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Airway epithelial responses to rhinovirus, coronavirus and cigarette smoke

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Citation

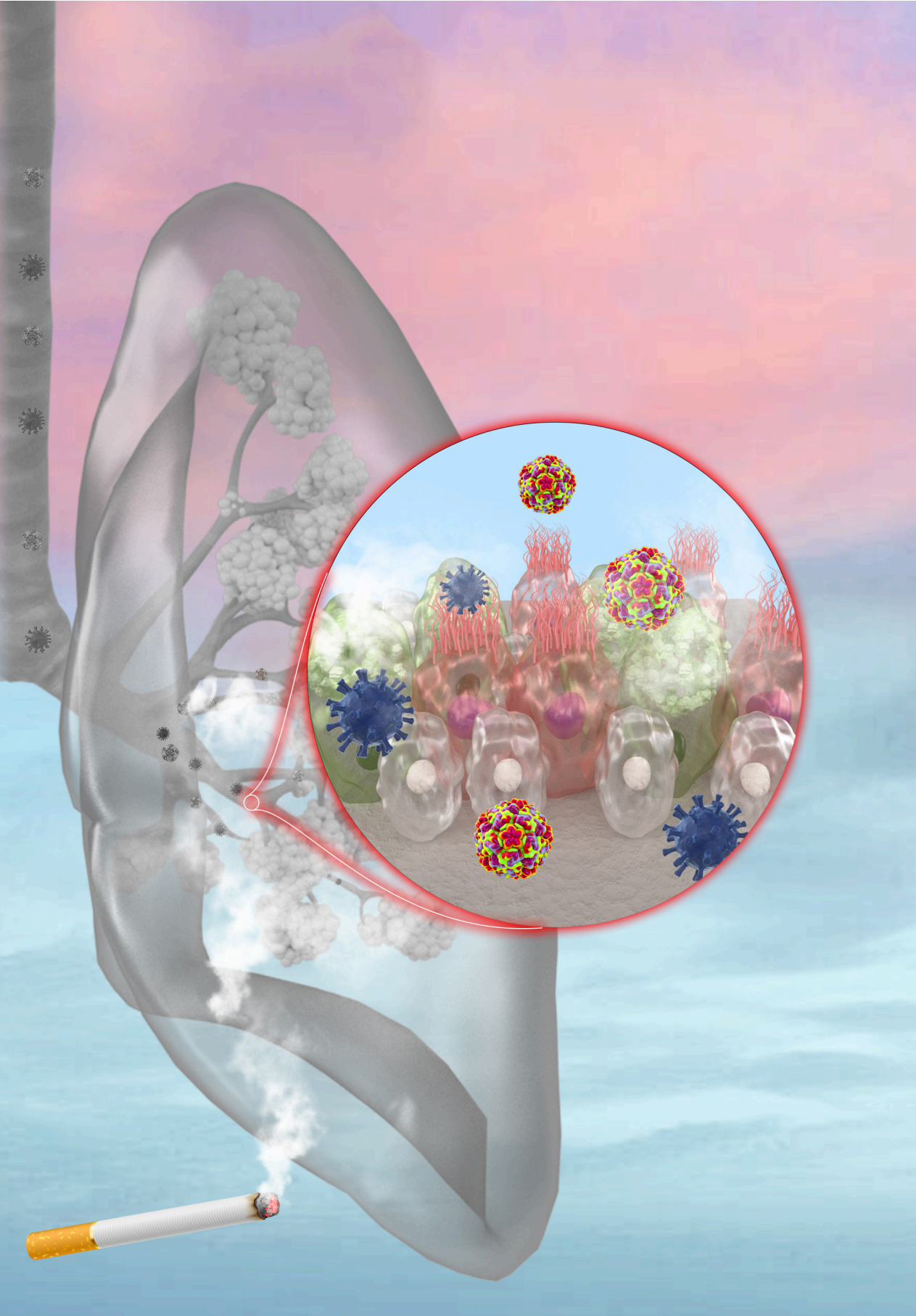
Wang, Y. (2023, January 26). *Airway epithelial responses to rhinovirus, coronavirus and cigarette smoke*. Retrieved from <https://hdl.handle.net/1887/3512925>

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Chapter 1

General introduction and outline of the thesis

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Introduction

Exposure to inhaled substances, especially pathogens, air pollution and cigarette smoke, has been linked to the development of many chronic lung diseases, including chronic respiratory diseases and lung cancer, resulting in millions of deaths globally (1, 2). Despite a variety of possible (preventive) measures and treatments, e.g. vaccination, smoking cessation and controlling air pollution, the medical and societal burden of chronic lung diseases remains high. Furthermore, the exact relation between exposure and disease development and progression is incompletely understood and may differ among individuals. Therefore, it is urgent to understand this link between exposure and respiratory health to find specific targets and biomarkers, supporting (individualized) prevention and treatment strategies.

Animal models have been widely used in basic and translational biomedical research, but ethical considerations, and increasing public and scientific concern related to the difficulty in translating results from animal models to humans, have resulted in discussions on the role of animal models in research (3). These ethical and scientific considerations have resulted in an increased interest in development and application of (human) cell or tissue culture models as an alternative to animal models. Specifically for studying inhaled exposures, there is a central role of the epithelial layer that lines the airways and alveoli, which acts as the initial responder to control and regulate host defense following exposure to inhaled agents (4). Therefore, reliable and representative epithelial models are considered essential to study the interaction between exposure to inhaled substances and the subsequent host response *in vitro*.

In what follows, I will focus on the role of cigarette smoke (CS) exposure and respiratory virus infections in the development of respiratory diseases, the central topic in this thesis, and provide an overview of the associated human airway epithelial cell culture models.

Hallmarks of inhaled insults

Inhalation of certain substances can be harmful to human health. Exposure to respiratory pathogens, including bacteria and viruses, as well as cigarette smoke and air pollutants contribute to a variety of chronic diseases. However, interactions between these exposures and the lungs are very complex, resulting in a series of both local and systemic cellular responses. Recently eight hallmarks of environmental insults were proposed that are related to the cellular and molecular mechanisms underlying these exposures: oxidative stress and inflammation, genomic alterations, epigenetic alterations, mitochondrial dysfunction, endocrine disruption, altered intercellular communication, altered microbiome communities, and impaired nervous system function (5), underscoring the complexity of the related processes.

Chapter 1

Smoking is generally considered as the most important risk factor for developing chronic lung diseases such as chronic obstructive pulmonary disease (COPD) (**Figure 1**). Increased oxidative stress and inflammation, that are among the aforementioned hallmarks, have been well documented in smoking-related respiratory diseases (6). Recent studies furthermore demonstrated a crucial role for mitochondrial dysfunction in the pathogenesis of smoking-related lung diseases (7, 8), which represents another of these hallmarks. Besides, early life exposure to cigarette smoke (CS) might affect lung development, and may increase a child's susceptibility to allergy and respiratory infections (9). (Excessive) inflammation caused by CS exposure leads to cell death, tissue damage and barrier dysfunction. Injured areas in the epithelial layer can be repaired, but abnormal repair may result in remodeling of the epithelial layer, such as an increase in the number of mucus-producing goblet cells or epithelial to mesenchymal transition (EMT), processes linked to COPD and lung cancer respectively (10). Not only smoking, but also air pollution induces excessive oxidative stress and a series of host responses (11). Based on these findings, treatment with antioxidants has been considered as a potential therapeutic strategy, but so far clinical benefits of such interventions are still a matter of debate (12). Evidently, quitting smoking or reducing exposure to CS and air pollution is the most crucial step for prevention and management of COPD, and asthma. In addition, clinical symptoms are reduced by bronchodilators [long-acting-beta-agonist (LABA) combined with a long-acting muscarinic antagonist (LAMA)] and corticosteroids, which are widely used in the treatment of COPD and asthma. However, most chronic pulmonary diseases are rather heterogenous, which increases the relevance for personalized treatment strategies.

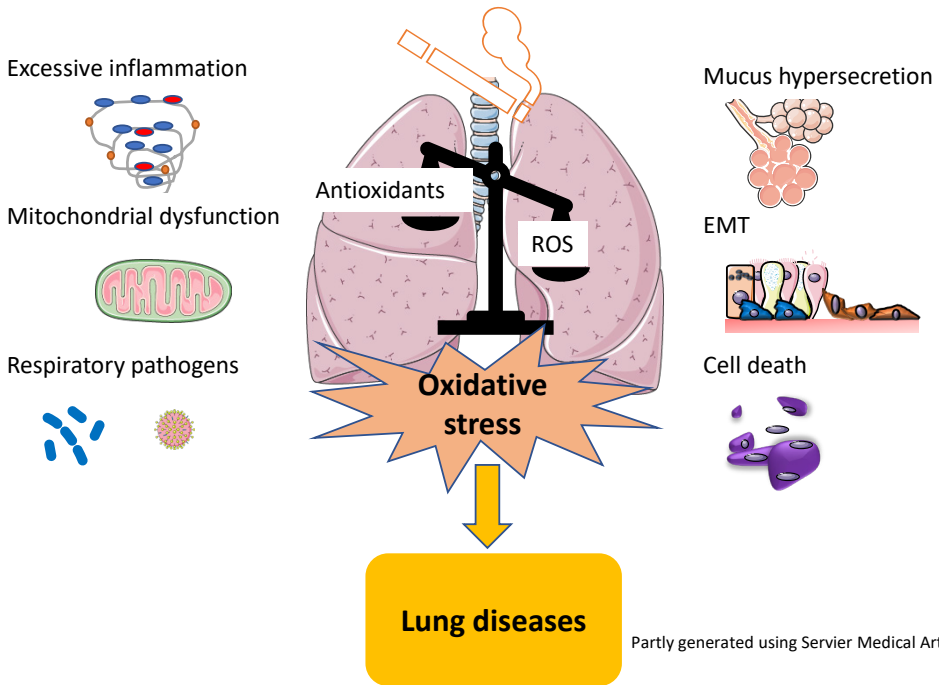


Figure 1. Cigarette smoke exposure partly drives the pathogenesis of chronic lung diseases via the regulation of oxidative stress. Oxidants and other constituents of inhaled cigarette smoke activate lung cells, including airway epithelial cells and macrophages, leading to production of endogenous reactive oxygen species (ROS) by mitochondria and by NADPH oxidases of inflammatory cells, resulting in an imbalance between oxidants and anti-oxidants. Excessive oxidative stress increases inflammation, causes mitochondrial dysfunction, increases susceptibility to pathogens, mucus hypersecretion, epithelial-mesenchymal transition (EMT) and/or cell death. Collectively, these mechanisms contribute to the development of chronic lung diseases. *The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.*

It has been widely reported that smoking increases the susceptibility to viral and bacterial infections, which further contributes to COPD exacerbations, defined as a sustained worsening of clinical symptoms (13). Rhinovirus (RV) is commonly detected during upper respiratory tract infections (URTI) and is usually associated with mild clinical symptoms. However, RV infection has also been linked to exacerbations of asthma and COPD (14-17). In addition, childhood RV infections have been associated with the risk of developing asthma (18). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the pandemic of coronavirus disease 2019 (COVID-19) is the latest newly emerging respiratory virus infection of global concern. The severity of symptoms caused by SARS-CoV-2 varies from asymptomatic to severe illness, even resulting in death (19). Variants of SARS-CoV-2 cause a wide range of disease severities in humans (20). Both infection with RV and

Chapter 1

SARS-CoV-2, as well as with other respiratory viruses, includes a series of events, such as host entry of viruses, viral replication and spread, initiation of subsequent immune responses and tissue damage, that are described in the next sections.

Pathogenesis of respiratory virus infections

The initiation of the infectious process starts with cellular entry of the particular virus following their attachment, for which the virus uses specific proteins and/or lipids. RV binds to host cells using capsid surface-exposed VP1, VP2 and VP3, depending on the RV type (RV-A, RV-B or RV-C) (21). Rhinoviruses are classified into *Rhinovirus A-Rhinovirus C* species while serotypes with *Rhinovirus A* and *B* were further specified into major and minor groups based on what cell receptor they bind(22). The RV major group viruses, including serotypes of all *Rhinovirus B* and 68 serotypes of *Rhinovirus A*, bind to the intercellular adhesion molecule 1 (ICAM-1) receptor, while the low-density lipoprotein receptor (LDLR) family is the target of RV minor group viruses (serotypes from the rest of *Rhinovirus A*) (23). The more recently identified *Rhinovirus C* targets *cadherin-related family member 3 (CDHR3)* as a cellular receptor (24). For SARS-CoV-2, the Spike (S) protein is largely responsible for initiating its entry into host cells. The S protein of SARS-CoV-2 consists of two subunits, S1 and S2; during the process of virus entry (25), the receptor-binding domain (RBD) of the S1 subunit binds to the cellular receptor, angiotensin-converting enzyme 2 (ACE2), and the S2 subunit not only anchors S protein to membrane but also mediates membrane fusion. Cleavage of the S protein enables mediate membrane fusion and is generally mediated by cell surface proteases, including transmembrane serine protease 2 (TMPRSS2) in the plasma membrane and cathepsins in the endosome (26). Ultimately, a fusion pore is created to deliver the viral genome into the cell cytoplasm.

Once inside cells, the respiratory viruses start the complex replication cycle in the host cells. As an example of SARS-CoV-2 replication (27), the translation starts following dissociation of nucleocapsid (N) protein from the viral genome and formation of polyproteins (pp1a and ppa1b) (27). These polyproteins are further processed into non-structural proteins via viral proteases and form replication organelles which consist of double-membrane vesicles (DMVs) derived from the endoplasmic reticulum (ER). Here, replication begins with synthesis of full-length negative-strand RNA and subgenomic (sg) RNA. Newly synthesized virus RNA and sgRNA escape from the organelles to form accessory proteins and reach the ER-Golgi intermediate compartment (ERGIC) for virion assembly. Finally, the formed progeny virions present in the lumen of ERGIC are released from the host cells.

Within the respiratory tract, respiratory viruses either remain localized in the upper respiratory tract, or spread from there to the lower respiratory tract including the airways and alveoli (28), which generally causes more severe clinical symptoms. In some cases, virus-induced lung injury allows viruses to invade into the bloodstream, which further accelerates dissemination of virus to other organs (28). For instance,

SARS-CoV-2 is not only found in the respiratory (and to a lesser extent the gastrointestinal) tract, but can also be found in multiple other organs, including the heart and kidney (25). Possibly, SARS-CoV-2 dissemination to the systemic compartment is mediated via monocytes, macrophages, and vascular endothelial cells present at the blood-air barrier in the gas exchange region of the lungs (29).

The immune system plays a pivotal role in the fight against respiratory viruses, and comprises the innate and adaptive immune system (**Figure 2**). RV and SARS-CoV-2 can be recognized by host cells using pattern recognition receptors (PRRs) present in membrane-bound form in the endosomes of cells, like toll-like-receptor (TLR) 3, 7 and 8, or in the cytosol, as is the case for retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5). These membrane-bound or cytosolic PRRs trigger various pathways to activate a series of antiviral and proinflammatory responses in host cells (30-32). This may restrict viral replication in the affected cells and/or recruit and activate immune cells. However, excessive recruitment of immune cells and related inflammation might lead to tissue damage and organ failure (33). RV infection in the upper respiratory tract does not cause severe local tissue damage, but upon spread to the lower respiratory tract, it may cause lower airway infection and inflammation, and even diffuse alveolar injury (34, 35). Damage to tissue and organs has been well documented in COVID-19 patients. Pulmonary injury, especially diffuse alveolar damage (DAD), including alveolar epithelial cell injury, is mostly detected in lung tissues of patients with severe COVID-19 (36). Besides lung injury and impairment of lung function, also acute kidney injury (37), failure of heart and liver (29), and neurologic manifestations (38) are found in critically ill COVID-19 patients. Altogether, immune responses control the process of virus infection, but may also result in extensive tissue injury contributing to the severity of the disease.

Besides the innate immune system, adaptive immune responses (antibodies and cell-mediated immune responses) mediated by B and T cells are also involved in defences against respiratory virus infections. B cells produce virus-specific antibodies, including those of the IgA, IgG and IgM class, whereas T cells can recognize the viral antigens presented by MHC class I and II, activating cytotoxic CD8⁺ and helper or regulatory CD4⁺ T cells respectively (39).

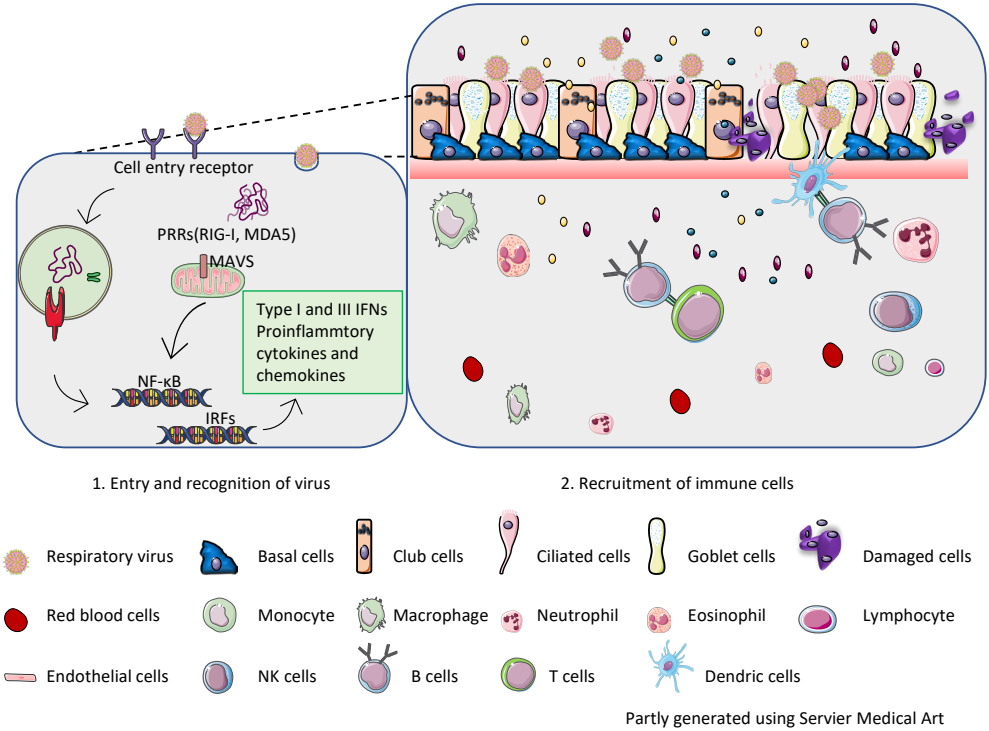
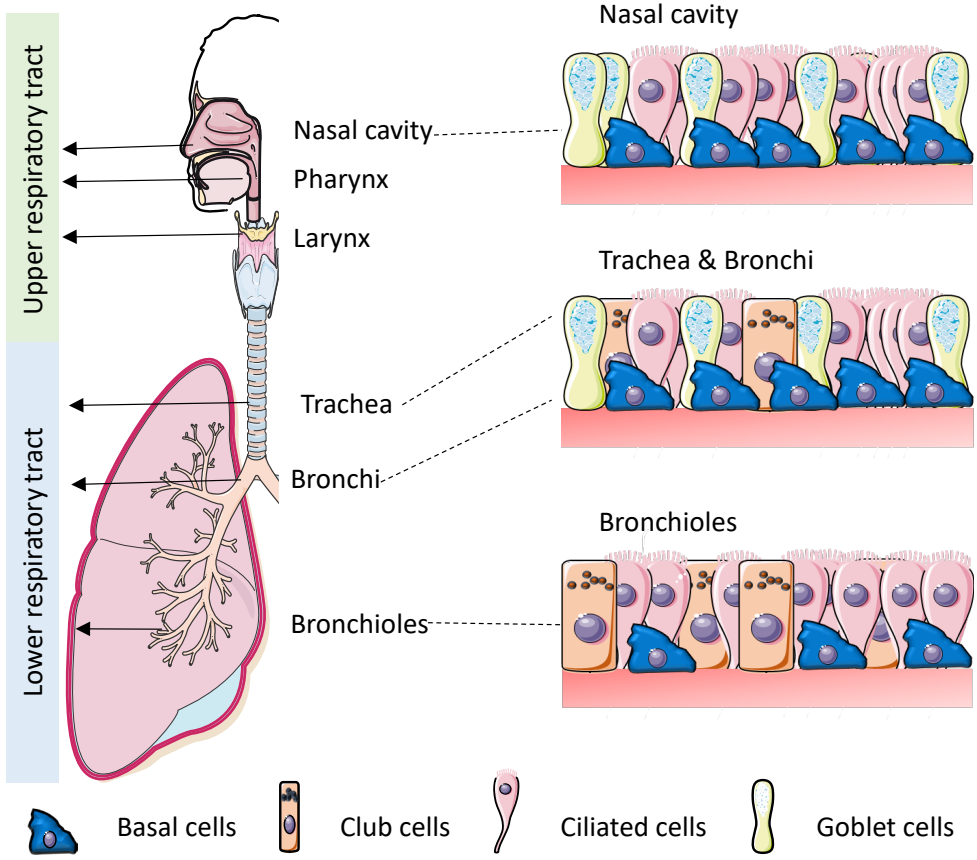


Figure 2. Overview of immune responses following respiratory virus infection of airway epithelial cells. 1. Entry and recognition of the virus: The initiation of the infectious process starts with entry of viruses into host cells (mainly respiratory epithelial cells). Respiratory viruses generally bind to receptors. After entry, respiratory viruses can be detected by pattern recognition receptors (PRRs) including membrane-bound toll-like-receptor (TLR) 3, 7 and 8, as well as cytosolic receptors, i.e. retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5). Once viruses are recognized, a series of responses are induced, such production of type I and III interferons and inflammatory mediators via activating interferon regulatory factors (IRFs) and NF-κB. 2. Recruitment of immune cells: airway epithelial cells activate immune cells, such as dendritic cells, natural killer (NK) cells, neutrophils and macrophages, which in response produce interferons, proinflammatory cytokines and chemokines. In the late stages of virus infection, airway epithelial cells together with innate immune cells recruit and regulate adaptive immune cells, resulting in production of virus-specific antibodies by B cell-derived plasma cells and activation of T cells. If this initial protective response overshoots, it may result in tissue damage. *The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.*

Whereas the pathogenesis of infection caused by many respiratory viruses shows substantial overlap, there are also marked differences that might explain the virus-specific unique clinical symptoms and disease development. Therefore, understanding the pathogenesis of individual viruses is essential for prevention, diagnosis and treatment.

The human airway epithelium

The human airway epithelium is the primary target for most respiratory viruses. It is composed of a combination of specialized epithelial cells that line the upper and lower respiratory tract and that are present in a pseudostratified layer in the major part of the airways (except for the smallest airways and the alveoli) (**Figure 3**). All major cell types have their own functions to act combinedly as immune sentinels against inhaled insults. The major cell-types include ciliated cells, mucus-secreting goblet cells, club cells and basal cells. More recently, a wider variety of additional, less abundant airway epithelial cell types were identified, such as ionocytes, tuft cells and neuroendocrine cells (40). In normal conditions, ciliated cells use cilia to remove external particles that are entrapped in mucus secreted by goblet cells, a process called mucociliary clearance. Club cells, previously named Clara cells, are multifunctional cells that secrete a variety of products amongst others, including club cell 10-kDa protein (CC-10), to protect the bronchiolar lining. Basal cells are identified as progenitor cells that have the capacity to self-renew and differentiate into other epithelial cell types. The proportion of each cell type in the epithelial layer varies according to the anatomical region of the lung (41). For instance, club cells are found especially in small airways but goblet cells are more abundant in larger airways. However, when exposed to inhaled insults or inflammatory processes, the airway epithelium can be damaged and the percentage of each cell type can also be changed. In smokers and COPD patients, typical airway epithelial changes occur depicted as goblet cell hyperplasia, probably triggered by smoking (42). In addition, transient epithelial cell types have been described, such as mucous ciliated cells, that are more abundant in asthma patients compared to healthy individuals (43). Also RV and SARS-CoV-2 infection have been shown to contribute to changes in epithelial cell composition, and furthermore increase mucin production and impair cilia function *in vitro* (44, 45). For example, single-cell RNA sequencing analysis identified differences in epithelial cellular composition of for instance the human nasopharyngeal mucosa between COVID-19 patients and controls (46). Since enhanced mucus is generally found in chronic airway diseases, blockade of mucus hypersecretion has been suggested as a potential therapy strategy (47). On the other hand, mucus is needed for mucociliary clearance. Therefore, optimizing mucociliary clearance by balancing mucus production, secretion and composition remains a challenge.



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Figure 3. Human airway epithelium lining the respiratory tract. The respiratory tract is divided into the upper (the nasal cavity, pharynx and larynx) and lower (trachea, bronchi, bronchioles and alveoli) respiratory tract. Human airway epithelial cells, primarily including basal cells, club cells, ciliated cells and goblet cells, constitute the airway epithelium that lines the upper and lower airways of the respiratory tract. *The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.*

Modelling lung tissue in vitro

Human airway epithelial cells can be isolated and cultured *in vitro* to generate a fully differentiated airway epithelium resembling that of the human lung. Nasal epithelial cells can be isolated from minimally invasive nasal scraping or brushing, whereas tracheal/bronchial epithelial cells are isolated from lung tissue derived from transplant programs, surgery or bronchoscopy (48). In the past decades, we have witnessed significant progress in the development of *in vitro* respiratory cell models, including air-liquid interface (ALI) static culture models, organ-on-chips,

3D organoids and precision-cut lung slices. Furthermore, the use of traditional immortalized or tumor cell lines has been gradually replaced by use of more representative primary cell cultures, or cell cultures derived from differentiated human induced pluripotent stem cells (hiPSC). Here, I will mainly introduce ALI-based primary airway epithelial cell models that are at the core of the research presented in this thesis. The other models are discussed in Chapter 7.

When primary airway epithelial cells are cultured on cell culture inserts and the apical side of the cultured epithelium is exposed to air, basal cells differentiate into luminal cells and form a typical pseudostratified structure. Importantly, this ALI-culture also allows exposure to airborne substances, relevant for studying responses to inhaled substances. Therefore, this ALI culture model was used to investigate changes in host defense and airway epithelial differentiation following CS exposure (49, 50). Furthermore, this cell culture model has also been widely employed in studies of respiratory virus infections to study e.g. cell tropism and virus-induced changes in cellular composition and functions. So far the model has been successfully used to identify airway epithelial target cells and receptors for RV and SARS-CoV-2 infections (51-53). Functional consequences of such infections in ALI cultures were found to include a decrease in barrier function (54, 55), loss of cilia (55, 56), and an increase in mucus secretion (45, 57) and pro-inflammatory mediators (58). In addition to providing opportunities to mimic the cellular composition and function of the human airway epithelium in health and diseases (as opposed to immortalized or tumour cell lines), detailed investigations into the contribution of e.g. single gene products are now also feasible in ALI models by the application of gene-editing technologies (59).

Outline of this thesis

The overall aim of the research presented in this thesis is to clarify the complex interactions between inhaled insults, including cigarette smoke and respiratory viruses (rhinovirus and SARS-CoV-2), and the human respiratory epithelium. In this thesis we employed primary airway epithelial cells cultured at ALI as the main cell culture model. Effects of CS exposure (**Chapter 2 and 3**), RV infection (**Chapter 3 and 4**) and SARS-CoV-2 infection (**Chapter 5 and 6**) on several epithelial functions, including mitochondrial (dys)function, host defense and mucin production, were investigated using this model.

In **Chapter 2**, we focused on CS-induced mitochondrial dysfunction in primary bronchial epithelial cells (PBEC) cultured at the ALI (ALI-PBEC) by assessing a comprehensive panel of key regulatory molecules involved in mitochondrial functions. Notably, multiple *in vitro* models of CS exposure were employed in this study, including submerged/undifferentiated and ALI/well-differentiated cultures, which were exposed to a soluble CS extract or whole CS (WCS) composed of gaseous components and particles. This way, we aimed to compare differences

Chapter 1

and similarities between the effect of CS on the regulation of mitochondrial metabolism in the various epithelial culture and exposure models. In **Chapter 3**, we investigated effects of WCS exposure and RV-A16 infection in ALI-PBEC using an unbiased RNA sequencing analysis to identify unique transcriptional responses of ALI-PBEC upon individual or combined WCS and RV-A16 exposure. In **Chapter 4**, we provided new insights into the regulation of mucin production and goblet cell formation following RV-A16 infection combined with drug treatment commonly used for reducing clinical symptoms of COPD and asthma patients, with a focus on tiotropium bromide and fluticasone propionate. **Chapter 5 and 6** illustrate how the pandemic shifted our research focus to investigate SARS-CoV-2 pathogenicity. Using our culture models, we aimed to characterize virus spread, localization, epithelial responses, and expression of SARS-CoV-2 (co-)receptors, as well the link between cellular composition and infection *in vitro* (**Chapter 5**). In **Chapter 6**, we sought to find the differences and similarities in the transcriptional responses of well-differentiated primary airway epithelial cell cultures in parallel infected with four different coronaviruses, including highly pathogenic SARS-CoV, SARS-CoV-2 and MERS-CoV and low pathogenic HCoV-229E. Finally, in **Chapter 7**, the main findings of the studies described in this thesis are summarized and discussed.

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Chapter 1

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Chapter 1

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