New aspects of allergic mechanisms and innate immunity in asthma. Alarmins as upstream mediators of asthma inflammation.

Ramu, Sangeetha

2023

Document Version:
Publisher's PDF, also known as Version of record

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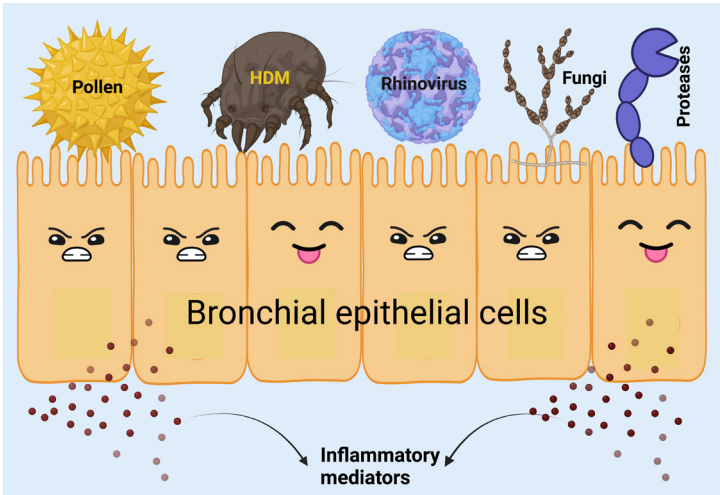
EXPERIMENTAL MEDICAL SCIENCE | FACULTY OF MEDICINE | LUND UNIVERSITY

Printed by Media-Tryck, Lund 2023 NORDIC SWAN ECOLABEL 3041 0903



LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Experimental Medical Science

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2023:73
ISBN 978-91-8021-413-1
ISSN 1652-8220

New aspects of allergic mechanisms and innate immunity in asthma

Alarmins as upstream mediators of asthma inflammation

Sangeetha Ramu

LUND
UNIVERSITY

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 2nd June 2023 at 13.00 in Segerfalksalen, Department of Experimental Medical Science, BMC, Lund

Faculty opponent
Prof. Madeleine Rådinger
University of Gothenburg, Krefting Research Centre,
Gothenburg

Organization LUND UNIVERSITY Faculty of medicine Department of experimental medical science Unit of respiratory immunopharmacology	**Document name** DOCTORAL DISSERTATION **Date of issue** 2 June 2023
Author: Sangeetha Ramu	Sponsoring organization

Title and subtitle: New aspects of allergic mechanisms and innate immunity in asthma: Alarmins as upstream mediators of asthma

Abstract

Asthma is a chronic inflammatory lung disease affecting over 300 million individuals worldwide. Both respiratory viral infections and aeroallergens have been identified as important risk factors for asthma. Rhinovirus (RV) infection has been recognized as a major cause of asthma exacerbations, and current research indicates that RV and allergens may have a synergistic effect, resulting in a higher risk of acute asthma exacerbations. Acute asthma exacerbations are characterized by worsened inflammation in the airways; this severe acute state currently lacks effective treatments and represents a significant unmet medical need.

The aim of this PhD thesis is to investigate the innate immune response of bronchial epithelial and smooth muscle cells to aeroallergens, allergic mediators, RV-infections alone and/or in combinations. We employed *in vitro* cultures of cell lines or primary human bronchial epithelial cells (HBECs) or bronchial smooth muscle cells (BSMCs) from healthy and asthmatic patients to study the regulation and molecular mechanisms of alarmins, anti-viral proteins and pro-inflammatory cytokines.

In summary, our results demonstrated that, HBECs release ATP and the pro-inflammatory cytokine IL-8, in response to stimulation with four different allergens; house dust mite (HDM), *Altenaria alternata* (Mugwort), *Betula pendula* (Birch) and *Artemisia vulagris* (Fungal). Only HDM induced uric acid release in HBECs as well as in our HDM-induced mouse model of allergic airway inflammation. Using specific inhibitors, we found that these responses were mainly dependent on allergen serine proteases. We further stimulated HBECs with the mast cell proteases tryptase and chymase and the results showed that these proteases induced ATP, IL-8 and IL-6 release and pre-treatment with tryptase and chymase reduced viral-induced IFN-β response. Reduced anti-viral response was associated with decreased pattern recognition receptors expression in HBECs. Further we confirmed that mast cell proteases can influence the epithelial integrity by reducing expression of tight junctional proteins expression. Next, we have investigated RV-induced IL-33 expression and regulating mechanisms in BSMCs from healthy and patients with asthma. Our results suggest that RV-induced IL-33 expression was higher in non-severe asthmatics compared to healthy and severe asthmatics. This response was mainly regulated through TLR-3 and activation of downstream signalling pathway TAK1 in BSMCs. We further show in a clinical RCT study that, house dust mite sublingual allergen immunotherapy (HDM-SLIT) increases viral-induced interferons and reduced alarmin IL-33 expression in HBECs. Our data suggest that allergic asthma patients who have a history of asthma exacerbations and recurrent respiratory infections could potentially benefit from AIT treatment.

In conclusion, our study has provided new understandings into how the interaction between allergens and viral infections influences the bronchial epithelial and smooth muscle cells to induce inflammation by producing alarmin cytokines as well as antiviral immunity in asthma.

Key words: Asthma, allergens, rhinovirus, airway structural cells,alarmins, proteases, anti-viral response, mast cells, allergen immunotherapy

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language** English
ISSN and key title 1652-8220		**ISBN** 978-91-8021-413-1
Recipient's notes	**Number of pages** 118	Price
	Security classification	

Signature Date 2023-04-19

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Cover photo: Features a stunning single sheet handmade paper cutting by **Roopalatha Varadaraj** featuring a human lung, flowers, and a bee.

Faculty of Medicine,
Department of Experimental Medical Science,

ISBN 978-91-8021-413-1
ISSN 1652-8220
Lund University, Faculty of Medicine Doctoral Dissertation Series 2023:73

Printed in Sweden by Media-Tryck, Lund University
Lund 2023

Dedicated to my mom

A dream is not that which you see while sleeping,
it is something that does not let you sleep.
-Dr. ABDUL KALAM

Table of Contents

List of papers

List of papers included in the thesis

I. **Ramu S**, Menzel M, Bjermer L, Andersson C, Akbarshahi H, Uller L. Allergens produce serine proteases-dependent distinct release of metabolite DAMPs in human bronchial epithelial cells. *Clinical & Experimental Allergy*. 2018 Feb;48(2):156-66.

II. **Ramu S**, Woehlk C, Nieto-Fontarigo JJ, Vázquez-Mera S, Menzel M, Malm Tillgren S, Cerps S, Akbarshahi H, Porsbjerg C, Uller L. Alarmin release in the bronchial epithelium of allergic asthma patients vary in response to different aeroallergens. *Manuscript in preparation*.

III. **Ramu S**, Akbarshahi H, Mogren S, Berlin F, Cerps S, Menzel M, Hvidtfeldt M, Porsbjerg C, Uller L, Andersson CK. Direct effects of mast cell proteases, tryptase and chymase, on bronchial epithelial integrity proteins and anti-viral responses. BMC immunology. 2021 Dec;22(1):1-2.

IV. **Ramu S***, Calvén J*, Michaeloudes C, Menzel M, Akbarshahi H, Chung KF, Uller L. TLR3/TAK1 signalling regulates rhinovirus-induced interleukin-33 in bronchial smooth muscle cells. ERJ open research. 2020 Oct 1;6(4).

V. Woehlk C*, **Ramu S***, Sverrild A, Nieto-Fontarigo JJ, Vázquez-Mera S, Cerps S, Pulga A, Andreasson LM, Eriksen LL, Dyhre-Petersen N, Menzel M, Klein KD, Hansen S, Uller L, and Porsbjerg C. Allergen Immunotherapy Enhances Airway Epithelial Antiviral Immunity in Patients with Allergic Asthma (VITAL Study): A Double Blind Randomized Controlled Trial. American Journal of Respiratory and Critical Care Medicine. 2023 Jan 26.

*Shared first author.

Papers not included in the thesis

1. Malm Tillgren S, Nieto-Fontarigo JJ, Cerps S, **Ramu S**, Menzel M, Mahmutovic Persson I, Meissner A, Akbarshahi H, Uller L. C57Bl/6N mice have an attenuated lung inflammatory response to dsRNA compared to C57Bl/6J and BALB/c mice. Journal of Inflammation. 2023 Dec;20(1):1-3.
2. Vanherle L, Lidington D, Uhl FE, Steiner S, Vassallo S, Skoug C, Duarte JM, **Ramu S**, Uller L, Desjardins JF, Connelly KA. Restoring myocardial infarction-induced long-term memory impairment by targeting the cystic fibrosis transmembrane regulator. EBioMedicine. 2022 Dec 1; 86:104384.
3. Cerps S, Sverrild A, **Ramu S**, Nieto-Fontarigo JJ, Akbarshahi H, Menzel M, Andersson C, Tillgren S, Hvidtfeldt M, Porsbjerg C, Uller L. House dust mite sensitization and exposure affects bronchial epithelial anti-microbial response to viral stimuli in patients with asthma. Allergy. 2022 Feb 3.
4. Porsbjerg C, Nieto-Fontarigo JJ, Cerps S, **Ramu S**, Menzel M, Hvidtfeldt M, Silberbrandt A, Froessing L, Klein D, Sverrild A, Uller L. Phenotype and severity of asthma determines bronchial epithelial immune responses to a viral mimic. European Respiratory Journal. 2021 Jan 1.
5. Nieto-Fontarigo, J. J., Tillgren, S., Cerps, S., Sverrild, A., Hvidtfeldt, M., **Ramu, S**., Menzel, M., Sander, A. F., Porsbjerg, C. & Uller, L. Imiquimod Boosts Interferon Response, and Decreases ACE2 and Pro-Inflammatory Response of Human Bronchial Epithelium in Asthma. 2021 dec 7, In: Frontiers in Immunology. 12, 743890.
6. Mogren S, Berlin F, **Ramu S**, Sverrild A, Porsbjerg C, Uller L, Andersson CK. Mast cell tryptase enhances wound healing by promoting migration in human bronchial epithelial cells. Cell adhesion & migration. 2021 Jan 1;15(1):202-14.
7. Menzel, M., **Ramu, S**., Calvén, J., Olejnicka, B., Sverrild, A., Porsbjerg, C & Uller, L. (2019). Oxidative stress attenuates TLR3 responsiveness and impairs anti-viral mechanisms in bronchial epithelial cells from COPD and asthma patients. Frontiers in Immunology, 2019,10, 2765.

8. Akbarshahi H, Menzel M, **Ramu S**, Mahmutovic Persson I, Bjermer L, Uller L. House dust mite impairs antiviral response in asthma exacerbation models through its effects on TLR3. *Allergy*. 2018 Jan 10.
9. Persson IM, Menzel M, **Ramu S**, Cerps S, Akbarshahi H, Uller L. IL-1β mediates lung neutrophilia and IL-33 expression in a mouse model of viral-induced asthma exacerbation. *Respiratory research*. 2018 Dec;19(1):16.

Selected abbreviations

AECs	Airway epithelial cells
ASM	Airway smooth muscle cells
AIT	Allergen immunotherapy
ATP	Adenosine triphosphate
BALF	Broncho alveolar lavage fluid
BEGM	Bronchial Epithelial Cell Growth Medium
BSMCs	Bronchial smooth muscle cells
COPD	Chronic obstructive pulmonary disease
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
dsRNA	double-stranded RNA
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H_2O_2	Hydrogen Peroxide
HDM	House dust mite
HMGB1	High mobility group protein B1
HRP	Horseradish peroxidase
HBECs	Human bronchial epithelial cells
IFNs	Interferons
IL	Interleukin
ILC2	Type 2 innate lymphoid cells
INFs	Interferons

MOI	Multiplicity of infection
MC_T	Tryptase positive mast cells
MC_{TC}	Tryptase and chymase positive mast cells
MC	Mast cell
mRNA	messenger RNA
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs	Nod-like receptors
PAMPs	Pathogen-associated molecular patterns
P2	Purinergic receptors
PEST	Pencilline streptomycine
poly(I:C)	Polyinosinic-polycytidylic acid
PRRs	Pattern recognition receptors
RLRs	Retinoic acid-inducible gene-I-like receptors
ROS	Reactive oxygen species
RAGE	Receptor for advanced glycation end products
RV	Rhinovirus
T2	Type 2
Th2	T helper 2
TJs	Tight junctions
TLRs	Toll-like receptors
TSLP	Thymic stromal lymphopoietin

Introduction

Asthma

Asthma is a chronic inflammatory lung disease that affects more than 300 million people worldwide and is responsible for over 250,000 deaths each year [1]. Asthma is a complex heterogeneous disease that includes multiple distinct disease phenotypes with different etiologic and pathophysiologic characteristics [2]. Individuals with asthma suffer from respiratory symptoms such as wheezing, coughing, shortness of breath, variable airflow limitations and airway hyper-responsiveness (AHR) to environmental irritants [3]. The diagnosis of asthma is based on the patient's history of characteristic respiratory symptoms, family history and lung function. Asthma diagnosis must identify a limitation in expiratory airflow and provide evidence of reversible obstruction [4]. Spirometry provides the most accurate measurement of variable airflow limitation or obstruction [5]. To demonstrate reversible airflow obstruction, guidelines recommend performing spirometry measurements (forced expiratory volume during 1st second (FEV1), forced vital capacity (FVC), and FEV1/FVC ratio) before and after the administration of a short-acting bronchodilator [6]. Asthma is often shown by at least a 12% increase in FEV1 (over baseline values) and at least a 200-mL increase in FEV1 over baseline after bronchodilator [7].

Risk factors of asthma

Asthma is a complex genetic condition, according to both family-based and twin studies [8]. The clinical manifestation of the disease and its related phenotypes, including bronchial hyperresponsiveness, atopy and increased IgE, are also known to be influenced by a variety of genetic and environmental variables [9]. Exposure to aeroallergens include dust mites, animal dander, cockroaches, mould, pollen, air pollutants and cigarette smoke are among the factors associated with an increased risk for development of asthma [10]. Lower respiratory tract infections with respiratory syncytial (RSV) virus and potentially with other viruses like

rhinovirus (RV) and meta-pneumovirus, or bacterial infections, strenuous activity, cold air, chemical fumes and strong emotions are all known to aggravate asthma symptoms [11] (**Figure 1**).

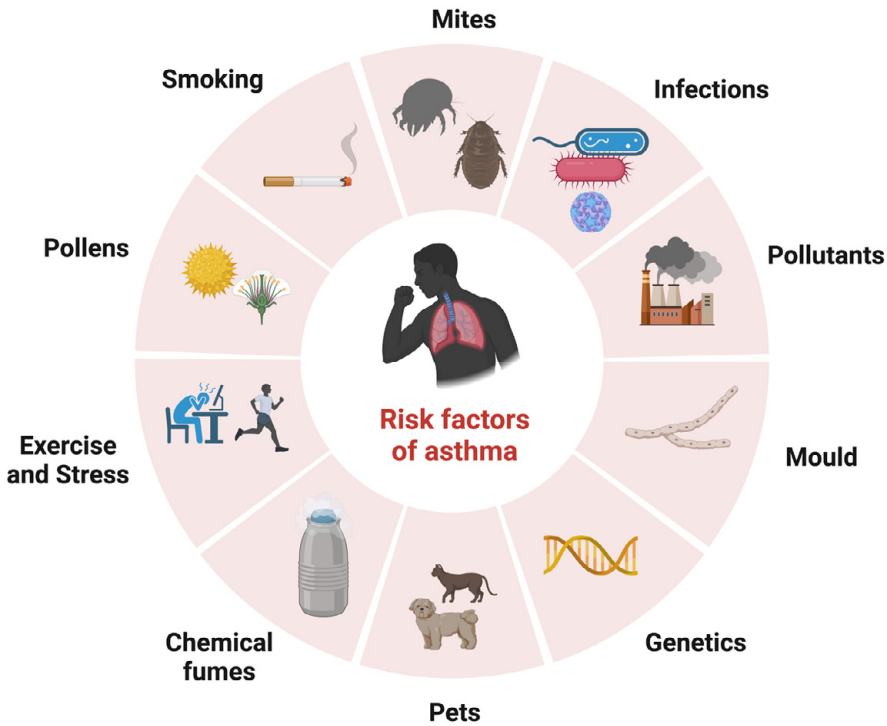

Figure 1: Examples of different risk factors of asthma. Biorender 2022.

Pathophysiology

The pathophysiology of asthma is complex and involves both innate and adaptive immune mechanisms that may lead to airway inflammation, intermittent airflow obstruction, and AHR [12]. Chronic airway inflammation subsequently results in airway edema, mucus hypersecretion, mucus trapping, and airway remodelling. Subepithelial fibrosis, sub-basement membrane thickening, increased airway smooth muscle mass, angiogenesis, and mucous gland hyperplasia all contribute to the remodelling of the airways, which leads to long-lasting structural alterations [13]. T helper (Th) 1, 2, and 17 and type 2 innate lymphoid cells (ILC2) responses, as well as underlying genetic predisposition, are involved in the

pathophysiology of how these known factors (**Figure 1**) cause persistent structural alterations in the different asthma phenotypes [11].

Phenotypes and endotypes

Rackemann first classified asthma phenotypes based on a single dimension and focused on easy classifications. That study defined two clinical asthma phenotypes, extrinsic and intrinsic asthma based on the involvement of allergens [14]. Today, it is increasingly understood that asthma is a heterogeneous syndrome that functions as an "umbrella term" for a number of different diseases aetiologies, each of which is characterized by a unique underlying pathophysiological mechanism [15]. According to Global Initiative for Asthma (GINA), specific "asthma phenotypes" are observable combinations of clinical, biochemical, and physiological traits, whereas an "asthma endotypes" refers to a subtype of a disease that is functionally and pathologically determined by a molecular mechanism or by treatment intervention [2].

Current approaches for phenotyping asthma include demographic and risk factors (such as age, obesity, and smoking), clinical (such as early/late onset, symptoms, exercise-induced, health status), response to treatment (such as inhaled and/or oral steroid sensitivity), airway obstruction (variable or partially fixed), trigger-related, bronchial hyperresponsiveness, exacerbations and allergy [16-18]. Asthma was classically considered to be an allergic, Th2-dependent inflammation because bronchial biopsies and/or bronchoalveolar fluid from asthmatics contained increased numbers of CD4+ lymphocytes, eosinophils, and mast cells [19]. However, T2-high and T2-low asthma has been identified as two subtypes of the condition based on the level of T2 inflammation. T2-high asthma is characterized by eosinophilic airway inflammation, which is linked to elevated fractional exhaled nitric oxide (FeNO) and/or elevated blood eosinophil counts, while T2-low asthma comprises neutrophilic asthma and paucigranulocytic asthma. Mixed granulocytic asthma is defined as the presence of eosinophilic and neutrophilic airway inflammation [20, 21]. Recently many studies revealed that ILC2 can produce IL-5 and IL-13 without the involvement of adaptive immune cells [22].

Allergic asthma

Allergic respiratory disorders such as allergic rhinitis and asthma are among the most common diseases worldwide, causing a considerable public health and economic burden due to morbidity and the impact on quality of life [23]. Allergic asthma, atopic dermatitis, allergic rhinitis, and food allergy are the most frequent allergic disorders affecting children, and their incidence has increased in recent years [24]. The onset and progression of allergic diseases are strongly influenced by an allergen sensitization to specific allergens. More than 50% of people with severe asthma also have allergic asthma [25, 26]. Patients who have allergic asthma or IgE sensitization to allergens are more prone to develop chronic, persistent inflammation in asthma [27]. As previously discussed, as regards risk factors of asthma, allergens such as house dust mites (HDM), animal dander, cockroaches, or fungus play a significant role in asthma inflammation. Additionally, asthma has been linked to seasonal pollen exposure [28]. The rationale for the role of allergens in asthma is that prolonged exposure to an allergen in a sensitized individual increases their chances of developing allergic asthma [29]. More than 80% of children who have asthma are also allergic to environmental allergens, with indoor allergens in particular playing a significant role in the development of asthma [30].

The airway epithelium

The airway epithelium of the upper (nasal) and lower (lung) airway serves as the first line of defence against inhaled foreign substances and is in direct contact with inhaled air and other compounds [31]. The distal airway epithelium is composed of multiple cell types that can be differentiated by their shape and function (**Figure 2**). The cells of the airway epithelium in the lower airways have a polarized and pseudostratified appearance and are comprised of secretory cells, basal cells, goblet cells, club cells, and ciliated cells. These are the most prevalent cell types in the airways, which include the trachea and bronchi [32]. The basal cells are the primary stem cells of the epithelium that facilitate epithelial regeneration. The apical surface is covered with ciliated cells, which help transport mucus through the airways and the ciliated cells work together with secretory cells that make mucus to trap and remove microorganisms and foreign material from the airways [33]. Type 1 and type 2 alveolar cells are more prevalent in the lower airways and alveoli [34].

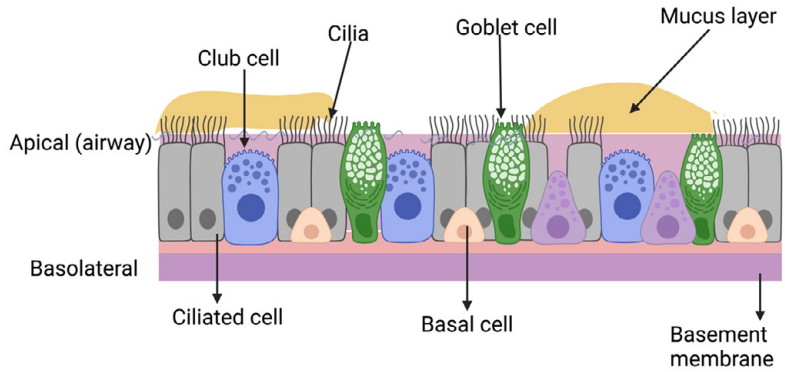

Figure 2: The structure and protective immune barrier capabilities of the human airway epithelium. Biorender 2022.

The airway epithelium plays an important role in onset and development of asthma. Additionally, it plays a crucial role in the initiation of host defence and the regulation of lung immune responses [35]. The primary function of the airway epithelium is to act as a physical barrier between the internal and external environment [35, 36]. The development of asthma is suggested to be influenced by reduced barrier function and increased goblet cell differentiation in the epithelium [37]. Airway epithelial cells (AECs) not only act as a physical barrier but most recently are recognised as an immune barrier initiating and regulating immune responses to inhaled substances by secreting a variety of molecules including alarmins, chemokines, cytokines, anti-viral proteins and extracellular matrix components [37-39].

Evidence suggests that epithelial cells play critical roles in the initiation, maintenance, and regulation of both innate and adaptive immune responses in the airways [40]. By forming a trans-epithelial interacting cellular network, dendritic cells (DCs) and airway macrophages work together as defenders against foreign particulate antigens [41]. During inflammatory and immune responses, AECs express pattern recognition receptors (PRRs) to initiate a host defense response, interact with DCs to control antigen sensitization, and release cytokines to attract effector cells [38, 39].

Airway smooth muscle cells

Airway smooth muscle (ASM) surrounds the airway tubes in humans, from the trachea to the smaller airways [42]. The pathophysiology of airway remodelling in asthma is most notable for the increased number and size of ASM. Remodelling has been reported in smooth muscle cells [43] and frequently involves physical alterations to the ASM, such as hypertrophy and/or hyperplasia, which increase ASM mass and, consequently, increase the thickness of the airway wall [44]. In people with severe asthma, it has been discovered that increases in ASM mass are linked to a decline in lung function compared to healthy individuals [45] (**Figure 3**).

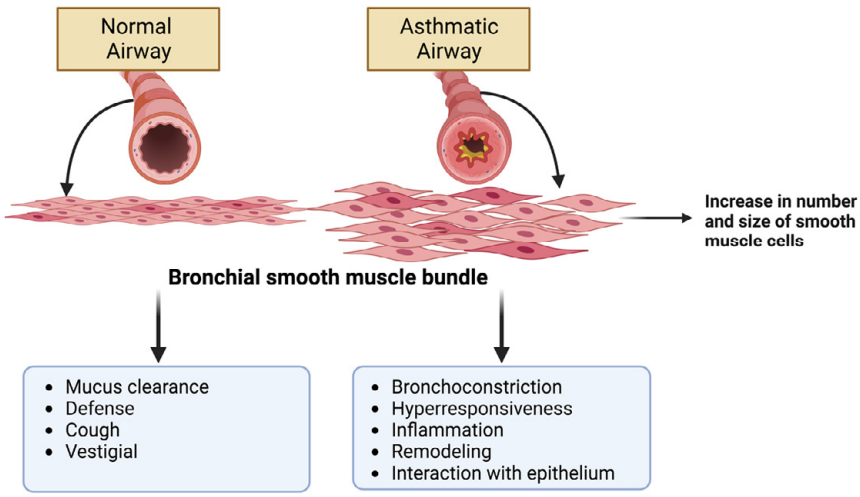

Figure 3: Features of airway smooth muscle cells of the normal and asthmatic airways. Figure adapted from Solway J, et al. N Engl J Med. 2007 [46]. Biorender 2022.

ASM cells are crucial for development, maintenance, and contraction of the airways. In addition to its contractile function, is recognized for its active role in the development of asthma. ASM can produce a variety of pro- and anti-inflammatory mediators in response to infection, inflammation, injury, and microbial products [47]. Interestingly, our research group showed for the first time a novel role for the ASM in the production if antiviral interferons and alarmins [48, 49]. ASM cells express a variety of cell surface components, including intergrins, costimulatory factors, and PRRs, which contribute to their ability to promote inflammation and regulate immune responses [50].

In line with this, the ASM has the ability to release a range of growth factors, chemokines, and cytokines that can promote the attraction and survival of inflammatory cells, such as mast cells (MCs) [51]. Human ASM in culture produces and releases eotaxin in response to a variety of inflammatory cytokines, including interleukin-beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) [52, 53] and ASM cells produce cytokines like transforming growth factor –beta (TGF-1β) to attract mast cells in vitro [54]. There is evidence that ASM cells produce inflammatory cytokines such as CCL5, IL-6, IL-8, CXCL10, MCP-1 and the T2 cytokines thymic stromal lymphopoietin (TSLP) and IL-33 [55].

Airway innate immunity

PRRs are necessary for initiating an innate immune response. These receptors are widely expressed by immune cells including dendritic cells (DCs) and macrophages and also non- professional immune cells such as epithelial, endothelial and smooth muscle cells [56]. There are many types of PRRs families, like toll-like TLRs, retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), and PARs [57]. Sensing of PAMPs and danger associated molecular patterns (DAMPs) by PRRs up-regulates the transcription of genes involved in inflammatory responses. These genes encode pro-inflammatory cytokines, type I interferons (IFNs), chemokines and antimicrobial proteins [57].

One of the early PRRs found in the innate immune system, which is crucial for inflammatory responses, were TLRs [58]. Based on the cellular localization of TLRs they determine the types of ligands and the recognition mechanism. TLR3, TLR7, TLR8 and TLR9 are expressed in the form of homodimers, which primarily recognize the nucleic acids of microorganisms. Other TLR1, 2, 4, 5, 6, 10 are expressed on the surface of immune or bronchial epithelial cells in the form of heterodimers or homodimers, recognizing the membrane components of pathogenic microorganisms, such as lipids, lipoproteins, and proteins [59, 60]. TLR3 was the first TLR to be linked to the recognition of viral nucleic acids. Despite TLRs 3, 7, 8, and 9 can access viral nucleic acids at the plasma membrane, they mainly reside in the endosome. Polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analogue of double-stranded RNA (dsRNA) that mimics viral infection and triggers antiviral immune responses by promoting the production of both anti-viral proteins (interferons) and

inflammatory cytokines, was initially identified as the recognition molecule (ligand) for TLR3 [61] including dsRNA molecule produced by ssRNA viruses during replication.

RNA viruses are also recognized by RLRs, which are intracellular receptors found in the cytoplasm. The RLR family includes at least three members: RIG-I, melanoma differentiation factor-5 (MDA5), and laboratory of genetics and physiology-2 (LGP-2). Recognition of viral RNA by RLRs triggers innate antiviral responses, primarily through the rapid induction of type I IFNs and inflammatory cytokines, which limit viral replication and coordinate an antigen-specific, adaptive immune response [62]. Both RIG-1 and MDA5 contain helicase and repression domains as well as two repeating CARD motifs at their N-termini. RIG-1 detects double-stranded RNA, triggering IRF3 and resulting in the production of type I IFNs. MDA5 and RIG-I share homology in their CARD and helicase domains but LGP2 lacks a CARD domain [63]. IFN-β release and the activation of the IRF3 transcription factor are both increased by the activation of RIG-I or MDA5, indicating that these two RLR proteins engage the same signalling pathway [63] (**Figure 4**).

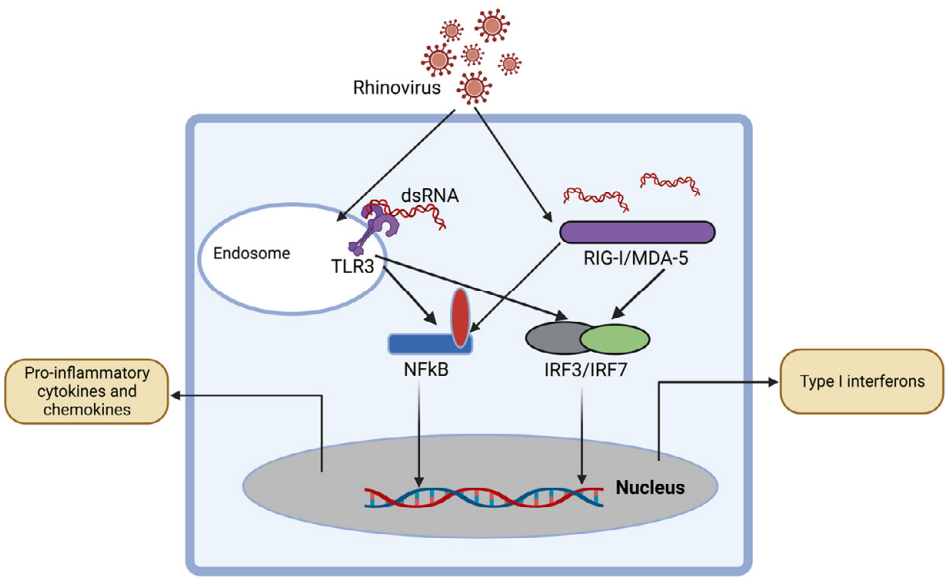

Figure 4: An overview of the signalling pathways which are activated by rhinovirus infection. Viral particles enter the cells and release dsRNA during their replication. dsRNA recognized by PRRs, such as TLR3 and the RIG-I-like receptors (RIG-I/MDA5) regulate various signalling pathwaya and leads to the activation of the transcription factrors NFkB and IRF3/IRF7. Activation of these transcription factors leads to production of pro-inflammatory and anti-viral cytokines. Biorender 2022.

Allergic airway inflammation

Allergic asthma involves the classical allergen-induced airway inflammation [64]. The asthmatic airway epithelium acts as a key source of epithelial alarmin cytokines such as IL-25, IL-33 and TSLP when exposed to allergens, pollutants, and other pathogenic substances, which cause Th2 cell polarization [65-67]. Recent studies have revealed that group 2 innate lymphoid cells (ILC2) play an important role in type 2 immunity and asthma immunopathogenesis [68-70]. ILC2 become activated in response to epithelial alarmin cytokines and release type 2 cytokines (IL-5 and IL-13) and prostaglandin [71], which enhance Th2-driven allergic reactions in the airways .

An increasing body of evidence supports the role of interaction between bronchial epithelial cells and dendritic cells (DCs) following allergen exposure during the initiation of asthmatic response [72]. When an allergen is inhaled, the professional antigen-presenting cells in the airways, DCs, present it to naive T-cells, which drives the formation of activated Th2 cells. The Th2 cells produce a number of cytokines that stimulate the production IL-4, IL-5, IL-13 among other pro-inflammatory cytokines that can also be produced by ILC2 [73]. Further, cytokines released by Th2 cells direct B cells to generate IgE. High-affinity receptors (FcεRI) on MCs and eosinophils captures IgE produced by B cells at the cell surface and allergens cross-link to the IgE causing MCs to produce cysteinyl leukotriene, prostaglandin D2, and histamine, which constricts the smooth muscles of the airways and increases microvascular permeability, mucus secretion, and inflammation [25] (**Figure 5**).

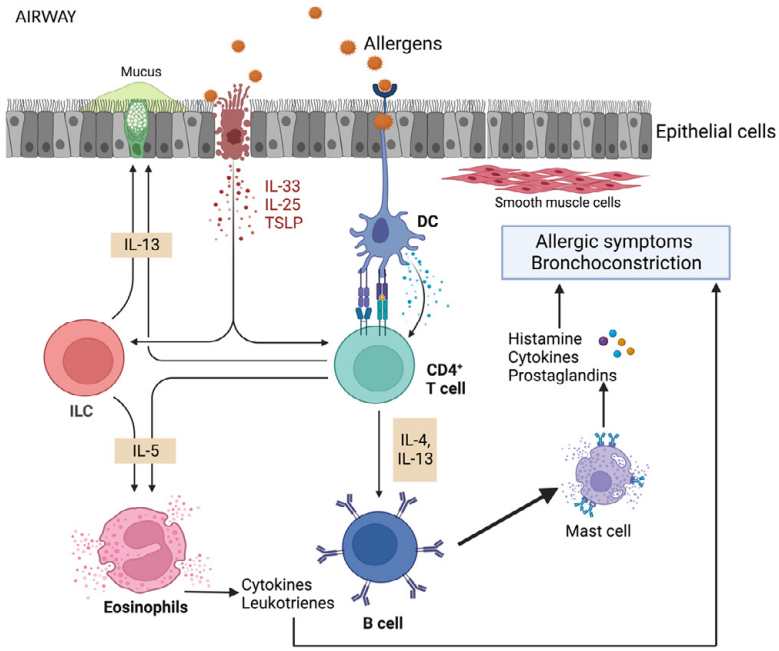

Figure 5: Allergens-induced airway inflammation. The bronchial epithelium are exposed to environmental allergens through inhalation. If these stimuli reach the epithelium, they cause the production of alarmins such as IL-33, IL-25, and TSLP. ILC2 become activated and release pro-inflammatory Type 2 cytokines like IL-5, IL-4 and IL-13 to induce eosinophil recruitment and mucus production, respectively. Dendritic cells function as a platform for the antigen-dependent activation of T helper cells, a kind of adaptive immune cell. Additionally, Th2 cells work with B cells to stimulate the creation of allergen-specific IgE and IgG1 antibodies, which attach to mast cells on mucosal surfaces. Repeated exposure to the allergen causes allergic inflammation, which in turn triggers the production of inflammatory mediators that impact the nearby tissues and cause the onset of symptoms. Figure adapted from [74], Biorender 2022.

House dust mite (HDM)

Approximately 50–85% of people who suffer from allergic asthma are sensitized to HDM [75, 76]. In particularly, two strains of HDM, *Dermatophagoides pteronisunnus* (Der p) and *Dermatophagoides farina* (Der f), are found all over the world. Der p is the strain of HDM that is most commonly found in most countries [77]. House dust mite faecal pellets and the mites themselves have the allergenic potential. More than 20 proteins found in HDM have been classified as allergens, including structural proteins and different enzymes [78]. The best-characterized mite-derived allergens have been categorized into groups that include serine (Derp3, Derp6 and Derp9) and cysteine (Derp1 and Derp3) proteases, non-protease lipid-binding molecules (Der p2), -helical proteins (Der p5), chitinases (Derp15 and Derp18) and structural molecules such as tropomyosin (Derp10) [79].

Although HDM contains a variety of proteases, the cysteine and serine proteases and their proteolytic activity has been mainly implicated for HDM allergenicity [80].

Asthmatic patients were exposed to HDM experience exacerbated bronchospasm and bronchial hyper-reactivity compared to mite-free environments [81]. The fact that HDM exposure and sensitization levels are reliable clinical predictors of asthma in *in vivo* provides additional evidence for the association between HDM and asthma [82, 83]. Furthermore, the fact that the interaction of mite sensitization or exposure, and respiratory virus infection worsens the asthma exacerbations as well as being the major cause of acute wheezing or hospitalization [84, 85] makes HDM of particular interest. Inhaling HDM can cause the proliferation of bronchial smooth muscle [86] and the presence of HDM-specific IgE was found in the sputum of asthmatics [87] are two major additional supporting factors that the connection between HDM and asthma.

Pollen

Allergenic pollen grains are typically produced by wind-pollinated trees, grasses, and weeds. They typically have a diameter of 10 to 100 μm, which enables them to enter both the upper and lower respiratory tracts [88]. The geographic distribution of these plants and the unique traits of the pollen grain, such as size, dispensability, and dynamic density, have a significant role in exposure and sensitization to pollens. Respiratory allergy reactions caused on by pollens have been more common and more severe in recent decades [89-92]. Due to their prevalence in the atmosphere [93] and allergenic potency [94], the following pollen allergens are of significant relevance in Europe: (*Betula alba or pendula),* alder *(Alnus incana),* hazel *(Corylus avellana),* cypresses *(Cupressus sempervirens),* olive *(Olea europaea),* mugwort (*Artemisia vulgaris and Platanus vulgaris),* ragweed (*Ambrosia artemisiifolia)*, and wall pellitory (*Parietaria Judaica)* [95].

Birch pollen, with Bet v 1 as the main allergen, and is responsible for high rates of sensitization in people with tree-pollen allergies [96]. Seasonal exposure to birch pollen can lead to allergic sensitization in those who are genetically predisposed, which is characterized by the differentiation of Th2 cells and the development of allergen-specific IgE antibodies increasing the course of the allergic diseases and causing allergic symptoms [97]. Epidemiological studies have shown that an increased risk of asthma

exacerbation is linked to the onset of the pollen season [98]. Intrinsic factors in pollen such as proteases, aqueous pollen proteins, lipids, nicotinamide adenine dinucleotide phosphate (NADPH) and antigens can trigger innate immune responses and cause allergic airway inflammation [99]. Also, pollen components may influence the epithelial barrier in addition to modulating immune cells. It has been proposed that pollen-released proteases disrupt the functioning of the epithelial barrier [100].

Fungi

Most allergenic fungus spores can penetrate deep into the lower airways of exposed persons due to their small size, which ranges from 3 to 8 μm [101, 102]. Fungi allergies affect roughly 3-10% of the general population in developed countries [103]. Most commonly, sensitivity to *Alternaria*, *Cladosporium*, *Aspergillus*, *Penicillium*, and *Fusarium* is observed. *Alternaria* are the most frequent moulds that cause asthma and increase diseases severity and mortality [104]. In addition to dust mites and pollens, fungi are also the main sources of sensitization in allergic respiratory diseases, with *Alternaria alternata* and its major allergen Alt a 1 being the most important and extensively studied in allergic diseases [105-108]. Previous studies have shown that the chitin component of fungal cell walls strongly activates the immune system, resulting in allergies and allergic asthma [109, 110]. The adverse effects of fungi on the respiratory tract are linked to a concurrent development of inflammation and disruption to the respiratory epithelial cells by non-allergenic proteins and toxins [111].

Allergen proteases

Allergenic proteins with broad structures and functions are responsible for allergenicity [112]. Enzymatic activity, especially protease activity of specific proteins, has been linked to allergenicity [113]. Allergenic sources, including pollen, house dust mites (HDMs), cockroaches, food, venom and fungus may contain protease allergens. A comprehensive list of protease allergens from different sources that have been identified and listed in WHO/IUIS allergen database (http://www.allergen.org/). Allergens proteases are mainly classified based on their protein structure or sequence similarities with serine, cysteine, aspartic or metalloproteases [114].

Protease-antiprotease balance

The maintenance of epithelial barriers in the airways depends on a fine balance between proteases and protease inhibitors, which disruption causes allergic sensitization and inflammation [115]. The significance of protease-antiprotease imbalance in asthmatic airways has been established by earlier studies [116, 117]. After exposure to an antigen, both early mast cell (MC) reactions, and late leukocyte activation, have been demonstrated to considerably increase the protease load in human airways [118]. The pathophysiology and the remodelling of the airways are associated with asthma and significantly influenced by this increase in proteolytic activity.

Disruption of epithelial barrier

There is evidence that the barrier function of the airway epithelium is impaired in patients with asthma [113]. The airway epithelium acts as a physical barrier by forming tight junctions (TJs), which block off the para-cellular space. For the integrity of the epithelial barrier, adherens junctions and apical TJs that keep airway epithelium together and maintain their characteristics. Zonula occludens (ZOs) proteins ZO1-3, occludin, claudins 1–5, and transmembrane adhesion proteins (beta-catenin, E-cadherin, and junctional adhesion molecule–1) are some of the proteins and receptors that interact to form TJs [119]. Previous studies showed that extracts from *Alternaria* species increased the epithelial permeability of cultured HBECs from asthmatic patients [120]. The transmembrane adhesion proteins E-cadherin (E-CDH), claudin-1, Occludin, and zonula occludens-1 (ZO-1) were demonstrated to be disrupted by proteases present in pollen diffusates, increasing trans-epithelial permeability. Tight junction disruption and increased trans-epithelial permeability allow allergens to enter epithelial sublayers, resulting in sensitivity to a wide spectrum of allergens [121].

Protease allergen as an inducer of T2 inflammation

T2 allergic immune responses have been linked to allergen protease activity [122]. Active proteases increased IL-33 levels in the lungs, whereas IL-33 deficient mice exhibited lower IgE/IgG1 levels and reduced eosinophils, suggesting the significance of IL-33 in protease-induced sensitization [122]. Furthermore, investigations have demonstrated that animals exposed via the nasal mucosa with active or inactive cysteine protease exhibited Th2 type lung hypersensitivity [123].

Activation of bronchial epithelial cells

The activation of PRRs such as TLRs and protease activated receptors (PARs) allows epithelial cells to detect and respond to inhaled allergens or proteases [124]. These activated receptor signals cause nuclear factor kB (NF-kB) or mitogen-activated protein kinases (MAPKs) activation, which in turn causes the transcription of many pro-inflammatory genes, including cytokines and chemokines and anti-viral responses [125, 126]. In allergic respiratory diseases, proteases have been demonstrated to promote pro-inflammatory cytokine production by epithelial cells via PARs [127]. Pro-inflammatory cytokines IL-6, IL-8, granulocyte macrophage colony-stimulating factor and monocyte chemotactic protein-1 were secreted by airway epithelial cells after exposure to mite, timothy grass pollen, or birch pollen extracts [128, 129]. HDM derived proteases Der p 1 and other Der p antigens have been found to directly promote the release of pro-inflammatory cytokines and chemokines from HBECs and airway epithelial cell lines through protease-dependent pathways [130, 131].

DAMPs or Alarmins

During epithelial cell damage, due to tissue injury or infections, the epithelium rapidly releases molecules which are already pre-stored inside the cells, that thus react with immune cells and trigger inflammation. These molecules can alert our body's defence of danger, and are therefore called endogenous danger signal or DAMPs or alarmins [132]. PAMPs and DAMPs are produced as a result of infection and cell damage or injury, and they activate PRRs in endosomes and on the cell membrane to trigger an inflammatory response. Similar pathways are activated by cytoplasmic PAMPs when they connect to NOD1 and NOD2 [133] (**Figure 6**). Further, these released molecules activate downstream multiprotein complexes known as inflammasomes, which then activate caspase-1 and cleave IL-1beta and IL-18 into active form [134]. In particular, it has been demonstrated that HDM activates the NLRP3 inflammasome through the PI3K/Akt pathway, which results in inflammation in asthma [135].

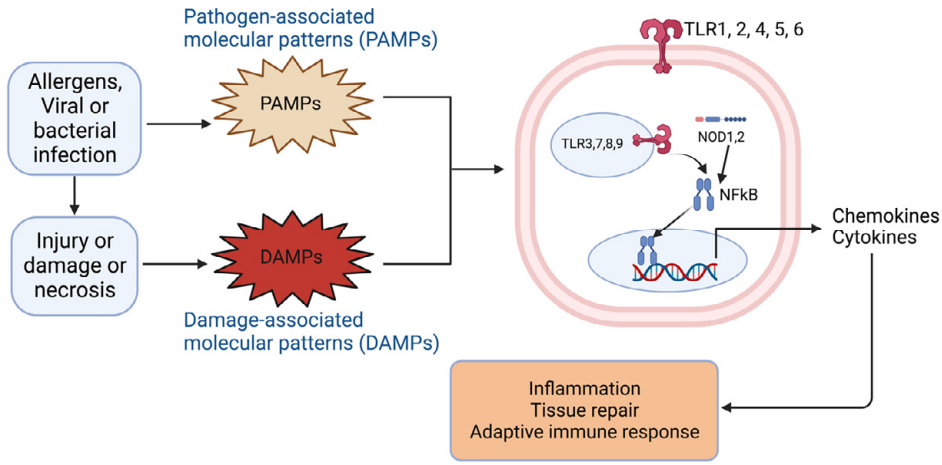

Figure 6: PAMPs and DAMPs are produced as a result of infection and cell damage or injury, and they activate TLRs in endosomes and on the cell membrane to trigger an inflammatory response. Similar pathways are activated by cytoplasmic PAMPs when they connect to NOD1 and NOD2.Figure adapted from Innate pathways of immune activation in transplantation. Journal of transplantation (2010). Biorender 2022.

Based on their origin DAMPs were classified to be cytosol, nuclear, mitochondria, or endoplasmic reticulum. Calcium-binding protein S-100, high-mobility group box protein 1 (HMGB1), or uric acid (UA) are examples of "Intracellular" DAMPs, which are a group of immunogenic chemicals produced by the breakdown of necrotic and apoptotic cells. "Extracellular" DAMPs are extra cellular matrix (ECM) components (glycoproteins, proteoglycans, or glycosaminoglycans) [136]. Previous studies showed that necrotic cells release DAMPs such as HMGB1, HSPs, and S100, which alter the innate and adaptive immune responses through interaction with pattern PRRs [136].

Metabolite DAMPs

Uric acid

UA has been regarded as a DAMP molecule and is synthesized in all cells by the degradation of purines from DNA and RNA [137]. Also, released from dying or damaged cells and a purinergic metabolite end product [138]. The accumulation of UA in tissues can cause gout, but soluble uric acid released by dead cells or injured cells acts as a danger signal to the immune system

[139]. UA levels increase in serum and can undergo the phase change of crystallization to monosodium urate crystals. UA crystals strongly trigger acute neutrophilic inflammation through stimulation of the NLRP3 inflammasome and release of interleukin-1β [140].

Role of UA in asthma

UA is recognized as an inflammatory mediator of allergic asthma and a cause of acute neutrophilic inflammation. At the start of an asthma attack, serum UA levels was increased and had a negative impact on asthmatics spirometric pulmonary functioning [141]. A previous study demonstrated that the Th2 cell adjuvant aluminium hydroxide, or alum, which is often co-injected with nontoxic antigens intraperitoneally to mice to induce allergic sensitization and experimental asthma, causes the release of UA in the peritoneal cavity [142]. Furthermore, allergen-challenged patients with asthma have higher amounts of UA in their airways [143]. Experiments in mice suggest that UA may play a role in the onset, pathogenesis, and immune-inflammatory aspects of allergic asthma [144]. Additionally, UA is constantly released on the mucosal epithelial tissues surface in the airways, with no apparent pathogenic effects [137]. Bronchial alveolar lavage fluid (BALF) UA levels were increased rapidly within 3 hours following a single airway exposure of mice to bromelain [145]. This data indicates that proteases play a major role in UA release in experimental mouse models of asthma.

ATP

Adenosine triphosphate (ATP) is known as a source of high energy phosphate bonds to support cellular metabolism. ATP can also be released extracellularly followed by cell death or damage, and acts as a danger signal to alert the immune system [146]. Plasma membrane-localized purinergic receptors P2X and P2Y recognize extracellular ATP and trigger the immune response [147]. Purinergic receptor activation in immune cells modulates the secretion of several pro-inflammatory cytokines, including IL-1β, IL-18, inducible nitric oxide synthase, and reactive oxygen species (ROS) [148-152].

Role of ATP in asthma

A number of immune cells, such as eosinophils [153, 154], mast cells [155, 156], dendritic cells [157, 158], and alveolar macrophages [159, 160] are activated by ATP, which plays a direct role in inducing airway inflammation.

Additionally, it has been demonstrated that the production of mucin is stimulated by ATP and adenosine, a breakdown product of ATP, in the epithelia, which plays a role in asthma [161, 162]. *In vivo* experiments have shown the importance of purinergic signalling in airway inflammation [163, 164]. Neutralising extracellular ATP in the lungs will reduce asthmatic inflammation [165, 166]. The extracellular accumulation of ATP seems to be critical for regulating the Ca2+ response and subsequent IL-33 release [167]. It was shown that the release of ATP from the airway epithelium is essential for *Alternaria*-evoked IL-33 mobilization from the nucleus and secretion into the extracellular milieu [168]. Allergen-challenged asthmatic patients have higher amounts of ATP in their airways [157]. Additionally, current findings demonstrated that extracellular nucleotides like ATP and UTP can reduce virally produced IFNs by suppressing TLR-3 and RIG-I in HBECs [169]. Higher expression levels of ATP and its receptors may be more effective targets for respiratory diseases, particularly asthma.

Epithelial Alarmins

The epithelial alarmin cytokines such as IL-25, IL-33 and TSLP are known regulate both eosinophilic dependent and non-dependent inflammation in asthma [170]. These alarmins are mainly released from the airway epithelium, and when they are released stimulate numerous effector cells (Th2 and ILC2) start to produce type 2 cytokines, IL-4, IL-5, and IL-13 [171]. Different allergic mechanisms, such as eosinophilic inflammation, immunoglobulin (IgG) class switching to IgE, stimulation of B-cell development, goblet cell metaplasia, and consequent mucus production, are initiated when type 2 cytokine expression is upregulated [74]. Epithelial alarmin cytokine (IL-25, IL-33 and TSLP) are significant mediators of inflammation during allergic diseases and may prove to be valuable targets for therapeutic intervention as a result of growing research.

IL-33

IL-33 belongs to the IL-1 family and is mainly expressed in the nucleus of endothelial and epithelial cells from different tissues [172]. Within the nucleus, IL-33 function as a nuclear factor regulating pro-inflammatory genes, such as IL-8 and IL-6. IL-33 acts as an alarmin and released during a programmed cell death such as, necroptosis and pyroptosis, activating

different immune responses [173]. IL-33 binds to its receptor ST2 [66], which is selectively and stably expressed on the cell surface of Th2 cells, CD4+ T cells, ILC2s and also other immune cells such as MCs, basophils, eosinophils, macrophages, DCs and natural killer cells [174]. Activation of ST2 (also called IL-1RL1) coupled receptor regulate signalling pathways based on the myeloid differentiation primary response gene 88 (Myd88) which further activates through nuclear factor kappa-B (NF-κB), c-Jun N-terminal kinase (JNK), and p38 MAPK cascades [175].

Role of IL-33 in asthma

IL-33 is one of the earliest cytokines to be produced in response to allergens [176] and has been shown to correlates with asthma disease severity [177]. IL-33 is augmented in the lung epithelium [178], airway smooth muscle [177] and BAL [179]. IL-33 and soluble form of ST2 levels are significantly higher in the blood and sputum of eosinophilic asthma patients compared to control groups [180, 181], as well as in those who are experience an acute exacerbation [180]. In moderate allergic asthma patients, exposure to inhaled allergens increases the expression of the ST2 receptor on eosinophils in blood and sputum. This increased receptor expression can be replicated by *in vitro* stimulation of eosinophils with IL-33 [182]. IL-33-deficient mice were used to show the significance of endogenous IL-33 in allergic inflammation, and it was reported that IL-33 was essential for both ovalbumin and protease allergen (papain)-induced airway inflammation [183]. *Alternaria* derived serine proteases induced IL-33 release, which in turn triggers the rapid onset of asthma exacerbations, results in significant inflammation [184]. In previous study, it was shown that the fungal allergen *A. alternata* might release IL-33 from airway epithelial cells through autocrine ATP signalling even in the absence of cellular necrosis. This finding suggests that IL-33 release may also occur in response to cellular stress [167].

In addition to airway epithelial cells, ASM cells may be a significant cell source for IL-33. We and other previously showed that, stimulation with TNF-α, interferon-gamma (IFN-γ), double-stranded RNA, ATP, and rhinovirus infection increase the production of IL-33 mRNA in primary human ASM cells [48, 52]. However, many of these effects seem to be brought on by IL-33 indirectly controlling ASM cells via immune cells such mast cells and ILC2s [185]. In comparison to healthy controls, asthmatic patients have considerably higher levels of IL-33 protein expression in ASM [177]. *In vitro* IL-33 stimulation or *in vivo* intranasal exposure to OVA have been shown to increase ST2 protein expression, which is relatively low

in both human and mouse ASM cells [186, 187]. Therefore, targeting the IL-33 signalling axis as a treatment strategy in this type of asthma inflammation may be beneficial.

TSLP

TSLP, an epithelial cytokine, was originally found in the thymic stromal cell line supernatant and was later demonstrated to maintain B cell line expansion over time [188]. TSLP can be produced by a variety of cells in the airways, including AECs, smooth muscle cells, DCs, basophils, and MCs [189]. TSLP is rapidly released from cells, triggering additional external and endogenous danger signals as well as aggravating inflammation. As TSLP is released, it binds to the TSLP receptor, which is made up of the IL-7 receptor chain-alpha and TSLP-γ, which are located on the majority of the cells in the airways [188, 190].

Role of TSLP in asthma

TSLP has been linked to the initiation and maintenance of inflammatory pathways in asthma [191]. Numerous triggers, including as mechanical damage, ligands for TLR3, TLR2 and NOD2, helminth infection, pro-inflammatory cytokines, and proteases such as trypsin and papain can cause epithelial cells to produce TSLP [192-194]. Viral infections can also lead to the production of TSLP in the lungs [195] and HBECs from patients with asthma have been shown to overproduce TSLP in response to TLR3 stimulation [196]. It is known that TSLP stimulates T2 inflammatory responses, and more recent research suggests that TSLP may also trigger non-T2 responses in asthma [38, 189]. TSLP can have a significant impact on a variety of cell types and signalling pathways to have a broad impact on airway inflammation due to its position at the top of the inflammatory cascade. Based on the previous evidence, TSLP orchestrates the effector activities of a wide range of myeloid and lymphoid cell types that play a role in inflammatory reactions in asthma [189]. TSLP expression is higher in the airways of asthma patients compared to healthy controls, and TSLP levels correlate with the expression of T2-attracting chemokines, disease severity, and risk of asthma exacerbation [197]. TSLP has been shown to be both essential and sufficient for the development of T2 cytokine-associated airway inflammation in animal models [198]. In a previous study, TSLP receptor (TSLPR)-deficient (Tslpr-/-) mice demonstrated noticeably lower airway inflammation upon OVA challenge, and local administration of anti-TSLPR

was reported to inhibit T2-mediated airway inflammation in asthmatic mice after OVA sensitization [199]. Moreover, local TSLP blockade abolished airway inflammation and structural remodelling in an HDM-induced mouse model [200] and reduced airway inflammation in allergic asthma patients [201]. Evidence from clinical trials in asthma patients treated with TSLP inhibitors has further supported the hypothesis that anti-TSLP therapy has wide anti-inflammatory properties [202, 203].

IL-25

Eosinophils and basophils have been identified as the primary sources of IL-25 in asthma patients. In addition, IL-25 is produced and secreted by a wide range of cells, including epithelial/endothelial cells, activated Th2 cells, alveolar macrophages, bone marrow-derived mast cells, and fibroblasts. For signal transduction, IL-25 binds to its receptor, which is composed of IL-17 receptors A (IL-17RA) and B (IL-17RB) [204]. It has been suggested that IL-25 is a type 2 cytokine that promote the release of IL-13, IL-5, and IL-4, which in turn prevents T helper (Th) 17 from differentiating and acts as an anti-inflammatory agent by suppressing Th17 and Th1 responses [205].

Role of IL-25 in asthma

Airway epithelial cells express IL-25 as a preformed cytokine and stored in the cytoplasm, thereby enable to rapidly release upon cell stimulation by environmental stimuli including allergens [206]. Asthmatic patients have high level of IL-25, both protein and transcript level in the airways compared to healthy controls [207]. It has been demonstrated that asthmatic individuals with higher IL-25 mRNA levels have more severe disease [208], indicating that this cytokine is a key inflammatory trigger. Extracellular release of IL-25 by airway epithelial cells regulates many other pathogenic features of asthma, including the recruitment of eosinophils, airway mucus over production and airway remodelling [209]. A recent study demonstrated that inhibiting IL-25 enhanced the antiviral response during respiratory viral infections in differentiated HBECs from asthmatics [210]. However, current studies from our group failed to detect any IL-25, either in the gene or the protein, in HBECs stimulated by allergens or infected with RV infection.

Mast cell and their mediators in allergic inflammation

MCs are myeloid cells that develop from hematopoietic stem cells in the bone marrow, travel through the circulation as progenitor cells, and differentiate into granular, tissue-inhabiting cells in the target tissue [211]. They are multifunctional cells that can recognize pathogen-associated molecular patterns (PAMPs) through TLRs, internal signals via cytokine and chemokine receptors, and antigens through IgE-FcεRI cross-linking [212-214]. MCs are crucial for tissue repair, homeostasis, host defense, and allergic inflammatory pathways. MCs degranulate and produce premade and *de novo* synthesised mediators including histamine, specific MCs proteases, proteoglycans, and cytokines, when they are stimulated by allergens or other environmental factors [215] (**Figure 7**). Tryptase and chymase are the most abundant serine proteases in the MCs granule. They have multiple effects on structural cells, which contributes to airway inflammation and remodelling [216]. Human MCs are divided into two major phenotypes based on their protease content. One phenotype contains tryptase only which is called Mast Cell Tryptase (MC_T) while a second contains tryptase, chymase, corboxypeptidase A and cathepsin G which is called Mast Cell Tryptase Chymase (MC_{TC}). Very few studies have been reported the rare populations containing only chymase called Mast Cell Chymase (MC_c) [217, 218]. MC derived serine proteases have important role in all phases of wound healing, including haemostasis, inflammation, proliferation and remodelling [219].

Increased numbers of MCs in the asthmatic airway, are a rich source of the neutral proteases such as tryptase and chymase, which can degrade basement membrane components [220]. Studies have been reported that increased MCs infiltration in airway epithelium and in the alveolar parenchyma [221, 222]. In addition, bronchial biopsies from asthmatic patients show MC degranulation within the epithelial layer [223, 224]. However, little is known regarding the role of intraepithelial MCs and their released mediator's contribution in HBECs. Current research aims to identify the relative importance of the beneficial and detrimental functions of MC proteases in allergic diseases. In a mouse model of asthma, knockdown of specific chymase *Mcpt4* −/− mice showed increased AHR, inflammation and smooth muscle volume which indicates a positive role in allergic inflammation [225]. In contrast, blocking of chymase and cathepsin G shows inhibition of both allergic and non-allergic inflammation in animal models [226] indicating that these enzymes are responsible for promoting inflammation. Similar to chymase, inhibition of tryptase reduced late-phase bronchoconstriction in

atopic asthmatics patients [227] and decreased eosinophilia and nasal symptoms in allergic rhinitis subjects [228, 229]. In contrast to their pro-inflammatory properties, tryptase has been shown to neutralise cytokines and matrix metalloproteinases and defence against bacterial infections [230].

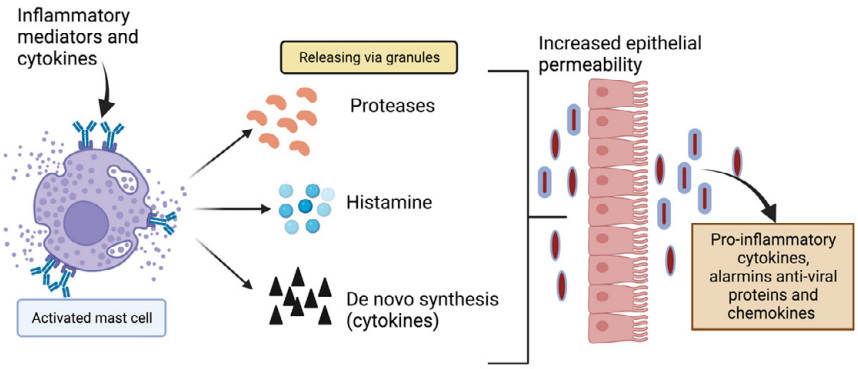

Figure 7: Mast cells produce pre-stored mediators as such proteases, histamine, and newly synthesized cytokines, which have an impact on the function of the epithelial barrier. Released mediators influence the production of alarmins, antiviral proteins, and pro-inflammatory cytokines in epithelial cells. Figure adapted from De Winter BY, et al. Biochim Biophys Acta. 2012. Biorender 2022.

Viral-induced asthma exacerbations

Asthma exacerbations continue to be a major cause of health-care demand and a significant financial burden on patients and society. Acute asthma exacerbations remain to cause significant treatment difficulties and frequently result in hospital admissions or visits to the emergency room, particularly in infants [231]. Viral infections can exacerbate asthma through a range of mechanisms, including increased serum IgE levels, epithelium injury or activation, impaired antiviral responses, altered host immunological responses, respiratory tract inflammation, and direct infection of the lower respiratory tract [232].

Respiratory viruses such as rhinovirus (RV), respiratory syncytial virus (RSV), influenza virus, parainfluenza virus, adenovirus, and coronavirus are most commonly associated with the development and worsening of asthma [233]. Where RV infection is most frequently reported to induce acute asthma exacerbations. RVs are incredibly diverse, and there are roughly 100 classical serotypes divided into RV-A, RV-B and RV-C [234]. Based on the

cellular receptors that they bind to, RV-A and RV-B are commonly identified as the major and minor groups. Intercellular adhesion molecule (ICAM) 1 is the receptor for the major group of RV-A and all of RV-B, the low-density lipoprotein receptor (LDL) is the receptor for the minor group of RV-A, and the cadherin-related family member 3 is the receptor (CDHR3) for RV-C [235, 236]. RV is taken up by receptor-mediated endocytosis, replicates in AECs, and is recognized by PRRs. The recognition of viruses by PRRs results in the release of a variety of pro-inflammatory cytokines and chemokines, as well as anti-viral interferons (IFNs) [237].

It is reported that rhinovirus infect healthy individuals and asthma patients with asthma at the same frequency but it is well known that the pathological effects of RV in asthma patients are far more severe than in healthy individuals. Studies have shown that anti-viral type I and III interferons were shown to be lower in RV-infected and dsRNA-stimulated AECs cultured from asthmatic patients compared to healthy controls [196, 238, 239]. In vitro, RV causes bronchial smooth muscle cells or airway epithelial cells to produce IL-33, TSLP and IL-25 [240, 241], and supernatants of RV-infected bronchial epithelial cells stimulate human T cells and ILC2 to generate type 2 cytokines [242]. In addition to viruses, allergens, bacterial infections, and pollutants can cause asthma exacerbations. A large body of epidemiologic studies supports the associations between RV infection, allergen exposure, and allergen sensitization with the development and exacerbation of asthma [85, 243]. RV infections and allergens can increase the production of IL-25 and IL-33 in airway epithelial cells, which promotes T2 inflammation and airway remodelling [210].

Anti-viral interferons

Interferons (IFNs) are a large group of pleiotropic cytokines that play an important role in host antiviral defences. They are classified into three types: type I, type II, and type III IFNs. IFN-α, IFN-β (IFN-α/β) (Type I) and IFN-λ (Type III) are produced in response to viral infection, and they activate transcription of IFN-stimulated antiviral genes (ISGs) in infected cells [244]. Upon viral infection, type I IFNs are produced and have antiviral effects through interacting with the IFN-α/β receptor (IFNAR), which is present on almost all cells [245, 246]. Released INFs bind to the IFNAR signal downstream to activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway. It then leads to the formation

of the ISGF3 transcription factor complex, which is made up of phosphorylated STAT1/STAT2 and IRF9. ISGF3 binding to IFN-stimulated response elements in the promoter region of ISGs increased the expression of many IFN target genes, including interferon regulatory factor 7 (IRF7) [247].

Several transcription factors, including NF-kB and especially IRF3/IRF7, regulate the production of type I and type III IFNs. The asthmatic epithelium has been impaired ability to produce IFN was initially shown by Wark et al. In their investigation, they discovered that HBECs from asthmatic patients had higher RV replication in vitro compared to healthy subjects, and that this was reflected in reduced and delayed IFN-β induction [239]. Similar findings of insufficient IFN-induction in RV-infected HBECs from asthmatic patients were later reported by Contoli et al. The authors also demonstrated that exacerbation intensity was inversely correlated with IFN-λ production using a human experimental model of RV exacerbation [238]. However, increasing evidence revealed that prior exposure to inhaled allergens decreases antiviral immunity in asthma and healthy HBECs [126, 248, 249].

Clinical treatments of asthma

The objective of asthma treatment is to control symptoms, prevent exacerbations, and reduce the risk of chronic airflow limitation and asthma-related death [6]. Major treatments prescribed are inhaled corticosteroids (ICS), long-acting beta-agonists, leukotriene modifiers and theophylline [250].

Inhaled corticosteroids (ICS)

ICS are the initial treatment for the asthma patients with infrequent and mild symptoms. ICS relief asthma symptoms by reducing the inflammation in the airways. At a molecular level, they function within the nucleus, interacting directly with DNA and result in suppression of transcription factors, such as NF-kB, which are involved in inflammation [251, 252]. Inhaled corticosteroids boost the synthesis of adrenergic receptors that are found on the smooth muscles of the airways, which in turn increases their potency [253].

Beta2-agonists

Long-acting beta-agonists added to these inhaled corticosteroids can reduced asthma symptoms even more [254]. Particularly, hospitalization and death are closely associated to asthma attacks that occur at night. Consequently, taking preventative measures against evening symptoms is a key treatment objective. Long-lasting beta2-agonists (LABA) are frequently prescribed for day use in conjunction with inhaled corticosteroids (when used alone, they can increase mortality), but they are also quite helpful for easing overnight symptoms and effectively lower the chance of symptom worsening in general [255].

Leukotriene modifiers

Leukotriene modifiers are another medication that may be taken together with inhaled corticosteroids. These leukotriene modifiers help to stop the activation of the CysLT 1 receptor as this receptor is responsible for several symptoms of asthma, such as increased mucus discharges and smooth muscle contraction [256].

Biologics

For some patients with severe asthma, the common controller medications might not be effective enough to control asthma symptoms on their own. In recent years, new medications called “biologics” have been approved for the treatment of severe asthma. These biologic therapies have provided auspicious targeted therapy [20]. Specific inflammatory pathways involved in the pathophysiology of asthma are the focus of biologic therapy, especially for individuals whose endotype is characterized by T2 inflammation. When considering biologic therapy, its crucial to distinguish between T2 high and low endotypes [257]. Studies have been done on monoclonal antibodies (mAb) that are directed against IL-4 [258], IL-5 [259] and IL-13 [260]. Asthma exacerbation rates were decreased, and lung function was improved as a result of blocking these T2 cytokines. The monoclonal antibody omalizumab, when administered in conjunction with other treatments, reduced the incidence of exacerbations by inhibiting plasma IgE [261-263].

Allergen Immune therapy

The ongoing inflammatory process and symptoms of asthma can be efficiently controlled by current treatments, but the underlying, dysregulated immune response is unaltered. The only aetiology-based treatment for allergic disorders that can modify the course of the disease is allergen immunotherapy (AIT), which has been shown to stop the development of new allergic sensitizations as well as the progression of existing allergic diseases [264]. Multiple immune-mediated processes are sequentially activated by effective AIT, resulting in the suppression of inflammation, development of allergen-specific tolerance, and a variety of therapeutic effects such as lowering the use of steroids, limiting asthma exacerbations and increase lung function [265-267]. Atopic individuals with IgE-mediated allergic rhinitis and/or allergic asthma caused on by inhaled allergens such pollen and HDMs are offered the choice of receiving immunotherapy if they have not responded well to anti-allergy medications or have experienced adverse drug side effects. AIT can be administered sublingually or subcutaneously (SLIT or SCIT) [268]. An earlier study shown that three years of continuous SCIT with grass pollen extract produced long-term improvements that persisted three years after the treatment was stopped [269]. Recently, Wohlk et.al, showed that AIT decreases respiratory infections that require antibiotic treatment as well as exacerbations of both seasonal and perennial allergic asthma [270].

Aim of the thesis

The main objective of the PhD thesis research is to understand the precise innate immune mechanism of virus, allergen, and mast cell mediator's interactions with airway epithelium and smooth muscle cells in asthma. The findings of this thesis will reveal novel targets implicated in the onset and progression of asthma and allergic inflammation, allowing us to establish new therapeutic strategies for the treatment of asthma exacerbations.

Specific aims:

- **Paper I and II**: Investigate and compare the release of the metabolic DAMPs (Uric acid and ATP), epithelial alarmins (IL-33 and TSLP) as well as anti-viral response in human bronchial epithelial cells exposed to different allergen stimulations *in vitro* and *in vivo*.
- **Paper III**: Study the effects of mast cell serine proteases, tryptase and chymase, on inflammatory cytokine profile and potential interactions with the anti-viral response in bronchial epithelial cells.
- **Paper IV**: Investigate and compare the specific involvement of pattern-recognition receptors and downstream signalling pathways in rhinovirus-induced IL-33 expression in bronchial smooth muscle cells from asthmatic and healthy individuals.
- **Paper V**: In a randomized control trials (RCT) study investigate the bronchial epithelial anti-viral response before and after HDM sublingual allergen immunotherapy (HDM-SLIT) treatment.

Material and Methods

This section provides an overview of the methodologies used throughout the entire thesis. Detailed descriptions of these procedures can be found in the material and method parts of the different publications and manuscripts.

In vitro cell culture models

The use of *in vitro* cell culture technique lowering the expense of using animal models will make research more affordable. Cell lines are frequently chosen because they provide a simple, inexpensive, and reliable platform. They can be cultured indefinitely and have a high rate of proliferation. Cell lines also have the advantage of being made up of pure cell populations, which greatly improves the reproducibility of experiments. However, they often do not accurately reflect what happens *in vivo*. Human primary cells might be a better option if wanting to represent the unique physiological function or disease phenotype. Both submerged and air liquid interface (ALI) cultures can be used to grow human bronchial epithelial cells. Although the ALI culture more closely resembles the *in vivo* situation, the cell culture model system must be chosen based on the research question. In this thesis, experiments are conducted with submerged cultures of primary bronchial epithelial or smooth muscle cells as well as commercial cell lines. The reason we choose for submerged cultures is that, in comparison to well-differentiated cell cultures, undifferentiated submersion cultures can reach higher levels of viral infection and, thus, enhanced cytokine expression and release [271].

Bronchial epithelial cell line

In paper, I and III bronchial epithelial cell line ware used. The human bronchial epithelial cell line Bronchial Epithelium transformed with Ad12-SV40 2B (BEAS-2B) was purchased from ATCC (Walkersville, MD, USA)

and was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% Fetal Bovine serum (FBS) and 1% Penicillin streptomycin (PEST) from Life Technology (Stockholm, Sweden). All incubations were carried out in air with an atmosphere of 5% CO_2 at 37°C. For the experiments, cells were seeded and incubated to 80-90% confluency in collagen coated (INAMED Biomaterials, CA, USA) 12-well culture plates (Nunc; Life Technologies, Stockholm, Sweden).

Primary human bronchial epithelial cells

The primary human bronchial epithelial cells (HBECs) were used in paper III and V, and were obtained from as a study in a collaboration project with Bispebjerg Hospital, Denmark. HBECs from asthmatic individuals were obtained through epithelial brushings by using fibre-optic bronchoscope. During bronchoscopy a standard sterile fibre-optic bronchoscope is inserted through the mouth or nose into the lungs. Material is then collected by brushings of the airways. Prior to culture, the brushings from the airways were vortexed and centrifuged.

Primary cultures of HBECs were established by seeding in collagen coated cell culture flask containing serum-free bronchial epithelial growth medium (BEGM, Lonza) supplemented with SingleQuots (Lonza) and 0.1% Primocin. Cells were cultured under standard conditions (5% CO_2 and 37°C). For experiments HBECs were used at passage 2 and seeded into collagen coated 12-well plates in BEGM medium and were grown to 70-80% confluence (Figure 11).

Bronchial smooth muscle cells

The primary human bronchial smooth muscle cells (BSMCs) studied in paper (IV) were obtained from Prof. Kian Fan Chung (Respiratory Medicine, Imperial College, London) as cryopreserved cells isolated from healthy, non-severe and severe asthma patients. Positive immunostaining with anti-calponin, anti-smooth muscle Beta-actin, and anti-myosin H Chain antibodies was used to define the BSMCs after growth from bronchial biopsies [272]. The BSMCs were cultured in tissue cultured flasks containing DMEM with 10% FBS, 1% PEST and 1% Amphotericin B (Life Technologies, Stockholm, Sweden) in 5% CO_2 and 37°C. For experiments, BSMCs were seeded into 6- or 12-well culture plates (Nunc, Life Technologies, Carlsbad, CA, USA) and at 80–90% confluence the growth

medium was replaced with DMEM containing reduced FBS (1%) for 24h prior to all experiments (**Figure 8**).

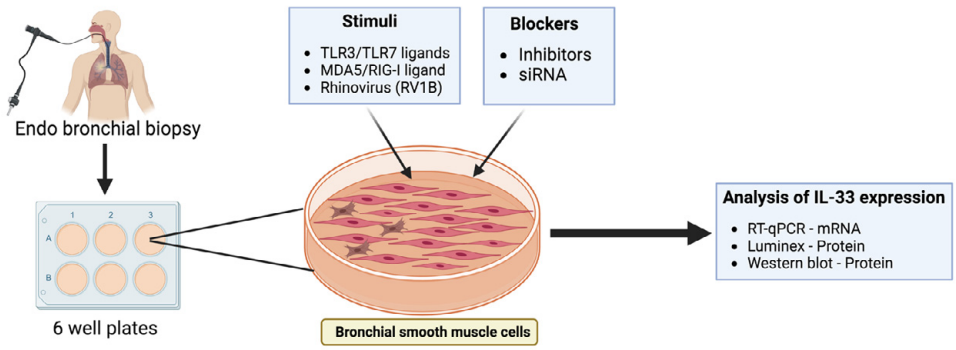

Figure 8: Submerged cell culture were used for bronchial smooth muscle cell cultures. Bronchial smooth muscle cells were infected with RV1B alone or used siRNA knockdown or pharmacological inhibitors to study the rhinovirus induced IL-33 regulating mechanism in bronchial smooth muscle cells. **Biorender 2022.**

Stimulation and inhibition of cellular responses

Allergens, proteases and TLR ligand stimulations in HBECs

In this thesis, four different aeroallergens were used to study the danger associated molecular patterns (DAMPs) or alarmin, pro-inflammatory cytokine release and anti-viral response in HBECs. The aeroallergens used were house dust mite (HDM), *Artemisia vulgaris* (mugwort), *Betula pendula* (birch) and *Altenaria alternata* (fungal), since the severity of asthma has been strongly associated with sensitization and exposure to these allergens. Allergen extract of *A. vulgaris*, *B. pendula* and *A. alternata* were purchased from Allergon (Angelhom, Sweden) and prepared as previously described [273]. The HDM whole extract used in paper I was acquired from GREER Laboratories (Lenoir, NC, USA), while for paper II, it was purchased from ALK-Abello (Denmark). In paper I, HBECs were stimulated with different doses of four different allergens for 1, 3, 6 and 24h (**Figure 9 (1)**).

To study anti-viral response, synthetic dsRNA analogue called polyinosinic-polycytidylic acid (poly(I:C)), was used to activate TLR3 in paper II-V. This synthetic dsRNA which mimics viral infection as rhinovirus produces

dsRNA when it replicates. dsRNA binds to the TLR3 within the endosome or RIG-I/MDA-5 in the cytoplasm. In paper II, HBECs were treated with TLR3 agonist poly(I:C), HDM (20 µg/ml) or *A. vulgaris* (1000 µg/ml) or *B. pendula* (200 µg/ml) or *A. alternata* (200 µg/ml) alone or pre-treated with four different allergens for 3 and 24h and then the cells were stimulated with 10 µg/ml of TLR3 agonist poly(I:C) for 24h (**Figure 9 (2)**). In paper III, HBECs were pre-treated with tryptase (0.5 µg/ml) or chymase (0.5 µg/ml) or poly(I:C) alone for 3h then the cells were treated with poly(I:C) for 3 and 24h (**Figure 9 (3)**). In paper V, HBECs from patients with allergic asthma were stimulated with poly (I:C) alone for 24h. Cell lysate and supernatants were collected after 3h and 24h for gene and protein analysis.

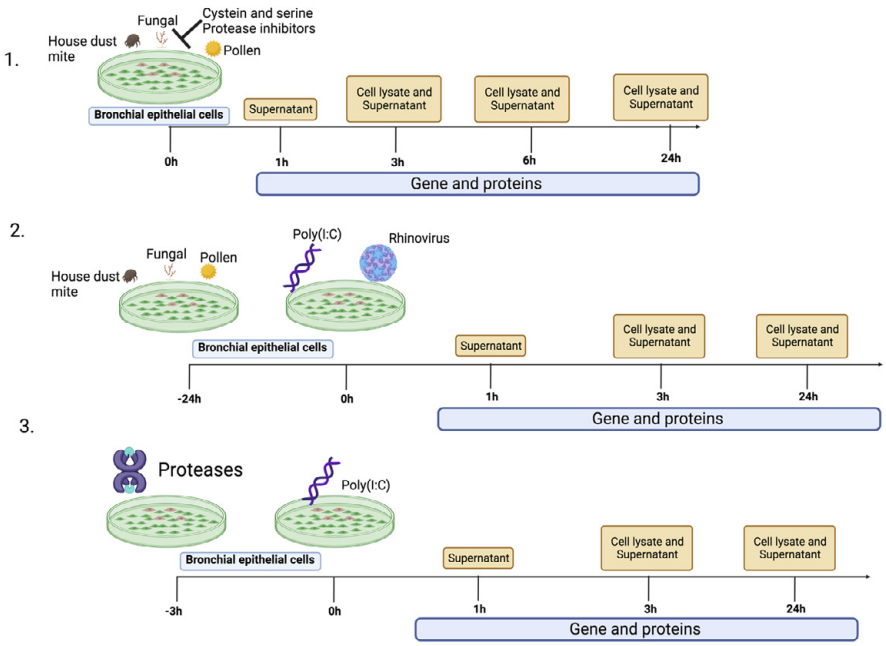

Figure 9: *In vitro* bronchial epithelial cell culture system. Biorender 2022.

Rhinovirus amplification and infection

Amplification of rhinovirus stock

Viral stocks of human rhinovirus RV1B (minor group) was generated by infecting monolayer cultures of HeLa cells at 33°C until cytopathic effects

were fully developed. Cells and the cell culture supernatants were collected, cells were disrupted by freezing and thawing, cell debris was pelleted by low-speed centrifugation, and the final supernatants were frozen at −80°C. Rhinovirus titration was performed on the frozen aliquots by exposing confluent monolayers of HeLa cells in 96-well plates to serial 10-fold dilutions of viral stock. Plates were cultured for 5 days in 4% minimal essential medium (MEM) at 37°C in 5% CO_2. Tissue culture infective dose 50% (TCID50)/ml values were determined after rhinovirus titration in confluent HeLa cells monolayer after fixation and staining with 0.1% crystal violet.

RV infection

HBECs and BSMCs infections were carried out using RV1B. In summary, cells were shaken at room temperature for 1 hour while being infected with 0.1 MOI RV1B for HBECs in paper II and 1 MOI RV1B for BSMCs in paper IV. Following the removal of the virus, cells were washed with PBS and incubated with fresh medium for the required periods of time at 37°C for further experiments.

siRNA knockdown and transfection

The effects of loss-of-function in specific genes have been extensively studied using targeted inhibition or knockdown of gene expression in cultured cells. One method for post-transcriptionally silencing individual gene expression is gene-specific degradation of mRNA. Using RNA interference (RNAi) technology is one of the most popular methods for generating such gene-specific RNA degradation. In paper IV, we used small interfering RNAs (siRNA) technique for gene knockdown. The cell cultures were 70-80% confluent when used for siRNA knockdown (Figure 10). For knockdown experiments, siRNA targeted against TLR3 (ID: s235), MDA5 (ID: s125361) or RIG-I (ID: s223616) or with non-specific siRNA (Ambion, Thermo Scientific, Waltham, MA, USA) at a final concentration of 10nM/L, was added to Lipofectamine RNAiMAX reagent in OPTI-MEM and incubated for 5 minutes at room temperature to form complexes before adding to the cells. The cells were used for experiments 48h post-transfection.

Pharmacological inhibitors

In paper IV, pharmacological synthetic inhibitors were used to block the proteins that regulate IRFs and NF-kB downstream signalling pathways in paper IV. The TBK1- mediated activation of IRF3 was blocked by 0.1 μM BX795 [274], IKK- mediated NFkB activation by 10 nM PS1145 [275] and TAK1 activation by 0.1 μM 5Z-7-oxozeaenol [276]. The cells were incubated at 37°C for 1h in the presence of all inhibitors and then infected with 1 MOI RV1B for 24h (Figure 10).

In vivo animal model of allergic airway inflammation

The mouse is increasingly being used in the development of *in vivo* asthma models. One clear advantage is that it is a non-endangered species with a large number of genetically defined inbred strains available at a reasonable cost. Above all, this species makes it possible to apply *in vivo* a wide range of immunological tools, including gene deletion techniques. The use of "knock-out" mice offers clear advantages for analyzing the functional role of a given cell or mediator in a complex setting, such as allergen-induced airway inflammation and its link to changed airway behaviour [277]. HDM-induced allergic asthma mouse model is a reliable and reproducible one that exhibits eosinophilic inflammation of the airways, production of Th2 cytokines, the presence of IgE specific for HDM, remodelling of the airways, and bronchial hyperreactivity, which are all features that are present in human allergic asthma [278].

In paper I, HDM-induced allergic asthma mouse model was used to investigate the uric acid (UA) release *in vivo*. The mice received intranasal (i.n) administration of 25 μg HDM extract 3 days/week for 3 weeks to induce allergic airway inflammation. Mice received saline as control. The Animal Ethics Committee at Lund University approved all the experiments performed (Ethical number: M36-13). At 5 hours following the final HDM challenge, phosphate buffered saline (PBS) was used to perform bronchoalveolar lavage (BAL) of the lungs. Total protein, UA release and total cell counts were assessed in BAL (**Figure 10**).

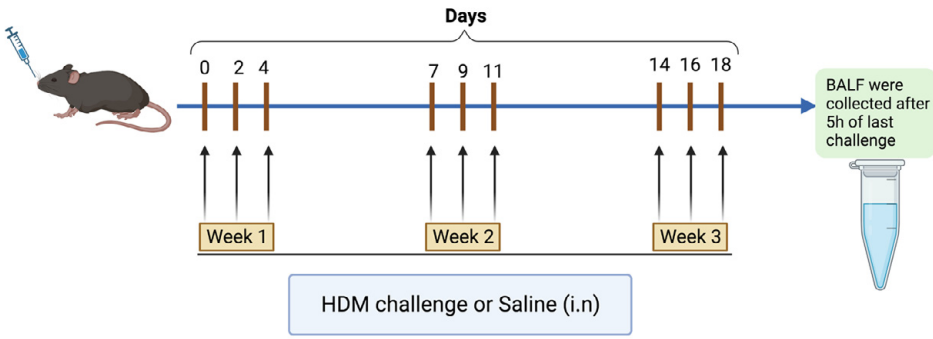

Figure 10: *In vivo* HDM-allergic asthma mouse model. Three weeks of challenges with either saline or HDM for 5 hours were given to wild type mice. Biorender 2022.

Assessing mRNA expression

All papers included in this thesis involve the technique Reverse transcription-quantification polymerase chain reaction (RT-qPCR) technique. This is one of a major technique to quantify the gene expression of our interest. In this method mRNA quantified by RT-qPCR can be either absolute or relative. Expression of target gene is normalised using a house keeping gene as a reference, which is stably expressed independent of tissue type, developmental stage, cell cycle state, or external signal. Within group comparisons were analysed according to the $^{\Delta\Delta}$Ct method by normalisation to the control sample [279].

Quantification of protein expression

Western Blot

In papers III and IV, the intracellular protein expression of IL-33 and PRRs was quantified using Western blot (WB). This is a commonly used analytical method for assessing protein expression. The WB method uses labelled antibodies to detect particular proteins that have been electrophoretically separated in a gel. WB can generate semi-quantitative and qualitative data on the target protein. It is a very useful method to use in cell and molecular biology. It enables to distinguish a single protein type among a mixture of proteins that have been isolated from cells. In addition, the protein size and

quantity can be determined. WB analysis was used in our experiments in order to detect and quantify the protein expression of IL-33, ZO-1, E-CDH and PRRs.

Extracellular protein measurements

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a method that enables sensitive and specific detection of soluble antibodies and antigens present in for example serum, BAL fluid or cell culture supernatants. In this thesis, extracellular release of IL-8, IL-33 and TSLP protein levels were measured in cell culture supernatants using commercially available sandwich ELISA 96-well microplates. All ELISA assays were performed according to the manufacturer's instructions with all the required reagents included in the kit (R&D Systems).

Luminex Multiplex ELISA

In paper, III, IV and V Luminex multiplex assay was used to measure the extracellular cytokine release in cell culture supernatants. Luminex multiplex assays use xMAP bead-based technology to simultaneously detect and quantify a variety of secreted proteins, such as cytokines, chemokines, and growth factors present in a sample. These beads are coded with fluorescent colour, which can be detected, measured and quantified. This technique was used in our experiments in order to detect multiple proteins or mediators released by the cell culture supernatants after different treatments. The multiple cytokine assay was performed according to the manufacturer instructions (R&D Systems, Abingdon, UK).

Metabolite DAMPs measurements

ATP measurements

To measure the ATP levels in cell culture supernatants, ATP Kit SL (BioThema luminescent assay, Handen, Sweden) was used. In whole thesis, the ATP levels were measured after 1h stimulation. The 40 μL samples were diluted in EDTA buffer and added to the 96 well plate. Then the equal amount of ATP reagent was added to the samples and measured light emission corresponding to sample ATP. Further, 10 μL of ATP standard was

added to the plate and the light emission was then measured. Very briefly, firefly luciferase catalyzes the ATP into Adenosine monophosphate (AMP), pyrophosphate (PPi), oxyluciferin, carbon dioxide (CO_2) and light and the light emission is measured fluorometrically.

$$\text{ATP} + \text{luciferin} + O_2 \longrightarrow \text{AMP} + \text{pyrophosphate} + \text{oxyluciferin} + CO_2 + \text{light}$$

Uric acid measurements

Amplex Red Uric Acid Assay Kit (Life Technologies, Eugene, Oregon, USA) was used to measure uric acid levels in cell culture supernatants. 50 µL of diluted samples, standard and control were added to separate wells of a microplate. Then the reaction was started by adding 50 µL of the Amplex red reagent/ horseradish peroxidase (HRP)/Uricase working solution to each microplate containing the samples, standards and controls. The microplate was incubated for 30 minutes at 37°C, while protected from light. The absorbance was measured in a microplate reader using absorbance at ~560 nm. The principle behind this assay is that uricase catalyzes the conversion of uric acid into allantoin, hydrogen peroxide (H_2O_2), and carbon dioxide. H_2O_2 reacts stoichiometrically with the Amplex Red reagent in the presence of HRP to produce the red fluorescent oxidation product resorufin, which is measured spectrophotometrically.

Summary of results and discussion

The studies included in this thesis have addressed allergic inflammation and innate immunity in asthma. Specifically, we studied if different allergens or allergic mediators induced alarmins and DAMPs release on both in an epithelial cell line and in HBECs from patients with allergic asthma and bronchial smooth muscle cells (BSMCs) from both asthma and healthy individuals. Additionally, we demonstrated that allergens and allergic mediator's exposure prior to viral infections affects the anti-viral and pro-inflammatory responses in bronchial epithelial cells. Further, we investigated RV-induced alarmin IL-33 expression and regulating mechanism in BSMCs from patients with asthma and healthy individuals. Finally, we hypothesized that HDM sublingual allergen immunotherapy (HDM-SLIT) improves the viral induced anti-viral IFN responses in HBECs from allergic asthma patients. In this section, the main results from the papers included in the thesis are summarized.

Different allergens and allergic mediators influences DAMPs, alarmins release and anti-viral responses in bronchial epithelial cells (**Paper I-III**)

Metabolite DAMPs and epithelial alarmins cytokines

Production of metabolite DAMPs release in HBECs after exposure to four different allergens

Alarmins such as extracellular ATP and UA have been implicated as important molecules in acute and chronic inflammation. Both UA and ATP levels were increased in asthma after allergen challenge in human and mice [280]. However, acute effects of different allergens or allergic mediators-induced alarmin release in HBECs were not examined. Hence, comparing

alarmin release after stimulation with different allergens and allergic mediators is of our interest.

The ability of four different allergens, HDM, *A. alternata*, *A. vulgaris* and *B. pendula* to induce metabolite DAMPs release in both bronchial epithelial cell line and primary HBECs from patients with allergic asthma were investigated in paper I and II. We demonstrated that, out of four different allergens, only HDM induced UA release in HBECs from both BEAS-2B cell line and in primary HBECs from patients with allergic asthma, as well as in BALF from our HDM-induced allergic mouse model. Interestingly, UA levels can be used as a biomarker for the severity of asthma exacerbations in patients since they are generated early in the immune response to protease allergens [145, 281]. It has been shown that human airway epithelial cells produce UA on a regular basis because epithelial cells have an active UA transport mechanism [282]. There is an evidence that Ormdl3 KO mice exhibit reduced UA production into the airways in response to *A. alternata* [144]. However, in BEAS-2B and primary HBECs exposed to *A. alternata*, we did not observe any UA release. A previous study demonstrated that exposure to HDM allergen enhances UA production from HBECs and that asthmatics had enhanced intrinsic UA synthesis [283]. Here we found that only HDM, when compared to other allergens used in this investigation, caused UA release both in BEAS-2B and primary HBECs from allergic asthma patients. However, additional research is required to fully understand the HDM-induced UA releasing pathway and its distinct function in comparison to other allergens in HBECs.

Nasal and bronchial human epithelial cells exposed to *A. alternata* cause rapid extracellular ATP release [284]. Inhibiting the P2 purinergic pathway or neutralizing ATP reduces the release of IL-33 and early innate Th2 immune responses to an allergen. These results indicate that extracellular ATP is crucial for different phases of T2 immune responses and inflammation in the airways [167]. Previous study showed that HDM allergens induced immediate release of a high quantity of ATP, which rapidly stimulates the release of Ca^{2+} from the endoplasmic reticulum by activating extracellular P2Y purinergic receptor signalling and allows activated keratinocytes to release IL-33 [285]. In our investigations, ATP release was observed in BEAS-2B cell line and was induced by all four allergens in a dose-dependent manner (Paper I) compared to control. Similarly, Suzuki et al., demonstrated that the aeroallergens HDM and OVA stimulated the release of ATP from human bronchial epithelial cell line 16-HBE14o- and

primary mouse trachea epithelial cells [286]. Further, compared to unstimulated cells, only birch pollen significantly increased the release of ATP in HBECs from allergic asthma patients, while HDM, *A. alteranta*, and *A. vulgaris* had no effect (Paper II). An earlier investigation revealed that treatment with *Alternaria* extract caused release of ATP that started about two minutes after addition to the apical surface of HBECs [284]. These results suggest that it is possible that we failed to assess ATP release in primary HBECs in response to *A. alternata* at the early time points. Collectively, airway epithelial cells are a source of extracellular ATP and may enhance the development of allergic asthma after exposure to inhaled aeroallergens.

Release of the metabolite DAMPs in HBECs is significantly influenced by allergen serine proteases

Proteases present in many allergens contribute to the pathogenesis of atopic asthma [113]. Proteolytic enzymes from allergens can directly activate AECs and hereby promote T2 inflammation via releasing numerous mediators [287]. Allergen proteases cause allergic airway inflammation and have been linked to the exacerbation of allergic responses due to their protease activity [184]. It has been suggested that the development of allergic Th2 responses is influenced by allergen protease activity [122]. Thus, addressing different allergens and their proteolytic activity with certain inhibitors may reduce inflammation caused by proteases.

In paper I, we wanted to investigate if allergen protease has any role in induction of metabolite DAMPs release in HBECs. Allergen extracts were incubated with cysteine (E64) or serine (AEBSF) protease inhibitors for 30 minutes then the HBECs were stimulated for 1, 3 and 24 hours. Our data showed that allergen-induced ATP and UA were largely reduced upon heat-inactivation of the allergens and blocking allergens protease activity with serine protease inhibitors significantly reduced allergens induced metabolite DAMPs in HBECs (**Figure 11**). Strikingly HDM-induced HBECs release of UA was completely blocked by serine protease inhibitors but not with cysteine protease inhibitor. A previous study revealed that the cysteine proteases, papain and bromelain can cause the release of UA in the airway lumen from mice [145]. However, in our investigation, HDM-induced UA release in HBECs was unaffected by cysteine protease inhibitor. Moreover, a study by Kale et al. discovered higher amounts of UA in the BALF of mice in response to active serine proteases like Per 10 compared to mice received inactive Per 10 and phosphate-buffered saline (PBS) [288]. These

findings shows that allergen extracts can increase airway inflammation through protease-dependent and protease-independent pathways in addition to their IgE-binding activities [113]. Altogether, targeting both exogenous (allergen proteases) and endogenous proteases such as tryptase and chymase may be an important for future therapies in allergic diseases.

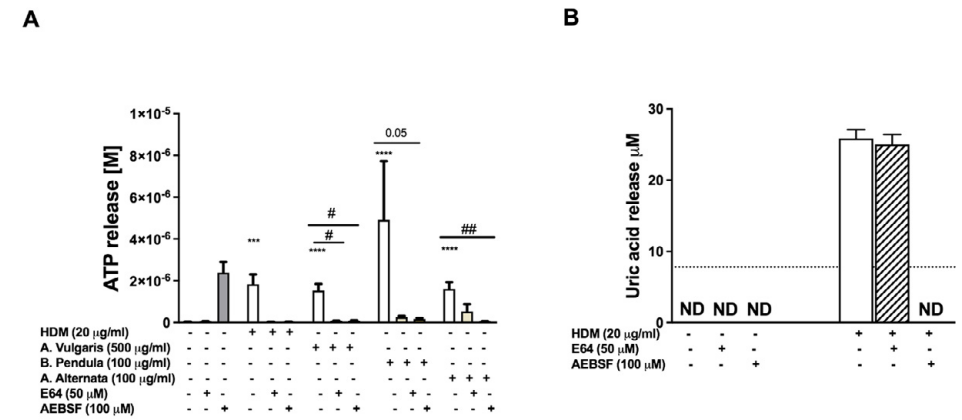

Figure 11: Serine protease inhibitor reduced metabolite DAMPs release in HBECs**.** HBECs were stimulated with an optimal concentration of HDM, *A. vulgaris*, *B. pendula* and *A. alternata* in absence and presence of AEBSF (100 µM; serine-protease inhibitor) or E64 (50 µM; cysteine-protease inhibitor). ATP was assayed in the supernatant at 1h (**A**), UA release was measured at 24h (**B**). ND (non-Detected). Data is presented as mean ± SEM, n=5-12 from 3-6 independent experiments. *P<0.05, ***P<0.001, ****P<0.0001 compared to respective control and #P<0.05, ##P<0.01 compared to allergen extracts alone and with inhibitors.

Mast cell proteases tryptase and chymase induced alarmin ATP release in HBECs

The pathogenesis of asthma is also greatly influenced by extracellular endogenous and exogenous proteases [289]. Tryptase and chymase, are endogenous serine proteases that are prevalent in mast cell granules, and strong activators of the PAR-2 [290]. In addition, a large number of aeroallergens related to asthma, including fungi and HDM, are exogenous proteases. Derp 3 and Derp 9 are two important HDM proteases that interact with PAR-2 to increase vascular permeability and detach epithelial cells [291]. In an animal model of chronic asthma, serine protease inhibitors reduced allergic airway remodelling, allergic airway inflammation, and AHR [289].

The finding that the allergen serine protease was the main cause of alarmin production in bronchial epithelial cells in paper I led to our investigation into

the impact of the MCs serine proteases tryptase and chymase on HBECs. Tryptase and chymase are the most prevalent MCs serine proteases, and both of these proteases have a number of effects on structural cells that contribute to airway remodelling and inflammation [216]. As was previously mentioned, within the airway epithelium, MCs are ideally situated to react on aeroallergens and pathogens and can promote an inflammatory response in the airways. Though many studies have been conducted on the involvement of MCs in the pathogenesis of airway diseases, whether the direct effect of MC serine protease tryptase and chymase on bronchial epithelial cells was unclear. In paper III, we wanted to investigate the direct effect of tryptase and chymase on inducing release of metabolite DAMPs, ATP and UA, in HBECs. Both BEAS-2B and primary HBECs were treated with two different doses of tryptase and chymase for 1 hour. We found that similar to allergens, both tryptase and chymase significantly increased extracellular release of ATP after 1 hour of stimulation in primary HBECs and BEAS-2B (**Figure 12**). An earlier study using HBECs demonstrated that protease treatment increased reactive oxygen species (ROS) and oxidant damage [292], and that oxidative stress could promote the release of ATP from primary pulmonary endothelial and epithelial cells [293]. UA levels were undetectable in both primary HBECs and cell lines stimulated with tryptase and chymase. This finding implies that MC proteases can result in epithelial stress, which triggers the release of the alarmin ATP from HBECs similar to allergens.

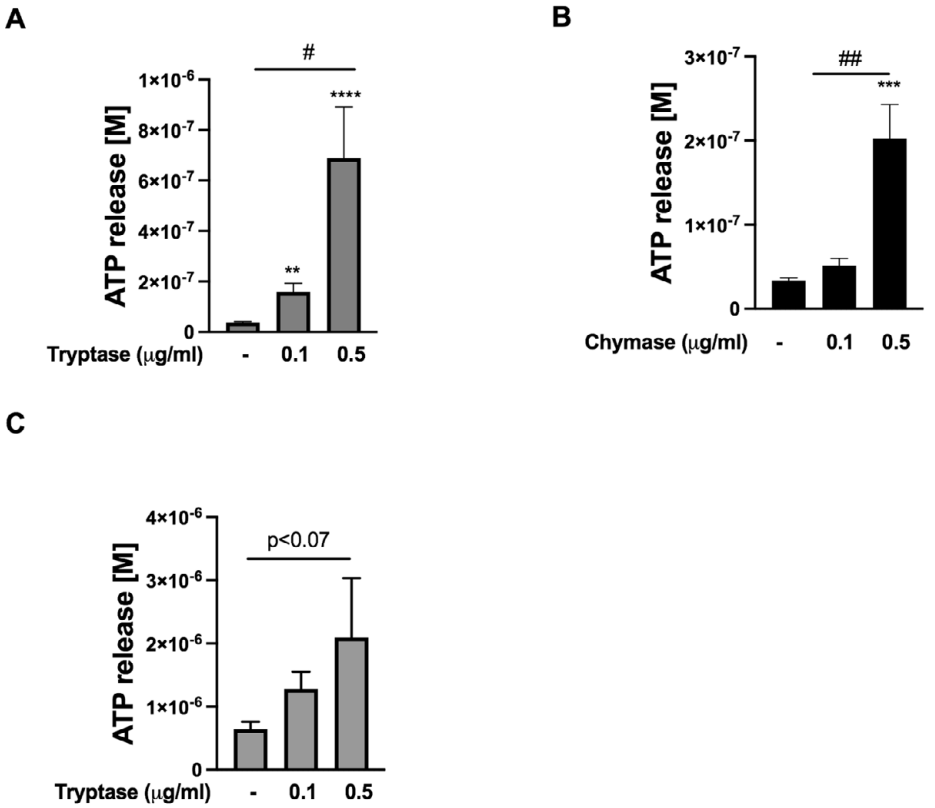

Figure 12: Mast cell proteases tryptase and chymase induced ATP release in both BEAS-2B and primary HBECs. BEAS-2B cells were treated with two different doses of tryptase and chymase and ATP levels were measured in cell culture supernatants after 1h. (**A**) Trypatse in BEAS-2B, (**B**) Chymase in BEAS-2B and (**C**) Tryptase in primary HBECs (n=3). Data are presented ±SEM. Figure adapted from paper III.

HDM and A. vulgaris induced IL-33 and TSLP expression in HBECs from patients with allergic asthma

Alarmins produced by bronchial epithelial cells, including IL-33, TSLP, and IL-25, can regulate both innate and adaptive immune responses [38]. In paper II, we investigated the epithelial alarmin cytokines release in response to four different allergens in primary HBECs from patients with allergic asthma. We demonstrated that HDM and *A. vulgaris* significantly increased TSLP and IL-33 mRNA expression at 3 hours in HBECs, whereas *A. alternata* and *B. pendula* do not cause any expression of IL-33 or TSLP. Further, HDM demonstrated both early (3 hours) and late (24 hours) release of IL-33 and TSLP protein and *A. vulgaris* showed only early protein release in HBECs

from patients with allergic asthma. Similar to IL-33 and TSLP gene expression we did not observe any release of IL-33 and TSLP in HBECs exposed to *A. alternata* and *B. pendula* (**Figure 13**).

In asthmatics, allergen exposure dramatically raised the production of alarmin cytokines, TSLP, IL-33, and IL-25, in the bronchial mucosa. This phenomenon was directly connected to the degree of increased airway obstruction after exposure [294]. Previous studies have demonstrated a role of *A. alternata* in triggering PAR-2-mediated release of TSLP [295] from bronchial epithelial cells, as well as release of IL-33 [167] and IL-25 [296]. However, similar to our findings, other studies have similarly failed to detect any IL-33 or TSLP release from either mouse lung epithelial cells treated to *Aspergillus fumigatus* [297] or HBECs exposed to Alternaria [298]. The timeframe and the various epithelial cells studied may be responsible for the discrepancies. Notably, previous study showed that the Per a 10, a major cockroach allergen with serine protease activity increased IL-33 and TSLP release in BEAS-2B [288]. In contrast to other allergens, HDM allergen contains a number of proteases that have potent proteolytic activity [299] that may contribute to the release of these alarmins in HBECs. The ability of HDM and *A. vulgaris* to cause the release of IL-33 and TSLP from bronchial epithelial cells supports the clinical importance of these aeroallergens in causing allergic airway inflammation in asthma [85, 300]. We were unable to detect IL-25 in our system, either for genes or proteins. Understanding the variations in epithelial alarmin release by the different allergens examined in this study warrants additional research.

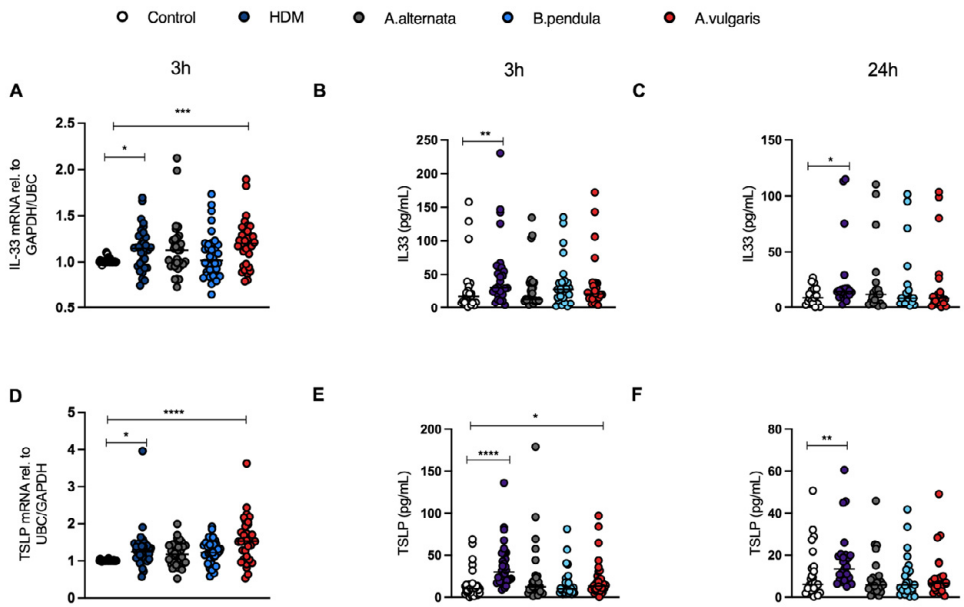

Figure 13: Different allergens induced epithelial alarmin cytokines response in HBECs from pateints with allergic asthma. HBECs were exposed to HDM, *A. alternata, B. pendula* and *A. vulagris* for 3 hours. Gene expression was measured by RT-qPCR and the protein release was measured by ELISA. **A**) IL-33 mRNA expression, **B**) IL-33 protein release at 3h, **C**) IL-33 protein lease at 24h, **D**) TSLP mRNA expression, **E**) TSLP protien release at 3h and **F**) TSLP protein release at 24h. Data were presented with median. Significant differences were defined as $p<0.05$. (Adapted from paper II).

Anti-viral and pro-inflammatory cytokines response

Both the development of asthma and acute asthma exacerbations are influenced by synergistic interactions between viral infections and allergic inflammation [301]. Interplay between allergen sensitization and viral infections have been related to the onset of asthma as well as exacerbations of pre-existing asthma [243]. Allergen exposure combined with viral infections enhanced the incidence of hospitalization [85]. However, little is known about how viral infection and aeroallergen exposure or allergic mediators interact to influence the pro-inflammatory and antiviral epithelial response in HBECs from patients with allergic asthma.

Different allergens and mast cell serine proteases modulate pro-inflammatory cytokine response in HBECs

Patients with atopic asthma had higher levels of pro-inflammatory cytokines than those with non-atopic asthma [302]. Epithelial cells release of pro-inflammatory cytokines and play a major role in inflammatory processes.

Increased cytokine release has a significant impact on allergic asthma development, according to previous studies [303]. Neutrophil inflammation may have a significant pathogenic role in those with severe asthma, and IL-8 has been associated with regulating neutrophilic inflammation [304]. IL-8 has been found to have a crucial role in the development of asthma by promoting airway remodelling and AHR [305, 306]. Our findings demonstrate that airway epithelial cells engage in an interaction with different allergens such as HDM, *A. alternata*, *B. pendula* and *A. vulgaris* that causes the production of IL-8. Moreover, serine protease inhibitors significantly reduced the allergens induced IL-8 gene expression (Paper I). Data suggested that the majority of common allergens that triggers asthma have intrinsic protease activities that contribute to sensitization and development of asthma [126, 307, 308].

In asthma, the pro-inflammatory cytokines IL-8, TNF-α, and IL-6 are crucial in controlling neutrophilic inflammation [309]. Neutrophils could produce immunopathology if these cytokines are not tightly controlled, which is necessary to maintain immunological tolerance. Pro-inflammatory cytokines that are overexpressed may cause inflammation in airways. Our recent study showed that pre-exposure to HDM reduced poly(I:C) induced IL-8, TNF-α and beta-defensin in HBECs and this can lead to secondary bacterial infections [249]. In paper II, we wanted to investigate whether different allergens exposure prior to viral infection modulate pro-inflammatory cytokine release in HBECs. We found that pre-treatment with *A. alternata* and *A. vulgaris* further increased the poly(I:C) induced IL-8 while HDM inhibited the gene expression of IL-8 caused by poly(I:C). *B. pendula* had no impact on the expression of IL-8 that is induced by poly(I:C). Fungal proteases have been linked to the production of pro-inflammatory cytokines and previous study demonstrated that these proteases were responsible for the enhancement of virus-induced cytokine productions by *A. alternata [126]*. The further induction of poly(I:C)-induced IL-8 expression in HBECs by *A. alternata* and *A. vulgaris* suggests that the interaction between allergens and viral infection may worsen symptoms by enhancing the inflammatory responses in the asthmatic airways.

Tryptase has been demonstrated to promote the production of the pro-inflammatory cytokine IL-8 by bronchial epithelial cells [310]. Consistence with the previous findings we also showed that the mast cell proteases tryptase and chymase induced the pro-inflammatory cytokine IL-8 and IL-6 in HBECs. Furthermore, we showed that MC proteases contributed to the

enhancement of poly (I:C)-induced IL-8 release in HBECs. This result is supported by Zhu et al., which showed that allergen proteases had a significant role in enhancing virus-induced cytokine production in bronchial epithelial cells [126]. According to our research, interactions between MC proteases and viral infections may increase the inflammatory response in asthmatic airways, particularly during a viral-induced acute exacerbation phase.

Viral induced IFN-β decreased by HDM and tryptase but further enhanced by A. alternata and B. pendula

Interferon secretion serves as the initial step in the clearance of viral infections, and it has been hypothesized that their underproduction causes viral-induced exacerbations [238, 239, 311]. Patients with asthma show severe deficits in the production of antiviral interferon after viral infections in their bronchial epithelial cells [239]. In a previous study, we demonstrated that TLR3 was involved in the regulation of HDM impairment of anti-viral IFN-β in both bronchial epithelial cells line and from asthma patients [248]. Asthma HBECs from both HDM+ and HDM- patients were exposed to HDM led to a dampening of Poly(I:C)-induced IFN-β production [249].

In paper II, we intended to study the impact of different allergens on the viral induced epithelial anti-viral response in HBECs from allergic asthma patients in order to further extend our prior findings with HDM. To study the different allergens effect, HBECs were pre-exposed to HDM, *A. alternata, B. pendula*, and *A. vulgaris* for 24 hours followed by stimulation with poly(I:C) or infected with RV1B for 24 hours. Our findings demonstrated that, in contrast to other allergens, HDM pre-exposure reduced poly(I:C) and RV1B triggered IFN-β in HBECs. In contrast to HDM, *B. pendula* and *A. alternata* exposure prior to poly(I:C) stimulation showed a tendency to further increase IFN-β gene expression in HBECs. A recent study by Gilles et al., reveals that pollen allergens play a function in the regulation of respiratory epithelial mediated antiviral immunity. Authors found that exposure to pollen during viral infections lowered the phosphorylation and translocation of interferon-related genes, which in turn reduced the release of pro-inflammatory chemokines and type I and III interferons [312]. Further, Zhu et al., reported a decreased innate immune response to respiratory viral infections after *A. alternata* exposure [126]. Among the blood immune cells of patients with birch allergies, an increased antiviral response associated with Bet v 1 stimulation was observed in a recent study [313]. The same authors also reported that seasonal exposure to environmental stimuli

generates an enhanced antiviral response in respiratory epithelia in patients with seasonal allergic rhinitis using proteomics [314]. One way that HDM decreased IFN-β and inflammatory cytokines may have contributed to secondary bacterial infections [249], while another way is that an overactive antiviral response to other allergens may have enhanced inflammatory responses that contribute to the disease phenotype. Overall, we found that different allergens affected HBECs differently in response to poly(I:C) stimulation. However, elucidating the exact mechanisms of allergens interactions with poly(I:C) or RV infection in HBECs requires further investigations.

Omalizumab, an anti-IgE treatment, has been shown in clinical trials to reduce asthma exacerbations and enhance quality of life in asthmatic patients [261-263], suggesting a link between inhibiting MC activation and increased antiviral capacity. Omalizumab treatment for asthmatic children has been demonstrated to lower exacerbation rates and increase levels of RV-induced IFN-α compared to placebo [315]. These finding indicate that MCs and MC proteases may have important role in the immune defence to reparatory viral infections. Our work in paper III showed that pre-treatment of BEAS-2B cells with tryptase, but not chymase, impaired poly(I:C) induced IFN-β mRNA expression in HBECs at 24h. We also found that reduced expression of IFN-β is partially associated with reduced expression of PRRs. Moreover, when tryptase protease activities were blocked by serine protease inhibitors, the expression of IFN-β and PRRs in HBECs was restored. Viral infections can directly or indirectly activate MCs to release histamine and serine proteases such as tryptase and chymase [316]. In our investigation, we showed that tryptase reduces poly(I:C)-induced HBECs production of IFN-β, while a more recent study also showed that histamine reduces viral induced airway epithelial cells production of IFNs [169]. These results imply that MC mediators reduced epithelial IFNs may enhance Th2 inflammation in the airways [317]. Our findings illustrated a mechanism by which tryptase reduced poly(I:C)-induced IFN-β by reducing PRRs expression. These factors collectively provide an explanation for how activated MCs and their granule proteases within the airway epithelium may change the innate immune response to viral infections.

Epithelial barrier proteins

Effects of Tryptase and chymase on epithelial barrier proteins

The airway epithelial barrier protects the lung from innate immune system from many external factors. Increased epithelium permeability, sub-epithelial damages, and tissue remodelling are all potential consequences of altered barrier function, which can also cause epithelial cell death, stress, or detachment from the basement membrane [318]. According to recent studies, the severity of asthma was correlated with an increase in epithelial barrier disruption [319]. Previous investigations have shown significant evidence that the proteases from allergens cause damage to the airway epithelial cells and increased permeability [113]. We intended to evaluate the expression of the tight junctional proteins E-cadherin and ZO-1, which are important components of the airway epithelial barrier, in HBECs in response to tryptase and chymase. In our investigation, tryptase and chymase both significantly reduced the expression of the proteins ZO-1 and E-cadherin. This finding is consistent with Xiaoying Zhou et al., [320] who demonstrated using immunostaining that tight junction proteins were disrupted by chymase in 16HBE cells [321]. These findings were further validated by gene expression, which revealed that tryptase but not chymase reduced the mRNA expression of ZO-1 and E-cadherin. These results suggest that an increase in MCs and their activation within the epithelium can damage the epithelial barrier functions by degrading cell junctional proteins. Further studies are required to investigate how the expression of epithelial barrier proteins is influenced by different allergens and MC proteases in HBECs cultured at the air-liquid interface (ALI).

TLR3/TAK1 signalling regulates rhinovirus-induced IL-33 in bronchial smooth muscle cells (**Paper IV**)

Not only HBECs, but BSMCs have also been shown to produce a variety of pro-inflammatory cytokines and alarmins *in vitro* [47]. Increased IL-33 expression in asthmatic ASM cells suggests that ASM cells may be a key source of IL-33 during inflammatory conditions [185, 187]. Previous studies have shown that human bronchial smooth muscle cells (BSMCs) express TLRs and release cytokines and chemokines in response to TLR agonists [322]. We recently demonstrated that RV infects BSMCs, which leads to the production of interferons and IL-33 [48]. However, it is unknown which signalling pathways are involved in the regulating mechanisms that cause RV to stimulate IL-33 production in BSMCs.

In paper IV, we compared the levels of IL-33 at baseline and RV-induced IL-33 gene expression in BSMCs from healthy controls, severe and non-severe asthmatics (NSA). We demonstrated that BSMCs from NSA express more IL-33 at baseline than healthy individuals. This result lends some relevance to a previous study by Prefontaine et al., which demonstrated that moderate asthmatics had higher levels of IL-33 in their bronchial epithelial cells than healthy controls [177]. Next, we investigated the RV-induced IL-33 expression in BSMCs from healthy, NSA and severe asthma. We showed that RV-induced IL-33 gene and protein are overexpressed in BSMCs from patients with NSA compared to healthy and severe asthma (**Figure 14**). Our findings suggest that even after RV infection, SA showed a lower induction of IL-33 than NSA. Chang et al., who also showed that BSMCs from SA patients, the majority of whom underwent maintenance treatment with oral steroids, produced less CCL11 in response to TNF-α than NSA patients [323]. Dexamethasone has been demonstrated to effectively block RV-induced IL-33 in BSMCs [48], suggesting that the decreased expression in SA BSMCs may be caused by increasing corticosteroid treatment.

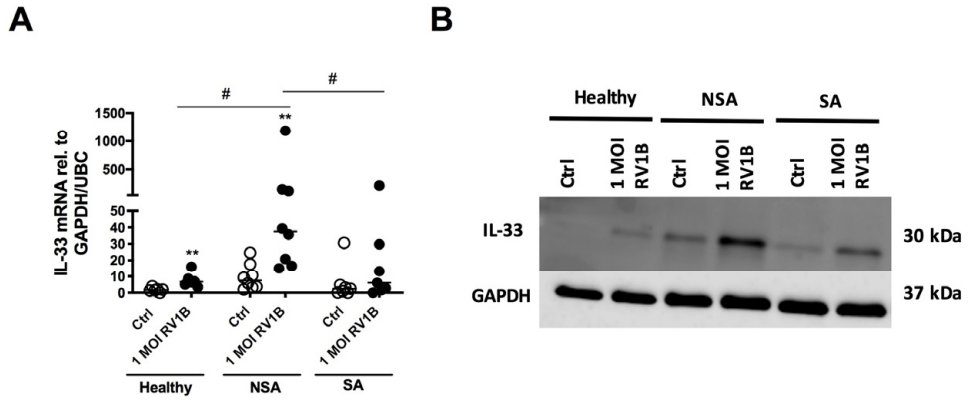

Figure 14: BSMCs from healthy and asthmatic subjects were infected with 1 MOI RV1B. (**A**) Gene expression of IL-33 and (**B**) representative western blot of IL-33 protein expression. Significant differences were defined as $p<0.05$ (Adapted from paper IV).

To further gain insight into the molecular mechanism underlying the RV-induced IL-33 in BSMCs, we studied the PRRs involvement of PRRs and the downstream signalling pathways. Our earlier study showed that activation of TLR3 by poly(I:C) and RIG-I-like receptors by poly(I:C)/lyovec, as well as RV infection, elevated the basal expression of IL-33 in BSMCs from both asthmatic patients and healthy controls [48]. This finding encourages additional investigation into the role of TLR3 downstream pathways in relation to RV-induced IL-33 in BSMCs. RV has been reported to activate not only TLR3, but also the RIG-I like receptors in BSMCs [49]. Using specific ligands and knockdown approaches, we confirmed the role of TLR3 in the regulation of IL-33. However, based on our data RIG-I and MDA5 appear not to be involved in RV-induced IL-33 expression in BSMCs.

Notably, we further showed that TAK1, but not NF-kB or TBK1, was implicated in RV infection-induced production of IL-33 in BSMCs by using known inhibitors of molecules likely engaged in downstream signalling cascades (**Figure 15)**. An earlier study showed that TAK1 is essential for the development of airway inflammation. [324, 325]. TAK1 has been shown to activate NF-kB and MAPK pathways, resulting in production of pro-inflammatory cytokines [326] and in airway remodelling through the enhancement of growth factor induced proliferation of airway smooth muscle cells [327]. Here we reported that RV-induced IL-33 expression in BSMCs is mediated by TAK1, suggesting that TAK1 is a novel therapeutic target for viral-induced airway inflammation.

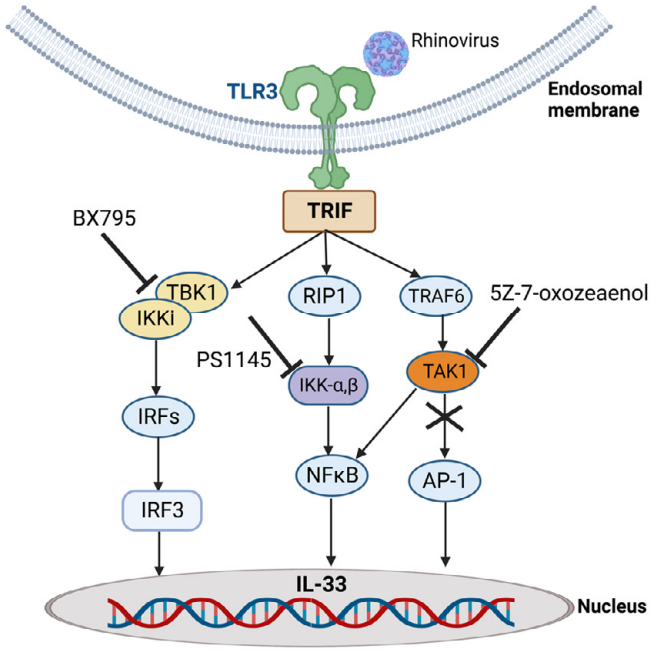

Figure 15: Proposed signalling mechanism involved in rhinovirus-induced IL-33 expression in bronchial smooth muscle cells (BSMCs). Recognition of RV1B by Toll-like receptor 3 (TLR3) leads to the recruitment of adaptor molecules that activate transforming growth factor (TGF)-β-activated kinase 1 (TAK1). Activation of TAK1 results in the activation of transcription factors that increase rhinovirus (RV)-induced IL-33 expression in BSMCs. (Adapted from paper IV). Biorender 2022.

The effect of HDM-SLIT on epithelial antiviral immunity in patients with allergic asthma (VITAL) (**Paper V**)

Allergen immunotherapy (AIT) causes differential immunomodulation that affects both the innate and adaptive immune systems [328]. AIT has been found to effectively prevent asthma and the development of new sensitizations in patients with allergic asthma [329]. HDM-SLIT has been shown to reduce AHR and improve symptoms in HDM-sensitized asthma patients [330]. Clinical studies have shown that AIT can lower exacerbation rates in patients with asthma, indicating that AIT may have an impact on antiviral responses [266]. Yet, the mechanisms of detrimental allergen-virus interactions [126, 248, 249, 331] and the function of AIT in their prevention in asthma have only just begun to be elucidated. However, it was unknown

if clinical AIT could have altered the epithelial anti-viral and inflammatory response in cultured HBECs from patients with allergic asthma.

In paper V, we investigated how HDM-SLIT affected epithelial anti-viral response and inflammatory cytokines in HBECs from patients with allergic asthma. HBECs from both placebo and AIT group were stimulated with poly(I:C) for 24 hours before and after treatment. Our results demonstrated that 24 weeks of treatment with HDM-SLIT significantly enhance the poly(I:C) induced bronchial epithelial production of IFN-β. The preventative effect of AIT reported on exacerbation and lower respiratory tract infections needing antibiotics, our results are in line with recent findings demonstrating an increased resistance to viral airway infections [270, 332]. On the other hand, the gene expression of IL-33 reduced in the cells stimulated with the poly(I:C) after HDM-SLIT compared to placebo (**Figure 16**). The decreased IL-33 response is consistent with earlier research showing that AIT reduces IL-33 and IL-25 levels to alleviate airway inflammation [333, 334]. We report a potential HDM-SLIT-induced alteration of the innate antiviral immune defense in the bronchial epithelium. We report novel insights suggesting that 24 weeks of HDM-SLIT increase bronchial epithelial antiviral type I and type III IFNs while decreasing epithelial alarmin cytokine IL-33.

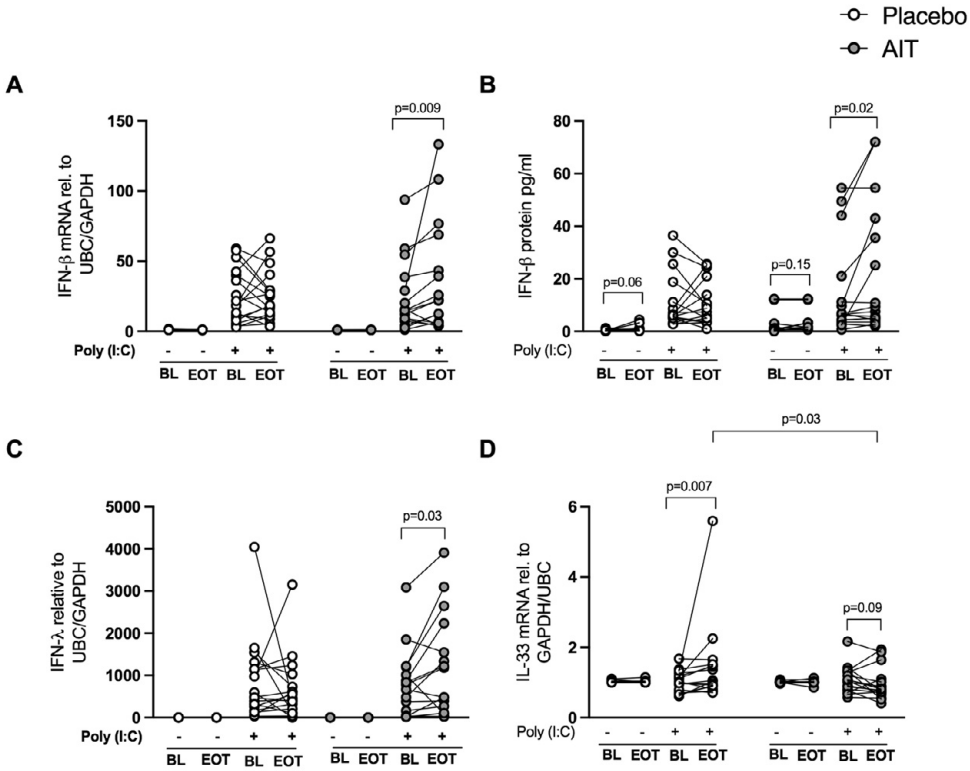

Figure 16: HDM-SLIT increased Poly(I:C) induced HBECs IFNs expression in patients with allergic asthma. EOT: End of treatment (week-24); BL: Baseline. HBECs from patients with allergic asthma were stimulated with 10 µg/ml Poly(I:C) for 24h. IFNs and IL-33 mRNA expression was quantified by RT-qPCR and protein release was measured by multiplex ELISA. (**A**) Gene expression of IFN-β, (**B**) protein releae of IFN-β, (**C**) gene expression of IFN-λ and (**D**) gene expression of IL-33. Placebo (n=17) and AIT (n=17). Significant differences were defined as $p<0.05$. (Adapted from paper V).

AIT reduces epithelial T2 inflammation, including IL-24 and CCL-26, as well as a decrease in inflammatory genes such as IL-8 [335]. Previous studies showed that, the AIT inhibits the development of Th2 cells and the production of Th2 cytokines, such as IL-4 and IL-5 [336-338]. After AIT, peripheral blood mononuclear cells (PBMCs) from HDM-sensitized allergic asthma patients produced less IL-5 and IL-13 [339], as well as thymus and activation-regulated chemokines in response to allergens [340]. AIT can therefore enhance the production of IL-10, TGF-beta, and IL-35 by regulatory T cells (Tregs) and inducible Tregs, which reduces the accumulation of Th2 cells in the airways [341]. Further, in our study we wanted to investigate whether 24 weeks of HDM-SLIT alter Th2 cytokine release in HBECs. We found that the 24 weeks of treatment with HDM-SLIT

dose not altered Th2 cytokines profile or no change in IL-10 within the treatment group or compared to placebo. However, a multiple factor, such as the nature of the allergen, the time of assessment, and the method of evaluation, may influence the contribution of Tregs in AIT to the suppression of Th2-type immune responses [342].

Conclusions

Based on the results obtained in papers I-V we can conclude the following

- Different aeroallergens caused the release of the metabolite DAMPs and the pro-inflammatory cytokine IL-8 in HBECs and this release was reliant on allergen serine protease activity. However, HDM stand out from other allergens by causing UA release in HBECs also in our HDM-induced allergic mouse model of asthma. Further, the bronchial epithelial alarmins IL-33 and TSLP were mostly produced by HDM and *A. vulgaris* in HBECs from patients with allergic asthma. Additionally, we demonstrated that pre-exposure with HDM for 24 hours decreases poly(I:C)-induced IFN-β and IL-8 expression, whereas *A. alternata* and *A. vulgaris* increase poly(I:C)-induced IL-8 expression in HBECs from patients with allergic asthma. The current investigation compared the ability of four different allergens in affecting on human bronchial epithelial cells, indicating a specific potential of HDM to elicit the production of UA and epithelial alarmins cytokines IL-33 and TSLP, as well as an impaired anti-viral response.
- MC proteases tryptase and chymase regulate the secretion of alarmin ATP and pro-inflammatory cytokines in HBECs. Moreover, both proteases may promote loss of epithelial integrity, which can lead to tissue remodelling, by affecting expression of tight junctional molecules. Additionally, we demonstrated that pre-treatment with tryptase impaired anti-viral proteins and PRRs expression in HBECs, and this effect was dependent on the protease activity of tryptase. These results may partly give a mechanistic explanation why atopic asthmatic patients are more susceptible to viral infections and more prone to viral-induced exacerbations [343].
- RV-induced IL-33 expression was higher in BSMCs from non-severe asthma patients than patients with severe asthma and healthy controls. Further, we report a novel mechanism of RV-induced IL-33

expression through a TLR3/TAK1 signalling pathway. These signalling molecules may contribute to the pathogenesis of viral-induced airway inflammation in asthma and may provide potential therapeutic targets for treating viral-induced asthma exacerbations.

- HDM-SLIT significantly boosted bronchial epithelial antiviral IFN-β and IFN-λ, but decreased IL-33 expression. These findings show that allergen immunotherapy improves antiviral immunity in allergic asthma patients at the level of the bronchial epithelium. Allergen immunotherapy may be a relevant potential future therapeutic strategy for viral induced asthma exacerbations. However, the mechanisms underlying the impairment and restoration of antiviral responses in patients with allergic asthma and the use of allergen immunotherapy need more investigation.

Future perspectives

Investigate the epithelial barrier function using air liquid interface (ALI) culture system

The epithelial barrier component is damaged or pathologically changed in a number of acute and chronic respiratory diseases including asthma. In asthma, the epithelial barrier is crucial for maintaining immunological activity at the epithelial site [37]. The air liquid interface (ALI) culture model enables HBECs to differentiate into a polarised epithelium with ciliated and mucous-producing cells that is more resembling of what happens *in vivo*. Therefore, it would be interesting to use this differentiated HBECs model in upcoming mechanistic studies of different allergens and viral-induced epithelial responses in health and disease.

The epithelial barrier homeostasis balance is disrupted in allergic asthma, which is characterized by loss of differentiation and diminished junctional integrity [344]. It has been demonstrated that sublingual allergen immunotherapy improves allergic asthmatic patient's airway wall thickness and remodelling [345]. A previous study found that specific allergen immunotherapy efficacy was associated with reduced ER stress and epithelial barrier dysfunction by lowering the expression of the epithelial cytokine IL-25, in mice [333]. In the future, we want to investigate whether 24 weeks of HDM-SLIT treatment could alter the HBECs epithelial barrier functions such as epithelial integrity and functionality. To do this, ALI cultures will be used to evaluate the expression of epithelial tight junctional proteins and trans-epithelial electric resistance (TEER) in HBECs before and after allergen immunotherapy using dextran permeability measurements.

The primary constituents of the airway epithelial barrier are tight junction proteins such as ZO-1 and E-cadherin [346]. In paper III, we investigated BEAS-2B expression of tight junctional proteins in response to MC proteases. There is disagreement about whether immortalized epithelial cell lines are the best method for examining the functions of the epithelial barrier. Thus, in further research, we intend to use ALI cultures of HBECs to examine

the expression of the barrier proteins in response to stimulation by MC proteases and different allergens. We are currently working to optimize the primary HBECs in ALI cultures from various asthma phenotypes.

Understanding the molecular mechanisms of allergen-induced alarmin release and anti-viral response

In papers I and II, we compared the four distinct allergens effects on UA release from primary HBECs from allergic asthma patients and bronchial epithelial cell lines as well as in our HDM-induced allergic mouse model of asthma. We discovered that only HDM was responsible for the release of UA in HBECs and BEAS-2B as well as in our HDM-induced mouse model of allergic asthma. Furthermore, we found that only pre-exposure to HDM lowered the virally produced IFNs response when compared to pre-exposure to mugwort, pollen, and fungal allergens. The extracellular nucleotides ATP and UTP can suppress the virally generated IFNs in HBECs, according to a recent study [169]. In future, we wanted to investigate whether the HDM-induced UA release affects the viral induced IFN response in HBECs. In both healthy and asthmatic HBECs, it will be interesting to see whether HDM-induced UA and decreased viral-induced IFNs response are related in any way. Another important aspect also need to address that all patients with allergic asthma used in paper II were allergic to HDM. Further, to determine if the lower IFN response to HDM was caused by an allergic background or if HDM interacted with bronchial epithelial cells differently than other allergens.

In paper V, we showed that HDM-SLIT increased the viral induced IFN-beta response in cultured HBECs from patients with allergic asthma. We and other have showed that allergens exposure prior to viral infections impaired IFNs response in HBECs were regulated through PRRs [126, 248, 249, 312]. Continuing from paper V, we will use the stored cDNA from the VITAL study to assess the change and expression in PRRs and transcription factors resulting in improved antiviral responses in HBECs before and after AIT.

It is also important to address in future studies by using specific allergen components from different allergens. Specifically, we wanted to use specific HDM components of *Dermatophagoides pteronissinus* with protease activity (Der p 1) and unknown enzymatic activity (Der p 2, Der p 5) to

elucidate exact mechanism of interaction of these HDM components with HBECs from asthma and healthy controls.

Mast cell protease and histamine effect on bronchial smooth muscle cells

The number of MCs present on allergic asthmatic human airway smooth muscle cells is higher than on non-allergic and healthy individuals [347, 348]. Due to their interaction with the smooth muscle in the airways, MCs are probably important players in affecting the bronchial smooth muscle tone and thus bronchial hyperresponsiveness. Mast cells infiltrate the airway smooth muscle bundles in asthma, where they engage in interactions with the smooth muscle cells. Studies have shown that, in situ and in co-culture with airway smooth muscle cells, MCs are constantly activated. This activation can cause MC degranulation and the release of T2 cytokines, which can further enhance epithelial driven inflammation and successive bronchoconstriction [349]. In the future, we wanted to investigate the effect of mast cell mediators (tryptase, chymase and histamine) on alarmins (IL-33 and TSLP) and pro-inflammatory cytokine release from bronchial smooth muscle cells from both healthy and asthmatic individuals in order to follow up on our findings in paper III.

Popular science summary

Asthma is one of the most common non-communicable chronic lung diseases. The symptoms of the disease are increased mucus production, coughing and shortness of breath. It is estimated that more than 300 million individuals worldwide suffer from asthma, making it a severe public health concern. The prevalence of asthma is higher in the western world, although incidence of asthma is increasing all around the world. Asthma is the most prevalent chronic disease in children and is listed as the fifth most common cause of death in this age group.

The goal of asthma therapy is to reduce and control symptoms. Glucocorticoids is the most common drug to reduce airway inflammation. Additionally, short-acting medications (relaxants) can be taken right away during an asthma attack to reverse the constriction of the smooth muscle in the airways and alleviate symptoms. Current therapies can effectively lower the ongoing inflammatory process and symptoms of asthma in the majority of patients, but there is no curative treatment available.

There are some groups of asthma patients that have severe disease and do not respond well to treatment. These patients might experience significant physical, psychological, economic, and social effects in their daily lives. The reason why some patient does not respond well to normal treatment options is that asthma is quite a mixed disease. For example, individuals with asthma have different types of underlying inflammation in their lungs, as well as different clinical aspects of the disease (e.g., what triggers it). These variations in asthma are known as "phenotypes." Asthma treatment is currently shifting toward aiming to treat the more dominant specific phenotype of asthma for individual patients rather than applying the same treatment plan for all patients.

The development of two key treatment approaches for asthma and allergies is ongoing. The first strategy, allergen-specific immunotherapy (AIT), seeks to reduce the allergy-driven diseases and has a long-term disease-modifying effect. The second strategy aim to stratify patients into a specific phenotype

and reduce specific pathological immune responses by using biological immune response modifiers. These drugs are called biologics.

Airway structural cells, such as epithelial and smooth muscle cells, are important in the pathophysiology of asthma in several ways inducing both innate immunity and airway inflammation. Environmental trigger factors for asthma include exposure to aeroallergens such as house dust mites, fungal, and pollen allergens, as well as respiratory viruses such as respiratory syncytial virus (RSV) and rhinovirus (common cold virus). Many aeroallergens contain proteases, which can activate and damage the epithelial barrier and increase allergen sensitization and airway infections. Airway epithelial cells (AECs) line the airways like a carpet and connect the outside and inside of the lung. These cells serve as the first line of defense against environmental stimuli and contain specific sensors that can identify these triggers through pattern recognition receptors (PRRs), including toll-like receptors (TLRs) or NOD-like receptors (NODs). The recognition or activation of various environmental stimuli by PRRs results in the production of several pro-inflammatory cytokines, anti-viral proteins, and alarmins. Alarmins or danger associated molecular patterns (DAMPs) are molecules that are normally stored within the cells, but when cells are damaged or stressed by environmental triggers, they release molecules into the extracellular environment. Once released into the extracellular space, they alert our immune system to regulate an inflammatory response.

The overall aim of this thesis is to investigate the innate immune response to aeroallergens and viral infections in airway structural cells. To carry out the studies, we have performed experiments with cultured bronchial epithelial and smooth muscle cells from the lungs of asthmatic patients. The first part of this thesis investigated effects of different allergens and allergic mediators on the metabolic DAMPs (ATP and UA), pro-inflammatory cytokines and alarmins cytokines (IL-33 and TSLP) release in bronchial epithelial cells. Next, we investigated exposure to allergens or stimulations with allergic mediators prior to viral infection to see if it altered the anti-viral response in bronchial epithelial cells from patients with asthma. Our results showed that allergens induced DAMPs release and pro-inflammatory cytokines which were blocked by serine protease inhibitors. Our findings might suggest that proteases within allergens are a key target for potential treatments for allergic inflammation in asthma.

The second part of the thesis is to investigate the rhinovirus-induced differential expression of alarmin (IL-33) expression and its regulating

mechanism in bronchial smooth muscle cells from both severe and non-severe asthma and healthy controls. Our results showed that non-severe asthmatics have higher expression of IL-33 than the severe asthma and healthy controls. Further, we demonstrated that rhinovirus-induced IL-33 expression in bronchial smooth muscle cells regulated through TLR3. Additionally, TLR3 activation results in downstream signalling molecules, which promotes gene expression. Next, we investigated the TLR3 downstream signalling pathways, and our results showed that TAK-1 downstream signalling pathway were responsible for RV-induced IL-33 expression in bronchial smooth muscle cells. These signalling molecules might be prospective therapeutic targets for the treatment of viral-induced asthma exacerbations since they might contribute to the pathophysiology of airway inflammation caused by respiratory viruses.

The final study of the thesis focuses on the question, whether house dust mite sublingual allergen immunotherapy (HDM-SLIT) could improve the epithelial anti-viral response in bronchial epithelial cells from patients with allergic asthma. We demonstrated that HDM-SLIT improves epithelial anti-viral response and tend to reduce the alarmin IL-33 expression. This indicates that allergen immunotherapy might be a potential treatment option for allergic inflammation and viral induced exacerbations in asthma.

Populärvetenskaplig sammanfattning

Astma är en av de vanligaste icke-smittsamma kroniska lungsjukdomarna. Symptomen är ökad slemproduktion, hosta och andnöd. Dessa symtom orsakas av ett överaktivt immunsvar i luftvägarna, vilket leder till inflammation. Det uppskattas att mer än 300 miljoner individer världen över lider av astma, vilket gör den till ett allvarligt folkhälsoproblem. Förekomsten av astma är högre i västvärlden, även om förekomsten av astma ökar över hela världen. Astma är den vanligaste kroniska sjukdomen hos barn och listas som den femte vanligaste dödsorsaken i denna åldersgrupp.

Målet med astmabehandling är att minska och kontrollera symtomen. Glukokortikoider är det vanligaste läkemedlet för att minska luftvägsinflammation. Dessutom kan kortverkande mediciner (avslappnande medel) tas direkt under ett astmaanfall för att minska sammandragningen av den glatta muskulaturen i luftvägarna och lindra symtomen. Nuvarande behandling kan effektivt dämpa den pågående inflammatoriska processen och astmasymtomen hos majoriteten av patienterna, men det finns ingen botande behandling tillgänglig.

Det finns en stor grupp astmapatienter som har en allvarlig sjukdom och som inte svarar bra på behandling. Dessa patienter kan uppleva betydande fysiska, psykologiska, ekonomiska och sociala effekter i sina dagliga liv. Anledningen till att vissa patienter inte svarar bra på normala behandlingsalternativ är att astma är en heterogen sjukdom. Till exempel har individer med astma olika typer av underliggande inflammation i sina lungor, såväl som olika kliniska aspekter av sjukdomen (t.ex. vad som utlöser den). Dessa variationer i astma är kända som "fenotyper". På senare tid har behandlingsstrategierna för astma ändrats och riktas mot att ge patienterna personlig behandling baserat på den specifika fenotypen av astma snarare än att tillämpa samma behandlingsplan för alla patienter.

Utvecklingen av två viktiga behandlingsmetoder för astma och allergier pågår fortfarande. Den första strategin, allergenspecifik immunterapi (AIT), syftar till att minska de allergidrivna sjukdomarna och har en långsiktig sjukdomsmodifierande effekt. Den andra strategin syftar till att indela

patienter i en specifik fenotyp och minska specifika patologiska immunsvar genom att använda biologiska immunsvarsmodifierare. Dessa läkemedel kallas biologiska.

Strukturella celler i luftvägarna, såsom epitelceller och glatta muskelceller, är viktiga i astmas patofysiologi. Utlösande faktorer i miljön inkluderar exponering för aeroallergener som kvalster, mögel- och pollenallergener, såväl som luftvägsvirus som respiratoriskt syncytialvirus (RS) och rhinovirus (förkylningsvirus). Många aeroallergener innehåller nedbrytande proteiner, så kallade proteaser, som kan aktivera och skada epitelbarriären och öka känsligheten mot allergen. Slemhinneceller kantar luftvägarna som en matta och förbinder insidan av lungan med den yttre miljön. Dessa celler fungerar som den första försvarslinjen mot yttre stimuli och kan identifiera utlösande ämnen genom igenkänningsproteiner (pattern recognition receptorer, PRRs), så kallade toll-like receptorer (TLR) eller NOD-like receptorer (NODs). Igenkännandet av olika miljöstimuli och aktiveringen av PRRs resulterar i produktion av flera pro-inflammatoriska cytokiner, antivirala proteiner och alarminer. Alarminer (damage associated molecular patterns, DAMPs) är molekyler som normalt lagras inuti cellerna, men när celler skadas eller stressas av yttre stimuli utsöndrar de ämnen till den extracellulära miljön. När de väl släpps ut i det extracellulära utrymmet, varnar de vårt immunsystem för att uppreglera ett inflammatoriskt svar.

Det övergripande syftet med denna avhandling är att undersöka det medfödda immunsvaret mot aeroallergener och virusinfektioner i luftvägarnas strukturella celler. För att genomföra studierna har vi gjort experiment med odlade luftvägsceller och glatta muskelceller från lungorna hos astmatiska patienter. Den första delen av denna avhandling undersökte effekter av olika allergener och allergiska ämnen på frisättningen av metaboliska DAMPs (ATP och UA), pro-inflammatoriska cytokiner och alarminer (IL-33 och TSLP) frisättning från luftvägarnas slemhinneceller. Därefter undersökte vi hur exponering för allergener eller stimulering med allergiska ämnen innan virusinfektion förändrade det antivirala svaret i bronkiala epitelceller från patienter med astma. Våra resultat visade att allergener inducerade DAMPs-frisättning och pro-inflammatoriska cytokiner som blockerades av serinproteashämmare. Våra fynd kan tyda på att proteaser i allergener är ett nyckelmål för potentiella behandlingar för allergisk inflammation vid astma.

Den andra delen av avhandlingen var att undersöka det rhinovirus-inducerade uttrycket av alarminet IL-33, och dess reglering i luftvägarnas

glatta muskelceller från patienter med olika svårighetsgrad av astma och friska kontroller. Våra resultat visade att astmatiker med mildare sjukdom har högre uttryck av IL-33 än svår astma och friska kontroller. Vidare visade vi att rhinovirus-inducerat IL-33-uttryck i glatta muskelceller regleras genom TLR3. Dessutom resulterar TLR3-aktivering i nedströms signalmolekyler, vilket främjar genuttryck. Därefter undersökte vi TLR3 nedströms signalvägar och våra resultat visade att TAK-1 nedströms signalväg var ansvariga för RV-inducerat IL-33 uttryck i bronkiala glatta muskelceller. Dessa signalmolekyler kan bidra till patofysiologin i luftvägsinflammation som orsakas av luftvägsvirus, och de kan vara potentiella terapeutiska mål för behandling av virusinducerade astmaattacker.

Den sista studien i avhandlingen fokuserar på frågan huruvida sublingual allergen immunterapi (HDM-SLIT) mot kvalster skulle kunna förbättra det antivirala svaret i luftvägsslemhinnan från patienter med allergisk astma. Vi visade att HDM-SLIT förbättrar slemhinnans anti-virala respons och tenderar att minska uttrycket av alarminet IL-33. Detta indikerar att allergenimmunterapi kan vara ett potentiellt behandlingsalternativ för allergisk inflammation och virusinducerade attacker vid astma.

அறிவியல் சுருக்கம்

ஆஸ்துமா என்பது மிகவும் பொதுவான நீடித்த, பரவும்/தொற்றும்தன்மை அல்லாத நுரையீரல் நோய்களில் ஒன்றாகும். சளி உற்பத்தி அதிகரிப்பு, இருமல் மற்றும் மூச்சுத் திணறல் ஆகியவை நோயின் அறிகுறிகள். உலகளாவிய ரீ தியில் 300 மில்லியனுக்கும் அதிகமான நபர்கள் ஆஸ்துமாவால் பாதிக்கப்பட்டுள்ளனர் ஆதலால் இது ஒரு கடுமையான பொது சுகாதார கவலையாக உள்ளது. மேற்கத்திய நாடுகளில் ஆஸ்துமாவின் பாதிப்பு அதிகமாக உள்ளது என்றாலும் உலகம்முழுவதும் ஆஸ்துமாவின் பாதிப்பு அதிகரித்துக்கொண்டே வருகிறது. ஆஸ்துமா குழந்தைகளில் மிகவும் பரவலாக காணப்படுகின்ற நோயாகும், மேலும் இந்த வயதினரின் மரணத்திற்கான ஐந்தாவது பொதுவான காரணியாக பட்டியலிடப்பட்டுள்ளது.

நோயின் அறிகுறிகளைக் குறைப்பதும் கட்டுப்படுத்துவதும் ஆஸ்துமா சிகிச்சையின் குறிக்கோள் ஆகும். காற்றுப்பாதை வீக்கத்தை குறைக்க குளுக்கோகார்டிகாய்டுகள் மிகவும் பொதுவான மருந்து. கூடுதலாக, ஆஸ்துமா தாக்குதலின் போது குறுகிய-செயல்பாட்டு மருந்துகள் (ரிலாக்ஸன்ட்கள்) உடனடியாக எடுக்கப்படலாம். இது காற்றுப்பாதையில் உள்ள மென்மையான தசையின் சுருக்கத்தை மாற்றியமைக்கவும் நோய் அறிகுறிகளை மட்டுப்படுத்தவும் உதவும். தற்போதைய சிகிச்சைகளின் மூலம் பெரும்பாலான ஆஸ்துமா நோயாளிகளின் ஆஸ்துமாவின் அப்போதைய அழற்சி செயல்முறை மற்றும் அறிகுறிகளை திறம்பட குறைக்கலாம் என்றாலும் குணப்படுத்தும் சிகிச்சை எதுவும் இல்லை.

சிகிச்சைக்கு பலனில்லாதுஆஸ்துமா நோயினால் கடுமையாக பாதிக்கப்பட்ட ஒரு பெரும் பகுதியினர் உள்ளனர். இந்த நோயாளிகள் தங்கள் அன்றாட வாழ்க்கையில் குறிப்பிடத்தக்க உடல், உளவியல், பொருளாதார மற்றும் சமூக விளைவுகளை அனுபவிக்கலாம். சில நோயாளிகளுக்கு சாதாரண சிகிச்சை முறைகள் சரியாக பலன் அளிக்காத காரணம், ஆஸ்துமா மிகவும் கலவையான நோயாகும். உதாரணமாக ஆஸ்துமா உள்ள நபர்களின் நுரையீரலில் பல்வேறு

வகையான அடிப்படை அழற்சி உள்ளன, அத்துடன் நோயின் வெவ்வேறு மருத்துவ அம்சங்கள் உள்ளது (எ.கா., எது தூண்டுகிறது?). ஆஸ்துமாவின் இந்த மாறுபாடுகள் "பினோடைப்ஸ்" என்று அழைக்கப்படுகின்றன. ஆஸ்துமா சிகிச்சையானது அனைத்து நோயாளிகளுக்கும் ஒரே சிகிச்சைத் திட்டத்தைப் பயன்படுத்துவதை விட, தனிப்பட்ட நோயாளிகளுக்கு ஆஸ்துமாவின் அதிக ஆதிக்கம் செலுத்தும் குறிப்பிட்ட பினோடைப்பை சிகிச்சை செய்வதை தற்போது நோக்கமாகக் கொண்டுள்ளது.

ஆஸ்துமா மற்றும் ஒவ்வாமைக்கான இரண்டு முக்கிய சிகிச்சை அணுகுமுறைகளின் வளர்ச்சி தொடர்கிறது. முதல் உத்தி, ஒவ்வாமை-குறிப்பிட்ட நோயெதிர்ப்பு சிகிச்சை, ஒவ்வாமை உந்துதல் நோய்களைக் குறைக்க முயல்வதால் நீண்டகால நோயை மாற்றியமைக்கும் விளைவைக் கொண்டுள்ளது. இரண்டாவது உத்தியின் நோக்கமாக நோயாளிகளை ஒரு குறிப்பிட்ட பினோடைப்பில் வகை படுத்தி, உயிரியல் நோயெதிர்ப்பு மறுமொழி மாற்றிகளைப் பயன்படுத்தி குறிப்பிட்ட நோயியல் நோயெதிர்ப்பு மறுமொழிகளை குறைப்பதை நோக்கமாகக் கொண்டுள்ளது. இந்த மருந்துகள் உயிரியல் என்று அழைக்கப்படுகின்றன.

ஆஸ்துமாவின் நோய்க்குறியியலுக்கு எபிடெலியல் மற்றும் மென்மையான தசை செல்கள் போன்ற காற்றுப்பாதை கட்டமைப்பு செல்கள் முக்கியமானவை. ஆஸ்துமாவுக்கான சுற்றுச்சூழல் தூண்டுதல் காரணிகளில் வீட்டில் தூசிப் பூச்சிகள், பூஞ்சை மற்றும் மகரந்த ஒவ்வாமை போன்ற ஏரோஅலர்ஜென்களுக்கு வெளிப்பாடு, அத்துடன் சுவாச ஒத்திசைவு வைரஸ் (RSV) மற்றும் ரைனோவைரஸ் (பொதுவான சளி வைரஸ்) போன்ற சுவாச வைரஸ்கள் ஆகியவை அடங்கும். நிறைய ஏரோஅலர்ஜென்களில் புரோட்டீஸ்ஸைக் கொண்டிருக்கின்றன. அவை எபிடெலியல் (தோல் மேல்புற செல்கள்) தடையைச் செயல்படுத்தி சேதப்படுத்தும் மற்றும் உணர்திறனை அதிகரிக்கும். (காற்றுப்பாதை) ஏர்வே எபிடெலியல் செல்கள் ஒரு கம்பளம் போன்ற காற்றுப்பாதைகளை வரிசைப்படுத்தி நுரையீரலின் வெளிப்புறத்தையும் உட்புறத்தையும் இணைக்கிறது. இந்த செல்கள் சுற்றுச்சூழல் தூண்டுதல்களுக்கு எதிராக பாதுகாப்பின் முதல் வரிசையாக செயல்படுகின்றன மற்றும் இந்த தூண்டுதல்களை அடையாளம் காணக்கூடிய குறிப்பிட்ட சென்சார்கள் உள்ளன. டோல் போன்ற ஏற்பிகள் (TLRs) அல்லது NOD போன்ற ஏற்பிகள் (NODகள்) போன்ற பேட்டர்ன் ரெகக்னிஷன் ரிசெப்டர்கள் (PRRs) மூலம் இந்த தூண்டுதல்களை அடையாளம் காணக்கூடிய குறிப்பிட்ட உணரிகளைக் கொண்டிருக்கின்றன. PRRகள் மூலம் பல்வேறு சுற்றுச்சூழல் தூண்டுதல்களை அங்கீகரிப்பது அல்லது

செயல்படுத்துவது, பல அழற்சி சார்பு சைட்டோகைன்கள், வைரஸ் எதிர்ப்பு புரதங்கள் மற்றும் அலாரமின்களை உற்பத்தி செய்வதில் விளைகிறது. அலார்மின்கள் அல்லது ஆபத்து தொடர்புடைய மூலக்கூறு வடிவங்கள் (DAMP கள்) பொதுவாக உயிரணுக்களுக்குள் சேமிக்கப்படும் மூலக்கூறுகள். ஆனால் செல்கள் சேதமடையும் போது அல்லது சுற்றுச்சூழல் தூண்டுதல்களால் அழுத்தப்படும் போது, அவை மூலக்கூறுகளை புற-செல்லுலார் சூழலில் வெளியிடுகின்றன. புற-செல்லுலர் சூழலில் வெளியிடப்பட்டதும், அவை அழற்சியின் எதிர்ப்பாற்றலை கட்டுப்படுத்த நமது நோயெதிர்ப்பு மண்டலத்தை எச்சரிக்கின்றன.

இந்த ஆய்வறிக்கையின் ஒட்டுமொத்த நோக்கம் காற்றுப்பாதை கட்டமைப்பு செல்களில் உள்ள ஏரோஅலர்ஜென்ஸ் மற்றும் வைரஸ் தொற்றுகளுக்கு உள்ளார்ந்த நோயெதிர்ப்பு மறுமொழியை ஆராய்வதாகும். ஆய்வுகளை மேற்கொள்வதற்காக, ஆஸ்துமா நோயாளிகளின் நுரையீரலில் இருந்து வளர்க்கப்பட்ட மூச்சுக்குழாய் எபிடெலியல் மற்றும் மென்மையான தசை செல்கள் மூலம் நாங்கள் பரிசோதனை செய்துள்ளோம். இந்த ஆய்வறிக்கையின் முதல் பகுதியானது, பல்வேறு ஒவ்வாமை மற்றும் ஒவ்வாமை மத்தியஸ்தர்களின் வளர்சிதை மாற்ற DAMPகள் (ATP மற்றும் UA), புரோ-இன்ஃப்ளமேட்டரி சைட்டோகைன் மற்றும் அலார்மின்கள் சைட்டோகைன்கள் (IL-33 மற்றும் TSLP) மூச்சுக்குழாய் எபிடெலியல் செல்களில் வெளியிடும் விளைவுகளை ஆய்வு செய்தது. அடுத்து, ஆஸ்துமா நோயாளிகளிடமிருந்து மூச்சுக்குழாய் எபிடெலியல் செல்களில் வைரஸ் எதிர்ப்பு எதிர்வினையை மாற்றியமைக்கும் வைரஸ் தொற்றுக்கு முன்னர் ஒவ்வாமை மத்தியஸ்தர்களுடன் ஒவ்வாமை அல்லது தூண்டுதல்களின் வெளிப்பாடுகளை நாங்கள் ஆராய்ந்தோம். ஒவ்வாமை தூண்டும் DAMPகள் வெளியீடு மற்றும் அழற்சிக்கு சார்பான சைட்டோகைன் ஆகியவை செரின் புரோட்டீஸ் தடுப்பான்களால் தடுக்கப்பட்டதாக எங்கள் முடிவுகள் காட்டுகின்றன. ஆஸ்துமாவில் ஒவ்வாமை வீக்கத்திற்கான சாத்தியமான சிகிச்சைகளுக்கான முக்கிய இலக்காக ஒவ்வாமை உள்ள புரோட்டீஸ்கள் இருப்பதாக எங்கள் கண்டுபிடிப்புகள் தெரிவிக்கலாம்.

ஆய்வறிக்கையின் இரண்டாம் பகுதியானது, ரைனோவைரஸால் தூண்டப்பட்ட அலார்மின் (IL-33) வெளிப்பாட்டின் மாறுபட்ட வெளிப்பாடு மற்றும் கடுமையான ஆஸ்துமா மற்றும் ஆரோக்கியமான கட்டுப்பாடுகளிலிருந்து மூச்சுக்குழாய் மென்மையான தசை செல்களில் அதன் ஒழுங்குபடுத்தும் பொறிமுறையை ஆராய்வதாகும். கடுமையான ஆஸ்துமா மற்றும் ஆரோக்கியமான கட்டுப்பாடுகளை விட கடுமையான ஆஸ்துமா

நோயாளிகள் IL-33 இன் அதிக வெளிப்பாட்டைக் கொண்டுள்ளனர் என்பதை எங்கள் முடிவுகள் காட்டுகின்றன. மேலும், TLR3 மூலம் கட்டுப்படுத்தப்படும் மூச்சுக்குழாய் மென்மையான தசை செல்களில் rhinovirus-தூண்டப்பட்ட IL-33 வெளிப்பாடு என்பதை நாங்கள் நிரூபித்தோம். கூடுதலாக, TLR3 செயல்படுத்தல் கீழ்நிலை சமிக்ஞை மூலக்கூறுகளில் விளைகிறது, இது மரபணு வெளிப்பாட்டை ஊக்குவிக்கிறது. அடுத்து, TLR3 கீழ்நிலை சிக்னலிங் பாதைகளை நாங்கள் ஆய்வு செய்தோம், மேலும் மூச்சுக்குழாய் மென்மையான தசை செல்களில் RV- தூண்டப்பட்ட IL-33 வெளிப்பாட்டிற்கு TAK-1 கீழ்நிலை சமிக்ஞை பாதை காரணம் என்பதை எங்கள் முடிவுகள் காட்டுகின்றன. இந்த சிக்னலிங் மூலக்கூறுகள் சுவாச வைரஸ்களால் ஏற்படும் காற்றுப்பாதை அழற்சியின் நோயியல் இயற்பியலுக்கு பங்களிக்கக்கூடும், மேலும் அவை வைரஸால் தூண்டப்பட்ட ஆஸ்துமா அதிகரிப்புகளுக்கு சிகிச்சையளிப்பதற்கான சாத்தியமான சிகிச்சை இலக்குகளாக இருக்கலாம்.

வீட்டு தூசிப் பூச்சி சப்ளிங்குவல் ஒவ்வாமை நோயெதிர்ப்பு சிகிச்சை (HDM-SLIT) ஒவ்வாமை ஆஸ்துமா நோயாளிகளிடமிருந்து மூச்சுக்குழாய் எபிடெலியல் செல்களில் எபிடெலியல் வைரஸ் எதிர்ப்பு எதிர்வினை மேம்படுத்துமா என்ற கேள்வியின் மீதான ஆய்வறிக்கையின் கடைசி ஆய்வு கவனம் செலுத்துகிறது. ஒவ்வாமை நோயெதிர்ப்பு சிகிச்சை ஆனது எபிடெலியல் வைரஸ் எதிர்ப்பு எதிர்வினை மேம்படுத்துகிறது மற்றும் எச்சரிக்கை சைட்டோகைன் IL-33 வெளிப்பாட்டைக் குறைக்க முனைகிறது என்பதை நாங்கள் நிரூபித்தோம். ஒவ்வாமை அழற்சி மற்றும் ஆஸ்துமாவில் வைரஸ் தூண்டுதல் அதிகரிப்புகளுக்கு ஒவ்வாமை நோயெதிர்ப்பு சிகிச்சை ஒரு சாத்தியக்கூறாக இருக்கலாம் என்பதை இது குறிக்கிறது.

Acknowledgement

The completion of this thesis would not be possible without an excellent team effort including numerous people and institutions. To them, I would like to express my sincere gratitude considering their crucial help.

Prior to all else, I would like to thank my main supervisor, **Prof. Lena Uller**. Lena, without whom this thesis would not even start. Thank you for introducing me to what biomedical research really is like with your smart insights and outstanding teaching supervision. I am incredibly grateful for her patience, support (both professional and personal), encouragement and enthusiasm, which has gone above and beyond expectations. I am very grateful that she believed in me and offered me the opportunity to pursue my PhD in her lab. Thank you!

I am deeply grateful to my co-supervisor, **Dr. Hamid Akbarshahi**, for his scientific assistance, remarkable constructive criticism and intellectual shrewdness and, most of all, for always be a tough craver. I truly believe I could not have done without your assistance in the lab.

Prof. Leif Bjermer, thank you for being my co-supervisor and for providing clinical samples.

I would also like to thank my co-supervisor, **Dr. Cecilia Andersson**, for giving me the chance to be a part of mast cell research. I am very appreciative and cherish our Uppsala meeting good memories and warm friendship.

I want to thank my half-time examiners, **Prof. Jonas Erjefält** and **Dr. Anna-Karin Larsson**. And a special thanks to **Jonas Erjefält** for cheering me up and spreading your positive energy around BMC D12.

I extend my special thanks to **Dr. Anja Meissner**. I have always admired your scientific competence and hard work in research. I have always enjoyed our “cell-lab” conversations.

I have always looked forward to be in the lab and have enjoyed working. This has been possible because of the following important people:

Jenny Calven, thank you for teaching me the perfection in the cell culture lab and technical details. For all your guidance with the bronchial smooth muscle cells even during your parental leave. I am so grateful for your guidance and encouragement. **Mandy Menzel**, thank you for being there for me from day one in the lab and for all your timely assistance and advice with my experiments and manuscripts. Additionally, I want to express my gratitude to you for sharing room with me during the conference and for inspiring me with your perfect timing and punctuality. **Irma Mahmutovic Persson,** you are the most excited person I have ever met. You continue to be an inspiration to me and thank you for your kindness and your friendship.

I want to express my gratitude to all of my lab members, past and present **Samuel Cerps** thank you so much for the pleasant experience in the lab, the several conferences, and the amazing discussions about basic immunology, **Sofia M** thank you so much "Anbey" for your friendship, the good times we spent together, and for sharing the Bio-render account, **Sofia T** it has been a pleasure working and sharing office space with you. A special thanks to you for introducing me to the Knäckebröd for my pre-lunch, **Fanny, Joakim**, **Frida**, **Omeyma, Ebba** and **Edwina,** for walking this PhD journey with me and for being such pleasant company.

Juanjo, thank you so much for all the support in the lab and laugh both in and outside work. Thank you so much in particular for the experience of the Spanish wedding; I will cherish those moments forever. I also want to thank **Fran, Sara**, **Moni** and **Pablo** for a wonderful experience in Spain and in the lab.

I am greatly appreciative of all my co-authors for a successful collaboration. I had the privilege to cooperate with very outstanding academic and generous persons that crucially contributed to the development of this work. I would like to express my gratitude to **Prof. Celeste Porsbjerg** at Bispeberg hospital in Copenhagen**, Asger** and **Morten,** for the clinical samples and interesting discussions during various meetings. Especially many thanks go to **Dr. Christian Uggerhoj Wöhlk**, for our collaboration around paper V all your help and excellent work experience especially with cell cultures.

Many thanks to **Lisette Eklund** and **Maria Palmkron** for their administrative assistance at EMV, in particular, for their tremendous emotional support throughout my visa application process. **Martin Nyström**

deserves special thanks for his technical assistance with IT and computer service.

A very special word of thanks goes to **Prem** and **Azra** exceptional persons that they are always present in good and bad moments, for their true friendship through long time.

Daisy and **Selvi**, thanks for being good friends, who always makes me laugh for the silliest of reasons. Thank you especially for the lunchtimes at D12 and the funny Insta reels (I hope both of you understand what I meant). Specially **Daisy** and **Gabriel** for our lovely time together.

Neha and **Manar,** Thank you so much for your friendship, love, support, lots of fun and good times over the years. Special thanks to **Manar** for the cookies, chocolates, and free health advices.

Special thanks go to **Björn Olde** and **Bengt-Olof Nilsson**, for their useful comments and advices throughout these years.

I would like to thank to all "**C12** and **D12 colleagues**" for creating such a nice and good environment, **Ellen Tufvesson**, **Sebastian Albinsson**, **Catarina Rippe, Karl swärd, Fredrik Leeb Lundberg, Katarzyna Krawczyk, Kreema, Fatima, Elisabeth, Sara Dhal, Li Lu, Katarzyna said Hilmersson, Katarzyna Kawka, Siv Svennson, Mario, Monika Bauden, Gayathri, Hodan, Renan, Julia, Lotte, Frank, Carlos, Martin, Carin, Francisco, Lena Thiman, Marie Wildt, Sara Rolandson,** and everyone else in D12. Thank you for all your support during my PhD and also for the Friday FIKA and fun during lunch.

Special thanks go to **Prof. Kaleel Razak** and my friend **Shafika**, my sister and well-wisher, for always encouraging me with your successes in both your personal and professional lives. Had fantastic memories of our time together watching "SURA" movie in LA, our trip to the Hollywood studio, Laguna Beach, and my first baby shower. My deepest love goes to **Haroon** and **Hassan**.

My close friends back in India, **Padma Meganathan** and **Subbulaxmi Pattamuthu**. Thank you so much for sending me the package from India and for the lovely vacation days we spent.

My dear friends from Lund and Malmö who have all made my life abroad so easy and never made me miss home a single day:

A big thank to **Veeresh** and **Ramesh**, for your support when I was alone in Sweden and all your timely help. **Saranya karthikeyan, Indhu Sanker, Rupa Bala, Belinda Deepan, Sathya Ramesh, Agatheesh, Sudar, Madhuri Paul, Priya Ram, Kumutha Athimoolam, Jeyasree Vishwakumar**, **Ashwini Kannan, Sunitha Mubarak, Deepa Sundar, Vignesh, Karthika Prem, Sudhakar, Chanchala Ram, Monalisa Panda, Vaishali Grish, Ranjana Sandeep, Chinmay, Kavitha Veguru, Madhavi Ravi, Sudha Parimala, Rathi Ramji and Farhana Jahid,** thanks a lot guys for considering me as one of your own many times. Thank you so much for all the fun times, be it lunch/dinner, birthday parties, ladies meet and kids dance practice sessions, all of them have been fun and only fun! Thanks a lot, everyone. And very special thanks to **Jeyasree Vishwakumar** and **Balamurugan** for correcting Tamil popular summary of my thesis. Special thanks go to **Roopsi** for the stunning cover image.

Finally, a word of appreciation goes to my entire family members for believing and supporting me throughout this PhD journey:

I want to express my gratitude to my uncle **Prof. Narayanan Muthu** and my sister **Ghandhimathi Narayanan** for helping to achieve my goals. Not just helping me, but also being my parents. I want to express my gratitude for everything you have done for me and for helping me pursue my studies. I could not have accomplished what I have up to this point without you both.

I intend to express my gratitude to my in-laws **Kurunthu** and **Karungammal** for their everlasting love and support. Particularly, my father-in-law, who I seen in my life being really supportive and encouraging his daughter-in-law's education and career. I feel really blessed to be a part of this family. I would also like to thank my entire family members: my brother in-laws **Mathiazhagan** and **Prabhakaran,** my co-sisters **Selvi, Maheswari,** my sister **Malar** and mama **Balamurugan,** my brother **Palani** and my anni **Parameshwari,** my sister in-laws **Revathi, Madhavi, Laxmi, Jamuna**, my niece **Rajapriya, Akash, Ajay, Karthika, Praveen, Naveen, Kiruthiga, Divya, Varun** and **Viven kutty.**

I wanted to thank my loving husband **Dr. Dharmalingam** for being there for me in both good and terrible circumstances and for bearing with all of my emotions. You are my main inspiration and pillar of my life. Without your

support, I could not have succeeded. I love ❤ you more than I can express in words.

My daughter **Akshaya Dharmalingam** has taught me how to be a fighter and never give up even when all the odds are against us, and I am grateful for her unconditional love. Over the years, I have learned so much from you. I thank you for being my mom during my difficult times.

Last but not least, my little princess **Ashvitha Dharmalingam**. I love you so much and I am so glad you entered my life.

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