Airway epithelial responses to rhinovirus, coronavirus and cigarette smoke

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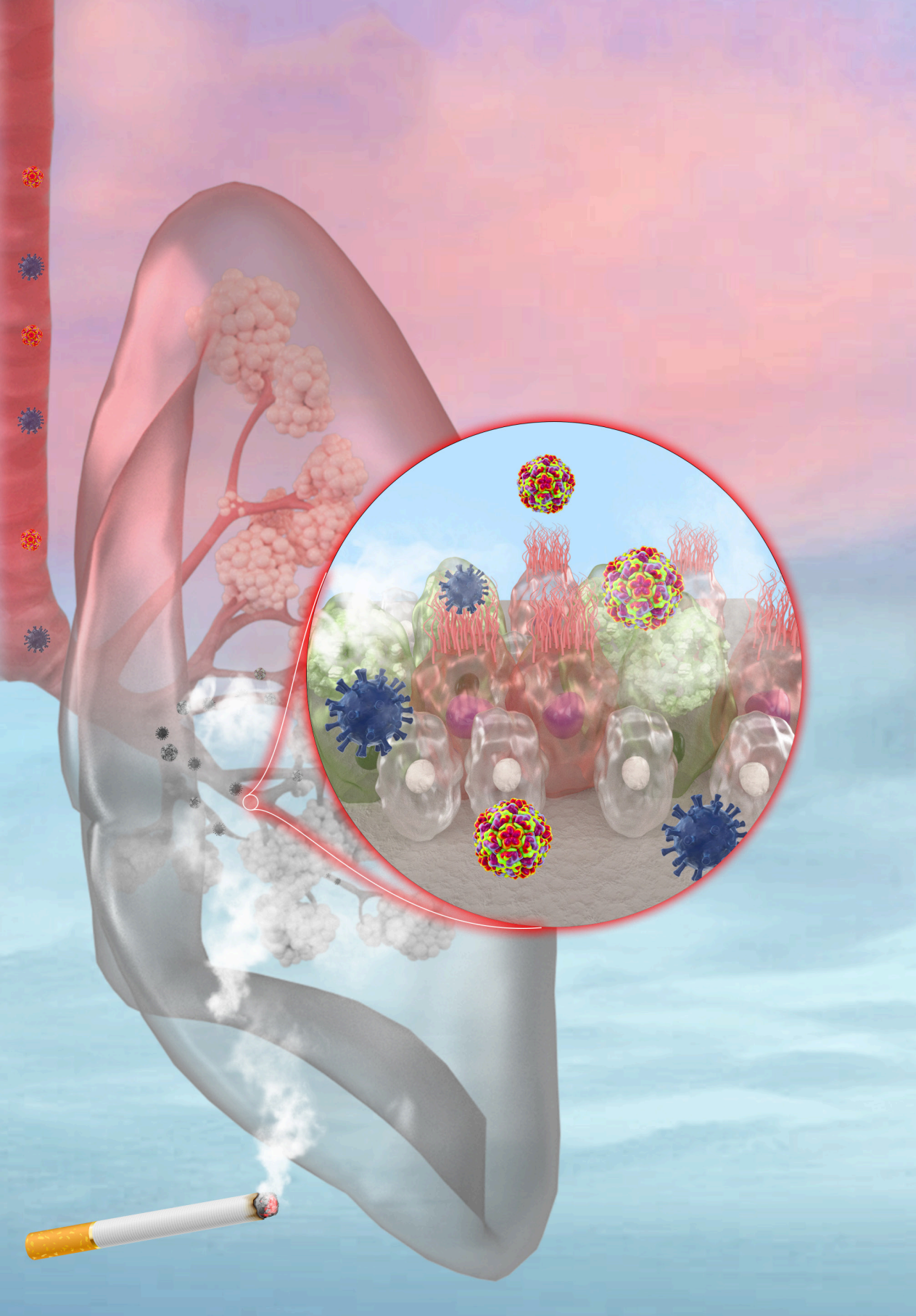

Chapter 7

Summary and general discussion

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Introduction

In this thesis, effects of inhaled toxicants and pathogens on human airway epithelium were investigated by exposing primary human (differentiated) airway epithelial cell cultures to cigarette smoke (CS) and respiratory viruses. These studies were performed to gain a better understanding of the roles of such epithelial responses in the development of chronic pulmonary diseases, and especially in acute exacerbations of asthma and chronic obstructive pulmonary disease (COPD), as well as in infections caused by SARS-CoV-2, the causative agent of COVID-19. The findings in this thesis provide insights into epithelial changes induced by such exposures, including production of inflammatory mediators, antiviral responses, oxidative stress, mitochondrial dysfunction, and epithelial remodelling. Understanding such responses is important, because so far there are limited options for therapeutic intervention that control pulmonary infections during acute exacerbations of chronic lung diseases. Interpersonal variability of clinical manifestation in patients with chronic lung diseases further hinders diagnosis of disease processes and development of targeted therapies. Therefore, understanding these cellular mechanisms may help discover new targets and biomarkers, supporting the identification of specific patients and contribute to individualized targeted therapies. In the first part of this chapter, the main findings of this thesis are summarized, followed by a discussion on how these findings could be used to support development of possible targeted therapies and how use of experimental animal-free *in vitro* lung models can further aid in the identification of therapeutic targets.

Overview of the main findings in this thesis

The human airway epithelium lining the respiratory tract plays an important role in protection against inhaled pathogens and toxicants (1, 2). Airway epithelial cells of the conducting airways do not only act as a physical barrier against such exposures, but also provide host defense by for instance mounting antimicrobial, inflammatory and immune responses (3). These defenses are often dysregulated in patients with chronic lung diseases, but how and why this occurs is not fully understood. To further understand the normal epithelial response and its dysregulation in diseases, we used cultured differentiated human airway epithelial cells as a model and studied the effects of variable exposures on the airway epithelium.

One of these exposures is active or passive smoking, which has been identified to increase the risk of lung cancer and other lung diseases like COPD, causing millions of deaths each year globally. However, the World Health Organization (WHO) predicts that only 4% of smokers will successfully quit smoking without professional support including cessation medication, due to the presence of addictive nicotine in tobacco products (as well as in electronic cigarettes). So far, it has therefore been proven difficult to prevent or reduce the development of smoking-related diseases,

also because successful smoking cessation does not fully normalize the risk of lung cancer, or reverse lung injury in COPD. Thus, discovering biomarkers, identification of specific therapeutic targets and understanding the cellular mechanism involved, are important steps to improve diagnosis and promote prevention and treatment of smoking-related diseases. In **Chapter 2**, we performed variable types of CS exposures of cultures with primary human bronchial epithelial cells (PBEC) (4) to mimic the effects of acute and long-term CS exposure and cessation on mitochondrial function, which is known to be dysregulated in e.g. patients with COPD. We combined this with mimicking damaged or intact airway epithelium by employing submerged or well-differentiated PBEC. We used our CS exposure cell models to investigate the expression of key molecules associated with mitochondrial function and metabolism post-CS exposure, including autophagy, mitophagy, mitochondrial biogenesis, mitochondrial contents, mitochondrial dynamics and the mitochondrial metabolic pathways. Overall, our findings demonstrate that CS exposure alters mitochondrial functions, robustly enhances autophagy and various markers of mitochondrial mitophagy and biogenesis. Comparison of such changes between multiple cell models indicated possible impacts of exposure concentration and duration, cell types and intact/impaired epithelium on mitochondrial functions, highlighting the importance of tailoring the *in vitro* model to the specific research question of – in this case - mitochondrial dysfunction. Furthermore, our findings on CS-induced autophagy that was observed in all models, align with those from previous studies that found a role for autophagy in a variety of lung diseases, including cancer, COPD and asthma (5), and support the notion that autophagy may be considered as a therapeutic target.

COPD exacerbations are characterized by an acute worsening of the patient's clinical condition, and are a major cause of disease progression and mortality. Apart from smoking, respiratory virus infections during COPD exacerbations have been associated with decreased lung function and increased susceptibility of the lung epithelium to secondary bacterial infection (6). Therefore, in **Chapter 3**, we wanted to investigate effects of CS exposure on epithelial susceptibility to rhinovirus-A16 (RV-A16) infection, by exposure of differentiated air-liquid interface (ALI) PBEC cultures to CS and RV-A16, separately and combined. In this way, we aimed to mimic virus-induced COPD exacerbations *in vitro*. Firstly, we showed that CS exposure increased susceptibility of ALI-PBEC to RV-A16 infection. Based on this finding we employed bulk RNA sequencing to analyse the responses of ALI-PBEC exposed to either CS or RV-A16 or the combination. We provide evidence that CS exposure may lead to higher susceptibility of bronchial epithelium to viral infection by altering pathways related to interferon responses, glycolysis and GDF15 signalling. Both CS exposure and RV-A16 infection were found to have their unique and combined effects on transcriptional responses of ALI-PBEC, including those related to WNT signaling, the complement system, inflammation and cell death, relating to the pathogenesis of COPD exacerbations. These results provide an explanation for the effect of CS

exposure on epithelial susceptibility to viral infection and help understand the pathogenesis of RV-triggered COPD exacerbations. In addition, this study widens the possible therapy strategies of virus-induced COPD exacerbations of inhibiting viral load by targeting interferons, glycolysis and GDF15, or targeting excessive inflammation and cell death.

In addition to exacerbations, mucus hypersecretion is also commonly observed in patients with COPD and other chronic respiratory diseases, and impairs mucociliary clearance which may contribute to small airway obstruction (7-9). Therefore in **Chapter 4**, we focused on investigating effects of RV infection on mucin production and modulation of this response by treatment with drugs used in the treatment of obstructive lung diseases (10). Both RV-A16 (major group RV) and RV-1B (minor group RV) were used to infect ALI-PBEC. The drugs we selected in this study were fluticasone propionate (inhaled corticosteroid) and tiotropium bromide (along-acting muscarinic antagonist, LAMA), which are widely used in the treatment of COPD and asthma. However, whether and how these drugs affect RV-induced mucin production in well differentiated ALI-PBEC is not clear. We showed that both RV-A16 and RV-1B infection cause excessive mucin production and secretion, partly by SPDEF-related pathways and ATP-mediated purinergic signalling, which was inhibited by pre-treatment with fluticasone propionate. In addition, treatment with tiotropium bromide was found to inhibit mucin secretion, but not its production. In contrast to these effects on RV-induced mucin production, both fluticasone propionate and tiotropium bromide did not affect viral replication. These findings give more insight into the mechanisms involved in RV-induced mucin production and help to understand the mechanism of action of fluticasone propionate and tiotropium bromide in COPD and asthma.

During this PhD project, the COVID-19 pandemic started, and therefore, as described in **Chapter 5**, we switched our focus to how severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, the causative agent of COVID-19) affects the human airway epithelium (11). Although a marked variation in clinical symptoms is generally found among COVID-19 patients, ranging from asymptomatic to severe disease and death, the detailed cellular mechanisms that explain this variation are not clear. Furthermore, a recent publication demonstrated the variation in viral infection between airway epithelium at different anatomical locations (12). Thus, we aimed to investigate whether differences in cellular composition affect viral infection at the initial phase. We used ALI-PBEC and ALI-primary human tracheal epithelial cells (PTEC) to investigate differences in anatomical locations on the susceptibility of the epithelium to viral infection. Epithelial cell cultures were differentiated for 3-5 weeks and actively skewed in cellular composition using specific compounds to investigate effects of cellular composition and cell differentiation on viral infection. Our study indicated that viral replication can be affected by changes in cellular composition, especially cells of the mucociliary system (ciliated cells, goblet cells and transient

secretory cells), differentiation status and anatomical origin of epithelial cells, which partly helps to explain variation in infectivity between individuals and between the anatomical locations.

Since, compared to other coronaviruses, infection with SARS-CoV-2 differs in transmissibility, host responses and pathogenicity, in **Chapter 6** we aimed to find identify the specific nature of the epithelial response to SARS-CoV-2 infection. To investigate this, we compared transcriptional responses of ALI-PBEC infected with the highly pathogenic SARS-CoV-2, SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), and the low pathogenic human coronavirus 229E (HCoV-229E) using RNA sequencing. In comparison with each other, we found that all coronavirus, except for SARS-CoV-2, increased expression of components related to JNK/AP-1 signalling, including *NR4A1*, *FOS* and *FOSB*. According to literature, the JNK/AP-1 signalling plays important roles in cell death, host responses to inflammatory cytokines and viral replication (13-16). Further experiments using JNK/AP-1 signalling-associated inhibitors showed that Dim-c-pphoh, a NR4A1 antagonist, decreased viral replication in Calu-3 cells infected with either SARS-CoV-2 or MERS-CoV. Furthermore, we observed that HCoV-229E induced stronger interferon responses than the more pathogenic coronaviruses, including SARS-CoV-2. These findings provide us a new insights into the pathogenicity of SARS-CoV-2.

Throughout the whole thesis, we demonstrate that exposure to inhaled substances causes complex host responses of human airway epithelium, including effects on inflammation, oxidative stress, WNT signalling, mitochondrial dysfunction and antiviral responses. Such responses are thought to contribute to development and progression of acute and chronic respiratory diseases. Evidently, prevention of airborne exposures to harmful substances is challenging, but important to prevent disease development. Thus, targeting the key events that are linked to the development of clinical symptoms is essential to improve patients' life. Based on our findings, we indicate some potential mechanisms and responses that could provide targets with therapeutic potential in the context of COPD and COVID-19. However, it is not clear to which extent these *in vitro* findings can be successfully translated into clinical application. Therefore, in what follows, I will discuss the ongoing clinical trials in which mechanisms and responses mentioned in this thesis are targeted.

Potential targets for treatment of COPD and COVID-19

Oxidative stress

The role of oxidative stress in the development of COPD is described in **Chapter 1**. In addition, increased oxidative stress also contributes to corticosteroid insensitivity in COPD and asthma patients (17). In **Chapter 2 and 3**, we observed that CS exposure

induced expression of oxidative stress-related genes and also modulated anti-oxidant gene expression, demonstrating the imbalance between oxidants and anti-oxidants triggered by CS exposure. Apart from exogenous oxidants, there are also several endogenous oxidants in the lung, such as reactive oxygen species (ROS) derived from inflammatory cells, including neutrophils, and those produced by a variety of oxidases, as well as mitochondrial ROS. Both exogenous oxidants (present in e.g. cigarette smoke and air pollutants) and endogenously produced oxidants increase inflammation, cause DNA damage and cell death, thereby driving the development of COPD. Targeting oxidative stress might therefore provide opportunities for new treatments of COPD patients (18). Oxidative stress also plays an important role in viral replication and viral mutations (19), which suggests that antioxidants may serve as antiviral agents. Preclinical studies have identified roles of antioxidants, antioxidant mimetics and associated inhibitors in reducing virus and CS-induced inflammation, lung injury and mucus hypersecretion (20). However, there are no approved drugs that effectively target oxidative stress so far. Some antioxidants are under evaluation in ongoing clinical trials for COPD and COVID-19 therapy, including thiol antioxidants (N-acetylcysteine, NAC) and NRF2 activators (Sulforaphane, Quercetin) for treatment of COPD, as well as Quercetin and vitamin C in treatment of COVID-19 (21). Despite the fact that basic and translational studies have provided clear evidence for oxidative stress as a suitable target for treatment of a variety of lung diseases, so far this has not resulted in an effective clinical treatment. Possibly current approaches do not effectively target lung-derived oxidants, because antioxidant defenses are complex and oxidants can be derived from a variety of sources, including mitochondria.

Oxidative stress may also result from ROS that are produced by mitochondria. Conversely, excessive ROS from a variety of exogenous or endogenous sources cause mitochondrial damage. In **Chapter 2**, apart from CS exposure-induced oxidative stress, mitochondrial dysfunction was also found in PBEC after CS exposure. Besides, mitochondrial dysfunction, increased oxidative stress is also found after SARS-CoV-2 and SARS-CoV infection (22, 23). Some pre-clinical studies have demonstrated inhibitory effects of mitochondrial-targeted ubiquinone (mitoQ) and mito-TEMPO on CS-induced mitochondrial dysfunction and oxidative stress-induced neutrophilic inflammation and inflammatory cytokines (24, 25). Although inhibition of mitochondrial ROS shows some benefits *in vitro* and *in vivo*, there are no mitochondria-targeted antioxidants and drugs used in clinical studies for COPD and COVID-19 yet. However, both mitoQ and SkQ1 (Visomitin) are or were used in ongoing and completed clinical trials for various indications, including aging-related diseases, chronic hepatitis C and chronic kidney disease (26). Some studies suggest to employ nanotechnology-mediated methods for targeting mitochondria in cancer therapeutics (27). Clearly, more studies are needed to investigate the impact of mitochondrial ROS and dysfunction on development of COPD and COVID-19, or on the potential of mitochondrial-targeted drugs for such diseases.

Inflammation and antiviral responses

Inflammation is the primary characteristic of COPD and COVID-19, that contributes to worsening of clinical symptoms. In **Chapter 3**, we observed that both CS and rhinovirus exposure increase gene and protein levels of inflammatory markers in ALI-PBEC. In **Chapter 5** we also found that SARS-CoV-2 infection increased inflammatory markers in ALI-PBEC. Therefore, research in exploring agents to reduce (aberrant) inflammation in this context is of importance. Anti-inflammatory drugs like corticosteroids have been used successfully in the treatment of COPD and in clinical trials related to COVID-19 to reduce inflammation. It is now recommended that systemic corticosteroids are used for hospitalized patients with COPD exacerbations (28) and multiple randomized trials confirm that systemic corticosteroids are helpful to reduce mortality in hospitalized COVID-19 patients, but no clear effect is found in non-hospitalized adults with COVID-19 (29, 30). Apart from that, recently more non-steroidal anti-inflammatory drugs (NSAIDs) have been tested. For example, clinical trials with selected monoclonal antibodies (mAb), like anti-IL-8 mAb (ABX-IL8), anti-IL-33 mAb (SAR440340) are ongoing for treatment of COPD, whereas for hospitalized patients with COVID-19 ongoing trials investigate the use of interleukin-1 receptor-associated kinases (IRAKs) inhibitor (PF-06650833) and anti-IL-6 mAb (Tocilizumab; also introduced in COVID-19 patient care in various countries) (www.clinialtrials.gov). Specifically in the field of respiratory viral infection, the role of antiviral responses in controlling viral replication and inflammation cannot be ignored. The existing therapy with subcutaneous injected IFNβ1a and IFNβ1b combined with orally hydroxychloroquine and Lopinavir/Ritonavir in clinical trials has been shown that treatment with IFNβ1a reduces time to clinical improvement (TTCI) without additional adverse events in severe COVID-19 patients (31). In **Chapter 5**, we found that there was a delayed induction of type I and III IFNs by SARS-CoV-2 in airway epithelial cells. Thus, exogenous administration of IFNs in the early phase of COVID-19 might help reducing viral load. Indeed, a phase 2, placebo-controlled randomized clinical trial has identified that treatment with pegylated interferon lambda helps reduce viral load and shorten the time of viral clearance in outpatients with COVID-19 (32). Altogether, targeting inflammation and antiviral responses, alone or in combination, shows potential for therapy of COPD (exacerbations) and COVID-19.

Other targets for therapy

Mucus hypersecretion is a key feature of many chronic respiratory diseases, causing obstruction of airways, leading to poor lung function and acute exacerbations (33). Blockage of mucin production and release can relieve clinical manifestations. In **Chapter 4**, we have demonstrated that drugs used in the treatment of COPD and asthma inhibit mucin production and secretion in rhinovirus infected epithelial cells. In this ALI-PBEC model, fluticasone propionate, an anti-inflammatory inhaled

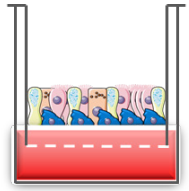
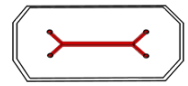
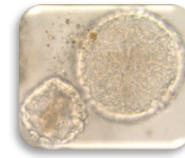
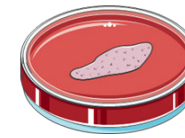
corticosteroid, decreased mucin gene expression, MUC5AC$^+$ goblet cell number and protein secretion. Furthermore, tiotropium bromide, a long-acting muscarinic antagonist (LAMA), showed an inhibitory effect on mucus hypersecretion. Apart from these drugs, additional modulators of mucin also have been tested in clinical trials, like BIBW2948 and Erdosteine (34). Unfortunately, these agents have not yet fulfilled clinical requirements. Considering the heterogeneity of chronic respiratory diseases, it will likely become essential to treat patients individually in a personalized fashion related to their specific needs. For this, more extensive characterization is needed to pinpoint differences between patients. Additional pathways could be explored to investigate their potential for therapeutic targeting. For instance, in **Chapter 6**, we illustrate a unique JAK/AP-1 signalling is not induced post SARS-CoV-2 infection compared to other coronaviruses, which might give new insights for SARS-CoV-2-target therapy.

Collectively, these studies show that whereas treatment of COPD and COVID-19 has markedly improved, there is an urgent need for clinical translation of these findings. Relevant and predictive preclinical models may help to make this translation.

The quest for animal-free respiratory virus infection platforms

The ultimate test of a novel intervention is the clinical trial and use of such interventions in regular patient care. In the past, many interventions developed in animal models of human disease, failed to make it to a successful treatment of human disease (35). To improve the translation of preclinical findings into a new treatment, it is essential to bridge the gap between *in vitro* and *in vivo* models. In this thesis, we primarily used well-differentiated airway epithelial cells cultured at the ALI as a model to study pathogenesis upon harmful exposures and related drug treatments. The platform used includes a characteristic pseudostratified mucociliary epithelial layer and provides the possibility of airborne exposures, mimicking those in the human respiratory tract. The ALI culture model and its exposure to airborne substances has been widely used in toxicology studies and in those aimed at unravelling disease pathogenesis and evaluation of novel treatments, by focusing on e.g. epithelial immune responses, changes in cellular composition and epithelial function. Despite these advantages, ALI cultures also have some limitations, including static medium conditions (opposed to microfluidic cultures), and the absence of a lung tissue-relevant microenvironment in the epithelial-only model (including lack of immune cells and other cells, and mechanical cues associated with airflow and breathing-associated stretch). Importantly, in **Chapter 2**, we showed that the type of CS-exposed epithelial cell model might affect the experimental outcomes. Therefore, it is necessary to characterize and validate the current models to understand their advantages and limitations, while simultaneously developing more complex human lung tissue-relevant model for studying exposures relevant

to chronic lung diseases with the ultimate aim to provide more reliable preclinical outcomes. This will furthermore aid in the transition from use of animal models to animal-free models. The development of more advanced patient- and disease-relevant models is essential for this transition, and during the past decades we have witnessed significant progress in cell culture models, including lung-on-chip, three dimensional (3D) organoids and precision-cut lung slices. In the following section, I will provide an overview of these advanced models (**Table 1**).

	ALI-cultures	Lung-on-chip	Lung organoids	Precision-cut lung slices
+	• Pseudostratified/ polarized epithelial layer • Optional co-culture	• Pseudostratified/ polarized epithelial layer • Microfluidic environment • Breathing-associated biomechanical forces (some models) • Optional co-culture	• Pseudostratified/ polarized epithelial layer • 3D self-assembly model • Tissue features	• 3D lung structure • Multiple cell interactions • Tissue features • Bronchoconstriction measurement
×	• No 3D or self-organization • Static culture • No breathing-associated biomechanical forces	• No 3D or self-organization • Large variety in designs • No high-throughput	• (Fluid-filled) lumen faced inside • Static culture • No breathing-associated biomechanical forces	• Short lifespan • Variation in slice size and thickness • No breathing-associated biomechanical forces

Table 1. Advantages and limitations of the advanced cell models. ALI-cultures, organs-on-chips, organoids and precision-cut lung slices each have specific advantages and limitations that are summarized in the table below the figure. For a description of the various models, see text. *The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.*

Lung-on-chip

Unlike static ALI-based cultures, micro-engineered organs-on-chips models allow culture of cells in a microfluidic environment, providing a continuous delivery of nutrients and removal of waste products, as well as – in selected models-inclusion of mechanical forces associated with breathing, such as airflow and breathing related stretch (1). Human lung-on-chip models usually incorporate adjacent air channel and medium channel (including blood vessels). Airway or alveolar epithelial cells can be seeded into one channel (together with e.g. macrophages) and the lower channel may include endothelial cells and allows infusion of other immune cells (36). Such

models have been used to investigate a variety of events, including respiratory virus infections. A human alveolus-on-chip model may contain vascular and epithelial cells and cocultured macrophages have been included to investigate the complex crosstalk between bacterial and viral pathogens and host cells (37). Recently, modelling human-to-human transmission of influenza virus infection *in vitro* has become feasible via passaging infected mucus droplets from chip to chip in human airway-on-chip model (38). In addition to studying viral infection and associated immune responses in the respiratory tract, tissue damage including lung/intestinal injury and endothelial cell damage are also investigated using human alveolus chips (39-41). Whereas respiratory pathogen-host cell interactions have been studied using organs-on-chips models, studies on smoking lung-on-chip models are limited. Benam et al. developed a small airway-on-chip connected to a "smoking" machine which allows cigarette smoke go inside and out the chip, resulting in similar molecular changes of the cultures compared to the findings in bronchoscopy samples from smokers (42). Whereas the development of organs-on-chips has progressed over the past years, most models only focus on emulating the cellular composition and organization of a single organ or tissue region, whereas mimicking the whole living organ still remains a distant aim, although attempts to create a body-on-chip are interesting next steps in the development (43). A large variety of challenges remain, including finding a suitable medium to culture all cell types and establishing a scaffold to mimic essential features of the anatomical location *in vivo.* Important developments have been reported in creating membranes on which cells can be grown, including fully biological membranes (44), curved substrates on which epithelial cells can be grown (45), development of body-on-chip models by connecting organ-chips (43), and the use of bioprinting to better mimic the airways and alveoli (46). These important developments will contribute to the development of more predictive lung-on-chip models for studying virus-host interactions.

Three-dimensional (3D) lung organoids

Organoids, 3D multicellular *in vitro* tissue mimics, are utilized to study respiratory virus infection. Self-assembly capabilities enable cells to simulate the structure and functionality of native organs, shortening the time to bypass animal studies. Recent studies identified that SARS-CoV-2 can not only infect airway organoids (47), but also alveolar organoids (48), brain organoids (49, 50), intestinal organoids (51), cardiospheres (52) and hiPSC-derived hepatic and pancreatic organoids (53). On the other hand, organoids are self-organized and self-renewing structures that could form an obstacle for respiratory viruses to efficiently enter inside lung organoids in which usually the epithelial layer is oriented towards an enclosed lumen inside the organoid. Therefore, alternative approaches are used for infecting organoids, including micro-injection into the lumen (54) or directly adding virus particles in the medium (53). However, variations in organoid size and cellular composition within

organoids complicate the reliable quantification of the number of viral particles per organoid. Studies have used several approaches to improve and better standardize the infection process. One of the methods applied has been to isolate the cells from the organoid cultures by enzymatic and/or mechanical dissociation allowing direct infection of single cells followed by reestablishment of organoid culture (48), or by adding dissociated organoids to insert cultures that can subsequently be infected (55). Another option is to reverse the orientation of the epithelial layer in the organoids inside-out resulting in the apical side being present on the outside (56). In addition, one recent report (47) used a system in which bronchial epithelial cells were infected when they were cultured at the ALI, and after infection organoid cultures were established from the infected ALI cultures. Such methods may better mimic natural respiratory virus infection and can be applied to broaden the utility of organoids. However, the typical organoids used in studies on respiratory virus infections only have epithelial cells and do not fully mimic the complexity of lung tissue, and furthermore lack the interaction with cells from other organs which could potentially be achieved by coculturing organoids derived from different anatomical sites in the future.

Precision-cut lung slices

To a large extent, precision-cut lung slices (PCLS) preserve 3D lung architecture, including maintaining relevant tissue structure and interaction among cells (such as immune cells, small airway epithelial cells and alveolar cells) (57). Therefore, PCLS are a good tool to study virus-host interaction and effects of inhaled exposure at the tissue or organ levels, and may have advantages over the use of tissue fragments obtained by non-standardized cutting. Generally, to generate PCLS (58), the freshly obtained lung tissues need to be solidified by infusion with warm low-melting agarose. Next, PCLS can be obtained using a vibrating microtome. PCLS derived from not only human lung but also from various animals (mainly mice) have been used (59-61). Specifically for use in studies on inhalation toxicology, a high-throughput system of PCLS was established by Mondonedo et al, which was used to test effects of acute cigarette smoke extract exposure under cyclic stretching conditions (62). Furthermore, to investigate SARS-CoV-2 infection, there have been recent attempts to improve the models by culturing sliced lung at the air-liquid interface in order to allow oxygenation (63). Drawbacks of the PCLS platform include the limited and variable viability of cells in culture, although methods for improved of viability upon prolonged culture (up to 20 days) have been developed using embedding in agarose as mentioned above. Furthermore, the impact of the cutting procedure on the quality and survival of cells, as well as the variation in thickness of slices between studies should be taken into account (64). Finally, the heterogeneity among tissue slices from the same donor is a cause for variation, but also an inherent advantage of the method. In summary, there is a need for further

improvement and standardization, as well as the analysis of results (including real-time imaging and deep-tissue imaging) obtained using the PCLS method (65).

Collectively, the development of these models and additional innovations (such as bioprinting, novel – decellularized - scaffolds) new models offers exciting new opportunities to improve the emulation of tissue in culture in healthy and diseased conditions. Yet, mimicking the complete human lung or even body with these models remains a challenge. In the coming years it is expected that more feasible, affordable, reproducible and realistic in vitro lung tissue models become available, which is of importance to reliably study the consequences of e.g. inhaled exposure.

Concluding remarks

Repetitive exposure to inhaled insults such as CS, respiratory viruses or air pollution, is the main factor contributing to development of various chronic respiratory diseases, lung cancer and respiratory infectious diseases. Additionally, inhalation of allergens by sensitized asthmatic patients may also result in major clinical symptoms. However, a cure for chronic respiratory diseases such as COPD or acute such as COVID-19 is not available, and the existing therapy primarily focuses on the prevention and reduction of clinical symptoms and mortality. Incomplete understanding of disease development might be one of the reasons why effective treatments are still largely lacking. Our findings in this thesis provide more insights into mechanisms affected by CS exposure as well as by RV or SARS-CoV-2 infection. These insights may contribute to the development of new therapeutic strategies for COPD and COVID-19. Furthermore, the studies presented in this thesis highlight how current *in vitro* human lung models provide a better understanding of the impact of inhaled toxicants and pathogens on lung tissue. The continuous improvement of patient- and disease-specific lung models is expected to accelerate the development and implementation of prevention and treatment strategies for lung diseases.

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