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Airway epithelial innate host defence in chronic obstructive pulmonary disease

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Citation
Amatngalim, G. D. (2018, October 11). *Airway epithelial innate host defence in chronic obstructive pulmonary disease*. Retrieved from https://hdl.handle.net/1887/66122

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Cover Page

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Title: Airway epithelial innate host defence in chronic obstructive pulmonary disease
Issue Date: 2018-10-11

CHAPTER 1

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

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Chin Med J (Engl). 2018 May 5;131(9):1099-1107

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

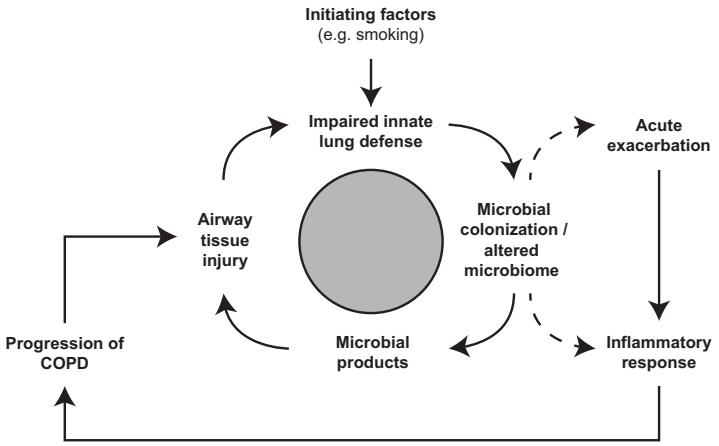


Figure 1. Vicious circle hypothesis of COPD. Model that explains the development and progression of COPD, focusing on a persistent cycle of microbial colonization and infections, inflammation and airway tissue injury. Adapted from: Mammen & Sethi, 2016 (23).

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

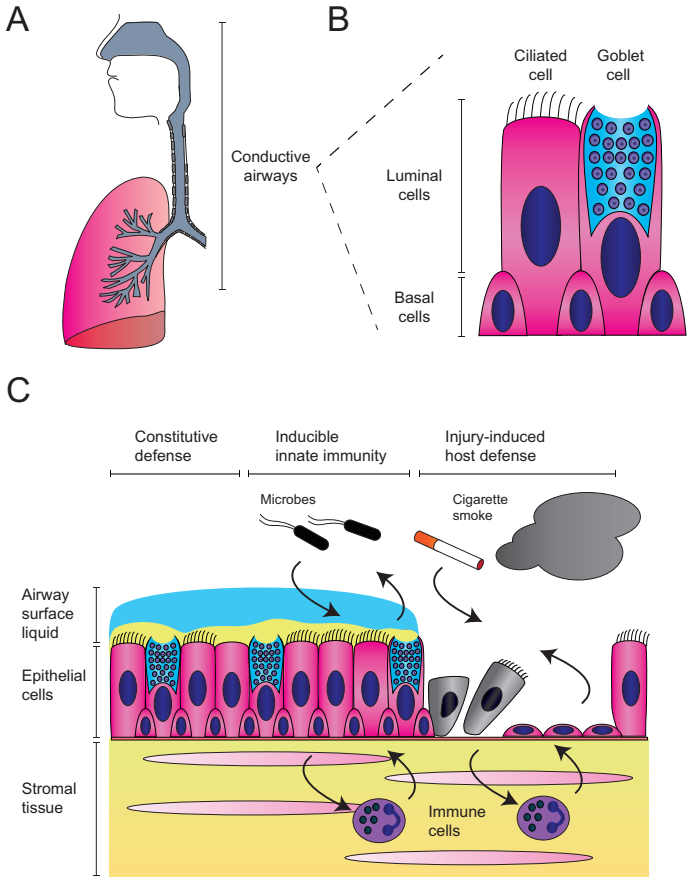


Figure 2. Schematic presentation of the airway epithelium. (A) The human respiratory tract, with the conductive airways highlighted in gray. (B) Composition of the pseudostratified airway epithelium, consisting of ciliated, secretory cells (i.e. goblet and club cells located in the upper and lower airways 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).

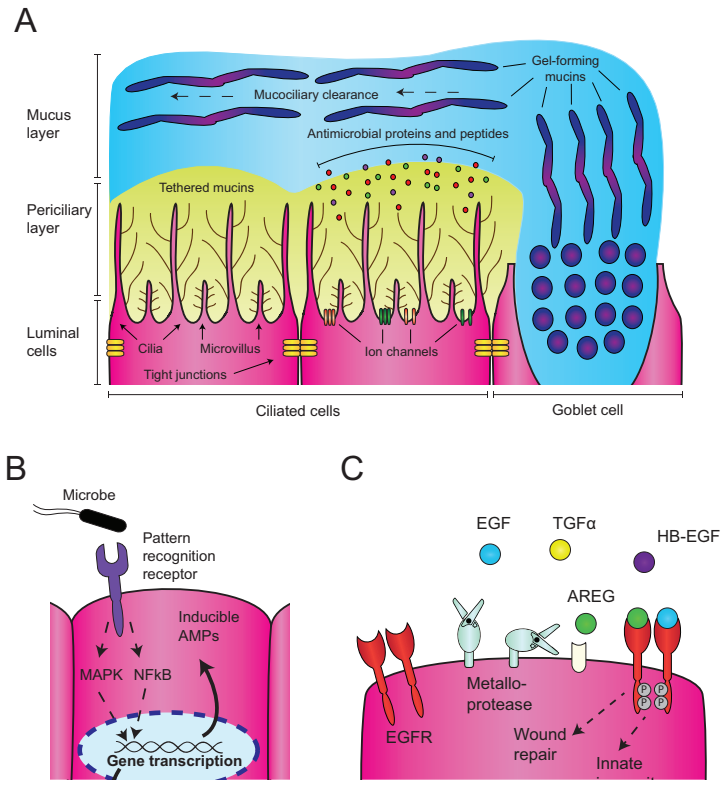


Figure 3. Airway epithelial host defense. (A) Constitutive host defense mechanisms of the luminal airway epithelium including barrier function, a cellular and tethered mucin barrier, defense via mucociliary clearance and secreted antimicrobial proteins and peptides, and regulation of airway surface liquid 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

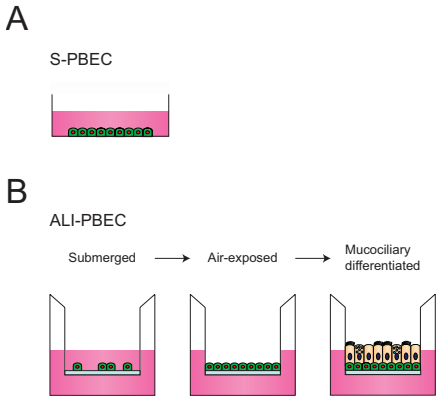


Figure 4. Airway epithelial cell cultures.
(A) Submerged cultured primary bronchial epithelial cells (S-PBEC) display an undifferentiated phenotype and resemble airway basal progenitor cells. (B) Culturing of undifferentiated cells on transwells at the air-liquid interface allows differentiation towards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

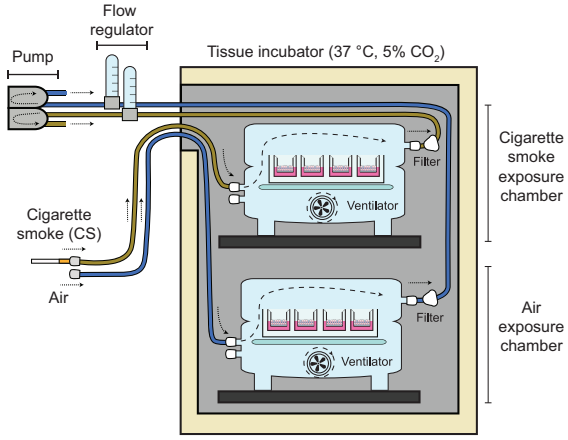
culture medium containing serum substitutes, or using a semi-defined culture medium including retinoic acid, air-exposed epithelial cells can differentiate in approx. 2-4 weeks towards a mature epithelium that includes ciliated and secretory cells (91). Differentiated ALI-cultures have been shown to 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.

A



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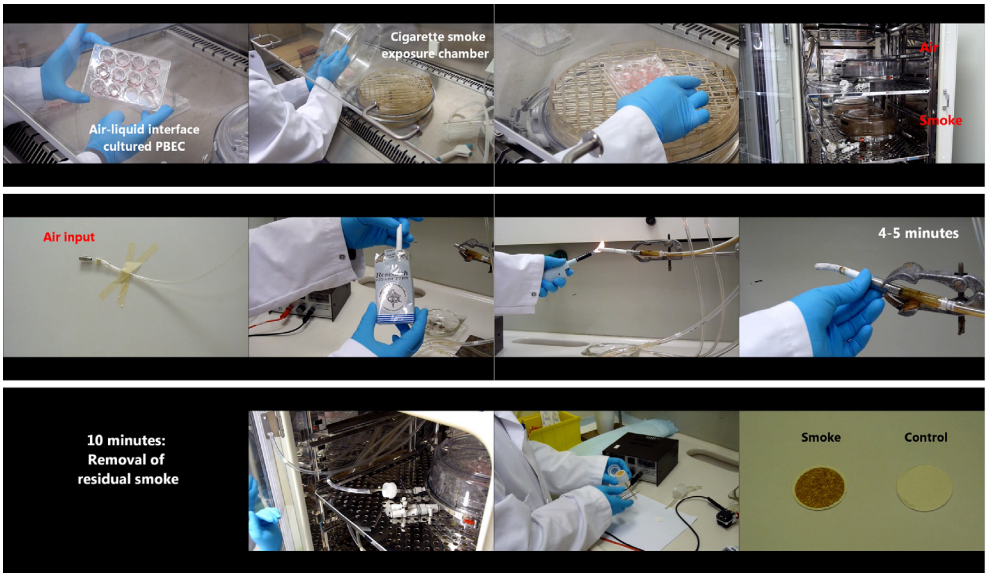

Figure 5. Whole cigarette smoke exposure model. (A) Illustration of the whole cigarette smoke exposure model setup. (B) In this model, epithelial cells are placed in modified hypoxic chambers in a tissue culture incubator at 37°C and 5% CO_2. During exposure, whole cigarette smoke (CS) derived from a 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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