Deciphering pediatric respiratory diseases using single-cell RNA sequencing

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In loving memories!

Declaration of Independence

Herewith I certify that I have prepared and written my thesis entitled *“Deciphering pediatric respiratory diseases using single-cell RNA sequencing”* independently and that I have not used any sources and aids other than those indicated by me.

Berlin, 02.04.2024 Jennifer Loske

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List of Publications

Discussed in this thesis:

Loske J*, Röhmel J*, Lukassen S*, *et al.* Pre-activated antiviral innate immunity in the upper airways controls early SARS-CoV-2 infection in children. **Nat Biotechnol.** 2022; 40(3):319-324; doi: 10.1038/s41587-021-01037-9.

Loske J*, Völler M*, *et al*. Pharmacological improvement of CFTR function rescues airway epithelial homeostasis and host defense in children with cystic fibrosis. **Am J Respir Crit Care Med.** 2024; doi: 10.1164/rccm.202310-1836OC.

Other publications with own contributions:

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Trump S*, Lukassen S*, Anker MS*, Chua RL*, Liebig J*, Thurmann L*, Corman VM*, Binder M*, **Loske J**, *et al.* Hypertension delays viral clearance and exacerbates airway hyperinflammation in patients with COVID-19. **Nat Biotechnol.** 2021; 39: 705-716.

Magalhães VG, Lukassen S, Drechsler M, **Loske J**, *et al.* Immune-epithelial cell cross-talk enhances antiviral responsiveness to SARS-CoV-2 in children. **EMBO Rep.** 2023 Oct 11:e57912. doi: 10.15252/embr.202357912. Epub ahead of print. PMID: 37818799.

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List of Abbreviations

ACE2	Angiotensin-converting enzyme 2
APOE	Apolipoprotein E
BCAM	Basal cell adhesion molecule
CCDC	Coiled-coil domain containing
CD	Cluster of differentiation
CDC20B	Cell division cycle 20B
cDNA	Complementary DNA
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
Cl^-	Chloride ion
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus Disease 2019
CTL	Cytotoxic T cell
CXCL8	Chemokine (C-X-C motif) ligand 8
DC	Dendritic cell
DDX58	DEAD box polypeptide 58
DEG	Differentially expressed gene
DHX58	DExH box helicase 58
DNAH5	Dynein axonemal heavy chain 5
ETI	Elexacaftor/tezacaftor/ivacaftor
F508del	Mutation of *CFTR* (deletion of phenylalanine at position 508)
FCGR3B	Fc Gamma Receptor IIIb
FOX	Forkhead box
GZM	Granzyme
HCO_3^-	Bicarbonate ion
IFIH1	Interferon induced with helicase C domain 1
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factors
ISG	Interferon stimulated gene
ITGAX	Integrin subunit alpha X
JAK-STAT	Janus kinase - signal transducer and activator of transcription
KRT	Keratinocyte
LGP2	Laboratory of genetics and physiology 2
MDA5	Melanoma differentiation-associated protein 5
MERS	Middle East Respiratory Syndrome
MHC	Major histocompatibility complex
MUC	Mucin
Na^+	Sodium
PBMC	Peripheral blood mononuclear cell
PRF	Perforin
PRRs	Pathogen recognition receptors
P. aeruginosa	Pseudomonas aeruginosa
RIG-I	Retinoic acid-inducible gene I
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SCC	Sweat chloride concentration
SCGB1A1	Secretoglobin family 1A member 1
scRNA-seq	Single cell RNA-sequencing
TCR	T cell receptor
TLR	Toll-like receptor
TMPRSS2	Transmembrane protease serine subtype 2
TP63	Tumor protein p63
VCAN	Versican

Summary

The respiratory system comprises specialized cell types that maintain homeostasis and facilitate air conductance and gas exchange. Disease states often involve dysregulation of this balance, leading to changes in cell composition and molecular profiles. Single-cell RNA sequencing (scRNA-seq) is a powerful tool for investigating disease-specific transcriptional changes in individual cells, shedding light on different epithelial and immune cell types and disease-associated alterations. By using non-invasive nasal swab samples, scRNA-seq provides a promising method to study airway pathophysiology and reveal disease mechanisms in the respiratory tract.

This thesis focuses on scRNA-seq data from the upper airways of pediatric and adult patients with corona virus disease 2019 (COVID-19) and children diagnosed with cystic fibrosis (CF), alongside healthy controls, for the study of disease progression and treatment responses of these respiratory diseases.

With the emergence of COVID-19 as a pandemic, the need to understand the molecular mechanisms underlying disease severity arose, particularly with regard to age-related differences in susceptibility to severe outcomes. By comparing the upper airway cells of healthy children to those of adults, this study revealed an already activated interferon response in conjunction with higher expression levels of pattern recognition receptors in children. This pre-activated antiviral cell state may facilitate a robust and rapid innate antiviral response upon infection, which likely protects children from severe disease progression.

CF is a genetic disorder caused by mutations on the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene. CF is a life-limiting condition that manifests with excessive mucus production and inflammation, significantly impacting quality of life. The triple combination therapy elexacaftor/tezacaftor/ivacaftor (ETI) has improved CF treatment, but its effects on transcriptional changes remained unclear. This study analyzed pre- and post-therapy samples of children with CF, investigating the effects of ETI on mucosal homeostasis following pharmacologic improvement of CFTR function. The results showed an increase in the expression of genes involved in interferon signaling and major histone compatibility complex in different epithelial cell types. Furthermore, the study revealed a decrease in the inflammatory

response, specifically related to the TNF and IL1 pathways, in macrophages and neutrophils of children with CF. These results highlight the advantages of ETI treatment in reducing the burden of CF and offer additional understanding of early stages of disease progression.

By introducing scRNA-seq of nasal swab samples to investigate COVID-19 resilience in children and the efficacy of triple combination ETI in CF treatment, this thesis significantly advanced the understanding of respiratory diseases at a molecular level. The research highlights how applying scRNA-seq to study the transcriptional landscapes of COVID-19 resilience among children and the outcomes of CFTR modulator therapies in CF can elucidate the pathophysiology of diseases and the mechanisms of treatment effects. As single-cell technology continues to evolve, its application will provide deeper insights into disease complexity and therapeutic advancements.

Zusammenfassung

Das respiratorische System besteht aus spezialisierten Zelltypen, die die Homöostase aufrechterhalten und den Lufttransport sowie den Gasaustausch ermöglichen. Krankhafte Veränderungen führen oft zu einer Dysregulation dieses Gleichgewichts, was Veränderungen in der Zellzusammensetzung und den molekularen Profilen zur Folge hat. Die Einzelzell-RNA-Sequenzierung (scRNA-seq) ist ein leistungsfähiges Verfahren zur Untersuchung krankheitsspezifischer transkriptioneller Veränderungen in einzelnen Zellen. Es gibt Aufschluss über verschiedene Epithel- und Immunzelltypen sowie krankheitsassoziierte Veränderungen. Durch die Verwendung nicht-invasiver Nasenabstriche bietet scRNA-seq eine vielversprechende Methode zur Untersuchung der Pathophysiologie der Atemwege und zur Aufdeckung von Krankheitsmechanismen in den Atemwegen.

Diese Arbeit untersucht den Krankheitsverlauf und die Behandlungsreaktionen von oberen Atemwegserkrankungen bei pädiatrischen und erwachsenen Patienten mit der Coronavirus-Krankheit 2019 (COVID-19) sowie bei Kindern mit Mukoviszidose im Vergleich zu gesunden Kontrollpersonen anhand von scRNA-seq-Daten aus den oberen Atemwegen.

Mit dem Auftreten von COVID-19 als Pandemie entstand die Notwendigkeit, die molekularen Mechanismen der Krankheitsschwere zu verstehen, insbesondere hinsichtlich altersbedingter Unterschiede in der Anfälligkeit für schwere Krankheitsverläufe. Diese Studie zeigt, dass in den Zellen der oberen Atemwege von gesunden Kindern im Vergleich zu Erwachsenen bereits eine Interferonantwort aktiviert ist und höhere Expressionswerte von Mustererkennungsrezeptoren vorliegen. Dieser voraktivierte antivirale Zellzustand könnte eine schnelle und robuste angeborene antivirale Antwort auf eine Infektion ermöglichen. Dadurch könnten Kinder vor einem schweren Krankheitsverlauf geschützt werden.

Mukoviszidose ist eine genetische Erkrankung, die durch Mutationen am *Cystic Fibrosis Transmembrane Conductance Regulator* (*CFTR*)-Gen verursacht wird. Sie äußert sich durch übermäßige Schleimproduktion und Entzündungen, was die Lebensqualität signifikant beeinträchtigt und die Lebenserwartung verkürzt. Die Dreifachkombination Elexacaftor/Tezacaftor/Ivacaftor (ETI) hat die Behandlung von

Mukoviszidose erheblich verbessert. In dieser Dissertation wurden Proben von Kindern mit Mukoviszidose vor und nach Beginn der Behandlung mit ETI analysiert, um die Auswirkungen auf transkriptionelle Veränderungen zu untersuchen. Die Ergebnisse zeigen eine Zunahme der Genexpression im Interferon-Signalweg und im Haupthistokompatibilitätskomplex in verschiedenen Epithelzellen. Zudem wurde festgestellt, dass die Entzündungsantwort abnimmt, die speziell mit den TNF- und IL1-Signalwegen in Makrophagen und Neutrophilen von Kindern mit Mukoviszidose zusammenhängt. Diese Ergebnisse zeigen, dass die Behandlung mit ETI hilft, die Belastung durch CF zu verringern, und liefern weitere Erkenntnisse über den frühen Krankheitsverlauf.

Durch die Einführung von scRNA-seq von Abstrichen der Nasenschleimhaut zur Untersuchung der COVID-19-Resistenz bei Kindern sowie der Wirksamkeit der Dreifachkombination ETI in der CF-Behandlung wurde im Rahmen dieser Dissertation das Verständnis von Atemwegserkrankungen auf molekularer Ebene wesentlich verbessert. Die Forschung zeigt, wie die Anwendung von scRNA-seq zur Untersuchung der transkriptionellen Landschaften der COVID-19-Resistenz bei Kindern und der Ergebnisse von CFTR-Modulator-Therapien bei CF die Pathophysiologie von Krankheiten und die Mechanismen der Behandlungseffekte aufklären kann. Mit weiterentwickelten Einzelzellentechnologien werden tiefere Einblicke in die Komplexität von Krankheiten und therapeutische Fortschritte möglich sein.

1. Introduction

1.1. Respiratory disease

The lung plays a central role in facilitating the exchange of oxygen and carbon dioxide, making it a fundamental part of human physiology and health. Studying the complexity of the lung is critical for advancing our understanding of respiratory diseases, which were identified as the third leading cause of death in 2019 [1]. Conditions range from mild illnesses like the common cold to more severe conditions such as asthma, chronic obstructive pulmonary disease (COPD), pneumonia and lung cancer [1-4]. Chronic respiratory diseases are often characterized by symptoms such as shortness of breath, coughing, wheezing and chest discomfort. These symptoms can lead to a reduced quality of life, limitations in daily activities and, in some cases, irreversible consequences. The development of chronic respiratory diseases can be influenced by environmental exposures, genetic factors and lifestyle choices [1-4]. An example of a chronic respiratory disease caused by genetic factors is cystic fibrosis (CF). CF is characterized by mutations in an anion channel, resulting in impaired function [5-7], and is recognized as one of the most lethal inherited diseases in White people [8]. The respiratory system is susceptible to infections by inhaled pathogens, including bacterial infection with *Pseudomonas (P.) aeruginosa*, a leading cause of lung infections, particularly in individuals with CF [8]. Viral infections also pose significant threats to respiratory health. Notable examples include influenza and coronaviruses such as Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) [9]. The recent Coronavirus Disease 2019 (COVID-19) outbreak highlights the impact of emerging respiratory viruses on global health and the urgent need for continued research, effective treatment and a comprehensive, multidisciplinary approach. Therefore, understanding the disease-specific pathological features and mechanisms underlying a respiratory disease is essential for an effective therapeutic intervention.

1.2. Respiratory system and cell composition

The respiratory system is a complex network of organs and tissues that is divided into upper and lower airways along with the respiratory zone within the lung. The

upper airways, which include the nasal cavity, pharynx and larynx, serve as the gateway to the respiratory system [10], while the lower airways, including the trachea and bronchi, repeatedly branch into smaller airways, facilitating air passage and ultimately leading to the alveoli, the small air sacs in the lungs [11-13]. The alveoli offer a large surface area for gas exchange, enabling oxygen to diffuse from the air into the bloodstream and carbon dioxide to be released from the blood into the air [11-13].

Along with their respiratory function, the airways filter and humidify inhaled air and serve as a crucial physical barrier against external environmental factors, such as allergens, pathogens or particulate matter. This barrier is maintained by a continuous layer of epithelial cells that can recognize and respond to airborne pathogens [14-17]. While innate and adaptive immune cells are essential for immunity, the airway epithelium also plays a significant role in host defense. For example, the respiratory epithelial cells have developed mucociliary clearance as its primary innate defense mechanism. This coordinated process helps to maintain airway homeostasis by eliminating trapped dust particles, pathogens and excess mucus [18, 19].

Epithelial cells:

The airway epithelium exhibits variations in cellular composition and function from the upper to the lower airways, reflecting its diverse roles in maintaining respiratory homeostasis. The upper airways are composed of cells of different sizes and functions, including predominantly ciliated, secretory and basal cells. In the lower airways, club cells, a subtype of secretory cells, become more abundant. In the bronchioles, the epithelium transitions to predominantly squamous cells with sparse cilia and finally in the alveoli to alveolar type I and II cells [13, 20-25]. This reflects a reduction in mucus clearance and an increased focus on gas exchange [23, 26]. Although there is evidence of a shift in epithelial cell populations and expression patterns [23-27], all major cell types can be found along the respiratory tract. Each cell type is characterized by unique gene expression patterns and distinct functions in order to perform the diverse functions of the respiratory system (Figure 1).

Ciliated cells have a central role in mucociliary clearance. They are terminally differentiated cells and are characterized by the expression of transcription factor *forkhead box (FOX) protein J1* (*FOXJ1*), which is involved in late ciliogenesis [28].

Additionally, the expression of *dynein axonemal heavy chain 5* (*DNAH5)* and *coiled-coil domain containing (CCDC)* genes contributes to the formation of multiple hair-like cilia. These cilia beat in a coordinated manner, facilitating the effective clearance of mucus from the airways [23, 29]. Furthermore, secretory cells, composed of club and goblet cells, are responsible for mucus secretion (*mucin* (*MUC) 5AC*, *MUC5B*) including different antimicrobial molecules like *secretoglobin family 1A member 1 (SCGB1A1)* and *SCGB3A1*. Notably, club cells possess a progenitor property, allowing them to undergo further differentiation into goblet and ciliated cells [30]. Beneath these specialized cells lie smaller ones known as basal or stem cells of the airways. They can be distinguished by high expression levels of the transcription factor *tumor protein p63 (TP63)* as well as *keratinocyte* (*KRT*) *5* and the *basal cell adhesion molecule (BCAM)*. Serving as the source for epithelial regeneration, basal cells exhibit a self-renewal capacity and the ability to differentiate into almost all other epithelial cell types, thereby coordinating the continual repair and maintenance of the cell type composition in the respiratory tract [31, 32].

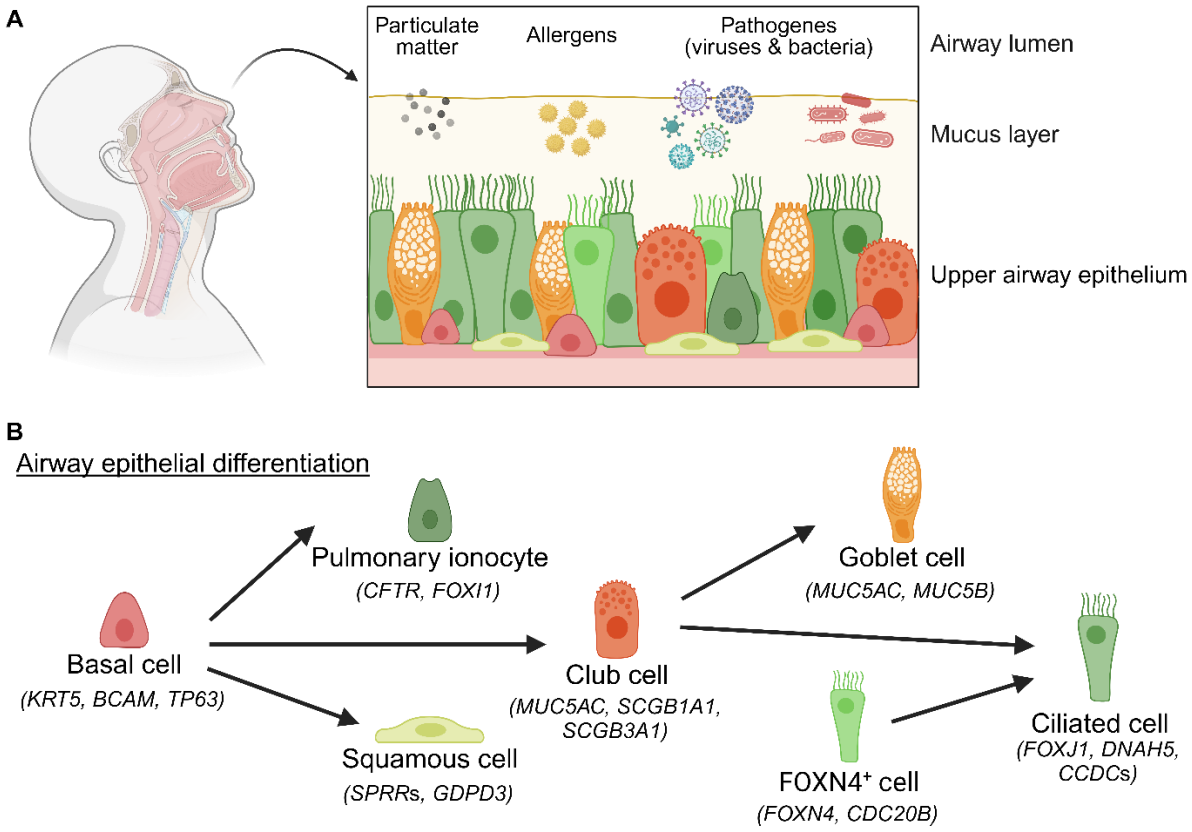

Figure 1: (A) Schematic of different epithelial cell types in the upper respiratory system and different environmental challenges that can affect the upper airways. (B) Depiction of airway epithelial cell differentiation and the characteristic expression features of the different epithelial cell types. Created with BioRender.com.

The influence of the smaller cell populations on the airway system should not be underestimated. The recent discovery of deuterosomal or FOXN4^{+} cells has added a new dimension to the understanding of airway cellular diversity [33]. They are an intermediate cell type between secretory and ciliated cells characterized by high expression of *FOXN4* and *cell division cycle 20B (CDC20B)*, a transcription factor and regulatory protein involved in amplification and organization of cilia formation [33]. Pulmonary ionocytes only account for 1% of the airway epithelium and are characterized by high expression levels of ion channels, especially *cystic fibrosis transmembrane conductance regulator* (*CFTR*), the gene causing the development of CF and the transcription factor *FOXI1*. They have gained attention for their pivotal role in regulating airway ions, fluidics and mucus properties [34, 35]. Their involvement highlights the intricate interplay between various cell types in maintaining the sensitive balance required for optimal airway function.

Immune cells:

To ensure the integrity of the airway barrier and appropriate immune responses a close interaction between the epithelial cells of the airways and resident or recruited immune cells is necessary. The immune cells can be broadly divided into two main categories: innate and adaptive immune cells (Figure 2), each of which perform different functions to protect airway integrity and health.

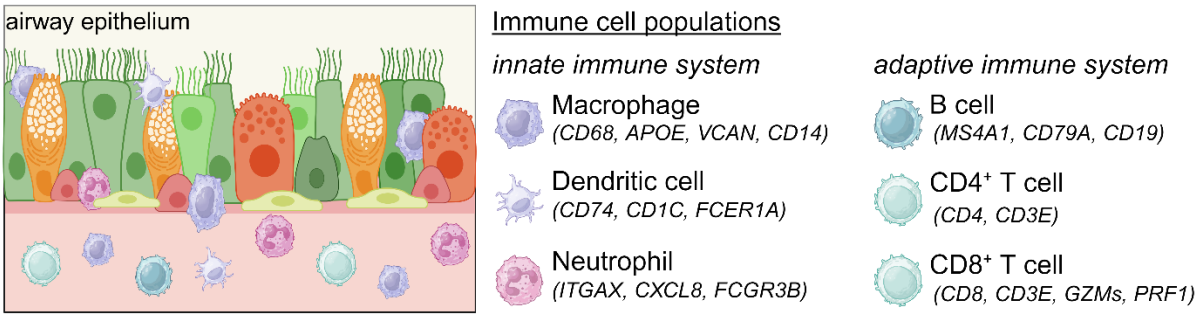

Figure 2: Overview of different immune cell types that can be found in the upper airways and their characteristic gene profile. Created with BioRender.com.

The innate immune system acts as the immediate, nonspecific responder against a wide range of pathogens [2]. One prominent innate immune cell type are macrophages, which act as phagocytes ingesting and neutralizing pathogens or particulate matter. They can be characterized by the expression of surface receptors *cluster of differentiation* (*CD) 14* and *68*, as well as *apolipoprotein E* (*APOE)* and *versican* (*VCAN)*. Together with dendritic cells (DC) they serve as professional

antigen-presenting cells, directing the activation of other immune cells, in particular of T cells [36]. In addition to macrophages, neutrophils also have phagocytic abilities and can be distinguished by the expression of the *Fc Gamma Receptor IIIb* (*FCGR3B)* and *integrin subunit alpha X* (*ITGAX)* receptors. Although having a short lifespan, they play a pivotal role as one of the initial responders among inflammatory cells. Their rapid migration to the site of inflammation is crucial for effectively controlling and eliminating pathogens. Furthermore, by releasing chemokines and cytokines such as interleukin (IL) 8 (encoded by *chemokine (C-X-C motif) ligand 8* (*CXCL8*)), neutrophils contribute to recruitment and activation of additional immune cells [37]. Together, they secret both pro- and anti-inflammatory molecules maintaining airway homeostasis, tissue repair and promote inflammatory responses, thereby closely interacting with the cells of the adaptive immune system.

The adaptive immune system is characterized as a highly specialized branch of immunity that provides a targeted and long-term defense against specific pathogens. It is characterized by its antigen-specific responses that promote an immunological memory, which enables a more effective but time-delayed defense against reinfection [38]. Adaptive immune cells include T and B lymphocytes.

T cells are responsible to induce cellular immunity and play a central role in the body's defense against recurrent infections. These specialized immune cells have T cell receptors (TCRs) on their surface that enable them to recognize specific antigens presented by cells with major histocompatibility complex (MHC) I or II molecules [39, 40]. As another characteristic of T cells, CD3 is necessary for the activation of downstream signaling pathways. Coupled to TCRs, these multiprotein TCR-CD3 complexes with their unique composition are essential for specific T cell activation and induction of the adaptive immune response [39, 40]. T cells can be further categorized based on their additional expression of surface receptor *CD4* or *CD8* [41]. $CD4^+$ T cells are activated upon interaction with MHC II complexes and differentiate into specific subtypes, including T helper cells and regulatory T cells. As T helper cells, $CD4^+$ T cells play a central role in regulating the immune system by secreting specific cytokines that activate other immune cells, such as B cells and cytotoxic T cells (CTL). In their role as regulatory T cells, $CD4^+$ T cells also maintain the balance between immune activation and suppression, prevent excessive immune responses and promote overall immune homeostasis [41, 42]. $CD8^+$ T cells, in

contrast, are directed by endogenous antigens presented on MHC I. Once activated, $CD8^+$ T cells undergo clonal expansion and differentiate into effector CTL. Through the release of cytotoxic molecules like perforin (PRF) and granzymes (GZMs), they target and eliminate damaged or infected cells, thereby minimizing the spread of pathogens. Additionally, a subset of memory $CD8^+$ T cells forms, providing long-term immunity and a rapid response upon re-exposure to the same antigen [43, 44].

The adaptive immune system has a second component that is executed by B cells. These cells express surface receptors, such as *CD19*, that bind to foreign antigens and activate the proliferation and differentiation of B cells. Matured B cells produce and release antibodies that recognize and neutralize pathogens. Additionally, B cells express MHC II molecules and are the third cell type which belongs to the class of professional antigen-presenting cells [38, 45, 46]. T and B cells in the adaptive immune system provide effective defense and long-lasting protection. The inclusion of memory cells promotes a rapid and enhanced response to known threats and strengthens the long-term resistance of the respiratory system.

1.3. Single-cell transcriptomic analyses

Advances in genomics have revolutionized our ability to understand biological systems [47]. Replacing previous reliance on microscopy or limited molecular analysis, the development of sequencing methods, including genome and methylome analysis, has provided more detailed insights into the molecular basis of life. Among these methods, analyzing the transcriptome is of great importance as it reveals the more dynamic interplay of genes and provides additional knowledge into cellular processes and their regulation. Thereby, it further improves our understanding of tissues, diseases and their underlying mechanisms.

Next-generation sequencing methods have significantly advanced this progress and are becoming more common-place in research as less time-consuming and more cost-effective options are developed. For a long time, many studies focused on bulk analyses as they used whole tissues or samples as input [48]. These are often based on the average of RNA expression across diverse cell populations, resulting in the loss of important information of individual cells [48]. A major breakthrough in this context was the emergence of single cell RNA-sequencing (scRNA-seq), which has opened up a new era of precision in simultaneously studying transcriptomic changes

in single cells [49]. This innovative technology has revolutionized our ability to characterize cell types, allowing for more insights into cellular heterogeneity within various tissues and organs, including the respiratory system [22-25, 32]. These studies not only extended our understanding of cellular differentiation and cell type specific gene expression in the airways, but also identified novel, rare cell types like FOXN4^{+} cells and pulmonary ionocytes [27, 34, 35]. Ultimately, scRNA-seq is not only improving our ability to study the homeostatic physiology of healthy tissues, but is also revealing previously unknown mechanisms of disease at the cellular level.

1.3.1. Deciphering diseases using single-cell transcriptomics

Using scRNA-seq technology provides a big amount of data that allows a valuable understanding of molecular mechanisms at single cell resolution. Moreover, the continuous refinement of scRNA-seq methods has enabled higher throughput, allowing for simultaneous analyses of large numbers of cells. This scalability has led to the development of molecular atlases that provide a fundamental understanding of the molecular landscape of different tissues of the human body, offering a comprehensive overview of the cellular components and their regulatory networks [22, 27, 50-54]. This technology enables direct comparisons between healthy and diseased tissues, shedding light on alterations in cellular composition and gene expression patterns underlying various pathological conditions. Its impact extends significantly across various domains, with profound implications in fields such as cancer research, developmental biology and immune response studies [55-57]. Importantly, it allows researchers to track the effects of different therapies on patients, thus entering an era of precision medicine with personalized treatment strategies. The continuous development of scRNA-seq methods and their application in various studies illustrates their indispensable role in the advancement of medical knowledge and therapeutic innovation [56].

Using scRNA-seq, this thesis addresses the intricate molecular landscapes of two critical respiratory diseases: the COVID-19 pandemic and CF. The aim was to uncover new insights into the underlying mechanisms of these diseases, advancing our understanding for improved diagnostic and therapeutic strategies. Each disease will be introduced in separate chapters.

2. Project I: COVID-19

2.1. COVID-19: Introduction

The global emergence of the novel coronavirus, officially termed SARS-CoV-2 [58], has triggered an unprecedented scientific and public health response since its identification in late 2019 [59-63]. This highly transmissible virus is responsible for COVID-19, a respiratory disease that rapidly crossed international borders and caused an enormous challenge to healthcare systems. It quickly led to a global pandemic and required exceptional public adaptations to counteract its rapid spread. Upon infection, the disease onset varies from mild symptoms to severe pneumonia and death. Notably, some individuals testing positive for SARS-CoV-2 remain asymptomatic [59, 64]. Several risk factors for a more severe disease outcome have been identified: primarily age, but also male sex and various comorbidities such as obesity, COPD, cancer, immunosuppression or hypertension [65-68]. Despite the lately observed significant decrease in disease burden due to the COVID-19 vaccination offer, immunization due to prior infections and the emergence of new and milder SARS-CoV-2 variants [69, 70], the high numbers that have been reported worldwide, with over 773,000,000 cases and almost 7,000,000 deaths [70], clearly outline the profound impact of COVID-19. This emphasizes the need for a better understanding of the disease and the underlying mechanisms that contribute to differences in disease susceptibility.

2.1.1. SARS-CoV-2 virus and its host response

SARS-CoV-2 is an enveloped, positive-sense RNA virus [60]. The main entry point on the host cell is the surface receptor angiotensin-converting enzyme 2 (ACE2). This interaction is mediated by the spike protein on SARS-CoV-2 which is later cleaved by transmembrane protease serine subtype 2 (TMPRSS2) [71]. Importantly, these entry factors are widely expressed by airway epithelial cells, especially secretory and ciliated cells, marking them the primary target cells [23, 72].

In response to viral infection, the host cell activates an early cellular innate immune response driven by pattern recognition receptors (PRRs) and the type I and III interferon (IFN) system, a crucial defense mechanism also acting against SARS-CoV-2 [73]. The initial step of the pathogen sensing process involves the detection of viral RNA by host cells. Therefore, epithelial and immune cells are equipped with

PRRs, such as the cytosolic proteins retinoic acid-inducible gene I (RIG-I, encoded by *DEAD box polypeptide 58* (*DDX58*)), melanoma differentiation-associated protein 5 (MDA5, encoded by *interferon induced with helicase C domain 1* (*IFIH1*)) and laboratory of genetics and physiology 2 (LGP2, encoded by *DExH box helicase 58* (*DHX58*)) and different toll-like receptors (TLRs) [74]. These PRRs allow the epithelial layer to rapidly recognize pathogens, activate and recruit other cells to initiate an early immune response. Upon recognition of viral RNA, a downstream cascade is initiated, which is characterized by the activation of interferon regulatory factors (IRFs) through phosphorylation. Once activated, binding of IRFs to their respective motifs induces expression and release of IFNs [75, 76]. Next, secreted IFNs can bind to their respective receptors on neighboring cells, activating the janus kinase - signal transducer and activator of transcription (JAK-STAT) signaling cascade, which ultimately leads to the expression of numerous IFN stimulated genes (ISGs) and cytokines [77-79]. This places the cells in an activated antiviral state that effectively inhibits viral replication and contributes to viral RNA degradation, providing a critical line of defense against pathogenic effects (Figure 3).

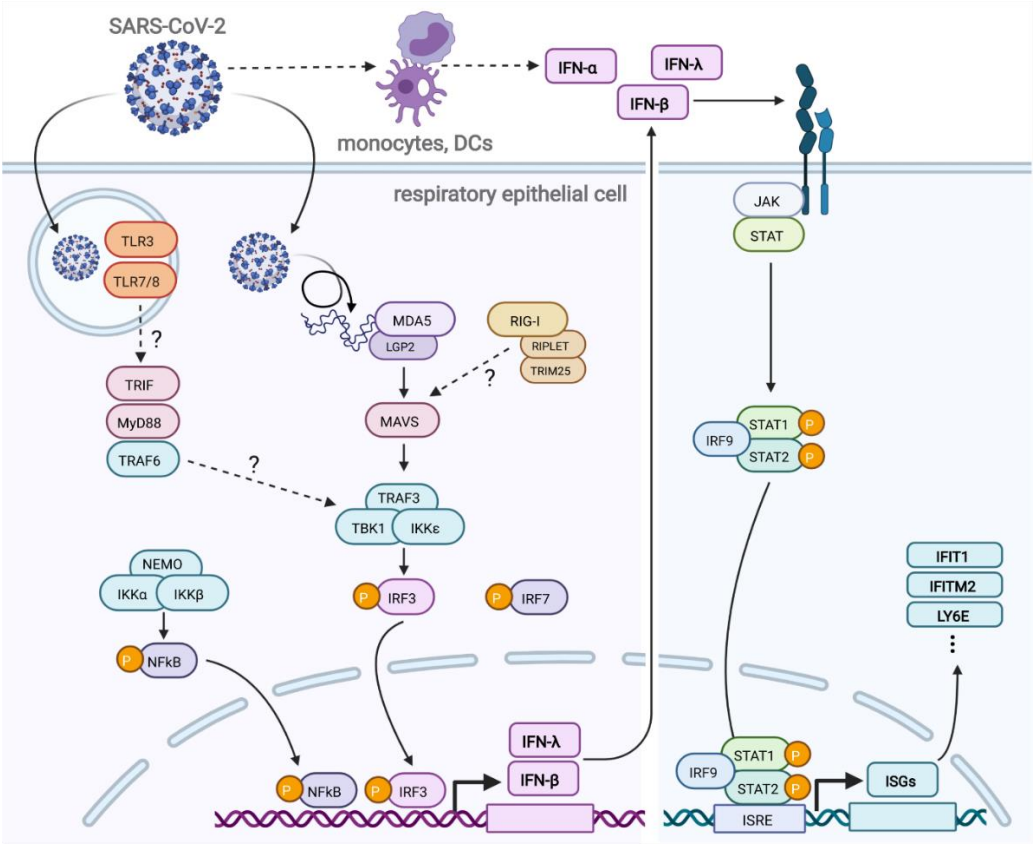

Figure 3: Schematic representation of the epithelial host response to SARS-CoV-2 infection, highlighting genes involved in viral sensing and downstream signaling leading to interferon expression and induction of interferon-stimulated genes (ISGs). A detailed description can be found in chapter 2.4.

In the case of SARS-CoV-2, several studies have shown a sensitivity to IFNs. Treatment with IFNs of different epithelial cell lines led to a reduction of viral replication [80-82] and in turn, inhibition of IFN signaling showed increased replication of SARS-CoV-2 in Calu-3 cells [80]. Moreover, a correlation of viral load and IFN response was identified in mild COVID-19 patients, while adults experiencing a critical COVID-19 disease progression showed an impaired or dampened IFN response [83-85]. In line, in clinical trials, early treatment strategies against COVID-19 included a combination of antiviral drugs and INF beta-1b [86].

2.1.2. SARS-CoV-2 resilience of children compared to adults

Given the unprecedented challenges caused by the COVID-19 pandemic, one notable aspect is the resilience shown by children compared to adults. The course of COVID-19 in children is generally asymptomatic or mild and only a minority of young patients require hospitalization [68, 87-91]. This difference is further emphasized by the low numbers of deaths occurring in children and adolescents with only 0.4% of all deaths reported with COVID-19 [92, 93]. At the beginning of the pandemic, the underlying reasons for the observed differences between children and adults in their response to COVID-19 were poorly understood. Investigations into viral transmission and load, comparing these age groups, did not unveil any significant differences in the expression levels of host-cell entry factors or initial viral load [90, 94-96]. While studies on viral load and host-cell entry did not explain childhood resilience, a decreased innate antiviral immune response in adults was found to lead to a more severe or critical disease outcome [96-98]. This thesis describes an important role of the interferon response as an initial step against viral infections that shows an age-dependent decline potentially contributing to differences in disease severity [99]. This hypothesis was supported by other studies at the time and was further confirmed by later studies [96-98, 100].

2.1.3. Exploring COVID-19 through scRNA-seq

Various research groups, including our own, have already demonstrated the impact of scRNA-seq in advancing our understanding of novel viral infections such as COVID-19. In studying SARS-CoV-2 infections, scRNA-seq has proven very helpful for identifying the main cell types expressing SARS-CoV-2 entry receptor *ACE2*, profiling transcriptomes and immune signaling pathways during disease

progression, studying changes in cell composition and differentiation trajectories and identifying the network of cell interactions in response to the virus [72, 101-108].

The first studies on COVID-19 focused primarily on bronchoalveolar lavage fluid and blood samples and investigated the activated immune response and the dysregulation of cytokines. These studies revealed an accumulation of immune cells that exhibit a strongly pro-inflammatory phenotype. In a pioneering work, our team established the first comprehensive cell atlas of epithelial and immune cells from the upper airways of adults with COVID-19 [108]. In line with previous studies, our results showed an increased presence of pro-inflammatory macrophages and highly activated CTLs. In addition, we observed impaired epithelial differentiation and, due to nasal swabs that allowed the study of both epithelial and immune cell expressions, we were able to show an increased interaction between these cell types in severe COVID-19 cases, possibly contributing to the enhanced immune response [108].

Shortly thereafter, further COVID-19 cell atlases were published, focusing on lung and other tissue samples from deceased COVID-19 patients [105, 106]. These studies not only confirmed the main findings observed in the upper airways, but also provided further details on the epithelial-immune interactions in the lower respiratory tract of COVID-19 patients. In particular, they reported a rapid development of pulmonary fibrosis, significantly advancing our understanding of the long-term complications faced by COVID-19 survivors [105, 106]. These atlases collectively contributed to a better understanding of the dynamics of COVID-19 disease and could serve as a resource for the development of therapies.

However, these previous studies have primarily examined severe to critical disease outcomes in adults. As a result, the molecular differences between children and adults in response to SARS-CoV-2 infection remained unclear, despite evident differences in the clinical disease course, leaving a gap in the understanding of the mechanisms involved across age groups.

2.2. Comparison of the nasal cellular landscape between children and adults

Therefore, in this study, single cells from the upper respiratory tract of children and adults who tested positive and negative for SARS-CoV-2 were compared. An age-

dependent shift in cell type composition and significant differences in gene expression patterns were observed. Notably, in uninfected children, there was an increase in host defense markers such as *IFIH1* and *DDX58*, along with increased IFN signaling and enhanced epithelial-immune cross talk, compared to healthy adults. Following infection, these responses were further activated, suggesting that children have an enhanced ability to recognize viral RNA and mount an immune response early in infection, potentially diminishing viral spread. Consequently, these findings suggest that children are primed for infections and have a more rapid immune response to pathogens, which may contribute to their observed resilience to COVID-19 compared to adults.

2.3. COVID-19: Declaration of own contribution

- establishment of scRNA-seq protocol in the lab
- project management and sample organization together with the clinicians
- sample preparation including single cell isolation from nasal swabs, scRNA library preparation and pooling for sequencing
- data handling including pre-processing, integration and analyses with initial support from Dr. Sören Lukassen
- creation and refinement of figures
- preparation and finalizing of the manuscript

2.4. COVID-19: Manuscript

Loske J*, Röhmel J*, Lukassen S*, *et al.* Pre-activated antiviral innate immunity in the upper airways controls early SARS-CoV-2 infection in children. **Nat Biotechnol.** 2022; 40(3):319-324; doi: 10.1038/s41587-021-01037-9.

3. Project II: Cystic fibrosis

3.1. Cystic fibrosis: Introduction

CF is an autosomal recessive disorder and the most prevalent genetic disease among the White population [8]. The disease typically manifests in early childhood, leading to frequent hospital visits and, in some cases, the need for oxygen therapy [5, 8, 109]. CF is caused by mutations in the *CFTR* gene, mainly expressed by epithelial cells throughout the body [6, 110-112], thereby manifesting as a multisystemic disease for which a cure is currently not available. Although CF affects various tissues in the body, its morbidity and mortality is primarily linked to progressive lung damage, resulting in impaired respiratory function [5-7]. Typical symptoms of people with CF include intensive mucus production, difficulty breathing, a persistent cough and recurrent infections. One of the primary consequences of the abnormal mucus properties is the clogging of the airways (Figure 4). This obstruction reduces the normal flow of air in and out of the lungs, significantly limiting the individual´s ability to breathe.

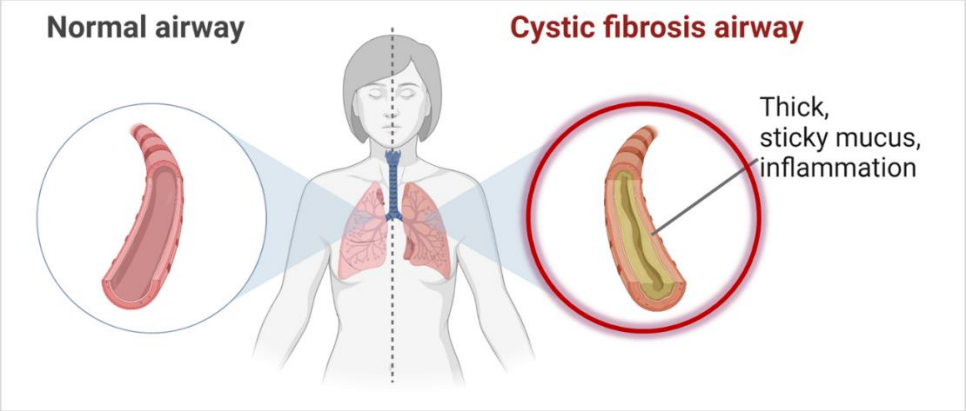

Figure 4: Overview comparing the physiology of the airways of healthy people (left) and cystic fibrosis patients (right). Created with BioRender.com.

Furthermore, the thick mucus in the airways provides an ideal environment for pathogens colonization, with *P. aeruginosa* emerging as the predominant bacterium [113-115]. Bacteria accumulation resulting in chronic respiratory infections is a hallmark of CF. It is associated with neutrophil infiltration and release of neutrophil elastase and IL8, two important modulators described in CF pulmonary infection [116-118]. The recurrent and persistent infections trigger a continuous cycle of inflammation, tissue damage and impaired lung function. Over time, this chronic inflammatory state leads

to an irreversible decrease in lung capacity, which is considered a decisive factor in the severity of the disease [5, 7, 109, 114, 119-121].

3.1.1. CFTR function and mutations

The *CFTR* gene encodes a transmembrane protein important to regulate ion and water transport across cell membranes as well as maintaining pH levels, especially within the epithelial cells lining the respiratory and digestive systems. CFTR is composed of distinct domains, including two nucleotide binding domains and two membrane-spanning domains. Despite the classification as an ATP-binding cassette transporter, CFTR is not an active transporter, instead, the opening of the two membrane-spanning domains, facilitated by ATP, forms a pore that enables the passive diffusion of ions, in particular chloride ions (Cl^-) and bicarbonate ions (HCO_3^-) [122-124]. CF results from various mutations affecting CFTR, leading to no protein or a less functional transmembrane protein. This impairment disrupts the sensitive balance of Cl^- and HCO_3^- within and outside cells. Consequently, a gradient is induced across cell membranes, leading to a reabsorption of water and other ions, such as sodium (Na^+) (Figure 5). This dehydration contributes to the characteristic formation of thick and sticky mucus [5, 7, 109, 113-115, 120, 121].

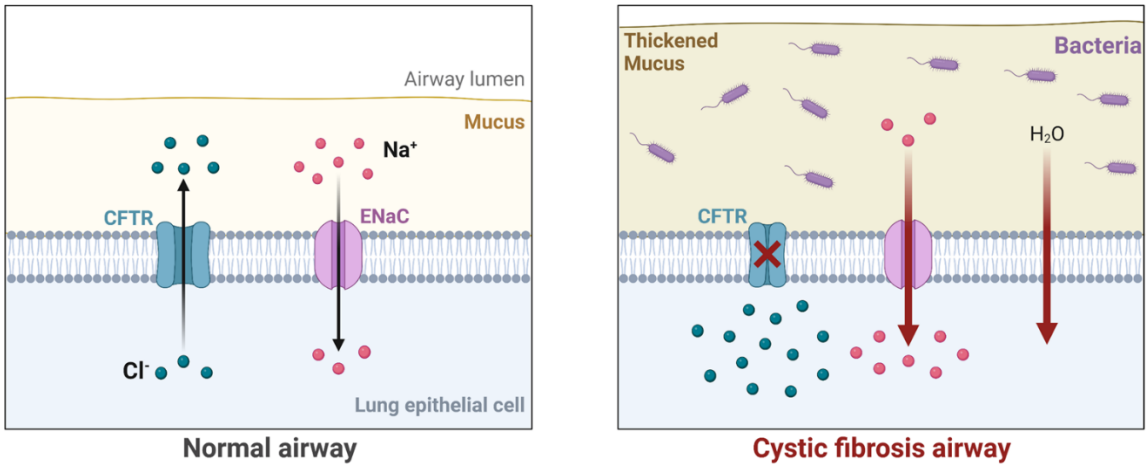

Figure 5: Molecular basis of cystic fibrosis transmembrane conductance regulator (CFTR) protein in healthy (left) and CF (right) cells. Blue dots represent chloride (Cl^-) and red dots sodium (Na^+) ions. Created with BioRender.com.

Until today, there are over 2,000 different *CFTR* mutations known, which can lead to diverse molecular outcomes as defective syntheses, impaired protein folding and lower numbers of CFTR protein and reduced functionality or no gating of anions [5, 8, 125]. The most common mutation is a deletion of a phenylalanine at position 508 (*F508del)*, which is present in at least one allele in up to 85% of CF patients

worldwide [8]. *F508del* is a loss of function mutation, leading to a miss folded CFTR protein and decreased insertion, function and stability in the apical cell membrane [123, 126].

3.1.2. Clinical assessment and diagnosis of cystic fibrosis

CF is diagnosed by a range of clinical measurements that are important for a comprehensive assessment of the impact of the disease on various physiological processes. A key diagnostic test is the determination of sweat chloride concentrations (SCC), with elevated levels considered a hallmark of CF [127, 128]. Pulmonary function tests, including spirometry and lung clearance tests, additionally help to monitor airway health and to assess the progression of airway obstruction. Furthermore, measurements of nasal potential difference provide insights into the functionality of the respiratory epithelium and contribute to a deeper understanding of CF pathophysiology [129].

Advancements in managing CF can be linked not only to enhanced treatment modalities but also to progresses in early detection practices. The implementation of newborn screening programs for CF, introduced in some European countries in the early 2000s and now adopted by increasing numbers of regions and nations [130, 131], has significantly contributed to the improved prognosis of the disease. Newborn screening is a critical tool for identifying CF-associated mutations and facilitating early diagnosis. Studies suggest that early detection can delay the onset of symptoms and reduce the impact on lung function. It can also limit the incidence of recurrent infections, thereby optimizing patient outcomes [8, 132]. Genetic testing is a fundamental part of CF diagnosis. The identification of specific mutations in the CFTR gene not only confirms the diagnosis but also guides personalized treatment of the disease [133].

3.1.3. Cystic fibrosis care and treatment

Despite the challenges presented by the disease, advances in treatment options have contributed to steady increases in the average life expectancy of CF patients. A decade ago, life expectancy was 38 years, but it has now reached around 56 years and continues to rise [8]. The management of CF typically involves a multidisciplinary approach, which includes airway clearance techniques, antibiotics,

nutritional support and, in some cases, lung transplantation. However, this treatment only delays disease progression [5, 109, 119].

In the past decades, many CF researching groups focused on improving treatment option for CF. In addition to treating the common symptoms, novel therapeutic strategies include gene therapies and modulators targeting the underlying genetic defects, holding promise for more effective intervention. High-throughput screening has played an important role in the rapid identification of potential drug candidates and therapeutic approaches [134].

Among the groundbreaking therapeutic developments are CFTR modulators, including ivacaftor, lumacaftor, elexacaftor and tezacaftor. Ivacaftor works as a potentiator, extending the opening time of the CFTR channel and thus allowing more ions to pass through [135, 136]. Later developments have introduced combination therapies, in which lumacaftor [137, 138] or tezacaftor [139, 140] work in conjunction with ivacaftor. These combinations focus on improving CFTR stability, correcting the folding defect specific for some mutations and improving protein transport to the cell surface, ultimately contributing to more effective treatment. However, these treatments only resulted in modest clinical benefits [137-140].

The recent approval of the triple combination of elexacaftor/tezacaftor/ivacaftor (ETI) was a milestone in CF therapy [141-143]. This pioneering triple combination promises great progress in the treatment of CF due to its multiple and synergistic therapeutic effects. Elexacaftor, beside tezacaftor, is another stabilizer binding to a different side of the protein, thereby additionally increasing the number of CFTR proteins at the cell surface (Figure 6).

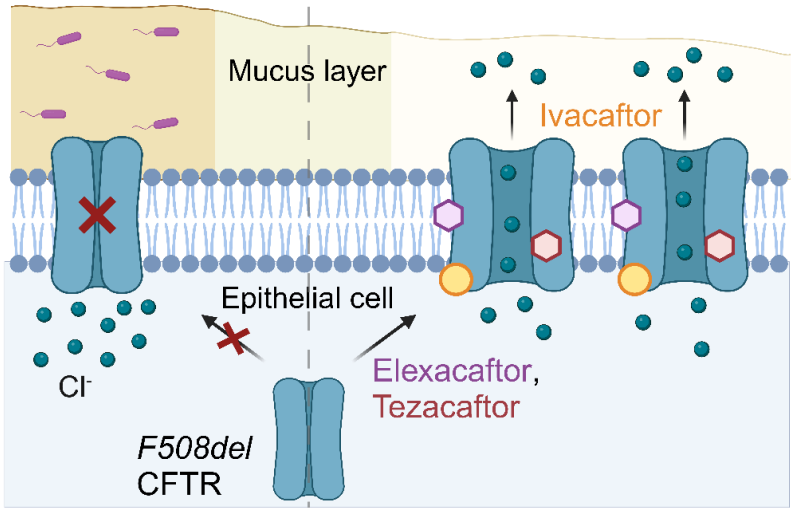

Figure 6: Enhanced function of *F508del* CFTR mutation following treatment with elexacaftor/tezacaftor/ivacaftor (ETI) combination therapy. Elexacaftor and tezacaftor stabilize the protein structure, facilitating its transportation to the transmembrane. Ivacaftor binds to a separate site on the protein, increasing the opening duration of the CFTR pore, thereby enhancing channel activity. This combined effect allows for a more efficient passage of chloride ions (Cl^-) across the transmembrane, approaching physiological homeostasis and consequent reduction in the thick mucus layer and susceptibility to infection characteristic of CF. Created with BioRender.com.

CFTR modulator therapies are specifically designed to correct the malfunctioning protein produced by the *CFTR* gene. Given that different mutations result in various defects in the protein, the modulators developed thus far are effective only in individuals with specific mutations. Therefore, ETI is only approved for patients with at least one *F508del* mutation, thereby providing a treatment options for up to 90% of CF patients [142, 144-148]. Preliminary data has demonstrated the remarkable success of ETI treatment, suggesting the potential for longer life expectancy and a reduction in CF-related complications. Several observational studies could show an increase in CFTR activity, reaching 40-50% when compared to normal CFTR function, significantly reducing the disease burden. Thus, patients treated with ETI showed improvements across several health parameters, including lung function, SCC, mucus viscoelasticity and reduced inflammation and infection [149-153]. The recent extension of studies evaluating the efficacy and safety of ETI to children was another significant achievement. This advance allows early intervention in disease progression, potentially preventing irreversible damage and optimizing the potential benefit of this highly effective therapy [145, 148, 154]. Although data collection is still ongoing and long-term studies are needed to fully understand the impact of the therapy, the current results elucidate the great impact of ETI on the lives of people with CF.

3.1.4. Insights into disease mechanisms in cystic fibrosis using scRNA-seq

While scRNA-seq has not been widely used in human studies in CF, where it has been used, it has demonstrated promise in advancing our understanding of the disease. A notable study focused on comparing cells extracted from lung tissue acquired during lung transplantation [155]. Through scRNA-seq analysis, Carraro *et al*. confirmed the expression of *CFTR* in lung epithelial cells and identified CF-related alterations in specific subsets of epithelial cell types and stages. They proposed an exhaustion of epithelial regeneration by reduced numbers of basal cells and a higher transition rate towards ciliated and secretory cells, mainly providing a molecular atlas to further validate and understand CF [155].

Furthermore, scRNA-seq has been employed to analyze sputum samples from CF patients, which shed new light on the airway immune landscape linked to CF pathology [156]. This study confirmed an elevated number of neutrophils and recruited monocytes in individuals with CF. Moreover, the findings revealed a pro-inflammatory yet relatively immature immune cell phenotype, shedding light into the intricacies of the immune response associated with CF [156]. These studies collectively underscore the potential of scRNA-seq in unraveling the cellular complexity and immune response of CF.

However, these studies primarily focused on the pathophysiological features of CF in adults. Studies of CF in children with a focus on the molecular mechanisms remain elusive.

3.2. Understanding the impact of ETI in children with cystic fibrosis

The primary objective of the study described in this thesis was to elucidate the cellular landscape and gene expression patterns in pediatric CF patients at single cell resolution. In addition, the analyses were extended to assess the impact of ETI treatment on airway restoration at the molecular level in these patients. To accomplish this, cells from nasal swabs from children with CF before and after treatment with ETI as well as healthy children were isolated and scRNA-seq analysis was performed. The results confirmed the CF phenotype as characterized clinically and showed a compromised epithelial host defense and a pro-inflammatory gene expression profile in immune cells. In addition, the study underlined the beneficial effects of ETI not only clinically, but also on the airway epithelium, as evidenced by

an enhancement of innate host defense mechanisms, such as IFN signaling and MHC complexes, together with a reduced activation of immune cells. These results highlight the success that can be achieved by early intervention in CF patients and offer promising prospects for restoring normal cellular function.

3.3. Cystic fibrosis: Declaration of own contribution

- project management and sample organization together with the clinicians
- sample preparation including single cell isolation from nasal swabs, scRNA library preparation and pooling for sequencing
- data handling including pre-processing, integration and analyses
- creation and refinement of figures
- preparation and finalizing of the manuscript

3.4. Cystic fibrosis: Manuscript

Loske J*, Völler M*, *et al*. Pharmacological improvement of CFTR function rescues airway epithelial homeostasis and host defense in children with cystic fibrosis. **Am J Respir Crit Care Med.** 2024; doi: 10.1164/rccm.202310-1836OC.

4. Extended summary, discussion and outlook

The development of single cell technologies enabled the analysis of individual cells with unprecedented resolution and depth. Conventional bulk analyses often mask cellular heterogeneity, hindering the comprehensive understanding of complex biological systems. In this thesis the advantages of utilizing scRNA-seq, in combination with a non-invasive approach for obtaining samples from the upper airway via nasal swabs, were employed to investigate various aspects of respiratory diseases in children.

Medical research often prioritizes adult subjects due to the easier acquisition of samples and consent and the more apparent disease manifestation in older populations [157]. However, studying the initial stages of disease is important for effective disease prevention and early intervention. Early treatment is key to preventing irreversible disease stages, particularly in children who have greater biological adaptability, making pathological changes potentially more reversible than in adults [119, 130, 132, 158-160]. Therefore, this thesis focuses on studying children with mild and early-stage COVID-19 and CF.

4.1. Unraveling disease complexity with single cell techniques

Since its introduction in 2009 [49], scRNA-seq has emerged as a powerful method for disease analysis [47], offering advantages over traditional techniques such as real-time polymerase chain reaction (qPCR), microarrays or bulk sequencing. While qPCR provides accurate amplification of specific genes, microarrays can already capture thousands of targeted genes but with limitations such as high background noise and narrow dynamic expression range [161]. In contrast, bulk RNA-seq has a considerable advantage as it provides a broad and unbiased representation of the whole transcriptome. This results in large datasets that offer insights into temporal patterns of gene expression [48] and allow the discovery of new gene models, such as splice variants [162]. However, bulk RNA-seq is based on the average gene expression profile of a heterogeneous cell population [48, 163]. Beyond the advantages of bulk sequencing, scRNA-seq enables the study of individual cells, facilitating the identification of rare cell populations and transitional states contributing to disease pathogenesis [22, 27, 34, 35, 108, 155, 156], which are often overlooked by traditional methods. For example, the recent discovery of pulmonary ionocytes through

scRNA-seq has shed light on their potential significance in CF research, given their distinctive feature of high *CFTR* expression [34, 35]. Additionally, scRNA-seq allows for the identification of cell-specific responses and molecular pathways involved in both health and disease.

Despite the significant advantages of scRNA-seq, it lacks the ability to capture detailed cellular organization within tissues. To understand cellular function, it is necessary to analyze their interactions in the microenvironment, where signaling pathways can be influenced by proximity and organization. Spatial transcriptomic methods, which capture gene expression patterns in tissue samples such as biopsies or organ slices, address this problem and are enabled by recent advances in next-generation sequencing and imaging [164, 165]. These techniques reveal molecular signatures that are spatially defined, providing insight into the distribution of cells within the tissue microenvironment and uncovering complex biological processes, such as cell-cell interactions, tissue development and disease progression [164, 165]. For instance, using a targeted spatial approach Rendeiro *et al.* demonstrated that SARS-CoV-2 primarily infects alveolar epithelial cells in the lung tissue. They observed an increase in hyper-inflammatory cell states near the infected cells and further associated severe disease progression with the closer proximity of fibroblasts and mesenchymal cells [166]. In the more severely infected lung compartments of deceased COVID-19 patients, spatial transcriptomics revealed an increased interaction between cytotoxic lymphocytes and pro-inflammatory macrophages [167]. Furthermore, elevated expression levels of genes associated with TNF signaling were observed in these regions, further enhancing our understanding of disease progression across tissue compartments [167]. Although a comprehensive, spatially resolved, whole transcriptomic single-cell lung atlas of COVID-19 or CF patients is not yet available, the recent release of the spatially resolved healthy lung atlas increases the comprehension of potential disease-related changes in lung tissue that spatial data can offer [168]. This atlas complements the scRNA-seq human lung cell atlas [22] and presents a more detailed resolutions of the lung architecture, including novel cell types, expression patterns and specialized cellular niches not previously explored in respiratory system studies [168]. Madissoon *et al*. propose associations between newly identified immune niches in the submucosal glands and disease progression [168]. Indeed, the submucosal glands

play a key role in the pathogenesis of CF, as they are mainly composed of mucus- and serous-producing cells [169, 170]. However, data on human cells derived from the submucosal glands at a single cell level is limited [26, 171]. Therefore, applying spatial transcriptomics to nasal or lung biopsies from patients with respiratory diseases like COVID-19 or CF can reveal unique disease structures, improve understanding of epithelial integrity and identify cell-cell interactions within microenvironments not captured by traditional single-cell methods.

It is important to acknowledge that RNA data is only a snapshot of gene expression at a specific time point and do not account for underlying factors such as epigenetic modifications, mutations and other regulatory mechanisms. Moreover, post-transcriptional modifications can significantly influence protein levels [172]. Due to the multifactorial nature of disease pathogenesis, complementing transcriptomic data with (epi-)genetic, proteomic and similar analyses can provide a more comprehensive understanding of complex cellular and biological processes that drive disease phenotypes [173, 174]. Therefore, integrating data of various layers of transcriptional and translational regulation, including also multiomic and spatial approaches, can further enhance our comprehension of respiratory physiology, genetic predisposition and disease. In the context of COVID-19, the wealth of publicly available data presents a unique opportunity to leverage the advantages of data integration, as demonstrated by initiatives like the COVID-19 Cell Atlas project [106]. Consequently, incorporating single-cell data that in the future will expand to further layers of transcriptional regulation, will broaden our understanding of the intricate network of factors that influence and promote respiratory diseases and thereby will facilitate identification of novel biomarkers and improve our ability to develop more precise therapeutic interventions.

4.2. Exploring respiratory diseases through nasal swab samples

Many studies of COVID-19 and other respiratory diseases have relied on blood samples because they are easily obtained in a clinical setting. However, as significant differences in host responses between the systemic and respiratory compartments exist [175-177], exploring alternative sample types also allowing the study of epithelial cells are necessary. Nasal or nasopharyngeal swabs have become a standard tool to screen for upper respiratory tract infections caused by

viruses, such as influenza or respiratory syncytial virus, or bacteria [178-184]. Their utility and efficacy were further demonstrated during the COVID-19 pandemic where they were used as a standard tool for detecting infections with SARS-CoV-2 [185-187]. Therefore, several groups, including our own, have recently highlighted the value of isolating single cells from nasal swabs for detailed transcriptomic analysis of upper respiratory tract diseases, offering additional knowledge of the molecular mechanisms underlying these infections [99, 107, 108, 175, 176, 188]. This highlights the potential of nasal swabs for the study of respiratory diseases as they can be collected non-invasively with minimal burden to the study participant [189], making them applicable in both clinical and research settings. Moreover, nasal swabs can be obtained with minimal training and equipment, unlike some sample types that require specialized instruments or preparation procedures, making them accessible also for sample collection in a variety of settings, including domesticated locations [179, 184, 190-192]. It also facilitates collection of follow-up samples [189], allowing researchers to track changes in gene expression or biomarker levels in longitudinal studies and monitoring of disease progression or treatment response. In particular for pediatric patients [185, 190-192], where compliance and sample availability can be challenging, nasal swabs provide an easy source to study airway cells. It minimizes discomfort [190-192] and avoids the need for sedation often required for more invasive procedures such as bronchoalveolar lavage or biopsy [193].

The upper airway epithelium, also highlighted by the findings of this thesis, shows similar major cell types and core gene expression patterns to those found in lower airway samples, suggesting that nasal swabs may serve as a reliable proxy for studying respiratory disease and enhancing our understanding of disease mechanisms. Nevertheless, it is important to consider the variability in the cell composition along the airways. Therefore, nasal swabs may not capture changes in lung specific cells. Single-cell studies of the human airway, including both upper nasal and lower bronchial cells, have revealed differences in cell type composition and gene expression profiles associated with sampling location [23-27]. These studies have identified distinct subtypes within the major cell types of ciliated, secretory and basal cells. In addition, they have reported proximal-distal gradients of cell types, including variations in the abundance of ionocytes, a rare cell type recently discovered with high expression of *CFTR* [34, 35]. According to these studies,

ionocytes are found to be most abundant in the upper respiratory tract. Therefore, studies that focus on the upper airway may provide a better opportunity to gain insight into the role of *CFTR*-high ionocytes [23, 27, 194]. In addition, gene expression patterns differ between the upper and lower airways. Genes commonly expressed in the upper airways are associated with developmental processes, cell specification and maturation, while those in the lower airways are more closely associated with differentiation or lung epithelium development [23, 27]. Considering these differences is important for a more complete understanding of the mechanisms underlying respiratory disease. Indeed, studies have shown a gradient of *ACE2* expression, the entry receptor for SARS-CoV-2 [71], along the airways with nasal cells exhibiting the highest levels [23], highlighting the importance of studying both nasal and lung samples in respiratory disease research. This is particularly important in diseases, such as COVID-19, where the nasal cavity serves as the primary site of infection.

It is important to be aware that, in addition to the anatomical aspect of sampling, the choice of tissue sampling method and dissociation protocol will have a significant impact on cell type proportions [23]. For example, biopsies yield cells from deeper anatomical structures, offering a more comprehensive insight into tissue composition and gene expression profiles compared to nasal brushes or swabs, which predominantly capture luminal cell types [23].

However, using the same sample type for both control and diseased subjects allows for direct comparisons that account for anatomical and methodological differences. Although obtaining lung specimens alongside nasal swabs can offer additional information, leveraging nasal swabs for sampling presents a non-invasive and accessible method that allows for analyses of respiratory diseases, particularly when implemented with careful study design and consideration of anatomical variations.

4.3. Innate host response in COVID-19 and cystic fibrosis

PRRs play a key role in innate immunity across various cell types, including airway epithelial cells. They are activated by the recognition of pathogen associated molecular pattern, which leads to the production of IFNs. IFNs, in turn, induce the expression of ISGs, which place the cells in an antiviral state. This coordinated response forms a line of defense against the infection caused by pathogens.

In this thesis, the particular importance of the innate host response in the control of infection by pathogens was highlighted. In healthy children, a higher number of innate immune cells and immune activation, as well as a primed antiviral response in epithelial cells prior to infection, likely contributed to their greater resilience compared to adults and to their protection against severe COVID-19. Subsequent studies confirmed a protective innate immune status against SARS-CoV-2 infections and provided additional evidence that this protection is mediated by enhanced early IFN and cytokine secretion in children [98, 100].

The study by Magalhaes *et al.* used different model systems to infer the differences in the immune epithelial cross talk between children and adults [100]. Using lung epithelial cells, they could show that pre-stimulation with IFN or inflammatory cytokines improved their responsiveness to SARS-CoV-2 infection. In addition, stimulated peripheral blood mononuclear cells (PBMCs) of children promoted a stronger antiviral response in lung epithelial cells compared to the response to adult PBMCs, which also led to more efficient clearance of SARS-CoV-2. Furthermore, immune cells in the upper airway of healthy children showed elevated cytokine expression, as well as stronger and more frequent interactions between children's immune and epithelial cells compared to those of adults. Thus, this study reveals further insights into age-related differences in immune regulation and supports the hypothesis presented in this thesis that a pre-activated innate immune response contributes to the protection of children from severe COVID-19 [100].

By applying a single-cell multiomic approach, Yoshida *et al.* analyzed upper and lower airway samples, as well as PBMCs, from individuals across different age groups, both with and without COVID-19 infection [98]. Their study further supports a heightened IFN response in the airways of children and adolescents, both before and after infection. Notably, Yoshida *et al.* observed an elevated production of IFNs by adult blood cells, underscoring the significance of the sample type and site of infection. Since the upper airways are the primary site of infection, an early and strong IFN response in this area may provide protective effects by enhancing viral clearance. In contrast, an excessive systemic IFN response could potentially lead to cytotoxic immune response, promote tissue damage and contribute to more severe disease courses [98].

Genome-wide association studies have identified a genomic region on chromosome 3, associated with overexpression of pro-inflammatory genes of innate immune cells, making it a risk factor for severe COVID-19 [195]. Interestingly, a recent study revealed differences in genetic predisposition in disease severity in blood samples between children and adults [196]. In contrast to adults, children with similar disease severity showed lower frequencies of the risk region on chromosome 3. However, the study observed variations in the *OAS* gene cluster among children [196], which is a component of IFN-mediated antiviral defense mechanisms [197], providing additional information of the underlying regulations for differences in disease severity between children and adults [196]. In summary, the resilience observed in children throughout the COVID-19 pandemic is likely due to a robust and early innate immune response, coupled with the presence of pre-activated antiviral epithelial cells that more rapidly recognize pathogens and thus provide better protection.

The presented findings for CF in this thesis indicate an impaired innate immune response characterized by enhanced inflammatory processes in macrophages and neutrophils in the upper airways of children with CF. The COVID-19 project has shown that upper airway cells of healthy children have a protective, pre-activated antiviral status which was impaired in CF patients. This may predispose CF patients to frequent infections and contribute to persistent inflammation, ultimately affecting the lung function. It is noteworthy that there is a significant reduction in MHC expression in epithelial cells, which may exacerbate the impairment of host defense mechanisms. Consistently, in other studies of CF, analyses of differentially methylated regions in CF lung macrophages have revealed potential associations with compromised innate immune cell function and phagocytosis in CF bronchoalveolar lavage [198]. Similarly, changes in DNA methylation patterns in nasal epithelial cells have been linked to genes involved in inflammatory responses, highlighting new potential gene targets and underlying regulatory mechanisms. This study identified methylation changes in genes encoding the receptor for TNFα [199], supporting the findings presented in this thesis as TNF signaling was found to be upregulated in the transcriptional data of CF children.

4.4. Susceptibility of cystic fibrosis patients to SARS-CoV-2

CF is characterized by excessive production of sticky mucus, which increases susceptibility to pathogens and leads to chronic lung infections. This persistent microbial presence contributes to an accelerated decline in lung function and compromises overall health [113-115]. In addition, viral co-infections such as with respiratory syncytial virus, rhinovirus or influenza, lead to exacerbation of respiratory symptoms and higher rates of hospitalization in CF patients [200-202].

Initially, a history of respiratory disease was considered a risk factor for severe COVID-19 outcomes [65-68] leading to concerns that individuals with CF might face a higher risk of developing severe respiratory complications. Contrary to expectations, later studies showed similar or slightly lower incidence of COVID-19 in CF patients compared to the general population without comorbidities [203-207]. Notably, severe COVID-19 in CF individuals tends to be associated with an advanced disease stage including poor lung function, diabetes, older age, pancreatic insufficiency or previous lung transplantation [203-206]. Yet, the majority of CF patients, particularly children, had mild or even asymptomatic COVID-19 [206].

Several factors potentially contribute to these observations. The CF population generally benefits from a younger demographic, heightened adherence to infection prevention practices and the employment of CF-specific anti-inflammatory treatments. The use of anti-inflammatory therapies specific to CF management, reduced levels of certain cytokines such as IL-6 and changes in the expression of the cellular entry factors for SARS-CoV-2, specifically *ACE2* and *TMPRSS2* [71, 203-208]. Lagni et al. demonstrated a reduction in SARS-CoV-2 replication after CFTR inhibition *in vitro*, suggesting a role of CFTR in regulating SARS-CoV-2 infection [209]. This finding was supported by evidence of decreased *ACE2* expression in CFTR-modified bronchial epithelial cells [210], as well as downregulation of *ACE2* and *TMPRSS2* in airway epithelial cells isolated from CF patients [211, 212], which likely limited viral entry and replication in this population.

The studies presented in this thesis did not include an analysis of SARS-CoV-2 entry factors or infection in CF patients. However, it may be of interest to investigate this further to understand the unexpectedly mild disease course of COVID-19 in the majority of CF patients.

The presented data shows a minor reduction in PRRs and decreased levels of IFN in CF children, which may indicate a worse outcome in the event of SARS-CoV-2 infection. However, it is important to note that the gene expression levels of CF children were not compared to those of healthy adults or CF patients during acute infection. This leaves open the possibility that although CF children exhibit lower expression levels of PRR compared to healthy children, they might still surpass those observed in healthy adults. Therefore, it is unclear whether these mechanisms protect CF children from severe COVID-19 infections or whether other protective differences are involved. As observed in the COVID-19 cohort, healthy children have a primed state for pathogen infection, which enables them to mount a faster immune response upon infection. Similarly, children with CF are also in an activated state due to exposure to bacteria, which may allow them to rapidly clear the virus after infection, potentially preventing severe disease. Including CF patients with acute SARS-CoV-2 infection and comparing the activation of viral host defense factors in CF patients versus children without comorbidities could contribute more insights into underlying disease mechanisms.

Moreover, other studies have suggested a protective effect of CFTR modulators, which reduce acute inflammation and improve mucociliary clearance in CF patients [149-153], potentially enhancing host defense against SARS-CoV-2 [208]. Analysis of the CF cohort treated with ETI revealed a significant increase in MHC molecules, viral sensing and IFN gene expression [188]. This finding may explain the reported benefits of ETI treatment in SARS-CoV-2 infections. Furthermore, infections with SARS-CoV-2 are associated with increased cytokine levels, which are reduced in CF patients treated with ETI, sometimes even to levels lower than those in healthy children, as shown in the presented data. However, additional research is necessary since ETI treatment was only approved in children after the peak of the COVID-19 pandemic and data on cases of infection in ETI-treated children are scarce.

4.5. Outlook

The data presented here have contributed to our understanding of molecular changes in the upper airway mucosa underlying different respiratory diseases during childhood, facilitated by the non-invasive nature of nasal swab sampling. However, some questions remain unanswered.

While the COVID-19 pandemic evolved, it became apparent that genetic variations of SARS-CoV-2 are linked to different disease severities [213, 214], not only across different age groups but also within pediatric populations [215]. The SARS-CoV-2 variants of concern identified by the World Health Organization, including Alpha, Delta, Gamma and later Omicron, emerged from the wild-type variant and were not included in the analyses of this thesis. It will be interesting to investigate the molecular differences in the host response and evaluate potential differences in innate host defense mechanisms of children infected with more severe variants.

Importantly, approximately 10% of COVID-19 patients experience persistent symptoms such as dyspnea, fatigue or cardiovascular impairments, commonly referred to as long COVID [216, 217]. Risk factors for long COVID include age, acute phase hospitalization and comorbidities. However, the impact of the disease extends beyond these factors and affects individuals of all ages and disease severities [218], including children, adolescents and those with mild or asymptomatic symptoms, who were not hospitalized during the acute phase of the disease [218-221]. These studies report that in some patients persistent symptoms have been observed up to 12 months after infection. Further investigation and comprehensive research into the molecular mechanisms are necessary to improve current diagnostic and treatment strategies.

Novel treatment options have significantly improved life expectancy and quality of life of CF patients [222, 223]. However, despite an identical *CFTR* mutation background, treatment responses vary, with approximately 20% of patients on ETI therapy continuing to experience symptoms associated with CFTR protein dysfunction [223]. Identifying those molecular changes that distinguish high responders from low responders would be highly important, as it could aid patient stratification. Additionally, comparing ETI outcomes across different age groups, particularly between adults and children, can provide further insights into disease progression dynamics.

In this project, some of the CF-associated changes, such as decreased MHC expression and the reduced IFN response, persisted despite three months of treatment with ETI, even in children with limited clinical impairment [188]. In this respect, the recent approval of ETI for children aged 2 to 5 years may be another

significant advance [145]. As this allows for an even earlier intervention in the disease course, it would be interesting to determine whether restoring CFTR function in preschool children or infants with CF would lead to a complete restoration of the innate epithelial immunity and amelioration of the pro-inflammatory response described in this thesis for school-aged children. Comprehensive longitudinal studies are necessary in the future to monitor the efficacy of the treatment and to potentially identify markers of early treatment failure.

Furthermore, while our analyses focused on the upper airways, extending validation efforts to the lower respiratory tract could offer additional insights. Due to the significant impact of CF on the digestive system [224], collecting samples from this region would allow for a better understanding of other organ systems affected by CF.

In summary, this thesis uses scRNA-seq of nasal cells from the upper airway to investigate the molecular mechanisms and cellular defense responses in children with COVID-19 or CF, both of which affect the respiratory system. These findings provide a great foundation for future research efforts aimed at deepening our understanding and developing precise interventions to reduce the burden of these respiratory diseases.

5. List of References

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