EPITHELIAL SIGNATURES IN RESPIRATORY DISEASE

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Epithelial signatures in respiratory disease
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Till min pappa, Dr. Torsten Ax

“Everything’s got to end sometime. Otherwise nothing would ever get started.” – Eleventh Doctor

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ABSTRACT

The epithelium of the human airways protects us against harm and helps maintain immune homeostasis. In respiratory diseases such as asthma and COPD, the functions of the epithelium are altered and can cause or contribute to disease progression. Additionally, these diseases are heterogeneous in regard to which inflammatory mechanisms and pathways are activated, thus creating inflammatory endotypes. Due to these differing endotypes, not all patients respond similarly to currently available treatments. Increased understanding of these endotypes will enable a precision medicine approach for respiratory diseases.

In this thesis, responses and functions of the airway epithelium in different possible inflammatory endotypes are investigated using a primary cell-based model system. Three types of epithelial signatures are established in response to inflammatory cytokines: gene expression, extracellular vesicle proteome, and miRNA expression. In **Paper I**, the IL-6 trans-signaling gene signature is used to identify a subtype of asthma patients with increased activation of this pathway in the airway epithelium. These patients demonstrate increased inflammation, epithelial barrier damage, and higher number of asthma exacerbations indicative of poorly controlled disease. This suggests that these patients could benefit from treatment blocking activation of the IL-6 trans-signaling pathway. In **Paper II**, extracellular vesicles released from epithelial cells stimulated with T2 and Th17 cytokines show proteomic differences related to airway disease-relevant processes. This is exemplified through the effect of the vesicles released under Th17 inflammatory conditions in promoting neutrophil migration. These findings enhance the knowledge about the contribution of epithelial extracellular vesicles in airway disease. In **Paper**

III, Th17 cytokines are shown to cause disruption of the airway epithelial barrier and induce the expression of several miRNAs predicted to target barrier-related genes. Preliminary results identify two miRNAs as possible candidates that interact with, and cause decreased levels of, mRNAs encoding proteins involved in formation of the epithelial barrier. This highlights the role of miRNAs as master regulators of genes important to airway epithelial functions.

Altogether, these studies show the diverse and fine-tuned responses and functions of the airway epithelium in inflammatory environments similar to what could be present in patients with respiratory disease. The results thus contribute to the understanding of endotype-specific processes taking place locally in the airways. Ultimately, increased knowledge of disease-driving mechanisms will lead to the development of novel treatments and biomarkers that can be used to improve the lives of patients with respiratory disease.

Keywords: airway epithelium, inflammation, gene expression, extracellular vesicles, miRNA
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Populärvetenskaplig sammanfattning

Längs med hela de mänskliga luftvägarna, från näsan till lungblåsorna, täcks ytan av epitelceller som bildar det så kallade luftvägsepitelet. Dessa celler skapar, likt huden, en tät barriär för att skydda oss mot sådant som kan vara skadligt i luften som vi andas in, såsom virus och luftföroreningar. Dessutom producerar luftvägsepitelet ett sekret, slem eller mucus, som fångar virus och andra partiklar, och viftande cilier på cellerna transporterar detta uppåt och ut från luftvägarna. Man vet också att luftvägsepitelet kommunicerar med andra celler, till exempel celler från immunsystemet, genom att släppa ifrån sig molekyler som påverkar dessa celler.

I de kroniska luftvägssjukdomarna astma och KOL (kroniskt obstruktiv lungsjukdom) är luftvägsepitelet påverkat och fungerar inte som det ska, detta på grund av bland annat den inflammation som pågår i sjukdomarna. Typen av inflammation skiljer sig åt mellan patienter, vilket kan vara orsaken till att de läkemedel som finns för astma och KOL idag inte fungerar för alla. Därför behöver man öka kunskapen om vad som pågår i de olika typerna av luftvägsinflammation för att kunna utveckla bättre mediciner samt biomarkörer så man vet vem som kan bli hjälpt av medicinen.

I den här avhandlingen har vi studerat hur en cell-modell av luftvägsepitelet svarar när det stimuleras med inflammatoriska proteiner, så kallade cytokiner, som representerar tänkbara typer av inflammation som kan förekomma i astma och KOL. På sikt tror vi att dessa svar kan användas för utveckling av mer specifika läkemedel och biomarkörer för luftvägssjukdomar. Vi har analyserat tre olika nivåer av svar; gen- och mikroRNA-uttryck i epitelcellerna samt proteiner i extracellulära vesiklar som epitelcellerna frisätter, och etablerat signaturer skapade av cytokinerna.

I det första delarbetet används gensignaturen orsakad av inflammationstypen IL-6 trans-signalering för att identifiera en grupp av astma-patienter där denna inflammationstyp är aktiverad. Dessa patienter har ökad inflammation i luftvägarna, en försämrad barriär-funktion och fler astma-attacker än övriga patienter. Detta antyder att det här är en grupp av patienter som inte hjälps av deras medicinering och därför skulle kunna hjälpas av läkemedel som blockerar just denna typ av inflammation.

I det andra delarbetet har vi isolerat extracellulära vesiklar som frisläppts från epitelcellerna, dessa vesiklar är små blåsor omgivna av ett membran och fungerar som kommunikation mellan celler genom bland annat de proteiner som finns i och på dem. Vi visar att proteinerna i vesiklarna varierar beroende

på om cellerna stimuleras med cytokiner kopplade till T2- eller Th17-inflammation. Nivåerna av proteinerna kunde också kopplas till olika processer som är relevanta i luftvägssjukdomar. En sådan process kunde vi också se exempel på i labbet då vesiklar frisläppta under Th17-inflammation kunde locka till sig neutrofiler, en immuncell som ofta finns i ökat antal vid Th17-inflammation. Denna studie föreslår att extracellulära vesiklar från luftvägsepitel kan både fungera som markörer för inflammation samt bidra till processer kopplade till luftvägssjukdom.

I det tredje och sista delarbetet fokuserar vi på hur mikroRNA kan bidra till en skadad eller icke-fungerande epitel-barriär. MikroRNA, eller miRNA, är korta RNA-molekyler som inte kodar för något protein. Istället binder de till och förhindrar mRNA(”messenger-RNA”)-molekyler från att användas till att göra protein, vilket i sin tur leder till lägre nivåer av detta protein. Vi visar att Th17-inflammation är skadligt för luftvägsepitelets barriärfunktion, vilket kan orsaka eller förvärra sjukdom. Vidare orsakar denna inflammation ökade nivåer av mikroRNA som förutspås att minska nivåerna av proteiner som är involverade i bildandet av barriärfunktionen. Resultaten i det här delarbetet, som fortfarande pågår, lyfter fram den nyckelroll som mikroRNA har i att reglera viktiga funktioner i luftvägarna.

Sammanfattningsvis förstärker de här studierna den viktiga roll som epitelcellerna har i att både bibehålla normaltillstånd i luftvägarna samt bidra till luftvägssjukdomar såsom astma och KOL. De gen-, protein- och mikroRNA-signaturer som uppstår i luftvägsepitelet som svar på inflammation kan användas för att identifiera processer som kan påverkas genom utveckling av nya mer specifika läkemedel. Dessutom kan de användas för att ta fram biomarkörer för att hitta de patienter som kan hjälpas av dessa läkemedel. Detta, så kallad precisionsmedicin, kommer innebära bättre behandling av astma och KOL och förbättra livet för patienter.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Epithelial IL-6 trans-signaling defines a new asthma phenotype with increased airway inflammation.**
Jevnikar, Z., Östling, J., **Ax, E.**, Calvén, J., Thörn, K., Israelsson, E., Öberg, L., Singhania, A., Lau, L.C.K., Wilson, S..J, Ward, J.A., Chauhan, A., Sousa, A.R., De Meulder, B., Loza, M.J., Baribaud, F., Sterk, P.J., Chung, K.F., Sun, K., Guo, Y., Adcock, I.M., Payne, D., Dahlén, B., Chanez, P., Shaw, D.E., Krug, N., Hohlfeld, J.M., Sandström, T., Djukanovic, R., James, A., Hinks, T.S.C., Howarth, P.H., Vaarala, O., van Geest, M. & Olsson, H.; Unbiased Biomarkers in Prediction of Respiratory Disease Outcomes study group
Journal of Allergy and Clinical Immunology, 2019; 143(2): 577-590.

II. **T2 and T17 cytokines alter the cargo and function of airway epithelium-derived extracellular vesicles.**
Ax, E., Jevnikar, Z., Cvjetkovic, A., Malmhäll, C., Olsson, H., Rådinger, M. & Lässer, C..
Respiratory Research, 2020; 21:155.

III. **Th17 cytokines mediate airway epithelial barrier dysfunction – A possible role for miRNAs.**
Ax, E., Weidner, J., Winslow, S., Lässer, C., Jevnikar, Z., Olsson, H. & Rådinger, M..
In manuscript.

List of papers not included in the thesis

Development of stable Vibrio cholerae O1 Hikojima type vaccine strains co-expressing the Inaba and Ogawa lipopolysaccharide antigens.
Karlsson, S.L., **Ax, E.**, Nygren, E., Källgård, S., Blomquist, M., Ekman, A., Benktander, J., Holmgren, J. & Lebens, M.
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Jansson, A.M., Csiszar, A., Maier, J., Nyström, A.C., **Ax, E.**, Johansson, P. & Holmberg Schiavone, L..
Journal of Biological Chemistry, 2017; 292(37).

The Airway Epithelium - A Central Player in Asthma Pathogenesis.
Calvén, J., **Ax, E.** & Rådinger, M..
International Journal of Molecular Sciences, 2020; 21(23).

Multi-omics links IL-6 trans-signalling with neutrophil extracellular trap formation and Haemophilus infection in COPD.
Winslow, S., Odqvist, L., Diver, S., Riise, R., Abdillahi, S., Wingren, C., Lindmark, H., Wellner, A., Lundin, S., Yrlid, L., **Ax, E.**, Djukanovic, R., Sridhar, S., Higham, A., Singh, D., Southworth, T., Brightling, C.E., Olsson, H.K. & Jevnikar, Z..
European Respiratory Journal, 2021; 58(4).

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Abbreviations

ALI	Air-liquid interface
AMP	Antimicrobial peptide
BAL(F)	Bronchoalveolar lavage (fluid)
CCL	CC motif chemokine ligand
CXCL	CXC motif chemokine ligand
COPD	Chronic obstructive pulmonary disease
CSF	Colony-stimulating factor
ELISA	Enzyme-linked immunosorbent assay
EV	Extracellular vesicle
FC	Fold change
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
HBEC	Human bronchial epithelial cells
HBEC-ALI	Human bronchial epithelial cells cultured at ALI
ICS	Inhaled corticosteroid
IgE	Immunoglobulin E
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
microRNA/miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
NGS	Next-generation sequencing
PCA	Principle component analysis
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNAseq	Ribonucleic acid sequencing
SEC	Size-exclusion chromatography
T2	Type 2 inflammation
T17	Th17 inflammation
TEER	Transepithelial electrical resistance
TGF	Transforming growth factor
Th	T helper (cell)
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
UTR	Untranslated region

Definitions in short

A list of short definitions of concepts as they are used within this thesis.

Airway epithelium	A layer of cells with differing functions that cover the airways and protect against harm from for example inhaled allergens and pathogens by producing mucus and antimicrobal proteins, form a tight protective barrier, and communicate with other structural cells and immune cells.
Asthma	Inflammatory disease of the airways with symptoms including wheezing and shortness of breath. The disease is triggered or worsened by e.g. allergens or infection.
COPD	Chronic obstructive pulmonary disease, progressive inflammatory irreversible lung disease commonly caused by smoking. Symptoms include shortness of breath and coughing.
Phenotype	A subset of patients characterized by observable, usually clinical, characteristics. For respiratory disease this includes e.g. late-onset asthma or frequent exacerbating COPD.
Endotype	A subset of patients characterized by underlying molecular mechanisms that cause disease manifestation. For respiratory disease this includes e.g. T2-driven asthma or alpha-1 antitrypsin deficient COPD.

T2	Type of inflammation encompassing cytokines IL-4, IL-5, and IL-13 and present in T2-high endotype in airway disease, usually related to presence of eosinophils and may also be linked to allergy.
T17	Type of inflammation encompassing cytokines IL-17A/F, IL-23, and TNFα, possible non-T2 endotype in airway disease, usually related to presence of neutrophils.
IL-6TS	IL-6 trans-signaling, possible non-T2 endotype wherein IL-6 signaling is mediated through the soluble IL-6 receptor and the signal-transducing receptor gp130.
Extracellular vesicle/EV	A particle enclosed by a lipid bilayer released from cells and can be involved in cell-to-cell communication.
miRNA	A short RNA strand involved in post-transcriptional regulation of gene expression by binding to target mRNA strands.

1. Introduction

The epithelium of the human airways is constantly challenged with possible pathogens and irritants in the thousands of liters of air that we inhale every day. To protect us, airway epithelial cells form a tight barrier and remove particles by trapping them in mucus and moving them up and out of the lungs through the work of beating cilia. But the epithelium is not only a barrier, it communicates with the cells of the immune system as well as other neighboring cells, helping to maintain homeostasis and alert the immune cells of present damage or pathogens. For this reason, the airway epithelium is heavily involved in respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) in the lower airways, as well as rhinosinusitis and rhinitis in the upper airways. In these instances, the epithelium can both cause and, through responding to the ongoing inflammation, drive or propagate the disease.

Asthma and COPD are heterogeneous diseases with disease subtypes where some patients have limited benefit from current available treatments. There is therefore a need to better understand this heterogeneity to develop more targeted treatment. Due to the involvement of the airway epithelium, the hypothesis of this thesis is that there are epithelial mechanisms and biomarkers specific for respiratory disease subtypes that can be used for the development of such targeted treatments, thus improving the lives of patients.

1.1. Respiratory diseases, asthma and COPD

More than 500 million individuals worldwide suffer from the chronic respiratory diseases asthma or COPD [1]. Asthma is more prevalent, making up approximately two-thirds of the total cases, and is the most common chronic respiratory disease in the world. COPD, however, has higher mortality and is the third leading cause of death globally with around 3 million deaths annually, compared to less than half a million for asthma [1, 2].

The main focus of this thesis is on asthma, a disease that affects the central airways and encompasses symptoms such as wheezing, shortness of breath, and coughing. The disease is heterogeneous with variations on multiple levels, including triggers, severity, and clinical characteristics. Usually, the symptoms or worsening of symptoms, known as an exacerbation or asthma attack, are caused by a trigger such as an allergen, a virus, irritants like perfume, cold temperature, or certain medications including aspirin [3]. These triggers cause or worsen an inflammation in the airways, leading to downstream effects including activation of airway smooth muscle cells, which cause the

bronchoconstriction behind the symptoms [4]. Onset of disease is most common during childhood, and caused by a combination of genetic and environmental factors that alter the immune system [4]. Asthma developed later in life is usually associated with different clinical characteristics such as absence of allergy and increased severity [5, 6]. Diagnosis is made based on symptoms and spirometry testing, usually showing that airflow limitation is reversible upon treatment with bronchodilator. There are also established biomarkers to assess the ongoing inflammation such as measuring the fraction of nitric oxide in exhaled breath (see more in 1.2. and 1.4.) [3].

Standard treatment for asthma are bronchodilators, which relax the constriction of the airway smooth muscle system, and can be used as needed when symptoms appear or as daily maintenance therapy. These are now commonly combined with inhaled corticosteroids (ICS), which dampen the ongoing inflammation, thereby diminishing the factor causing the symptoms [3]. The aim of these treatments are to control the symptoms and reduce the risk of severe exacerbations. If this is not achieved, the dose of bronchodilator and ICS is increased [3]. Up to 25% of individuals with asthma show limited response to these treatments and are classified as severe or difficult-to-treat asthmatics [3, 7]. This patient group is associated with higher morbidity and reduced quality of life which affects their productivity in society and, thus, negatively influences the health economics of asthma [8]. To help these patients, the commonly prescribed treatment is oral corticosteroids (OCS), and long-term use of corticosteroids may cause severe side effects such as osteoporosis. Another option is more targeted treatment such as antileukotrienes or biologics targeted against specific types of inflammation (see 1.2.) [3].

Symptoms of COPD includes shortness of breath and coughing, and sometimes also with excessive phlegm production. In contrast to asthma, the shortness of breath, or reduced airflow limitation, is usually not reversible [2]. However, some individuals have some reversibility and overall a clinical phenotype similar to asthma, hence there are discussions in the field about a potential asthma/COPD overlap disease [3, 9, 10]. COPD is most commonly caused by long-term inhalation of noxious gases, such as smoke from cigarettes or from burning of solid fuels for cooking and heating in poorly ventilated homes, and is therefore usually developed later in life [2, 11]. As with asthma, COPD is also related to chronic inflammation, but the disease is spread throughout both central and peripheral parts of the airways, where it can cause destruction of the alveoli, known as emphysema [2]. Similar to asthma, COPD is a heterogeneous disease with a variety of causes and manifestations of disease [12, 13].

Diagnosis of COPD is made based on a combination of symptoms, exposure to the risk factors such as cigarette smoke, and that spirometry shows airflow limitation despite treatment with bronchodilator [2]. If a COPD patient is a smoker, the best treatment is smoking cessation. Following that, standard treatment consists of bronchodilators, which may be combined with anti-inflammatory treatment such as ICS even though there is limited evidence for response to corticosteroids in COPD [2]. Additionally, COPD is often associated with increased bacterial colonization [14-16] and/or serious illness due to viral infection, therefore antibiotics and vaccines are also common treatments [2].

The heterogeneity of both asthma and COPD, combined with the fact that there are limited curative or disease-transforming treatments, highlights the need for further and deeper understanding of disease-driving mechanisms. This may aid in identifying patient subpopulations based on disease mechanisms, as well as developing biomarkers and new treatments that address these mechanisms in individual patients.

1.2. Inflammatory endotypes

Several studies have investigated the heterogeneity of asthma and COPD through data collected in large cohorts comprised of patients and healthy control subjects. Through clustering methods based on clinical and/or molecular data, these studies have identified subpopulations with specific phenotypes [5, 17-27]. A phenotype is defined as an observable characteristic, which in the context of respiratory disease could be e.g. late-onset asthma, allergic asthma, or frequent exacerbating COPD [28, 29]. Further, studies have also found different types of inflammatory patterns, or inflammatory endotypes, a concept which is more established in asthma than in COPD [13, 30-32]. Endotypes are thus molecular mechanisms, such as different types of inflammation, that can differ between individuals with the same phenotype.

An early step towards defining an endotype classification in asthma was the identification of subtypes based on which granulocytes were present and at what levels in induced sputum; eosinophils, neutrophils, or a mixture of the two [33]. These cells are thus markers of what inflammation is present in the airways, and it is the mechanisms causing or related to this inflammation that define the inflammatory endotypes [25, 34]. An alternative model representing the heterogeneity in chronic respiratory disease is that of ‘treatable traits’, where traits, such as airway hyperresponsiveness, eosinophilia, or bacterial colonization, could provide a means of treating respiratory disease regardless of specific disease diagnosis [35].

Thus, inflammatory endotypes represent one way of addressing heterogeneity in respiratory disease. Generally, the endotypes are defined as T2/T2-high or non-T2/T2-low, where the latter is likely to comprise several more specific endotypes (**Figure 1.1.**). The endotypes encompass mechanisms and markers that lead to improved understanding of disease as well as development of more precise treatment. Difficulties, however, lie in the translational work to uncover the mechanisms and in how the biomarkers representing these can be identified, accessed in a non-invasive manner, and used in the clinic.

Currently, clinical characterization of inflammatory endotypes mainly consist of biomarkers such as fraction of exhaled nitric oxide (FeNO), sputum differential cell counts (eosinophils and neutrophils), and blood eosinophil levels [33, 36, 37]. These methods come with varying levels of difficulty and are mostly used to identify the T2 endotype, hence additional biomarkers are needed to better define inflammatory endotypes in asthma and other respiratory diseases.

1.2.1. Type 2 (T2)

Initially believed to be the only type of asthma, T2-driven/T2-high asthma is now recognized as the most common [38] and the most studied endotype. In this endotype, T helper type (Th2) cells, eosinophils, and innate lymphoid type 2 cells (ILC2s) drive the ongoing inflammation through the cytokines IL-4, IL-5, IL-9, and IL-13. This was classically coined allergic asthma due to the relationship between T2 inflammation and allergy and the common concomitant presence of allergen-specific IgE antibodies and atopy in asthma, although eosinophilic/T2 asthma without allergy does exist [39, 40]. Non-allergic eosinophilic asthma is usually linked to a late-onset of disease [39, 40], and/or could be mediated by epithelial alarmins that are released upon epithelial damage [41, 42] (see 1.4.1.3.).

The inflammatory cascade in allergic T2 airway inflammation is initiated by inhalation of allergen which is presented by dendritic cells to T cells, causing them to differentiate to Th2 cells. Th2 cytokines released by these cells stimulate B cells to produce allergen-specific IgE, which in turn triggers degranulation of mast cells. Further, IL-5 promotes maturation and survival of eosinophils, and IL-4 and IL-13 have effects on the airway epithelial cells [4, 39] (see 1.4.1.). All these processes can be enhanced, or caused, by ILC2s, that respond to the epithelial alarmins and release IL-4, IL-5, and IL-13 [43-45].

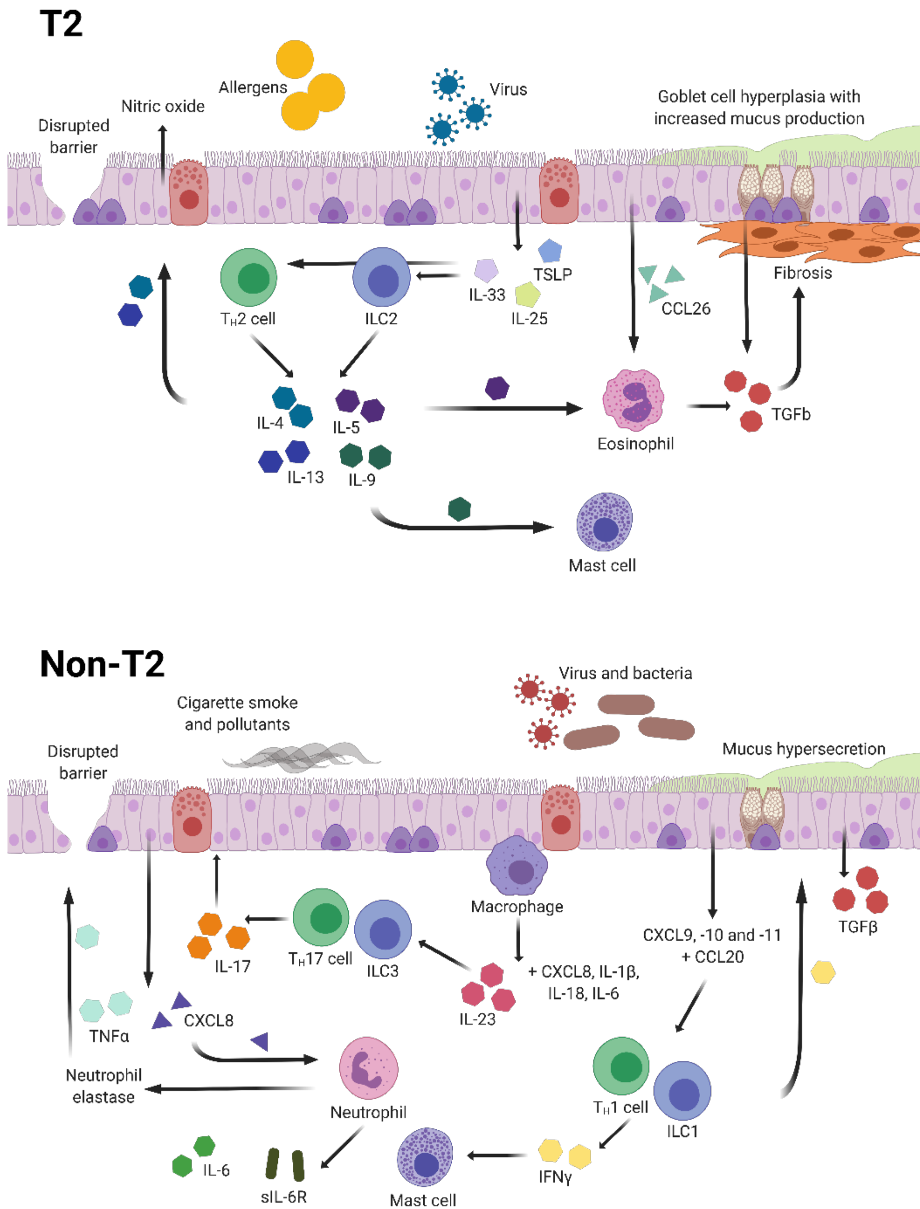

Figure 1.1. *Cells, mediators, and processes involved or implicated in T2 (upper panel) and non-T2 (lower panel) inflammatory endotypes of respiratory disease. [Created with BioRender.com]*

Generally, individuals with the T2 endotype respond well to standard asthma treatment with ICS, a connection that has been known since 1958 [38, 46]. However, some do not, which may be due to high levels of inflammation or corticosteroid resistance caused by, for example, defects in the glucocorticoid receptor functionality or a genetic predisposition [47, 48]. There is also evidence that this resistance may be mediated by the epithelial alarmin thymic stromal lymphopoietin (TSLP) (see 1.4.1.3.) acting on ILC2s in T2 inflammation [49]. More advanced treatment for this endotype has been developed through the use of monoclonal antibodies which mainly target components of T2 signaling family [47, 50, 51]. For allergic asthma, allergen immunotherapy and anti-IgE antibodies are additional options for treatment [39].

Some patients with COPD also have features of T2 inflammation and atopy, an increased tendency towards allergic reactions, is a risk factor for COPD being present in approximately 20% of COPD patients [9]. Additionally, 15-40% of COPD patients have airway eosinophilia linked to a reversible airflow limitation as well as exacerbations which can be prevented by corticosteroids [2, 9, 13]. These individuals may be described as having asthma-like COPD, or fall within the term of the postulated asthma/COPD overlap.The mechanism for eosinophils in COPD is uncertain but increased levels of IL-5 have been found and there may be mechanisms mediated by epithelial alarmins (see 1.4.1.3.) [13]. There is also direct evidence of T2 inflammation in COPD through the increase of IL-13 in airway T cells compared to healthy individuals and the identification of a T2 signature in airway epithelial cells [9] (see more in 1.4.).

1.2.2. Non-T2

Those individuals with asthma where an ongoing T2 inflammation can not be detected, or is only present to a lower degree, are coined as Non-T2/low-T2 asthma [3, 47]. Here, the inflammation is characterized by other inflammatory cells and cytokines that thus may comprise specific endotypes. The most commonly discussed inflammation in non-T2 is Th17/T17 which is usually related to neutrophilia and is the predominant type of inflammation in COPD (see 1.2.2.1.). There are different indications as to whether these possible endotypes are more or less severe than T2. Nevertheless, non-T2 asthma is less responsive to corticosteroids and there are currently no biologics or other specific treatments available for these patients [3, 39, 47, 52, 53]. However, recent results show that an antibody targeting TSLP, an epithelial alarmin, can decrease the number of exacerbations in severe asthma regardless the type of inflammation [54].

In addition to T2 and Th17/T17 inflammation, it is likely that other types of inflammation exist, both in asthma and in COPD. These additional inflammatory types need to be studied in order to develop targeted therapies and identify the patients where those therapies would be the most effective.

1.2.2.1. Th17/T17

In a Th17, herein called T17, endotype T helper type 17 (Th17) cells, neutrophils, and the cytokines IL-17A/F, IL-23, and TNFα are commonly present in the airways and involved in driving inflammation [39, 55]. T17 inflammation in asthma is usually less responsive to corticosteroids and there are indications that corticosteroids can shift the type of inflammation from T2 to T17 [56-59], where the effect on stimulating neutrophils may be potentiated by bronchodilators [60]. A recent study has shown that metabolic inhibition can make T17 inflammation more susceptible to corticosteroid treatment, due to the metabolic activity being high in IL-17 producing T cells during airway inflammation [61]. These findings could explain why severe asthma, representing around 10% of all individuals with asthma, can be associated with more IL-17 and neutrophilic inflammation [55, 62, 63]. However, it is debated whether neutrophils in asthma are just a phenomenon due to corticosteroid treatment or if the neutrophils are disease-driving [62, 64].

Mechanistically, exposure to irritants, pollutants, pathogens, cigarette smoke, or allergens injures the airway epithelium, causing it to release alarmins as well as neutrophil and Th17 cell stimulating mediators [39, 55, 62]. This together with an altered innate immune system establishes the inflammation with release of IL-17, that further triggers the release of mediators from the epithelium (see 1.4.1.), and presence of neutrophils [55, 65]. TNFα can be released by cells of the immune system as well as epithelial cells upon inflammation or infection [66, 67]. It is associated with increased levels of neutrophils, possibly due to direct attraction, and increase in TNFα in asthma is also linked with airway hyperresponsiveness [66-68].

As T17 inflammation in respiratory disease is insensitive to corticosteroids, there is a need for more targeted therapy. Available treatments to date consist of theophylline, which is bronchodilating and decreases the neutrophil attractant CXCL8, and antibiotics, but these are associated with concerns related to dosing and chronic use [39]. Attempts at targeting members of the T17 signaling family using monoclonal antibodies or small molecules have not yet been as successful as their T2 counterparts [39, 50, 51]. This may, at least in part, be due to a lack of precision medicine strategies to identify likely responders (endotyping of patients) [52, 62].

The inflammation in COPD is most commonly neutrophilic, involving mechanisms similar to those described above where exposure to noxious gases, oxidative stress, bacteria, and viruses trigger the release of neutrophil stimulating and attracting mediators, including TNFα, from the airway epithelium [2, 9, 13]. In parallel, macrophages are stimulated to produce IL-23 and IL-1β, which causes Th17 cells and ILC3s to secrete IL-17, which in turn may be important in the development of emphysema [2, 12, 13]. IL-17 also induces the release of CXCL8/IL-8 from airway epithelial cells which further attracts neutrophils [13]. Importantly, this inflammation is, as in asthma, unresponsive to treatment with corticosteroids [13].

1.2.2.2. IL-6 trans-signaling

Several studies have identified increased levels of IL-6 in both asthma and COPD, with some differences regarding if the levels were increased locally in the airways, systemically in the circulation, or both [69-75]. In asthma, the increase has been found in different subtypes of the disease, however it appears to be most associated with severe, non-T2, and/or neutrophilic asthma [70-72, 74, 76] as well as with an increased risk for exacerbation [69]. High systemic levels of IL-6 have been linked with metabolic dysfunction and obesity in severe asthma [77]. Further, IL-6 was found to be negatively correlated with lung function in both asthma and COPD, and in the latter it was linked to an increased mortality [70, 72, 73].

IL-6 is a pleiotropic cytokine that has long been considered a general marker of ongoing inflammation. It is mainly produced by cells from the innate immune system but it can also be produced by epithelial cells in response to stimuli causing cellular stress or damage such as pathogens or allergens [70]. Increased levels in the airways is thus reflective of local inflammation and indicative of epithelial damage [70, 72]. Classical signaling of IL-6 is through membrane-bound IL-6 receptor (IL-6R) which requires the ubiquitously expressed signal-transducing receptor gp130 for initiation of the intracellular signaling cascade. The IL-6R is expressed by immune cells and hepatocytes, but alternative splicing or shedding by proteases can release a soluble form, sIL-6R, enabling signaling of IL-6 in all cells expressing gp130 through what is called trans-signaling (IL-6TS) [78-81]. Macrophages and neutrophils have been identified as the primary source of sIL-6R, which is released after various inflammatory stimuli including C-reactive protein, CXCL8, and infection as well as lipopolysaccharides [70, 82-85]. sIL-6R has, as IL-6, been found to be elevated in asthma and COPD [86-90]. Two genetic variants in the gene encoding IL-6R appear to cause an increase in sIL-6R and have been associated with increased asthma risk and severe asthma, respectively [70, 91, 92].

The effects of IL-6 includes prevention of apoptosis in T cells as well as promoting both Th2 and Th17 cell differentiation and inhibiting the action of regulatory T cells [62, 70, 78]. Additionally, depending on cellular source, IL-6 can be involved in the production of IgE and mucus as well as establishing eosinophilic or neutrophilic influx to the airways [93, 94]. IL-6TS controls immune cell infiltration into tissues [78], and specifically drives a switch from neutrophils to mononuclear cells, e.g. monocytes and T cells, through increasing the levels of CCL2 [83]. As an effect of infection-induced release of sIL-6R, IL-6TS can augment the inflammation caused when toll-like receptors are activated by e.g. microbe-derived ligands [95]. These functions highlight the role of IL-6/IL-6TS in contributing to airway inflammation and suggest that this may represent an endotype of its own.

Under normal conditions, the activity of IL-6TS is controlled by a soluble form of gp130, working as a scavenger receptor. Thus, this serves as a possible therapeutic opportunity specific for IL-6TS, which has been pursued in pre-clinical studies of asthma [87, 96] and has shown success in clinical studies of inflammatory bowel disease [97]. Studies of an have shown that it is able to increase the number of regulatory T cells and decrease the presence of other T cells, however this also affects the beneficial effects of IL-6 in acute immune responses [78]. This treatment did not meet the endpoints, comprised of early and late asthmatic responses to allergen, in a small clinical study of asthma [98]. However, the anti-IL-6R antibody did show effect when administered to two young patients with non-allergic asthma and carrying a genetic variant of the IL-4 receptor (*IL4RA*^{R576}) which drives a mixed Th2/Th17 inflammation [99].

1.3. The human airways and the airway epithelium

The human respiratory system is divided into the upper and the lower respiratory tract. The upper tract is comprised of the nose and mouth below which the lower respiratory tract starts with the trachea. At the end of the trachea sits the carina, the first branching of the conducting airways, where the two main bronchi lead further into the right and left lung. Each bronchus is then, on average, split over 22 generations into primary, secondary, and tertiary bronchi followed by bronchioles and ending in the alveoli where the gas exchange occurs [100].

Covering the outside of the central airways, the trachea and main bronchi, are cartilage rings which provide structure and integrity. Contraction of the conducting airways is enabled by airway smooth muscle (ASM) cells, which cover the airways all the way down to the terminal bronchioles [101].

Mediators such as histamines released by mast cells, nitric oxide released by the airway epithelium, as well as neurotransmitters cause the contraction [102, 103]. Increased contraction of the ASM, due to increased release of or increased sensitivity to mediators, is what causes airway/bronchial hyperresponsiveness in asthma leading to shortness of breath [4, 102]. Close to the ASM cells are fibroblasts, which produce extracellular matrix (ECM) components, providing anchor points for cell adhesion, including the basement membrane, and regulation of airway stiffness. The production of ECM, along with differentiation and increased proliferation of fibroblasts, is altered in asthma and leads to airway remodelling [104]. Additionally, ASM cells also produce ECM and can undergo differentiation and increased proliferation, further contributing to airway remodeling [4, 104]. Nutrients for the cells of the respiratory system come, as for other organs, from the blood circulation through vessels made up of endothelial cells. These vessels also branch off into thin capillaries around the alveoli to allow for the gas exchange.

On top of the basement membrane, covering the inside of the whole respiratory system, are epithelial cells (**Figure 1.2.**). Epithelial cells cover all surfaces of the body which are exposed to the environment, including, for example, the lungs, the skin, and the gastrointestinal tract. Based on the location in the airways, the epithelial cells are of different types and structure. In the central airways, the cells form a pseudostratified epithelium comprised of ciliated cells, secretory goblet and club cells, and the progenitor/stem cell-like basal cells that are able to differentiate into the other cell types upon injury [105-108]. The secrectory cells produce, among other molecules, antimicrobial peptides and mucus and are, together with the ciliated cells, responsible for clearance of pathogens and particles through the mucociliary escalator [109, 110]. Additionally, more rare or novel epithelial cell types have been identified including the pulmonary neuroendocrine cell [111] and the ionocyte [112, 113]. Most of these cells continue into the more distal airways, but a specialized cellular organization in the form of submucosal glands, which are like mucus factories, are only present in the cartilaginous airways [114]. In the alveoli, there are two types of epithelial cells, called alveolar type 1 (AT1) and alveolar type 2 (AT2) cells. AT1 cells are long and thin and responsible for the gas exchange with the capillary system, and AT2 cells produce surfactants to reduce surface tension and can also differentiate to AT1 cells if needed [101].

After gas transport, the key function of the airway epithelium is to protect against infections and other possibly harmful agents such as pollutants. As mentioned above, the mucociliary escalator clears the airways by trapping particles and pathogens in the mucus and the beating cilia moves the mucus up and out through the mouth and nose [109, 110]. If this system does not function properly, the second line of protection is in the form of the epithelial barrier

where the tight connections between the epithelial cells prevent entry to the underlying tissues and circulation (see 1.4.1.2.). Thirdly, the airway epithelium plays an important part in bridging between innate and adaptive immunity by releasing inflammatory mediators in response to potential threats or in disease (see 1.4.) [115, 116].

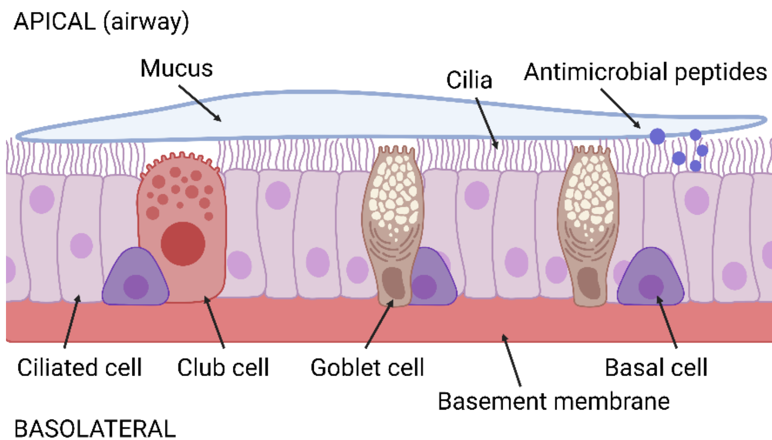

Figure 1.2. *Main cell types, structure, and protective functions of the central airway epithelium. [Created with BioRender.com]*

1.4. The epithelium and airway inflammation

As indicated above, the airway epithelium does not only serve as a barrier against the environment, but is involved in cross-talk with the immune system [115, 116]. In healthy airways, the epithelium contributes to maintaining homeostasis [107, 117], whereas in disease it can cause and/or drive the pathology [107, 117, 118]. During inflammation, the epithelium is affected by the mediators present, such as cytokines, leading to effects including a dysfunctional barrier, an altered composition of epithelial cell types, and changes in the epithelial secretome [119]. The epithelium can also respond directly to inhaled agents such as the asthma-associated rhinovirus [120, 121], and many genetic associations with airways disease have been linked to the epithelium [116, 122, 123]. For these two reasons, the epithelium may also be upstream in the disease process.

Several studies have investigated the role of the epithelium in inflammatory respiratory diseases, especially the T2 and T17 asthma endotypes described above, and the possibility of using epithelial gene signatures and epithelium-derived proteins for patient stratification and biomarker discovery [17, 56, 124]. The identification of a gene signature (*POSTN*, *CLCA1*, *SERPINB2*) and

periostin as a protein biomarker induced in airway epithelial cells in response to cytokines associated with T2 inflammation was a breakthrough in asthma patient segmentation [124], but the utility of serum periostin as a single clinical biomarker for T2 asthma has been limited [74, 125, 126]. A similar approach using gene expression patterns induced by Th2 and Th17 cytokines in airway epithelial cells (*in vitro*) was then used to demonstrate the reciprocal regulation of T2 and T17 inflammatory pathways in asthma [56]. The same was also found in a cohort study using the same epithelial T2 and T17 gene signatures, here the individuals with IL-17-high asthma had characteristics resembling psoriasis with epithelial dysfunction and upregulated antimicrobial response [127]. An IL-17A-induced epithelial signature has also been used to identify a subgroup of COPD patients who are unresponsive to steroid treatment [128]. These findings clearly demonstrate that signatures generated *in vitro* can be used to define human disease.

1.4.1. Effects of inflammation on the epithelium

In inflammatory airway disease, present cytokines and other inflammatory mediators affect the epithelium. These responses include alterations in cellular phenotypes of the epithelium, changes in secretion of mucus and other antimicrobial defenses, and damages to the airway epithelial barrier. In response to such insults, the epithelium releases alarmins, chemokines, and other mediators that can affect immune cells as well as other structural cells, thus contributing to disease processes.

1.4.1.1. Cell phenotypes, mucus, and antimicrobial defenses

One phenomenon in respiratory disease is airway remodeling, whereby the numbers and phenotypes of cells present across the airway wall are altered as a response to inflammation and damage, making the airways more rigid and less functional with regards to e.g. mucociliary clearance [104, 118]. Changes to the epithelial compartment during remodeling includes detachment of cells, impaired barrier function, fewer ciliated cells, and goblet cell hyperplasia [118]. Additionally, IL-6 and other asthma-related cytokines can induce senescence in epithelial cells which can further contribute to remodeling. Epithelial cells can, in addition to fibroblasts, produce extracellular matrix components, which can become skewed in response to T2 and T17 cytokines to promote fibrosis [104].

A cytokine implicated in airway remodeling is TGFβ, which classically is involved in immune regulation and is increased in asthma [129, 130]. Sources of TGFβ in the airways includes eosinophils and fibroblasts, moreover, alveolar macrophages, whose functions are altered in asthma, can activate TGFβ in its latent form [104, 118, 131]. It can also be released from the

epithelium in response to increased mechanical stress (which is associated with remodeling), damage, allergens, infection, and/or cytokines such as IL-13, IL-17, and IL-33 [104, 118, 129]. TGFβ then causes the airway remodeling by inducing growth and differentiation of the epithelium and activation of the epithelial-mesenchymal tropic unit [104, 130].

Goblet cell, or mucous, hyperplasia leads to increased mucus production and can be induced by T2 cytokines and TGFβ [104, 130]. A new epithelial cell state in the form of mucous ciliated cells has recently been identified, it was shown that T2 cytokines are behind this differentiation which ultimately leads to increased mucus production [132]. Excessive mucus production, mainly composed of the proteins MUC5AC and MUC5B, can also be caused by T2 cytokines as well as TNFα, TGFβ, and neutrophil elastase released by neutrophils [13, 118]. Periostin, a protein induced in epithelial cells by T2 cytokines, may further increase mucus production [133] in addition to its implications in fibrosis mechanisms [134]. This mucus hypersecretion has been found in both asthma and COPD and has been linked to increased coughing and exacerbations in patients along with lung function decline [110, 135-138].

In addition to pathogen-trapping mucus, epithelial cells provide antimicrobial defense through the production of e.g. antimicrobial peptides (AMPs) stimulated upon activation of toll-like receptors [139]. Examples of these are lysozyme, defensins, S100 proteins, and they can be grouped based on their antimicrobial mechanism of action [139, 140]. Additionally, chemokines CCL20 and CXCL14, which are produced by epithelial cells, have been shown to have antimicrobial functions [141-143]. The production of AMPs is impaired in T2 inflammatory diseases, whereby IL-4 and IL-13 cause a decrease in expression of their corresponding genes in epithelial cells, and decreased levels of AMPs have been found in airway samples from individuals with asthma and chronic rhinosinusitis as well as allergic rhinitis [140, 144-146]. On the other hand, cytokines can increase the expression of some AMPs, including CCL20 and β-defensin 2 which can be induced by both T2 and T17 cytokines, and S100A7, -8, and -9 which are induced by IL-17 [139, 140, 147, 148]. An increase in AMPs have been found in the sputum of COPD patients where this increase correlated with neutrophil levels [149], thus linking the ability of T17 cytokines to induce AMPs.

1.4.1.2. Epithelial barrier

The physical protection created by the epithelium is mediated by the epithelial barrier, made up by strong protein-protein interactions within anchoring junctions and desmosomes between neighboring cells [150]. The tight junctions are found near the apical side of the cells, where occludin, claudins

and junctional adhesion molecules (JAMs) anchor to cingulin and tight junction/zonula occludens (ZO) proteins in the cytoplasm [150]. Further below are the adherens junctions, comprised of the transmembrane protein E-cadherin which binds to p120-, β-, and α-catenin [151]. The desmosomes provide cell-cell adhesion and stability by anchoring the cytoskeletal intermediate filaments of neighboring cells through the proteins desmoglein, desmocollin, plakoglobin, and desmoplakin. The intermediate filaments also bind to the hemidesmosomes, including the integrin α6β4, which attach the cell to the basement membrane [152].

Several of these proteins are decreased or disorganized in asthma and COPD [153-157], making the barrier dysfunctional and more permeable. The importance of the barrier is supported by the findings of genetic associations between asthma and barrier-related genes, including *PCDH1, CDHR3,* and *ORMDL3* [123].

Specifically, it has been shown that IL-4 and IL-13 inhibits expression of occludin, claudin-18, ZO-1, E-cadherin, and β-catenin in airway epithelial cells, leading to an impaired barrier [158-162], an effect that may be mediated by histone deacetylases [157]. Additionally, mast cell chymase reduces expression of occludin, claudin-4, ZO-1, and E-cadherin [163], and eosinophil-derived exosomes from asthmatics cause epithelial cell apoptosis and impaired wound healing [164], which may cause further barrier damage. A barrier disrupting effect can also be caused by IL-6, TNFα, and IFNγ, whereas there are conflicting findings with regards to the effect of IL-17 on the airway epithelial barrier [165-169]. In a mouse study using models of eosinophilic, neutrophilic, or mixed granulocytic airway inflammation, all three were found to have decreased epithelial barrier proteins, with strongest effect seen in the neutrophilic model [170].

1.4.1.3. Epithelial alarmins, chemokines, and other mediators

Upon damage, challenge with virus, allergens, or a pro-inflammatory environment, the airway epithelium releases the alarmins TSLP, IL-33, and IL-25. These alarmins have been found to be upstream mediators of T2 airway inflammation and, thus, pursued as targets for monoclonal antibody treatment [171, 172]. There are also variants in the genes encoding TSLP and IL-33 that have been linked to asthma [173-175]. All three alarmins are constitutively expressed by the airway epithelium, and stimuli, such as the action of proteases, causes their release as well as increased production. Additionally, cytokines such as TNFα, IFNγ, IL-4, IL-5, and IL-13 can upregulate their expression [171, 176, 177]. Effects of the epithelial alarmins includes activation of eosinophils, stimulation of dendritic cells to promote Th2 cell differentiation, and to increase production of T2 cytokines. IL-25 prevents

Th17 cell differentation and IL-33 can also drive a Th1 response. One main function of epithelial alarmins is the ability to activate ILC2s, causing them to release large amounts of T2 cytokines, thereby promoting T2 inflammation [171, 178].

The epithelium also responds to inflammation by releasing chemokines which attract immune cells to the airways. One important chemokine in T2 inflammation is CCL26 which is a strong inducer of eosinophil migration and is highly expressed by the epithelium in response to IL-13 [179-181]. Another CC chemokine expressed by the epithelium is CCL20, mentioned above for its antimicrobial effects, which is induced by IL-17, IL-1β, TNFα, IL-4, and IL-13 [142, 182, 183]. The T2 cytokines have been shown to be weaker inducers of CCL20 [184] whereas the induction by TNFα may be enhanced by glucocorticoids [59]. CCL20 is chemotactic for Th17 cells and neutrophils [184, 185], supporting a role for this chemokine in the T17 endotype. Two additional neutrophil-attracting mediators produced by airway epithelial cells, and strongly induced by IL-17 and TNFα, are CSF3 and CXCL8/IL-8, both of which exhibited increased expression associated with severe asthma [55, 56, 186, 187].

An important biomarker of airway inflammation is the gas nitric oxide, which can be measured in exhaled breath and is an established clinical biomarker (fraction exhaled nitric oxide, FeNO) for the T2 endotype [188, 189]. Nitric oxide is produced by inducible nitric oxide synthase (iNOS/NOS2) which is upregulated in the airway epithelium in response to cytokines. The strongest effect on iNOS was from the T2 cytokine IL-13 [190, 191], hence FeNO being a marker for T2. Importantly, the production of nitric oxide can be repressed by corticosteroids, enforcing the usefulness of FeNO as a biomarker [192, 193].

1.4.2. Epithelial extracellular vesicles

In addition to single or soluble mediators released from the epithelium, a more complex signaling package comes in the form of extracellular vesicles (EVs), nano- to micro-sized vesicles that are released from cells. EVs have been found to be secreted from most cells studied so far and can be found in several body fluids such as bronchoalveolar lavage fluid (BALF), induced sputum, blood, and urine [194-197]. Classically, EVs have been categorized to three different classes depending on their release mechanism; exosomes, microvesicles, and apoptotic bodies. Exosomes, the most studied class, are 50-150 nm in diameter and are of endosomal origin as they are produced and stored in multivesicular bodies that when fusing with the plasma membrane releases the vesicles into the extracellular environment. Microvesicles are larger, 50-1000 nm in

diameter, and are formed by shedding from the plasma membrane. Apoptotic bodies are the largest, ranging from 50-5000 nm in diameter, and are a result from apoptotic cells that have undergone fractionation. EVs can also be classified depending on their size and/or density such as small EVs or light EVs, and further what surface markers they have. The surface markers and the lipid composition of the EV membrane may also reflect the origin of the vesicle [195].

EVs contain functional molecules such as proteins, mRNA, and microRNA, which can be shuttled between cells and organs and alter the recipient cell phenotype [195, 198, 199]. Furthermore, the EV cargo is representative of the EV-producing cell and may therefore serve as biomarkers of disease, a possibility which has been pursued in cancer research [195, 200]. The biological role of EVs and their potential as biomarkers in inflammatory airway disease is gaining interest and studied by several groups.

EVs isolated from the airways show differential cargo and properties between diseased and healthy individuals. This includes differences in cargo or their ability to induce production of inflammatory mediators or migration of immune cells [144, 201-206]. Epithelial cells are identified as a major source of extracellular vesicles in the airways [199, 204, 207-209]. The epithelium-derived EVs can be coated with mucins which may mediate their ability to neutralize influenza virus [199, 210]. Furthermore, the release of epithelial EVs has been shown to be increased following stimulation with IL-13 or upon mechanical stress as is present in airway remodeling [207, 209]. A recent study has shown that epithelium-derived small EVs can be coated with IL-33, providing a mechanism for release of IL-33 from non-necrotic cells [211]. This was also the second study showing that decrease of EV release using a small molecule inhibitor alleviated T2 inflammation in mouse models of airway disease [211, 212]. IL-13 treatment additionally can alter the miRNA cargo of epithelial EVs as well as their ability to attract immune cells [204, 207]. Epithelial cells stimulated with cigarette smoke was found to release increased numbers of EVs [213, 214], and additionally such EVs could enhance IL-8 release from naïve epithelial cells [215]. These findings suggest that epithelial EVs are important contributors to pathological processes in airway disease.

1.4.3. Translational regulation through miRNAs

Control of the production of all proteins discussed above can be mediated by miRNAs (microRNAs), ~22 nucleotide long RNA strands encoded in the genome and important post-transcriptional regulators of gene expression. miRNAs are expressed as pri-miRNAs that form a hairpin structure, this is then processed to a pre-miRNA that is transported from the nucleus to the

cytoplasm. There, the loop is cut off, generating a duplex consisting of the -5p and -3p forms of the miRNA, of which one strand is usually more biologically active than the other. Binding of the duplex with the protein Argonaute generates the silencing complex after release of one of the strands. The seed region of the miRNA then recognizes and binds to the 3' untranslated region (UTR) of their target mRNAs. This interaction classically causes degradation or translational repression of the mRNA, both leading to decreased levels of the encoded protein. Adding to this important regulation by miRNAs is the fact that one miRNA can target several mRNAs, and one mRNA can be targeted by several miRNAs [216]. Therefore, the role of miRNAs in pathological mechanisms in respiratory disease is gaining interest. In addition, miRNAs can be released to the extracellular environment where they are commonly found in extracellular vesicles (described in 1.4.2.) or bound to lipoproteins. This protects the miRNAs from degradation, making them attractive as biomarkers [217].

There are several studies that have established differential expression of miRNAs in individuals with versus without airway disease (reviewed in [218-221]). However, a limited number have focused on the epithelium [222-231], and even fewer have performed mechanistic studies. Involvement of miRNAs in mucus production and airway surface liquid composition has been shown in both asthma and COPD, with models supporting the T2 and T17 endotypes, respectively. A recent study showed by CRISPR/Cas9 knockout that miR-141, a member of the miR-200 family which is increased in asthma epithelium following allergen challenge, contributes to IL-13-induced goblet cell differentiation and, thus, increased mucus production [232]. In COPD, the airway surface liquid may be perturbed by cigarette smoke-induced miR-101 and miR-144, which target the chloride channel CFTR (cystic fibrosis transmembrane conductance regulator) [233].

Furthermore, miRNAs have been found to have both pro- and anti-inflammatory functions in the airway epithelium. In neutrophilic inflammation, miR-146a(-5p) was shown to be anti-inflammatory and decreased in asthmatic epithelium, whereas miR-629-3p was pro-inflammatory and increased in epithelium in severe asthma [234, 235]. The two miRNAs had opposite effect on the production of CXCL8 which is involved in promoting neutrophilia, and although miR-146a(-5p) was induced by IL-17A and/or TNFα as well as IL-4 *in vitro*, the reason for it failing to suppress inflammation in disease may be due to altered epigenetic regulation [235].

Although some studies have found that altered expression of miRNAs in the epithelium cannot be restored by corticosteroid treatment [226, 228], including

the miR-34/449 family which is repressed by IL-13 and decreased in asthma [226], evidence for the opposite has also been shown. This is the case for miR-320d which is increased in asthma in remission and in COPD after corticosteroid treatment [229, 236]. miR-320d was found to be anti-inflammatory through its ability to decrease production of GM-CSF (CSF2) and cigarette smoke extract-induced CXCL8 in airway epithelial cells [229, 236]. In eosinophilic asthma and in epithelial cells treated with IL-13, miR-181b-5p had decreased repression that could be restored with corticosteroids, this enabled targeting and decreased levels of SPP1 which further decreased expression of proinflammatory cytokines [224]. Additionally, six miRNAs were shown to be dysregulated in severe asthma and transfection of these into epithelial cells could recapitulate asthma characteristics, such as increasing levels of TNF and preventing glucocorticoid repression of inflammation [223], reflecting mechanisms and the limited response to corticosteroids in severe asthma. These studies highlight the important, yet complex, contributions of epithelial miRNA expression to airway disease pathogenesis, affecting both cell phenotype and inflammatory cascade.

2. Aim

The aim of this thesis was to establish airway epithelial signatures at different "omics" levels generated in response to disease-relevant stimuli *in vitro*, and to determine the presence of these signatures in patient cohort data as well as their involvement in disease-driving mechanisms.

Specific aims:

- Establish gene signatures and biomarker profiles in epithelial cells generated in response to disease-relevant cytokine stimuli and use such signature/-s for stratification of patients with respiratory disease.
- Isolate airway epithelium-derived extracellular vesicles and determine how their cargo and function is altered in the context of an inflammatory environment.
- Define the epithelial microRNA expression upon cytokine stimulation and how microRNAs could influence local airway disease processes.

3. Methods

The methods applied in the papers included in this thesis will be discussed here. For more details, please see corresponding section of the papers themselves.

3.1. *In vitro* model of the airway epithelium

The backbone of the work of this thesis relies on an *in vitro* model of the human central airways, consisting of an air-liquid interface (ALI) culture of normal primary human bronchial epithelial cells (HBECs). This model is established using cells isolated from the tracheal-bronchial region of deceased or living donors, the latter in the form of bronchial biopsies or brushings. The cells are then expanded in culture, usually up to passage 2, before being frozen or used immediately. At this stage, the cells mainly consist of basal cells, which are similar to stem cells and can be expanded and differentiated to the other cells that make up the pseudostratified epithelium of the central human airways [108]. The cells are seeded on Transwell culture inserts and remain covered with media until confluent, at which the media on the apical side of the cells is removed completely and the media on the basolateral side is changed to a differentiation media (**Figure 3.1.**).

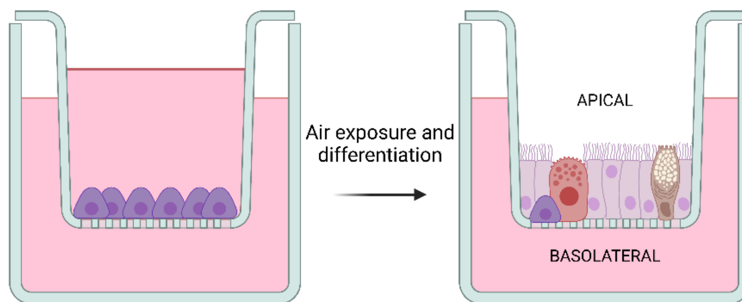

Figure 3.1. *Schematic over air-liquid interface culture of airway epithelial cells. [Created with BioRender.com]*

After 3-4 weeks at ALI, the cells have undergone full mucociliary differentiation, forming a polarized pseudostratified epithelium with ciliated cells, mucus-producing goblet cells, basal cells, and the junctional proteins between these cells create a strong barrier. These features can be assessed visually, using a simple light microscope, where the confluence of the cells, the movement of cilia, and the presence of mucus are visible. Additionally, the morphology and distribution of cells can be studied using histology and immunohistochemistry. Assessing the tight and adherens junctions and the barrier formed can be done either by an electrode, which measures the

transepithelial electrical resistance (TEER), or by fluorescently-labelled (usually fluorescein isothiocyanate /FITC) dextran, that is allowed to pass from the apical to basolateral side and measured using for example a plate reader [237] (see **Paper III** for detailed description). There are also more advanced methods for studying the cells such as confocal imaging and tracking cilia movement by high resolution video analysis [238].

When sampling ALI cultures, there are three main sources: conditioned media from the basolateral side, the apical side that can be washed using phosphate-buffered saline (PBS) or media, and the cells themselves which can be formalin-fixed or lysed in buffers for isolation of, for example, RNA or proteins (**Figure 3.1.**).

ALI cultures of primary HBECs have been shown to be highly similar to the *in vivo* airways on transcriptional, morphological, and phenotypic levels [108, 239-241], although there are indications that alterations to the cells, including e.g. perturbed miRNA expression, may occur during culturing [108, 242]. Still, supporting its validity, differences between individuals with and without disease can be observed in ALI cultures of cells from these individuals [108, 157, 243-245].

However, many features and functions of the airway epithelium *in vivo*, both in health and in disease, is dependent on direct or indirect interaction with other cells such as underlying fibroblasts and infiltrating immune cells [108, 117, 246]. Therefore, to fully recapitulate the human airway epithelium, co-culture models with for example fibroblasts, T-cells, or neutrophils have been employed and are under development [108, 237, 246-248]. However, as the aforementioned cells and the epithelial cells require different culture conditions, co-culture models are challenging and, to date, not the standard for the majority of labs studying the airway epithelium. Additionally, the ALI model itself is costly, time consuming, and sensitive to contamination since antibiotics/antimycotics are not always used. Also, the donor variation due to the primary origin of the cells needs to be considered. Finally, ALI cultures of primary airway epithelial cells have been known to be difficult to transfect or genetically modify to a required degree, but new methods are being established, especially through the use of CRISPR/Cas9 [232, 249, 250].

Some of the features related to the airway epithelium can also be recapitulated by culturing bronchial epithelial cell lines such as BEAS-2B and Calu-3, either as submerged monolayers or on Transwell inserts. The use of cell lines comes with a lower cost, is less time-consuming, has less variability, and allows for easier manipulation such as transfection of cells. However, there are

limitations when it comes to their ability to form a tight barrier and/or functional cilia [108, 237].

3.2. Modelling airway inflammation

The *in vivo*-mimicking properties of ALI cultures of primary HBECs also enables their use in modelling airway disease, yielding improved translatability of *in vitro* results [108, 251]. Therefore, diseases where the conditions are wholly or partly known can be modelled through these cells. The disease conditions can be in the form of, for example, mechanic pressure, allergens, viruses or viral mimics, and cigarette smoke [108, 209, 247, 252-254]. In this thesis, as in many other studies, the cells were stimulated with cytokines, which can be done both basolaterally and/or apically. Here, basolateral stimulation of ALI cultures was used, which is more technically feasible and suitable for longer stimulations, this would also reflect cytokines that are released from tissue-resident or infiltrating lymphocytes present basolaterally from the epithelial cells [119]. The cytokines were chosen to represent types of inflammation that are, or may be, present in individuals with respiratory disease such as asthma (see 1.2. and **Table 3.1.**).

Table 3.1. *Cytokines reflective of different types of inflammation used in stimulation of bronchial epithelial cells.*

Inflammation type	Cytokine/-s
Type 1	IFNγ
Type 2/T2	IL-4 + IL-13 or IL-5
Th17/T17	IL-17A + TNFα
IL-6TS	IL-6 ± sIL-6R
T regulatory	TGFβ1

In this project, the cells used were from donors without respiratory disease (normal HBECs), but alternatively, cells from asthmatic or COPD donors could have been used. However, as mentioned above, it is uncertain if all phenotypic features are preserved during culturing and differentiation. Additionally, when studying mechanisms and features related to different disease endotypes, the donor has to be well-characterized with regards to the present endotype, and such cells are difficult to obtain. Therefore, the use of normal HBECs provides a more general baseline upon which the disease is modelled through applied conditions.

3.3. Different levels of -omics analysis

For the establishment of epithelial signatures to be used for patient stratification or identification of mechanisms contributing to disease, different types, or levels, of -omics analysis have been used in this project and these are described below.

3.3.1. Transcriptomics of gene expression

In all studies within this thesis, epithelial gene, or mRNA, expression signatures have been used (see **Paper I** for details). These were established through basolateral stimulation of the HBEC-ALI cultures described in 3.1. and 3.2. using inflammatory cytokines for 24 hours. After this, cells were lysed in a phenol-guanidine based lysis buffer which ensures sufficient lysis and homogenization of the ALI cultured cells, which is otherwise difficult to achieve as the cells are tightly attached to each other and covered with a protective mucus layer. Additionally, this lysis method usually ensures a high yield of good quality RNA, which is needed for more advanced analyses.

To isolate the RNA, a commercial column-based kit was used, and after assessment of quality and concentration of samples, libraries for next-generation sequencing (NGS) were created and sequenced. NGS is a highly sensitive method for identification of nucleic acid sequences which can then be aligned to the annotated human genome, and the number of reads, or counts, per annotated sequence allows for quantitation of gene expression. NGS has become more widely available and high throughput, however it is dependent on specific instrumentation and knowledge in data processing. Alternatively, microarrays can be used for the same purpose. These are easier to use and analyze and usually less expensive depending on size and type of array, but they require a larger amount of sample, are biased with regards to which sequences they are designed for, and do not have the high resolution that NGS provides [255, 256].

Once the number of reads is obtained, algorithms and software are used to calculate differential expression for each cytokine stimulation by comparing it to the data from non-stimulated cells (see 3.6.1.).

3.3.2. Transcriptomics of miRNA expression

As described in the Introduction, miRNAs are gaining interest as important regulatory mediators, and possibly also biomarkers and targets, in respiratory disease. Compared to protein-coding genes/mRNA, the workflow for miRNA analysis is less established and still under development. Here, as detailed in **Paper III**, miRNAs were isolated from cells stimulated and lysed as described

above in 3.3.1. and transcriptomic analysis was done by NGS. NGS allows for quantification of known as well as possible novel miRNAs. The possibility of identifying novel miRNAs is one advantage of NGS compared to microarray analysis of miRNAs. Additionally, microarrays are dependent on hybridization between nucleotide strands which may be less efficient for miRNAs due to the shorter strands [257]. From the data, processing and calculations are done in a similar manner as gene-based NGS, see 3.6.1. for details.

3.3.3. Quantitative PCR (qPCR)

For validation of NGS data and for further experiments in this thesis, mRNA and miRNA expression were assessed by quantitative PCR using the Taqman system (Thermo Fisher). Isolated RNA is converted to complementary DNA (cDNA) through reverse transcription. The cDNA is then mixed with reaction buffer, Taq polymerase, and the Taqman assay for the target of interest or a housekeeping target allowing for relative quantification. The Taqman assay contains one forward and one reverse primer unique for the target and a probe that binds specifically to a sequence between the primers, usually spanning exons which reduces the risk of detecting genomic DNA contaminants. The probe has a fluorophore on one end and a quencher on the other so that when the Taq polymerase extends the primer by copying the annealing strand, the quencher is cleaved off allowing for detection of the target gene.

For each PCR cycle, the signal from the fluorophore is detected, allowing the generation of a curve from which the cycle where the fluorescence passes a threshold can be defined for each sample (the C_T). By using the C_T values for the target and the housekeeping gene (HKG) for both the treated and the untreated control samples, the differential expression defined as fold change (FC) can be calculated using the following equation:

$$FC = 2^{-ddC_T}$$
$$ddC_T = dC_T(treated) - dC_T(control)$$
$$dC_T = C_T(target) - C_T(HKG)$$

qPCR is a standard method in most labs, and the availability of pre-designed primers and probes makes it easy to use and reproducible between labs. With the use of different fluorophores it is possible to multiplex, allowing more data to be generated from a limited amount of sample. Disadvantages of qPCR include the possibility of incomplete reverse transcription when generating the cDNA, this is an issue especially for short RNA species such as miRNAs, where, additionally, the binding between cDNA and primers+probe may be limited.

3.3.4. Quantitative proteomics

For the isolated epithelium-derived extracellular vesicles (EVs) (see 3.4.), quantitative proteomics were applied in order to compare the protein cargo of EVs from different conditions. This work was done by the Proteomics Core Facility at the University of Gothenburg, and details can be found in **Paper II**.

The method used was Tandem Mass Tag (TMT) mass spectrometry (MS) which utilizes an isobaric labeling system that allows for quantitation of the peptides within a sample and relative quantitation between samples. Isobaric labels are chemical tags that contain atoms of different isotopes at certain sites whereas their overall mass is identical. After digestion of the protein samples, they are labeled using one unique tag for each sample and combined into a pool which is fractionated to reduce complexity and enables the identification of low-abundant proteins. The equal masses of the tags means that tagged peptides from the same protein elute together in the liquid chromatography that precedes the first MS, and further fragmentation of these peptides cleaves the tags, leading to different masses of the tagged peptides in the second MS, and thus the relative intensity of a single peptide in different samples can be obtained [258]. The pooling of samples thus allows for higher throughput and also eliminates variation that can occur if the samples are run separately. Additionally, pooling increases the total amount of protein which allows for better fractionation. For these reasons, the TMT method reduces time and cost. The peptide tags allow for relative quantification between samples, however, the results only contain proteins present in all samples, meaning that proteins that might be unique within a sample can not be detected using this method.

Using certain thresholds for the obtained data combined with fragmentation databases, which include the information about the TMT system used, peptides can be identified and relatively quantified. The fold change usually presented for this data is the same as the ratio between for example treated and control samples.

3.4. Isolation and characterization of extracellular vesicles

There are several different methodologies that can be applied for the isolation of extracellular vesicles (EVs). The choice is dependent on desired type, purity, and yield of vesicles as well as type and amount of sample, available infrastructure, throughput, down-stream application, and skillset. There are two aspects of purity when it comes to isolation of EVs: an isolated EV population which has low levels of contamination of non-EVs, such as soluble

Table 3.2. *Some different methods for isolation of extracellular vesicles (EVs) with their basis and outcome shortly summarized.*

Method	Basis	Recovery	Specificity
High speed ultracentrifugation	Single centrifugation step using very high speed.	High	Low
Polymer-based precipitation	Can be purchased as kits, particles of certain size range are trapped in polymer which is then pelleted at low g-force. Easy to use, good for small samples, but also traps e.g. lipoproteins.	High	Low
Differential centrifugation	Sequential centrifugation with increased g-force (ultracentrifugation), usually up to ca 100 000×g. Separation based on volume and other physical properties. High molecular weight proteins mixed with smaller EVs.	Intermediate	Intermediate
Size exclusion chromatography	Gel-packed column, sample passed through by gravity or by using a pump. Separates based on size.	Intermediate	Intermediate
Affinity capture	Beads coated with e.g. antibodies binding to antigens on the EV surface.	Low-Intermediate	Intermediate-High
Density gradient centrifugation	Sample loaded onto gradient with varying viscosity and then centrifuged, also high g-force. Separates based on density, removes soluble proteins.	Low	High
Centrifugal filters	Spin columns with filters that can be used at lower g-force.		
	Low molecular weight cut-off	High	Low
	High molecular weight cut-off	Intermediate	Intermediate
Microfluidics	Several different categories based on affinity capture, sieving, or trapping.	TBD	TBD

proteins, and/or an isolated EV population which has low variation with regards to EV characteristics, such as size [259]. Due to these aspects, each methodology has its pros and cons and prioritizing one aspect will most often compromise another. See **Table 3.2.** for summarized aspects of different methods (adapted from MISEV 2018 [259] and Cañas et al [260]), which can be used on their own or in combination with each other to achieve the desired outcome. However, as mentioned above, the choice of method/-s is up to each researcher and should depend on research question and application as obtaining an absolutely pure EV population is usually not necessary [259]. Therefore, characterization (described in 3.4.2.) of the obtained EV population is important in order to describe the outcome of the applied isolation methods and for drawing conclusions from downstream experiments.

3.4.1. Isolation

In this project, a sequential isolation protocol using density gradient centrifugation followed by size exclusion chromatography was used (see **Paper II** for details). This workflow was previously developed in the lab for the isolation of EVs from plasma samples [261], where the combination of methods that first separate based on density and then based on size allows for an EV population with low contamination of lipoproteins, soluble proteins, and protein aggregates. Since the method was developed for plasma samples, it was possible to isolate EVs from smaller volumes, but at the same time it is more difficult to scale up. Other drawbacks of the protocol used were that it is time-consuming and requires an ultracentrifuge.

To generate the material required for EV isolation, apical washes from an entire 24 well Transwell plate (see 3.1.) were pooled for each condition and larger aggregates and cell debris moved by short low-speed centrifugation. This means that fairly large amounts of cells and associated materials were required. Therefore, by finding that the stimulation-induced gene expression returned to baseline after one week in cytokine-free medium, one plate with cells could be used up to three times in order to isolate more EVs.

3.4.2. Characterization

In addition to the broader characterization of the isolated vesicles, in the form of quantitative proteomics as described in 3.3.4., the EVs were analyzed with regards to their size distribution and morphology, the number of isolated vesicles, as well as the presence or absence of vesicular or cellular markers. The results from these analyses gave information about what type of vesicle had been isolated, the yield, the degree of contamination, and what their possible role could be *in vivo*. The detailed methods can also be found in **Paper II**.

For size and number of vesicles, Nanoparticle tracking analysis (NTA) was used. This method is based on videos that capture light scattering from the particles, in this case vesicles, and a software then measures the Brownian motion of each particle. The speed of movement is related to the particle size which can then be calculated using the Stokes-Einsten equation [262]. There are several different methods for assessing vesicle size and concentration, each with their own strengths and weaknesses due to inherent properties that can lead to varying results [263]. NTA has a low limit of detection of around 70 nm in vesicle diameter while being fairly quick to perform [263], but it is less suitable for confidently determining the size of small vesicles.

Morphology of vesicles was studied by electron microscopy, this also provides information about size of vesicles and can identify very small vesicles, but is more labor intensive than NTA [263]. Additionally, electron microscopy can give information about purity of the isolated sample with regards to the presence of large amounts of protein aggregates and/or cellular debris that has not been successfully separated during isolation.

The presence, or absence, of certain vesicular and cellular markers are commonly assessed in order to characterize the isolated vesicles with regards to identity, purity, and, to some extent, origin. In this project, Western blot was used to detect the amount of the protein flotillin-1, which is part of lipid rafts and involved in vesicular trafficking [264] and is used as a marker for multiple types of EVs [259, 265]. Additionally, the isolated samples were analyzed for the endoplasmic reticulum marker calnexin, which should preferably be absent to indicate a sample free from contamination of cells [259].

3.4.3. Functionality of EVs

After quantitative proteomics of the isolated epithelial EVs (see 3.3.4.), the obtained data was analyzed by pathway analysis (see more details in 3.6.5.). Such analysis can provide suggestions into what possible role, or functionality, the EVs could have *in vivo* by indicating biological pathways and processes that may be affected by the differential levels of proteins in the EVs. Specifically, in this project, we were interested in how EVs from airway epithelial cells stimulated with T2 or T17 cytokines (see 3.2.) differed compared to EVs from non-stimulated cells, as well as to each other.

Among other findings, the pathway analysis suggested that neutrophil movement could be differently affected by the proteins in the EVs. As this could be important in the establishment of neutrophilia in T17 inflammation (see 1.2.2.1.), this was studied *in vitro* through the use of a chemotaxis chamber (see **Paper II** for details). The neutrophils used for these experiments were

isolated from peripheral blood of healthy volunteers using a magnetic bead-based system which binds to and removes all non-neutrophil cells. This method is quicker and easier than more traditional methods based on density gradient centrifugation, but may subject the sensitive neutrophils to stress that could activate them causing the release of neutrophil extracellular traps [266]. Once isolated, the neutrophils were added to the upper wells of a micro chemotaxis chamber, separated from the lower wells by a thin 8 µm membrane, coated in gelatin to allow for the migration of the neutrophils [267]. This chemotaxis system has small-volume chambers (50 µl in the lower wells, 30 µl in the upper wells) that reduces the amount of material needed for the experiment. This low volume was beneficial in this project where the EVs added to the lower wells were of limited supply. However, the small volumes, the overlay of the thin membrane, and the final assembly of the chamber system introduces the risk of potential errors through varying volumes, overflow, bubbles, and holes in the membrane. Additionally, the number of migrated cells were counted manually which takes time and neutrophils are short-lived. In an attempt to handle this, only one neutrophil donor per day was studied, four replicates of each condition were used with the conditions separated by empty wells, and the samples were transferred to individual tubes before counting to avoid evaporation.

3.5. Functional analysis of miRNAs

To validate interaction between miRNAs and their mRNA targets, as identified by target prediction (see 3.6.4.), two methods, pull-down and transfection, were used in this project.

3.5.1. Pull-down experiments

In a pull-down experiment the aim is to validate that a miRNA of interest directly binds to a predicted mRNA, or simply an mRNA of interest, in a specific cell type. Here, this was done using a protocol adapted from Gao et al [268] (see **Paper III** for details). Cell lysates from HBEC-ALI were incubated with miRNA mimics containing a biotin tag, the mimics along with any mRNA bound to them are then pulled down using magnetic beads coated with streptavidin. The RNA can then be isolated and the mRNA analyzed by qPCR as per normal (see 3.3.1. and 3.3.3.). The fold change of the analyzed mRNA is normalized against a negative control mimic, thus allowing quantification of the enrichment achieved through the pull-down. The drawbacks of this method includes that the RNA yield is low and that follow-up experimentation is needed to confirm the physiological importance of the miRNA-target interaction.

3.5.2. Transfection of miRNA mimics

If a binding interaction between a miRNA and an mRNA has been confirmed by pull-down as described above, the downstream effect on the mRNA can be analyzed by transfecting cells with the same miRNA mimic. A decrease in level of the mRNA in transfected cells compared to a control then indicates that the miRNA has caused degradation of the targeted mRNA. Alternatively, the level of translated protein from the mRNA can be analyzed where a decrease would indicate an effect from the miRNA on either mRNA degradation or inhibition of translation.

As ALI cultures of HBECs are difficult to transfect [250], in this project, a simplified model of the airway epithelial barrier based on HBECs was developed (see **Paper III** for details). Here, the cells are kept covered with differentiation medium from day 4 after seeding on the Transwells rather than exposing them to air. At day 6-7, the cells have formed a strong barrier, but in-house experiments thus far, indicate that they are still amenable to transfection. This is likely due to the cells remaining in a basal cell state, similar to the standard 2D/submerged model of HBECs where transfection is less of a challenge. However, as the cells have not differentiated, their phenotype with regards to miRNA expression was found to be different and therefore transfection of antagomirs in addition to mimics was not pursued.

3.6. Bioinformatics/*in silico* and statistical analyses

The emergence of "big data", to which -omics data belongs, comes with a need for advanced analysis methods using algorithms and databases that together can be described as bioinformatics. These tools enable, for example, indication of cellular or molecular pathways that may be differentially affected under certain conditions and the clustering of samples or patients in subgroups with shared characteristics. The results can then be used for hypothesis generation and design of new experiments as well as identification of novel, or more specific, drug targets and biomarkers for a certain disease or patient subpopulation.

For several steps in such analyses, included some of those described below, the software R [269] was used in this work. R is a software environment for computational analysis as well as creation of graphics of data for which a large number of algorithms have been developed. This includes processing and differential expression calculation of NGS data, and these algorithms can be combined with the user's own coding into scripts.

3.6.1. Differential expression

For the NGS data acquired in this project, differential expression, expressed as fold change (usually log2 transformed), of each mRNA/miRNA per stimulated condition versus the control condition was calculated in R using the DESeq2 package [270]. For data with a large number of variables, as in NGS where each mRNA /miRNA is a variable, multiple-comparison analysis is used to identify variables that are significantly differentially expressed. In DESeq2, Benjamini-Hochberg correction is used which calculates the adjusted *p*-value, or *q*-value, as a so-called false discovery rate (FDR), where a value less than 0.05 is seen as significant. This means that if 100 variables are differentially expressed, one can expect, or assume, that 5 of them are false positives.

3.6.2. Heatmaps/hierarchical clustering

A common type of graphical visualization of data with many variables is in the form of heatmaps. This allows for easier interpretation of data, especially when it is combined with some sort of clustering method. Clustering reveals patterns and groups of variables and/or conditions based on, for example, differential expression data. To generate this, R, GraphPad Prism, and TIBCO Spotfire were used in this project. Of these softwares, GraphPad Prism is the simplest to use whereas R is more difficult. R and Spotfire can both be used for hierarchical clustering using different methods, however the latter is easier to use.

Hierarchical clustering entails grouping, or clustering, together samples or variables based on shared similarities. This can aid in identification of subgroups of patients or what variables are most different between groups of samples. To do this, the distance between any two observations (samples or variables) in a multidimensional space is calculated using a metric based on the type of data. In this project, the Euclidean distance was used, which is common for continuous variables such as gene expression. Next, the pairwise distances are used to calculate the distance between sets of observations using a linkage criterion. Here, the commonly used unweighted average linkage clustering (UPGMA) was applied, where the average of the distance between all pairs within two clusters is calculated. The clusters are then commonly presented using a dendrogram, a tree-like structure with branches, which can be combined with the heatmap.

3.6.3. Principle Component Analysis

Another way to visualize data with a very large number of variables, which also shows clusters on either sample or variable level, is Principle Component Analysis, or PCA. PCA is a means of dimension reduction of a large data set

where each variable can be regarded as a dimension and therefore graphical representation by plotting all variables against each other is impossible beyond three variables. The dimension reduction is performed by the creation of a new coordinate system with vectors reflecting the pattern of the data. These vectors, or principle components, then become new variables onto which the original data is projected. The first principle component should explain as much of the variance of the data as possible and thereafter decreasing with each one. This usually leads to the possibility of using the first 2-3 principle components for plotting in a 2D or 3D graph respectively, and allows for the visualization of clusters of samples or variables that have shared properties. The principle components can also be used for further calculations and modeling, with using more components than 2-3 to explain more of the variation of the data [271, 272].

3.6.4. miRNA target prediction

As described in 1.4.3., miRNAs are post-transcriptional regulators of protein expression where one miRNA can target several mRNA molecules and one mRNA molecule can be targeted by several miRNAs. It is therefore of interest to identify the targets of miRNAs in order to perform pathway analysis (see 3.6.5.) or to select miRNAs that target a gene, or set of genes, of interest. There are several databases available that provide lists of miRNA targets, in this project miRDB [273], TargetScan [274], and miRWalk [275] were used. The information held in these databases is based on either experimental data (usually luciferase-based assays), analysis and calculations of sequence complementarity and strength of interaction between the miRNA and the mRNA, and/or transcriptomics data. Experimental data is usually considered more trustworthy, whereas the latter methods provide more predicted, or putative, targeting.

3.6.5. Pathway analysis

An additional method to draw conclusions or generate hypotheses from omics data is by performing pathway analysis. By having lists of protein-coding genes/miRNAs/proteins, pathway analysis tools can provide information on which biological processes, cellular compartments, diseases, upstream/downstream regulators, or signaling pathways with which these components are associated. If many of the components from the list are associated with a certain pathway, that pathway is considered to be enriched and can be of biological importance. Adding differential expression of these components can then help to further indicate whether these pathways are activated or inhibited under the condition from which the data was obtained. These results can then form the basis for further experiments looking more closely at the pathways suggested/identified.

There are several tools available for pathway analysis, both online versions and commercial software. They all rely on their own knowledge base comprised of collated data and networks covering the pathways, their components and functionality. Choosing which tool to use depends on objective, skills, and cost. Usually there are filters or settings to be used when performing the analysis which can affect the size, trustworthiness, and usability of the output.

3.6.6. Statistical analyses

To aid in drawing conclusions from data, different types of statistical analyses are commonly applied in all areas of science. The most commonly used analyses will test the probability that the null hypothesis - that there is no difference between groups - is true. Generally, a *p*-value of less than 0.05 is considered low enough to be able to reject the null hypothesis, meaning that there is most likely a difference between groups. There are many statistical tests and models that can be used and the choice depends on properties of the data, such as if there are two or more groups to be compared or if the observations are repeated measurements. Depending on the size of the data and how it was collected, additional tests, such as multiple-comparison analysis described under 3.6.1., are usually applied.

The calculations compare the mean value and variation of the group data and, therefore, a minimum of three replicates per group are needed in order to calculate the variation. Ideally, since these statistical tests are based on the assumption that the collected data is representative of a larger population, the more replicates performed the better.

In this project, for data obtained from methods other than NGS, ANOVA, analysis of variance, was applied. ANOVA is a generalisation of the *t*-test and suitable for comparison between the means of two or more groups. These analyses were performed in GraphPad Prism which guides the choice of model, including tests for multiple comparison, depending on the nature of the data and what comparison/-s that are to be made.

4. Results and Discussion

The main findings of **Papers I-III** are summarized and discussed in this chapter. Unpublished results of relevance are also included.

4.1. Inflammatory cytokines induce unique gene signatures in airway epithelial cells

Since the airway epithelium is now recognized to have a significant role in the immune system and in respiratory diseases [115, 117], where it responds to mediators present during inflammation [119], it has become intensively studied with the purpose of identifying disease mechanisms and biomarkers for patient stratification. Seminal studies by Woodruff, et. al. and Choy, et. al. showed that gene signatures generated from cytokine-stimulated airway epithelial cells *in vitro* can be used for this purpose [38, 56, 124]. This led us to the aim of establishing gene signatures from cells stimulated with cytokines reflective of different types of immune responses that may be involved in airway disease, including the T2 and Th17 cytokines used by Woodruff and Choy. These signatures could then be used to identify patients with subtypes of asthma or COPD as well as serve as a source to identify disease-driving mechanisms.

For this purpose, we used the well-defined, *in vivo*-mimicking, model of air-liquid interface cultured primary human bronchial epithelial cells (HBEC-ALI). The cells were stimulated with cytokines reflective of Type 1 (IFNγ), Type 2/T2 (IL-4+IL-13 or IL-5), Th17/T17 (IL-17A+TNFα), IL-6TS (IL-6±sIL-6R), or T regulatory (TGFβ1) immune responses. Gene expression signatures were then established using next-generation sequencing, comparing stimulated versus non-stimulated cells.

The induced genes under each stimulation contained several genes unique to each signature as well as protein-coding genes known to be related to the modelled immune responses, thus validating the experimental set-up (**Figure 4.1.**). The IFNγ-induced genes included the chemokines *CXCL9*, *-10*, and *-11*, which bind to the receptor CXCR3 and can recruit lymphocytes, mainly Th1 cells and cytotoxic T cells [276]. A subtype of severe asthma presenting with Type 1 inflammation has been found and these individuals had higher levels of CXCL9, CXCL10, and CXCR3 in the airways [277]. In COPD, these three chemokines are increased [278] and levels of CXCR3-expressing T cells have been shown to be different between males and females, suggesting a possible role for this axis in sex differences that are associated with variations in disease severity [279].

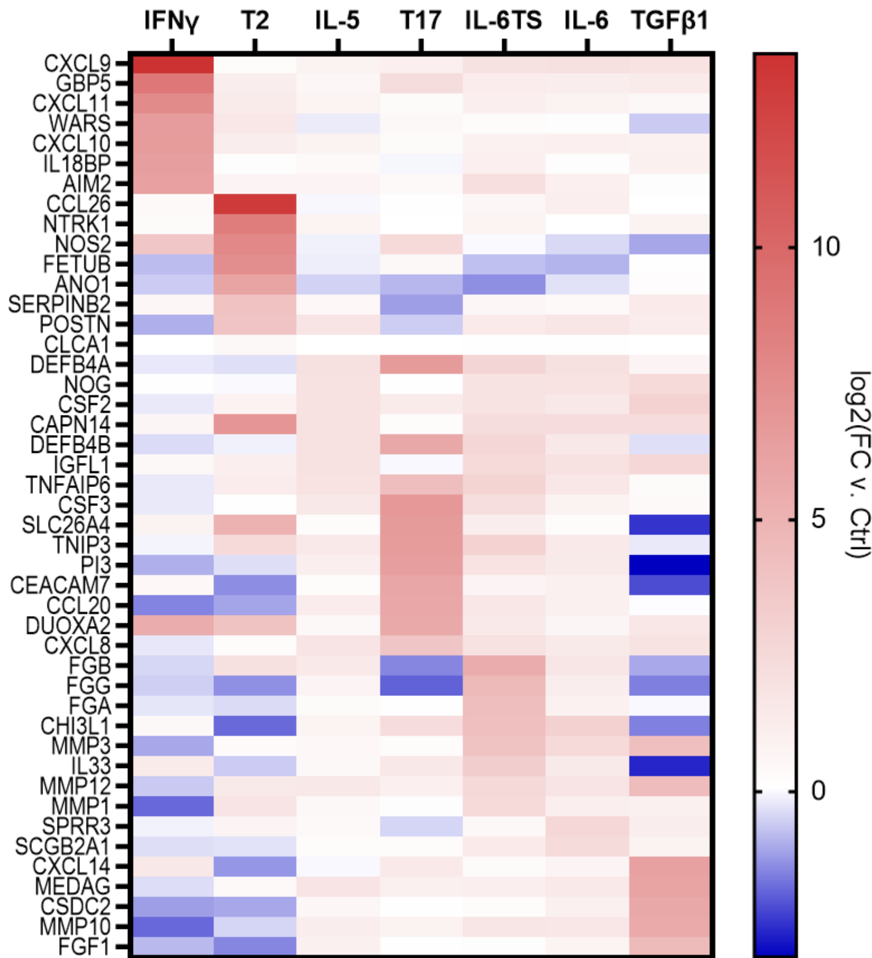

Figure 4.1. *Gene expression signatures induced by cytokines in primary human airway epithelial cells cultured at air-liquid interface. Data presented as log2 of fold change versus control/non-stimulated. [T2, T17, IL-6TS, and IL-6 signatures are part of results from* ***Papers I-II****, remaining is unpublished data]*

The most increased gene upon IL-4+IL-13 (hereafter called T2) stimulation was *CCL26*, a strong eosinophil-attracting chemokine known to be induced by IL-13 [180, 181], followed by *NOS2*, which produces reactive nitric oxide that can be used as a marker for T2 inflammation [189, 190]. Additionally, *SERPINB2*, *POSTN*, and *CLCA1*, as part of the T2 signature established by Woodruff et al [124], were also increased, albeit *CLCA1* did not reach statistical significance. The chloride channel CLCA1 is involved in mucus production, and recent studies have shown that this function, as well as the

expression of MUC5AC, is dependent on anoctamin 1/TMEM16A, which we also found to be increased on gene level (*ANO1*) by T2 cytokines [280, 281]. Further, T2 cytokines caused upregulated expression of *FETUB*, encoding fetuin-B, a protease inhibitor with implications in inflammation where increased levels has been linked to eosinophilia and disease severity in COPD [282, 283].

For IL-5, the overall expression of induced genes were lower than for IL-4+IL-13. However, the most increased protein-coding gene was *DEFB4A*, followed shortly by *DEFB4B*, paralog genes encoding the AMP β-defensin 2 which is induced by inflammatory conditions and can activate dendritic cells [139]. Similarly increased was *NOG*, encoding the protein noggin, which is involved in organ development through inhibition of bone morphogenic protein, this inhibition promotes epithelial cell proliferation during airway repair [284, 285]. This could thus cause hyperplasia of epithelial cells, however when this inhibition is lacking, the phenotype of the cells could become abnormal [286], highlighting the importance of accurately controlling developmental pathways. There was also increased expression of *CSF2*, or GM-CSF, a stimulator of production, activation, and survival of neutrophils and eosinophils [287] that is increased in asthma and COPD, especially in severe asthma where it also correlated with sputum eosinophilia [288].

Under T17 inflammatory conditions, modelled by IL-17A+TNFα, we found, similar to Choy et al [56], that the most induced gene was *CSF3*, and there was also increased expression of *CXCL8*/*IL8*, both involved in attracting and stimulating neutrophils and implicated in severe asthma [186]. Another mediator chemotactic for neutrophils which was induced by the T17 cytokines is *CCL20*, which also attracts Th17 cells [184, 185]. The second most induced gene was *SLC26A4*, encoding the anion transporter pendrin, which was also increased by T2 cytokines. Pendrin is upregulated in mouse models of asthma and COPD as well as individuals with allergic rhinitis, chronic sinusitis, and asthma [289-291]. It has been shown to be involved in mucus production and airway hyperresponsiveness, effects which can be diminished by small molecule inhibitors [289, 292].

IL-6 ± sIL-6R increased the levels of *CHI3L1*, or YKL-40, a chitinase-like protein which has been implicated in lung function, inflammation, and remodeling, and increased YKL-40 is associated with resistance to steroid treatment [32, 293-296]. A polymorphism in the *CHI3L1* gene has been associated with risk of asthma as well as increased levels of the protein [297]. Increased levels have been found both locally and in circulation in multiple studies of asthma and COPD where it has been associated with increased severity and exacerbations as well as non-T2 inflammation [31, 32, 88, 293-

296, 298, 299]. CHI3L1, together with periostin or eosinophil-derived neurotoxin, may function as markers for asthma-COPD overlap [298, 300]. Additional remodeling-related genes induced by IL-6TS includes the matrix metalloproteinases *MMP1*, *-3*, and *-12*, as well as the epithelial alarmin *IL33*, which have all been implicated in airway remodeling in severe asthma and COPD [88, 301-304].

Being involved in airway fibrosis and remodeling, TGFβ1 also caused increased expression of *MMP3*, *-10*, and *-12*, as well as fibroblast growth factor *FGF1* which increases epithelial and airway smooth muscle cell proliferation and is increased in the bronchial epithelium in COPD [305, 306]. Furthermore, TGFβ1 induced the chemokines *CXCL13* and *-14*, which are chemoattractants for various immune cells and have been associated with asthma [307, 308]. CXCL14 may also be involved in airway remodeling and fibrosis due to its effect on cellular growth and fibrogenesis [309-311] in addition to its previously mentioned function as an antimicrobial peptide [141].

The expression of a selection of the top genes induced by the different stimuli was validated by qPCR. Additionally, the secretion of the corresponding protein was also increased, as analyzed by enzyme-linked immunosorbent assay (ELISA) (**Figure 4.2.**). One limitation of the sequencing described above is that it was only performed in a single donor, as was some of the validation work. However, the experiments were repeated multiple times and for the T2, T17, and IL-6TS signatures, gene and protein expression was validated in multiple donors (see **Paper I** and **Paper II**). Of special interest, the T2 and T17 gene signatures were found to be highly correlated to the protein content of EVs released from stimulated epithelial cells, as identified by proteomics in **Paper II**. This thus also serves as validation of our gene signatures.

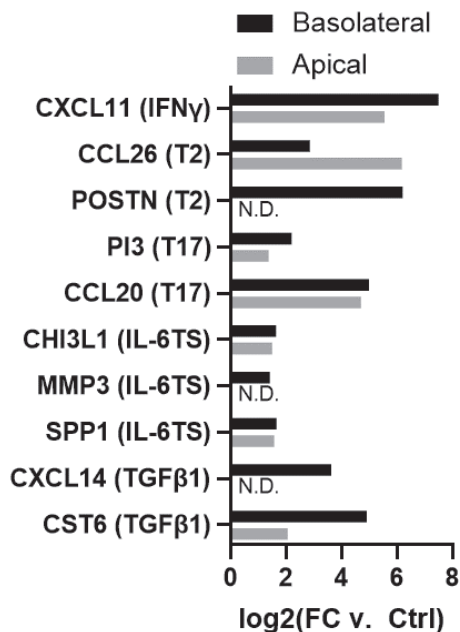

Figure 4.2. *Cytokine stimulation increases the release of proteins corresponding to induced genes to the basolateral and/or apical compartments. Data presented as log2 of fold change versus control. [Unpublished data]*

Altogether, the gene signatures induced by cytokines in HBEC-ALI are reflective of the modelled immune responses and replicate previous findings of cytokine-induced signatures in airway epithelial cells as well as provide novel signatures that can be further analyzed. Moreover, they showcase the plethora of mediators expressed by the airway epithelium in inflammatory settings, supporting its important role in homeostasis as well as in disease. Additionally, the gene signatures indicate mechanisms and pathways that may be unique for a specific inflammatory endotype. Thus, these signatures may be used in the future for patient stratification approaches as well as the development of novel specific treatments and biomarkers. This is exemplified in the following sections with results from **Paper I**, **II**, and **III** where the T2, T17, and IL-6TS signatures have been used.

4.2. Epithelial IL-6 trans-signaling signature is enriched in a subpopulation of asthma patients

Due to the increased levels and implications of IL-6 and its soluble receptor (sIL-6R) in asthma (see 1.2.2.2.). In **Paper I** we set out to determine if a subgroup of asthma patients have airway inflammation related to the combined signaling of IL-6 and sIL-6R, in the form of IL-6 trans-signaling (IL-6TS), and if this signaling could be identified using an epithelial signature.

To identify genes unique for IL-6TS, the IL-6TS and T2 gene signatures, established as described in 4.1., were compared against each other, showing that the majority of genes were non-overlapping. The most induced genes under IL-6TS were then used for hierarchical clustering based on epithelial transcriptomic data from 103 individuals with asthma from the U-BIOPRED cohorts, where 8 of the genes (*TNFAIP6*, *PDE4B*, *IL1R2*, *S100A9*, *S100A8*, *S100A12*, *CHI3L1*, and *SPP1*) co-clustered and defined a subset of 17 patients where these genes were increased (Figure 2A in **Paper I**). This subset, referred to as IL-6TS-high, did not have higher levels of circulating IL-6 and sIL-6R (Figure 2B in **Paper I**), indicating that the increased genes are due to local pathway activation. A similar cluster of patients was found in the Wessex Asthma Cohort, using sputum levels of proteins corresponding to 5 genes related to the IL-6TS signature (CHI3L1/YKL-40, MMP3, CXCL8/IL-8, CCL4/MIP-1β, and IL-1β, Figure 6 in **Paper I**).

Clinically, the IL-6TS-high subset was distinct from the remaining individuals through the experience of frequent exacerbations, suggesting that something up- or downstream of IL-6TS is driving exacerbations. The link between exacerbations and IL-6 has been observed before [69]. However, depending on if increased IL-6 is present in circulation or locally in the airways, the

mechanisms behind may differ, since circulating IL-6 is a marker for systemic inflammation that may be due to comorbidities [71, 77]. The increased exacerbations related to IL-6TS in the airways may be due, in part, to the higher numbers of T cells and macrophages in the submucosa of these patients, which is in line with the finding that IL-6TS promotes immune cell infiltration [78]. In the IL-6TS-high U-BIOPRED subset, there were more IL-6TS-high patients that had high levels of blood and sputum eosinophils than the rest of the cohort, despite low expression of the T2 signature genes *POSTN, CLCA1,* and *SERPINB2*. However, in the IL-6TS-high Wessex subset the levels of sputum neutrophils was also increased. Although high IL-6/sIL-6R, especially in the airways, has mostly been linked to neutrophilia [70, 72, 312], there are studies showing no difference with regards to granulocytic phenotype [75, 93, 94]. In the subsets studied here, the higher eosinophil levels may be related to the IL-6TS-induced expression of epithelial IL-33, which can activate and stimulate eosinophils [313, 314].

Mechanistically, we found evidence for IL-6TS causing decreased epithelial barrier function. This was demonstrated *in vitro* by a decrease in transepithelial electrical resistance (TEER) and in the epithelium of the IL-6TS-high subset there was decreased expression of barrier-related genes such as *CLDN18*, a lung specific claudin previously linked to asthma [315], and *TJP1*/*ZO-1* (Figure 4 in **Paper I**). Thus, an impaired barrier may be involved in driving disease in IL-6TS-high patients, where the increased exposure of subepithelial tissue to inhaled pathogens may promote inflammation and exacerbation. The possible role of pathogens may also be the upstream cause of IL-6TS, as the IL-6TS-high subset had increased expression of genes related to Toll-like receptor (TLR) signaling (e.g. *TLR2*, *TLR4*, *MYD88*, *CXCL8/IL8*, *CCL4/ MIP-1β*, and *IL6*). TLRs are activated by pathogen-associated molecular patterns and infection has been shown to cause the release of sIL-6R, most likely from macrophages and neutrophils [70, 83]. The link between increased airway bacterial colonization and local IL-6TS was also seen in a cluster of both asthma and COPD in a larger cohort study [26]. Recently, we have also shown that an IL-6TS-high subset can be found among COPD patients that also had colonization of *Haemophilus*. Furthermore, *Haemophilus influenzae* caused increased release of sIL-6R from neutrophils, possibly through formation of neutrophil extracellular traps [316].

In summary, we have shown that a subset of patients with asthma, and also COPD, display activation by IL-6TS (IL-6+sIL-6R) in airway epithelium. The local source of sIL-6R may be neutrophils responding to infection and the resulting IL-6TS-induced airway epithelial barrier dysfunction could expose underlying tissue to infectious pathogens. These pathogens cause inflammation and propagation of the cycle with release of more sIL-6R. The

increase in exacerbations in patients with IL-6TS activation suggests that this group may benefit from treatment decreasing the activity of this pathway.

4.3. Extracellular vesicles can be isolated from the apical side of airway epithelial cells

Extracellular vesicles (EVs) have been shown to carry unique signatures in airway diseases, as well as have functional roles in disease-driving mechanisms. Additionally, the epithelial cells have been identified as a key source of EVs in the airways (see 1.4.2.). Therefore, in **Paper II**, we wanted to isolate and characterize EVs released from our HBEC-ALI cultures in order to study how these may differ in, and contribute to, inflammatory airway disease.

To do this, apical washes and conditioned basolateral media were collected from plates with HBEC-ALI cultures and EVs were isolated using a protocol developed in our lab for the isolation of EVs from plasma [261]. Reasons for using this protocol was the similarity between sample sizes. Additionally, the sample matrices may share similarities in the form of the presence of aggregated proteins such as mucins in the apical washes. The protocol is based on sequential use of different types of isolation techniques in order to yield a pure population of EVs. First, the sample is layered on a density cushion, which after centrifugation yields a band containing vesicles, thereby separating these from the majority of protein aggregates and cell debris. This band is then loaded onto a size exclusion chromatography (SEC) column, where the EVs elute earlier than soluble proteins based on their size difference. Eluted fractions containing EVs can then pelleted by ultracentrifugation depending on downstream application.

Analysis of the fractions eluted from the SEC showed that no EVs could be isolated from the basolateral compartment of the HBEC-ALI cultures, the same was found when attempting to isolate EVs using ultracentrifugation as a single method. This was surprising to us as there are reports on basolaterally released EVs from HBEC-ALI cultures [204, 317]. Possible reasons for our findings are only speculative, but perhaps the combination of the filter of the Transwells and extracellular matrix produced by the epithelial cells in our culture conditions prevent the release of EVs into the basolateral media. However, EVs from the apical side of epithelial cells may be reflective of EVs present locally in the airways, where they might be involved in homeostatic or disease-causing processes, functions that have been suggested previously [201, 208]. Additionally, the apically released EVs isolated here may be similar to those

isolated from airway-derived samples such as BALF and induced sputum [196, 197, 201, 202, 205], thereby possibly having a role as biomarkers.

The vesicles were found in fractions 7-12 from the SEC and were pooled. The vesicles were then thoroughly characterized, showing that they were ≤ 100 nm in diameter, free from large protein aggregates and cell debris, and positive for the vesicular marker flotillin-1, but negative for the endoplasmic reticulum marker calnexin (Figure 2 in **Paper II**). Additionally, we could show that the EVs contain RNA shorter than 200 nucleotides, however, the properties related to this content is yet to be determined.

Next, we used proteomics to define the protein cargo of our isolated EVs. This analysis identified >1300 proteins, out of which 100 were top-ranking EV-associated proteins as listed in vesicle databases. More than half of the total proteins were associated with the term "extracellular exosome" when gene ontology analysis were performed. Additionally, terms related to the endosomal pathway were associated to the epithelial EV proteome. Thus, these findings altogether show that we have isolated a pure population of small EVs from the apical side of polarized airway epithelial cells. Moreover, our data suggests that at least a part of the EVs are of endosomal origin, as is the case for EVs classified as exosomes.

4.4. T2 and T17 cytokines differentially alter the protein cargo and functionality of epithelial EVs

To establish how stimulation mimicking T2 or T17 inflammation alters the epithelial EVs, we isolated and characterized EVs isolated from cells stimulated with T2 (IL-4+IL-13) or T17 (IL-17A+TNFα) cytokines (**Paper II**). The size and analyzed protein markers of these EVs were similar to those released from unstimulated control cells. Interestingly, the number of released EVs was higher for both types of stimulation compared to control. This is in line with previous studies showing an increase in the number of EVs released from epithelial cells after treatment with IL-13, as well as cigarette smoke extract and hydrogen peroxide [207, 213-215, 318], and indicates that the epithelium responds to a non-homeostatic environment by releasing more EVs that may further affect other cells [207, 214, 215].

The epithelial cells do not only respond to cytokines by releasing more EVs, the protein cargo of these EVs is also altered as determined by quantitative proteomics. Principle component analysis of the proteins in the EVs released from T2 or T17 stimulated epithelial cells show that their cargo differs from that of EVs from control cells as well as from each other (Figure 5A in **Paper**

II). Additionally, as mentioned in 4.1., this protein cargo correlated to the corresponding gene expression signatures which were largely non-overlapping.

For T2-derived EVs, the most abundant protein was NOS2, producer of the T2 biomarker nitric oxide. Biochemically functional NOS2 has been identified in nasal lavage EVs [144], showing that EVs may contribute to the levels of nitric oxide in the airways. We further found decreased levels of the antimicrobial protein S100A7 in these EVs, S100 proteins are decreased in allergic diseases [144, 146], possibly making these individuals more susceptible to infections.

In T17-derived EVs, neutrophil-stimulating CSF3 was the most increased protein, indicating a possible role for EVs in neutrophilic inflammation. Pendrin (SLC26A4) was found to be increased in both types of EVs, as was the case on gene level in the cells. As pendrin is involved in mucus production, it could thus be involved in this process regardless of endotype. This demonstrates that EVs isolated from T2 or T17 stimulated epithelial cells contain protein signatures that may distinguish between T2- or T17-driven inflammation.

To further identify what roles the EVs from cytokine stimulated airway epithelial cells could have in disease-related processes, we performed pathway analysis on the EV proteomes. This revealed that proteins in EVs from cells stimulated with the T17 cytokines, such as CSF3, TNFAIP3, TNIP1, and SAA1, are involved in pathways related to neutrophil recruitment. These pathways were, thus, predicted to be activated, whereas the opposite was seen in the analysis of the proteome from EVs from T2-stimulated cells.

This was further confirmed *in vitro* by using a chemotaxis chamber, where T17-EVs promoted migration of more neutrophils than did the T2-EVs (Figure 6C in **Paper II**). Interactions between airway EVs and immune cells have been shown previously, including the ability of nasal EVs to induce migration of monocytes, neutrophils, and NK cells *in vitro* [144]. Specifically for airway epithelial EVs, these may induce chemotaxis of macrophages and neutrophils and affect the functions of dendritic cells (reviewed in [319]).

Additionally, epithelial EVs were released in increased numbers in models of acute respiratory distress syndrome and could induce a pro-inflammatory phenotype in alveolar macrophages, furthermore, these EVs could cause disease characteristics when administered to naïve mice [320]. Likewise, a pro-inflammatory phenotype in macrophages was also induced by EVs released by airway epithelial cells in response to *P. aeruginosa* [321].

Altogether, these results show that bronchial epithelium-derived EVs reflect an inflammatory environment and further that these EVs may be involved in processes contributing to airway disease, such as nitric oxide and mucus production as well as communication with immune cells, such as promoting migration. As the EVs in our study were isolated from healthy donor cells stimulated with endotype-reflective cytokines, it would be interesting to compare them with EVs isolated from epithelial cells of well-characterized individuals with asthma. Such results, together with our data, could shed more light on the role of epithelial EVs in airway disease and how they could be exploited to develop improved treatments.

4.5. Sequencing identifies differentially expressed miRNAs in cytokine-stimulated epithelial cells

As the role of miRNAs has become a growing interest in the field of respiratory disease, both as biomarkers and as key drivers of disease-causing mechanisms (see 1.4.3.), we decided to expandon our gene expression signatures (as described in 4.1.) and determine the miRNA signatures in the same samples. To do this, we used the fraction containing RNA molecules shorter than 200 nucleotides that was separated during the isolation of RNA used to generate the gene signatures. These small RNAs were then converted to libraries and subjected to small RNA sequencing. This means that these two datasets are paired and can be directly compared to each other, such as when verifying the expression of genes predicted to be targeted by miRNAs.

After removing miRNAs with low number of counts from the sequencing, 430 miRNAs were available for calculation of differential expression. PCA of the mean counts after normalization showed relevant biological clustering (**Figure 4.3.**), exemplified by the close proximity between IL-4+IL-13 and IL-5, as well as between IL-6 and IL-6TS. In the first two principal components, TGFβ1 was distant from the rest of the treatments, suggesting a differing miRNA signature under this cytokine.

For almost all stimuli, miR-9-5p was the most increased miRNA (**Figure 4.4.**). miR-9-5p has been shown to be anti-inflammatory by leading to decreased release of pro-inflammatory mediators, possibly through repression of NFκB [322-325]. miR-9-5p is decreased by cigarette smoke exposure in airway epithelial cells [326] and additionally, decreased levels were found in BAL vesicles from severe asthma where miR-9-5p negatively correlated with BAL neutrophils [327]. The opposite was, however, found in a study showing increased levels of miR-9 in sputum from individuals with neutrophilic asthma,

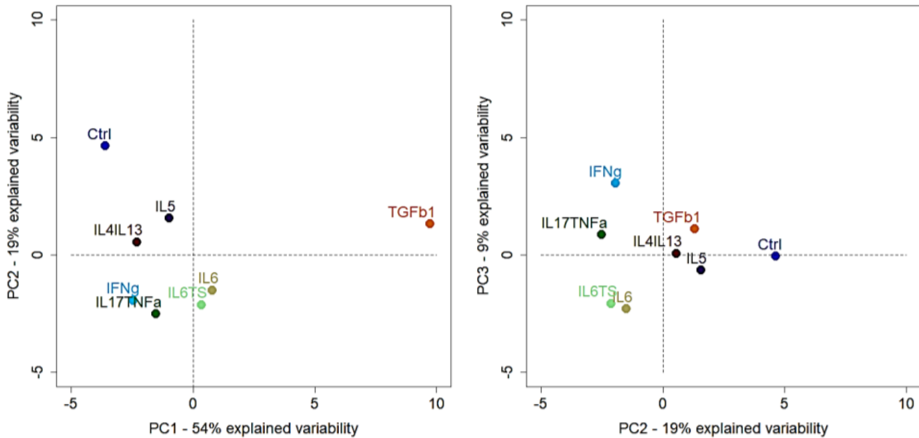

Figure 4.3. *PCA (principle component analysis) of mean normalized counts from sequencing of miRNA in cytokine-stimulated epithelial cells. Left panel shows principle components (PC) 1 and 2, right panel shows PCs 2 and 3. [Unpublished data]*

and inhibition of miR-9 restored corticosteroid response in a mouse model of severe asthma [328]. Furthermore, miR-9-5p promotes cell proliferation [325, 326] while maintaining epithelial cell identity [329], thus, potentially being involved in the difficult balance between epithelial cell repair and hyperplasia. This indicates that the level and effects of miR-9-5p, as supported by the different expression across our conditions, may depend on disease or asthma endotype.

miRNAs that are reported to be differentially expressed in asthma includes the miR-34/449 family (-34a, -34b, -34c, -449a, -449b, and -449c) of which the -5p forms were decreased in bronchial epithelial cells from individuals with asthma as well as by IL-13 *in vitro* [226, 330]. However, increased levels of miR-34c-5p and -3p, as well as no difference in miR-449, have also been found in bronchial biopsies from asthmatics [222, 228]. In another study, the effect of IL-13 on the expression of miR-34a and miR-34b *in vitro* varied depending on timepoint and what compartment was analyzed [204]. Additionally, increased levels of this miRNA family were associated with mucus hypersecretion in COPD [231]. In our data, the -3p forms of miR-34b, -34c, and -449b were increased under several cytokine stimuli. This again shows possible inflammation- or disease-related differences in miRNA expression, and possibly different regulation and function of -5p and -3p forms.

Another group of miRNAs implicated in asthma is the miR-17~92 cluster that has paralog clusters in miR-106a~363 and miR-106b~25 [218, 331]. Members of these clusters, including miR-19a-3p, miR-19b-3p, miR-20b-5p, and miR-363-3p, were differentially increased in our cytokine-stimulated epithelial

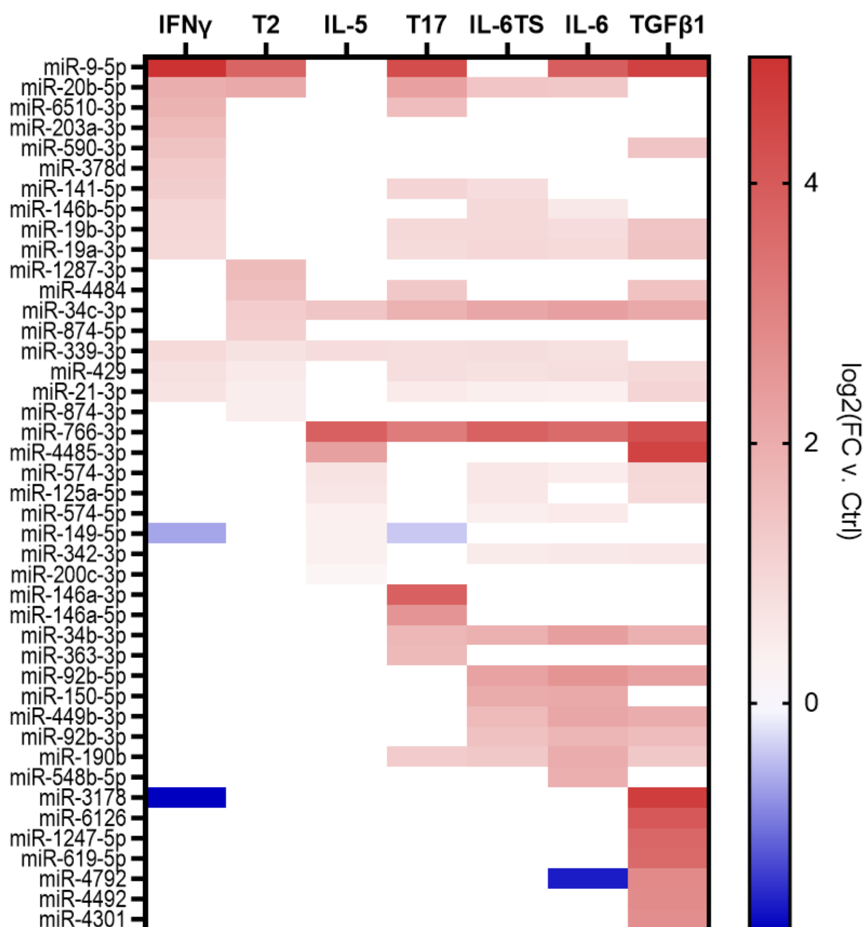

Figure 4.4. *miRNA signatures induced by cytokines in primary human airway epithelial cells cultured at air-liquid interface. Data presented as log2 of fold change versus control/non-stimulated, non-significant values are assigned as 0. [Unpublished data]*

cells. The miR-17~92 cluster is involved in activation of Th cells [332] but studies have shown opposing functions for the different members. This is exemplified by miR-19a and -19b where the former was increased in allergic inflammation and promoted the production of T2 cytokines whereas the latter was decreased and found to target the gene encoding TSLP [218]. As above, these findings highlight the complex role of miRNA in respiratory disease along with the possibility that they are highly specific targets and markers of pathological processes.

4.6. A possible role for miRNAs in Th17 cytokine-mediated airway epithelial barrier disruption

A dysfunctional, disrupted, or damaged airway epithelial barrier, is present in some patients with respiratory disease and is usually related to decreased levels of barrier-related proteins (see 1.4.1.2.). As decreased protein expression can be caused by miRNA/-s targeting the corresponding mRNA, in **Paper III**, we aimed to determine the role of miRNAs in driving inflammation-induced airway epithelial barrier disruption.

First, by comparing the barrier-disrupting effects caused by our different inflammatory stimuli using paracellular flux of fluorescent dextran, we found the T17 cytokines to be strong disruptors of the airway epithelial barrier *in vitro* (Figure 1A-B in **Paper III**). This was further shown to be mediated by an additive effect of the two cytokines, IL-17A and TNFα. As an impaired epithelial barrier is commonly associated with more severe asthma [154, 244, 253], which in turn can be linked to the T17 endotype [55, 57, 63], our results support a direct link between T17 inflammation and an impaired barrier. Previous studies, including our own studies of IL-6TS, have shown the ability of multiple cytokines to impair the epithelial barrier [157, 167-169, 333-335]. In this study, such effects appeared to come later and were less prominent than that caused by the T17 stimulation. Different outcomes in studies of epithelial barrier integrity can partly be due to which method that is used, such as flux of fluorescent dextran or transepithelial electrical resistance (TEER). TEER, albeit quick and non-invasive, is more sensitive to environmental fluctuations as well as the presence and activity of cellular ion channels [336]. However, it is clear that the epithelial barrier can be disrupted in multiple inflammatory settings, but the mechanisms and kinetics may differ.

Second, we performed bioinformatic analysis on the top 30 miRNAs induced by the T17 cytokines, using miRNA target prediction databases combined with our gene expression data (as described in 4.1.) and a list of barrier-related genes identified from relevant publications [150, 157, 315, 337]. This revealed that 20 of these miRNAs were predicted to target at least one barrier-related gene that, in turn, had decreased expression under the same stimulation. However, by using qPCR, only two miRNAs, miR-146a-3p and miR-363-3p, could be validated. The reason for this lower validation success may be technical, as there has been evidence of poor agreement between screening techniques such as NGS and qPCR for miRNAs [338]. Alternatively, or additionally, the donor-to-donor variation can be prominent for miRNAs which are tightly regulated. Still, miR-146a-3p and miR-363-3p stand out as interesting miRNAs since they were only increased under the T17 stimulation (**Figure 4.4.**).

Additionally, their increased expression was, as the barrier damage, found to be mediated by IL-17A and TNFα in combination, suggesting a link between these miRNAs and the observed phenotype.

miR-146a-3p and miR-363-3p were predicted to target a total of 13 barrier-related genes with half of them being shared between the two miRNAs. Seven of these genes (*CLDN8*, *DSP*, *JAM3*, *MPDZ*, *PCDH1*, *PTEN*, and *TJP1*) were validated by qPCR and shown to be decreased upon stimulation with IL-17A ± TNFα. To confirm interaction between the two miRNAs and these predicted targets, pull-down experiments were performed. This demonstrated that miR-363-3p interacted with mRNA transcripts of *DSP* and *PTEN*, and possibly the remaining genes with the exception of *JAM3* where levels could not be determined. In the miR-146a-3p pull-down, there was only a trend towards an interaction with *TJP1* (Figure 4 in **Paper III**).

Next, we set out to determine miRNA targeting of the barrier-related genes in a cellular context by using transfection of miRNA mimics. Due to overlap in predicted targets for the two miRNAs, we used mimics for miR-146a-3p and miR-363-3p both alone and in combination. Since fully differentiated HBEC-ALI cultures are difficult to transfect [250], we used a simplified model of the epithelial barrier comprised of largely undifferentiated HBECs (see 3.5.2.). Based on our preliminary results, with this model, we could achieve dose-dependent increased levels of our transfected miRNAs (Figure 5 in **Paper III**). Furthermore, this transfection was associated with decreased levels of the target genes, which was mainly driven by the miR-363-3p mimic alone and in combination with miR-146a-3p mimic.

Decreased levels of *CLDN8*, *PCDH1*, and *PTEN* or their corresponding proteins have been detected in asthma and/or COPD or were shown to contribute to the pathology of these diseases in *in vitro*/*in vivo* models [162, 170, 337, 339-342]. *PCDH1* has been genetically associated with asthma [343] and could, along with *CLDN8*, be involved in corticosteroid-mediated improvement of the epithelial barrier [340, 344]. There are some miRNAs identified to be involved in mechanisms directly or indirectly linked to epithelial barrier function. miR-19a, miR-744, miR-203, and miR-221 are found to be differentially expressed in asthma and may affect the proliferation of epithelial cells [225, 227, 345-347]. The miR-200 family (-200a, -200b, -200c, -141, and -429), the miR-34/449 family (-34a, -34b, -34c, -449a, -449b, and -449c), and miR-205 are involved in epithelial cell differentiation such as regulation of epithelial to mesenchymal transition [226, 348].

The strongest effect from the two miRNAs examined in this study was seen by miR-363-3p, a relatively unknown miRNA. One study found increased levels

of miR-363-3p in bronchial biopsies from asthmatics [236], and a preliminary study linked it to airway smooth muscle contractility [349]. Additionally, the miR-106a~363 cluster, which is paralogous to the miR-17~92 cluster involved in immune system regulation [331, 332], has been linked to T17 inflammation through regulating Th17 cell differentiation [350]. This work, together with our preliminary findings in epithelial barrier dysfunction, suggests that miR-363-3p may have multiple roles in T17-driven airway inflammation.

The other miRNA increased by T17 cytokines was miR-146a-3p, the passenger strand to the more well studied miR-146a-5p which was also increased by T17 cytokines (**Figure 4.4.**). miR-146a has been previously implicated in inflammatory diseases [351], with mechanisms related to airway inflammation, remodeling, and mucus hypersecretion [231, 352, 353]. A direct relationship with the epithelial barrier was demonstrated through a positive effect on nasal epithelial barrier function [354]. miR-146a-5p can be induced by different inflammatory stimuli in airway epithelial cells [234, 352, 354, 355]. Generally, miR-146a-5p has been described as anti-inflammatory, and, relevant for T17 inflammation, it is predicted to target the mRNA encoding IL-17A [356]. There are conflicting results on the levels of this miRNA in respiratory diseases. Reduced levels of miR-146a-5p were found in epithelium of smokers and individuals with asthma, where in the latter this was associated with a contribution in establishing neutrophilia [234, 330]. In contrast, increased levels were found in epithelial cells from asthma as well as COPD patients [230] and increased circulating levels have been associated with severe asthma as well as allergic asthma [355, 357]. miR-146a is therefore likely to contribute to multiple processes in inflammatory airway disease, with possible different roles for the -3p and -5p strands.

5. Conclusions

In this thesis, the diverse responses and functions of the airway epithelium in respiratory disease have been demonstrated through the establishment of three types of signatures: gene expression, extracellular vesicle proteome, and miRNA expression. These signatures were reflective of the different inflammatory stimuli used in these studies, modelled to correspond to different inflammatory endotypes in diseases such as asthma and COPD. Within the signatures are genes, proteins, or miRNAs corresponding to mechanisms and markers that can be used as a starting point for further studies.

Such possible applications of these epithelial signatures were exemplified in this thesis through identification of; an IL-6TS-high subset of asthma (**Paper I**), the role of extracellular vesicles in the T2 versus T17 endotypes (**Paper II**), and how miRNAs may contribute to epithelial barrier dysfunction (**Paper III**).

In **Paper I**, a subset of asthma patients with activation of IL-6TS in the airway epithelium were identified. These patients show evidence of poorly controlled disease through an increase in exacerbations, possibly mediated by submucosal inflammation and an impaired airway epithelial barrier. Therefore, these patients may benefit from treatment decreasing the activity of the IL-6TS pathway.

In **Paper II**, extracellular vesicles released from the apical side of airway epithelial cells were shown to be representative of the epithelial response to T2 or T17 inflammation and could, therefore, serve as markers of these disease endotypes. Further, these vesicles may contribute to disease-associated processes, such as immune cell migration. These findings enhance the understanding of local mechanisms in airway inflammation that can enable the development of improved and targeted therapies in respiratory disease.

In **Paper III**, it was demonstrated that Th17 inflammation is disruptive to the airway epithelial barrier and that this could be mediated by the increase in expression of several miRNAs predicted to target barrier-related genes. Preliminary results identified miR-146a-3p and miR-363-3p as candidates for mediating the barrier-damaging effect. This supports the hypothesis that miRNAs are involved in barrier dysfunction in inflammatory airway diseases such as asthma.

6. Future perspectives

Since the airway epithelium has an important role in maintaining homeostasis as well as contributing to disease, it is a valuable source of therapeutic targets in respiratory diseases and may also be valuable for biomarker discovery. Specifically, since the airway epithelium responds and contributes differently depending on stimuli, such as inflammatory conditions studied in this thesis, these biomarkers and targets may contribute to the development of precision medicine for airway disease endotypes.

The composition and functions of the airway epithelium is an area of intense study that has seen great progress, especially through the development of single-cell RNA sequencing. This has identified novel epithelial cell types and cell states that have distinct properties and may be important contributors to a disease setting [107, 117]. These include the ionocytes, which are rare cells that have high expression of *CFTR*, suggesting that they are key cells in cystic fibrosis where the chloride transporter encoded by this gene is absent or non-functional. Further, mucous ciliated cells, which co-express markers for ciliated cells and goblet cells, appear to only occur in asthmatic airways where they can be markers of mucous cell hyperplasia [107, 117]. Moreover, novel identified functions within the airway epithelium includes the ability to sense and clear the airways of apoptotic cells, inflammatory memory mediated by epigenetic mechanisms, and the presence of a circadian clock that cause variations in inflammatory functions throughout the day [117]. Exactly how these cell types, cell states, and functions are altered or contribute to disease is under investigation.

In this thesis, the airway epithelial responses to inflammation, as present in the respiratory diseases asthma and COPD, has been the focus. Our results exemplify how the epithelium responds under selected inflammatory conditions with alterations in gene, miRNA, and protein signatures. Those alterations are further related to mechanisms such as an impaired epithelial barrier and cross-talk with immune cells. How these, and similar, findings can be taken further is dependent on aspects including relevant pre-clinical models, availability of rich patient cohort data, and the possibility of characterizing patients using novel minimally invasive sampling techniques along with technical advancements in analysis of EVs and miRNA for example.

It is clear that the respiratory diseases asthma and COPD are heterogeneous and that there are patients in need of better treatment options than what is currently available. To help these patients, the mechanisms behind the heterogeneity, the endotypes, need to be elucidated as they may serve as a basis for the development of new therapeutics and/or biomarkers. This is the goal of

translational, or bench-to-bedside, research which is reliant on highly relevant pre-clinical models. For such research in respiratory disease, and especially of the airway epithelium, the gold standard *in vitro* model is epithelial cells cultured at air-liquid interface (ALI), as was used in this thesis. To obtain a fully differentiated airway epithelium mimicking the human airways, the cells need to be of primary human origin [108, 239]. However, this system has several drawbacks related to cost, time, donor variation, and difficulty to perform high-throughput screening of, for example, drug candidates. The ALI cultures have historically been difficult to genetically manipulate through traditional transfection, but recent advancements with new protocols have shown success, especially with the application of CRISPR/Cas9 [232, 249]. Additionally, the epithelial cells used for ALI cultures have generally been isolated from the human tracheo-bronchial region which is a relevant region for asthma, but less relevant for COPD where the disease is mainly related to the small airways. The use of small airway epithelial cells in ALI cultures has been less common, but is gaining traction due to the development of improved protocols and cell culture media [358, 359]. Moreover, the function of the airway epithelium *in vivo* is highly reliant on the presence of other cell types that the epithelial cells interact with, such as fibroblasts that also provide additional extracellular matrix for the epithelial cells to grow on and interact with, and immune cells with which the epithelial cells communicate [119, 360].

Hence, improved models of the airways are needed and are under development. These new models include co-culture systems based on the ALI culture, but combined with other cells such as fibroblasts, endothelial cells, eosinophils, neutrophils, T-cells, or macrophages [108, 251]. In addition, epithelial cells may be co-cultured with bacteria, to mimic interactions between the epithelium and the crucial microbiota of the airways as well as to study mechanisms related to infection and the effects of antimicrobial agents [248, 251, 361-363]. The challenge with these model systems is largely that of the different media requirements for the various types of cells. Additionally, the use of a porous membrane still separates the cells and, thus, may not enhance the *in vivo*-mimicking properties of the ALI system. Another model is that of airway organoids, where pluripotent stem cells or primary airway epithelial cells are allowed to form and differentiate in a 3D structure that more closely resembles the architecture of both the central and distal human airways. Moreover, organoids have an improved capability for studying genetic variants, either already present in the donor cells or introduced through e.g. CRISPR/Cas9, but the use of stem cells requires the application of a differentiation protocol that may be lengthy and complex. They key advantage of airway organoids over ALI cultures is the possibility for use in high-throughput screening. However, protocols need to be improved in order to generate reproducible organoids of

sufficient quantity for screening [108, 284]. Finally, biomedical engineering has led to the development of microfluidic "airway-on-a-chip" systems [364]. Here the epithelial cells can still be cultured and differentiated on a porous membrane, but the media underneath the cells is flowing, simulating the blood flow. Furthermore, the bottom side of the membrane can be seeded with endothelial cells and immune cells can be added to the basolateral side to study interactions and migration [365]. These chips are still quite complex to use, but they can shed light on complex biological processes and further improvements could make them more widely used [366]. Fascinatingly, there is the proposed possibility of connecting systems representing different organs, creating the "body-on-a-chip" [364] where an airway-chip could be connected to a liver-chip where "inhaled" drugs could be metabolized and circled back to the airway. These chips, alone or in combination, may be able to replace, at least partly, the use of animal models in some aspects of research.

Translating findings from pre-clinical models into the human disease setting relies on data from cohorts with thoroughly characterized individuals. As exemplified in this thesis, gene signatures generated from endotype models *in vitro* can be used to identify patients that may represent this endotype among a cohort of individuals with varying disease characteristics. However, access to such data is not accessible to all researchers, and the type of -omics data contained within is usually restricted to genomic and transcriptomic with a focus on expression of protein-coding genes. Proteomic and miRNA transcriptomic cohort data is less frequent than gene expression data, most likely due to the increased difficulty of performing such analyses. Additionally, it is of further applicability if proteomic and/or transcriptomic data is generated from samples obtained from the affected organ rather than from the circulation. For respiratory diseases, this poses a challenge since sampling cells from the central or distal airways is highly invasive as well as unpleasant and may pose a risk for the individual. Hopefully, the generation of various types of -omics data and sampling of the airways will become more feasible, making cohort data broader as well as accessible for more researchers.

If a disease endotype is to be the target for precision medicine, it has to be confidently identifiable within an individual patient, preferably through the use of one or several biomarkers that have to be assessable in a clinical setting [74, 367]. As mentioned above, direct sampling of the lower human airways is generally invasive with methods requiring bronchoscopy and consisting of bronchial brushings and biopsies, as well as bronchial and bronchoalveolar lavage [367]. A minimally invasive central airway sample is induced sputum, which is valuable for determining immune cell population, inflammatory mediators, and bacterial colonization of the airways [367, 368]. Some features of respiratory disease, especially of allergic asthma, can also be reflected in

less invasive samples from the nose, as described by the united airway concept [367, 369, 370]. Alternative methods under development include naso- and broncho(ab)sorption whereby filter strips are used to absorb nasal or bronchial secretions from which soluble mediators can be analyzed [371, 372]. Even though bronchoabsorption requires bronchoscopy, it is likely to be less unpleasant for the individual as no mechanical damage is induced in the airways. Importantly, these absorption techniques are not associated with extensive dilution which is the case for lavage techniques and further, the samples can be taken from a precise location and paired with a biopsy or brushing sample. For analytes that can be detected in the nose, there is also the possibility for an individual to take the sample themselves in their home. This sampling method can then be repeated and samples can be stored in the fridge/freezer to allow for monitoring over one or several days. A final example is that comprised of non-invasive methods analyzing exhaled air, where the assessment of FeNO, as described in 1.4.1.3., is included. These methods now can include the eNose (electronic nose) which detects volatile organic compounds, creating a fingerprint signature that can be related to airway inflammation [367, 373]. Non-volatile analytes, such as phospholipids and proteins, can be analyzed from particles in exhaled air (PExA) that can be collected using specific breathing techniques and combined with different proteomics techniques [374, 375].

From these sample collection methods, there is the possibility to retrieve and analyze multiple types of biological classes, including, as studied in this thesis, mRNAs, extracellular vesicles, proteins, and miRNAs. These can then be used for studies aimed to increase disease understanding or serve as biomarkers of respiratory disease. For studies in the lab, these classes still possess difficulties with regards to isolation, purification, and analysis. These difficulties also hinders their possible use as biomarkers as, to date, the methodology is too complex to be feasibly used in a clinical setting. However, as previously described, technical improvements with regards to isolation and purification techniques as well as analyses including -omics approaches are likely to diminish these difficulties. Thus, these tehnical advances will lead to further knowledge about respiratory diseases and the possibility to develop precision medicine that ultimately will improve the lives of patients.

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