level of TGF-β1. Inversely, *WFDC12* expression increases upon differentiation and is high in the luminal cells of restored epithelium. This expression is higher in non-COPD compared to COPD airway epithelial cells.

is furthermore unknown how comparable the levels of WFDC12 are between epithelial cells and macrophages and therefore whether lower levels in COPD epithelium are compensated or overruled by the enhanced numbers of inflammatory macrophages in the lungs of COPD patients. Further research examining both the functional properties of WFDC12 from airway epithelial cells, and studying the expression of this protein in macrophages and other immune cells in COPD is therefore required to investigate this.

Based on our findings, we propose the following model (Figure 6) in which WFDC12 expression is dynamically regulated in the airway epithelium. In intact epithelium, WFDC12 is constitutively expressed in luminal cells, and the protein is released upon exposure to microbes. WFDC12 is furthermore induced by TNFα/IL-1β, and suppressed by TGF-β1, which may be derived from by the epithelium itself, or by immune or stromal cells. Upon epithelial injury, death and/or shedding of luminal cells may result in exposure of the underlying basal cells to invading micro-organisms, which may result in the induction of WFDC12 in these basal cells. Upon epithelial repair, TGF-β1 initially suppresses airway epithelial WFDC12 expression. When repair progresses, TGF-β1 levels decline, whereas WFDC12 expression by luminal cells increases. This constitutive expression of WFDC12 in the luminal cells of restored epithelium is lower in COPD airway epithelial cells when compared to non-COPD smokers.

ACKNOWLEDGMENTS

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CHAPTER 10

Antimicrobial peptides and innate lung defenses: role in infectious and non-infectious lung diseases and therapeutic applications.

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ABSTRACT

Respiratory infections are a major clinical problem, and treatment is increasingly complicated by the emergence of microbial antibiotic resistance. Development of new antibiotics is notoriously costly and slow, and therefore alternative strategies are needed. Antimicrobial peptides, central effector molecules of the immune system, are being considered as an alternative to conventional antibiotics. These peptides display a range of activities, including not only direct antimicrobial activity but also immunomodulation and wound repair. In the lung, especially airway epithelial cells and neutrophils contribute to their synthesis. The relevance of antimicrobial peptides for host defense against infection has been demonstrated in animal models, and is also supported by observations in patient studies, showing altered expression and/or unfavorable circumstances for their action in a variety of lung diseases. Importantly, antimicrobial peptides are active against micro-organisms that are resistant against conventional antibiotics, including multidrug resistant bacteria. Several strategies have been proposed to use these peptides in the treatment of infections, including direct administration of antimicrobial peptides, enhancement of their local production and creation of more favorable circumstances for their action. In this review, recent developments in antimicrobial peptides research in the lung and clinical applications for novel therapies of lung diseases are discussed.

INTRODUCTION

Respiratory infections are a major clinical problem. According to the World Health Organization (WHO), lower respiratory infection was the fourth leading cause of death worldwide in 2012 (1). In addition to pneumonia and bronchitis, lower respiratory tract infections also markedly contribute to chronic inflammatory lung disorders such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and asthma. Although antibiotic treatment is considered as the most effective medical intervention currently available, there is a growing concern regarding the increase of microbial antibiotic resistance. Antibiotic resistance is associated with marked morbidity and mortality, and also poses an increasing economic burden. Therefore, it is now considered as a top public health threat for which novel approaches are needed. In addition to stimulating the prudent use of antibiotics, there is an urgent need for new antibiotics. However, it has proven increasingly difficult and extremely costly to develop novel antibiotics resulting in a limited pipeline for new antibiotics. Therefore, many pharmaceutical companies have put a hold on their antibiotic research activities.

Several alternatives for antibiotics have been proposed, and in a back-to-nature approach new strategies are being developed, including the use of bacteriophages, probiotics and antimicrobial peptides. Antimicrobial peptides (AMPs) are effector molecules of the immune system, and research has demonstrated that these peptides may serve as potential alternatives for conventional antibiotics. AMPs display broad-spectrum antimicrobial activity against bacteria, fungi and viruses, including multidrug resistant (MDR) micro-organisms and those present in biofilms. Production of AMPs by e.g. airway epithelial cells and neutrophils is one of the varieties of mechanisms used by the lung to deal with the continuous largescale exposure to numerous inhaled pathogens. In this review we will discuss the natural role of AMPs in lung host defense and their therapeutic potential in the treatment of lung diseases.

ANTIMICROBIAL PEPTIDES AND THEIR ROLE IN HOST DEFENSE AGAINST RESPIRATORY INFECTIONS AND IN CHRONIC LUNG INFLAMMATION

Originally discovered for their (direct) antimicrobial actions, AMPs are nowadays increasingly recognized for their miscellaneous qualities ranging from antimicrobial to anti-biofilm, anticancer and immunomodulation. AMPs are part of the evolutionary conserved innate immune system, and are abundantly produced in the lung and other mucosal tissues, where they act as the first line of defense against infections (2) . Over 2000 naturally occurring AMPs have been identified so far (http://aps.unmc.edu/AP/main.php), and also humans express various AMPs. These AMPs have overlapping actions, but often are active against different pathogens, at different locations in the body with different mechanisms of action. The main AMPs that are detected in lung tissues and secretions, are neutrophil α-defensins/human neutrophil peptides (HNPs), human β-defensins (hBDs) and the cathelicidin hCAP18/LL-37 (3, 4). In the lung, not only the smaller AMPs but also other larger antimicrobial proteins are produced, including lysozyme, lactoferrin, secretory leukocyte proteinase inhibitor (SLPI), elafin and RNase 7 (5) . Several cell types contribute to production of AMPs in the lung. Airway epithelial cells represent a major source of these peptides in the lung, producing e.g. hBD-1, hBD-2, hBD-3 and LL-37. These AMPs are expressed either constitutively or are

induced by microbial exposure, injury, cytokines, growth factors produced during wound repair, or (micro)nutrients such as vitamin D 4 . Also, myeloid cells such as neutrophils and macrophages, contribute to the presence of AMPs in the lungs. In particular, neutrophils that are attracted during inflammation are the primary source of HNPs and LL-37. These AMPs act intracellular within the phagolysosome to kill ingested micro-organisms, or are secreted or released in complex with DNA in structures known as neutrophil extracellular traps (NETs) (6). Recent studies highlight that AMPs such as LL-37 may also stimulate formation of NETs and stabilize these by providing protection against nuclease-mediated degradation, and thus contribute to this recently identified mechanism of host defense (6, 7).

The presence of these AMPs contributes to innate lung defense by displaying a variety of mechanisms of action, including direct antibacterial and antifungal activity mediated by lysis of micro-organisms through formation of transmembrane pores or by impairing bacterial viability by affecting processes such as cell wall biosynthesis (8). In addition, AMPs display antiviral activity against a range of viruses (9). Besides their direct antimicrobial activity, AMPs have an active role in shaping the immune response in the lung, displaying pro- and anti-inflammatory properties, chemotactic activity, inducing expression of cytokines and chemokines and modulating dendritic cell maturation (10). Moreover, they can promote wound healing by enhancing airway epithelial cell repair and promoting angiogenesis (11, 12). The detailed contribution of AMPs to innate defenses against infections has been clearly demonstrated in a variety of animal model studies. In the fruit fly Drosophila melanogaster, which only has an innate immune system, abrogation of microbial induced expression of AMPs resulted in enhanced infections and reduced host survival (13). In line with this, aerosol treatment with a bacterial lysate or a combination of microbial ligands, provides protection against a broad-spectrum of respiratory pathogens in a murine model (14). This protection was not dependent on immune cells, but mediated by lung epithelial cells through induced expression of AMPs. An example of the importance of microbial induced expression of AMPs in host defense is observed in patients with Crohn's disease that have a polymorphism in the gene encoding the cytosolic microbial pattern recognition receptor nucleotide-binding oligomerization domain-containing protein 2 (15). This polymorphism impairs intestinal expression of AMPs, thereby contributing to enhanced infections and chronic inflammation.

Whereas these studies highlight the relevance of AMPs for host defense in general, a variety of studies also provide direct evidence for a protective role of AMPs in lung infections. This is well-illustrated by research on the cathelicidin peptide LL-37 and the cathelicidin mouse orthologue (cathelicidin-related antimicrobial peptide) CRAMP. Transgenic overexpression of LL-37 in mice provided protection against Pseudomonas aeruginosa infection (16). Conversely, CRAMP deficient mice were more sensitive to Gram-negative bacterial pneumonia (17). Similar in vivo studies also support a role for human LL-37 and murine CRAMP in host defense against influenza A respiratory viral infections (18). Moreover, in individuals with vitamin D deficiency, reduced expression of LL-37 expression may increase susceptibility to tuberculosis infections (19). Similar model studies have highlighted the role of e.g. β-defensins in respiratory infections (reviewed in (20)).

In addition to a role in respiratory infections, aberrant expression and activity of AMPs is also associated with non-infectious lung diseases (Table 1). Airway epithelial expression of hBD-

2 is reduced in smokers, patients with COPD, and CF lung disease, which could be a reason for increased microbial colonization and infections in these diseases (21-23). Also allergic airways inflammation has been shown to reduce AMP expression (24). Moreover, reduced pH of the airway surface liquid as observed in infants with CF, may impair the activity of AMPs, as demonstrated in a porcine CF model and in vitro (25, 26). Importantly, not only the pH of the local environment but also other local conditions may impair the antimicrobial activity of AMPs, including AMP degradation by microbial and host proteases (27, 28), and inhibition of AMP activity by salt, microbial polysaccharides, F-actin and DNA from dying cells, and mucus (29).

Table 1. AMPs and their relevance for lung diseases

AMP	**Localization (main cell types)**	**Changed expression in lung disease**
Neutrophil α-defensins (Human neutrophil peptides [HNP]1-4)	Neutrophils	• Asthma: increased systemic expression of HNP-1 in especially neutrophilic asthma (58) • Asthma: increased BAL HNP1-3 levels in RV infection (34) • Bronchiolitis obliterans syndrome: increased HNP1-3 in BAL(33) • COPD: HNP-1 and HNP-2 increased in BALF(31); sputum HNP1-3 associated with COPD severity (30) • Cystic fibrosis: high levels of HNP1-3 in sputum (59)
β-defensins	Mainly airway epithelial cells	• Acute pneumonia: smoking associated with reduced hBD-2 in pharyngeal washings (22) • COPD: Increased hBD-1 expression in central airways in COPD (60) • COPD: hBD-2 expression is decreased in central airways (21) and sputum and BAL(61), and increased in peripheral lung tissue(62) • Cystic fibrosis: decreased hBD-2 levels correlate with disease severity (23) • Diffuse panbronchiolitis: increased hBD-2 in plasma and hBD-1 and hBD-2 in BAL(63)
hCAP18/LL-37	Neutrophils and airway epithelial cells	• Bronchiolitis obliterans syndrome: increased levels in BAL(33) • COPD: increased expression in small airways in smokers and especially smokers with COPD(64) • Cystic fibrosis: increased levels in BAL correlate with disease severity(23) • Lung cancer: increased expression(65)

In contrast to the impaired expression and activity of airway epithelial AMPs in COPD and CF, increased neutrophilic inflammation in these diseases is associated with increased levels of neutrophil-derived HNPs and LL-37 (23, 30, 31). Moreover, exacerbations in COPD caused by bacterial infections, or induced experimentally upon rhinovirus infection, are associated with further increased levels of neutrophil-derived AMPs in the lung (28, 32). Similar to

COPD and CF, increased neutrophilic inflammation correlated with enhanced levels of neutrophil-derived AMPs in neutrophilic asthma, bronchiolitis obliterans syndrome, and interstitial lung disease (33-35). In these diseases, especially neutrophil-derived AMPs might contribute to lung injury and inflammation through their pro-inflammatory activity and cytotoxic properties at high concentrations. These studies illustrate that both insufficient as well as excessive levels of AMPs may contribute to lung disease development and progression. Low levels of AMPs or impairment of their activity may increase susceptibility to infection, alter the microbiome and impair wound repair, inflammation and immunity. In contrast, highly increased levels of AMPs may contribute to inflammation, uncontrolled immune cell recruitment, tissue injury and an altered microbiome. This illustrates that homeostasis of AMPs in the lung is important and that lack or excess of AMPs both have detrimental effects. Therefore, monitoring levels of AMPs in respiratory secretions or tissue in inflammatory lung diseases may provide important information on disease pathogenesis. Furthermore, when designing clinical trials using AMPs, these consequences of inappropriate levels of AMPs need to be taken into consideration.

AMPS AS CANDIDATES FOR DRUG DEVELOPMENT

Use of AMPs as therapeutics seems a valid option based on their multiplicity of actions against multidrug resistant (MDR) pathogens as discussed above. Furthermore, their antibiofilm qualities are extremely relevant in a hospital setting where biofilms (including those with MDR micro-organisms) on both biotic surfaces (such as the airways) as well as on implants or mechanical ventilators are notoriously therapy resistant (36). However, because of the variety of antimicrobial and other activities, the therapeutic window of these peptides is probably different for each peptide and each disease. As discussed in the previous section and illustrated in Figure 1, a relative deficiency as well as excessive AMP expression and/or activity may be detrimental. Several factors need to be taken into account that could affect the therapeutic success of AMP-based therapeutic strategies: 1) type of AMP used, 2) route of administration and 3) local environment. However, a threat to this use is the potential development of pathogenic resistance against these peptides (37). Especially the development of resistance against endogenous peptides would be detrimental for the host defense against infection. However, as AMPs are ancient molecules that have diverse actions, complete resistance as detected in modern antibiotics is not expected to occur (38). Another potential risk could be interference of externally administered AMPs with microbiota. This is important since the composition of the microbiome is highly relevant to the development and progression of chronic lung disease (39). However, especially since the microbiome also needs to be protected against unwanted actions of endogenous AMPs, it may not be surprising that the healthy microbiome appears to be relatively protected against AMP-mediated killing (40).

Proof-of-principal for therapeutic efficacy of endogenous AMPs in lungs has been delivered by over-expressing LL-37 in the lungs of mice or by direct administration of this peptide to the lungs. This resulted in reduced bacterial load and enhanced survival of the infected mice (16, 41). AMPs were also active against P. aeruginosa in a rat model of CF where both reduced pathogenic load and anti-inflammatory activity was observed by these peptides (42). Alternatively, one could rebalance endogenous production of AMPs, for instance when

expression of these are compromised during disease. Several inducers of endogenous AMP expression have been discovered over the last 10 years, of which sodium- and phenylbutyrate have shown promising activities in clinical trials (43, 44). The success of vitamin D treatment, another inducer of AMP expression, may be most pronounced in those patients with severe vitamin D deficiency (45, 46) .

Despite the array of naturally occurring AMPs, little progress has so far been made with therapeutic development of these endogenous peptides. This may in part be explained by AMPs not displaying optimal activity in an inflamed and infected lung. Especially in CF, the loss of function of the cystic fibrosis transmembrane conductance regulator (CFTR) has multiple consequences that may affect endogenous AMP activity, including direct effects on the pH in the airway surface liquid as mentioned before and indirect effects caused by inflammation, cell death and mucus hypersecretion. Therefore, the discovery of small molecules that restore CFTR function may have important implications for local AMP activity in the lungs of CF patients treated with such compounds. Furthermore, in the lung a rather different environment exists compared to for example skin, gut or tissue. So peptides possibly have to be designed for the specific microenvironment they have to be functional in to withstand protease activity or for example unfavorable pH. Furthermore, the local environment can also alter AMP activity (47), which is another feature that needs to be taken into consideration as AMPs might work in one organ and not in another.

The limited success of AMP-based therapy so far may also be explained by the fact that these AMPs have several disadvantages for therapeutic use in addition to the loss of local activity, such as a short half-life and toxicity. An alternative and perhaps also complementary strategy is the development of improved substitutes for AMPs. Innate defense regulators (IDRs) are a group of synthetic peptides inspired by various naturally occurring peptides with the aim to enhance their immunomodulatory activity for therapeutic use (48). So far, IDR peptides were shown to reduce pathogen load and inflammation in mice infected with (MDR) M. tuberculosis (49) and were successful as a vaccine strategy in mice and cotton rats against RSV (50). Furthermore, research also focuses now on alternative compounds based on AMPs (e.g. peptidomimetics) for exogenous administration. Peptidomimetics are compounds that have favorable qualities compared to AMPs, though at lower production cost and without protease sensitivity (51). Both in vitro and in mice, AMPs mimetics have been shown to have favourable effects against for example oral candidiasis (51, 52) .

In addition to the component itself, the route of administration needs to be wisely chosen also taking into consideration which activity of the AMP is targeted. When aiming for direct antimicrobial activity, most likely inhaled administration is preferred with these peptides over systemic administration. The immunomodulatory effects of AMPs seem more robust and could probably also contribute to their activity upon systemic administration, if toxicity allows this type of delivery. Administration can be further enhanced for example by combining AMPs with exogenous surfactant, which was demonstrated to be a promising possibility for improved delivery of AMPs to the lung (53). Alternatively, also for the inducers of endogenous AMPs, their effectiveness can be improved by enhancing their delivery (54). Lastly, combining AMPs with antibiotics shows promising results in the fight against (MDR) microorganisms as synergy is observed in their antimicrobial actions (55, 56). This synergy

may in part be explained by the ability of AMPs to increase bacterial membrane permeability to antibiotics, as recently demonstrated by the ability of LL-37 to potentiate the penetration of azithromycin into MDR bacteria (57) .

Altogether, successful management of an AMP(-based) therapy seem challenging due to the high variety of factors that need to be taken into consideration. However research shows innovative solutions to optimally target AMPs for therapeutic success. Whereas in the past, several clinical trials have evaluated the effect of AMP administration for the treatment of respiratory diseases, the current focus in ongoing clinical trials is on treatments, which induce the endogenous expression of AMPs (www.clinicaltrials.gov).The encouraging trend observed in e.g. studies with vitamin D and phenylbutyrate will hopefully show promising results further stimulating research into this direction.

CONCLUSION

Research on AMPs has flourished in the past decades, but so far has not resulted in major breakthroughs in the treatment of respiratory infections. This is partly explained by the low cost and effectiveness of conventional antibiotics for the majority of patients. As a result, new antibiotic strategies are usually reserved for patients with complicated infections, and as a result the potential market is still limited. In this review we have highlighted why AMPs may be interesting candidates for novel antibiotic strategies, and shown that the multitude of activities displayed by AMPs may both be an advantage of their use, but also poses specific challenges. There are three promising strategies for their use: direct application of AMPs or AMP-inspired compounds, enhancement of local production and improving local conditions for AMPs actions (Figure 1). In addition, further research on the mechanisms underlying

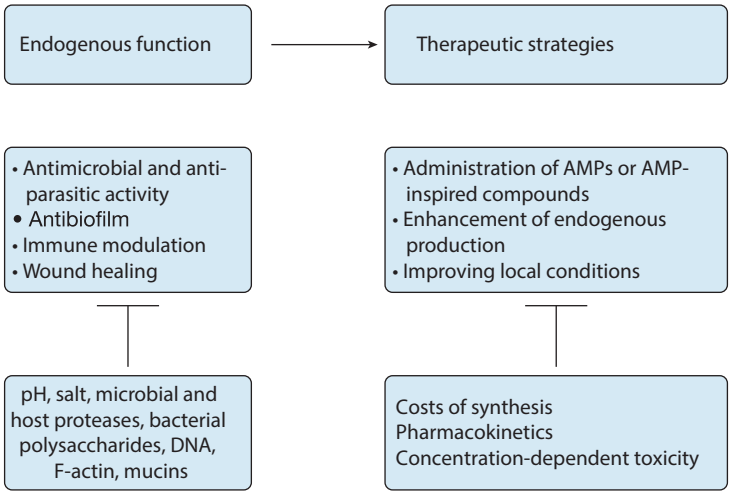

Figure 1. Endogenous functions and possible therapeutic strategies for using AMPs in the treatment of infectious or inflammatory lung diseases. Although AMPs offer an attractive alternative to conventional antibiotics, various factors that may limit their use need to be considered. AMPs = antimicrobial peptides.

deficient AMP expression and/or activity in chronic lung diseases is needed, and may also lead the way to the discovery of novel treatments.

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CHAPTER 11

Summary and Discussion.

SUMMARY AND GENERAL DISCUSSION

This thesis describes a collection of studies which examined the effects of cigarette smoke (CS) and COPD disease status on airway epithelial innate host defense. In this last part, first an overview is given of the main findings of this thesis. Next, the role of alterations in airway epithelial innate host defense will be further discussed, focusing on its role in microbial colonization and infection, airway inflammation, and epithelial remodeling. Finally, a model will be proposed in which the role of airway epithelial cells in COPD will be summarized, according to the vicious circle hypothesis (1).

OVERVIEW OF THE MAIN FINDINGS IN THE THESIS

Antimicrobial proteins and peptides (AMPs) play an important role in the host defense of the lungs. In **Chapter 2** (2) it is summarized that this defense mechanism is altered in COPD and therefore may contribute to the disease. Excessive neutrophilic inflammation in COPD leads to enhanced levels of neutrophil-derived AMPs, i.e. LL-37 and alpha-defensins, which may promote inflammation and airway tissue injury and remodeling. In contrast to neutrophils, AMPs produced by the airway epithelium are suppressed by smoking or in COPD. Therefore, this may contribute to the increased susceptibility to microbial colonization and infections in patients.

In the next series of experimental studies described in this thesis, it was explored how airway epithelial expression of AMPs and other host defense mediators are affected by smoking and COPD disease status.

In **Chapter 3** (3) it is described that expression of the antimicrobial protein Ribonuclease 7 (RNase 7) was selectively induced upon microbial stimuli of undifferentiated basal cells (BCs) but not in differentiated cell cultures. Moreover, BCs present in differentiated cultures also produced RNase 7 upon transient injury caused by CS exposure. This was mediated by activation of the epidermal growth factor receptor (EGFR) and the downstream MEK/ERK1/2 signaling transduction pathway.

The microbial-induced expression of well described AMPs, i.e. human beta-defensin 2 (hBD-2), CCL20, LCN2 and S100A7, and antibacterial activity of airway epithelial cells was investigated in **Chapter 4** (4). Here we first observed an impaired antibacterial activity towards non-typeable *Haemophilus influenzae* (NTHi) in COPD airway epithelial cultures, when compared to non-COPD controls. In line with this, we furthermore detected lower NTHi-induced mRNA expression of the AMPs hBD-2 and S100A7. Next, we observed that CS exposure reduced airway epithelial expression of hBD-2, CCL20, LCN2 and S100A7 in both NTHi-stimulated COPD and non-COPD cultures. In contrast, expression of the pro-inflammatory mediators IL-8 and IL-6 persisted upon epithelial exposure to NTHi and CS. This selective impairment of AMPs expression was related to suppression of NFκB signaling, while inflammatory mediator expression was likely increased due to persistent MAPK/AP-1 signaling

.

Next to microbial-induced AMPs, we further investigated in **Chapter 5** the expression of constitutively expressed AMPs and other host defense proteins in airway epithelial cells. We observed that human beta-defensin 1 (hBD-1) was highly expressed in undifferentiated airway epithelial cells, while the expression was reduced upon cell differentiation. In contrast, other AMPs and host defense proteins, i.e. SLPI, SPLUNC, LPLUNC, and PIGR, were increased in differentiated cultures and were furthermore found to be restricted in luminal epithelial cells. Using a chronic CS exposure model, we further demonstrated that impaired epithelial differentiation caused by CS was accompanied by a reduced expression of luminal cell-restricted host defense proteins. This differentiation-dependent effect could be mimicked by blocking the Notch signaling pathway

.

In addition to expression of innate immune mediators, we further determined the effects of cigarette smoking on airway epithelial injury and repair, which is described in **Chapter 6** (5). Here we examined the effect of CS on the airway epithelial barrier integrity and wound repair using a calcium switch assay and mechanical injury model respectively. CS exposure caused a delay in epithelial repair in both models. In contrast, combined epithelial injury and CS exposure further enhanced RNase 7 expression. Increased number of BCs were observed at the wounded edge of CS exposed injured cultures, thereby suggesting that CS reduced BC migration. It was furthermore shown that CS-induced oxidative stress contributed to impaired wound repair. Moreover, oxidative stress increased downstream MEK ERK1/2 signaling independent of EGFR, thereby causing a further increase in RNase 7 expression.

As CS exposure of epithelial cells caused transient impairment in epithelial repair while causing low cytotoxic effects, we further examined in **Chapter 7** the effect of CS on activation of the cytoprotective integrated stress response (ISR) in airway epithelial cells. We observed that CS caused transient activation of the ISR in differentiated airway epithelial cells, which could be reproduced in undifferentiated cells exposed to CS extract. In addition we observed that CS-induced mRNA expression of the ISR target genes GADD34 and CHOP was higher in COPD cultures compared to non-COPD controls and negatively correlated with lung function. In addition, we described how CS-induced oxidative stress can induce expression of GADD34 via an ISR-independent mechanism. In the discussion, we speculated that enhanced oxidative stress-dependent expression of GADD34 is needed to promote the production of inflammatory mediators by airway epithelial cells, and therefore may contribute to airway inflammation in COPD.

Next to epithelial injury and repair, we further explore the potential influence of CS on airway tissue remodeling in **Chapter 8** (6). Here we described that the surface bound matrix metalloprotease ADAM17 contributed to increased mRNA expression and shedding of soluble IL-6 receptor (sIL-6R) and amphiregulin (AREG). Basolateral protein shedding of sIL-6R and AREG mediated by ADAM17 was significantly higher in COPD cultures. In addition to ADAM17, we furthermore observed that CS-induced EGFR signaling was involved IL-6R and AREG mRNA and protein expression, thereby further demonstrating the importance of EGFR in airway epithelial inflammation and repair/remodeling.

In **Chapter 9** we further examined the expression of the novel innate immune mediators WFDC12 in airway epithelial cultures. Similar to RNase 7, WFDC12 expression was only observed in undifferentiated cultures upon microbial stimulation. However, CS exposure did

not affect the expression in differentiated cell cultures, as was seen with RNase 7. Stimulation with the pro-inflammatory cytokines IL-1β and TNFα did however increase the expression of WFDC12 in differentiated cells, whereas TGFβ1 attenuated the expression. Constitutive expression of WFDC12 increased upon mucociliary differentiation, and was furthermore enriched in the luminal airway epithelial fraction of differentiated cell cultures. Moreover, we observed lower WFDC12 expression in COPD cultures compared to non-COPD controls, which correlated with lung function.
Finally, in **Chapter 10** (7) it is discussed how the impaired antimicrobial defense system in COPD but also other noninfectious and infectious lung diseases can be therapeutically targeted. Here it is mentioned that the COPD patients may be treated with synthetic AMPs, AMP–inspired molecules or through induction of endogenous AMPs to prevent microbial infections and colonization.

ROLE OF IMPAIRED AIRWAY EPITHELIAL ANTIBACTERIAL DEFENSE ON MICROBIAL COLONIZATION AND INFECTIONS IN COPD

The increased susceptibility of smokers and COPD patients to microbial infections raised the question whether airway epithelial innate host defense is attenuated. It has been shown that mucociliary clearance is impaired in response to CS and in COPD (8). Although this function is indeed critical for airway host defense, it has been shown in primary ciliary dyskinesia patients, which display impaired mucociliary clearance, that other host defense mechanisms allow sufficient protection against microbes (9). Studies in cystic fibrosis (CF) suggest that an additional impaired activity of antimicrobial proteins and peptides (AMPs) causes a more severe susceptibility to microbial colonization and infections (10). Therefore, we have focused on the airway epithelial defense functions mediated by AMPs, as this mechanism is largely neglected in COPD. We have observed that the expression of AMPs is differentially regulated in intact epithelial cells, upon microbial exposure, and upon injury and repair. CS and COPD disease status have different effects on the expression of these different classes of AMPs, which will be discussed further.

Constitutively produced AMPs

As shown in the seminal study by *Smith et al.*, the airway surface liquid of differentiated airway epithelial cultures from healthy individuals displayed intrinsic antibacterial activity towards respiratory pathogens (11).This defense against low number of microbes was seen without prior stimulation with pro-inflammatory mediators, suggesting that differentiated airway epithelial cells have a constitutive expression of mediators that provide baseline antibacterial defense. In line with this it is shown in **Chapter 5** that several AMPs, i.e. SLPI, SPLUNC1 and LPLUNC1, are highly expressed at a steady-state level in differentiated airway epithelial cells and allocated to the luminal airway epithelial cells. Similar to mucociliary clearance, alterations in luminal airway epithelial cell differentiation or composition may affect the expression of constitutively produced AMPs in COPD. We have shown that chronic CS exposure attenuated the expression of luminal cell-restricted AMPs by suppressing cell differentiation. In contrast to the effects of CS, we did not observe differences in the expression of constitutively produced AMPs and baseline antibacterial activity between COPD and non-COPD cultures (**Chapter 4 (4)**). This is likely caused by the low disease severity in examined

patients, whereas in more severe (GOLD IV) patients the expression might be abrogated due to persistent epithelial remodeling in culture (12, 13).

Persistent epithelial remodeling, i.e. basal cell hyperplasia and squamous metaplastic lesions, may attenuate expression of luminal cell-specific AMPs. Moreover, goblet cell metaplasia, for instance mediated by the cytokines IL-13, IL-4, IL-17 (14), may alter the expression of AMPs that are restricted to a specific luminal cell type. A previous study in our laboratory has shown that SLPI is not significantly altered in goblet cell enriched cultures (15). However, it was shown by others that SPLUNC1 and LPLUNC1 are respectively absent or restricted to goblet cells (16). In addition to the AMPs examined in this thesis, *Tanabe et al.* have also shown that the antibacterial protein $sPLA_2$ is increased upon ciliated cell differentiation and lost in goblet cell enriched cultures (17). Moreover, as shown in a secretome analysis of differentiated airway epithelial cultures (18), also other antimicrobials such as dermcidin are constitutively produced by differentiated airway epithelial cells (19). This further suggests that a broader spectrum of other AMPs are also regulated by cell differentiation and restricted to luminal cells. Further research is therefore required to understand the broad composition of constitutively-produced AMPs in differentiated airway epithelium and how epithelial remodeling may affect this. This for instance may explain the selective colonization of certain microbes in remodeled airway lesions, which might be caused by a favorable micro-environment shaped by altered expression of constitutively produced AMPs (20). In conclusion, alterations in airway epithelial differentiation due to smoking and persistent epithelial remodeling in COPD may result in a weakened constitutive host defense due to impaired expression of luminal cell-restricted AMPs. This may lead to onset of microbial outgrowth and early colonization of opportunistic respiratory pathogens in the lungs of smokers and COPD patients.

Microbial- and cytokine-induced AMPs

Next to constitutively produced AMPs, microbial infection may lead to the further induction of other types of AMPs in the airway epithelium upon activation of patter recognition receptors and downstream innate immune signaling pathways. In previous studies it was shown that CS exposure reduced the antibacterial defense against opportunistic pathogens of cultured airway epithelial cells (21). This finding corresponded with impaired expression of the AMP hBD-2 in CS-exposed cultures and with the attenuated expression of hBD-2 in tracheal washing of smokers with pneumonia. Extending these findings, we have shown in **Chapter 4** (4) that COPD airway epithelial cells displayed reduced antibacterial activity compared to non-COPD smokers, which corresponded with impaired expression of several microbial-induced AMPs including hBD-2. This finding was furthermore in agreement with observational studies in bronchial tissues from COPD patients, in which lower hBD-2 expression persisted after smoking cessation (22). We were unable to identify the molecular mechanism causing a reduced antibacterial activity of COPD airway epithelial cells, and therefore further research remains needed to determine this. However, we did gain more insight in the mechanisms underlying the impaired antibacterial activity of airway epithelial cells in response to CS. First, we observed that not only expression of hBD-2 was attenuated by CS, but also a panel of other AMPs (4). This suggests that the attenuated antibacterial activity of airway epithelial cells by smoking is the result of abrogation of several microbial-induce AMPs in addition to hBD-2. Next to impaired expression in response to microbial stimuli, we also observed that CS impaired the expression of AMPs upon stimulation with

TNFα and IL-1β (unpublished data). This suggests that attenuated expression of AMPs is not due to effects of CS at the receptor level, but due to alterations in common downstream innate immune signaling pathways. Supporting this, we found that CS impaired the NFκB signaling pathway, which in previous studies was shown to be critical in regulating epithelial expression of several AMPs (23-25). In a previous study it was shown that CS-induced oxidative stress contributed to impaired expression of hBD-2 (26). Therefore it is likely that reactive oxygen species (ROS) mediates suppression of NFκB signaling via post-translational modifications of upstream kinases (27, 28). It has previously been shown that reduced hBD-2 expression in the airways of COPD patient was localized in the upper, but not in the lower airways (22). This might be explained by differences in the density of cigarette smoke particles to which the upper and lower airways are exposed, and suggests that a certain threshold is present at which antibacterial defense is impaired. It has been shown that diesel exhaust may also impair expression of hBD-2 in differentiated airway epithelial cells (29). Therefore, attenuation of the inducible antibacterial defense system in airway epithelial cells may be a common target of air pollutants. In summary, the microbe-induced expression of AMPs is a second defense mechanism that may prevent the further outgrowth of microbes when the constitutive airway epithelial defense system is overwhelmed. Attenuation of this defense system in COPD, because of smoking or exposure to air pollutants, may increase the susceptibility to microbial infection. This mechanism may therefore contribute to microbial infections in smokers or COPD patients during disease exacerbations.

Injury and repair-induced AMPs

Although airway BCs are normally quiescent, findings in **Chapter 3** (3) and **5** suggested unique host defense responses by these cells in the context of epithelial injury and repair (30). hBD-1 has been described in previous study as a constitutively expressed AMP (21). Although this is indeed the case, we observed that hBD-1 was highly expressed in undifferentiated cultures and relatively reduced upon differentiation. This suggests that BCs display a high expression of hBD-1 upon initial phases of epithelial regeneration that may contribute to epithelial defense upon injury. Interestingly, when comparing COPD and non-COPD cell cultures, we observed that hBD-1 expression was significantly higher in COPD cultures (unpublished data). This corresponded with an observational study, which demonstrated enhanced levels of hBD-1 in sputum samples of COPD patients compared to healthy controls (31). Similar to hBD-1, we furthermore described in **Chapter 3** (3) that the antimicrobial protein RNase 7 was highly expressed by cultured BCs. Although we did not observe a significant higher expression of RNase 7 in COPD cultures (unpublished data), we observed that CS exposure increased RNase 7 expression in BCs of differentiated cells. This was related to a transient impairment in the epithelial barrier integrity, and could be further enhanced upon epithelial wounding as described in **Chapter 6** (5). This, also suggests that RNase 7 is a damage-induced AMP, which is increased in activated BCs. Indeed, stimulation of undifferentiated airway epithelial cells with NTHi, increased the expression of RNase 7 and other microbial-induced AMPs, i.e. hBD-2, CCL20, LCN2 (**Chapter 3** (3)). Therefore, BCs in damaged epithelium may display more broad antimicrobial activity that may prevent microbial colonization and infection during wound repair. In summary, increased epithelial injury in COPD and smoking is reflected by the enhanced expression of BC-produced AMPs. Moreover, the epithelial restricted expression of hBD-1 and RNase 7 in BCs of damaged epithelium, suggests that these proteins may be used as selective biomarkers that may reflect epithelial injury in smokers and COPD. The

functional contribution of hBD-1 and RNase 7 on the antibacterial defense in the injured lung requires further investigation. However, it can be speculated that the enhanced expression of these AMPs in smokers and COPD may contribute to a favorable micro-environment that leads to selective microbial outgrowth as mentioned in **2.2** (20). Another explanation, is that hBD-1 and RNase 7 do not provide sufficient protection, as the expression of other AMPs is reduced. For instance CS suppresses microbial- and cytokine-induced AMPs. Furthermore, cigarette smoke may modulate vitamin D3-induced antibacterial responses, which require further study. Interestingly, it has been shown that EGFR activation in keratinocytes leads to expression of human beta-defensin 3 and LCN2 (32). These responses are lacking in airway epithelial cells exposed to CS. This differential response between keratinocytes and airway epithelial cells might be due to tissue dependent EGFR-mediated responses. However, another explanation is that CS selectively modulates downstream EGFR signaling transduction, leading to a selective impaired expression of certain AMPs.

AIRWAY EPITHELIAL INFLAMMATORY RESPONSES

In addition to AMPs, we furthermore determined the expression of pro-inflammatory mediators as indicator of epithelial inflammatory responses. In particular the role of the chemoattractant CXCL8/IL-8 is of importance, as it reflects the contribution of airway epithelial cells in neutrophilic inflammation in COPD (33, 34). In this section the role of CS-induced airway inflammation will be discussed, highlighting the importance of epithelial injury and repair as cause of inflammation, and the contribution oxidative stress.

Cigarette smoke-induced inflammation as part of epithelial repair

In line with previous studies (35, 36), we have shown that CS exposure increases IL-8 expression in airway epithelial cells via activation of ADAM17/EGFR signaling transduction (**Chapter 3,6,8** (3, 5, 6)). In contrast to RNase 7, we observed IL-8 expression in both luminal and basal cells of differentiated cell cultures (3). It has been shown that EGFR is selectively expressed by BCs (37). Therefore, it can be speculated that luminal epithelial IL-8 expression is indirectly mediated via currently uncharacterized BC-interactions. EGFR-dependent release of secreted factors from BCs may for instance cause induction of inflammatory responses in the luminal airway epithelium. Further research is needed to understand the potential basal-luminal cross-talk, which might be targeted to dampen the inflammatory response in the epithelium. We furthermore showed that combined exposure of CS with either microbial stimuli or injury further enhanced IL-8 expression (**Chapter 4 and 6** (4, 5)). Microbe-induced activation of the innate immune system mediates pro-inflammatory responses by airway epithelial cells in large part via NFκB signaling transduction. However, also based on observations by others (38-40), it can be questioned if the NFκB system has an important role in airway epithelial inflammatory responses during cigarette smoking. As discussed in paragraph **2.3**, the NFκB-dependent innate immune responses seems to be suppressed by CS, thereby contributing to microbial colonization and infections. In contrast, sustained activation of MAPK signaling and the downstream AP-1 family of transcription factors have likely a more prominent role in the expression of inflammatory mediators in the airway epithelium.

EGFR and MAPK/AP-1 have a critical role in mediating tissue recovery after injury of

epithelial tissues. Therefore, it can be speculated that CS-induced inflammation is part of the epithelial repair mechanism, whereas immune cells such as neutrophils normally contribute to airway host defense at the site of injury. Although epithelial recovery and resolution of inflammation would normally occur, the repetitive injury caused by cigarette smoking will prevent this from happening. This may help to explain chronic neutrophilic inflammation in smokers, and its contribution to COPD development. Sustained expression of IL-8 in regenerating airway epithelium, is supported by previous studies showing elevated expression of IL-8 in a xenograft model (41). Also in **Chapter 6** (5), we observed enhanced expression of IL-8 in CS-exposed airway epithelial cells with an impaired barrier integrity. In addition, we have shown in **Chapter 5** that IL-8 expression is elevated upon chronic exposure of airway epithelial cells during cell differentiation. This furthermore suggests a relation between an injured or repairing state of the airway epithelium and expression of pro-inflammatory cytokines.

In addition to the role of epithelial repair mechanisms, inflammatory responses may also result from a loss of anti-inflammatory mediators expressed by the luminal airway epithelium, i.e. SLPI and CC10/CC16 (42, 43). These secreted factors may normally act in intact epithelial layers as inhibitory signals to maintain airway homeostasis, while loss of these mediators upon luminal cell depletion may act as a negative signal to promote inflammatory responses in BCs, but also stromal and immune cells. Similar to SLPI and CC10, WFDC12 described in **Chapter 9** may also act as such an anti-inflammatory mediator, based on its high expression in differentiated luminal cells. However, further research is needed to evaluate this.

We did not detect differences in CS- and microbial-induced inflammatory mediator expression between COPD and non-COPD airway epithelial cultures (44). Since these cultures were derived from current and former smokers, it needs to be noted that we did not compare these cultures to those of healthy never-smokers, and possibly the induction of these mediators is lower in healthy airway epithelium. Moreover, it possible that in contrast to differentiated cells, enhanced inflammatory responses may persist in undifferentiated cultures. This was shown in a previous study by *Schulz et al.* (45), which observed enhanced inflammatory mediator expression of undifferentiated airway epithelial cells from COPD patients compared to non-COPD smokers. Similar findings have also been reported in studies comparing airway epithelial cultures from CF patients and non-CF controls, whereas it was shown that enhanced inflammatory responses were only seen in undifferentiated CF airway epithelial cells (46, 47). This raises the interesting possibility that especially in injured epithelium, dominated by basal cells, differences between epithelium from patients and controls becomes apparent. Therefore, further studies are required, in which the importance of airway epithelial repair and differentiation status will be determined in the expression of inflammatory mediators in COPD cultures, as this will further reflect the importance of injured on airway epithelial inflammatory responses.

Role of CS-induced oxidative stress in inflammation

Reactive oxygen species present in smoke, and induction of oxidative stress may also contribute to airway inflammation upon smoking and in COPD. CS-induced oxidative stress causes a delay in epithelial repair as shown in **Chapter 6** (5) and therefore may lead to sustained inflammatory responses mediated by repairing signals. Moreover, oxidative stress

can increase MAPK signaling, thereby enhancing the expression of inflammatory mediators via these pathways. As speculated in **Chapter 7**, oxidative stress may furthermore control the translation of inflammatory mediators through induction of GADD34. In previous studies, it has been shown that GADD34 controls protein translation of IL-6 and interferons upon viral infection by temporary suppression of the integrated stress response (48, 49). Similar to this, CSC-induced oxidative stress may promote the translation of pro-inflammatory cytokines in airway epithelial cells via MAPK p38-dependent expression of GADD34. This mechanism may explain our recent findings in which airway inflammation is reduced in CS-exposed GADD34 deficient animals (unpublished data). However, further research is needed to confirm the contribution of GADD34 in regulating airway epithelial inflammatory responses after cigarette smoke exposure.

AIRWAY EPITHELIAL REMODELING

The airway epithelium is distinct from other mucosal tissues by its low steady-state turn-over in the lungs of healthy individuals (50). This is reflected by the quiescent state of BCs, which act as the main progenitor cells. Observations made in airway tissues from smokers, demonstrated that BCs have an enhanced turn-over (51), and it is hypothesized that the renewal capacity of airway BCs is limited (52). Smoking may therefore accelerate the aging of the airway epithelial tissues (53), as impaired regenerative capacity of BCs may underlie epithelial remodeling in smokers and COPD.

As mentioned in previous sections, EGFR has a prominent role in airway epithelial responses upon CS exposure. EGFR expression is largely restricted to BCs and plays a key role in wound repair (37, 54). Different ligands can activate EGFR, i.e. epidermal growth factor (EGF), amphiregulin (AREG), transforming growth factor-alpha (TGFα), and ligand-dependent activation of EGFR has been shown in recent studies to have a prominent role in airway epithelial remodeling. EGF and AREG are both enhanced in the airway epithelium of smokers, however the cells that express the ligands are different. EGF is mainly expressed by ciliated airway epithelium, whereas AREG is mainly expressed in BCs (37, 55). It has been shown *in vitro* that AREG and EGF are both critical in affecting airway epithelial remodeling. EGF is released by cultured epithelial cells upon exposure to CS extract, moreover exogenous stimulation of airway epithelial cells with EGF during differentiation causes a squamous cell phenotype (37). We have demonstrated in **Chapter 8** (5) that CS exposure of airway epithelial cells enhanced mRNA expression and ADAM17-dependent shedding of AREG protein, which was more prominent in COPD epithelial cultures. This enhanced release of AREG may further contribute to epithelial remodeling, as it has been shown by that AREG released by BCs, together with EGF, may affect epithelial differentiation by promoting basal cell hyperplasia and goblet cell metaplasia (55). These observations made *in vitro* were supported by a transcriptome analysis, demonstrating EGFR-dependent remodeling of the small airways of smokers and COPD patients resulting in a more proximal airway epithelial phenotype in the distal airways(56). Despite enhanced EGFR signaling, and aberrant epithelial differentiation during chronic CS exposure, we did not observe goblet cell metaplasia in our experimental model in **Chapter 5**. This might be due to a the lack of EGF expression in the luminal airway epithelial cells, which we have not addressed. Moreover, the experimental setup of the chronic

CS exposure model could have been to restricted. In our model, we observed transient and mild injury induced by CS exposure with no differences in the epithelial barrier integrity. EGF released from luminal airway epithelial cells may only affect BCs upon extensive injury, as has been shown in the case of Neuregulin (57). Therefore, it would be interesting to determine the effect of additional epithelial injury on epithelial differentiation in the chronic CS exposure model. This could for instance be achieved by incubating cells with calcium-free medium to abrogate the epithelial barrier integrity or by mechanical wounding. In addition, we did not determine the influence of chronic cigarette smoke exposure on airway epithelial cell cultures that were well-differentiated. This may lead to different alterations in epithelial differentiation compared to chronic smoke exposure in undifferentiated cells, and therefore requires further study.

Microbial products have also been shown to contribute to goblet cell metaplasia, and these were absent in our model which may help to explain the lack of goblet cell metaplasia in our CS-exposed cultures. Another explanation for the lack of goblet cell metaplasia might be the additional contribution of immune cells in airway epithelial remodeling, which are absent in our *in vitro* system. Indeed, it has been well established that cytokines such as IL-13, IL-4 and IL-17, derived from T-lymphocytes or innate lymphoid cells, may induce goblet cell differentiation (14). However, also neutrophils, the predominant cell type attracted by smoking may contribute to epithelial remodeling. As discussed in **Chapter 2** (2), neutrophils produce the AMPs LL-37 and alpha-defensins, which have been shown to modulate airway epithelial cell responses. LL-37 has been shown to mediate transactivation of EGFR by mediating matrix metalloprotease dependent shedding of EGFR-ligands (58). The ligands responsible for this are unclear, however it is possible that LL-37-mediated EGFR activation may contribute to epithelial remodeling. In addition, expression of HNPs was associated with squamous lesions in airway tissues from smokers, and it has been shown that HNPs can induce mucin expression and promote proliferation in airway epithelial cell lines (59-61). Therefore, further research examining the influence of neutrophil-derived secreted factors, together with CS exposure, may give further insight in the mechanisms underlying epithelial remodeling in smokers and in COPD.

THE VICIOUS CIRCLE HYPOTHESIS REVISITED: ROLE OF THE AIRWAY EPITHELIUM

Collectively, the studies presented in this thesis support an important role for the modulatory effect of CS on airway epithelial function in the development and progression of COPD. It is speculated that progressive impairment of airway epithelial host defense, resulting from smoke exposure, may act as an important driver in the onset of COPD in susceptible smokers. Such a suppression of constitutive expressed AMPs in luminal airway epithelial cells together with impaired mucociliary clearance may allow early microbial colonization. Microbial outgrowth and/or changes in the airway microbiota subsequently leads to activation of microbe-induced innate immune responses. Smoking may causing an imbalance in inflammation and host defense, thereby selectively decreasing microbe-induced expression of AMPs while enhancing inflammatory responses. This may be more pronounced in COPD patients, since it was found that expression of AMPs is also reduced in absence of CS exposure. Smoking and

additional damage caused by microbes or immune cells leads to further damage to the airway epithelium. This results in extensive injury, leading to loss of luminal cells and activation of BCs. In part via EGFR, activation in BCs further promotes inflammatory responses while wound repair is delayed due to smoking. Moreover, the combined effect of smoking on EGFR-mediated repair and immune cells leads to aberrant epithelial differentiation. This process will take place repeatedly until the airway epithelial tissues are persistently altered, as seen in severe COPD patients, likely due to epigenetic imprinting.

CONCLUDING REMARKS

Collectively, the studies presented in this thesis emphasize that inflammation, host defense and epithelial repair are interconnected processes that are affected by smoking and disturbed in COPD. Furthermore, the studies suggest that in particular targeting epithelial repair in COPD may have beneficial outcomes on inflammation and host defense and may be an effective approach to halt COPD progression. Since inflammation in COPD is at least part resistant to steroid treatment (62), and steroids are known to impair wound repair and lung host defense (63, 64), this furthermore stresses the potential benefit of targeting airway epithelial repair.

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ADDENDUM

Nederlandstalige samenvatting
List of publications
Curriculum vitae
Dankwoord

NEDERLANDSTALIGE SAMENVATTING

Onze longen hebben de essentiële taak om via de ademhaling te zorgen voor de uitwisseling van zuurstof en koolstofdioxide. Deze uitwisseling van gassen is verstoord in patiënten met COPD (chronic obstructive pulmonary disease; chronische obstructieve longziekte), een longziekte die wordt gekenmerkt door blijvende schade aan de longblaasjes en luchtwegen, en vaak gepaard gaat met een ophoping van slijm in de luchtwegen. Deze beide ziektebeelden, die leiden tot een afname in de functie van de longen, worden respectievelijk emfyseem en chronische bronchitis genoemd. Roken is de voornaamste risicofactor voor de ontwikkeling van COPD. Echter, niet alle rokers ontwikkelen de ziekte, wat aanduidt dat bijkomende genetische en omgevingsfactoren een rol spelen. Naast symptomen als kortademigheid, vermoeidheid en ophoesten van slijm, wordt COPD gekenmerkt door een verhoogde gevoeligheid voor luchtweginfecties. Infecties door schadelijke bacteriën (en virussen) dragen bij aan de ontwikkeling van ontsteking en blijvende schade aan het longweefsel. Het oplopen van een luchtweginfectie kan daarnaast leiden tot een longaanval, waarbij de functie van de longen in zeer korte tijd scherp achteruitgaat en zelfs kan leiden tot de dood van een patiënt. Naast patiënten met COPD hebben ook rokers een verhoogd risico op het krijgen van een luchtweginfectie. Dit suggereert dat er een causaal verband is, waarbij roken zorgt voor een onderdrukking van afweermechanismen in de longen. Welke afweermechanismes verstoord zijn, is echter grotendeels onduidelijk. Om deze reden is fundamenteel onderzoek naar de moleculaire en cellulaire afweerprocessen in de longen en de luchtwegen van groot belang. Dit is ook belangrijk omdat het kan leiden tot nieuwe inzichten hoe infecties in COPD behandeld kunnen worden.

In gezonde longen zorgt het slijmvlies van de luchtwegen voor de voornaamste afweer tegen bacteriën. Het slijmvlies bestaat uit een laag luchtwegepitheel, dat zorgt voor het filteren van ingeademde lucht tijdens het transport naar de longblaasjes. Het luchtwegepitheel bestaat uit verschillende soorten cellen met ieder een eigen unieke functie. Secretoire epitheelcellen, bestaande uit slijmbeker (goblet)- en clubcellen, zorgen voor de aanmaak van een dunne laag slijm op het oppervlak van het epitheel. Aan dit slijm blijven bacteriën en andere ingeademde micro-organismen en deeltjes kleven, wat leidt tot het zuiveren van ingeademde lucht. Trilhaarepitheelcellen zorgen voor het verwijderen van het slijm uit de luchtwegen naar de keel, een continu "zelfreinigend" proces dat vergelijkbaar is met de werking van een lopende band. Naast de aanmaak en het verwijderen van slijm, bieden luchtwegepitheelcellen ook bescherming tegen infecties door het produceren van antimicrobiële eiwitten. Dit zijn moleculen die bacteriën doden of de groei van bacteriën remmen, en die dus kunnen worden gezien als de lichaamseigen antibiotica. Naast de aanmaak van een beperkte hoeveelheid antimicrobiële eiwitten door epitheel dat niet wordt geprikkeld, vertoont het epitheel een verhoogde productie van deze eiwitten na het herkennen van bacteriën door speciale receptoren. Deze verhoogde productie treedt voornamelijk op bij de nesteling en groei van bacteriën in de luchtwegen en deze bacteriën en andere micro-organismen zorgen ook voor de aanmaak van ontstekingsfactoren door het epitheel. De ontstekingsfactoren zorgen voor het aantrekken van afweercellen uit het bloed naar het ontstoken weefsel. Deze afweercellen voorkomen de verdere verspreiding van een luchtweginfectie naar het longweefsel, door bacteriën te doden en op te ruimen.

In intact epitheel zorgen secretoire- en trilhaarcellen voor de voornaamste verdediging door het epitheel. Ernstige schade aan het epitheel, bijvoorbeeld door roken of tijdens een infectie, kan echter leiden tot celdood van secretoire- en trilhaarcellen. Herstel van het epitheel is in dit geval cruciaal, om infecties van het onderliggende longweefsel te voorkomen. Naast secretoire- en trilhaarcellen, bestaat het epitheel ook uit een groep stamcellen. Deze cellen bevinden zich op het basaal membraan, de scheidlijn tussen het epitheel en het onderliggende bindweefsel, en worden daarom basaal cellen genoemd. In tegenstelling tot secretoire en –trilhaarcellen, kunnen basaal cellen zich vermenigvuldigen en transformeren tot nieuwe secretoire- en trilhaarcellen, aan de hand van een proces wat differentiatie genoemd wordt. In onbeschadigd epitheel zijn basaal cellen niet actief, echter bij epitheelschade zorgen ze voor herstel van de epitheel laag en tevens voor het aantrekken van afweercellen uit het bloed. Bij het herstel van het epitheel zorgen basaal cellen eerst voor het sluiten van een epitheelwond, door cel migratie en vermenigvuldiging van het aantal basaal cellen. Daarna differentieert een aantal basaal cellen tot secretoire- en trilhaarcellen.

Het luchtwegepitheel heeft dus een zeer belangrijke rol in de afweer tegen bacteriën in gezonde longen. De vraag is of onderdrukking van de functies van epitheel een rol speelt in de ontwikkeling van COPD. Eerder onderzoek heeft aangetoond dat verstoring van de aanmaak en verwijdering van slijm in de luchtwegen (vooral in de kleine luchtwegen) een belangrijke oorzaak is van de ontwikkeling van COPD door chronische bronchitis. Een verhoogde slijmproductie en afname in de trilhaarfunctie van het epitheel, die samen leiden tot de ophoping van slijm in de luchtwegen, wordt in eerste instantie geprikkeld door het roken van sigaretten. Echter na verloop van tijd zijn deze veranderingen als het ware geprogrammeerd in de epitheelcellen, wat leidt tot een blijvende ophoping van slijm in de luchtwegen. Er wordt verondersteld dat dit plaatsvindt door het aanhouden van een vicieuze cirkel van roken, infecties en longschade. In deze vicieuze cirkel zorgt slijmophoping door roken voor de nesteling en groei van bacteriën in de luchtwegen. De daaropvolgende detectie van bacteriën door receptoren op het epitheel leidt tot de productie van ontstekingsfactoren, die zorgen voor de aantrekking van afweercellen uit het bloed. Alhoewel deze cellen normaal gesproken cruciaal zijn voor de afweer tegen micro-organismes, zorgen ze tijdens chronische ontsteking voor schade aan het longweefsel. Veranderingen in de aanmaak en afvoer van slijm door het epitheel vormen dus een belangrijk mechanisme in de ontwikkeling van COPD. Het is evenwel onduidelijk hoe bacteriën de luchtwegen kunnen koloniseren, ondanks de aanwezigheid van afweercellen uit het bloed. Dit kan mogelijk verklaard worden door een verandering in de aanmaak van antimicrobiële eiwitten door het luchtwegepitheel. Of en hoe dit proces verstoord is tijdens de ontwikkeling van COPD, is grotendeels onduidelijk. In dit proefschrift is daarom onderzocht of het roken van sigaretten leidt tot een afname in de productie van antimicrobiële eiwitten door het luchtwegepitheel, en of epitheelcellen van COPD patiënten een verminderde antibacteriële activiteit hebben. Naast deze vraagstelling is onderzocht of het epitheel nog andere afweerfuncties heeft die mogelijk verstoord zijn in COPD patiënten of door het roken van sigaretten. Dit is onderzocht door verschillende vormen van afweer door het epitheel te bestuderen: i) epitheel afweerfuncties die continu actief zijn en dus voor een basale vorm van afweer zorgen, ii) een afweer na nesteling en groei van bacteriën gedurende een infectie en iii) afweer door basaal stamcellen na schade aan het epitheel.

De afweer door het luchtwegepitheel is onderzocht aan de hand van experimenten met gekweekte cellen. Naast celkweken met alleen basaal cellen, is er ook gebruik gemaakt van een kweekmodel met gedifferentieerde luchtwegepitheelcellen. Hierin worden basaal cellen gekweekt in lucht-blootgestelde condities, wat leidt tot differentiatie in secretoire en trilhaarcellen. De afweerfuncties van het gekweekte epitheel zijn bestudeerd na blootstelling aan sigarettenrook, stimulatie met bacteriën en na het beschadigen van het epitheel. Door epitheelcellen van COPD patiënten te vergelijken met het epitheel van rokers zonder COPD, probeerden we meer inzicht te krijgen in het eventuele verschil tussen deze twee groepen.
In hoofdstuk 2 is er literatuur vooronderzoek gedaan naar de rol van antimicrobiële eiwitten in de ontwikkeling van COPD. Eerdere studies wijzen erop dat het luchtwegepitheel mogelijk minder antimicrobiële eiwitten produceert door schadelijke effecten van het roken. Dit was medebepaald aan de hand van experimenten met gekweekt luchtwegepitheel, blootgesteld aan sigarettenrook. Daarnaast is in studies met luchtwegweefsels van COPD patiënten aangetoond dat ook na het stoppen met roken er een verlaagde expressie van antimicrobiële eiwitten waarneembaar was. In tegenstelling tot de productie van antimicrobiële eiwitten door het epitheel, is er een toename van vergelijkbare eiwitten geproduceerd door afweercellen uit het bloed. Dit is het gevolg van chronisch ontsteking in de luchtwegen van COPD patiënten. Het type antimicrobiële eiwitten van afweercellen uit het bloed en epitheelcellen verschilt niettemin van elkaar. Daarnaast wordt verondersteld dat de eiwitten van afweercellen uit het bloed bijdragen aan longschade in COPD. Kort samengevat, tonen eerder uitgevoerde studies dus aan dat er een verschuiving is in de balans van het type antimicrobiële eiwitten in de longen van rokers en COPD patiënten. Waar, in het geval van het epitheel, een verminderde productie mogelijk bijdraagt aan een verhoogde gevoeligheid voor infecties.

Aangezien het onduidelijk was of basaal cellen een andere afweeractiviteit vertonen ten opzichte van intact gedifferentieerd epitheel is dit in hoofdstuk 3 onderzocht. Dit onderzoek werd uitgevoerd aan de hand van een vergelijking van gekweekte basaal cellen en gedifferentieerd luchtwegepitheel, beide gestimuleerd met bacteriën. Zowel basaal cellen en gedifferentieerd luchtwegepitheelcellen vertoonden een toename in expressie van bepaalde antimicrobiële eiwitten na blootstelling aan bacteriën. Echter naast een overlap, observeerden we dat alleen basaal cellen in staat waren om een specifiek antimicrobieel eiwit te produceren. Dit eiwit, genaamd RNase 7, was in eerdere studies omschreven als een belangrijke beschermer tegen infecties van bacteriën op de huid. Naast een verhoogde expressie na bacterie stimulatie, vonden we dat RNase 7 ook geproduceerd werd door basaal cellen na schade van intact epitheel door sigarettenrook. Dit proces bleek onderdeel te zijn van het epitheelherstel proces, waarbij activatie van een bepaalde receptor, de epidermal growth factor receptor (EGFR), betrokken was. Samenvattend hebben we in deze studie aangetoond dat basaal cellen in het luchtwegepitheel, naast een stamcelfunctie, ook een mogelijke bijdrage leveren aan de afweer tegen bacteriën door de aanmaak van het antimicrobiële eiwit RNase 7.

In hoofdstuk 4 is er onderzocht of de aanmaak van antimicrobiële eiwitten na bacterie blootstelling anders was in gedifferentieerd luchtwegepitheel van COPD patiënten, vergeleken met cellen van rokers zonder COPD. Bij dit onderzoek bleek inderdaad dat epitheelcellen van COPD patiënten een verminderde genexpressie hadden van de antimicrobiële eiwitten β-defensin 2 en S100A7. Overeenkomend met dit resultaat, zagen we in andere experimenten een verminderde antibacteriële activiteit van gekweekte COPD epitheelcellen ten opzichte

van rokers zonder COPD. In zowel epitheel van COPD patiënten als van rokers, vonden we een remmende werking van sigarettenrook op de expressie van antimicrobiële eiwitten. Maar in tegenstelling tot de afname van antimicrobiële eiwitten, veroorzaakte sigarettenrook samen met bacterie blootstelling een toename in ontstekingsfactoren. Dit geeft dus aan dat het roken van sigaretten een tegengestelde werking heeft op de afweerfunctie van het epitheel, waarbij er een afname is in antibacteriële activiteit en een toename in ontsteking. Dit effect komt overeen met de toename in de gevoeligheid voor infecties, en chronische ontsteking in de luchtwegen, in zowel COPD patiënten als rokers.

Hoofdstuk 5 omschrijft een studie waar de invloed van roken onderzocht werd op de productie van antimicrobiële eiwitten tijdens de differentiatie van basaal cellen. In dit hoofdstuk tonen we eerst aan dat gedifferentieerde luchtwegepitheelcellen een bepaalde groep antimicrobiële eiwitten specifiek aanmaakt, die nauwelijks geproduceerd worden in basaal celkweken. Gedurende de differentiatie van basaal cellen onderdrukt sigarettenrook niet alleen de epitheeldifferentiatie, maar (daarmee) ook de aanmaak van deze eiwitten, wat leidt tot een verminderde antibacteriële activiteit van het epitheel. Deze studie toont dus een direct verband aan tussen de onderdrukking van de differentiatie van het luchtwegepitheel door sigarettenrook en de afweer tegen bacteriën door antimicrobiële eiwitten.

In hoofdstuk 6 wordt de invloed van sigarettenrook op het herstel het epitheel verder bestudeerd. Hier is gebruik gemaakt van epitheelschademodellen, waarin het epitheel mechanisch verwond werd, of waarbij de verbindingen tussen epitheelcellen tijdelijk verbroken werden door calcium weg te nemen. In beide modellen vonden we dat sigarettenrook zorgde voor een afname in epitheelherstel. In tegenstelling tot deze afname in het herstel, vonden we dat sigarettenrook de ontstekingsreactie en afweer door basaal cellen juist verhoogde. Dit werd veroorzaakt door oxidatieve stress, veroorzaakt door sigarettenrook. Kort samengevat toont deze studie aan dat sigarettenrook een tegengestelde werking heeft op het epitheel tijdens schade, waarin het herstel geremd wordt en ontsteking juist toeneemt.

In hoofdstuk 7 wordt de invloed van roken op activatie van de integrated stress response (ISR) in luchtwegepitheelcellen bestudeerd. ISR is een cellulair mechanisme dat mede bepaalt of een cel na het oplopen van schade in leven blijft of doodgaat. In het onderzoek vonden we dat ISR geactiveerd werd in gekweekt luchtwegepitheel na blootstelling aan sigarettenrook. Dit effect was sterker in het epitheel van COPD patiënten dan in het epitheel van rokers zonder COPD. Daarnaast vonden we een negatieve relatie tussen de expressie van bepaalde eiwitten gerelateerd aan de ISR en de longfunctie van patiënten. Deze bevindingen suggereren dat een toename van de ISR door het roken mogelijk een rol speelt in de ernst van COPD. Echter hoe dit precies in zijn werk gaat moet nog verder onderzocht worden.

In hoofdstuk 8 wordt er verder onderzocht hoe sigarettenrook ontsteking- en wondherstelresponsen door het luchtwegepitheel beïnvloedt. In dit hoofdstuk hebben we specifiek gekeken naar de werking van het eiwit ADAM17, wat betrokken is bij het vrij knippen van ontsteking- en groeifactoren die zich op het oppervlak van het epitheel bevinden. Blootstelling van luchtwegepitheelcellen aan sigarettenrook bevorderde de activiteit van ADAM17, wat leidde tot het vrij knippen van soluble IL-6 receptor (sIL-6R) en amphiregulin, respectievelijk een ontstekings- en groeifactor. De activiteit van ADAM17 was hoger in

luchtwegepitheel cellen van COPD patiënten ten opzichte van rokers zonder COPD. Daarom is het goed mogelijk dat dit mechanisme een rol speelt bij de ontwikkeling van ontsteking en verstoring van het epitheelherstel in COPD.

Naast ontstekingsfactoren produceren luchtwegepitheelcellen ook moleculen die ontsteking in de longen kunnen remmen. Hoofdstuk 9 omschrijft een studie waarin de expressie van een mogelijk nieuw ontstekingsremmend eiwit, WFDC12, onderzocht is. Basaal cellen, maar niet gedifferentieerde epitheelcellen, vertoonden een verhoogde expressie van WFDC12 na stimulatie met bacteriën. Ten opzichte van basaal cellen hebben gedifferentieerde cellen echter een intrinsiek hoge expressie van WFDC12, wat onderdrukt kon worden na stimulatie met de groeifactor TGF-β1. Aangezien voorgaande studies hebben aangetoond dat expressie van TGF-β1 verhoogd is in luchtwegepitheelcellen van COPD patiënten, is het dus mogelijk dat TGF-β1 voor een onderdrukking zorgt van WFDC12 in COPD. Dit wordt ondersteund in experimenten waarin we de expressie van WFDC12 hebben vergeleken in gekweekt luchtwegepitheel van COPD patiënten en rokers zonder COPD. Uit deze vergelijking bleek inderdaad dat de expressie van WFDC12 lager was in celkweken van COPD patiënten ten opzichte van rokers zonde COPD. Daarbij vonden we een correlatie tussen de mate van WFDC12 expressie en longfunctie. Vervolgonderzoek is nodig om te onderzoeken of de verlaagde expressie van WFDC12 bijdraagt aan ontstekingsreactie in de luchtwegen van COPD patiënten.

In hoofdstuk 10 is uiteengezet hoe een afname in de expressie en functie van antimicrobiële eiwitten in COPD en andere longziekten behandeld zouden kunnen worden. Hier wordt aandacht besteedt aan mogelijk therapieën met synthetische antimicrobiële eiwitten of daarvan afgeleide moleculen. Daarnaast wordt de mogelijkheid besproken om de productie van antimicrobiële eiwitten door cellen in de longen therapeutisch te bevorderen.
De studies in dit proefschrift hebben geleid tot nieuwe inzichten in de afweermechanismen van het luchtwegepitheel. Hierin kunnen we onderscheid maken in specifieke afweermechanismes van gedifferentieerde, secretoire en trilhaarepitheelcellen, en basaal cellen. Er is daarnaast onderzocht hoe deze mechanismen beïnvloed kunnen worden door het roken van sigaretten, of persistent aanwezig zijn in het epitheel van COPD patiënten. Sigarettenrook onderdrukt de expressie van antimicrobiële eiwitten van het gedifferentieerde epitheel. Daarnaast hebben luchtwegcellen van COPD patiënten een intrinsiek verlaagde antibacteriële activiteit vergeleken met rokers zonder COPD. In tegenstelling tot antimicrobiële eiwitten, is de expressie van ontstekingsfactoren verhoogd. Deze bevinding geeft aan dat er in het epitheel sprake is van een disbalans in afweermechanismen die bijdragen aan een verhoogde gevoeligheid voor infecties en de ontwikkeling van chronische ontsteking in de longen. Sigarettenrook zorgt naast veranderingen in de afweerreacties en verstoring van deze balans, ook voor schade aan het epitheel. Ondanks onderdrukking van epitheel herstelfuncties, is er een toename in productie van ontstekingsfactoren, mogelijk door toedoen van de basaal cellen. Deze bevindingen geven aan dat de ontwikkeling van ontsteking selectief in stand wordt gehouden tijdens schade en herstel van het luchtwegepitheel.

In navolging van ophoping van slijm, is er dus ook een onderdrukking van andere afweermechanismes door het luchtwegepitheel, die mogelijk bijdragen aan de verhoogde gevoeligheid voor bacteriële luchtweginfecties. De indirecte afweer, waarin afweercellen uit

het bloed worden aangetrokken door ontstekingsfactoren, blijft desondanks in stand. Het roken van sigaretten zorgt dus voor de ontwikkeling van de vicieuze cirkel van infecties, ontsteking en schade, waarbij het luchtwegepitheel een centrale rol speelt. Een afname in directe afweermechanismen, en een toename in productie van ontstekingsfactoren, lijken beide direct verbonden met het oplopen van schade en afwijkend herstel van het epitheel door roken. Therapieën die gericht zijn op de bevordering van het herstel van het epitheel kunnen dus mogelijk leiden tot een verlaagde gevoeligheid voor infecties en onderdrukking van ontsteking. Ter aanvulling kan de verminderde expressie van antimicrobiële eiwitten behandeld worden door therapieën met synthetische antimicrobiële eiwitten of daarvan afgeleide moleculen.

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CURRICULUM VITAE

Gimano D. Amatngalim is geboren op 22 mei 1987 te Leidschendam. Na het behalen van het VWO diploma (Veurs Lyceum, Leidschendam) startte hij in 2005 met de Bachelor opleiding Biomedische Wetenschappen aan de Universiteit Leiden. Als onderdeel van deze studie volgde hij een onderzoekstage bij de onderzoekgroep van Prof. dr. Pieter Hiemstra (afdeling Longziekten, Leids Universitair Medisch Centrum). Na het behalen van het Bachelor diploma in 2008, startte hij met de Master opleiding Biomedical Sciences aan de Universiteit Leiden. Tijdens deze opleiding werden onderzoeksstages gevolgd bij de onderzoeksgroep van Prof. dr. Bob Hancock (department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada) en Prof. dr. Peter ten Dijke (afdeling Moleculaire Celbiologie, Leids Universitair Medisch Centrum). Na het behalen van het Master diploma in 2010, startte hij onder begeleiding van Prof. dr. Pieter Hiemstra met het promotieonderzoek bij de afdeling Longziekten in het Leids Universitair Medisch Centrum. Een deel van het promotie onderzoek heeft hij uitgevoerd in de onderzoeksgroep van Prof. dr. Stefan Marciniak (Cambridge Institute for Medical Research, University of Cambridge, Verenigd Koninkrijk), gefinancierd door een short-term research fellowship van het Longfonds. Momenteel is hij werkzaam als postdoc in de onderzoeksgroep van dr. Jeffrey Beekman (afdeling Kinderlongziekten, Wilhelmina Kinderziekenhuis; Regenerative Medical Center, Universitair Medisch Centrum Utrecht).

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