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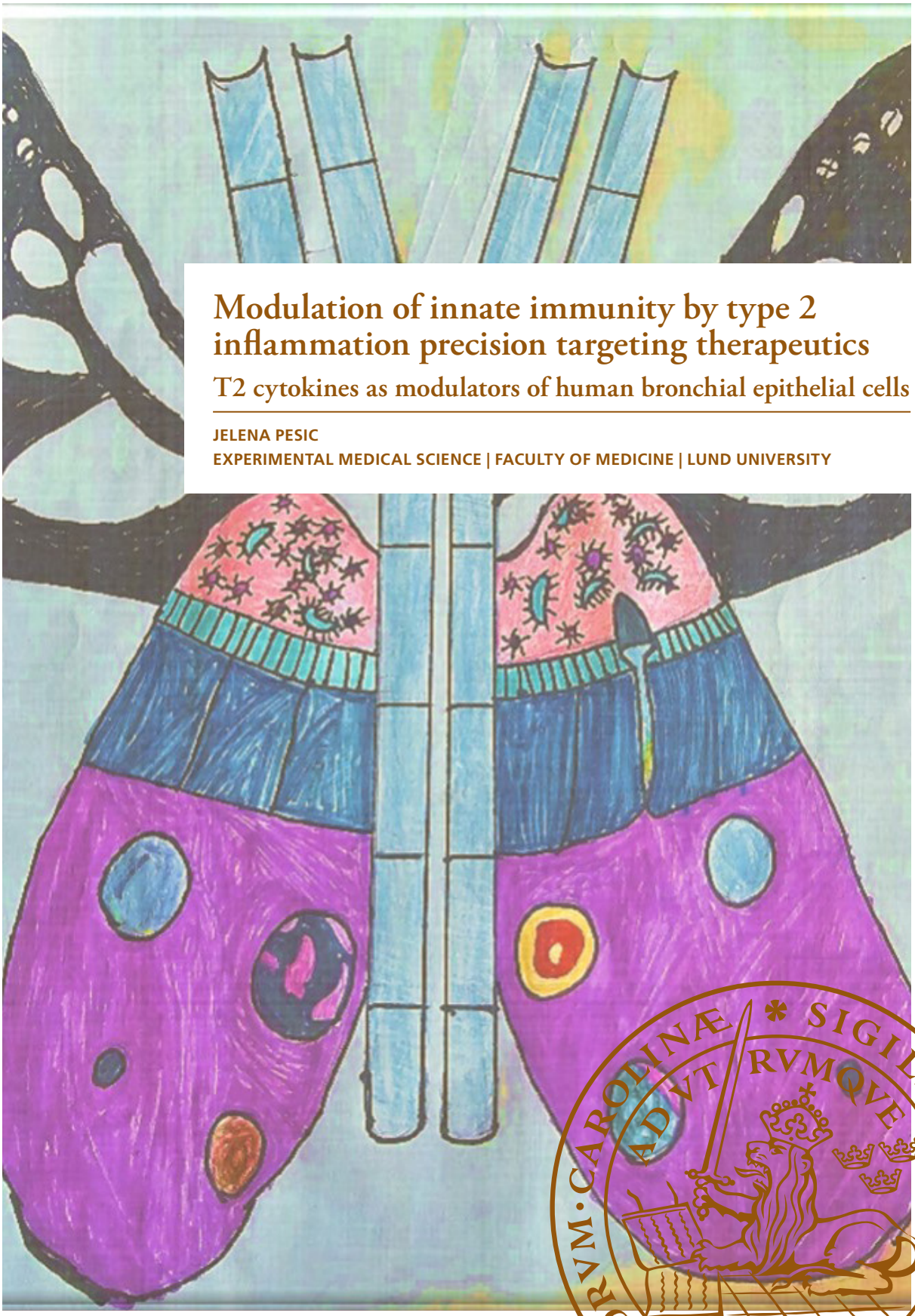

Modulation of innate immunity by type 2 inflammation precision targeting therapeutics

T2 cytokines as modulators of human bronchial epithelial cells

JELENA PESIC
EXPERIMENTAL MEDICAL SCIENCE | FACULTY OF MEDICINE | LUND UNIVERSITY

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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 12th June 2025 at 09.00 in Segerfalksalen, Department of Experimental Medical Science, BMC, Lund

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Title and subtitle: Modulation of innate immunity by type 2 inflammation precisison targeting therapeutics: T2 cytokines as modulators of human bronchial epithelial cells

Abstract:

Airway epithelium, formed by epithelial cells lining human airways, is a critical barrier against inhaled allergens, viruses, and pollutants. Beyond being a physical barrier, it functions as a first-line defense by secreting alarmins, inflammatory cytokines, and chemokines, thereby activating other immune cells. In respiratory diseases like asthma and chronic obstructive pulmonary disease (COPD), the epithelial barrier's function is compromised, partly due to persistent inflammation.

This doctoral thesis primarily investigated the impact of type 2 (T2) cytokines, particularly IL-4 and IL-13, on bronchial epithelial cells (BECs) and their antiviral responses using translational *in vitro* air-liquid interface (ALI) cultures. These studies involved epithelial cells from healthy individuals and patients with asthma and COPD. The cytokines were introduced to BEC cultures exogenously, with and without rhinovirus (RV) infection. Additionally, we developed a co-culture system with innate lymphoid cells (ILC2) and BECs to explore the immunological interaction between these cells, given that ILC2s are potent sources of T2 cytokines.

The research further investigated the mechanisms of IL-4 receptor alpha (IL-4Rα) monoclonal antibody (mAb) treatment under T2 inflammatory conditions, focusing on its influence on viral-induced exacerbations.

Our studies indicated that BECs from individuals with asthma showed increased inflammatory responses upon acute exposure to IL-4 and IL-13. This was evidenced by increased levels of eosinophil chemoattractant (CCL26) and alarmins (TSLP) while maintaining antiviral responses. These cytokine-induced effects were attenuated by IL-4Rα mAb. In settings of chronic T2 cytokine exposure or within the BEC/ILC2 co-culture model, a reduction in TSLP over time was noted, alongside altered antiviral responses.

The findings aim to elucidate the mechanistic effects of T2 inflammation and IL-4Rα mAb inhibition specifically on airway epithelial cells, contributing to a deeper understanding of how anti-IL-4Rα treatment assists in improving lung function and reducing exacerbations in patients. Our novel data on decreased levels of CCL26 (Eotaxin-3) in both asthma and COPD may explain why asthma and COPD patients with eosinophilia respond well to IL-4Rα treatment.

Key words: Asthma, COPD, epithelium, IL-4, IL-13, rhinovirus, airway structural cells, alarmins, antiviral response, innate lymphoid cells, IL-4Rα inhibitors

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Cover photo: Illustrated by my 11-year-old son, **Nemanja Pesic** and 9-year-old dotter **Andrea Pesic.** A Human Lung Depicted in Butterfly Wings Enhanced with Epithelial and Immune Cells, Featuring an Immunoglobulin (IgG) Antibody (Ab) as the Butterfly's Body.

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"The most beautiful thing about a cell is that it knows what it is supposed to do and does it without being told."

Lewis Thomas

Dedicated to my family

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Papers not included in the thesis

1. Petrova T, **Pesic J**, Pardali K, Gaestel M, Arthur JSC. p38 MAPK signalling regulates cytokine production in IL-33 stimulated Type 2 Innate Lymphoid cells. *Sci Rep 2020 Feb 26;10(1):3479*.

2. Åstrand AB, Hemmerling M, Root J, Wingren C, **Pesic J**, Johansson E, Garland AL, Ghosh A, Tarran R. Linking increased airway hydration, ciliary beating, and mucociliary clearance through ENaC inhibition. *Am J Physiol Lung Cell Mol Physiol 308: L22–L32, 2015.*

3. Risse F, **Pesic J**, Young S, Olsson LE. A texture analysis approach to quantify ventilation changes in hyperpolarised ^{3}He MRI of the rat lung in an asthma model. *NMR Biomed 2012 Jan;25(1):131-41.*

4. Deierborg T, Staflin K, **Pesic J**, Roybon L, Brundin P, Lundberg C. Absence of striatal newborn neurons with mature phenotype following defined striatal and cortical excitotoxic brain injuries. *Exp Neurol 2009* Sep;219(1):363-7.

5. Nygren J, Wieloch T, **Pesic J**, Brundin P, Deierborg T. Enriched environment attenuates cell genesis in subventricular zone after focal ischemia in mice and decreases migration of newborn cells to the striatum. *Stroke 2006 Nov;37(11):2824-9*

Abbreviations

ALI	Air - liquid interface
APC	Antigen presenting cell
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BECs	Bronchial epithelial cells
CBF	Cilia beating frequency
CCL26	Eotaxin-3
CDHR3	Cadherin-related family member 3
cGAS	Cyclic GMP–AMP synthase
COPD	Chronic Obstructive Pulmonary Disease
CT	Computed Tomography
DAMPs	Damage-associated molecular patterns
dsRNA	Double-stranded RNA
EAO	Early asthma onset
FeNO	Fraction of Exhaled Nitric Oxide
FEV1	Forced Expiratory Volume
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HMGB1	High mobility group box 1 protein
HRV	Human rhinovirus
ICAM	Intracellular adhesion molecule
ICS	Inhaled corticosteroids
IFNs	Interferons
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IKK	IkappaB kinase complex
IL	Interleukin
ILC2	Innate Lymphoid Cells type 2
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
JAK	Janus kinase
LABA	Long-acting beta agonist
LAMA	Long-acting muscarinic antagonists

LAO	Late Asthma Onset
LDLR	Low-density lipoprotein receptor
mAb	Monoclonal antibody
MAVS	Mitochondrial antiviral signaling
MCC	Mucociliary clearance
MDA5	Melanoma differentiation-associated protein 5
mDCs	Myeloid dendritic cells
MLEC	Mouse lung endothelial cells
MOI	Multiplicity of infection
mRNA	Messenger RNA
MSCs	Mesenchymal stem cells
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cell
NKT	Natural Killer T Cells
PAMPs	Pathogen-associated molecular patterns
PGE2	Prostaglandin E2
PNEC	Pulmonary neuroendocrine cells
PRRs	Pattern recognition receptors
RLRs	Retinoic acid-inducible gene-I-like receptors
RSV	Respiratory Syncytial Virus
RV	Rhinovirus
sEV-mDCs	Small extracellular vesicle-mature dendritic cells
ssRNA	Single stranded RNA
STAT	Signal transducer and activator of transcription
ST2	Suppression of tumorigenicity 2
T2	Type 2
TBK1	TANK-binding kinase 1
Th2	T helper 2
TLRs	Toll-like receptors
TNFα	Tumour Necrosis Factor Alpha
TSLP	Thymic stromal lymphopoietin
T reg	Regulatory T cell
TYK2	Tyrosine Kinase 2
VCAM-1	Vascular cell adhesion protein 1
WAT-MSCs	White adipose tissue-resident multipotent stromal cells

Introduction

Asthma

Asthma is a persistent inflammatory condition of the airways, exhibiting symptoms like wheezing, difficulty breathing, chest constriction, and coughing. These symptoms are often variable and can be resolved either naturally or with medical intervention. Asthma affects people across all age groups and represents a major global health issue, affecting over 300 million individuals worldwide [1]. The pathological mechanisms of asthma are complicated and differ across various phenotypes, influenced by diverse environmental factors, age groups, obesity, and genetic elements. Beyond airway inflammation, new evidence indicates that processes independent of inflammation also play a role in the pathogenesis of asthma [2].

Early-onset asthma (EOA) and late-onset asthma (LOA) represent two distinct phenotypes of asthma, different in clinical presentation, pathophysiology, and treatment response (**Figure 1**). The age of 12 years is often used to distinguish these phenotypes [3]. EOA generally begins during childhood or adolescence, and tends to have a higher genetic predisposition, mostly in cases with reduced lung function, suggesting genetic variations based on onset age and lung capacity [4], while LOA typically starts in adulthood. EOA is more commonly associated with atopy and IgE-mediated allergic reactions [5]. EOA and LOA exhibit distinct inflammatory patterns. EOA often involves a Th2-driven inflammation including eosinophils and IgE, while LOA might involve less eosinophilic inflammation and can be linked with obesity [6]. Patients with LOA are more likely to experience conditions like chronic rhinosinusitis, nasal polyps, and obesity compared to those with EOA [6]. These comorbidities differently impact disease control.

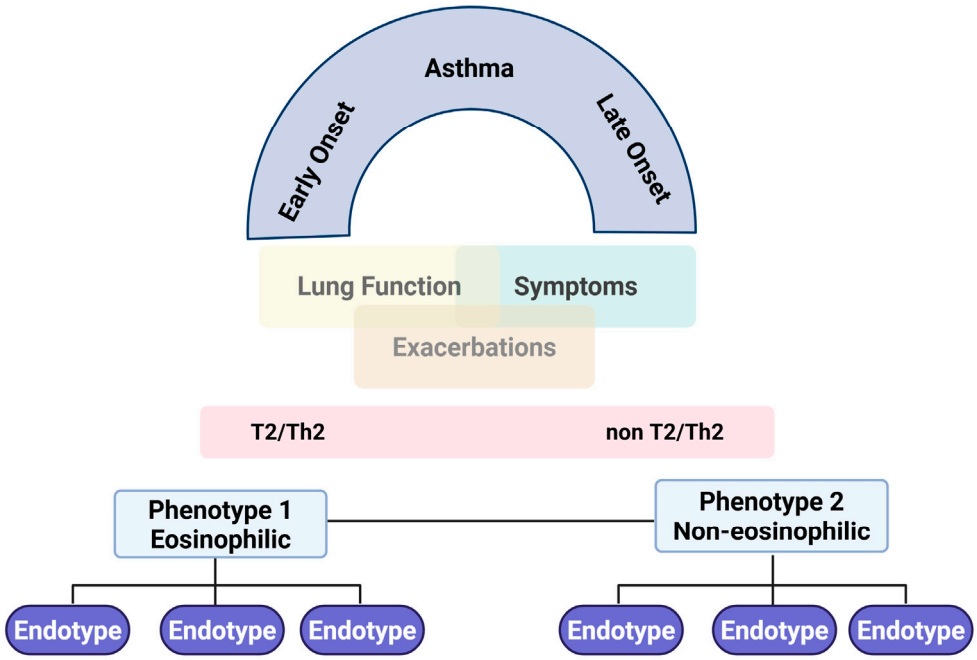

Figure 1: Schematic representation of asthma and the spectrum of inflammation type and endotypes underlying clinical phenotypes. T2 = type 2; Th2 = T-helper type 2. Adapted from Carr T. et al. 2018 [7].

The concept of different asthma phenotypes highlights the existence of subgroups within asthma, each driven by unique pathophysiological mechanisms, referred to as endotypes, focusing on defining asthma entities based on known or presumed mechanisms related to the disease. These include factors such as clinical features, biomarkers, genetics, histopathology, lung function, and treatment response [8]. Unlike phenotypes, which may vary over time or due to treatment, endotypes are relatively stable groups, characterized by distinct genetic or molecular traits. However, studies suggest that patients can move from one endotype to another and are dynamic events influenced by external and internal features.

Type 2 (T2) high and T2 low asthma represent two broad phenotypes of asthma, distinguished by different inflammatory pathways and treatment responses (**Figure 2**). Understanding the distinctions between T2 high and T2 low asthma is essential for customizing treatment strategies for individual patients, underscoring the importance of personalized medicine. T2 high asthma is linked with increased levels of T2 inflammation, typically involving Th2 lymphocytes, type 2 innate lymphoid cells (ILC2s), mast cells, basophils, eosinophils, and production of T2 cytokines (IL-4, IL-5, IL-13) that promote eosinophilic airway inflammation [9]. Clinical biomarkers for

T2 high asthma include exhaled nitric oxide (FeNO), serum IgE, as well as blood and sputum eosinophils [10]. T2 high asthma often correlates with allergic asthma, marked by an IgE-mediated reaction to allergens [11]. Patients with T2 high asthma generally exhibit a positive response to biological therapies targeting specific T2 cytokines [12].

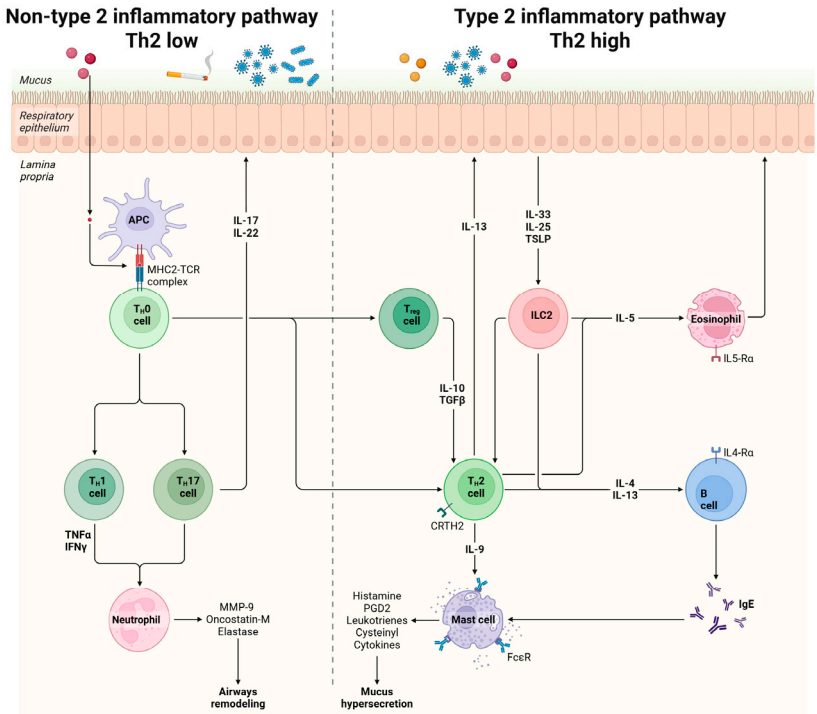

Figure 2: Mechanism of Th2 high and Th2 low asthma (non Th2) and activation of immune cells (Created in https://BioRender.com).

T2 low asthma also referred to as non-T2 asthma, is characterized by low or absent T2 inflammation. This type may involve neutrophilic inflammation and is less commonly linked with allergic responses [10]. Identified by IL-17 and tumour necrosis factor α (TNF-α), which facilitate neutrophil migration to the lungs [9]. Recognizing T2 low asthma typically involves identifying the absence of elevated biomarkers seen in T2 high asthma. T2 low commonly is associated with factors like obesity, smoking, environmental pollutants, viral or bacterial infections, and aging [13]. The treatment for T2 low asthma is less defined due to its heterogeneity and the lack of specific targeted therapies. Physicians often focus on controlling symptoms and preventing exacerbations with inhaled corticosteroids and other non-biologic treatments. If T2 low asthma with neutrophilic inflammation is a real endotype is currently under debate.

Classification of asthma severity (intermittent, mild persistent, moderate persistent, and severe persistent) is based on symptom frequency, lung function, and the need for rescue medication [14]. T2-high asthma can be present from mild to severe, however individuals with T2-high asthma tend to exhibit more severe asthma characteristics. This phenotype is often associated with a higher degree of airway inflammation, more frequent exacerbations, and a greater impact on quality of life compared to T2-low asthma. T2-high asthma, with its distinct inflammatory profile and clinical manifestations, may require targeted treatment approaches, including biological therapies designed to inhibit specific pathways involved in T2 inflammation [10, 15, 16]. When symptoms in asthma patients worsen significantly with difficulty breathing or shortness of breath, wheezing, persistent cough, and chest tightness, they are defined as asthma exacerbations, commonly known as asthma attacks. Causes of exacerbations can be due to exposure to allergens, air pollutants or most common respiratory viral infections. Asthma exacerbations can be life-threatening and frequently result in hospitalization [17]. Viral-induced exacerbations are a significant concern in children, accounting for over 80% of exacerbations in this population, with respiratory syncytial virus (RSV) and rhinovirus (RV) being the most common problems.

Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) like asthma are both obstructive lung diseases that share some similarities in symptoms, which can sometimes make it challenging to distinguish between the two conditions (**Figure 3**). COPD is a progressive condition that poses a significant public health challenge due to its widespread prevalence resulting in high levels of illness and death [18]. COPD is characterized by persistent airflow obstruction that is generally progressive and associated with airway inflammation [19]. The primary cause of this inflammation is often the inhalation of harmful particles, such as cigarette smoke, but other factors like air pollution also play a role. COPD is known for its heterogeneity, with tobacco smoking being the primary environmental risk factor [19]. Also, genetics such as alfa1-antitrypsin deficiency are known to be risk factors for COPD. Recently, “pre-COPD” has been merged as a potential early stage of COPD that is identified by irregular spirometry results or expressive emphysema on computed tomography (CT) in the absence of airflow obstruction. In patients experiencing emphysematous pre-COPD, lung examinations already reveal a reduction in small airways and signs of airway remodeling, even when there are no evident physiological obstructions in the airways [20].

Within the COPD patient population, some individuals exhibit signs of type T2 inflammation, which includes increased eosinophil counts and changes in the expression of specific T2 inflammatory markers [21]. Although T2 inflammation is commonly associated with asthma, it is also present in a subset of COPD patients and can occur both in stable phases and during exacerbations [22]. Some COPD patients exhibit evidence of both local and systemic T2 inflammation [23, 24], which is driven by inflammatory cytokines such as IL-4, IL-5, and IL-13, produced by Th2 cells and innate ILC2 [25]. Exacerbations of COPD contribute significantly to the overall burden of the disease, and they are believed to be a primary factor in disease progression, particularly the decline in lung function [26]. Respiratory viral infections play a major role in triggering these exacerbations [27, 28]. Therefore, the development of new COPD treatments that can prevent exacerbations is a critical objective, in addition to enhancing lung function and improving quality of life.

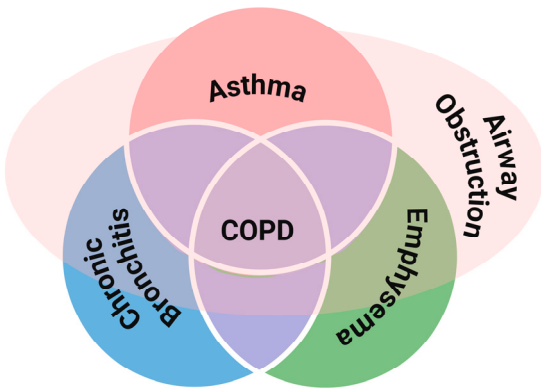

Figure 3: Asthma and COPD are both obstructive lung diseases that share some similarities in symptoms, which can sometimes make it challenging to distinguish between the two conditions. Adapted from Nakawah MO et al. 2013 [29, 30].

Bronchial Epithelial Cells (BECs)

Structure

The human respiratory system is divided into two main parts: the upper and lower respiratory tracts. Conducting airways, which extend from the trachea to the proximal end of small bronchioles are lined by pseudostratified respiratory epithelium. The bronchial airway epithelium cells (BECs) act as a crucial barrier, functioning as the first line of defence against various threats such as pollution, viruses, fungi, and bacterial infections [31]. The airway epithelium is a dynamic multicellular structure (**Figure 4**)

that adapts to environmental changes. This pseudostratified epithelium includes three main types of cells: ciliated cells, mucus-producing goblet cells, and basal cells [32]. Basal cells are cuboidal in shape, attached to the basement membrane, and play a fundamental role in maintaining respiratory homeostasis and epithelial regeneration after injury. Basal cells are particularly important as they drive differentiation into most epithelial cell types in the airways. They serve as the primary stem cells of the airway, capable of self-renewal following injury [33] and differentiating into various essential cell types, such as goblet cells, club cells, ciliated cells, tuft cells, pulmonary neuroendocrine cells (PNECs), and pulmonary ionocytes [34-36].

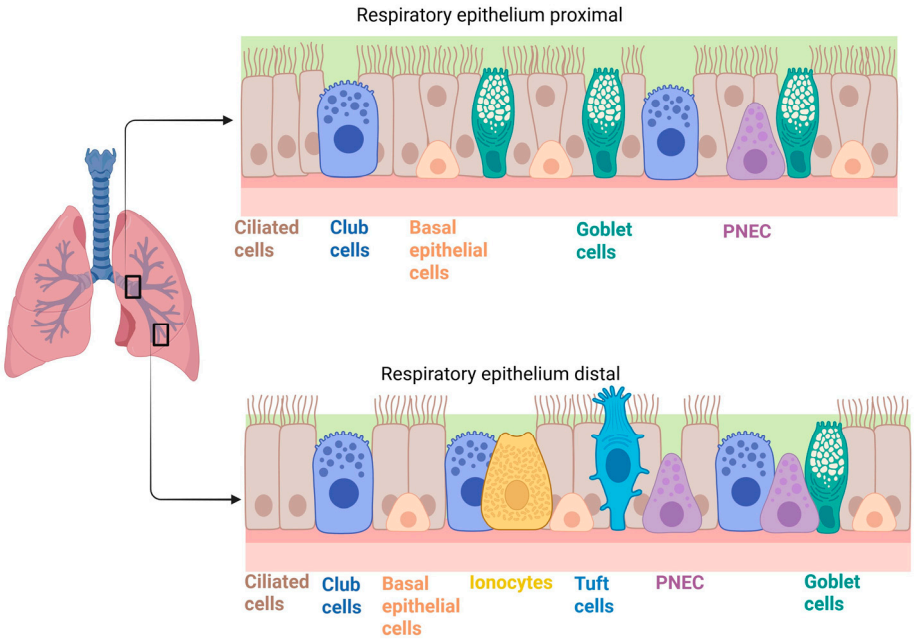

Figure 4: Multicellular structure of airway epithelium (Created in https://BioRender.com).

Club cells, formerly known as Clara cells, are dome-shaped and primarily found in the respiratory bronchioles [37]. These cells can also function as stem cells where they differentiate into both ciliated and goblet cells and have the ability to revert to basal cells if necessary to facilitate epithelial repair [38] when basal cells are injured or lost [39, 40]. They also can act as secretory cells. Ciliated cells are columnar and spread throughout the airways, originating from either club or goblet cells [41]. They are key to maintaining airway homeostasis by trapping and expelling microorganisms, mucus, and debris. This process is known as mucociliary clearance (MCC) [42]. Goblet cells, named for their

goblet-like shape, are the primary producers of mucus in the airways. Along with ciliated cells, they facilitate effective MCC. Like ciliated cells, goblet cells derive from club cells through alternative transcriptional pathways [43]. Of particular importance for distal epithelium are rare cell types like Tuft cells (also called brush cells), neuroendocrine cells, and ionocytes, each implicated in various disease processes [44]. BECs are the primary targets of respiratory viruses and other external agents. When exposed to different molecular patterns, such as allergens, viruses, and pollutants, the airway epithelium activates specific cellular pathways. This response is tailored to the type of exposure, enabling the determination of the most appropriate immune response at the mucosal barrier [45]. When activated by different triggers, the airway epithelium initiates the release of pro-inflammatory mediators known as alarmins [46, 47].

Decoding the Interplay: Early Host Responses to Virus

Human Rhinovirus

Human Rhinovirus (HRV) is a member of the *Picornaviridae* family and the *Enterovirus* genus, known for causing most common colds. The virus has an icosahedral protein capsid and a positive, single-stranded RNA (ssRNA) genome. The capsid is composed of four proteins: VP1, VP2, VP3, and VP4, which coat the RNA genome. Non-structural proteins are involved in viral genome replication and assembly [48]. HRV exhibits significant genetic diversity, with over 150 strains identified through serology or sequencing. These strains are categorized into HRV-A and HRV-B, which are common in humans, and HRV-C, associated with more severe diseases. The major receptor group for HRV-A and HRV-B serotypes uses the intercellular adhesion molecule 1 (ICAM-1) (**Figure 5** ①) for host cell infection [49]. Twelve HRV-A serotypes utilize the low-density lipoprotein receptor (LDLR) (**Figure 5** ②), and HRV-C viruses bind to human cadherin-related family member 3 (CDHR3) [50]. HRV primarily infects the upper respiratory tract, through both ciliated and nonciliated epithelial cells, leading to patchy infection sites.

Pattern Recognition Receptors (PRRs)

The innate immune response plays a crucial role in defending against viral pathogens, primarily through pattern recognition receptors (PRRs) that detect viral infections [51]. These include Toll-like receptors (TLRs), the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS); and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) [52]. TLRs are pivotal in sensing viral pathogen-associated molecular patterns (PAMPs)

within the extracellular compartment and endosomes (**Figure 5** ③). RIG-I and melanoma differentiation-associated gene 5 (MDA-5), are members of the RLR family, and exhibit differential recognition of RNA viruses [53], with RIG-I implicated in the detection of a broad range of viruses [53]. RIG-I and MDA-5 are activated by different types of viral RNAs, with RIG-I primarily responding to ssRNA or 5'-triphosphate dsRNA and MDA-5 to long dsRNA virus species (**Figure 5** ③). This enables a broad and effective immune response against various RNA viruses. RIG-I remains inactive in the cytoplasm under normal conditions but undergoes a conformational change upon viral infection, leading to ATP-dependent dimerization. The activated multimeric form of RIG-I or MDA-5 then interacts with the downstream adaptor protein mitochondrial antiviral signaling protein (MAVS). Engagement of RIG-I or MDA-5 with MAVS activates the IKK-related kinase, TBK1/IKK-ε (**Figure 5** ④), which activates transcription factors IRF3/IRF7 (**Figure 5** ⑤), resulting in the transcription of interferons type I and III (**Figure 5** ⑥).

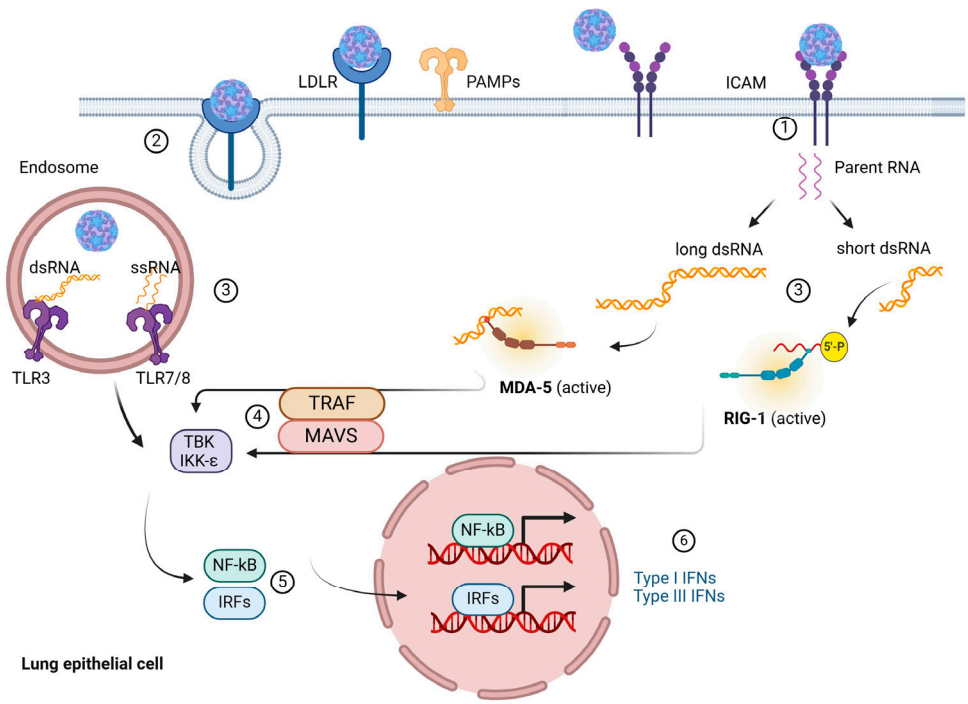

Figure 5: Specific virus strains bind to either ① ICAM-1 or ② LDLR. ③ MDA-5 and RIG-I both serve as cytoplasmic dsRNA receptors but distinguish their ligands in part by size; MDA-5 binds to long dsRNA, whereas RIG-I binds short dsRNA. ③ Among the TLRs, TLR3 recognizes dsRNA, TLR7/8 recognizes endosomal ssRNA. ④ PRR activation initiates downstream signaling TRAF and TBK IKK pathway that in turn activates transcription factors ⑤ IRFs (IRF-3, IRF-7), and NF-κB. The resulting expression of type I and III IFN genes ⑥ affect the innate immune response to confer pathogen resistance and enhance the adaptive immune response to infection (Created in https://BioRender.com).

Interferons

After PRRs recognition of viral infection, the initial sign of an immune response to a viral infection is the release of type I interferons (IFNs). Type I IFNs are part of a cytokine family that includes one subtype of IFN-β, 13 subtypes of IFN-α, as well as IFN-ω, IFN-κ, IFN-ε, and IFN-ν. The presence of multiple IFN-α genes and the fact that nearly all viruses have proteins that hinder the production or response to type I IFNs highlight their critical role in the antiviral immune response.

IFN-β is the first type I IFN to be induced following viral detection. For the transcription of IFN-β mRNA to occur, three groups of transcription factors must bind to the IFN promoter's regulatory domain: NFκB, activating transcription factor 2 (ATF2)/c-Jun, and interferon regulatory factors 3 and 7 (IRF-3 and IRF-7). The activation of these factors in response to viral infection is initiated by either the RLR or TLR systems. Signaling of type I IFNs through their receptor promotes the transcription of numerous interferon-responsive genes (ISGs) that inhibit viral replication and boost the immune response. Secreted type I IFNs engage the IFN-α/β receptor complex (IFNAR), consisting of the transmembrane subunits IFNAR1 and IFNAR2, which are on the surface of every nucleated cell. The recognition of type I IFNs can stimulate the production of additional type I IFNs and other inflammatory cytokines. When IFN-α or IFN-β triggers the dimerization of IFNAR subunits, it activates the kinases Jak1 and Tyk2, which phosphorylate the STAT transcription factors. This process leads to the formation of STAT homodimers (STAT1) and heterodimers (STAT1 with STAT2). Phosphorylated STAT1 and STAT2, together with IRF-9, form a complex known as interferon-stimulated gene factor 3 (ISGF3), which moves to the nucleus and activates ISG transcription [54, 55]. Type III IFNs (IFN-λ) are also produced shortly after infection and, though less studied than type I IFNs, they perform similar functions. Like type I IFNs, type III interferons (IFN-λ), which include IL-29, IL-28α, and IL-28β in humans, are expressed by various cell types in response to viral infections or TLR ligand stimulation and have similar effects to those observed with type I IFNs [56]. The receptor for IFN-λ (IFN-λR) consists of one IFN-λR chain and one IL-10Rβ chain. IFN-λR also signals through the JAK-STAT pathway [57, 58]. Expression of IFN-λR appears to be limited to non-hematopoietic cells, including epithelial cells.

Viral-induced asthma and COPD exacerbations

Acute exacerbations are a primary cause of asthma-related morbidity, mortality, and healthcare costs, and they present significant challenges for treatment and prevention. These exacerbations are primarily triggered by respiratory viruses, with human RV being

the most common cause. RV accounts for about two-thirds of all viral-induced asthma exacerbations in children and adults [59-61]. Despite the prevalence and impact of viral-induced asthma exacerbations, the mechanisms underlying these exacerbations are not fully understood. Experimental models of rhinovirus-induced asthma exacerbations have been developed, offering insights into the pathogenesis and potential therapeutic targets [62]. IFN-β was found to be particularly deficient in asthmatic patients compared to others [63], and IFN-λ production of which was also deficient *in vitro* and related to asthma exacerbation severity *in vivo* [64]. These studies highlight novel mechanisms that contribute to the increased susceptibility of individuals with asthma to RV infections. Acute exacerbations, especially those associated with virus infection, have been shown to be associated with neutrophilic inflammation [65], However, the presence of eosinophils suggests the possibility of mixed inflammation. Viral infections can amplify the T2 high immune response, leading to further activation of these pathways, which can result in severe asthma exacerbations. The presence of T2 airway inflammation has emerged as a key independent risk factor for exacerbations, and this provides important insight into the pathophysiology of acute asthma. In population samples of adults, elevated blood eosinophils are associated with an increased risk of exacerbations [66]. The presence of active T2 airway inflammation and its measurement with biomarkers such as blood eosinophils and FeNO provides a clear indication of risk for future exacerbations [67]. Non-type 2 inflammation in asthma has been linked to dysregulation of innate immune responses, including the activation of inflammasome pathways and development of airway neutrophilia [68, 69].

Epithelial alarmins

Thymic Stromal Lymphopoietin (TSLP)

Thymic Stromal Lymphopoietin (TSLP) acts as an alarmin by being rapidly released from epithelial cells, thereby inciting further exogenous and endogenous danger signals and exacerbating inflammation. This rapid release and action highlight its role as an early mediator in immune responses thus given its name alarmin. In humans, TSLP has two main isoforms: a short isoform typically expressed under basal conditions and a longer isoform that is induced in response to inflammatory stimuli [70, 71]. The effects of TSLP are utilized through binding to the high-affinity TSLP receptor (TSLPR) complex that consists of a TSLP-binding chain and the IL-7Rα subunit [72]. The TSLPR is widely expressed among hematopoietic cell populations. Studies have shown that TSLP can significantly induce the development, expansion, and effector functions of various immune cells, thus activating both the innate and adaptive

immune systems. In response to pathogenic stimuli or mechanical injury, TSLP exacerbates inflammation by activating multiple effector cells involved in the immune cascade, such as ILC2 and myeloid dendritic cells (mDCs) [73, 74]. Additionally, TSLP influences $CD4^+$ and $CD8^+$ T cells, regulatory T cells (Tregs), B cells, mast cells, natural killer T (NKT) cells, monocytes, $CD34^+$ progenitor cells, eosinophils, basophils, and airway smooth muscle cells [75-77]. TSLP plays a key role in the pathobiology and pathophysiology of chronic respiratory diseases, such as COPD and asthma. It's particularly observed TSLPs' role in driving airway inflammation through the release of T2 cytokines [78]. TSLP expression is elevated in the airways of individuals with asthma, increases with asthma severity and, is induced in response to allergens and certain respiratory viruses [79, 80]. Since TSLP promotes the differentiation of naïve T lymphocytes into Th2 cells by activating antigen-presenting cells (APCs) [79, 80], it is now considered a crucial link between innate antiviral epithelial immunity and the Th2 atopic immune response [79, 80]. TSLP also plays a significant role in regulating airway immune responses during early life. There is evidence that the human infant airway epithelium shows strong TSLP production when exposed to viral stimuli [81]. In young children, viral-induced airway TSLP production is associated with recurrent viral respiratory disorders during early infancy [82]. These findings align with mechanistic studies in animal models that link virus-induced TSLP production in early life to the later development of allergic airway inflammation, particularly during infections by RSV [83] and RV [84].

IL-33

IL-33 is naturally expressed in the nuclei of epithelial, endothelial, and fibroblast-like cells. It functions as an alarmin and damage-associated molecular patterns (DAMPs), being released upon cell damage or stress to alert the immune system. IL-33 binds to the ST2 receptor on various immune cells, including Th2 cells, mast cells, and ILC2s, driving intense proliferation and the production of cytokines (e.g., IL-5, IL-4, IL-13, GM-CSF, and amphiregulin), chemokines (e.g., eotaxin), and peptides [85]. IL-33 exists in two forms: a full-length form (pro-IL-33) [86] and a mature form. The mature form of IL-33 is produced by the cleavage of pro-IL-33 by caspases, enabling it to exert its biological function. IL-33 is implicated in the pathogenesis of several diseases, including asthma, allergic inflammation, autoimmune diseases, and cancer. It is a key player in allergic conditions since can induce Th2-type immune responses [87]. However, IL-33 stimulation alone leads to limited activation of ILC2 cells. More robust activation occurs when IL-33 is combined with co-stimulatory cytokines, such as IL-2, IL-7, or TSLP [88]. This means that the responsiveness to IL-33 and the cytokine production profiles vary depending on tissue type and species [88-90].

IL-25

IL-25 is another pleiotropic cytokine and alarmin, also known as IL-17E, and it is part of the IL-17 family that is found with Th2 cells and activated mast cells [91-93]. IL-25 is constitutively expressed in the lungs, indicating its potential role in maintaining pulmonary homeostasis. Although IL-25 was first discovered as being secreted primarily by Th2 cells, various tissues and cell types including lung and colon epithelial cells, alveolar macrophages, eosinophils, basophils, and mast cells are recognized as sources of IL-25. Similar as other alarmins, IL-25 can enhance downstream T2 cytokine expansion, including IL-4, IL-5, and IL-13, and increase IgE level and eosinophil count, to promote T2 immunity [93, 94]. Like the effects of TSLP, these IL-25 effects have the potential to regulate T2-dependent asthma. RV can also induce the production of IL-25 and IL-33 in the lungs, suggesting a role for IL-25 in the immune response to viral infections [95].

TSLP, IL-25, and IL-33 have each been independently described as key activators of T2 immunity, particularly by stimulating ILC2s [88, 96, 97]. There is also substantial evidence indicating that these cytokines contribute to allergic responses by directly interacting with various immune cells, including T cells, dendritic cells, macrophages, and ILC2s [98, 99]. They are activated by IL-33, released from damaged epithelial cells in response to parasitic infections or allergens, which triggers eosinophilic inflammation [100]. Additionally, IL-25, secreted by tuft cells, stimulates ILC2s to enhance mucus production [90, 100, 101]. Findings from murine studies suggest that IL-33 may have a more dominant role than IL-25 or TSLP in triggering allergic inflammation in the lungs through ILC2 activation [102]. TSLP, a pivotal cytokine for ILC2 differentiation, is thought to be essential for initiating and sustaining airway remodeling during chronic allergic asthma [103]. However, targeting all three simultaneously inhibited development and progression of the disease [104].

Key Players in T2 Asthma and COPD

The type 2 cytokines IL-4, IL-5, and IL-13, are pivotal contributors to the underlying inflammatory processes and pathophysiology in Th2/T2 high asthma [105].

IL-4 and IL-13

IL-4 is crucial for the differentiation of naive T cells into Th2 cells, making it a predominant cytokine that promotes Th2 cell differentiation and the production of

downstream cytokines, including IL-5 and IL-13. IL-4 is involved in B-cell activation of IgE isotype switching, which binds to allergens and triggers allergic reactions [106]. Aside from its role in promoting IgE isotype switching and the polarization of naïve T cells into Th2 cells, IL-4 also triggers the expression of vascular cell adhesion protein 1 (VCAM-1). This expression facilitates the migration of T cells, monocytes, basophils, and especially eosinophils to areas of inflammation. Additionally, IL-4 stimulates the expression of mucin genes, which leads to an increase in airway mucus production [107]. IL-4 is produced by Th2 cells, mast cells, and basophils. In summary, IL-4 is a major cytokine produced by Th2 cells that contributes significantly to the pathophysiology of Th2 asthma by mediating the allergic and inflammatory responses of the disease. IL-13 shares functions with IL-4, including promoting IgE production [108]. It is also involved in promoting mucus production (goblet cells hyperplasia) [109] and bronchial hyperresponsiveness. Plays a role in the structural changes in the airways associated with chronic asthma, such as fibrosis and increased smooth muscle mass [110]. IL-13 has been detected in biological fluids and tissues of patients with asthma, underscoring its role in disease pathogenesis. In patients with asthma and rhinitis, there was a significant increase in IL-13 transcripts and secreted proteins in the allergen-challenged bronchoalveolar lavage (BAL) compared with the saline-challenged controls, whereas the expression of IL-13 transcripts was not detected in the BAL of healthy subjects challenged with the same dose of allergen [111]. In the lung, there is evidence that epithelial cells produce IL-13 [112]. Also, it is produced by other cell types, including Th2 cells, follicular helper T cells, ILC2s, eosinophils, mast cells, and basophils [113]. Fractional exhaled nitric oxide (FeNO) is a biomarker of T2 inflammation in asthma and is significantly influenced by IL-13. FeNO levels are indicative of the activity of IL-13 and other T2 cytokines in the airways [114].

IL-4 and IL-13 receptors

IL-4 and IL-13 interact with two receptor complexes and share a common receptor subunit. IL-4 binds to its specific receptor complex, which includes the IL-4 receptor alpha chain (IL-4Rα) and the common gamma chain (γc), forming the type I receptor [115]. Initially, IL-4 attaches with high affinity to the IL-4Rα subunit, leading to the dimerization with the γc subunit and subsequent activation of the JAK-STAT6 signaling pathway [116]. Both IL-4 and IL-13 engage with the shared type II receptor complex composed of IL-4Rα and IL-13Rα1 (**Figure 6**). While the type I and II receptors signal intracellularly via STAT6, the IL-13Rα2 subunit was originally thought to function as a decoy receptor due to its short cytoplasmic tail [117]. However, some studies have shown that cell membrane-bound IL-13Rα2 may possess signaling capabilities linked to tissue remodeling responses that are likely independent

of STAT6 [118]. IL-4 and IL-13 signaling, as mediated by STAT6, therefore contributes to the multiple pathologic features of asthma including eosinophilic inflammation, airway hyperresponsiveness, subepithelial fibrosis, and excessive mucus production [119].

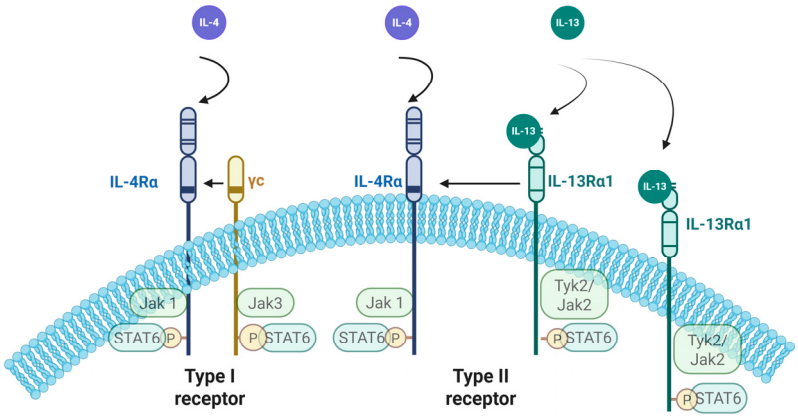

Figure 6: IL-4/IL-13 ligation of type I and type II receptors (Created in https://BioRender.com).

IL-5 cytokine and receptor

IL-5 has pleiotropic effects on a variety of target cells, including B cells, eosinophils, and basophils. This cytokine plays a crucial role in regulating immune responses, particularly by supporting the development and function of eosinophils, which are central to promoting inflammation in allergic diseases like asthma. IL-5 is primarily responsible for the growth, differentiation, recruitment, activation, and survival of eosinophils. These cells are key players in the body's defense against parasites and are significantly involved in allergic reactions and asthma. In T2 asthma, IL-5 contributes to airway inflammation by promoting the accumulation of eosinophils in the lungs. This results in bronchial hyperresponsiveness, mucus production, and airway remodeling, all of which are hallmark features of asthma [120]. IL-5 is produced by both hematopoietic and non-hematopoietic cells, such as T cells, granulocytes, and natural helper cells. IL-5 binds specifically to the IL-5 receptor alpha chain (IL-5Rα), resulting in the formation of oligomeric receptor complexes with the beta chain (βc) [121], leading to the initiation of cytoplasmic phosphorylation events. The IL-5R is composed of two subunits: IL-5Rα, which binds IL-5 with high specificity, and βc, which is shared with other cytokine receptors and is crucial for signal transduction.

Eotaxins

Asthma severity and symptoms are associated with high pulmonary eosinophil counts. Eosinophil trafficking into the airways involves a complex interplay of cells and chemotactic factors, including IL-5, and eotaxins 1-3 (CCL11, CCL24, and CCL26) [122]. CCL11, CCL24, and CCL26 are CC chemokines with structurally diverse profiles, yet all engage the CCR3 receptor to stimulate eosinophil chemotaxis. Besides eosinophils, CCR3 is expressed in basophils [123], mast cells [124], and some Th2 cells [125], which are also predominant cells at allergic inflammatory sites. All three eotaxins have variably been reported to be increased in epithelial cells from endobronchial biopsies of patients with mild asthma and in response to allergen challenges [126, 127]. Specifically, increased CCL24 and CCL26 mRNA levels have been documented in airway epithelial brushings of asthmatic patients [128]. This upregulation is associated with decreased lung function, elevated sputum eosinophil counts, and more frequent asthma exacerbations. The link between CCL26 and asthma has been established in several studies, highlighting its role in disease development [129]. Studies demonstrate that both IL-4 and IL-13 are the central stimulus for CCL26 expression in human bronchial epithelial cells [24, 130] with elevated IL-13 levels noted in the bronchial mucosa and sputum of patients with severe asthma. Both IL-4 and IL-13 induce eotaxins through activation of the IL-4Rα receptor/STAT-6 pathway. Epithelial cells secrete CCL26 in response to T2 cytokines, and CCL26, in turn, attracts CCR3 expressing cells, including eosinophils, basophils, and Th2 lymphocytes [128]. The significant role of epithelial cells in the pathogenesis of asthma, and the importance of the interaction between CCL26 and the T2 cytokines IL-4 and IL-13 and their receptors in the inflammatory response, suggest that the modulation of CCL26 production may provide a novel therapeutic opportunity for reducing airway inflammation in asthma and other eosinophilic diseases. Moreover, increased CCL26 levels are observed in patients with other eosinophilic diseases, such as atopic dermatitis, chronic rhinosinusitis, and eosinophilic esophagitis, indicating an early role for CCL26 in the pathogenesis of eosinophilic diseases [131-134].

ILC2 cells

Innate lymphoid cells (ILCs) represent a diverse group of non-T, and non-B lymphocytes that originate from common lymphoid progenitor cells and are characterized by their lack of antigen-specific receptors [135]. Based on their production of distinct cytokines, the transcription factors driving their development, and unique phenotypic markers, ILCs are classified into three major groups:

- T-bet$^+$ ILC1s, which secrete type 1 helper T cell (Th1) cytokines like IFN-γ in response to interleukins IL-12, IL-15, and IL-18 [136].
- GATA3$^+$ ILC2s, which produce Th2 cytokines such as IL-5, IL-9, and IL-13, as well as amphiregulin (Areg), when stimulated by alarmins [137, 138].
- Rorγt$^+$ ILC3s, which secrete Th17-associated cytokines such as IL-22 and IL-17A in response to signalling through IL-23 and IL-1β [139-141].

ILC2s were first identified in 2010 as IL-13-producing non-B/non-T innate effector cells that play a critical role in T2 immune responses, especially during helminth infections [142]. During development, progenitor cells migrate to peripheral tissues in the fetal stage, where they undergo further differentiation and maturation, confirming that ILC2s are tissue-resident cells [143]. Rather than mounting antigen-specific responses, ILC2s respond to a diverse range of environmental factors, including cytokines, neuropeptides, lipid mediators, hormones, and nutrients. Following these signals, ILC2s secrete a variety of cytokines, including IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, GM-CSF, and Areg. Through the production of these cytokines, ILC2s play a role in the pathogenesis of several diseases, including parasitic infections, lung diseases, allergic conditions, autoimmune disorders, metabolic syndromes, and cancers [90, 142, 144-150]. Remarkably, ILC2s are believed to be significantly more potent than CD4$^+$ T cells in inducing T2 cytokines, with estimates suggesting that ILC2s produce 10 times more cytokines per cell compared to T cells [83]. ILC2s, play fundamental roles in lymphoid tissue development, the initiation of inflammation, and more complex immune processes, such as bridging the gap between innate and adaptive immunity and contributing to chronic inflammation [151, 152].

Initially, ILC2s were identified in adipose tissue [90]. However, they are now recognized as being distributed in various organs exposed to the external environment, including the digestive tract, respiratory system, and skin, as well as within other tissues such as the liver, muscle, and brain [153]. ILCs increased presence in the gut, lungs, or skin is associated with the severity of several chronic diseases, including asthma, atopic dermatitis, chronic COPD, and inflammatory bowel disease [88, 96, 97]. Notably, ILC2s play a pivotal role in T2 airway inflammation mediated by innate immunity [135] and assist in the repair of airway tissue damage by producing amphiregulin following influenza virus infection [147]. Emerging studies also link ILC2s to lung fibrosis, COPD exacerbations, and lung cancer [154]. These findings highlight the diverse roles of ILC2s and their involvement in the pathogenesis of several lung diseases.

Interestingly, ILC2s appear to be essential during early life for lung development and play a role in lung repair and remodelling after injury. However, their numbers increase

significantly in the lungs during infections or damaging responses that need epithelial repair. During an allergen challenge of allergic asthmatic, the increase of ILC2 in the lungs has been shown to be mediated by recruitment from the bloodstream [155]. While this increase can facilitate tissue repair, it can also lead to chronic disease phenotypes, potentially contributing to lung tissue destruction [96, 156-158]. The overexpression of specific cytokines by ILC2s early in the immune response can drive eosinophilia (via IL-5), mucus hypersecretion (via IL-13), and lung remodelling (via Areg).

ILC2s are known to express receptors for epithelial-derived cytokines, alarmins, including IL-25, IL-33, and TSLP. However, the precise contribution and relative importance of these cytokines in shaping the ILC2 phenotype remain unclear. ILC2s play a critical role in maintaining tissue homeostasis. For example, ILC2s contribute to adipose tissue browning, which may help prevent metabolic disorders [159]. Additionally, they are pivotal during parasitic infections, with ILC2-derived IL-13 being essential for helminth expulsion and subsequent tissue repair [160]. ILC2s are also involved in repairing damaged lung epithelium after viral infection by releasing IL-13 and Areg [83, 137, 161].

However, ILC2s can also drive pathological conditions by inducing allergic immune responses when exposed to allergens in tissues such as the skin, lungs, and intestine. ILC2-derived IL-5 and IL-13 are critical mediators in the development of allergic diseases, including asthma, atopic dermatitis, and food allergies [97, 162-166]. ILC2s are thought to act as initiators of these allergic responses, sensing tissue-derived alarmins early on. This is followed by the involvement of adaptive immune responses, including Th2 cells and IgE, which dominate the later stages of allergic reactions [167].

Emerging evidence suggests that distinct subsets of ILC2s may play specialized roles in tissue repair and disease, with different contributions to disease phenotypes. ILC2s exhibit plasticity, allowing them to adapt to the immune environment and differentiate into various subtypes based on the context. The early induction of pathogenic ILC2 responses may have long-lasting effects, shaping the lung environment into adulthood and contributing to the progression of chronic diseases associated with aging. For example, respiratory insults during early life are linked to an elevated risk of developing asthma and COPD due to persistent lung remodelling [168-170].

Furthermore, ILC2s are implicated in acute asthma and COPD exacerbations [95]. During viral infections, their numbers increase, leading to heightened inflammation, tissue damage, and, in some cases, the transition of ILC2s into pro-inflammatory ILC1s [96, 154]. As the lung ages, its function declines, characterized by decreased elasticity and altered immune responses [171]. In this context, ILC2 activation exacerbates viral-

induced injury, particularly in individuals with pre-existing conditions. While ILC progenitors in aged mice are increased in the bone marrow, their maintenance and functionality in the lung are diminished [172]. Although several pulmonary diseases are associated with aging, the precise role of ILC2 activity in influencing the severity of these conditions remains unclear.

Asthma and COPD Treatments

The primary aim of asthma treatment is to control symptoms, prevent exacerbations, and reduce the risk of chronic airflow limitation and asthma-related mortality. In most patients with asthma, good disease control can be achieved using standard inhaled treatments (ICS). GINA guidelines recommend that all adults, adolescents, and children over 5 years with a diagnosis of asthma should be treated with regular or (for mild asthma) as needed ICS-containing treatment to control symptoms and exacerbations. However, about 5-10% of patients included in the global population of asthmatic individuals experience various subtypes of inadequately controlled and difficult to treat asthma [173]. Patients with severe asthma experience persistent symptoms or frequent exacerbations that need to continue oral glucocorticoid therapy, or both, despite adequate treatment with high-dose ICS, long-acting β_2-agonists (LABA), and long-acting muscarinic antagonists (LAMA) [173]. In these patients, add-on treatment, which includes biological therapies, is needed to reduce the disease burden.

Inhaled Corticosteroids

Inhaled corticosteroids (ICS) are a class of medication commonly used in the management of asthma and other respiratory conditions (e.g. COPD). They are considered one of the most effective long-term control medications for asthma because they help reduce inflammation and swelling in the airways, making it easier to breathe and preventing asthma symptoms and attacks. ICS interact directly with DNA inside the nucleus, suppressing inflammation-related transcription factors like NF-kB. Additionally, ICS enhances the synthesis of adrenergic receptors in the smooth muscles of the airways, thereby increasing their effectiveness. ICS are an essential part of asthma and COPD management for many patients, helping to maintain control and improve quality of life by reducing the underlying inflammation that contributes to symptoms.

Long-acting β_2-Agonists

Long-acting β_2-Agonists (LABAs) are a class of medication used in the management of asthma and COPD. They are primarily used as part of a combination therapy with ICS to provide long-term control of symptoms. LABAs work by stimulating β_2 adrenergic receptors in the airways, leading to muscle relaxation and bronchodilation. LABAs play a crucial role in maintaining long-term control of asthma when used appropriately with ICS, improving symptom management, and reducing the need for rescue medications.

Long-acting Muscarinic Antagonist

Long-acting muscarinic antagonists (LAMAs) are a class of bronchodilator medications primarily used to manage COPD and, in some cases, asthma. They help to keep the airways open by blocking the action of acetylcholine, a neurotransmitter that can cause airway constriction. LAMAs improve lung function and reduce COPD exacerbations. LAMAs are sometimes combined with LABAs for enhanced bronchodilation.

T2 high asthma treatment: from phenotypes to precision medicine

In patients with severe asthma where standard controller medications are insufficient, biologics have emerged as a promising treatment option. These therapies target specific inflammatory pathways, focusing on individuals with a T2 inflammation endotype. Distinguishing between T2 high and low endotypes is crucial when considering biological therapy. However, the most recent biological treatment (Tezepelumab) being approved has reported effects in both T2 high and low asthma [174]. Monoclonal antibodies (mAb) targeting IL-4, IL-5, and IL-13 have been shown to decrease asthma exacerbation rates and improve lung function [175]. Omalizumab (anti-IgG), reduces exacerbations by inhibiting plasma IgE when used in combination with other anti-asthma therapy [175].

Anti IL-4Rα (Dupilumab)

IL-4 and IL-13 cytokines are the main drivers of Th2 high asthma. Dupilumab, a monoclonal antibody, is approved for the treatment of moderate-to-severe T2 asthma. It targets the IL-4 receptor alpha subunit, effectively blocking both IL-4 and IL-13 signaling pathways (**Figure** 7) [176]. In an efficacy and safety study involving individuals with glucocorticoid-dependent asthma, Dupilumab reduced the required

corticosteroid dose by 70%, compared to a 42% reduction in the placebo group. Additionally, patients treated with Dupilumab experienced a 59% lower rate of severe asthma exacerbations compared to the placebo group [177]. In patients with moderate-to-severe uncontrolled asthma, Dupilumab was tested at two doses (200 mg and 300 mg every two weeks). Both doses resulted in approximately a 50% reduction in asthma exacerbation rates compared to placebo groups. The most significant reduction in exacerbations was observed in patients with elevated eosinophil counts (≥ 150 cells/μL) compared to placebo [178]. Dupilumab has also been tested in patients with COPD demonstrating T2 inflammation, as indicated by elevated blood eosinophil counts. Those treated with Dupilumab experienced fewer exacerbations, improved lung function, enhanced quality of life, and less severe respiratory symptoms compared to those receiving a placebo [23].

Anti-IgE (Omalizumab)

Omalizumab was the first biologic therapy approved for patients with severe persistent asthma [179]. It is a recombinant humanized monoclonal IgG1 antibody that binds to the Fc fragment of free IgE, forming IgE-multimers (**Figure** 7). This action blocks the activation of mast cells and other IgE-mediated pathways [180]. Omalizumab is approved as an add-on treatment for adults and children aged six years and older with moderate-to-severe asthma [181].

Anti IL-5 (Mepolizumab, Reslizumab) and anti-IL-5R (Benralizumab)

IL-5 is a cytokine that significantly enhances eosinophil viability. Two therapies blocking IL-5 activity, Mepolizumab, and Reslizumab, are approved for severe asthma treatment. Mepolizumab was the first anti-IL-5 therapy approved for severe eosinophilic asthma (**Figure** 7). In a clinical trial involving patients with a history of two or more exacerbations in the prior year while on high-dose ICS plus bronchodilator therapies, Mepolizumab reduced the number of exacerbations requiring corticosteroid therapy and/or emergency department visits when compared to a placebo group [182]. Furthermore, in a separate trial involving participants requiring oral corticosteroids at enrolment, those treated with Mepolizumab experienced a reduction in daily corticosteroid dose compared to placebo [183]. Reslizumab has been shown to reduce exacerbation rates and increase time to exacerbation compared to control subjects [184]. In a study with participants having blood eosinophil counts of ≥ 400 cells/μL, Reslizumab increased pre-bronchodilator FEV1 at 16 weeks compared to baseline values, with a dose-dependent effect [185].

Benralizumab is anti-IL-5Rα inhibitor (**Figure 7**). In addition to inhibiting the receptor and the downstream activation of the pathway, the Fc portion of the antibody results in Fc receptor-mediated phagocytosis and clearance of all cells exhibiting IL-5R [186]. During a phase 3 clinical trial that involved patients with two exacerbations per year while on high-dose ICS and LABA, subjects were divided into 4- or 8-week dosing intervals, stratified by blood eosinophil counts. Patients with counts ≥ 300 cells/μL had fewer exacerbations and improved pre-bronchodilator FEV1 compared to the placebo group in both dosing schedules [187]. However, those with counts < 300 cells/μL demonstrated no difference in exacerbation frequency across dosing intervals relative to placebo [187]. Another trial targeting more severe asthma patients confirmed similar outcomes: reduced annual exacerbations and improved prebronchodilator FEV1 in patients with counts ≥ 300 cells/μL [188]. Interestingly, a reduced exacerbation rate was noted in those with counts < 300 cells/μL, although no improvement in pre-bronchodilator FEV1 was observed compared to placebo.

Anti-alarmins (Tezepelumab)

The triad of TSLP, IL-33, and IL-25 is recognized as a hallmark of the asthmatic airway epithelium in adults [189]. Among these epithelial cytokines, TSLP is the first to be explored as a potential therapeutic target for asthma in adults [190]. Blocking TSLP (**Figure 7**), a cytokine secreted by epithelial cells, initiates allergic inflammation, has been evaluated in limited clinical trials and is undergoing further consideration in severe asthma [191]. In a clinical study involving adults with uncontrolled asthma, patients who received Tezepelumab had lower rates of clinically significant asthma exacerbations than those who received a placebo, independent of baseline blood eosinophil counts [190]. Tezepelumab also showed a significant effect on exacerbation rates in patients with allergic and eosinophilic asthma [192]. IL-33 cytokine activates ILC2 cells and promotes Th2 responses; currently, biological agents that inhibit IL-33 activity are being studied [193]. Anti- IL-33, Tozorakimab is a novel therapeutic agent with a dual mechanism of action that blocks reduced IL-33 and oxidized IL-33 activities offering the potential to reduce inflammation and epithelial dysfunction in human disease [194].

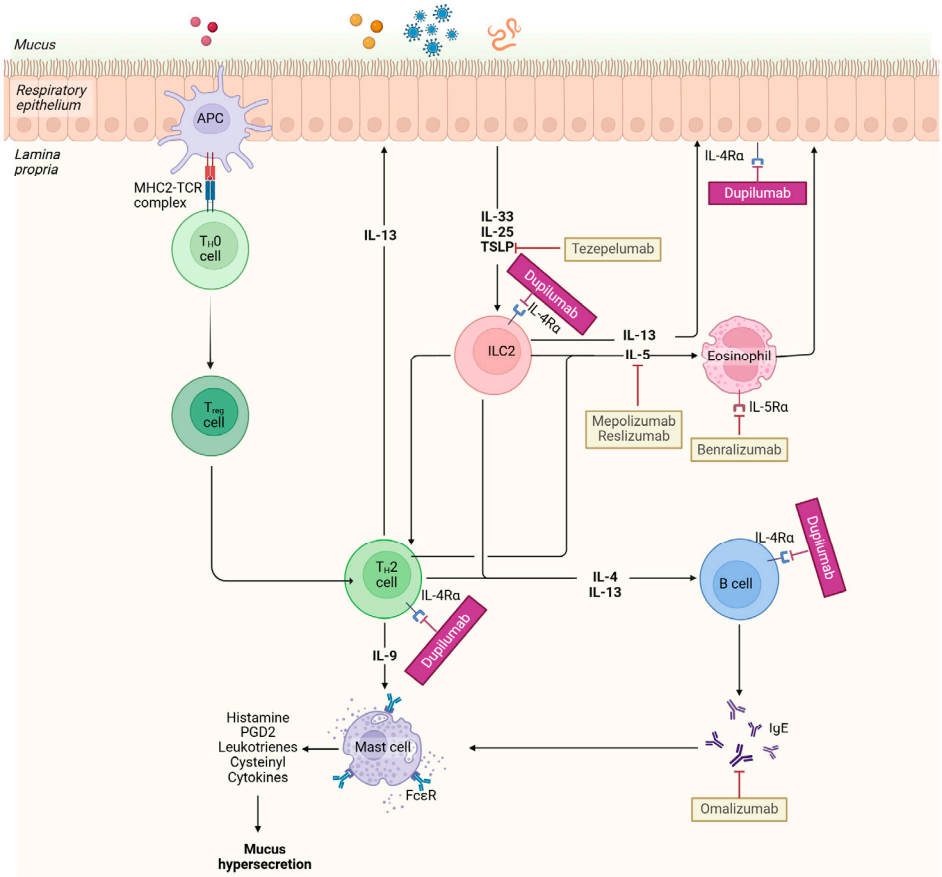

Figure 7: Current biological treatments for severe asthma. Dupilumab (anti-IL-4Rα), Mepolizumab and Reslizumab (anti-IL-5), Benralizumab (anti-IL-5Rα), Tezepelumab (anti-TSLP). (Created in https://BioRender.com).

Aims of the thesis

The primary objective of this PhD thesis was to investigate the effects of T2 cytokines on bronchial epithelial cells (BECs) and their antiviral responses using translational *in vitro* ALI cultures. The studies included epithelial cells obtained from healthy individuals and patients with respiratory diseases (asthma and COPD). T2 cytokines were added to the BEC cultures exogenously with or without RV. In addition, using a co-culture system with ILC2 cells and epithelial cells we also investigated the immunological crosstalk between these two cell types. Furthermore, we explored the underlying mechanisms of IL-4Rα monoclonal antibody (mAb) treatment under T2 inflammatory conditions, with a focus on elucidating its impact on viral-induced exacerbations. The findings of this thesis aim to reveal and explain the mechanistic effects of T2 inflammatory conditions and the IL-4Rα mAb inhibitor specifically on airway epithelial cells. This research contributes to a better understanding of how the anti-IL-4Rα clinical treatment effect is involved in improving lung function and reducing exacerbations in patients.

Specific aims:

- **Paper I**: Examine acute and chronic impacts of T2 cytokines on bronchial epithelial cells derived from healthy individuals and asthma patients. Explore the underlying mechanisms of IL-4Rα mAb therapy in acute T2-driven inflammatory conditions and during RV using a translational *in vitro* model of differentiated airway epithelial cells (ALI).
- **Paper II**: Investigate the effects and underlying mechanisms of IL-4Rα mAb treatment on bronchial epithelial cells from COPD patients under T2 inflammatory conditions, both in the presence and absence of rhinoviral infection, using a translational *in vitro* model (ALI).
- **Paper III**: Development of a co-culture system of human Innate Lymphoid Cells type 2 (ILC2) and bronchial epithelial cells fully differentiated at the air-liquid interface (ALI) to elucidate the interactions and crosstalk between these cell types. By assessing the impact of epithelial responses on ILC2 activity and vice versa, we gain deeper insight into their interplay within disease contexts.

Material and methods

This section offers an overview of the methodologies utilized throughout the thesis, as well as the advantages and challenges associated with the *in vitro* systems used. Descriptions of these procedures, in more detail, are available in the materials and methods sections of the individual publications and manuscripts.

In vitro cell culture models

Using primary cells for *in vitro* experiments offers several important benefits for achieving accurate and relevant research outcomes. Primary human cells provide a closer approximation to actual human biology compared to animal models or immortalized cell lines, which can have significant differences in cellular physiology and response to treatments. These cells maintain the genetic and epigenetic characteristics of the donor individuals, capturing the genetic diversity and complexity found in human disease populations. Primary human cells are particularly useful for modeling complex diseases and understanding the underlying mechanisms, especially for conditions that are hard to replicate in animal models. Using primary cells from specific patient cohorts can help tailor treatments and understand individual responses, covering the way for personalized therapeutic strategies.

ALI culture

In paper I, II, and III the air-liquid interface (ALI) cultures were used. These cultures are broadly used and recognised as a valuable tool for lung research due to their ability to mimic the physiological conditions of the human respiratory tract harbouring some features of the physical barrier. ALI cultures facilitate the growth of primary human bronchial epithelial cells (HBECs) in a manner that promotes extensive mucociliary differentiation. This results in an *in vitro* model that closely represents the *in vivo* airway, making it highly relevant for respiratory research [195]. ALI culture models can replicate the typical features of the respiratory tract *in vitro*, both in healthy and diseased states. This ability helps reduce the need for animal experiments. The ALI system allows

the establishment of a culture environment that is more representative of *in vivo* conditions compared to other culture systems. This is particularly useful for mechanistic studies of respiratory epithelial cells and drug interventions [195]. The ALI method is advantageous for promoting cell differentiation and optimizing the morphological and histological characteristics of airway epithelium cells, making it a critical tool for developing *in vitro* respiratory models as well to study interactions between respiratory pathogens and the host. However, there are different challenges that we as scientists are faced with when using ALI cultures. The ALI culture system requires significant time and resources, leading to lower and slower throughput. Differentiating cells in ALI cultures is a time-consuming and costly effort. Successfully handling and differentiating cells in ALI cultures demands rigorous expertise, patience, and persistence. There is considerable variability in how cells from different donors respond to challenges or treatments. This variability can introduce alteration in experimental results between donors and complicate data interpretation.

To develop a fully differentiated ALI culture, dissociated epithelial cells (NHBE/D-HBE-As, Lonza) are initially expanded in store scale in a submerged culture (passage 3) before being differentiated on a porous membrane insert (0.4 µm, 24 well permeable supports). After seeding, human airway epithelial cells are cultured and submerged in the medium (PneumaCult™-Ex Plus Medium) which is applied to both the apical chamber (inside the insert) and the basolateral chamber (below the insert). Once the cellular monolayer reaches confluency, the medium is replaced with an ALI differentiation medium (PneumaCult™-ALI Basic Medium plus supplements), added only to the basolateral chamber. During differentiation, the cells develop basal epithelial cells that remained close to the insert membrane, while ciliated and goblet cells orient towards the air (**Figure 8A**). Following the differentiation of the cells, the apical chamber, along with the cell layer, is then exposed to air.

Human Innate Lymphoid Cells type 2 (ILC2)

In paper III, ILC2s were exploited as a source of T2 cytokines to replicate T2 asthma conditions. The isolation of peripheral blood mononuclear cells began with magnetic labeling, followed by the separation and depletion of lineage-positive cells (mature hematopoietic cells such as T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, erythroid cells, and their committed precursors). The depletion of lineage-positive cells resulted in the enrichment of untouched stem and progenitor cells (unlabelled lineage-negative cells) which were quantified and subjected to live-dead staining, followed by antibody staining for fluorescence-activated cell sorting (FACS). Ultimately, the isolated ILC2s from peripheral blood were characterized as Lin^-,

$CD127^+$, $CD161^+$, $CRTH2^+$, and c-$Kit^{-/+}$ [196]. The yield of cells post-isolation was low (between 5,000 to 40,000 cells retrieved from 400 mL of blood) and insufficient for co-culture. Consequently, the sorted ILC2 fraction was expanded [197] (**Figure 8B**). Expanded ILC2 cells were transferred to 24 well plates underneath the insert membrane of ALI cultures (**Figure 8C**). Combination media was used for co-cultures. The viability of cells was daily checked by microscope and supernatant was collected (**Figure 8E**). A schematic summary of the experimental procedure is in **Figure 8**.

Working with ILC2s *in vitro* presents several challenges due to their absence in peripheral blood. The low amount of these cells means that initial isolation yields insufficient quantities for direct experimental use, necessitating an expansion phase to obtain viable numbers. Moreover, the proliferation of ILC2s during in vitro culture is highly variable and significantly influenced by the individual donor's characteristics. This donor dependency can lead to inconsistencies in experimental outcomes, as the growth rate and responsiveness of ILC2s may vary between samples. Differences in genetic background, health status, and environmental factors of donors all impact the behavior of ILC2s in culture, complicating efforts to standardize experimental conditions. Additionally, maintaining the functional phenotype of ILC2s during expansion may be challenging. Prolonged culture or inadequate conditions may lead to altered cell characteristics, which could affect their activity and function in assays.

ALI cell culture stimulation and treatment

ALI cultures in Paper I and II were stimulated acutely (**Figure 9A**) or chronically (**Figure 9B**) by the basolateral addition of human recombinant proteins IL-4 and IL-13 to mimic T2 inflammation in these experiments. Additionally, the cells were treated with either an IL-4Rα therapeutic monoclonal antibody (mAb) or human IgG4 Ab as control (**Figure 9A**).

Human rhinovirus and cell culture infection

Human rhinovirus serotype 1B (HRV1B, # H1611A) was purchased from Virapur (San Diego, USA). HRV stocks were generated by infecting H1-Hela cells the resulting supernatant was Polyethylene glycol (PEG) precipitated, rinsed with PBS, and re-suspended in DMEM, 1% Penicillin/Streptomycin Fungizone, 5mM MgCl, 20 mM Hepes Buffered Saline pH 7.2. A retained vial was thawed, and infectious titer was determined by TCID50 assay. The infectious titer by TCID50 was 6.3×10^9 infectious units per ml. ALI cultures were infected with HRV1B (MOI 0.1 or 1) apically (**Figure 8D and Figure 9**). Infection was carried out for 3.5h at 33^{0}C. Cultures were washed out with DPBS (w/o Ca, Mg) moved to a 37^{0}C incubator, and harvested 24h after

infection (**Figure 9**). During co-culture, supernatant was collected on various days, and cells were harvested on day 7 post-infection (**Figure 8E**).

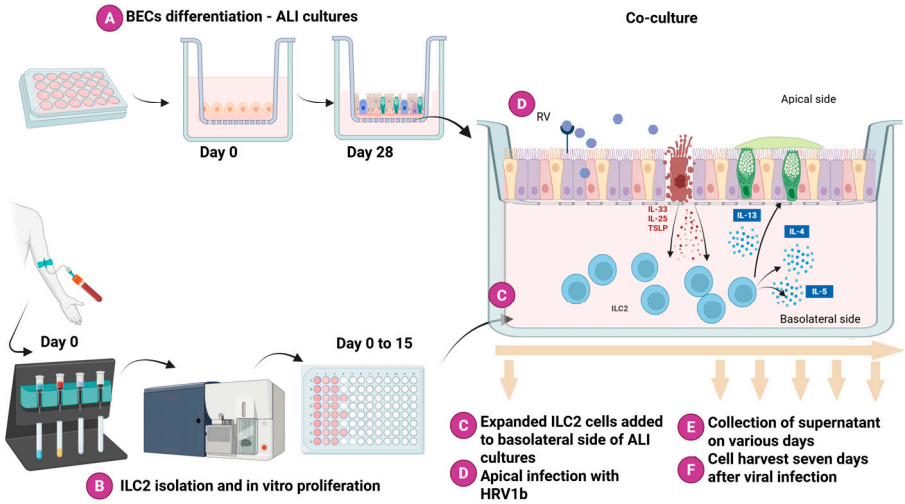

Figure 8: Summary of experimental procedure for ALI/ILC2 co-culture: A) Differentiation of ALI cultures B) Isolation and expansion of ILC2 cells C) Introduction of expanded ILC2 cells to fully differentiated ALI cultures D) RV infection (apical) E) Collection of supernatant and F) Cell harvest (Created in https://BioRender.com).

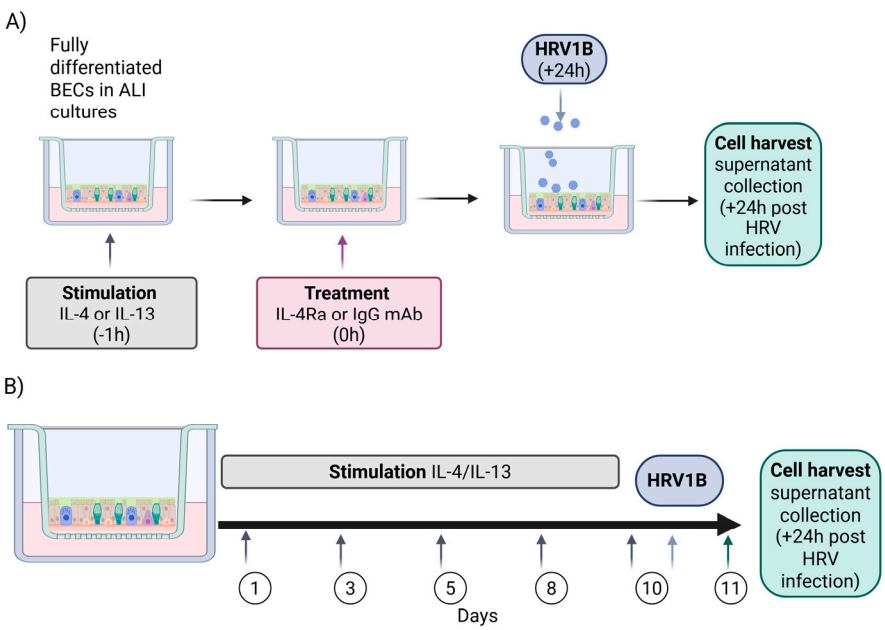

Figure 9: Experimental design A) Acute stimulation of ALI cultures with T2 cytokines, treatment with IL-4Rα mAb and viral infection B) Chronical stimulation of ALI cultures with T2 cytokines, and viral infection (Created in https://BioRender.com).

Assessing mRNA expression

The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) technique is used frequently, and it is a major method for quantifying gene expression. In this technique, mRNA levels can be quantified as either absolute or relative. The expression of the target gene is normalized using a housekeeping gene as a reference. These reference genes are stably expressed, regardless of tissue type, developmental stage, cell cycle state, or external signals. The 2-ΔΔCT method was employed to calculate relative changes in gene expression based on data obtained from real-time quantitative PCR experiments. The data are presented as the fold change in target gene expression normalized to the geomean of control genes (GAPDH and HPRT1) and relative to the vehicle (untreated) or IgG-treated cells [198].

Quantification of protein expression

Multiplex immunoassays based on electrochemiluminescence

To determine protein concentration in the assay, U-Plex, Meso Scale Discovery (MSD) was used. The MSD technique is a powerful tool offering quantifiable assessments with high sensitivity, multiplexing capabilities, large dynamic range, and efficient use of sample volumes for the detection and quantification of a wide range of biomolecules.

The technique employs electrochemiluminescence (ECL) detection, which involves the use of electrodes to stimulate luminescence from labels attached to the target molecules in the presence of an electric potential. This method is useful for measuring cytokines present at low concentrations in biological samples and can measure multiple analytes simultaneously within a single sample. The technique offers a broad dynamic range, enabling the quantification of analytes across several orders of magnitude without the need for multiple dilutions. Due to its high sensitivity, the MSD technique can be performed with minimal sample volumes (<50 µL of sample for up to 10 cytokines).

Physiological and Pathological Assessments of ALI Cultures

Measurements of Cilia Beating Frequency

In manuscript III, cilia beating frequency (CBF) was used to assess the physiological changes in ALIs due to possible interference and T2 cytokines of ILC2s in co-culture. CBF was measured by using a Nikon Eclipse Ti2 microscope with an integrated incubator and Cilia-X software for batch CBF analysis. The measurements and analysis are performed according to manufacturer instructions.

Histology

In manuscript III, histology was used to provide insights into the normal structure and function of tissues as well as pathological changes associated with diseases or stimulation. The fixation, paraffin embedding, and Alcian Blue (AB) staining process for ALI cultures preserves tissue architecture for histological analysis. Fixation stabilizes cellular components, followed by dehydration through graded alcohol washes and clearing in xylene. The tissue is then infiltrated with paraffin wax and sectioned using a microtome, providing a medium for thin sections suitable for microscopy. AB staining visualizes acidic polysaccharides, aiding in identifying mucus production and goblet cells in the epithelial layer [199].

AI technology

In this thesis, I applied advanced AI technologies to improve the clarity and precision of writing. Specifically, AstraZeneca's internal ChatGPT was helpful in refining and improving the sentences throughout the document. Additionally, the Research Assistant (RA) played an essential role in identifying relevant information and sourcing references that supported the thesis. I have processed the generated text and take full responsibility for the content.

Results and Discussion

In T2-driven airway diseases, epithelial cells are continuously exposed to excessive T2 cytokine production, impacting both the epithelial cells and the innate immune responses. In healthy epithelium, viral infection triggers normal interferon (IFN) and alarmin epithelial response. Conversely, in diseased epithelium, individuals experience more severe symptoms and exacerbations. The critical question is whether and how the innate response contributes to these exacerbations. To address this, we used our 3D in vitro model, ALI cultures, to explore the mechanism that could explain the source of viral-induced exacerbations in asthma and COPD patients. Several groups showed that IFNs are impaired in asthma patients [200] and that T2 cytokines can further reduce the IFN response [201].

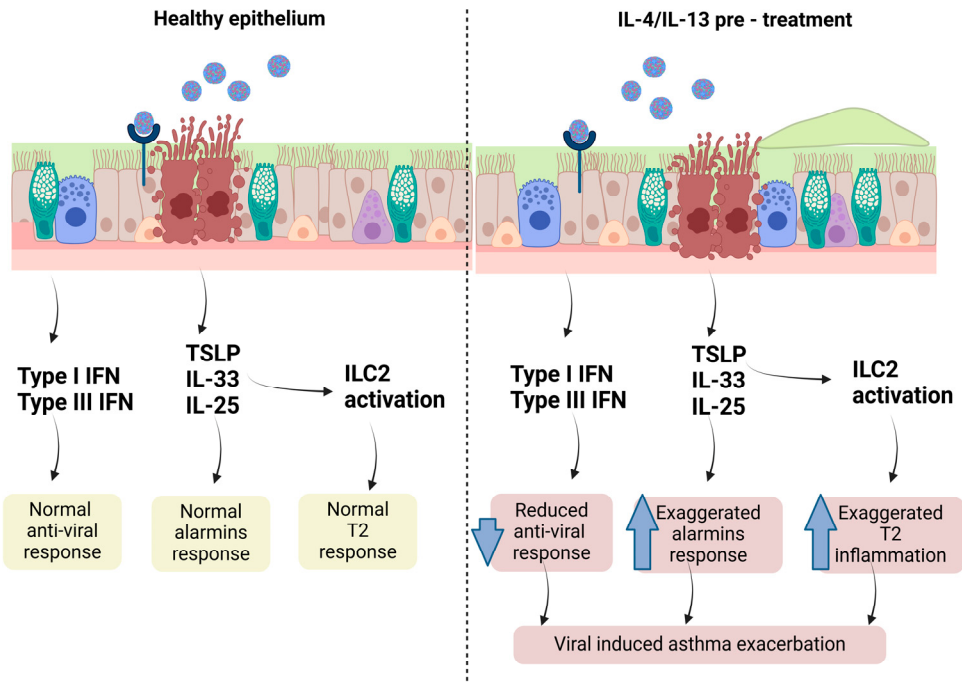

Figure 10: Hypothesis: antiviral and alarmins response in healthy and IL-4/IL-13 pretreated BECs (Created in https://BioRender.com).

Overall hypothesis for this thesis: healthy epithelium can mount a proper and balanced T2 antiviral response whereas diseased epithelium exhibits an exaggerated epithelial response with alarmin production, increased T2 inflammation, and reduced antiviral defense (**Figure 10**).

Effects of Acute and Chronic T2 Cytokine Exposure on Healthy *vs.* Asthma BECs (Paper I and III)

Our primary aim was to investigate the alarmin and antiviral responses in IL-4 and IL-13 stimulated bronchial epithelial cells (BECs) from both healthy and asthmatic patients, using an air-liquid interface (ALI) culture model. We examined the effects of both acute and chronic stimulation in these BECs (**Paper I**). To ensure activation of the IL-4/IL-13 pathway, we focused on CCL26 expression, as CCL26 is previously known to be directly affected by IL-4 and IL-13 [202]. Our experiments confirmed the upregulation of both the CCL26 gene and protein in the cultures (**Figure 11**), in response to T2 stimulation independent of RV infection. We identified a significant difference in CCL26 protein responses between healthy and asthmatic BECs, suggesting that in asthmatic individuals, this chemoattractant enhances eosinophil migration to the lungs with expression correlating with asthma severity [128].

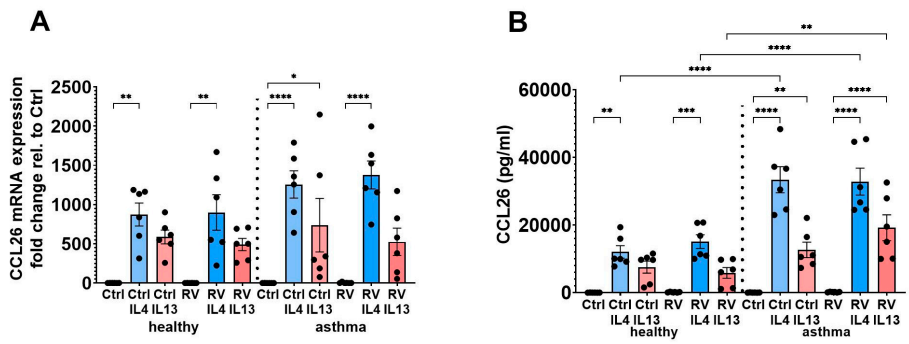

Figure 11: CCL26 A) mRNA and B) protein activation by T2 cytokines in healthy and asthma BECs.

To further explore our hypothesis, we investigated the acute effects of IL-4 and IL-13 on alarmin responses in BECs from healthy and asthmatic individuals. We observed an increase in TSLP in asthmatic BECs after viral infection and an additional significant increase in combination with either IL-4 or IL-13 stimulation (**Figure 12A**). Additionally, the IL-33 response was elevated in asthmatic BECs after IL-4 and IL-13 stimulation and viral infection (**Figure 12B**). IL-25 levels were significantly higher in

asthmatic BECs compared to healthy ones following IL-4 and IL-13 stimulation and viral infection (**Figure 12C**). These findings collectively confirm our hypothesis that a T2 cytokines environment leads to an increased epithelial alarmin response.

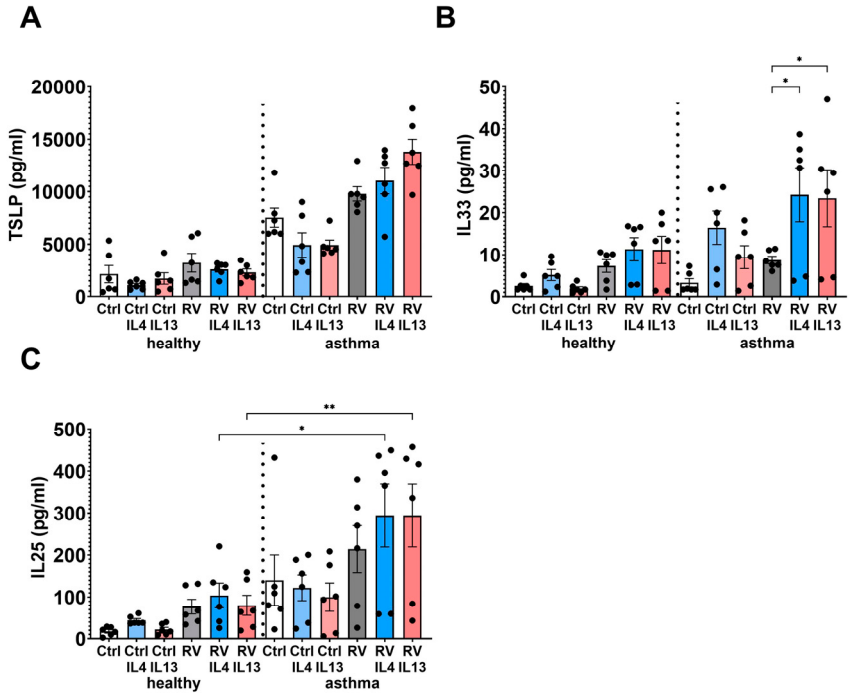

Figure 12: Effect of acute IL-4 and IL-13 stimulation and viral infection on BECs from healthy and asthma individuals A) TSLP B) IL-33 and C) IL-25 responses.

Our results motivated an investigation into the alarmin response after chronic exposure to a combination of IL-4 and IL-13 in both healthy and asthmatic BECs. We demonstrated that chronic exposure to T2 cytokines resulted in a significant reduction of TSLP in asthmatic BECs (**Figure 13A**). Contrarily, we did not observe significant changes in IL-33 and IL-25 levels in healthy or asthmatic BECs (**Figure 13B-C**). These findings led us to review the literature, and it was surprising to discover how little is known about the direct effects of T2 cytokines on alarmins, as research typically focuses on the downstream effects of alarmins. In 2008, Zhang et *al.* [203] reported that IL-13 stimulation for 10 days significantly decreased TSLP mRNA and protein levels, which we confirmed with our findings. While TSLP expression is initially elevated in asthmatic cells *in vitro* it is decreased with chronic IL-13 stimulation. The elevated baseline TSLP may suggest a genetic or epigenetic predisposition to increased TSLP expression in individuals with asthma. While TSLP may initiate a T2 response,

persistent IL-13 activity could down-regulate TSLP production, thereby dampening its effect. Our work confirms the previously published data and supports T2 cytokines playing a role in regulating TSLP activity.

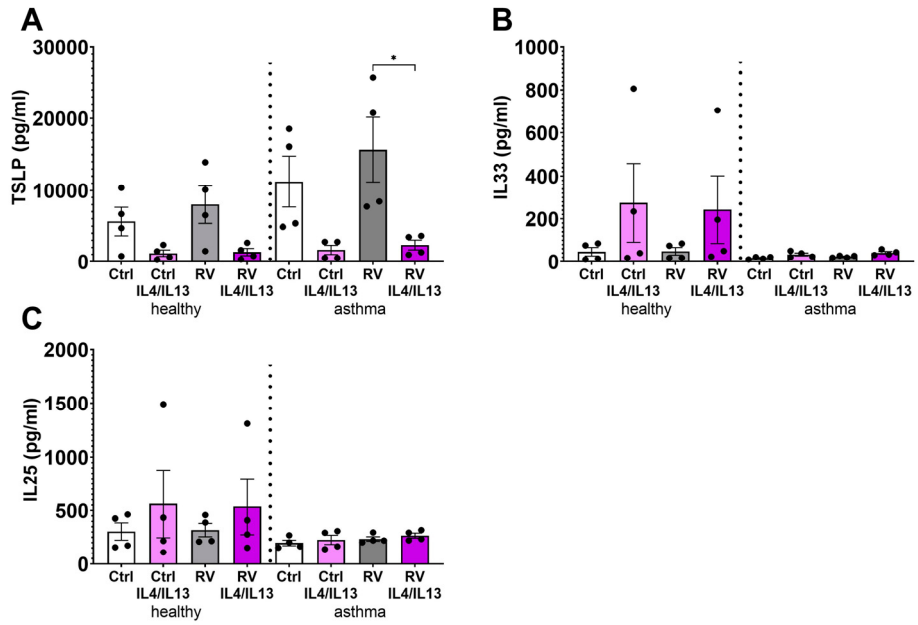

Figure 13: Chronic exposure to T2 cytokines and alarmins rsponse A) TSLP B) IL-33 C) IL-25

The effect of IL-13 on β2-adrenergic receptor (β2AR) desensitization in human airway epithelial cells has been evaluated, suggesting that IL-13 can cause receptor desensitization [204]. In our study, chronic stimulation with IL-13 might lead to desensitization of the IL-13 receptors on the bronchial epithelial cells, which means that these receptors become less responsive to IL-13 over time. This can result in decreased signaling and reduced induction of TSLP expression. Furthermore, prolonged exposure to IL-13 has been hypothesized to create a plastic epithelial phenotype that is profibrotic through continuous secretion of soluble mediators, which could include reduced TSLP expression as part of the altered signaling landscape [205].

Cells often have built-in negative feedback mechanisms that regulate cytokine production and prevent overexpression that could lead to continuously persisting inflammation. Chronic exposure to IL-4/IL-13 might activate such feedback loops that suppress TSLP production as a regulatory homeostatic measure. Also, long-term cytokine stimulation can cause changes in gene expression through epigenetic mechanisms such as methylation or histone modification, potentially leading to

reduced expression of the TSLP gene. These mechanisms can vary based on the experimental conditions, such as the concentration of IL-4/IL-13, the duration of exposure, and the specific characteristics of the bronchial epithelial cells used in the study. Further experimental investigation would be necessary to determine the exact cause in a particular setting.

However, in our co-culture system as detailed in **Paper III**, we observed a decrease in TSLP levels starting from day 3 in the co-culture of BECs and ILC2s, involving BECs from both healthy subjects and asthma patients (**Figure 14**). By day 5, this decrease became statistically significant in co-cultures with asthma BECs. These findings further support the concept of chronic exposure to T2 cytokines leading to a reduction in TSLP levels. It's important to note that while **Paper I** involved the exogenous addition of T2 cytokines, in **Paper III**, in the co-culture, the T2 cytokines were produced by ILC2s, which in turn seemed to affect TSLP levels.

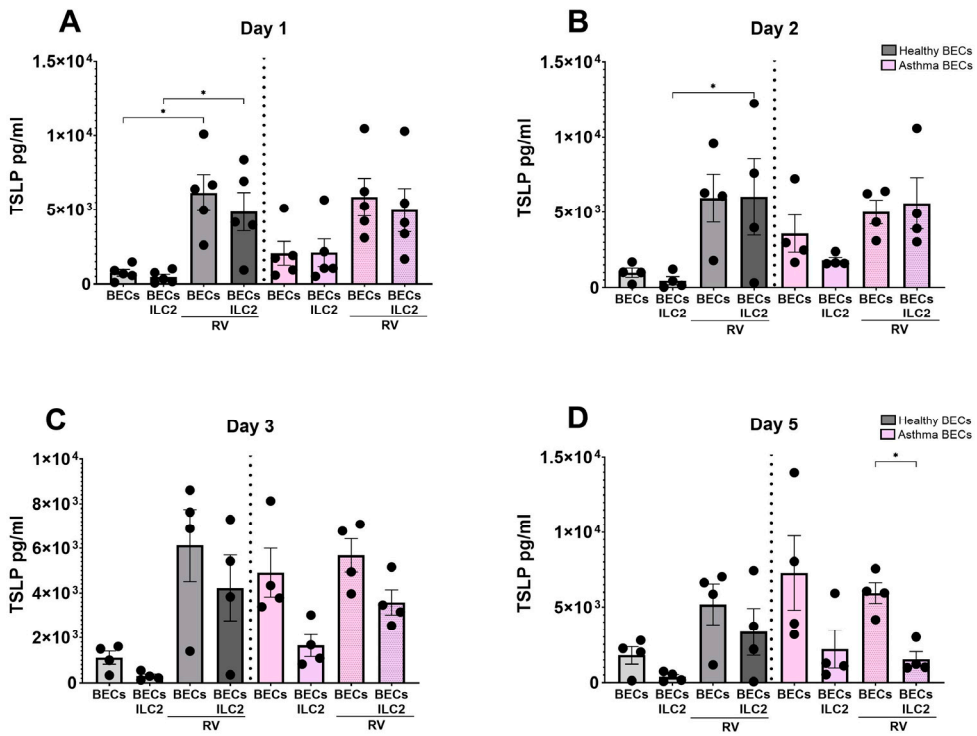

Figure 14: TSLP response over time in the co-culture model. Initial baseline levels were recorded A) on day 1, followed by observations B) on day 2 C) day 3 and D) day 5.

Our second major observation regarding the antiviral response was the inability to detect impairment of IFNs in the BEC cultures (**Paper I and II**) following acute

stimulation (**Figure 15A-B**). We tested several viral strains and investigated different post-infection time points; however, we were unable to replicate published results [201]. This discrepancy could be attributed to various factors, such as the source of the epithelial cells, differences in culturing systems, and the limited number of donors, all of which may have impacted our experiments. However, after chronic stimulation with IL-4 and IL-13, followed by viral infection in asthmatic BECs, we observed a significant increase in IFNβ compared to healthy BECs (**Figure 15C**). Notably, the increase in IFNβ gene expression was observed after viral infection, with an additional, significant increase demonstrated when T2 cytokines were combined with a viral infection.

We also investigated type III interferons (IFNλ1) and found that T2 cytokines, in combination with viral infection, decreased the IFNλ1 gene expression (**Figure 15D**). This is an interesting finding because type I and type III interferons are generally expected to have similar dynamics and be released simultaneously. However, it is worth noting that type I interferons can induce a prolonged response, which may lead to host cell damage [206]. This differential regulation might be due to some protective mechanism to balance the antiviral response while minimizing potential harm to the host cells.

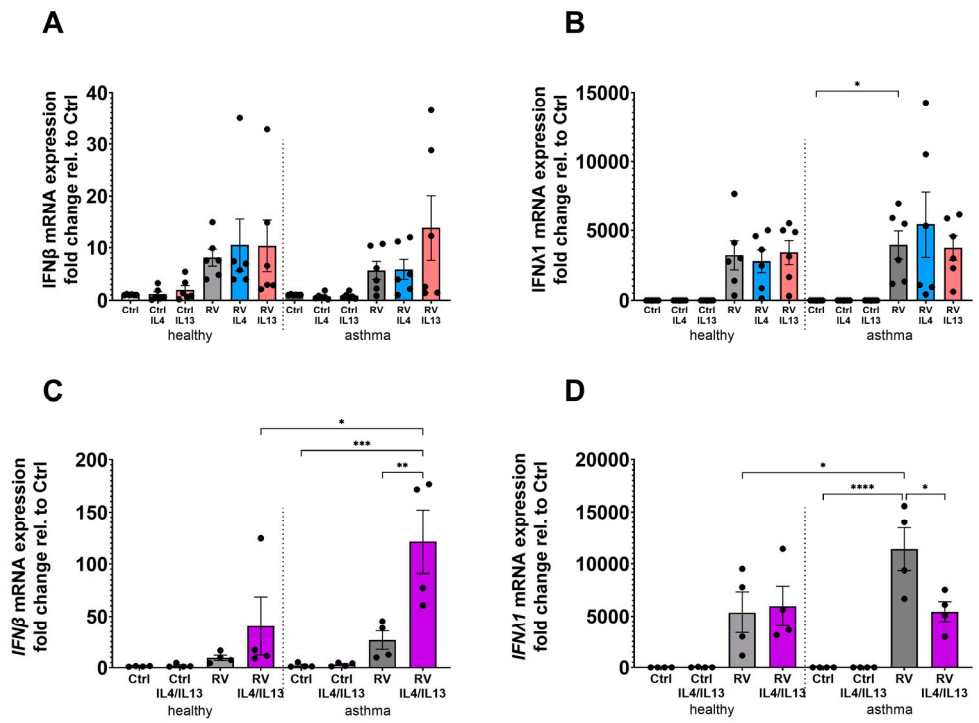

Figure 15: IFN response following acute (A-B) and chronic (C-D) exposure to T2 cytokines and viral infection in healthy and asthma BECs. A-B) Demonstrate the effects of acute exposure to T2 cytokines C) Significant increase in IFNβ gene expression in asthma BECs D) Significant decrease in IFNλ1 gene expression in asthma BECs.

It has been proposed that in asthmatic patients, the IFN response to RV is impaired, leading to increased viral replication and delayed cell death. However, a protective role of type III IFN in asthma has also been shown, suggesting a distinctive role of the different IFN responses in asthma [63]. Asthmatic patients demonstrate deficient type I and type III interferon production in response to viral infections, which could explain increased susceptibility in allergic asthma. This deficiency is linked to molecular mechanisms such as IgE/FcεR1 cross-linking inhibiting virus-induced IFNα responses [207, 208]. In well-controlled asthma patients, rhinovirus-induced IFN production was not found to be impaired, suggesting that antiviral mechanisms might not be intrinsically impaired in asthma [209].

These publications and our findings indicate that the IFN response in asthma patients can be reduced or altered in certain contexts, particularly in response to viral infections, which may contribute to the increased susceptibility to exacerbations seen in these patients. However, the response can vary, and in some cases, such as well-controlled asthma, IFN response may not be significantly impaired. This suggests a complex interplay between the IFN response and asthma pathophysiology, which may vary among individuals and under different conditions, and explains the differences we observed in our studies.

Uller *et. al* showed asthma BECs expressed less IFNβ and more TSLP mRNA and protein in response to dsRNA than BECs from subjects without asthma [210]. In asthma patients, blocking TSLP activity using Tezepelumab *in vitro* reduced airway epithelial inflammatory responses, including IL-33 and T2 cytokines in response to viral challenge, without affecting the antiviral host resistance [211]. However, the direct effect of TSLP *in vitro* on IFNβ and IFNλ1 in BECs was not previously investigated. We, therefore, initiated an additional pilot study where we directly stimulated healthy BECs with TSLP and subsequently infected them with a virus to investigate the gene expression of IFNβ and IFNλ1. Our results revealed that exogenously added TSLP significantly reduced the IFNβ gene expression (**Figure 16A**), whereas IFNλ1mRNA remained unchanged (**Figure 16B**). Moreover, IL-33 did not affect the antiviral responses (**Figure 16A-B**). From these findings, we conclude that the exogenously added TSLP inhibits the IFNβ mRNA without affecting IFNλ1.

The relationship between TSLP, T2 cytokines, and IFNβ suggests a complex interplay in immune regulation. The inhibition of TSLP by IL-4 and IL-13, which are key T2 cytokines, implies a regulatory mechanism where the suppression of TSLP could lead to increased levels of IFNβ. Conversely, high levels of TSLP could result in decreased IFNβ levels, indicating that TSLP can negatively regulate the IFNβ response. This regulatory axis highlights the balance between different cytokines in modulating

immune responses, where TSLP can influence the level of IFNβ, a critical cytokine in antiviral defense and immune regulation. Unfortunately, we did not follow up stimulation with TSLP and performed a study in diseased BECs.

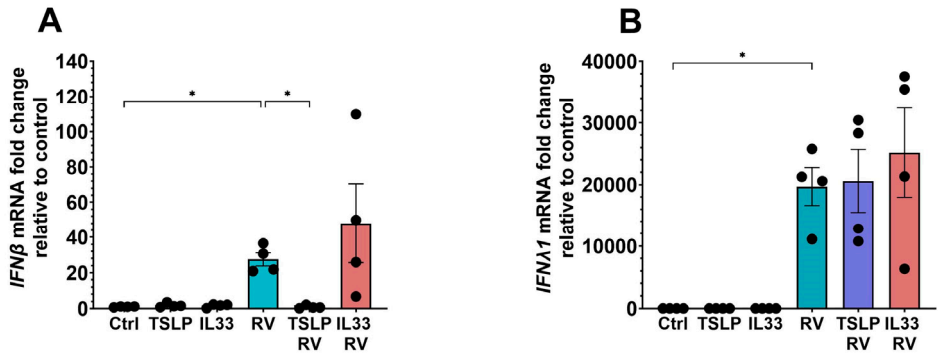

Figure 16: IFN response in healthy BECs after TSLP and IL-33 stimulation A) TSLP stimulation significantly reduces the IFNβ response. B) TSLP has no effect on the IFNλ1 response (unpublished data).

Towards precision medicine in asthma and COPD: Targeting T2 cytokines (Paper I and II)

We next investigated the effects of an IL-4Rα mAb on the inflammatory (CCL26 and alarmins) and antiviral (IFNs and MDA-5) responses induced by the T2 cytokines in RV-infected asthmatic and COPD BECs using a translational *in vitro* model of differentiated airway epithelial cells (**Paper I and II**). Treatment with IL-4Rα mAb effectively inhibited IL-4 and IL-13-induced CCL26 protein release and gene expression on the background of RV infection in asthma (**Figure 17A**) and COPD BECs (**Figure 18A-B**). IL-13-induced TSLP secretion in asthma BECS was significantly reduced by IL-4Rα mAb treatment (**Figure 17B**). IL-4Rα mAb treatment did not significantly affect the expression of IFNβ (**Figure 17C**) or the cytosolic pattern recognition receptor MDA5 following T2 stimulation and viral infection (**Figure 17D**). We observed a similar response in COPD BECs, where IL-4Rα mAb treatment preserved natural antiviral gene expression (**Figure 18C-F**) [212].

In the present studies, we demonstrated that IL-4Rα mAb effectively modulates the bronchial epithelial immune response to RV under a T2 environment (T2 cytokine stimulation), reducing CCL26 in both asthma and COPD BECs. Since CCL26 is a chemokine critically involved in recruiting eosinophils to the airways, a key feature of

airway inflammation in T2 high asthma [213], it emerges as another crucial driver of airway inflammation and hyperresponsiveness targeted by anti-IL-4Rα therapy. The observed increases in eosinophil blood counts observed in Dupilumab treated patients with asthma, chronic rhinosinusitis, and atopic dermatitis treated with Dupilumab [214] may be due to reduced CCL26 release from the airway epithelium disrupting the chemoattractant signal required for blood eosinophils to migrate to the tissue, in turn resulting in increased levels of blood eosinophils. This phenomenon has been observed in a preclinical *in vivo* model, where IL-4Rα blockade prevented lung tissue entry of eosinophils by attenuating chemotaxis and endothelial activation [215, 216]. Our findings align with these observations and identify inhibition of CCL26 release from the lung epithelium as one potential mechanism of action behind the therapeutic efficacy of IL-4Rα biologics in asthma. By reducing and normalizing key inflammatory mediators such as CCL26 and TSLP, while preserving essential antiviral defense mechanisms, IL-4Rα blockade offers a balanced approach to managing asthma and COPD symptoms and preventing exacerbations. Our studies provide valuable insights into the modulation of inflammatory and antiviral responses in human BECs by the T2 cytokines during RV infection, and the effect of IL-4Rα blockade.

Typically, *in vitro* experiments including pharmacological treatment are initiated before stimulation. This methodology is adopted to ensure that the therapeutic agents have sufficient time to exhibit their effects before the cells are subjected to any inflammatory or pathological stimulus. Such an approach, while useful for understanding the potential prophylactic benefits of a treatment, doesn't fully capture the treatment dynamics once a disease has been established. In our study, the anti-IL-4Rα antibody was deliberately not administered prophylactically, as we sought to replicate a more clinically relevant model of diseased lungs. The decision was based on the understanding that in actual disease scenarios, treatments are often applied after the onset of symptoms and the establishment of the disease rather than prior. By mimicking this sequence of events, we aim to better understand the efficacy and dynamics of therapeutic interventions in conditions parallel to those present in diseased lungs.

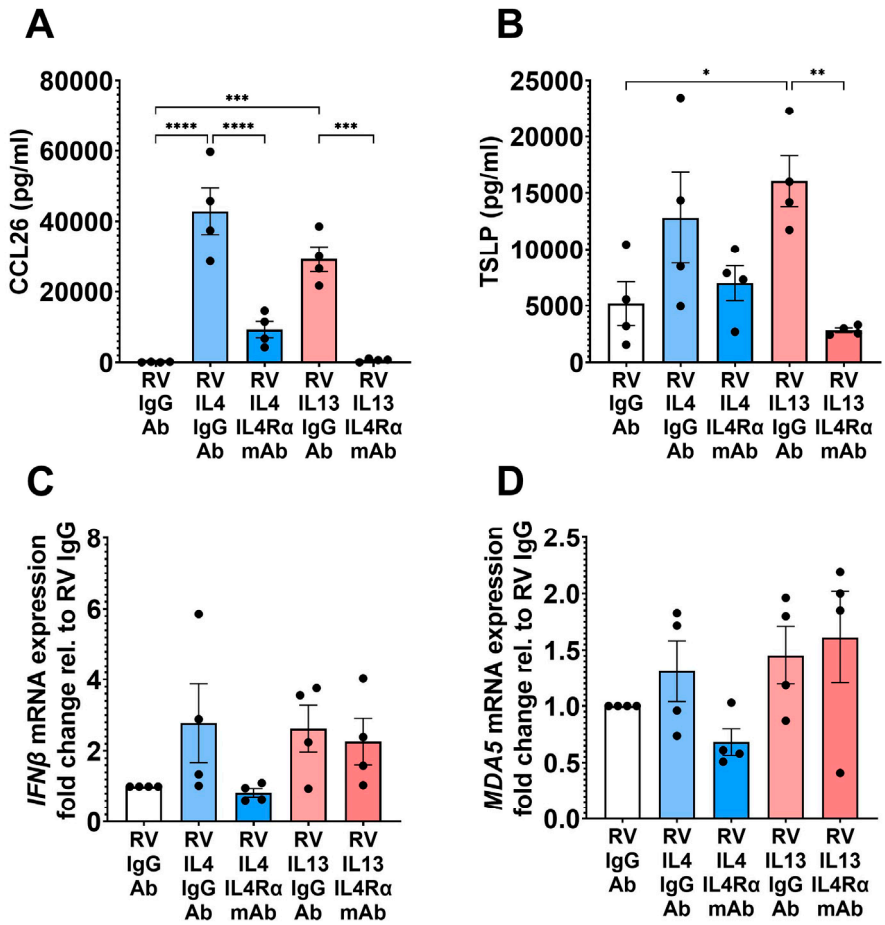

Figure 17: Asthma BECs and IL-4Rα mAb treatment effect after IL-4 or IL-13 stimulation and viral infection on A) CCL26 protein B) TSLP protein C) IFNβ mRNA and D) MDA-5 mRNA.

This approach provides valuable information on how treatments might perform in real-world conditions, where interventions occur after disease symptoms present, offering potential pathways for enhancing therapeutic strategies in clinical settings. According to the reviewer from a recent revision of our excepted paper [212] in COPD BECs our novel data was supplement to Dupilumab treatment in COPD patients [21], adding new possible mechanisms for Dupilumab and control of exacerbations.

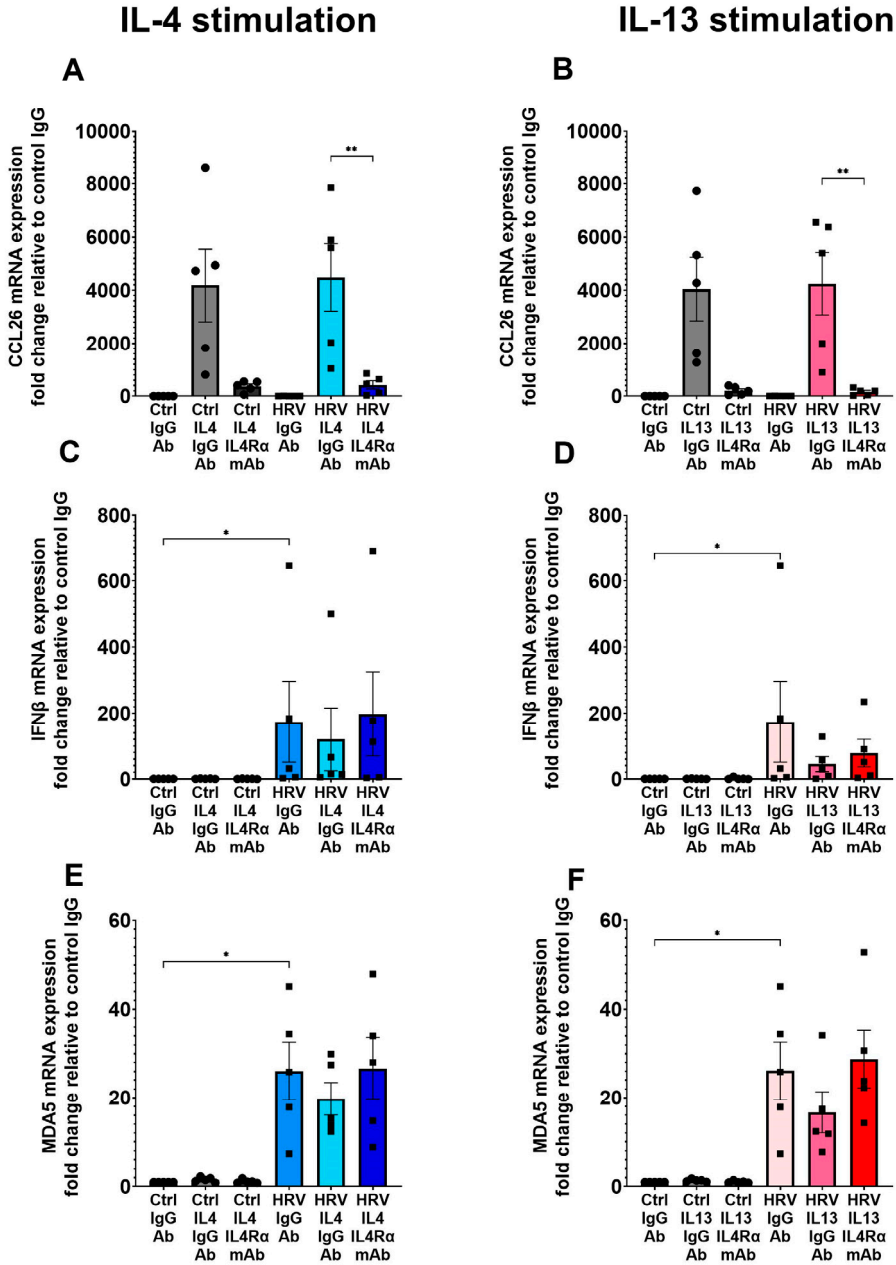

Figure 18: COPD BECs and IL-4Rα mAb treatment effect after IL-4 or IL-13 stimulation and viral infection on: A-B) CCL26 gene expression, C-D) IFNβ gene expression, E-F) MDA-5 gene expression.

Modeling Respiratory Defense: Epithelial and ILC2 Crosstalk in Action (Paper III)

To explore the natural source of T2 cytokines and their effects on BECs, we developed a co-culture model consisting of ILC2 cells and BECs derived from both healthy individuals and asthma patients. This approach allows us to closely mimic the diseased lung environment, providing a more translational research model. ILC2 cells are potent producers of T2 cytokines, making them ideal candidates for co-culture systems with BECs from both healthy and asthmatic sources.

Due to the rarity of ILC2 cells in blood, we expanded them *in vitro* to increase their availability. Before initiating co-culture experiments, we compared the marker expression of expanded cells with freshly isolated ILC2 cells. Sorting using FACS confirmed no differences in marker expression between blood-derived and expanded ILC2s (**Paper III**). During the expansion, we evaluated their ability to produce T2 cytokines, and the results confirmed their capacity to produce IL-4, IL-13, and IL-5 (**Paper III**). Additionally, in the expansion protocol, we incorporated a day of ILC2 cell starvation to ensure that they produce only baseline levels of cytokines before being introduced into the co-culture.

To further qualify ILC2 cells, we assessed whether expanded ILC2 cells could be stimulated with alarmins and/or a virus, followed by measurement of T2 cytokines in the supernatant, in a subsequent study. The findings showed that the virus alone did not enhance T2 cytokine production. However, a cocktail of alarmins, either alone or in combination with RV, significantly elevated the production of IL-4, IL-13, and IL-5 on various days (**Figure 19**).

Upon co-culturing BECs with ILC2 cells, we assessed the BECs' response to alarmins and the effect of ILC2s on alarmin expression. As detailed in **Figure 14**, ILC2 cells specifically decreased TSLP expression in asthmatic BECs. This, together with our previous results, suggests that T2 cytokines released by ILC2 cells may be directly involved in this reduction. Additionally, we evaluated T2 cytokine release, finding that IL-5 and IL-13 were significantly increased from day 3 in co-culture (**Figure 20**), while IL-4 was released at lower levels (data not shown).

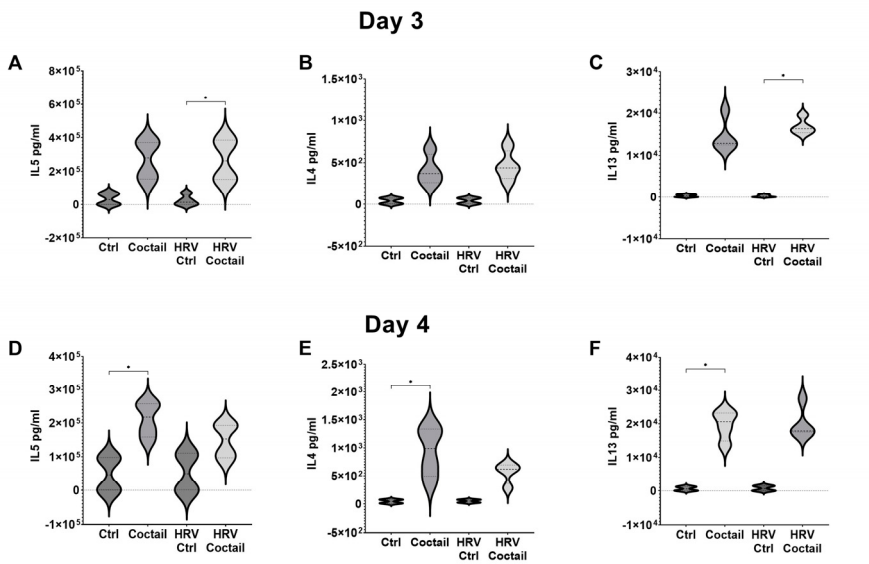

Figure 19: ILC2 T2 cytokine release after *in vitro* alarmin stimulation and RV infection A-C) IL-5, IL-4, and IL-13 release on day 3 D-F) IL-5, IL-4, and IL-13 release on day 4.

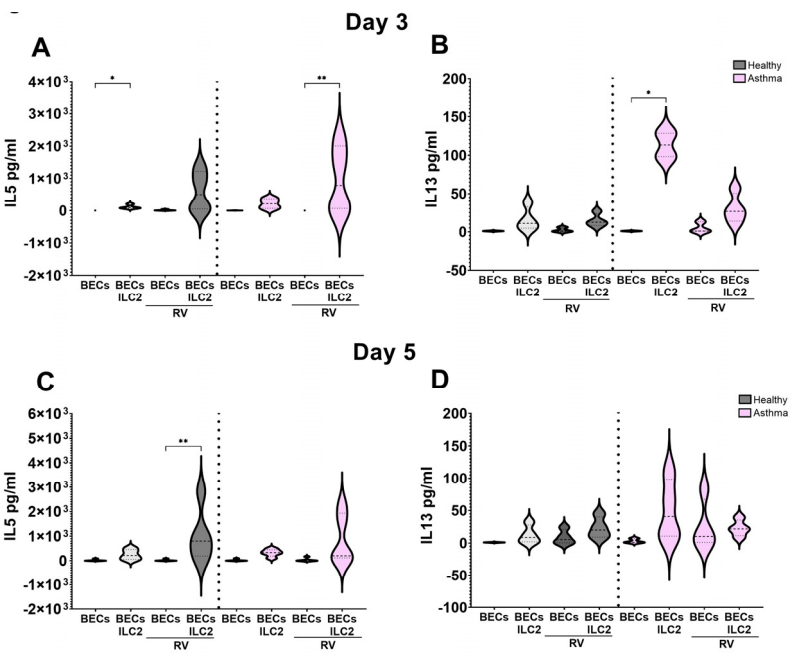

Figure 20: IL-5 and IL-13 release in an epithelial ILC2 co-culture model A-B) day 3 and C-D) day 5 post viral infection.

When we examined antiviral responses, in our co-culture model, we observed no effect of T2 cytokines on IFNλ1 response on day 3 or 5 (**Figure 21**). In general, we found a trend towards a lower expression of IFNλ1 in asthma BECs. As an additional measure of epithelial cell activity, we assessed the cilia beating frequency (CBF). Measurements were taken on day 2 and day 6, however no significant differences in CBF were observed between any of the conditions in the co-culture. Still, upon examining histology sections, clear differences in mucin expression were evident between healthy BECs and the co-culture with ILC2 (**Figure 22A-B**), as well as between asthmatic BECs and the co-culture with ILC2 (**Figure 22C-D**).

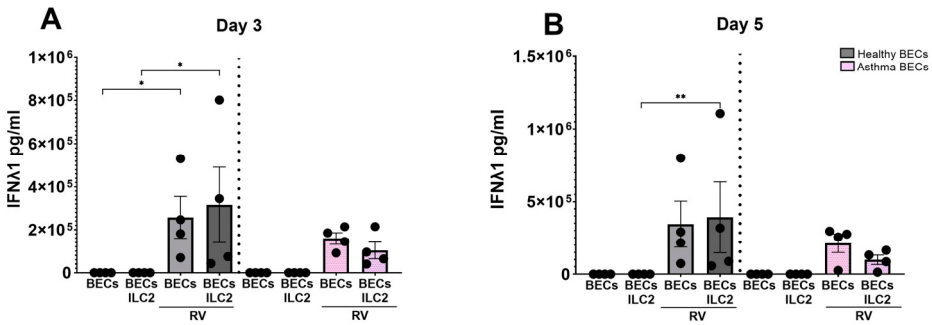

Figure 21: Assessment of IFNλ1 release in an epithelial ILC2 co-culture model.

		BECs	BECs + ILC2 (co-culture)
A)	Healthy BECs donor		
B)	Healthy BECs donor		
C)	Asthma BECs donor		
D)	Asthma BECs donor		

Figure 22: Histology evaluation of the co-culture model A-B) Healthy BECs only and with ILC2s in co-culture C-D) Asthma BECs only and with ILC2s in co-culture.

The interactions between ILC2 cells and various other cell types in co-culture systems have been extensively studied, revealing insights into their roles in immune responses,

tissue repair, and disease pathogenesis. Their complex interactions with different immune and non-immune cell types in co-culture systems elucidate the multifaceted roles of ILC2s in immune regulation, tissue repair, and disease mechanisms. Mouse ILC2s and airway smooth muscle (ASM) co-culture, anti-HMGB1 mitigated ILC2-induced ASM proliferation in a co-culture model. This indicates the role of HMGB1 in ILC2-mediated ASM cell proliferation [217]. Co-culturing mouse ILC2s with $CD4^+$ T cells promote the proliferation of $CD4^+$ T cells and the production of T2 cytokines. When IL-4-deficient ILC2s are co-cultured with wild-type $CD4^+$ T cells, there is a significant decrease in IL-5 and IL-13 production compared to co-culturing wild-type ILC2s with $CD4^+$ T cells. This suggests that ILC2-derived IL-4 plays a key role in ILC2/$CD4^+$ T-cell interaction [218].

ILC2 and small extracellular vesicle-mature dendritic cells (sEV-mDCs) in co-culture, ILC2's GATA3 levels decrease. Introducing a PGE2 receptor antagonist into the co-culture can reverse the negative effect of sEV-mDCs on ILC2 [219]. Co-culture of mouse lung endothelial cells (MLEC) with ILC2 significantly decreased MLEC death, suggesting a direct protective effect of ILC2 on MLEC in sepsis. The protective effect was abrogated when a neutralizing antibody against IL-9 was added to the ILC2-MLEC co-culture system challenged with LPS and TNFα [220]. The direct co-culture between ILC2 and mesenchymal stem cells (MSCs) identified that white adipose tissue-resident multipotent stromal cells (WAT-MSCs) can serve as a reservoir for IL-33, particularly in response to cellular stress, but also provide additional signals for sustaining ILC2. Consequently, ILC2-derived IL-4 and IL-13 feedback induce eotaxin secretion from WAT-MSCs, supporting eosinophil recruitment. Thus, MSCs create a niche for dynamic interactions with ILC2s, supporting a T2 immune environment in WAT [221]. Co-culture of B cells and ILC2 significantly increased the frequency of $EBI2^+$ B cells, indicating a reciprocal activation between these cell types [222].

These studies exemplify ILC2s significant influence on the proliferation and cytokine production of $CD4^+$ T cells and facilitate protective effects on endothelial cells during inflammatory challenges. The findings demonstrate the reciprocal activation between ILC2s and B cells, as well as the important niche provided by mesenchymal stem cells that fosters a type-2 immune environment. Notably, the role of critical signaling molecules, such as IL-4, IL-9, and HMGB1, further underscores the complexity of immune communication.

However, most studies on ILC2s have been conducted using mouse models, and there are notable differences between mouse and human ILC2s. These differences reflect variations in immune system development, tissue localization, and potential function. Such discrepancies can be attributed to species-specific immune system characteristics and the chronological differences in fetal immune development between mice and

humans. The mechanisms of cell-cell interactions between ILC2s and other cells, such as adipose stem cells (ASCs) and mesenchymal stem cells (MSCs), which support ILC2 function in the peripheral microenvironment, may also vary between these species. These interactions are critical for the formation of an ILC2 niche and are influenced by major sources of IL-33 and TSLP. In addition, mouse studies have shown that ILC2s prime dendritic cells (DCs) to induce Th2 differentiation. However, ILC2s also seem capable of directly influencing T-cell responses, suggesting a complex interplay that might differ between mice and humans. All this underscores the uniqueness of our co-culture experiments involving human ILC2s and BECs. Overall, our results advance our understanding of ILC2 dynamics in immunological contexts and suggest potential therapeutic targets for modulating immune responses in various diseases. In the future, we could also explore interaction between ILC2s from asthma or COPD patients and BECs from the same patients. This would be the perfect model for further understanding the diseases and differences between the patients.

Summary

This PhD thesis explored the effects of T2 cytokines on BECs isolated from healthy and diseased lungs and investigated early innate immune responses, using a translational *in vitro* model (ALI cultures). The studies address important differences in the T2 cytokine effect on BECs from diseased and healthy lungs and the treatment potential of IL-4R-α inhibition. The findings suggest a novel mechanism by which IL-4Rα mitigates exacerbations and reduces features of T2 inflammation. This mechanism involves reducing levels of alarmins and eotaxin-3 (CCL26), potentially having an impact on reduced eosinophil migration to the lungs, and simultaneously preserving antiviral responses to rhinovirus (**Figure 23**).

The study also created and tested a new co-culture approach to study the direct interactions of T2 cytokines from immune cells with BECs (**Figure 23**). ILC2 are potent producers of T2 cytokines in the lungs. The model verified the consequences of chronic stimulation of exogenous T2 cytokines on the epithelial TSLP release and the antiviral response (IFNλ1). Moreover, this co-culture approach could be used for future therapy assessments including not only IL-4Rα mAb but also anti-TSLP, anti-IL-33, anti-IL-5 treatments, and iJAK inhibitors, etc.

In summary (**Figure 23**), this thesis demonstrated that T2 cytokines affect the first line of innate immunity responses after rhinoviral infections and that blocking IL-4Rα indirectly reduces alarmins and eosinophil chemoattractant but maintains antiviral response, therefore providing one more means of managing respiratory diseases exacerbations. Our novel data on reduced levels of CCL26 in both asthma and COPD may explain why both asthma and COPD patients with eosinophilia respond well to IL-R4α treatment (Dupilumb).

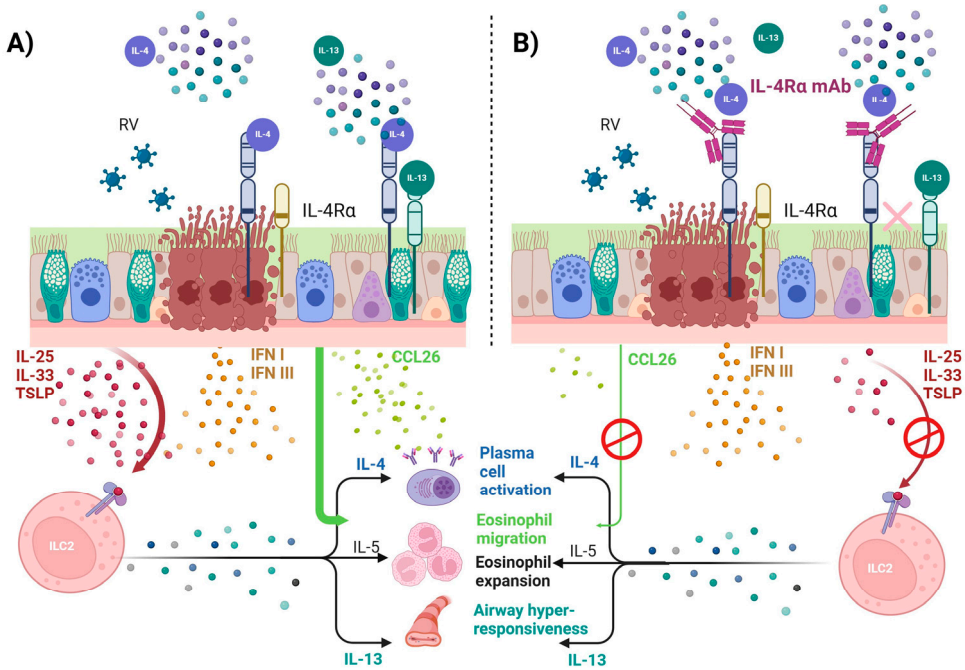

Figure 23: Thesis summary: A) T2 cytokines effect on diseased BECs in the presence of the viral infection and B) IL-4Rα mAb treatment effect on diseased BECs in T2 enviroment and viral infection.

Future perspective

There is a substantial unmet medical need in respiratory diseases, emphasizing the need for novel treatments to help patients with COPD and asthma. Given the impact of climate change on lung health, where the airway epithelium is a culprit cell for these environmental challenges, patients with asthma and COPD may suffer more in the future and improved treatments will be a demand. Since some patient groups develop severe disease or experience periods with flare-up of symptoms, exacerbations, and not responding to conventional treatments, precision medicine approaches are much needed to target specific mechanisms. Recent developments in biological therapy for asthma have pointed towards the role of the airway epithelium in initiating and orchestrating T2 inflammation. The knowledge gained from this dissertation not only expands our knowledge of T2 cytokine dynamics in respiratory disorders but also sets the groundwork for novel treatments that might greatly help patients. Thus, targeting epithelial-derived cytokines could be a superior strategy leading to a better effect on preventing exacerbations and broader clinical effects than targeting downstream cytokines and immune cells. Blocking IL-4 and IL-13 cytokine signaling by targeting IL-4Rα is an established therapeutic approach for the treatment of asthma and emerging for the treatment of COPD. Still, an increased mechanistic understanding of the role of this pathway in T2 inflammation and rhinoviral infection using advanced *in vitro* model systems is needed. Thus, an increased immunological understanding of the role of this pathway in T2 inflammation and rhinoviral infection was needed by utilising advanced *in vitro* models. Future studies addressing the efficacy and immunological differences between inhaled and systemic biologics for the same pathway will be interesting. Not only can the local deposition of the mAb in the airways be beneficial but this route of administration could also lead to less costs for the society and easier administration for the patient. Another interesting possibility for the future is to combine a cocktail of different biologicals to achieve even broader effect. Some companies have ongoing programs developing a combination of anti-TSLP and anti-IL-R4α for the inhaled route. Another option is to combine treatment with the available systemic biologicals to investigate if a better treatment response is achieved. However, targeting the epithelial-derived cytokine TSLP with Tezepelumab (AstraZeneca together with Amgen) showed for the first time effect in two different

endotypes of severe asthma, both T2 high and T2 low. However, a recent study in COPD failed to meet the primary endpoint [223]. Another aspect to consider for the future is when in time to start treatment with biologicals, perhaps we today start to treat too late when the disease is already too severe.

The novel co-culture system developed in this thesis represents a significant advancement in understanding the interactions between innate immune cells and epithelial cells. This model opens new possibilities for the screening of pharmaceutical compounds, providing a platform to identify treatments that enhance antiviral activity while minimizing inflammation. Future research can use this co-culture model to evaluate various combination therapies that may yield synergistic effects on viral immunity and inflammation control.

Moreover, the co-culture model facilitates the investigation of interactions between ILC2s and BECs isolated specifically from asthma or COPD patients. By allowing cells from the same patients to be studied, this model offers a promising avenue for personalized medicine. It enables the customization of treatments based on individual cytokine profiles, potentially leading to more effective and tailored therapeutic strategies.

Furthermore, the established co-culture model supports long-term treatment applications, providing a robust framework for continuous research on chronic conditions and their management. Overall, this innovative approach not only enhances our understanding of cell-cell interactions but also has the potential to significantly impact patient-specific treatment plans in the future. Looking forward, researchers must prioritize the advancement of complex cell model technology, because these models can offer a more comprehensive understanding of diseases.

Populärvetenskaplig sammanfattning

Astma och KOL (kronisk obstruktiv lungsjukdom) tillhör våra folksjukdomar och är komplexa sjukdomar som kan påverka patienterna på olika sätt. Det gör att två patienter med samma diagnos kan ha helt olika behov av behandling beroende av sjukdomens svårighetsgrad. Under de senaste åren har betydande framsteg gjorts för att identifiera vilka astmapatienter som kan dra nytta av nya biologiska läkemedel. Nyligen godkändes behandling av biologiskt läkemedel för KOL. Biologiska läkemedel är antikroppar riktade mot specifika cytokiner, proteiner som är involverade i sjukdomsprocessen. På så vis får man bättre effekt och mindre biverkningar jämfört med om man behandlar med höga doser anti-inflammatoriska läkemedel.

Luftvägsepitelet är som en matta, lungans skydd mot den yttre världen som är utrustade med flimmerhår och slem. På så sätt kan allt vi inandas, särskilt små ämnen som allergen, luftföroreningar och virus och bakterier stoppas från att ta sig in i kroppen där de annars kan starta i gång inflammatoriska processer i våra luftvägar. Luftvägsepitelet är därför centralt när det gäller att starta i gång inflammation genom att frisätta cytokiner. Patienter med astma och KOL har kroniska inflammationsprocesser i sina luftvägar som gör att de upplever symptom som andnöd, bröstsmärta, trötthet och hosta. Tidigare forskning har visat att epitelets cytokiner har en roll i att starta i gång dessa symptom. Samtidigt som epitelet producerar sjukdomsalstrande cytokiner har det också en viktig roll när det gäller att producera ämnen som skyddar oss mot infektion. Luftvägsvirus, där förkylningsvirus är oerhört vanliga och sprids snabbt i samhället kan orsaka stora problem hos patienter med astma och KOL. Det finns minst 160 olika sorters förkylningsvirus och virusen muterar snabbt vilket gjort det omöjligt att utveckla ett vaccin.

Min forskning i detta doktorandprojekt har syftat till att fördjupa förståelsen för epitelets roll i hur olika delar av immunförsvaret samverkar vid en infektion med förkylningsvirus samt med inflammatoriska cytokiner, IL-4 och IL-13 som är typiska T2 cytokiner. Jag har särskilt studerat medfödd immunitet, receptorer som känner igen förkylningsvirus, antivirala proteiner, cytokiner och hur dessa samverkar med den kroniska inflammation som kännetecknar olika sjukdomsfenotyper vid astma. Vi har

också utforskat potentialen hos ett biologiskt läkemedel som godkänts för både astma och KOL för att utvärdera om det påverkar patienternas immunförsvar vid.

I den här avhandlingen har vi använt en cellmodell av luftväggsepitel som kallas för ALI (Air Liquid Interface). Den här modellen är en blandning av olika epitelkomponenter som finns lungans naturliga epitelbarriär och innehåller också typ av celler som kallas för bägarceller som producerar slem. I studie I har vi utforskat hur cellmodellen svarar när den stimuleras med inflammatoriska proteiner så kallade, cytokiner. Vi har analyserat cellernas svar i modellen och hittat att vissa proteiner är förhöjds om cellerna ursprungligen kommer från astmapatienter. Vidare har vi behandlat cellerna i modellen med ett biologiskt läkemedel och upptäckte att den biologiska behandlingen kunde minska de förhöjda nivåerna av dessa proteiner.

I studie II har vi undersökt effekten av den biologiska behandlingen på celler i vår modell som kommer från KOL patienter. Här också kunde vi bevisa den positiva effekten av behandlingen som återigen har sjunkit ner upphöjda proteiner. Från studie I och II kunde vi dra slutsats att vi har bevisat en ny mekanism hur behandlingen påverkar astma och KOL patienter och bidrar till deras välbefinnande.

I studie III skapade vi och testade ut en ny cellmodell som är mer komplex då den förutom epitel även innehåller en typ av immuncell som kallas för ILC2. Denna modell ville vi utveckla för att kunna den direkta interaktionen mellan cytokiner som frisätts från immunceller (ILC2) och dess påverkan på epitelet. ILC2 är kraftiga tillverkare av inflammatoriska cytokiner i lungorna. Modellen bekräftade konsekvenserna som vi sett i arbete I och II av kronisk stimulering med cytokiner på epitelet och växlingar i proteintillverkning i celler från sjuka människor. Dessutom skulle denna modell kunna användas för att i framtiden testa nya behandlingar för astma och KOL.

Avslutningsvis har resultaten från denna avhandling bidragit till en djupare insikt om verkningsmekanism för ett biologiskt läkemedel i olika inflammatoriska miljöer och vid infektion av förkylningsvirus. Vi har utvecklat ett modellsystem där bronkepitel odlas tillsammans med en potent immuncell, ILC2 vilket kan ge möjlighet att bättre utvärdera farmakologiska substanser och kan främja utvecklingen av nya terapier för respiratoriska sjukdomar där det finns ett medicinskt behov.

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About the author

JELENA PESIC began her academic journey in Biomedical Education at Lund University, where she earned her master's degree. Her career took a pivotal turn towards the pharmaceutical industry when she joined AstraZeneca in Gothenburg, specializing in in vivo lung imaging. Jelena later transitioned to the Respiratory Department, where she focused on in vitro research, contributing as a Senior Research Scientist to drug development for lung diseases.

After 15 years at AstraZeneca, Jelena pursued an industrial PhD program supported by the Swedish Foundation for Strategic Research. Project involved collaboration between AstraZeneca and Department of Experimental Medical Sciences at Lund University under the supervision of Professor Lena Uller. Her research focuses on the impact of type 2 (T2) cytokines, particularly IL-4 and IL-13, on bronchial epithelial cells (BECs) and their antiviral responses utilizing translational in vitro air-liquid interface cultures (ALI). Working with epithelial cells from healthy individuals and patients with asthma and COPD, her studies explored into the mechanisms of IL-4 receptor alpha (IL-4Rα) monoclonal antibody (mAb) treatment under T2 inflammatory conditions, with a focus on viral-induced exacerbations.

This work enhances our understanding of T2 inflammation and IL-4Rα mAb inhibition, shedding light on how this treatment improves lung function and reduces exacerbations in patients. Jelena's contribution significantly advances the field of respiratory health, offering potential pathways for more effective therapeutic strategies.

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