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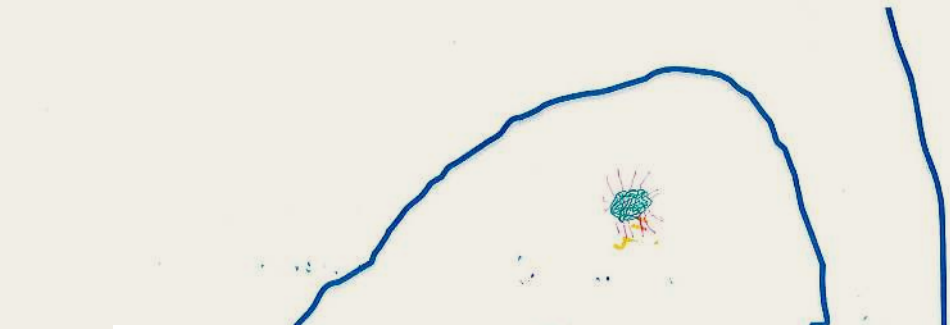

Translational studies of viral-induced asthma and COPD exacerbations

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LUND
UNIVERSITY

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Abstract

Exacerbations of asthma and COPD are mainly caused by viral infections, where majority are represented by the common cold, rhinovirus. These are severe acute episodes in the disease and need effective treatments. Worldwide, the huge economical burden and suffering of several hundred million of patients are in need of novel drugs to treat and prevent the high rate of morbidity and mortality. To develop novel treatments new models are needed, that can mimic clinical features of asthma and COPD exacerbations, to elucidate the molecular mechanisms involved in causing and maintaining respiratory exacerbations caused by viral infections.

This thesis aims to develop *in vivo* models and also using both *in vitro* and *in vivo* settings to study exacerbations. The goal was then to further investigate drug interventions, or gene knockouts, to elucidate the mechanisms involved in the signalling pathways leading to exacerbations. For this purpose, primary human bronchial epithelaial cells (HBECs) and mice have been used. Firstly, primary HBECs donated from asthmatics and COPD patients were cultured and stimulated with a viral surrogate dsRNA, mimicking rhinoviral replication, inducing cytokine expression. Simultaneously drug substances were applied to study the anti-inflammatory effects while cytokine expression of TSLP, IFNβ, TNFα and CXCL8 was observed measuring both mRNA expression (RT-qPCR) and protein production (ELISA). Also transcription factors NF-κB and IRF3 were studied (Western blot). The substances tested were small-molecular inhibitors called RES as well as Capsazepine (CPZ) and Simvastatin. The further work in this thesis involved development of an asthma exacerbation model that involved allergen provoked allergic inflammation that was superimpose by dsRNA for induction of exacerbation. The allergen challenge involved Ovalbumin in the first study, while the other two studies involved house dust mite (HDM). Leukocytes in bronchoalveolar lavage fluid and lung tissue were studied, as well as gene expression (RT-qPCR) and protein release (ELISA, Immunohistochemistry) of various cytokines and induction of pattern recognition receptors; TLR3,RIG-I and MDA5. The last *in vivo* study explored the effects of gene knockout of pro-inflammatory IL-1β, while using HDM-triggered experimental asthma model with superimposed dsRNA-triggered exacerbation.

The results showed that both CPZ and RES substances inhibit viral-induced cytokine production of TSLP as well as IFNβ in both HBECs from asthmatic and COPD patients. Also the pleotropic effects of Simvastatin explored in COPD HBECs stimulated with dsRNA, showed anti-inflammatory effects, where TSLP and IFNβ production was inhibited dose-dependently and more effectively compared to inhibition with steroids. RES and CPZ exerted their function through NF-κB inhibition while Simvastatin rather exerted its effects through IRF3. The asthma exacerbation model showed induction of Th_2 upstream cytokines TSLP, IL-33 and IL-25 being significantly induced at exacerbation, giving synergistic effects from both allergic provocation and dsRNA. Also PPRs were shown to increase at exacerbation. The IL-1β knockout mice showed less inflammation and leukocyte tissue infiltration as well as less apoptosis and necrosis compared to wildtype mice. Most interestingly, the Th_2 upstream cytokine induction in wild type mice seen at exacerbation was not altered in the KO mice. This thesis explored both *in vitro* and *in vivo* models, revealing important and potential drug target molecules such as TSLP, IL-33 and IL-25 involved in triggering or maintaining of viral-triggered respiratory exacerbations. Interestingly enough, IL-1β seems to be involved in the regulation of all three Th_2 upstream cytokines in asthma exacerbation and might also serve as an option of treatments. Drug intervention using small-molecular inhibitors or already existing drugs possessing pleotropic effects, could be another considered option for future development of combination therapy.

Key words: Asthma, COPD, Exacerbation, dsRNA, Th_2, virus, dsRNA, TSLP, IL-33, IL-25, IL-1β, HDM

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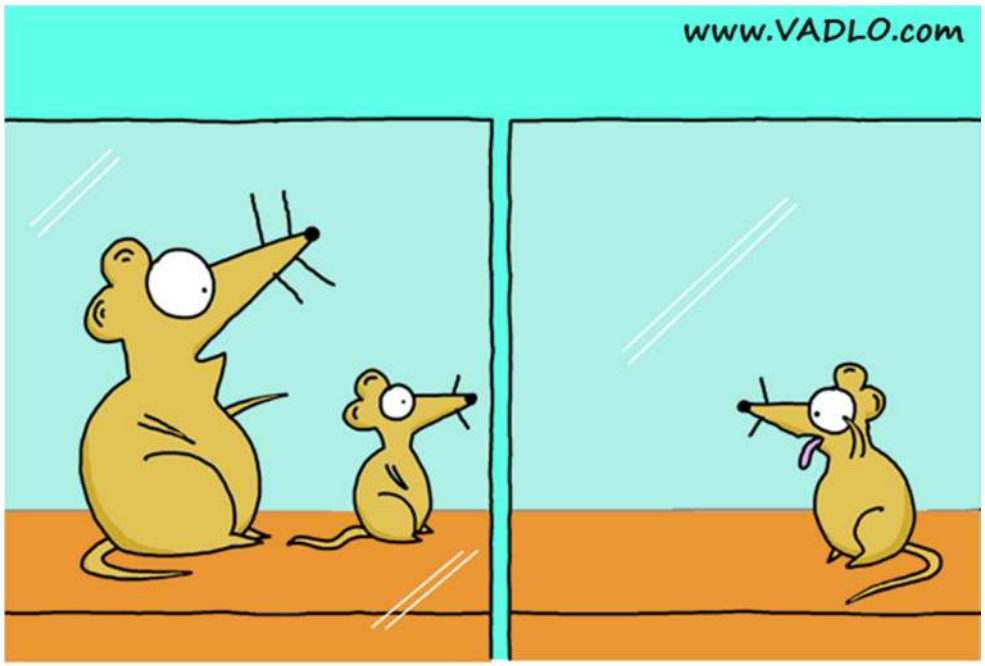
"Criticism is something we can avoid easily by saying nothing, doing nothing, and being nothing"

- Aristotle



"Don't play with him, he is **Wild Type**."

This thesis is dedicated to my family

..with your love and support..

..you give me strength to reach infinity!!

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Abbreviations

AHR	Airway hyper responsiveness
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCL	Chemokine (C-C motif) ligand
COPD	Chronic obstructive pulmonary disease
CXCL	chemokine (C-X-C motif) ligand
DAB	3,3'-Diaminobenzidine
DAMPs	Damage/danger associated molecular patterns
dsRNA	double-stranded RNA
ELISA	Enzyme-linked immunosorbent assay
FEV_1	Forced expiratory volume in 1 second
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GINA	Global Initiative for asthma
GOLD	Global Initiative for obstructive lung disease
HBEC	Human bronchial epithelial cells
HDM	House dust mite
ICAM-1	Intercellular adhesion molecule-1
ICS	Inhaled corticosteroids
IFN	Interferon
IgE	Immunoglobulin E
IκB-α	Inhibitory-kappa-B alpha
IL	Interleukin
IRF	Interferon regulatory factor
KO	Knock-out

LDH	Lactodehydrogenase
MCP-1	Monocyte chemoattractant protein-1
MDA5	Melanoma differentiation-associated protein 5
MMP	Matrix metalloproteinase
mRNA	messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor kappa B
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
Poly(I:C)	polyriboinosinic:polyribocytidylic acid
PRRs	Pattern recognition receptors
RANTES	Regulated on activation, normal T cell expressed and secreted
RIG-I	Retinoic acid inducible gene 1
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
RV	Rhinovirus
SAR	Structure activity relationship
Th	T helper
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha
TRPV1	Transient receptor potential vanilloid 1
TSLP	Thymic stromal lymphopoietin
WT	Wildtype
18S	18 Svedberg units (ribosomal RNA)

Introduction to respiratory exacerbations

The definition of the word ‘exacerbation’ is worsening of a disease or increase in its symptoms [1, 2]. Respiratory exacerbation is episodic worsening of the inflammatory disease that occurs in patients already having an on-going chronic inflammation, such as in patients with asthma and chronic obstructive pulmonary disease (COPD) [3, 4]. Exacerbation can be triggered by different stimuli, such as: pathogens, allergens, chemicals, pollution, cold air or exercise among other factors. However, the majority of exacerbations triggered in both asthma and COPD are caused by respiratory viral infections, where the most abundant virus is the common cold virus; rhinovirus[5].

Exacerbations in asthmatics and COPD patients worldwide are estimated to cost the society huge amounts [6], while the patient suffering is countless. Existing therapy is not enough to adequately treat exacerbations, making development of new drugs warranted. The difficulties in developing new therapies lies in the facts that good models for studying exacerbation are currently lacking, and the underlying mechanisms that initiate and drive exacerbations are not fully elucidated. Asthma and COPD are two separate diseases with different causes. At times these two distinct diseases appear to overlap, where both diseases display chronic inflammation and recurrent exacerbations. In particular, upon viral-induced exacerbations, both asthmatics and COPD patients have, to date, alternative options other than increasing their mainstay steroid treatment in addition to broncho-relaxing drugs to manage the symptoms, since there is no optimal therapy, to date.

Due to the lack of optimal treatment for exacerbations, the urge for additional research is warranted to be able to find new therapies. In this thesis we sought to elucidate new key-mechanisms and molecular pathways by establishing model systems with translational features. Therefore, we have used both *in vivo* and *in vitro* models to study the main causes and mediators that play a major role in triggering and maintaining of exacerbations. In order to do this, initially human bronchial epithelial cells, donated from asthmatics and COPD patients were used.

Epithelial cells are the main cells lining the airways and act as the first barrier towards the outer environment. Primary cell cultures have the advantage over cell lines by providing a model system where disease phenotypes remain in the culture dish. Using these cells enabled a model for investigation in how epithelial cells interact with the external environment and how their function is changed when interacting with allergens, virus or drug intervention. This experimental setting is limitless in viral mimic- and drug-testing observations and can lead to the discovery of key-mechanisms and molecules as potential drug targets for future drug development.

The bronchial epithelial cells possessing phenotypic characteristics of asthma or COPD disease were stimulated with the rhinoviral-mimic; dsRNA in cell cultures, while intervention with substances was performed targeting transcription factor inhibition. Thus, affecting inflammatory markers that could possibly be produced by these cells upon a viral infection, mimicking an exacerbation that occurs in the patients' lungs.

We further used a similar setting of mimicking rhinoviral infection as previously performed in bronchial epithelial cells, henceforth by employing an *in vivo* model, where we developed a suitable exacerbation mouse model with disease characteristics resembling human asthma exacerbation. This provided another dynamic and the possibility to study immune regulation and responses on a more advanced level where many interactions and mechanisms were present, involving several different cell types.

In addition to developing experimental asthma exacerbations in mice this thesis also studies induction and expression of cells and cytokines. Lastly, this thesis involved knockout mice lacking the expression of the central pro-inflammatory cytokine IL-1β. Henceforth, this mechanistic study with cytokine knockout enabled another dimension where exacerbation was studied, being able to observe cytokine-dependent effects on exacerbation outcome.

This thesis includes papers that touch upon some of the most important markers and cells involved in rhinoviral-induced exacerbations. Our research strategy for this thesis has been to use both *in vitro* systems with human primary bronchial epithelial cells but also *in vivo* experimental asthma exacerbation model studying clinically relevant markers for translational interpretation.

Background

Airways and chronic inflammation

The lung is a complex organ and is extremely heterogeneous, where the anatomy and function of the structural cells and tissues differ gradually along the way descending down towards the alveoli, where the vital gas exchange occurs. The main purpose for the lung is to breathe in oxygen and exhale of excessive carbon dioxide [7]. During these physiological processes, the lung is constantly exposed to the potentially harmful particles existing in the external environment [8]. In the upper airways, mucosal lining and cilia are present, which have a protective function, trapping potentially harmful particles being from entering the lung. These particles are then transported upwards by cilia movement and can eventually be removed from the airways by sneezing or coughing; this process is referred to as the mucociliary transport [9, 10]. The first ciliated barrier cells are the epithelial cells. Figure 1 demonstrates location of the epithelium as well as the peripheral descending airways with alveoli structure.

Since epithelial cells are exposed to the external environment, they are also equipped with a secretory mechanism (mucus, cytokines, chemokines and growth factors) as a secondary defence to the barrier function [11-14]. Immune cells are constantly patrolling the airways in a close association to the airway lumen and the lung parenchyma, ready to combat invading pathogens and protect the lung when needed. The antigen presenting cells (APCs), alveolar macrophages together with dendritic cells, are constantly present in conjunction with the epithelium where they are activated upon tissue injury or pathogen invasion. Upon inflammation or infection more immune cells, such as granulocytes and lymphocytes, are induced to infiltrate the lung tissue and lumen. Inflammation of the airways can arise due to different reasons, however, structural cells of the lung and the immune cells recruited from the circulation system, all work together towards resolving the inflammation and removal of the harmful invading particles, whether they be pathogens or chemical pollutants.

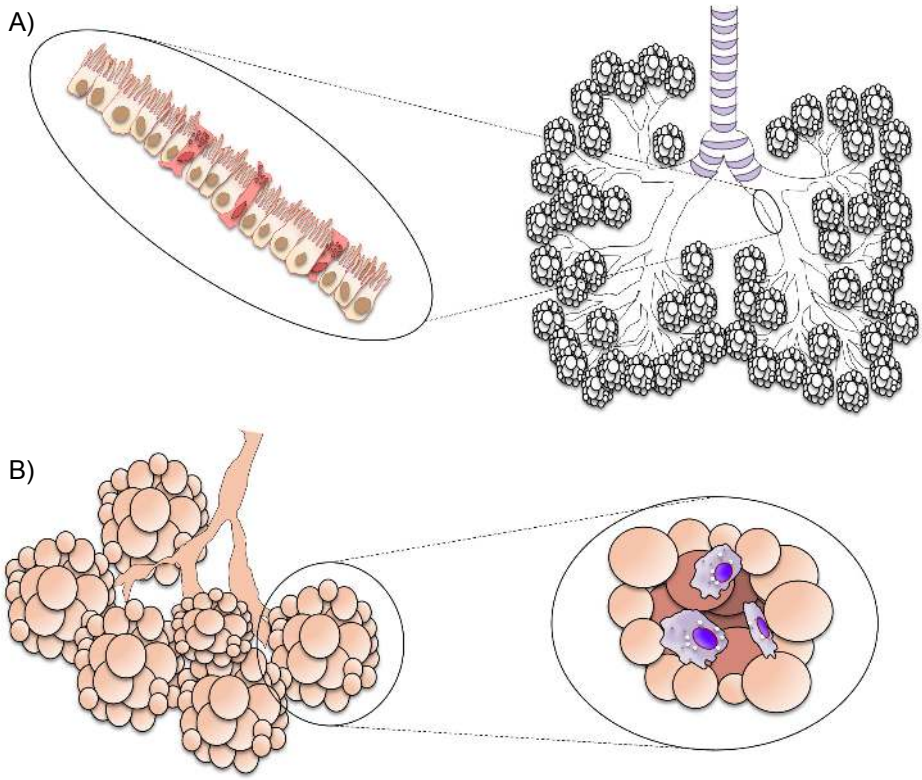

Figure 1:
The structural and functional view of the airways. The schematic picture shows A) epithelium with ciliated and mucus producing cells lining the brochial tubes, and B) the gas-exchanging alveoli with alveolar macrophages patrolling the the airways.

The temporary inflammation is necessary and needed in order to protect us from harmful pathogens. Therefore, immune cells or secreted molecules originating from the immune system are subsequently navigated to the tissue site upon infection or irritant exposure [11, 13, 15]. When the inflammation becomes chronic and persistent, then we are at risk for becoming seriously ill. The released mediators during the inflammatory process will become harmful and impact the normal function of the body's cells and tissue if the inflammation persists, which then results in chronic inflammatory diseases.

Two major chronic inflammatory lung diseases, asthma and COPD, are explored in this thesis, and described more in detail below. The prevalence of asthma and COPD has an increasing trend over decades [3, 4, 16] and, as common denominator, both diseases result from environmental and genetic predisposition. Both asthma and COPD seem to exhibit unbalanced immune response at the same

time as impaired immunity. The extremely important gas exchange occurring in the distal airways; in the alveoli sacs, where only one cell layer exists between the air lumen and the blood stream. This area is extremely affected by cell destruction or cellular hypertrophy during chronic inflammation as seen in asthma and COPD [17, 18].

Obstruction emerging from airway constriction and inflammation gives rise to lumen narrowing [19], in addition air-trapping [20] and mucus overproduction [21], leading to mucus plug formation [10], also contribute to breathing difficulties. The most used method for monitoring and evaluating lung function is through the use of spirometry [3, 4]. Forced expiratory volume is measured during the first second (FEV_1), as well as forced vital capacity (FVC), forced expiratory flow and FEV_1/FVC ratio can be measured and used to better predict disease severity. In addition to spirometry, more complicated measurements as Impulse Oscillometry, CO-diffusion and N_2-washout can be used in order to further explore peripheral airway obstruction and gas exchange [22-25]. These methods are invariably used in clinical routine but often used for research purpose to gain more knowledge, and when studying asthma and COPD.

Complex interplay of cells, cytokines and mediators are involved in triggering and maintaining of chronic inflammation in COPD and asthmatic airways. Several cell types, being both immune cells and structural cells, express many of the chemotactic cytokines. The released cytokines then give rise to autocrine and paracrine loops of mediators being released and cascade events of cell activation and infiltration occur, events being initiated and maintained in the chronic inflammation.

An immune response typically involves activation of T lymphocytes by the APCs, which then produce various cytokines and create a certain cytokine-milieu based on the type of cytokines released. This, in turn reflects the stimulus initially being sensed by the APCs as simplified drawing in Figure 2. The typical cytokines involved, are usually separated as Th1 and Th2 cytokines and often a balance shift is discussed when referring to chronic inflammation, though sometimes a Th1 and Th2 milieu can co-exist. Th1 associated cytokines are mainly IL-2, IL-12 and IFN-γ while the main Th2 cytokines are IL-4, IL-5 and IL-13 [26-28]. Recently, subsets of T cells such as Th17 and ILC2 cells have gained increasing attention among the different phenotypes occurring in both asthma [29-32] and COPD [33, 34]. IL-17 cytokines (IL-17A and IL-17E), and additional epithelial derived cytokines Thymic stromal lymphopoietin (TSLP), IL-33 and CCL5 [27, 28, 35-38] among several others are considered important and involved in Th2 immunity and exacerbations. The involved key-regulators can be seen in Figure 2.

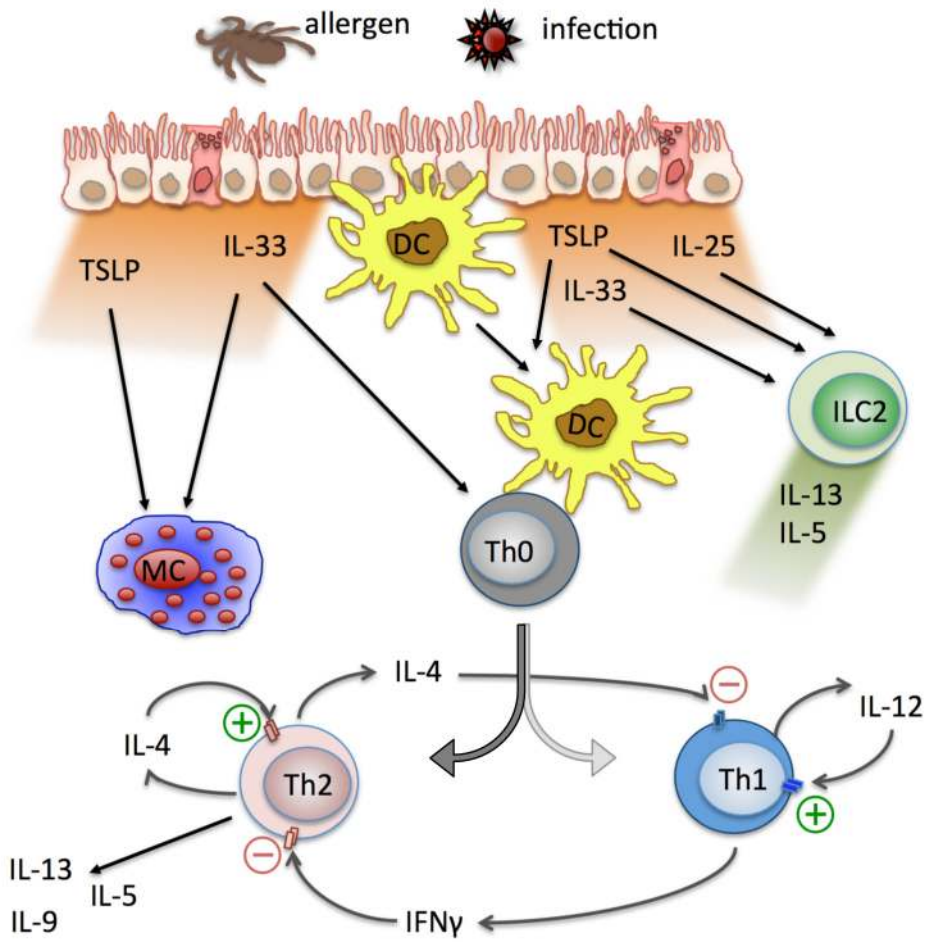

Figure 2:
Allergens, viral infection or other potentially harmful stimuli are able to activate the epithelium and induce further downstream effects such as priming dendritic cells (DC). Epithelial derived cytokines are able to influence the T cell activation from naïve Th0 into development of Th1 or Th2. In addition mast cells (MC) and innate lymphoid cells (ILCs) can also play an important role in immune responses.

Asthma

Asthma is a lung disease with chronic inflammation of the airways [39], affecting almost 10% of the population in developed countries. The worldwide estimation is that over 300 million people are affected [3, 16, 40], and it is predicted to increase to around 400 million by the year of 2025 [40, 41]. Asthma can develop at any age, but sometimes patients can recover completely during adolescences [42-45]. Typical symptoms of asthma relate to an increased AHR with variable airway

obstruction that may be reversed spontaneously or after medication. The clinical symptoms include chest tightness, wheezing, coughing and overall difficulty to breathe, as well as excessive mucus production [10, 21]. The respiratory difficulties with narrowed bronchi can be explained due to muscle contraction of the smooth muscle cells surrounding the airways. The bronchoconstriction decreases the lumen area, where the passage of the respiratory air occurs. Chronic inflammation of the tissue also makes the airways appear more narrow, and at the same time the goblet cells in the airways contribute to increased mucus production in the lumen, adding further difficulties to breathing, as shown in Figure 3.

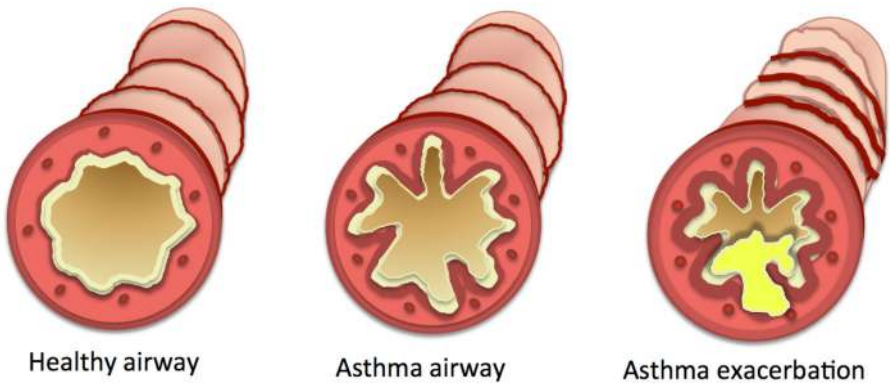

Figure 3:
A schematic drawing of the airway lumen in a healthy individual (left), compared to an asthmatic with chronic on-going inflammation (middle), and finally asthmatic airways during an asthma exacerbation (right).

Both genetic background [46, 47] and environmental exposure, such as allergens, pollution [48, 49], tobacco smoke, but also respiratory infections [50-54] early in life are major discussed risks for the development of asthma [55]. To determine the asthma diagnosis [56], patients usually have experienced reoccurring symptoms and often in association to medical history together with family-associated atopy [46, 57-59], the final diagnosis is confirmed by performing spirometry measurements and an available asthma questionnaire if necessary [60-63]. Standardised FEV_1 improvement with 12% and over 200mL after bronchodilatory inhalation is considered as having reversible airflow obstruction. The standardised guidelines that help to categorise and describe asthma and severity are called “Global Initiative for Asthma” (GINA), and are often used to estimate disease state and how to improve management [3].

Not long ago asthma was considered as one homogeneous disease. The different severity degrees of asthma were classified as mild, moderate or severe asthma. Nonetheless, asthma has been differentiated as controlled asthma, poorly controlled or uncontrolled asthma, meaning that some patients did not experience

symptom relief although they frequently used their medication [64-66]. Recently, the asthma disease has been described as existing as different phenotypes [30-32] and is nowadays considered more of a syndrome, rather than one specific disease [67]. Different types of asthma could differ in early- or late onset, obesity-associated asthma, cold-triggered asthma, eosinophilic dominant or neutrophil dominant asthma. Clearly, there are also patients overlapping in two or more of these phenotypes, as observed during performed cluster analyses studies from different cohorts [68-70]. Around 70% of asthmatics are atopic, having a predisposition of Immunoglobulin E (IgE)- and eosinophilic inflammation in this population [71, 72]. Nevertheless, childhood asthma is more or less atopic and associated with Th2 type immune responses [42, 71-73]. The non-atopic asthma seems to evolve later in life and is also more severe, but not related to allergens and in addition is difficult to treat [72, 74].

Overall, to achieve control over asthma disease, as well as acute asthma symptoms, patients are treated with β2-agonists while the long-term treatment for managing the inflammation is dampened with inhaled corticosteroids (ICS) [3]. Asthma disease affects the whole lung, from large airways to the small airways, although the symptoms of airway closure and hyperresponsiveness is most pronounced in the larger bronchi [3] that can be reversed spontaneously or with the help of the bronchodilators [20]. Between 5-10% of patients using ICS do not respond to the treatment and symptoms in severe asthma persist and more frequent wheezing and nocturnal awakening may occur despite long-acting bronchodilators and ICS [20, 75-77]. In addition, also use of leukotriene receptor antagonists have been explored in asthma, by blocking the leukotriene driven inflammation this treatment has proven to be effective in some patients alone or in combination with ICS [78, 79].

Asthma symptoms arise due to airway responses by different cells, mediators and cytokines involved. CD4+ Th2 cells producing IL-4, IL-5 and IL-13 will subsequently give rise to a so called Th2 milieu, which is allergen-induced and initially regulated via epithelial cell sensing and APC processing of recognised molecular structures and patterns from the pathogens or allergens triggering the immune response [80, 81]. Eventually involvement of cells such as eosinophils, dendritic cells, mast cells, basophils, and in addition macrophages and neutrophils which will enhance the immune response further aiming to clear the injurious irritant [26, 82]. Once the different immune cells infiltrate the lung tissue and are activated, they release different mediators and cytokines to further activate surrounding cells. Additional cells will also migrate from the circulatory system. This is what causes the inflammation. Different immune cells also contain vesicles and granules, which can be released extracellularly. The content of the granules possesses the tremendous ability to affect the airway smooth muscle cells to contract but also the more toxic and acidic granules will induce tissue damage [83-

85]. A few of the major mediators to note of, which are released from the main asthma related immune cells include eosinophil granules which contain reactive oxygen species (ROS) such as superoxide, eosinophil peroxidase, and lipid mediators such as eicosanoids leukotriene D_4 (LTD_4) or prostaglandin PGE_4 [84, 85]. Mast cells are another important cell type in asthma disease, known to possess the powerful effect of being both a cytokine releasing cell, but most importantly they are usually associated with the smooth muscle cell contracting mediators such as histamine, but also leukotrienes and prostaglandins [83]. As evidently, neutrophils are being increasingly associated with severe types of asthma, involving neutrophilic and eosinophilic mixed sputum, as well as bronchoalveolar lavage (BAL) in patient samples [37, 86-89]. Neutrophils, also present at viral-induced exacerbation in asthma [90, 91], are able to release granules filled with content such as myeloperoxidase, elastases, proteases and defensins among others [92]. Not only the short-term effects of increased immune response and tissue damage is mediated by the immune cell activation and degranulation, but the long term effects occur. The continuing effects of further increased tissue damage as well as increased repair rate, eventually tip the balance of events, which finally leads to fibrosis, remodelling and cell hypertrophy as well as mucus overproduction [10, 93-98]. As summarised in Figure 4 below, all these processes occurring in asthma may then be further exacerbated upon allergen-, smoke-, injury-induction or infection of the airways.

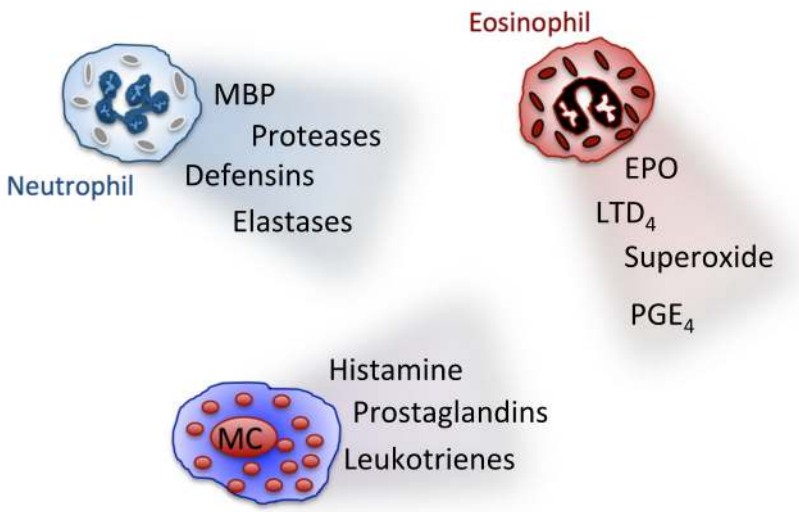

Figure 4:
Immune cells involved in asthma and asthma exacerbations, and their main released mediators. Mast cell (MC), Major basic protein (MBP), Eosinophil peroxidase (EPO), Leukotriene D_4 (LTD_4), Prostaglandin E_4(PGE_4).

COPD – Chronic obstructive pulmonary disease

In lungs of patients with COPD, two different clinical manifestations are observed in the small airways; emphysema including destruction of the alveolar ducts, and fibrosis with the excessive tissue repair which results in stiffness of the tissue [96]. These two phenomena can even appear in close association irregularly scattered within the same lung tissue part. On the organ level, this is experienced as reduced recoil, meaning loss of stretch, but also stiffness of the lung making it difficult to breathe and mainly to exhale the respire air volume [7, 99, 100]. Bronchitis, inflammation of the larger airways, is also present in COPD lung and is expressed as tissue swelling and mucus overproduction, however, this mainly occurs in the larger airways, while emphysema is more present in the alveoli (small airways) [100]. COPD is a persistent disease with progressive airflow and airway obstruction, which is non-reversible in contrast to asthma.

The increasing morbidity and mortality in COPD is estimated to be the 3rd leading cause of death worldwide [4, 16, 99, 101]. COPD brings physical but also psychosocial damage and is a huge economical burden, although the main concern is the reduction of quality of life in these patients. There is no cure for COPD, only symptoms can be relieved using anti-inflammatory ICS [102] as muscle relaxing drugs for mainstay therapy, similarly to what is used in asthmatic patients. The most severe stages of COPD also involve oxygen treatment [103].

As observed in asthma, different types of COPD can be displayed depending on the risk factors, source of disease initiation, and genetic background, but also how the disease manifests in terms of clinical symptoms and whether symptom relief or medication resistance occurs. [96, 104, 105]. These are some of the various factors that will affect the long-term lung function of these patients. In COPD, there are guidelines, similar to GINA, used to assess disease severity/progression named the Global Initiative for Chronic Obstructive Lung disease (GOLD). It is mainly based on the FEV_1 measurements [4]. A patient is diagnosed with COPD, if the FEV_1 is lower than 80% and the ratio of FEV_1 to forced vital capacity (FVC) is lower than 0.7 [4]. Although, these guidelines were found not to reflect the clinical overall symptoms and disease status in the COPD patients. Therefore, to better assess a real clinical view of the patient stages of this disease, newly updated guidelines have been created [106], which take into account FEV_1, symptoms, comorbidity and also exacerbation frequency [4, 106].

Up to 90 % of COPD patients are current or former smokers [4]. The disease occurs also in people suffering from occupational dust or chemicals, environmental pollution [101], and those having a mutation in the Alpha-1-antitrypsin gene[4], which leads to a similar destruction of the lung tissue seen in smokers. Previously, COPD was mainly associated with cigarette smoke but it is

really a disease that develops due to constant injury and overwhelming lung tissue repair that becomes chronic by causing damage at the same time as tissue remodelling [100]. Hence, the immune cells are activated and infiltrate into the injured and toxic exposed tissue, thus further inducing injury and toxicity, as shown by the schematic drawing in Figure 5.

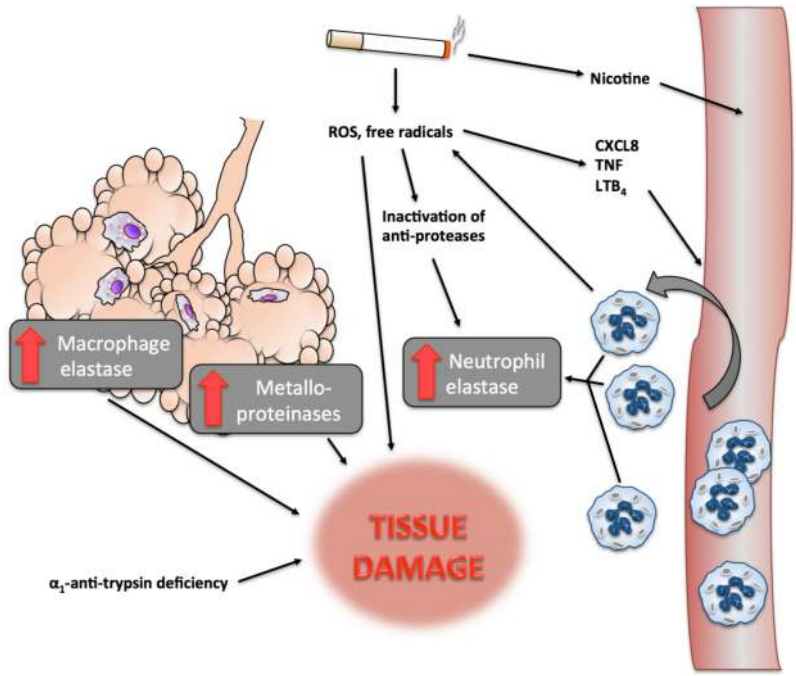

Figure 5:
Schematic drawing showing the damaging effects of cigarette smoke. Released mediators such as proteases and elastases that cause the damage in the lung tissue are released by the alveolar macrophages and neutrophils that migrate to the lung from the circulation system. Reactive oxygen species (ROS).

COPD has mainly been associated with increased cytokine induction including CXCL8 in humans and CXCL1 cytokine induction seen in mouse models mirroring COPD [107, 108]. These cytokines are potent chemotactic mediators effecting the neutrophilic infiltration into the tissue from the circulation system. As described in the previous section, neutrophils are major granulocytes releasing cells containing proteases as well as defensins. In addition, they are able to phagocytose as well as attack pathogens through formation of neutrophil extracellular traps (NETs) [109]. In the milieu of a COPD patient's lung, the major pathological mediators are the proteases known to be released from macrophages and neutrophils that infiltrate the lung and contribute with tissue damage, eventually leading to lung tissue destruction and emphysema. The ROS generated upon smoke exposure of the lung tissue also contributes to an imbalanced

protease-activity [110-115]. In addition, TGFβ is a cytokine often mentioned in the pathology of obstructive airway disease. TGFβ is released by epithelial cells as well as immune cells [116, 117], and a mouse study showed the possibility of IL-13 released by Th2 cells to activate TGFβ in a fibrosis model [118]. TGFβ is able to stimulate the fibroblasts that eventually will give rise to imbalance of proliferating extracellular matrix (ECM) components such as collagen among others, leading to fibrotic tissue formation [119].

Viral-induced exacerbations of asthma and COPD

Respiratory viral infection is the most common cause of exacerbation in asthma and COPD [5, 120, 121]. Between 40-50 % of all exacerbations occurring in COPD patients are caused by viral infections, while 80% of all asthma exacerbations in children are associated with respiratory viruses and 50-75% of the cases corresponding in adults [122, 123]. Among all viral-triggered exacerbations, 65% of them are associated with rhinovirus [4, 6, 124-126]. Due to the high occurrence of rhinoviral-triggered exacerbations in both asthma and COPD, many people are affected worldwide with effects on morbidity and mortality, and therefore new therapeutic options are warranted. There is no optimal treatment for these acute respiratory conditions, only mainstay therapy dose is increased and a combination of therapies are used for symptom management, as the definition of exacerbation implies; “events that require a change in treatment because of a change from the patient’s previous status” [1].

Many *in vitro* and *in vivo* studies are performed considering optimisation and improvement of asthma and COPD models, where the allergen-triggered experimental asthma or smoke-induced COPD is investigated using different models [107, 108, 127, 128]. Nevertheless, to date, there are few, if any suitable laboratory models aiming at studying exacerbations [129], which occur widely and frequently in these two diseases in humans. Therefore, exacerbations in asthma and COPD remain a highly topical research field where there is a need for novel translational models that will eventually develop towards new improved therapy.

Unmet medical need

Currently, there is no specific therapy available to treat exacerbations occurring in neither asthmatics nor COPD patients. Mainstay treatments in both asthmatics and COPD patients available on the market are those that function as symptom relievers, but do not cure disease [130]. Only anti-inflammatory steroids, inhaled or orally administered, and airway relaxing drugs such as long-acting or short-

acting β_2-agonists are the mainstay treatments [4, 131-133]. These regularly used medications manage to stabilize disease symptoms, but are only increased in frequency and dose, once an exacerbation occurs.

The use of β_2-agonists reverse the smooth muscle contraction in the airways, thus opening up the airways and enabling better airflow. Also by using ICS, in combination or alone, more long-term effects can be achieved. Optimal dose and combination therapy can thus decreasing the inflammation in the lungs, as well as decreasing the mucus secretion, tissue swelling, cytokine release from the epithelium and in turn immune cell recruitment [102]. Even if the anti-inflammatory ICS decrease the inflammation, they do not guarantee patients will not experience future exacerbations [6, 104, 130, 134]. Likewise, the use of ICS can only be increased to a certain level and also high dosing may lead to adverse effects [135, 136]. Still all patients do not respond to the steroid treatment in terms of symptom relief, which results in hospitalisation [137, 138]. The combination therapy of different pharmaceuticals administered in these two patient groups might differ although the concept stays the same; principally the approach of broncho-relaxing and steroid treatment is kept.

Airway cells and rhinovirus

As mentioned previously, the epithelial cells lining the airways are the first structural cells in contact with the external environment inside the lung: they form a mechanical barrier while sensing different particles and pathogens present in the respiratory air [139]. This makes the epithelium the first line of defence but also the main target for rhinoviral infection. Therefore, these cells have the ability to mount different responses early on, as the first cells being exposed to different stimuli, but most importantly and pathologically relevant due to this power of possessing the key regulatory role of up-stream events, epithelial cells also set the surrounding milieu. This in turn is detrimental for the downstream effects, involving cells and cytokines, but most importantly affects the fate of health state or disease depending on the existing stimuli but most notably the combination of triggers and genetic background [14].

Already during the 1860s, asthma researcher Salter tried to describe the worsening of asthma disease as attacks that could last a few days, nowadays described as exacerbations, still to date, we are in need of a way to elucidate the mechanism of exacerbation [140, 141]. It was believed for many years that the rhinovirus, now knowing to be responsible for the majority of the viral-induced respiratory exacerbations, was thought to not affect the lower airways [5]. Rhinovirus thrives at 33°C in the nasal mucosa where it mainly infects cells and replicates [142]. Thus, the word “rhino” originates from the Greek language meaning “nose”, as it

has both named the animal rhinoceros, as well as the nasal-infectious; rhinovirus. Nowadays, it is known that the rhinoviral infections do also descend further down into lower airways at 35-37°C where it resides, and causes these worsening periods in both asthmatics and COPD patients when infecting the bronchial epithelium [143-148]. Science moved large steps forward in this field once the PCR method was invented and viral replication became evident when analysing bronchial epithelial cells [149-151]. Subsequently, more population studies have been performed where rhinoviral infection peaks could be associated with increased exacerbation frequency [5].

The rhinovirus operates by invading the host cells, most likely the epithelial cells, via membrane receptors. The major group uses the ICAM-1 receptor while the minor group uses the LDL receptor for entry into cells via endocytosis and can then access the host cell machinery for replication [152-154]. Rhinovirus is single stranded RNA (ssRNA) enclosed by a capsid, and during its replication cycles dsRNA is produced, which is the ligand for Toll-like receptor 3 (TLR3) but can also be sensed by other receptors called RIG-I-like receptors (RLR), located in the host cell [142, 155, 156].

Once the epithelial cells are able to sense a pathogen or when mechanical injury occurs, various mediators will be released. In this case, the epithelium serves as a major upstream regulator of immune response and thus induces downstream signals that will propagate further and induce cells of the innate and adaptive immune responses. Aiming for targeting various cytokines in chronic airway inflammation would be an exceptional strategy for drug development. Due to cytokines regulating the fate of both immune cells and structural cells, these mediators are of great interest to control. In particular, epithelial derived cytokine blocking would provide further upstream inhibiting effects such as influencing a complete pathway or agonist as far up as possible in a signalling cascade. Accordingly, therapy would be able to interfere with distinct inflammatory processes while enclosing many downstream pathological effect [157]. Therefore, it is detrimental to focus study on the epithelial cells but also cytokines in relation to the innate receptors, and by using relevant models to do so.

Lung innate immune mechanisms and pattern recognition receptors

As mentioned, both the structural epithelium and immune cells express different immune receptors called pattern recognition receptors (PRRs). This family of receptors are able to sense so-called pathogen associated molecular patterns (PAMPs), which are different molecules or epitopes of pathogens or allergens. Various PAMPs can be recognised by different PRRs depending on what the PRR is able to recognise; bacterial fragments, lipopolysaccharide (LPS), viral RNA,

DNA and so forth [158-160]. The focus in the thesis has been on dsRNA-sensing receptors due to their involvement in rhinoviral-induced exacerbations in both asthma and COPD. The dsRNA-sensing TLR3, as well as cytosolic RLRs Retinoic acid inducible gene I (RIG-I) and Melanoma differentiation associated protein 5 (MDA5) were investigated in this thesis. Other important innate receptors not studied in this thesis are for instance Dectin or Protease activated receptor 2 (PAR2) to mention others, which are possibly involved in allergen- and viral-triggered interacting signals [8, 161-165].

Upon activation of PRRs the downstream pathways are activated towards transcription factor induction following gene expression of cytokines. The cells of the first line of defence are very potent producers of both anti-inflammatory and pro-inflammatory cytokines, as well as anti-viral interferons. Some PRRs are able to recognise other molecules besides pathogens such as endogenous molecules commonly generated during inflammation or injury. These molecules are then called damage associate molecular patterns (DAMPs), being equally- if not more - immunogenic compared to PAMPs. This is an excellent feature evolved through evolution, due to the fact that dead and mechanically damaged cells or intracellular content needs to be cleared by the immune system in order to protect the body from accumulating non-functional or old cells. These PAMPs and DAMPs thus serve as alarmins and will trigger an immune response by the innate immune system before the adaptive immune system can assist with a delayed although specific response. Another part of the immune system besides alerting and activating immune cells is the ability to induce cell death and clearance of unwanted cells. There are several important pathways involved in the so-called cell death regulation and different forms of induced cell death occurs for maintaining homeostasis, although sometimes they are the direct link to disease when not operating correct. Few studies have emerged regarding the control of programmed cell death and potential importance in exacerbation in chronic lung disease [166].

Evidently, exacerbations in asthma and COPD can be caused by many different stimuli, and in addition the genetic predisposition in combination to the environmental milieu together give rise to lung diseases. Mechanisms driving these events are not fully revealed and this thesis studies some of the important pathways and potential targets that might be responsible in initiation of exacerbation as well as driving the reaction forward. Innate immune mechanisms as well as cytokine profile in different model systems have been the focus of this thesis.

Molecular signalling; PRRs and transcription factors

PRRs are tightly regulated by their endogenous expression or capability of being induced, which then signals further downstream in the cell and engages different internal pathways. The main pathways studied in this thesis either involve signalling via NF-κB or IRF3 transcription factors (also displayed in Figure 6). NF-κB is activated upon TLR3 activation involving the adaptor molecule TIR-domain-containing adapter-inducing interferon-β (TRIF) and the final result is translocation into the nucleus of the NF-κB subunits where they dock onto DNA and initiate the transcription. Transcription of cytokines is then initiated, but also newly produced transcription factors, as well as up-regulation of specific mRNA for additional cellular receptors. The transcription factor IRF3 is regulated in a different manner, where actual phosphorylation rather than subunit-dissociation occurs, in turn for initiating transcription of immune-modulatory genes. IRF3 mainly gives rise to interferon production, while NF-κB typically induces TNFα and CXCL8. TSLP was shown to be induced through activation of both transcription factors. As this cytokine has a key-regulatory role in priming dendritic cells, it exerts a pathological role in asthma and COPD [167, 168].

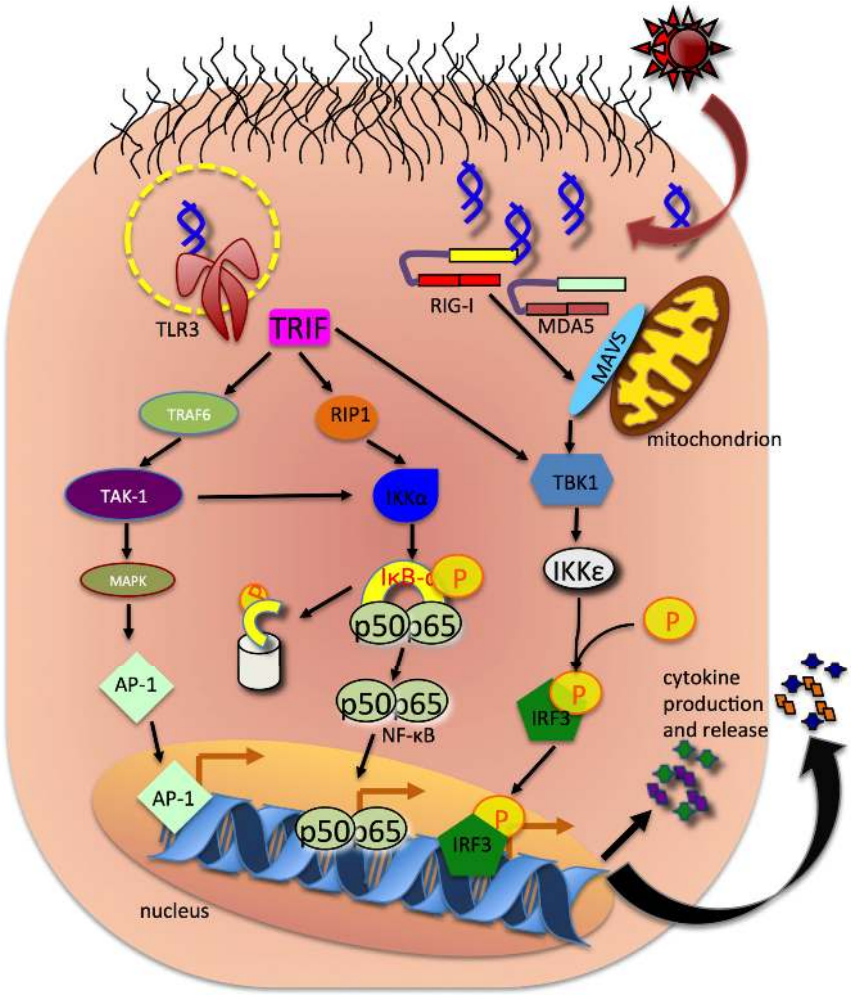

Figure 6:
Schematic drawing of the signalling pathways downstream of the three main studied PRRs, upon viral RNA stimulus.

Th2-upstream cytokines

In our mouse models of asthma exacerbation, TSLP as well as two other important Th2-upstream cytokines were highlighted, namely IL-33 and IL-25. All three cytokines are epithelial-derived cytokines possessing the property of being among the first cytokines released by the epithelium and further inducing downstream effects on structural and immune cells, and directing them towards a Th2 immune response [169-171], hence the collective expression and umbrella term "Th2-upstream" is used. Besides exerting their separate effects via autocrine or

paracrine stimuli on immune cells or structural cells, all three Th2 upstream cytokines can activate the newly explored ILC2 cells [172, 173]. The ILC2 corresponds to a small fraction of the total amount of T cells in the lung, yet they possess an incredible property to influence Th2 immune response in the airways [29, 174]. Due to pathological link of TSLP in both asthma and COPD, various anti-inflammatory compounds were tested in this thesis using *in vitro* settings to potentially inhibit or reduce dsRNA-induced TSLP expression in primary HBECs donated from asthmatics and COPD patients. While TSLP was studied in all five papers included in this thesis, the other two Th2-upstream cytokines IL-33 and IL-25 were only investigated in the last two paper (IV and V). All three Th2 upstream cytokines are described briefly in separate sections.

TSLP

TSLP was originally discovered in the culture supernatants of a mouse thymic stromal cell line [175, 176] and thereafter the TSLP receptor (TSLPR) was discovered [177]. Subsequently, it was shown that TSLP binds to the complex of two surface receptors, the low affinity TSLPR and the IL-7Rα chain, which provides the functional link and induces a signalling cascade, mainly involving the transcription factors STAT5, JAK 1 and 2 [177-179]. The production of TSLP is mainly restricted to the surface-type cells such as airway- and gut epithelium and skin [180], but also produced by fibroblasts, smooth muscle cells, and immune cells such as dendritic cells and mast cells [181-183]. The TSLPR is on the other hand broadly expressed on different cell types such as dendritic cells, eosinophils, macrophages, basophils, B and T cells as well as structural cells such as epithelial cells and smooth muscle cells [184-189]. This hub-cytokine is therefore involved in key-regulatory mechanisms where it activates dendritic cells to produce CCL17 and CCL22, which will attract CD4+ T cells [182, 190, 191]. Dendritic cells primed by TSLP produce the Th2 effects such as production of IL-4, IL-5 and IL-13 [182, 184]. Also direct T cell activation by TSLP occurs where it plays an important driving role in recruitment and activation of mast cells and eosinophils and B cell activation inducing IgE-production [190, 192, 193]. Thus, TSLP might serve both as a link between the innate and adaptive immune system as well as producing the possible development of Th2 directed immune response upon a viral infection [194, 195].

Overproduction of TSLP has been linked to pathological effects in several inflammatory diseases such as in skin disorders found in the lesions in patients with dermatitis [196-198] as well as allergic rhinitis [195, 199] and marked overproduction of TSLP has been shown in both asthma and COPD [200-202].

IL-33

The latest member included in the IL-1 family is the IL-33 cytokine. As recently as 2005, IL-33 was first identified as a ligand for the ST2 receptor [203]. It has been identified to be stably expressed on the surface of Th2 cells, but not Th1 cells [204]. This finding connected the ST2 receptor and thus IL-33 signalling pathway to Th2 mediated responses without involvement of cytokines IL4 or IL-5 [205, 206]. The signalling of IL-33 seems to demand an associated receptor complex consisting of ST2 and the IL-1R accessory protein (IL-1RAP) [203, 207], where it activates the cascade chain of signalling events involving TIR domain; MyD88, IRAK1/4 and subsequently, NF-κB and ERK1/2, p38 and JNK1 [203, 208, 209]. The receptor enabling signalling is mainly expressed in macrophages [210], NKT cells [211], basophils [212], mast cells [209, 213], eosinophils [214], dendritic cells [215] as well as Th2 cells [204] and ILC2s [216]. While IL-33 itself is mainly expressed by the lining cells such as endothelium and epithelium, it is also expressed by fibroblasts and smooth muscle cells [208]. In addition, induced expression by macrophages and mast cells is seen upon infection and inflammation [217, 218]. IL-33 has also been shown to possess the augmenting role of exacerbating bronchoconstriction due to its possibility to interact with the mast cells and induce degranulation as well as affect storage and synthesis of mast cell mediator serotonin in an allergic mouse model [219].

IL-33 seems to exert dual function with both possessing a protective role in the cardiovascular system [220] as well as involvement in inflammation and disease [221]. Among the first studies involving IL-33 neutralisation showed reduced airway inflammation in a mouse model [222], but also diseases developed in mouse models, such as lupus or arthritis, were reduced or supressed by IL-33 blocking antibodies [223, 224]. Also, ulcerative colitis patients showed elevated expression of IL-33 in the inflamed mucosa [225]. Regarding airway disease; increased levels of IL-33 were observed in smooth muscle cells from patients with severe asthma [226]. In addition, Jackson and colleagues recently showed the IL-33 dependent Th2-inflammation during rhinoviral infection to cause the asthma exacerbation [227]. Another study showed increased ST2 and IL-33 in induced sputum from asthmatic children [228]. In addition, a study conducted in asthmatics having an exacerbation showed elevated levels of the soluble ST2 protein in exacerbating asthmatics compared to controls. The raised serum concentrations of ST2 correlated with the severity of the exacerbation [229].

It has recently been revealed that IL-33possesses the dual role of disease enhancer and protective mediator. In addition, IL-33 function differs and at the same time also operating within different cellular compartments. The processed protein, released by cells serving as a cytokine with immune-modulatory role, as well as nucleus stored IL-33 in its full length is on the other hand, released upon stress, injury or cell rupture. Therefore also named an alarmin [230, 231].

IL-25

IL-25 has not gained as great attention regarding Th2 upstream effects as much as the other two previously mentioned proteins TSLP and IL-33. IL-25 is also known as IL-17E and belongs to the IL-17 family of cytokines [232] and is considered important in a more AHR-inducing manner as well as involvement in remodelling with sub-epithelial thickening [233-235], above Th2 cytokine induction [236-238]. Both immune cells as well as structural cells express IL-25; although significantly elevated levels were also found in bronchial mucosa in asthmatics as compared to control subjects [239, 240]. Yet, IL-25 is constitutively expressed by eosinophils and basophils from both allergic and healthy individuals, although IL-5 is able to potently initiate abundant levels of IL-25 [241]. As functional IL-17A signalling requires a receptor complex of two receptors, it seems that also IL-25 signals through a receptor complex composed of IL-17RA and IL-17RB [242]. The signalling downstream of IL-25 points to the involvement of transcription factors GATA3 [241] and STAT6 [238].

Involvement of pro-inflammatory cytokines in exacerbation

On-going chronic inflammation might involve key immune regulatory cytokines such as the studied Th2 upstream cytokines, and serve as good drug targets for future treatment in asthma and COPD exacerbations. Although pro-inflammatory cytokines and chemokines that will attract and activate effector immune cells, also being induced at exacerbation, it might be as important to investigate and elucidate the full spectrum of involved molecular mechanisms. Nevertheless, huge focus of this thesis has been to find potential key targets in asthma exacerbation, where TSLP and IL-33 have gained large focus, yet other potentially involved cytokines such as TNFα, CCL2, CCL5 and IL-1β could also have a possible large contributing role in asthma and COPD exacerbations. As shown in paper IV all four cytokines were increased at exacerbation, while TNFα also increased after allergen exposure as well.

The important CCL5 is known to be induced in bronchial epithelium during RV infection [35], and was induced at exacerbation in our study in paper IV. CCL5 is one of the key-chemotactic and activating cytokines for eosinophils as well as being involved in T cell recruitment since it serves as a ligand for CCR3 receptor, which is expressed on both eosinophils and T cells [243]. While eosinophilia is largely associated with allergy and Th2 immune response, the pro-inflammatory IL-1β on the other hand is mostly associated with macrophages and the Th1 immunity [244]. Despite this association, epithelial cells from asthmatic subjects showed larger expression and production of this cytokine [245]. In a murine model, IL-1β showed to be involved in inflammation with leukocyte infiltration,

mucus overproduction, as well as production of CXCL1, MMP-9 and MMP-12 [246]. In the lungs of COPD patients IL-1β correlated negatively with FEV_1 [247], indicating this cytokine was a marker of clinical aspects in both asthma and COPD. Therefore, when it comes to the aim of elucidating the key involved mediators in exacerbation of both asthma as well as COPD, IL-1β became an obvious mediator for further detailed investigation.

IL-1β

As this cytokine is mainly associated with pro-inflammatory and auto-inflammatory events, the potential role in asthma or COPD exacerbations was not clearly defined until recently. Nevertheless, immunohistochemical staining of IL-1β showed increased expression in asthmatic airways compared to controls. In addition, COPD patients' serum levels of IL-1β have shown to be elevated. [248]. However, the link to respiratory exacerbations was never discussed widely in neither of these two diseases until recently where a collaboration of an Australian and a Chinese lab with Fu and colleagues suitably showed the correlation of IL-1β together with systemic inflammation as risk markers for future exacerbations in both asthma and COPD [249].

IL-1β belongs to the IL-1 cytokine family just as the more recent emerged cytokine member IL-33, although their function may differ greatly regarding their contribution to exacerbation in airways of asthmatics and COPD patients [250]. IL-1β is produced by both macrophages as well as epithelial cells, and is the ligand for the surface membrane bound IL-1R, as well as being able to bind to the soluble receptor form sIL-1R, being a regulatory molecule of free circulating IL-1β [250, 251]. In addition, the IL-1 receptor antagonist; IL-1Ra can bind to the same sites as IL-1β on to IL-1R although it is not able to induce the signalling pathway. Increase of IL-1β can either be managed by decreased production of the cytokine itself or increased expression of the naturally occurring antagonistic IL-1Ra. Synthetically produced IL-1β antagonist is called Anakinra and is regularly used in patients suffering from arthritis, gout as well as type 2 diabetes [244, 252, 253]. Studies point to the elevated IL-1β not being the main concern in the case of auto-inflammatory diseases, instead the conditions point to an imbalance of IL-1β/IL-1R [254, 255].

IL-1β signals through the MAPK involving NF-κB or AP-1 transcription factors, leading to the mRNA production of IL-1β. Once IL-1β is produced as mRNA and translated, it is considered as biologically non-active pro-IL-1β form, which demands activation by proteolytic cleavage via caspase-1 [253, 256] activity or by neutrophilic released elastases and metalloproteases [257-259]. The caspase-1 in turn needs activation via inflammasome formation and pro-caspase-1 cleaving involves the NLRP3 complex [260]. The NLRP3 can in turn be enhanced in activation upon DAMPs and alarmins released from damaged and leaking cells,

such as ATP or Uric acid [260-263], or by viral infection [264]. Summary of NLRP3 function involved in IL-1β processing is displayed in a schematic drawing, Figure 7.

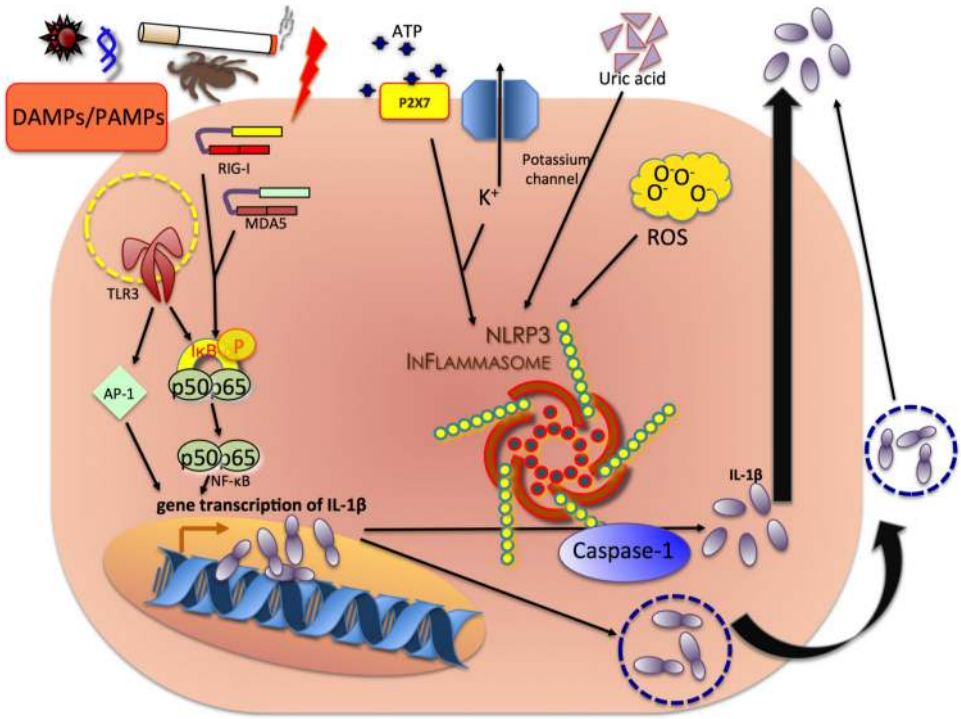

Figure 7:
Post-transcriptional IL-1β production generates the so-called pro-IL-1β protein that is not bioactive and requires proteolytical cleaving by caspase-1. The caspase-1 in addition is necessary to activate via NLRP3 inflammasome formation and cleaving of pro-caspase-1 into an active enzyme. NLRP3 activity can be enhance via extracellular ATP, Uric acid, ROS or other injury/stress response.

Translational *in vitro* and *in vivo* models of viral-induced exacerbations

In this thesis different experimental models have been utilised and discussed in order to find new targets and molecular mechanisms potentially involved in causing and driving exacerbations in obstructive lung diseases. Primary cells donated from both asthmatics and COPD patients have directly been a source of human material to study the phenotype in diseased airways. Here, while introducing dsRNA to the chronic inflammatory phenotypic background, different drugs have been used to inhibit transcription factors and investigate the further effects on cytokine expression in both bronchial epithelial cells from asthmatic as well as COPD donors. This opportunity to study patient material compared to working with cell lines, brings the *in vitro* setting towards more translational interpretation.

Another step further in this work involves development of experimental asthma by using two different allergens and protocols to be able to further study experimental asthma exacerbations. In addition to this, the obtained model was then used to study parameters known to be associated with human asthma exacerbations. Animal models have been used for decades and are necessary to use when the *in vitro* or *ex vivo* systems can no longer be used to study different cell interactions and events occurring at tissue level or compensatory or inhibitory mechanisms that can occur in the whole organ or systemically in an individual. Mouse models are considerably inexpensive and easy to handle, while having the possibility to knock-down certain genes and therefore studies with isolated effects, cells or cytokines can be performed [265].

dsRNA as a challenge agent of exacerbation

The focus of this thesis is viral-triggered exacerbation and in particular the human rhinovirus generates dsRNA during its replication [142]. To be able to study respiratory exacerbations triggered by rhinoviral infection, RV1β infection or use of synthetically available molecules known as Poly(I:C), was applied. This type of stimulus, synthetic dsRNA, is widely used as a viral mimic in both *in vitro* and also *in vivo* studies [266-268], and gives the opportunity for better control of dose and timepoint and exposure rate compared to usage of real virus, which replicates differently between batches, and mouse strains. Nevertheless, studies using rhinovirus have mentioned the difficulty with using real RV for infection of cells or animals [269]. In addition, the virus replication and experiments involving viruses require more effort and cost and might be difficult to perform at all

laboratories available. This makes the viral stimulus less reproducible. Also, we have shown in paper III, the ability to generate similar results regarding the initiation of exacerbation in allergic experimental allergic asthma, either by using superimposed RV1β or dsRNA on top of the allergen-induced lung inflammation.

Primary cultures of human bronchial epithelial cells

Using *in vitro* settings is both convenient and cost-productive. Especially, work with epithelial cell lines provides more easily-handled and highly reproducible experiments, giving more accurate results. On the other hand, studies performed in cell cultures might be oversimplified and may lose the translational value, or just do not behave substantially as the primary cells in sense of producing a particular cytokine or responding to a stimuli or a drug in the same way as the airway cells. Bronchial brushings, where cells are sampled from human airways during a bronchoscopy, provide both cell material to work with for investigation of particular mechanism or pathway activation, but most of all: the possibility to actually study a specific disease phenotype expressed in the airway cells, which brings great value and translational results to be interpreted.

When working with patient donated primary HBECs, importance was to focus and evaluate mechanisms of dsRNA-induced cytokine and transcription factor interplay by using different inhibitors. This setting has been extremely valuable since patient material was used that still possesses the disease phenotype in cell cultures as originally had existed in the lung tissue. In addition, the use of epithelial cells, as the first responders upon pathogen interaction in a lung, showed their important role as upstream regulatory cells, and their function was skewed and obviously changed when comparing responses by HBECs from healthy with disease. Numerous studies have recently proven the importance of the epithelium as powerful upstream regulatory cells [12, 14], not to forget the ability to produce the so-called upstream-Th2 cytokines TSLP, IL-33 and IL-25 [12, 169].

Animal models of asthma and asthma exacerbations

We have used mouse models (study III-V) to strengthen the knowledge of innate immune response and mechanistic pathway involvement by using mouse models that mimic the human asthma exacerbations. Both the well-known and frequently used allergen ovalbumin has been used, as well as the more translational and currently emerging models of using house dust mite (HDM) extract as allergen challenges [270]. Mice do not develop asthma spontaneously, but we are able to use allergens to induced experimental asthma-like symptoms and by adding the viral component directly on top of the allergic inflammation created beforehand,

we are able to study events similar to those occurring during asthma exacerbations in a human lung [265, 271].

Previously published data on exacerbation in mouse models has been slight. The latest studies of exacerbation involving HDM combined with dsRNA by Clarke and colleagues were similar to our HDM model, although they used a high dose of dsRNA, which might be considered excessive [272]. Another research group aiming to study asthma exacerbations in HDM-model combining superimposed real rhinoviral infection were not able to produce the exacerbation expected in their asthma exacerbation model, probably due to the viral component not did not producing the anticipating effects in the mice [273]. In addition, both studies had their main focus to test therapeutics and also focused on aspects such as airway hyperresponsiveness (AHR) or cytokines in their studies [272, 273]. None of these studies looked into the Th2 upstream cytokines IL-33, IL-25 and TSLP, in a relevant HDM- and dsRNA-induced asthma exacerbation model as we performed in paper IV. At the same time we were able to provide a unique study of the Th2upstream cytokines while developing a new translational asthma exacerbation model in mice.

From the translational point of view it has been important to finally develop allergen-induced experimental asthma in mice and at the same time study the molecular mechanisms by knock-down of a gene coding for the pro-inflammatory cytokine IL-1β, to see whether it could play a role in exaggerating the on-going inflammatory response in the lung before and during an exacerbation.

Future treatment for asthma and COPD

As the mainstay treatments available on the market for both asthmatics and COPD patients are those that function as symptom relievers, they do not cure asthma or COPD [130]. Lately, new treatment strategies have emerged, such as blocking antibodies [274, 275] for neutralising IgE antibodies or cytokine blocking drugs affecting the Th2 cytokines IL-5 and IL-13 [276], although the cytokine blocking drugs are still newcomers from clinical trials [277]. The blocking anti-IL-5 antibodies were successful in reducing the eosinophilic inflammation when tested in the optimal subgroup of asthma, possessing the high eosinophilic inflammation [70, 278]. Blocking the Th2 cytokines would provide effects on IL-4, IL-5 and IL-13 known to be involved in Th2 cell recruitment and activation, induction of AHR and mucus hyper-secretion and remodelling [279, 280]. This generated Th2 milieu in the lung tissue and lumen involving eosinophils for instance, is optimal to target using IL-5 blocking drugs such as Mepolizumab [70, 278]. Yet the IL-5 blocking

intervention is unsuccessful in neutrophilic dominant severe asthma or non-eosinophilic COPD [18, 281].

Severe asthma, mainly being the neutrophilic dominant asthma does not respond to ICS nor would it benefit from IL-5 blocking antibodies. IL-17 has shown involvement in this type of refractory asthma where patients showed elevated levels of neutrophils in association to eosinophilia present and induce airflow obstruction and ICS resistance [282, 283]. Anti-IL-17 has been up for discussion for treatment of IL-17-high type of asthma [18, 284].

Although, these blocking antibodies towards certain cytokines are only partly a solution, since they target a specific type of asthma or COPD patient subgroup, the urge to find new treatments remain and research involving relevant and translational models are warranted, not least to combat the exacerbations occurring in asthmatics and COPD patients.

Due to the selective effects shown by the Th2 blocking antibodies, such as Omalizumab (anti-IgE), mepolizumab (anti-IL-5), lebrikizumab (anti-IL-13) [285-289], also more controversially considered drugs, possessing pleotropic effects are also now being tested in asthmatics and COPD patients, in hope to reduce the worse inflammatory features. This would in turn reduce the progressive remodelling and lung function decline over time. A few of these drugs are for instance the anti-bacterial macrolides [290-294]; as well as various types of statins [295], normally used for cholesterol lowering [296]. As shown in the second paper of this thesis, simvastatin showed to function in an anti-inflammatory matter independent of the cholesterol-pathway.

Importance for future drug development regarding chronic inflammation as well as the urge to reduce the bronchoconstriction of the airways in asthma and COPD, would be beneficial if the same compound could produce both effects. In addition, if the drug molecule size is in the lower range of compounds, it would be able to reach further down into the peripheral airways, the better. In this thesis, the first paper presented the use of small-molecular inhibitors, with the ability to affect both the bronchial contraction as well as serving in an anti-inflammatory matter by reducing cytokine production such as TSLP, TNFα, CXCL8 but also interferons.

Molecules originally found in nature inspire the design of small molecule inhibitors. Nature-associated molecules that can be modified and synthetically re-made are used more frequently nowadays. In the first paper, capsazepine and capsazepinoids called RES were designed with inspiration from the naturally occurring capsaicin, which is the substance found in the spicy chilli peppers where they contribute to the hot sensation [297, 298]. Capsaicin is a naturally occurring agonist for the Transient receptor potential vanilloid 1 (TRPV1) receptor while capsazepine functions as an antagonist for the TRPV1 receptor. Although, the anti-

inflammatory and broncho-dilatory effect exerted by capsazepine was independent of TRPV1 antagonism [299].

We have pointed out few targets in this thesis, to study their involvement in exacerbations as well as using blocking agents or knockout mice aiming to elucidate their complete mechanisms and their impact on other cells and mediators being active at exacerbation.

Aims of the thesis

The overall aim of this thesis was to develop and use translational *in vitro* and *in vivo* models to study the effects and molecular mechanisms potentially involved in viral-induced exacerbation of asthma and COPD.

Specific aims addressed in paper I-V

- Paper I explores epithelial anti-TSLP and anti-IFNβ effects of capsazepine and novel capsazepine-like broncho-relaxants using primary bronchial epithelial cells from asthmatic and COPD donors.

- Paper II tests the hypothesis that simvastatin may inhibit dsRNA-induced TSLP in human primary bronchial epithelial cells obtained from COPD and healthy donors.

- Paper III employed dsRNA challenges *in vivo* in mice with established experimental OVA-induced allergic lung inflammation to develop a model of viral induced exacerbation of asthma.

- Paper IV tests whether dsRNA challenges in mice with HDM-induced experimental asthma would produce important translational features of asthma exacerbations.

- Paper V studies the role of IL-1β on inflammatory features in an HDM-induced asthma exacerbation model.

Methods and experimental settings

The methodology section in this thesis aims to give an overview of the main methods and techniques used in paper I-V. A more detailed description of the materials and methods used can be found in each paper, but is also summarised in Table 1, at the end of this section.

Ethical approval

In the first two studies (paper I-II), human patient material in the form of bronchial brushings was included for *in vitro* studies. The studies were performed in compliance with the Helsinki Declaration [300] and patients signed a written informed consent and received information regarding the study before brushings were obtained by bronchoscopy. All examination and monitoring of the patients, as well as sample collection and sample handling, were initially approved by the "Regional ethical review board in Lund".

The *in vivo* experiments were performed after approved ethical application by the "Animal Ethical Committee in Lund". Mouse models were performed in accordance with the GLP standards, with clear endpoints in case acute illness would demand termination.

Primary Human Bronchial Epithelial Cells (HBEC)

Primary bronchial epithelial cells were obtained from healthy donors as well as patients with asthma or COPD. The bronchial brushings were obtained during bronchoscopy using a fiberoptic bronchoscope and a nylon cytology brush to collect bronchial epithelial cells from the second- and third generation bronchi, according to published standard guidelines [301, 302]. The patient characteristics are presented in paper I and II.

The bronchial brushings were dispensed in PBS and following vortexing and centrifugation cells were collected and cultured in T25 flasks with growth medium

and hormonal supplements optimal for epithelial cell growth. The submerged monolayer cell cultures were rinsed with PBS after attachment and then fresh medium was substituted. Confluent epithelial cells were then passaged into a T75 flask and subsequently into 12-well plates, where experiments were performed. Firstly, before initiating cell experiments, the growth medium was replaced with basal medium without growth supplements, 24h prior to treatment to be able to stimulate the cells in a quiescent state within the same cell cycle phase.

Stimulation with dsRNA

Cells were exposed to the synthetic dsRNA molecule to mimic rhinoviral replication. Bronchial epithelial cells were stimulated for 2, 3 or 24 h with dsRNA alone or in combination with different compounds, as mentioned in the initial two studies; paper I and II. All dsRNA treatments were performed using one optimal concentration of 10 μg/mL, which was chosen based upon an initial dose-response experiment in our laboratory, but also from literature with previous use of dsRNA [267, 303]. The non-stimulated samples were only kept in culture medium alone representing the negative control, while dsRNA dissolved in sterile water served as the main stimuli, the positive control, to which everything was compared to. Figure 8 shows a simplified drawing of the HBEC stimuli *in vitro*.

Therapeutics in airway diseases

Prior to- and in combination with dsRNA stimulation different compounds were used to study the different molecular mechanisms in primary HBECs. The different compounds used in paper I and II were Dexamethasone, Simvastatin, capsazepine and derivates of capsazepine called RES-substances described in detail below. Also other compounds have been used but not focused on in the thesis since they only served as internal controls, such as mevalonate as negative control, or the inhibitor TANK-binding kinase 1 (TBK1) and IκB kinase ε (IKKε); BX795, that is known to inhibit effects downstream of IRF3 and thus IFNβ production. Dimethyl sulfoxide (DMSO) has also been used in the *in vitro* studies, where it served as an internal vehicle control, since it was used as solvent for the treatment substances, and added alone in the same concentration as used in the highest substance treatment.

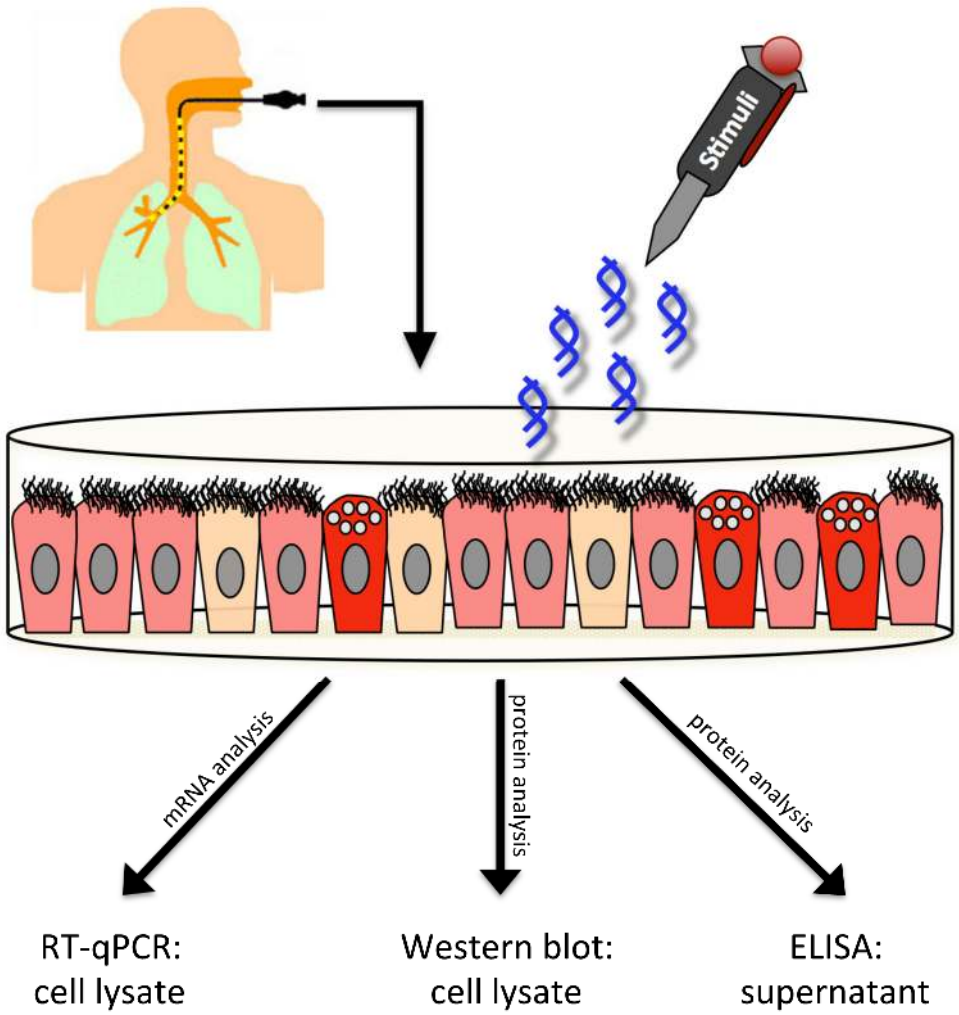

Figure 8:
Simplified drawing of the stimuli performed *in vitro*, using HBECs obtained from asthmatic and COPD patients, as well as healthy subjects. The experiment was terminated after 2, 3 or 24h with dsRNA stimuli, followed by supernatant collection for protein analysis (ELISA), and also cell lysate sampling for protein (Western blot) or gene (mRNA) analysis (reversed trancription-quantitative polymerase chain reaction (RT-qPCR),

Dexamethasone

This steroid has been used as a positive control in the second *in vitro* study to compare the unknown effects of the Simvastatin treatment. Dexamethasone molecular backbone can be observed in Figure 9 below.

Statin treatment in lung disease

Simvastatin is a cholesterol-lowering drug prescribed to patients with high blood cholesterol levels, and exerts its function by inhibiting the HMG-CoA reductase [304]. Simvastatin treatment in patients with COPD showed, unexpectedly, improvement of the lung related symptoms, and reduced risk of exacerbations. [305-307]. Various statins can be used to test the pleotropic anti-inflammatory effect nevertheless, in this thesis in paper II, only Simvastatin was used to treat HBECs in combination with dsRNA stimuli. In Figure 9, simvastatin is shown, among other compounds investigated in the thesis, demonstrating their different molecular structures.

Small-molecular inhibitors

The strategy to reduce inflammation, by way of inhibiting inflammatory cytokines, with a therapeutic drug also able to induce broncho-relaxation was explored in paper I. This study was a structure-activity-study where different compounds were tested for their broncho-dilatory and anti-inflammatory properties. These small-molecular inhibitors are Capsazepine as well as different forms of the capsazepinoid-like molecules named RESPIR substances.

CPZ - Capsazepine

Capsazepine used in paper I, for bronchorelaxation and anti-inflammatory purpose was optimised beforehand. The dose used for treatment of the HBECs was initially tested for dose-response and also optimal time point of incubation regarding the pre-treatment before addition of dsRNA. The evaluation of dose and exposure time pointed at the optimal 1 h pre-treatment prior to dsRNA addition, at doses ranging from 3, 10 to 30μM [303].

RES - RESPIR

RES substances were chemically designed to mimic the behaviour of the initially studied Capsazepine [303]. These molecules were provided and designed by the company Respiratorius AB in Lund, as a follow-up on previously designed RES compounds [299, 308-312]. The particular RES substances used in paper I are displayed in Figure 9. Respiratorius AB tested and evaluated the substances regarding broncho-dilatory effects while the anti-inflammatory property exerted in dsRNA-stimulated HBECs was performed in our laboratory.

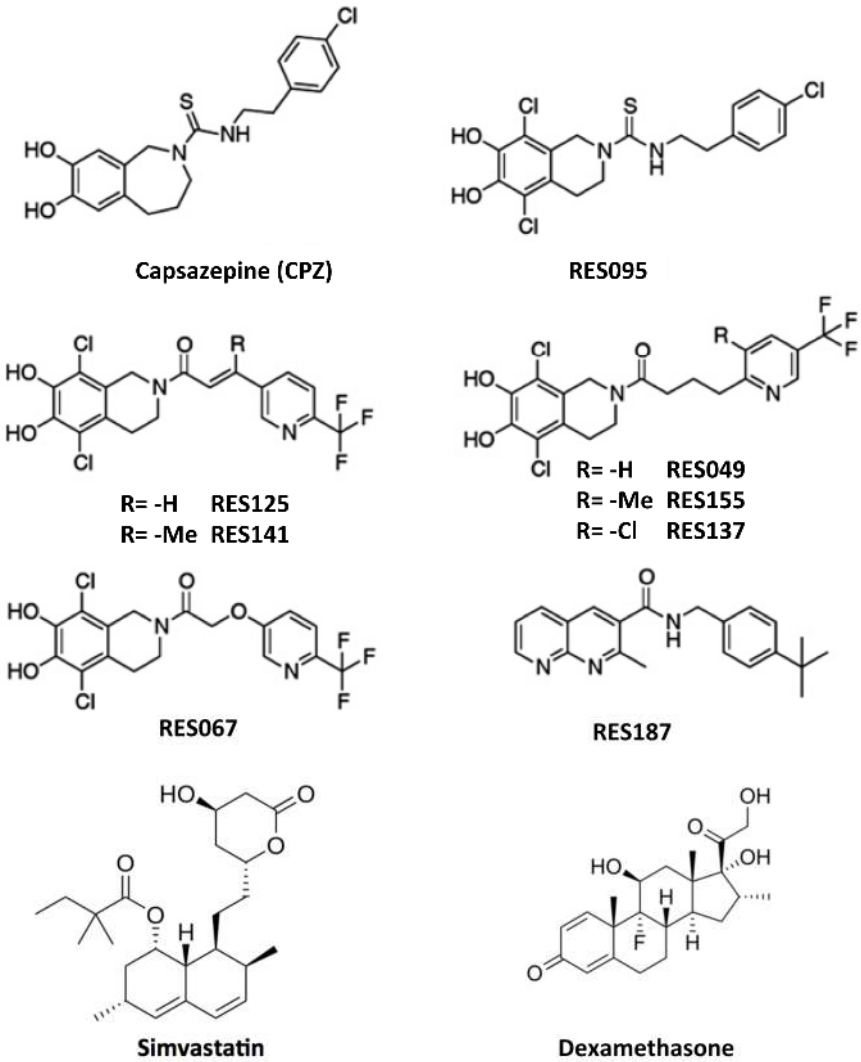

Figure 9:
Molecular structure of the different compounds used as treatment in addition to dsRNA stimuli in HBECs. CPZ and RES substances were used in paper I and simvastatin and dexamethasone were applied in paper II .

Mouse models of asthma exacerbation

In paper III-V, two different animal models of experimental asthma were used. The purpose was to produce allergic asthma in mice and to further provoke viral-induced asthma exacerbation, by introducing the synthetic dsRNA on top of the already established allergic asthma. The first *in vivo* study, presented as paper III, was the only study where real Rhinovirus was used in one group of mice for the exacerbation provocation, when administered on top of the allergic asthma. This was repeated in a second group of mice, although then using allergen-provocation with addition of superimposed dsRNA-stimuli. In paper III, initially dsRNA was administered to naïve mice without allergen challenge to study the effects induced by dsRNA alone.

The second *in vivo* study, presented as paper IV involved a new type of allergen and challenging protocol, intranasal HDM was also employed in the last study. The last study, presented as paper V, contained both wild type (WT) and knockout (KO) mice, and explored the mechanisms involved in exacerbations from the point of view where the pro-inflammatory cytokine IL-1β was present (WT) or absent (KO).

Allergen challenges

Two different allergens were used for provocation of experimental asthma in the mice. First, the well-known and commonly used chicken egg protein; ovalbumin (OVA), was used during the allergen challenge, presented in paper III. OVA was first introduced in association with a carrier adjuvant, aluminium-hydroxide, and administered as intraperitoneal injection (i.p) to generate a sensitisation towards the allergen. Fourteen days after the sensitisation, OVA was again administered, although diluted to 1% in Saline and aerosolised via aerosol-chambers over pressure at 4 bar, during 30 minutes each day, for four consecutive days. Control animals received Saline alone in the aerosol-chamber and were sensitised and treated according to the same procedure (Figure 10).

Study IV and V were both composed according to the same allergen challenge and protocol for provocation of asthma exacerbation. Now the more translational and clinically relevant allergen; house dust mite (HDM) was used, and administered 3 times per week, during 3 weeks for establishing allergic asthma, via one single administration route; intranasal inhalation. HDM was used as whole body extract from species *Dermatophagoides pteronyssinus*, diluted in Saline to a concentration of 1 mg/mL. Administration volume of HDM or Saline was in total 25 μL.

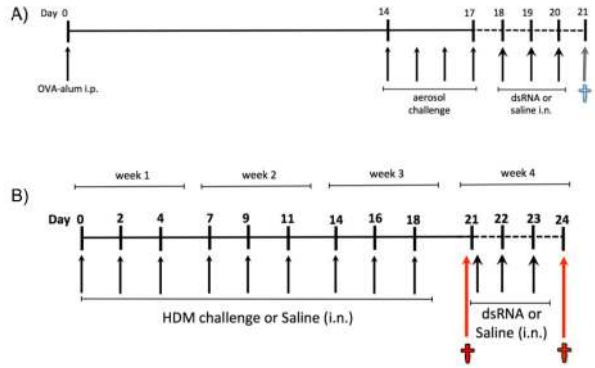

Figure 10:
Study design of the various protocols used in paper III-V. (A): the allergen provocation in paper III involved intraperitoneal (ip) injection of OVA-alum as sensitisation followed by four OVA aerosol challenges. Further, the dsRNA provocation was performed directly after the allergen-challenge, creating experimental asthma in mice. (B): the challenging protocol applied in paper IV and V, using HDM allergen for provocation of experimental asthma (3 weeks). This was followed by dsRNA administration to induce the exacerbation phase.

Experimental asthma exacerbation

As in the *in vitro* experiments involving dsRNA stimuli to mimic the rhinoviral infection, the same treatment was applied *in vivo*, in the different studies with allergen provoked asthma mouse models. Mice received a concentration of totally 50µg or 100µg of dsRNA. Before applying the dsRNA in mice with allergen-induced experimental asthma, the synthetic dsRNA was administered intranasally to naïve mice at different time points and at different doses, to evaluate the optimal concentration and duration of stimulation in mice. The exposure protocol for contained timepoints of 24h, 72h and 96h dsRNA. The 72h-exposure involving 100µg per intranasal administration and three administrations in total, once per day for three consecutive days, was chosen as the most optimal dose and time-point as most similar mimic in relation to the common cold duration and response according.

Experiment termination

The experiments were terminated 24h after the last administration, according to the protocol shown in Figure 10. Mice were given an overdose of Pentobarbital sodium, subsequently BALF was collected and the lungs were taken for further analysis. The left lung was fixed in 4 % formaldehyde while the right lung lobes were weighed and snap frozen in liquid nitrogen and stored at –80°C until analysis by RT-qPCR, Western blot or ELISA. To be able to analyse the lung tissue with

these techniques, lungs were first homogenised using different protocols, described more in detail in each method paragraph below.

Analysis of bronchoalveolar lavage fluid (BALF)

Bronchoalveolar lavage was carried out by initially performing tracheostomy and connecting a small tube to the trachea entering the airways, following inflation of 1 mL ice-cold PBS solution into the alveoli, through the bronchus tree, and collecting the fluid back in a collecting tube. The BAL is performed using gravity dependent lavage bench, and not by syringe – to preserve the lung tissue and avoid pressure damage and blood leakage into the airways and lavage samples.

Once the BALF was sampled, it was kept on ice until it was spun down at 1200rpm and 4°C, for 10 min. The supernatant was collected, and then stored at –80°C until further use. The cell-containing pellet was re-suspended with PBS and cells were counted with an automatic Cellcounter. This was followed by cytospin of 50,000 cells per glass slide, then cells were stained with May-Grünwald and Giemsa staining and lastly a differential cell count was performed using a microscope, and counting 400-600 cells per slide [313].

Total protein analysis

The BALF supernatants were taken for analysis of total protein concentration, reflecting a general inflammatory marker and a measurement of plasma exudation into the lumen. For this analysis a BCA kit was used, where BSA with known concentrations was used for the standard curve, for evaluation of protein concentration of all proteins in the sample, referred to as total protein.

LDH analysis

LDH is a well known although unspecific marker of necrotic cells [314]. Once the cells burst and cellular content is leaking out to the extracellular space, LDH among other molecules can be found in the extracellular space. LDH was analysed in BALF, to determine the grade of necrotic cells in the lumen.

Extracellular ATP analysis

Extracellular ATP is considered an alarmin, and a danger signal, which is able to activate immune responses, through purinergic receptors [261]. Since ATP has important energy transducing function and is kept in the intracellular compartment in healthy cells, once released or leaking into the extracellular space the molecule is immunogenic [315]. ATP was measured in BALF, as an indicator of cell membrane integrity.

Uric Acid

Another danger signal, one of the so-called DAMPs is the uric acid crystals formed when the stressed or infected cells are leaking uric acid into the extracellular space. This is then interpreted by surrounding cells as a warning signal that invading pathogens or other stressors are awaking an immune response, involving many different immune responses such as phagocytosis by the macrophages, activation of complement system or involvement of the inflammasome to trigger further release of inflammatory cytokines [260].

Lung tissue analysis

Homogenising samples

Frozen lung tissue was homogenised for evaluation by RT-qPCR, Western Blot or ELISA. The mechanical degradation of the lung tissue was performed using an OmniPrep Rotor Stator Generator with rotating blade and in addition the lung was placed in an enzymatically degrading lysis buffer with protease inhibitors.

Lung homogenate samples were prepared differently depending on the analysis method that followed. Preparing samples for RT-qPCR analysis, lysis buffer from the RNA extraction kit was used. While, samples prepared for protein analysis employing ELISA were lysed with lysis buffer including protease inhibitors, and protein analysis with Western Blot demanded sample preparation with modified RIPA lysis buffer.

Histology

Following experimental termination and lavage, the left lung lobe was dissected and perfused with fixative 4% formaldehyde. After fixative treatment and paraffin embedding, lung tissue was sectioned 4 μm thick and stained. For general tissue visualisation and inflammation assessment, simple background staining with Haematoxylin and Eosin (H&E) was performed. All stained sections were scanned with ScanScope.

Immunohistochemistry

For specific staining of proteins or cells of interest immunohistochemical technique was applied, following background staining with H&E. The sections were first blocked with 5% serum, following overnight incubation at 4°C with primary antibody. Then rinsing and incubation with secondary antibody enabled visualization by using 3,3'-diaminobenzidine (DAB). Simultaneously, another tissue section was used as negative control on the same glass slide, where the same staining procedure was performed although without incubation with primary antibody.

Entirely, three different immunohistochemical stainings were done among study III-V; visualising TSLP, IL-33 or neutrophils in the lung tissue sections. The results were either just shown as representative photos for each stimulus group in the study or also additional quantification was performed using ImageScope software and intensity of the staining was detected by the program, then presented per tissue area mm^2. Stained neutrophils were either manually counted using a microscope or by the program quantification.

Gene analysis

Both cell lysate from *in vitro* experiments using HBECs and lung homogenate from mice were analysed for various genes of interest coding for structural proteins, cytokines and receptors of the innate immune system (PRRs). At first RNA was isolated from cell lysates or lung homogenates, using a commercial RNA extraction kit, followed by reverse transcription (RT) of the RNA sequence into cDNA. By using Mastermixes, primers and buffers together with cDNA optimal conditions for the polymerase chain reaction (PCR) took place, described more in detail below.

RT-qPCR

Following RNA isolation, concentration of RNA in each sample was estimated using NanoDrop. This was followed by RT of 1-2µg total RNA into cDNA, and finally quantitative PCR was performed.

Both SYBR Green probe and TaqMan probes were used and human or mouse primers purchased from either PrimerDesign or Qiagen.

The PCR product was detected by a sequence detection system from with standard cycling parameters and thermocycling with real-time detection of the PCR products. After the PCR was performed Cq/Ct values were recalculated employing ΔΔCT method in Excel and then the gene expression was quantified. Gene levels were expressed as fold change in relation to a stably expressed gene referred to as a reference gene. Genes of interest from the human samples were analysed in the experiments using primary HBECs (presented in paper I and II), where two different reference genes were used coding for Ubiquitin (UBC) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Nevertheless, only one reference gene coding for 18S was applied for the mouse gene analysis, performed in studies presented in paper III-IV. The gene expression was lastly presented as normalised to its control sample at each time point.

Protein analysis

Analysing the protein expression or release in the samples, two different methods were used: Western blot and sandwich ELISA. The preparation of samples differed depending on the origin of sample and analysing method.

Western Blot

For assessing the protein expression in cell lysate or lung homogenate, samples were prepared in lysis buffer including complete protease inhibitor cocktail. Western blot was applied in order to quantify proteins situated in the cytoplasm or being released.

Totally, transcription factors, receptors, enzymes and cytokines were analysed by western blot, among study I-V. Following lysing and homogenisation of the cells and tissues, respectively, samples were diluted in sample buffer and equal amount sample was loaded on a 10% polyacrylamide gel and electrophoresed. The separated proteins on each gel were blotted onto a Polyvinylidene fluoride (PVDF) membrane or nitrocellulose membrane and blocked in 5% non-fat dry milk diluted in TBS-T. The membrane was then incubated in primary polyclonal antibody over night in 4°C, followed by washing steps and then secondary antibody incubation for 2-3h at room temperature. All primary antibodies were purchased from Cell signalling, while the secondary antibodies were horseradish peroxidase-conjugated IgG antibodies, from FischerScientific or R&D Systems. The reference genes GAPDH, β–actin or β-tubulin were used as loading control. Proteins of interest were detected with substrate and after exposure to chemiluminescence using Li-Core system and software ImageStudio protein bands were visualised. The band density was calculated from the optical density and ratio was obtained after calculations in Microsoft Excel.

ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) was employed for protein analyses in collected cell supernatants, as well as lung tissue homogenates and BALF. The cell supernatants as well BALF samples were directly analysed using the ELISA kit without any dilutions, while the lung tissue samples were pre-treated with buffer containing protease inhibitors, homogenised and centrifuged before the supernatant fraction was diluted and analysed by ELISA. Also total protein determination was performed in homogenates, thus being able to correct the tissue ELISA analysis towards the total amount of protein in each sample. The

results were finally presented in relation to total protein concentration for each sample, respectively.

Statistical analysis

All statistical calculations were performed with the software GraphPad Prism, version 5.0 and 6.0. Data are presented as mean and SEM or median with IQR. Significance levels were set to *p<0.05; **p<0.01; ***p<0.001.

Mann-Whitney test and Wilcoxon test was used to compare variance between groups. The *in vivo* data were expressed as mean and SEM unless otherwise stated and statistical test assessed in this study was unpaired t-test, after assumed Gaussian distribution. Normal distribution was initially tested by performing Kolmogorov–Smirnov.

Experimental summary

In Table 1 below, a short summary of the five papers is provided as an overview. More details, time points, study layouts and concentrations can be found in appendix with all five papers, paper I-V.

Table 1:
Summary of each study presented as paper I-V, including species, gender, disease phentype and also which concentrations of dsRNA were used. In addition, either allergen provocation was performed in case of *in vivo* experiments or drug intervention using different compounds in the *in vitro* studies.

Paper	Study performed in:		Gender:	Disease:	dsRNA	Intervention ‡ or Allergen-challenge*
I	Human	Primary HBEC	♂,♀	Asthma, COPD	10 µg/mL	RES-compounds, Capsazepine ‡
II	Human	Primary HBEC	♂,♀	Healthy smokers, COPD	10 µg/mL	Dexamethasone, Simvastatin ‡
III	Mouse	BALB/c WT	♀	Asthma	50-100 µg	OVA aerosol *
IV	Mouse	C57BL/6 WT	♂	Asthma	50-100 µg	HDM challenge *
V	Mouse	C57BL/6 WT/IL-1β$^{-/-}$	♂	Asthma	100 µg	HDM challenge *

Results and discussion

The major findings from the original published papers (study I-IV) and the submitted manuscript (study V) are summarised and discussed below.

The need of new therapeutics

As previously mentioned, the mainstay treatment for asthma and COPD are in most cases not sufficient to treat periods of exacerbations. In most cases increased doses of inhaled corticosteroids are used together with long acting or short acting β2-agonists as bronchodilators. Often oral treatment with Prednisolone is used as well as macrolide antibiotics [316, 317].

The chronic inflammatory lung diseases asthma and COPD have been studied awhile although rarely involving the viral component, which is known to be the major cause of respiratory exacerbations [5, 318]. Therefore, relevant models to study the exacerbation phase are lacking and research has yet not solved this issue of developing optimal therapeutics for managing these acute and recurrent conditions [6, 134]. That lack of optimal therapy for the viral-caused exacerbations may depend on the absence of suitable experimental models. Yet, just developing new models and studying new mediators of events occurring in any disease will not give rise to new medication just because it is a new model exploring something new and generating previously unknown data. If the experimental models are not clinically relevant or at least have a translational value, then it will just be another new model. Despite having thought through the optimal experimental layout for any *in vitro* or *in vivo* project, it will still be just a model that can never deal with all the important facets normally present in a certain disease [265, 271, 319-321].

The strategy of this thesis has been to include both *in vitro* and *in vivo* experiments, thus study molecular pathways and interpret findings from diseased bronchial epithelial cells and in addition use more complex setting such as *in vivo* models exploiting the whole animal. In our newly developed asthma exacerbation model we applied two different allergens, and in addition exploring the effect of KO of an important cytokine; IL-1β to see what role it particular could have at

exacerbation. Nevertheless, also the key-regulatory role of the epithelium was investigated, using the primary HBECs donated by patients with either asthma or COPD, and this is discussed further in the following sections.

Lung cytokine expression and transcription factors (I-II)

Cytokines are produced as mediators from structural cells in the lung as well as by the immune cells in response to many different stimuli including, viral infections. The bronchial epithelium remains a major cellular source of lung cytokines [322], not only inducing inflammation, but can also serve as amplifying molecules of the already on-going inflammation [276]. The strategy to block cytokine release or their agonist function using specific receptor blockers is therefore a cleaver strategy - to start with. Especially targeting the epithelial derived cytokines would be a beneficial approach, due to their important immuno-regulatory role, secretory properties and due to their constant exposure towards the external environment. The problem is to find a cytokine target that can affect as many pathological pathways as possible while keeping the side effects as few as possible. Thus not interfere with the immune system and general tissue functions that needs to work undisturbed.

In paper I and II, in this thesis, different pharmacological agents were used with the aim to inhibit bronchial epithelial production of the hub-cytokine TSLP. This cytokine has been shown to drive pathological events in both asthma and COPD [167, 202]. The increased expression of TSLP in asthmatic subjects has shown to be correlated with both Th2 cytokines as well as disease severity [201]. TSLP, is shown to be elevated in bronchial cells from asthmatic patients compared to healthy subjects in response to dsRNA stimulation [267], and is also reported increased in children with rhinoviral infection [323]. Also, TSLP was shown to increase upon RSV infection of airway epithelial cells, where it induced a Th2 immune response towards the infection [194]. The infection-triggered expression, among other non-infectious allergen or mechanical stressors, being able to induce TSLP, makes this cytokine hugely relevant to study in this thesis. Especially since it has been suggested as a mediator of viral infection occurring early in life, thus being the contribution to asthma development [323, 324]. Nevertheless, in addition to rhinoviral infection TSLP is also released upon allergen exposure [325-327] cigarette smoke exposure [328], diesel exhaust [181, 329, 330], or upon mechanical injury of the epithelium [331] thus can induce pathological immune response in synergy with the other epithelial derived cytokines or alone.
Besides the chronic inflammation present in the airways of asthma and COPD patients, also the bronchoconstriction is a major problem for these patients. Since

broncho-relaxing drugs are regularly taken by asthmatic patients as well as being actively used by COPD patients too, a small SAR study was performed in paper I, where different compounds were designed and tested to evaluate their capacity to block inflammatory cytokines as well as dilating constricted bronchi (paper I). In this study capsazepine (CPZ) and newly synthesised derivates of CPZ called RES-substances were investigated. The aimed blocking of TSLP expression and thus release by the primary HBECs upon dsRNA-stimuli was successful in both asthmatic and COPD donor cells. Simultaneously, the broncho-relaxing properties of the small-inhibitory molecules were tested. By initially applying LTD_4 for airway-constriction and combining then with small-molecular inhibitors, the remaining contraction could be assessed.

Broncho-dilatory effects

In Figure 11 below, the broncho-relaxing properties of the different RES and also CPZ are shown. The broncho-dilatation that was measured in paper I, was presented as % remaining contraction (%RC) after LTD_4-induced contraction. Previous studies exploring the effects of the substance CPZ tested various concentrations of either 10μM or in the range of 3-30μM [299, 303], therefore only one dose of 10μM CPZ was used in paper I. The relaxing property shown by CPZ was approximately 55 % remaining contraction, hence reduced the LTD_4 induced contraction halfway. This dilatation was exceeded by all the RES substances at the concentration of 10μM, and even lower concentration at 1μM of particularly RES187.

By developing bronchodilators, while producing small molecules able to inhibit cytokines such as TSLP, can improve further and eventually lead to improved drug targets with dual role in asthma and COPD exacerbations. The importance is not to impact on the important anti-viral interferon production, which all tested substances in our small SAR study, unfortunately did.

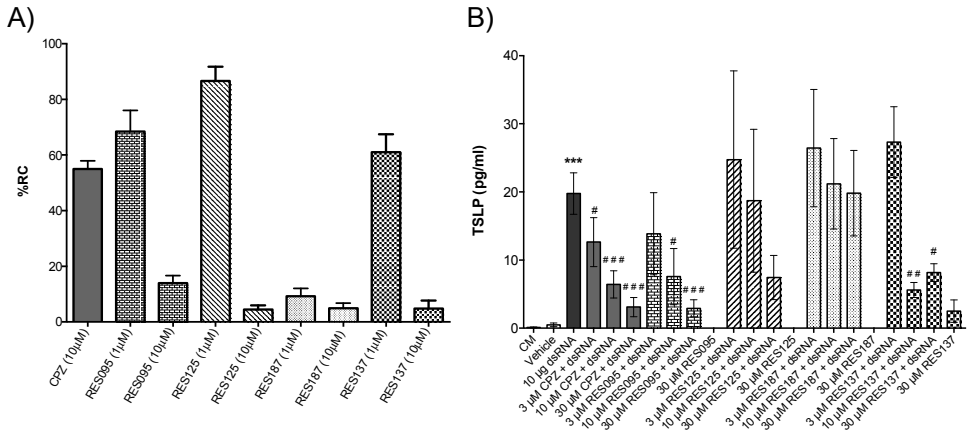

Figure 11: The substances CPZ (10µM), RES095, RES125, RES187 and RES137 were tested for their broncho-relaxing properties in contracted airways (A) and anti-TSLP effects were explored by the substances in primary HBECs donated from asthma and COPD donors.

The SAR study reveals that the broncho-relaxing effect and the anti-inflammatory effect of the CPZ derivates may depend on different parts of the molecular backbone. The different RES-substances varied in their structure (Figure 11), also exerting their effects in various magnitudes in the HBECs upon stimulus and bronchoconstriction. The anti-TSLP effect provided by CPZ and RES095 for instance was similar, although differed tremendously regarding the broncho-dilatory properties.

NF-κB and IRF blockers/inhibitory treatment

In addition to the bronchodilating and anti-TSLP effects obtained by CPZ and RES substances these compounds also reduced the transcription factor NF-κB (paper I). In paper II we further explored whether the cholesterol-lowering drug simvastatin would have the same effect on TSLP production, and if it acts via reduction of NF-κB activity (paper II). This assumed pleotropic effect exerted by statins [307], have been observed elsewhere using mouse macrophages and observing effects on adipose tissue but mainly studies involving OVA-challenged mice have tested different statins such as Pravastatin and Pitavastatin besides Simvastatin [332-335]. The assumed anti-inflammatory effects by statins explored in the various mouse studies could be confirmed, while on the other hand a newly published study in NEJM involving nearly 900 COPD patients indicated that the Simvastatin treatment did not affect the exacerbation rate over a period of more than 20 months [336]. In paper II we demonstrate that dsRNA-induced TSLP, and unfortunately also anti-viral IFNβ are decreased by Simvastatin in HBECs from

both COPD patients and healthy smokers. In contrast, minor or no inhibitory effect was shown at mRNA or protein level of TNFα and CXCL8. The TSLP expression presented as median with IQR, shown in Figure 12 below, was significantly reduced with the highest dose of Simvastatin treatment after dsRNA-induced release.

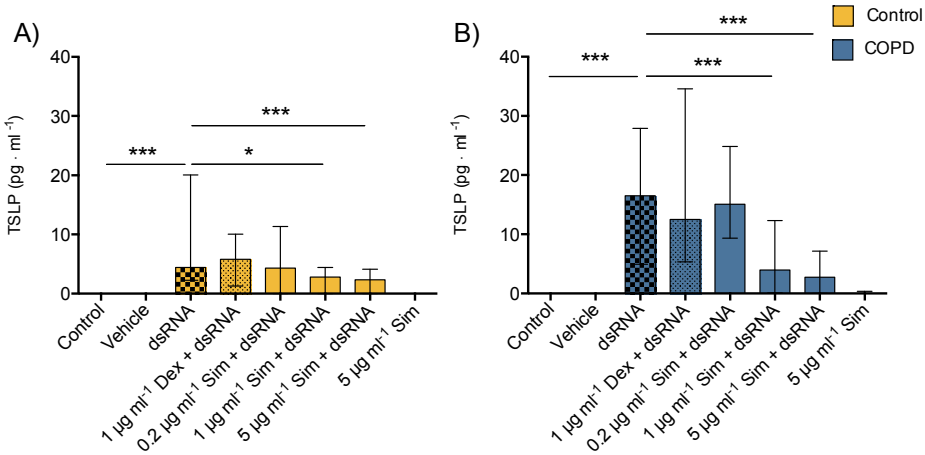

Figure 12:
Protein release of TSLP, from healthy smokers (Control) with yellow bars and COPD in blue bars. The striped bars indicating dsRNA-triggered TSLP release, being significantly increased compared to non-stimulated in both healthy and COPD. Remarkably, TSLP was almost 4 times more induced in HBECs from COPD patients compared to the healthy smoker controls, upon dsRNA stimuli. Simvastatin subsequently managed to reduce the TSLP release in a dose dependent manner.

The included dexamethasone treatment served as a reliable control. Evidently, the anti-TSLP effect by Simvastatin was greater compared to the effect exerted by dexamethasone. The exceeded effect by CPZ and simvastatin in relation to dexamethasone, make these newly designed compounds highly relevant to study for potential future anti-inflammatory compounds. The desired blocking of TSLP production was successful in both paper I and II, although also the unwanted interferon inhibition occurred in both studies by CPZ and simvastatin, respectively. Constitutively elevated levels of TSLP have been described in the airways of both asthma and COPD patients [167, 337, 338]. In addition, the expression of TSLP increases upon dsRNA or rhinoviral stimuli [303, 339].

By performing Western blot and analysing NF-κB, as performed in paper I, where it was dose dependently inhibited by CPZ (Figure 11 above), was now not affected by the simvastatin treatment at any dose tested (Figure 12) in paper II. Research groups have shown that NF-κB is initiated in order to induce TSLP mRNA expression [340-342]. Abe and colleagues showed anti-inflammatory effects

through IRF3 inhibition in macrophages, when using Pravastatin and Pitavastatin [332], whereas Kato *et al.* discussed the potential involvement of both NF-κB and IRF3 for TSLP mRNA expression using siRNA studies [342]. We clearly demonstrated in paper II, also explored in Figure 13 below, that IRF3 was successfully and significantly decreased in a dose dependent manner by simvastatin treatment after dsRNA-induction in primary HBECs.

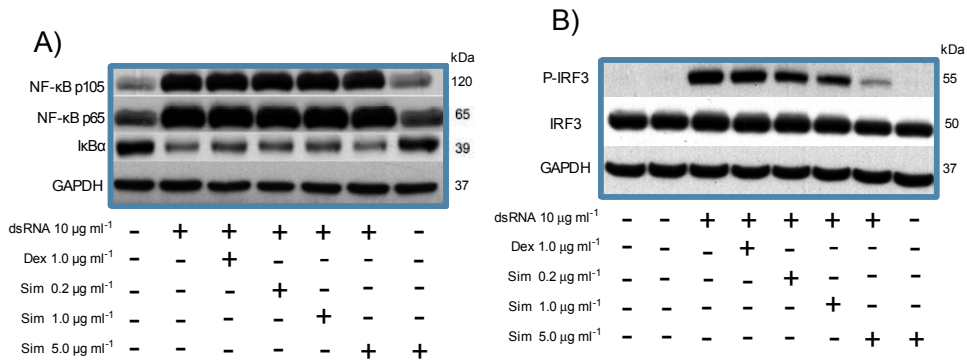

Figure 13:
Representative Western blot figures indicating no inhibitory effect of transcription factor NF-κB on any of the subunits or the accessory protein IκB-α by Simvastatin treatment (A). The transcription factor activity of IRF3 (phosphorylated IRF3) was on the other hand decreased dose-dependently (B).

Mevalonate independent effects by Statins

The anti-inflammatory effects by simvastatin were not dependent on the mevalonate pathway, since addition of recombinant mevalonate did not affect the simvastatin-inhibitory effects on dsRNA-induced TSLP production in HBECs, compared to the treatments without mevalonate.

Discover new targets for future drug development

In paper I we found that dsRNA-induced TSLP was inhibited and that the reduction of TSLP was associated with reduction in NF-κB expression by CPZ and RES substances. In contrast, in paper II we found that IRF3 was the transcription factor being involved in the anti-TSLP action by simvastatin. The different effects of CPZ and simvastatin shown in paper I and II seemed to exert their effect by inhibiting different pathways, although both leading to TSLP and IFNβ inhibition. Different structures of the molecules give them their different properties. Also other properties that might influence the inhibition of transcription factor in various directions, such as the use of patient material from different

cohorts, mainstay therapies the patient is currently using, but also time points and doses of the stimuli *in vitro*.

Being able to use small molecules for dilatory purpose would be beneficial and are important to deliver into the peripheral airways where obstruction also may occur. Also, in the small airways is where diffusion rate might be affected even further if the compounds do not reach the inflamed and constructed sites of the airways [17, 299]. Lately, also the small airways have been recognized as being a huge inflammatory contribution as part of the pathology in airway disease, due to they correspond to the huge surface area within the lung as well as their active immuno-regulative role including the peripheral immune cells being present at the site [17, 343-345]. This has been known in COPD [346], but asthma has mainly been associated with the large airway pathology and bronchoconstriction, until recently [347, 348]. By producing future inhalation drugs in the lower size range, the administration would reach further down the airways and give as local effects as possible, also increasing efficacy while reducing side effects [136, 137, 349]. If the drug can function as broncho-dilatory at the same time as anti-inflammatory, the more optimal the treatment strategy is. As shown in paper I, both Capsazepine and various RES compounds had slight different effects on the bronchi and cytokine production. These results point towards interesting data, yet at an early stage towards drug development.

We and others have shown that primary bronchial epithelial cells can provide a methodological advantages since the disease phenotype is preserved in these cells in contrast to bronchial epithelial cell lines being commonly used for *in vitro* studies. The SAR study presented in paper I, as well as the study of pleotropic effects of a drug such as simvastatin is of great interest and potential for future drug target development and new thinking when it comes to therapeutics in progress. Use of the drug pleotropic effects, or design of derivates of molecules occurring naturally in the nature might be provided to patients sooner as therapeutics compared to traditionally manufactured with many regulatory stages of trials.

Pro-inflammatory cytokines promote cell-recruitment

With a great focus on epithelial derived hub-cytokine TSLP, both paper I and paper II addressed the need to block this Th2 priming cytokine since it has been shown to promote upstream affects of several cytokines but also accordingly induction of cells downstream of its signalling pathways [168, 350]. Studies performed involving co-cultures or condition media treatment have demonstrated the regulatory role of TSLP and its ability of providing cross-talk effects between

different cell types [351-353] Additionally, taken together that TSLP exerts the key-regulatory role of priming immune cells and that the TSLPR is widely expressed on various cell types, makes TSLP into a superb target for drug intervention. TSLP blocking strategies have already been performed in mice as well as in clinical trials [354-356]. As showed in our studies, in paper I and II, also producing anti-TSLP effects. Unfortunately also the important IFNβ was inhibited simultaneously by simvastatin, which was not desired due to protective effect of the interferons upon viral infections.

The important interferons possess anti-viral function and initiate responses interfering with viral replication, thus eliminating viral infections more efficiently. The interferon deficiency in asthmatics has been pointed out as an important reason for viral-induced exacerbations [267, 318, 357-360]. Several studies show and discuss the lowered interferon expression upon viral infection or dsRNA stimuli in asthmatics, while at the same time TSLP was shown to increase in these patients instead [167, 267, 339, 342]. This might partly give rise to the exaggerated response in asthma and COPD patients while dealing with a respiratory viral infection. Importantly, recently shown in a study by Sykes and colleagues, that HBECs from well-controlled asthmatics did not have a deficient interferon response upon rhinoviral infection [361].

Cytokines and their chemotactic properties are crucial for the cell recruitment, although the cell migration is also able to cause more damage than protection of the tissue. Therefore, despite using valuable patient material and investigating potential blocking agents in the first two initial studies, it is important to approach the complex interplay of epithelium with the systemic effects and other immune cells playing a big contributing role in asthma and COPD exacerbations. We therefor developed an *in vivo* model involving allergen-provoked inflammation in mice, to study the cell recruitment, which followed cytokine expression and release. Also, important clinical markers were analysed to be able to compare the obtained animal model with the clinical relevance and the translational value of our exacerbation model.

Obtaining the suitable model of asthma exacerbation (III-IV)

The use of different allergens when obtaining a suitable asthma model in mice has been discussed over the last decade [265, 319, 362, 363]. It is important to keep in mind that any allergen used for the purpose to provoke experimental allergic asthma in animals such as rodents, must be interpret with caution [271, 364]. Mice are not normally prone to develop asthma and by designing the provocation

settings it is relevant only depending on what the outcome is for the study. Many important studies conducted in animal models have indicated promising results, which have been taken further in human clinical trials in association to drug development. Nevertheless, more or less majority of the clinical studies have been unsuccessful indicating completely different outcome compared to the expected. The use of animal models can therefore show similar reactions, although not the same receptors, cells or mechanism functions as in humans. Sometimes even the same end-product of a produced protein might involve completely different signalling molecules downstream of the initial receptor-ligand interaction [365, 366]. Depending on what the aim of the study is, the study design and choice of animal and model must be picked wisely. We chose to work with both OVA-challenged and HDM-challenged model, using doses of allergens and timepoints of stimulation as such; that would induce enough allergic response, although still being able to produce superimposed effects upon dsRNA provocation on top of the induced experimental asthma.

When developing the first mouse model of asthma exacerbation, we employed the commonly used and well known OVA allergen in Balb/c mice, which has been used frequently for asthma provocation in mouse studies worldwide for many years [270, 367, 368]. We chose to work with this robust and trusted allergen as the already studied Th2 upstream cytokine TSLP has shown to be induced in this model [198, 337, 369]. Overall discussion has been whether the OVA model is considered to be more aggressive and that it produces a more acute phase of the experimental asthma, rather than chronic inflammation. Nevertheless, induction of TSLP using the OVA-model would provide the optimal allergic immune response that we aim to study. Notably, TSLP has shown to be detrimental for development of Th2 allergic response, as observed in mouse models [198, 369, 370]. One study demonstrates that TSLPR KO mice exhibit strong Th1 response with high levels of IFN-γ, IL-12 and IgG, while low levels of IL-4, IL-5 and IL-13 were expressed. In addition, these mice failed to develop allergic inflammatory response towards allergens. Once the KO mice received naïve CD4+ cells, then ability of an allergic response was restored [370].

In our mouse model in paper III, both dsRNA administration, but also real rhinoviral infection was performed to produce an exacerbation. Both stimuli on top of the induced allergic experimental asthma showed similar effects, giving the confirmation and verification that dsRNA is a reliable stimulus to use for rhinoviral mimicking effects (paper III). Other studies using the OVA-challenged allergic background and subsequently administrating dsRNA in mice, all managed to produce a mouse model of asthma exacerbation [371-373]. One research group compared the add-on effects of LPS and dsRNA after OVA-challenges and showed that the significantly high increase of AHR measured in mice receiving both OVA and dsRNA was synergistically high in comparison to only dsRNA- or

OVA effects alone [371]. Whether the focus was to elucidate the role of NK cells in Th2 response [372]; or involvement of TRIF-pathway in asthma exacerbations, including KO mice [373] explored in other studies, yet the OVA-dsRNA combined administration was successful for exacerbation induction.

Nevertheless, continuing with the last two *in vivo* studies (paper IV and V) another allergen was used for asthma provocation. Here, the HDM whole body extract from Greer laboratories was employed, which was shown to be the most potent among various HDM-extracts to evoke experimental asthma, when given intranasally in a recent comparison study [374]. Importantly, atopy in humans is strongly associated with development of asthma, and considering that up to 85% of asthmatics have positive allergy test towards HDM [375], make the use of this allergen relevant to include in mouse models of experimental asthma. Therefore, we included HDM challenges in the last two studies (paper IV and V) but also due to the fact that different facets of the asthma disease were more reflected in the HDM-triggered asthma model. Key-findings from both OVA-triggered and HDM-triggered asthma model were; increased total protein exudation in the BAL as well as eosinophilic infiltration into the tissue was obtained. Both the protein exudation and eosinophilia are typical clinical features of human allergic asthma [376, 377], and discussed in previous articles that bring up the importance of an animal model mimicking the human disease [265, 363, 364]. The stimuli protocol and allergen we used in the last two studies (paper IV and V) was a more translational model and provides a reliable platform for the use of studying specific events involved in asthma exacerbations, that could be more associated with exacerbations in human.

Allergens and administrative rout

Both OVA-triggered and HDM-triggered asthma can be used to provoke experimental asthma in mice, but it is extremely important to consider the purpose of the study and also the nature of these two different allergen-challenging protocols and what they create. OVA has been criticised by many, yet still used by even more researchers [270]. The feature of this allergen-challenge gives a relative aggressive inflammation, with an emphasis on the eosinophilic asthma and massive airway obstruction in majority of the studies. The human asthma is poorly reflected in its true disease symptoms and not to mention the resolving effects seen in our study as well as observed by others, probably due to it is an acute inflammation after all. Nevertheless, this is a good model for studying inflammatory markers being involved in asthma triggering. To use OVA and to create a platform of asthma-like features and then further study the exacerbation caused by superimposed dsRNA or rhinoviral stimuli, which after all is the main purpose of this thesis. The disadvantage of using the OVA-challenge protocol for creating experimental asthma is the administrative rout of intraperitoneal

injections and the need of an adjuvant, normally being aluminiumhydroxide, to actually trigger the immune system in a proper fashion [378]. Due to the administrative route, use of aluminiumhydoxide adjuvant and the obtained acute and clinically irrelevant type of inflammation that this allergen-challenge entails, makes the OVA-model non-translational, yet short and reliable to use.

dsRNA-induced asthma exacerbation (III-V)

The superimposed effect created by combining allergen and the effects of rhinoviral infection, produced asthma exacerbation in our model enabling studies for treatment targeting, as presented in paper III, IV and V. The superimposed effects observed in our model were elevated total protein and LDH levels in BALF as well as immune cell infiltrates in both BALF and tissue. Also, the expression of several cytokines was highly induced only at exacerbation, showing synergistic effects constituted by the combined allergen-challenge and dsRNA stimuli.

To study the asthma exacerbation in an animal model, with the aim to both develop a reliable model, which can produce important translational features as well as being able to study key-regulatory cytokines as potential drug targets and at the same time keeping the optimised protocol for asthma exacerbation triggering as simple and reproducible as possible, is at all times easier stated then performed. As discussed by Persson *et al*. already back in 1997 [320], was still current in 2006 and brought up by Wenzel and Holgate [321]; extensively used mouse models are employed although the major features of asthma are not always demonstrated and are of importance to include and evaluate when developing an allergic asthma model in mice. One such thing to mention is the plasma exudation, which reflects the general inflammatory state and is a major clinical feature in human asthma as seen in both OVA- and HDM-challenged mice presented in this thesis in paper III and paper IV, shown as total protein estimated in the BALF. As shown in Figure 14 below, the allergen challenge with both OVA and HDM induced significant increase of total protein in BALF.

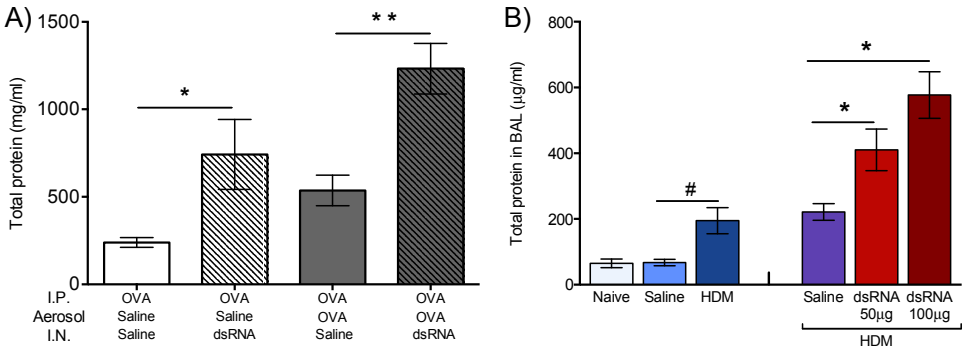

Figure 14:
Total protein measurements in BALF reflecting the general inflammation as protein exudation. OVA/OVA/dsRNA and HDM/dsRNA correspond to the exacerbation phase (paper III,) OVA-model (A) and (paper IV) HDM-model (B), respectively.

Another important clinical marker that we have focused on as well is the released LDH in BALF, obtained in all *in vivo* studies. The LDH in contrary to the total protein was observed to increase only at exacerbation (Figure 15).

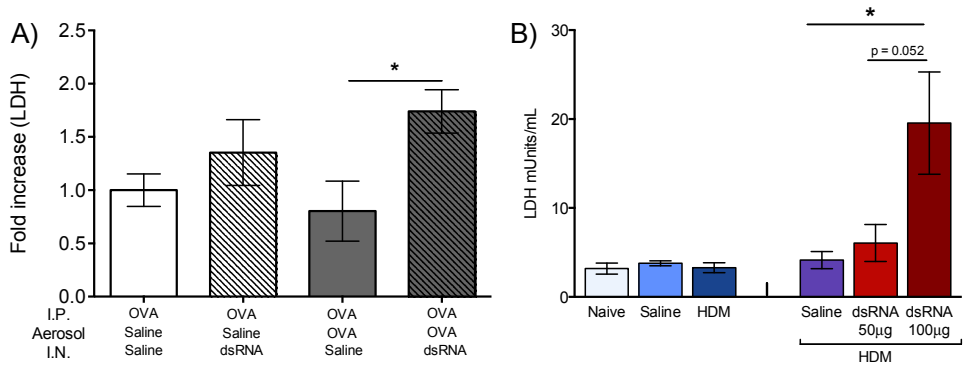

Figure 15:
LDH levels measured in BALF from two studies paper III (A) and IV (B) showed that only high dose of superimposed dsRNA administration induced the significant increase of LDH.

In addition, other presented data in this thesis from the three animal studies that together reflect the exacerbation model and the relevance it brings while studying exacerbation. That is, results such as increased inflammatory score evaluated from tissue sections (paper III), epithelial cell shedding (paper IV) but also importance of balance regarding cell clearance and cell-death, which was studied through assessment of Caspase activation, more specifically; levels of cleaved Caspase-3

in lung tissue homogenates (paper V). All these different parameters taken together may help to see the whole picture and suggesting new target opportunities for future studies to analyse more in detail and eventually may be used as markers to predict disease severity. Other more specific inflammatory cytokines investigated in the mouse exacerbation model such as TNFα, chemokines CXCL1; CCL2; CCL5 and CCL11 but also interferons were investigated.

Most importantly, when studying cytokines with focus on TSLP, also other two Th2-upstream cytokines were observed; IL-33 and IL-25. In addition, IL-1β has also been studied, while not being a typical Th2 cytokine, but extremely central for inflammation induction as well as amplifier of the triggered inflammation response, which is discussed in the fifth and last paper of this thesis.

The trio of upstream Th2 cytokines (IV-V)

Th2 cytokines have gained increasingly importance and attention over the last years [170]. As stated in the discussion above from the *in vitro* data, the first barrier and first line of defence; the bronchial epithelial cells, possessing the immuno-regulatory role in the airways are able to respond to any exogenous as well as endogenous trigger [162]. The bronchial epithelial cells are also the main producers of the Th2-upstream cytokines TSLP, IL-33 and IL-25 together with other immune cells being induced. Due to this, these three cytokines would be optimal drug targets for future exacerbation treatment. Although, studies aiming to block any of the Th2 upstream cytokines or using mouse strains of knockout genes of any of them, have clearly shown the important role of TSLP, IL-33 and IL-25 in chronic inflammatory lung disease [170]. Yet the debate goes on regarding which cytokine is the most important and most detrimental for asthma development or main driver of exacerbations [234, 379]. In addition, anti-TSLP blocking drugs were discussion already 10 years ago [380, 381], where it was brought up that this hub-cytokine could be optimal target for treatment of asthma and allergies suitably being upstream of IL-4, IL-5 and IL-13 and as well possessing the role of priming dendritic cells for finally production of Th2 milieu in the lung. Zhang and group members managed to block TSLP in an allergic mouse model using OVA-challenges [356] and reduce allergic disease severity through actions in the dendritic cells. Recently the concept of blocking TSLP in human studies showed positive effects. Although not fully established on which mechanisms of action these effects were induced [354, 355] but also another aspect needs to be brought up; namely the importance of blocking these cytokines and what can be the detrimental events following? IL-33 for instance, has shown to be protective in associations with heart and vascular function, where high sST2 or low IL-33 levels

were associated with poor recovery, observed in myocardial infarction models [382, 383] Furthermore, TSLP has shown to be important in gut mucosal immunity [384], in contrary being highly induced in the dermatitis disorders such as psoriasis and allergic dermatitis [385]. As observed in paper III and IV, also showed in Figure 16 below, the expression of TSLP and IL-33 increased after allergen challenge alone and were further elevated at exacerbation.

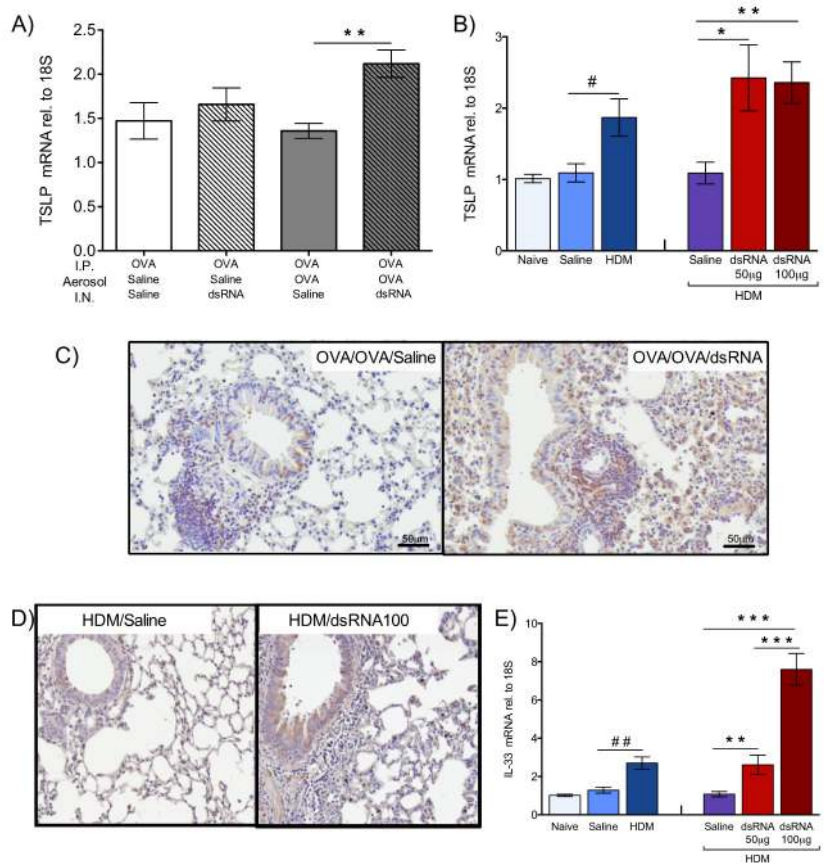

Figure 16:
The Th2 upstream cytokines TSLP and IL-33; gene expression and immunohistological pictures demonstrating the induction of these cytokines at exacerbation. TSLP mRNA expression in paper III (A), TSLP mRNA expression in paper IV (B), TSLP stained in lung tissue in paper III (C), IL-33 stained in lung tissue in paper IV (D), mRNA expression of IL-33 in paper IV (E).

IL-25 is not as extensively studied as the previously mentioned two upstream cytokines, few studies look into IL-25 in allergic asthma but few studies address the role of IL-25 at asthma exacerbation [386]. It is known that this cytokine is involved in airway remodelling and AHR, but mainly contributes to an

eosinophilic allergic asthma, which already can be reduced by the IL-5 and corticosteroid treatment [70, 136, 278]. A most recent clinical study evaluating the responsiveness of corticosteroids showed that the group with greatest AHR and blood eosinophilia was the subset of individuals with elevated epithelial IL-25 expression [233]. ICS improved FEV_1 in these patients but not in those subjects that expressed low levels of IL-25. In paper IV of this thesis IL-25, was significantly induced at exacerbation, but only showed a tendency towards increase when assessed in paper V. This cytokine might be induced at an earlier time point and also has an earlier peak of expression and release, than we were able to detect in our present exacerbation model. Gregory and Lloyd have shown that HDM challenges induce the Th2 cytokines IL-4, IL-5 and IL-13 to peak at second week of allergen administration [163]. IL-25 might be in the same time-window of induction. Below is a schematic drawing of the three studied Th2 upstream cytokines and their relation to surrounding cells, both structural cells as well as induced- and activated immune cells in the epithelium and interstitium (Figure 17).

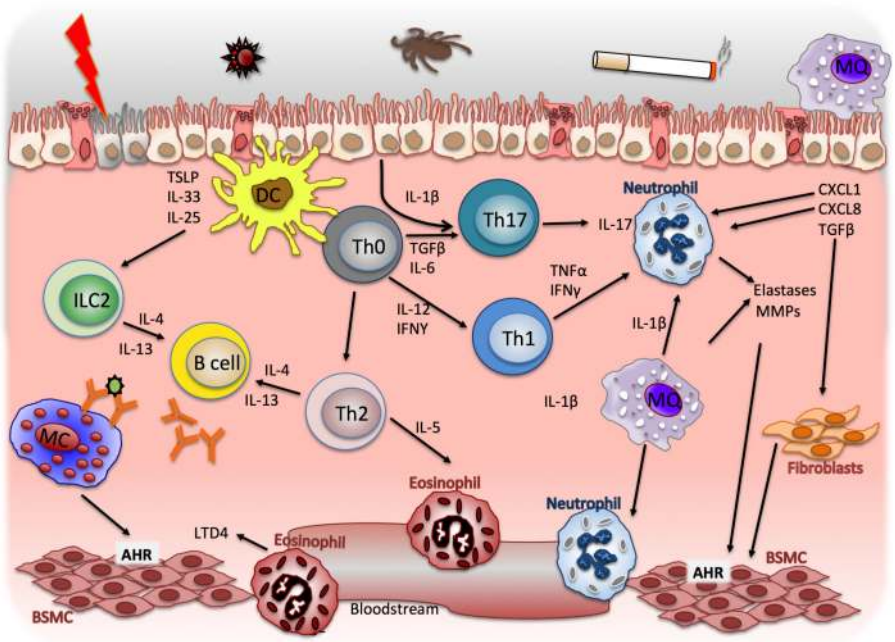

Figure 17:
A scematic draving of how the different immune cells and structural cells interplay by migrating and releasing mediators , that may contribute to exacerbations.

Other options for targeting exacerbations?

Importance of the Th2 immune response cannot be denied when observing the actions of IL-4, IL-5 and IL-13 and their contribution to AHR, the hypersecretion of mucus, inflammation of the airways and long-term effects such as remodelling and hyperplasia, events constituting the hallmarks of asthma. The severity of the disease and the exacerbation frequency go hand in hand. To rule over the Th2 cytokines, the epithelial derived Th2-upstream cytokines have lately emerged as the better options to target. What if the Th2 immunity cannot be controlled or treated among all the different phenotypes of atopic asthma, and what about the non-allergic asthma? Should we focus on the exaggerated immune response regardless being Th2, Th1 or even Th17 driven? And considering the exacerbations occurring in COPD patients; can there be any possible treatment that will slow down the destruction of the airways and lower the inflammation and bronchitis, without interfering with the healing process? Is the exacerbation just a response that needs to be diminished and controlled not necessarily blocked or modified into Th2/Th1? Regardless if it is allergen triggered or induced by mechanical injury, toxicity, cigarette smoke or viral infection, pro-inflammatory cytokines may be an option for new target treatments, if only to be used for the severe cases of chronic inflammatory disease or during exacerbation phase.

The fifth and last study in this thesis is presented as a submitted manuscript and paper V. Here, we explore the role of the pro-inflammatory cytokine IL-1β, also being a member of the IL-1 cytokine family as IL-33. Although, in contrast to IL-33; IL-1β is not associated with allergy or possessing a priming role of immune cells, instead autoimmunity and infection-induced expression is the primary function of this cytokine.

Pro-inflammatory cytokines involved in exacerbations

In our search beyond Th2 cells and cytokines, another cytokine emerged as a potential drug target, the pro-inflammatory IL-1β [387]. This cytokine was observed elevated only at exacerbation and not by the allergen-challenge alone, in our study presented in paper IV (Figure 18). This cytokine is also increased in both asthma and COPD patients when studying sputum, as well as tissue staining of asthmatic airways [245, 249].

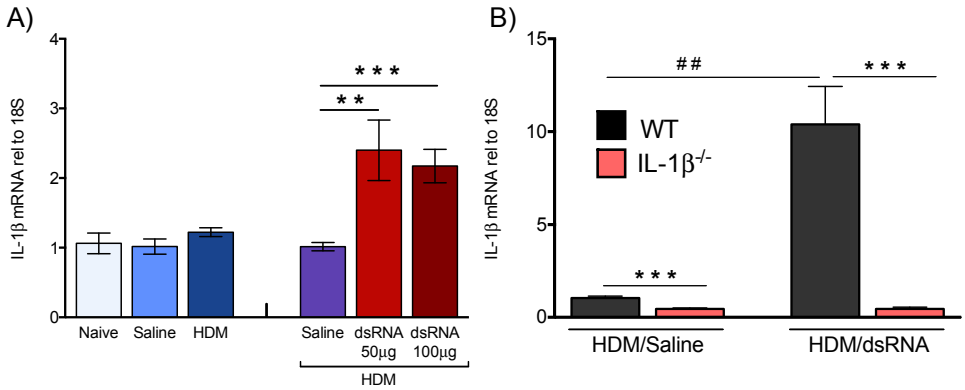

Figure 18:
The gene expression of IL-1β was only induced at exacerbation and not by allergen challenge alone, shown In paper IV (A), while in paper IV this gene was only analysed at exacerbation, showing the increase in WT mice while not being expressed in the KO groups (B).

In the last study (paper V), the IL-1β knockout mice were exposed to the same provocation protocol as in previous published model (paper IV) reflecting the asthma exacerbation. In this last study included in the thesis, the role of IL-1β was explored at exacerbation and its potential effects on the upstream Th2 cytokines as well as other general inflammatory parameters. Interestingly, paper V demonstrated IL-1β knockout influencing on both total protein and total immune cells assessed in BALF. Both parameters were significantly induced at exacerbation in the wildtype mice, while indicating towards a tendency of lower amounts of both proteins and cells in the KO mice (Figure 19).

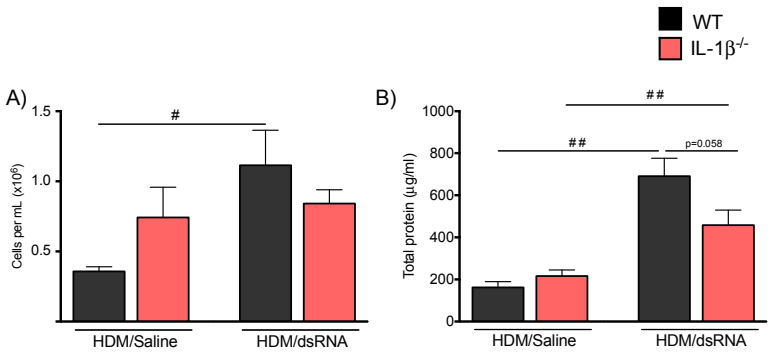

Figure 19:
Total protein (A) and total cells (B) in BAL showed form paper V. Both parameters were significantly increased at exacerbation in the WT mice while indicating lower induction in the KO mice.

Importantly, also the Th2 upstream cytokines being induced in the WT mice at exacerbation as studied in paper IV, were not altered in the IL-1β KO mice. (Figure 20).

Major affected parameters in both BALF and tissue were the neutrophilia and the neutrophilic chemokines; CXCL1 and TNFα. In addition, also less cell death and reduced induction of the PPRs was observed. The exaggerated immune response seemed to be orchestrated only by the knockout of this cytokine, thus IL-1β dependence.

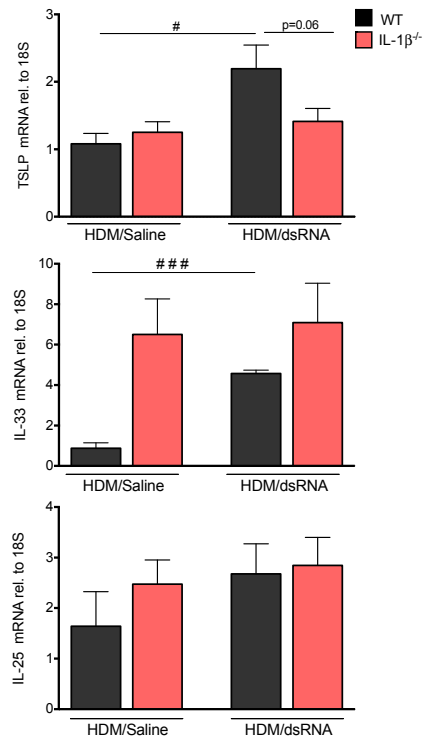

Figure 20:
Th2 upstream cytokines were analysed by RT-qPCR from lung homogenate samples, paper V. TSLP and IL-33 increased significantly at exacerbation, while the KO mice had unaltered cytokine expression. IL-25 showed a tendency towards induction at exacerbation.

In a recent study performed by Yang and co-workers, the dependence of IL-1β was shown to be detrimental for ICAM-1 up-regulation during inflammation [388], as well as adhesion receptor regulation has been associated with IL-1β [389]; both being detrimental for cellular infiltration from the blood stream into the tissue upon inflammation [390]. The importance of IL-1β may not completely come as a surprise since studies conducted in both patients and animal models

have indicated the link between the neutrophilia and IL-1β [391]. This important role that IL-1β displayed in paper V, was reflected on both cell activation, infiltration as well as in regulating cell death; indirectly has an impact on clearance and possible generation of DAMP and PAMP and thus affecting PPR activation [9, 162, 392]. As the histological stainings in our study (paper V) indicate, the general inflammation was hugely affected in the KO mice, at exacerbation. The lack of expression of IL-1β reduced the total cellular infiltration observed in the tissue, and more specific, the neutrophil staining showed clearly that neutrophilia was lowered (Figure 21).

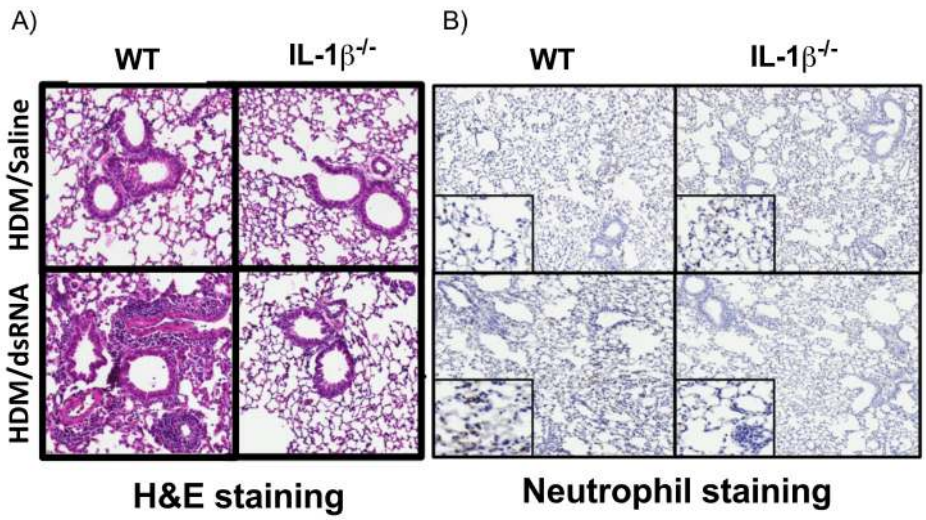

Figure 21:
General inflammation and cells infiltrating the lung at exacerbation shown by representative photos of Haematoxylin&Eosin staining (A) and specific staining of neutrophils (B) obtained by immunohistochemistry.

Importantly, also mucus secretion being another important facet of asthma, and especially at exacerbations, can also be regulated by the IL-1β. In paper V, we showed that the main mucin: *MUC5ac* gene expression was significantly increased at exacerbation in the WT mice and significantly lower in the IL-1β -KO mice (Figure 22).

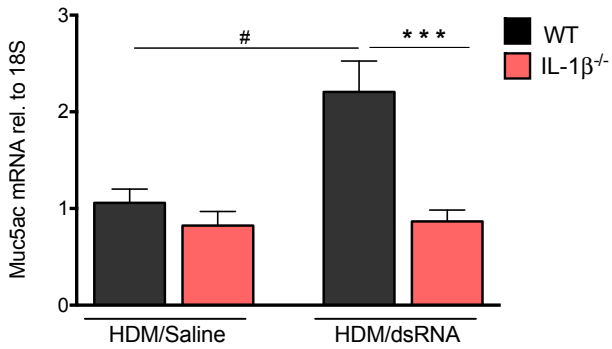

Figure 22:
Gene expression of MUC5ac analysed in paper V. Muc5ac was significantly induced at exacerbation while not being altered in the KO mice.

Hereby presenting several important events such as inflammation, neutrophilia, cytokine expression, mucin regulation as well as cell death in tissue and lumen being regulated by the presence of IL-1β. By blocking IL-1β cytokine may be another possible therapeutic strategy when research and development fails to provide optimal drugs for all the different phenotypes of asthma and COPD, especially the more severe types, not least at exacerbations. Particularly, the neutrophilic dominant severe asthma being difficult to treat would benefit from IL-1β blocking treatment. To date, the anti-IL-1β treatment already exists and is commonly used in patients with rheumatoid arthritis, gout and even type 2 diabetes [252]. The already available and synthetically made anti-IL-1β is an analogue for the naturally occurring IL-1Ra called Anakinra, with the function of neutralising free circulating IL-1β. The safety trials already performed during the manufacturing and testing of Anankinra has already been approved. Therefore, when treating sever cases of exacerbations, use in patients with massive inflammation and mucus plugging, might be one complementary treatment approach to involve IL-1β in addition.

How to elucidate the complex interaction of cells and mediators in respiratory exacerbation?

Knowing the complexity of the event that respiratory exacerbations include, yet we have been able to bring the knowledge forward. This thesis studies the Th2 upstream cytokines, where inhibitory substances were used as well as KO mice to elucidate mechanistics, involved in exacerbation of asthma and COPD. Importance of TSLP; IL-33 and IL-25 has been brought up and in connection to the releasing

cells that possess the power of immune-regulation; the epithelial cells. The fact that well-controlled asthmatics do not exhibit interferon deficiency upon rhinoviral infection, might be the clue towards the constantly mentioned epithelium and its importance [361]. The bronchial epithelial cells obtained from these well-controlled asthmatic patients probably have managed to keep the interferon production complete, due to intact barrier function. Again, pointing towards the importance that when the integrity of the epithelium is kept, other functions work properly as well, such as interferon expression and release. Recent study by Sekiyama *et al*. pointed out the association of glucocorticosteroid use and enhanced epithelial barrier integrity [393]. This could be linked with the difficult to treat corticosteroid refractory asthma, being mainly associated with neutrophilic presence and also high levels of Th17 [394-397].

The severe neutrophilic asthma has showed a positive correlation of disease severity and IL-17A [398, 399]. Likewise, IL-1β showed to affect Th17 cells differentiation and expansion [391] and in particularly the Th2 memory CD4+ cells that produce IL-17 showed to promote exacerbation in asthma [400]. IL-1β blocking is also known to reduce neutrophilia, which is a common side affect in Anakinra treatment, resulting in increased infections in some patients due to the lowered amount of neutrophils [252]. Moreover, connecting the fact that IL-1β is involved in induction of mucin expression showed in previously published studies [401, 402], as well as observed in paper V of this thesis. This gives IL-1β increasingly central role considering mucus-overproduction in asthma and COPD exacerbations. Lastly, we clearly showed that both apoptosis and necrosis seemed to be dependent on the expression of IL-1β, explored through increased cleaved caspase-3 and LDH release by necrotic cells.

Has the tissue damage reached a certain level of tolerance until the damage-repair balance is totally crashed? If the bronchial epithelium is already under constant repair, yet also continuously loosing the integrity by allergen-interaction and presence of environmental pollution, not to mentioned viral infection induced damage. Protease and elastases excess released by the already present granulocytes, such as in long-term neutrophilic severe asthma. These various events might in turn tip the balance further towards high cell death and insufficient clearance of infiltrated immune cells and dying cells (epithelial or immune cells).

Study performed by Besnard and co-workers showed the importance of the inflammasome in Th2 inflammatory disease, where the NLRP3 was displayed required in order developing asthma in mice and that absence of adjuvant in allergen sensitisation is dependent on this pathway [403]. This highlights the important role of the IL-1β cytokine as well as endogenous release of DAMPs and alarmins into the extracellular space upon necrosis, being an important link in severe asthma. Figure 23 below provides a proposed map of events that might be

involved in initiating exacerbation in already inflamed lung tissue of asthmatics or COPD patients, from the IL-1β point of view.

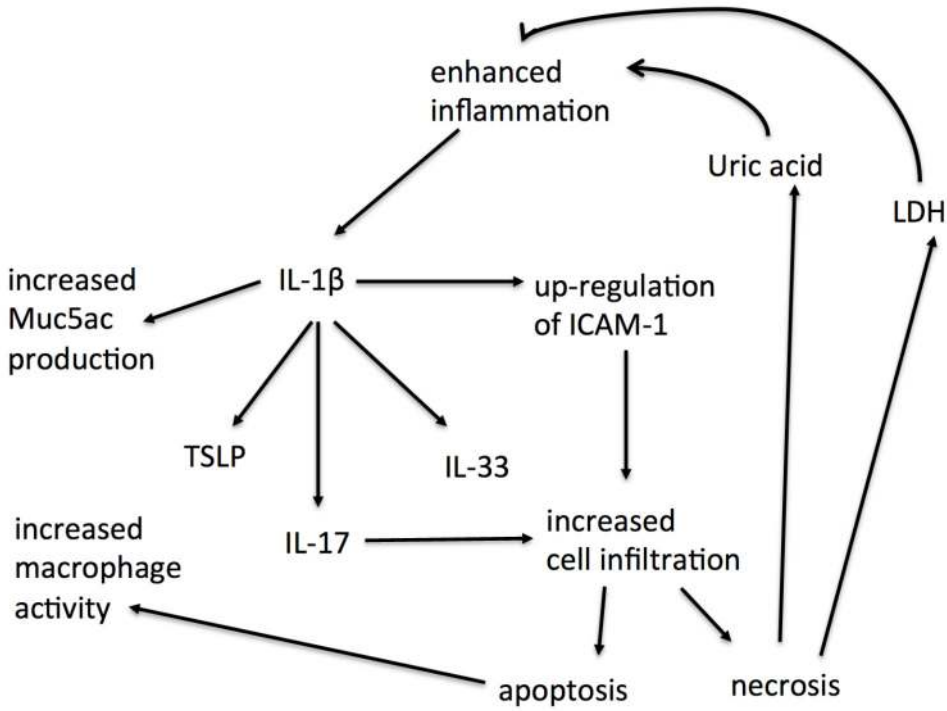

Figure 23:
Proposed actions of IL-1β, regulation that may affect triggering of exacerbation. The different parameters are shown to be affected by IL-1β KO observed in this thesis (paper V), or shown by others previosly published.

Summary of major findings

Paper I: Capsazepine and derivates of this compound reduced dsRNA-induced TSLP expression and production in primary HBECs from asthma and COPD donors involving NF-κB inhibition. This drug effect was accompanied by reduced IFNβ expression and production.

Paper II: Simvastatin inhibited TSLP as well as IFNβ through pleotropic effects, independent of the mevalonic pathway. Anti-TSLP effects of simvastatin were more potent than those shown by steroid treatment and through inhibition of IRF3 and not NF-κB, observed in primary HBECs donated from COPD patients and healthy smokers.

Paper III: Development of a new asthma exacerbation model using OVA challenges for asthma development and in addition applying superimposed dsRNA or rhinoviral stimuli to provoke exacerbation in mice. Expression of TSLP was exclusively induced at exacerbation and general inflammation such as lung inflammatory score, BAL total proteins and LDH being induced by dsRNA or allergen alone was synergistically increased by both exacerbation stimuli at exacerbation phase.

Paper IV: Further development of an asthma exacerbation mouse model in mice using a human allergen, HDM, combined with additional dsRNA provocation to evoke exacerbation. Allergen triggered eosinophilia was sustained and further accompanied by neutrophilic induction in BALF. Total cells, total protein in BAL and tissue cytokines; TSLP, IL-33 and TNFα were induced by allergen challenge alone and further increased at exacerbation. However, ATP, LDH, all three PRRs (RIG-I, MDA5 and TLR3) and cytokines IL-25, IL-1β, CCL5, CCL2 were only increased at exacerbation.

Paper V: HDM- and dsRNA-triggered asthma exacerbation in WT and IL-1β KO mice. KO mice showed less induced BAL and tissue cell infiltration compared to WT mice. Significantly induced TSLP and IL-33 were not altered in KO mice at exacerbation. Mainly, neutrophilia and neutrophil chemokines CXCL1 and TNFα were reduced in the KO mice. In addition, both cell necrosis and apoptosis were less induced in the KO mice at exacerbation, compared to WT.

Conclusions and final remarks

In the first two papers of this thesis TSLP gain the main focus during mechanistic studies in the HBECs, while the *in vivo* studies have been assessing the trio of upstream-Th2 cytokines and shown that they are exclusively increased at exacerbation (even if minor increase upon dsRNA- or allergen alone, exacerbation gave synergistic effects on the cytokine-trio). The important regulation of Th2 upstream cytokines might be the leading option in exacerbations and among them TSLP preferentially as the blocking target, due to IL-33 serves as protective mediator in other organs, while IL-25 mainly regulates similar events such as eosinophilia and AHR, exerted by IL-5 and IL-13 presence.

This thesis has mainly brought up the issue of respiratory viral-triggered exacerbations, occurring in chronic inflammatory diseases such as asthma and COPD. The acute flare-ups occurring once the already inflamed lung tissue is striked by a viral infection, have shown to exaggerate the immune response, to the extent, where the immune system induces more harm than protection. These acute exacerbations are in need of better management, but in order to come up with new therapeutic treatment we need to identify both the underlying inflammatory disease as well as further develop the proper and translational models. Different phenotypes of both asthma and COPD have been discussed, the viral sensing receptors and downstream effector cells and released mediators, all events connected to the epithelium.

All three Th2 upstream cytokines do affect events occurring in asthma disease such as AHR, eosinophilia, mucus-hypersecretion and hyperplasia, finally inducing remodelling over time. These events are the hallmarks of the disease and are mainly through the action of downstream regulation of IL-4, IL-5 and IL-13. Studies aiming to block one of the upstream cytokines have also mentioned their specific roles in causing pathology, but also discussing the synergy they possess [241, 331]. IL-25 not getting as large focus as IL-33 and TSLP, might depend on the fact that this cytokine was recently discovered [404], as well as the fact that this cytokine mainly regulates the AHR and eosinophilic migration and remodelling. Inhibitors of the IL-25 pathway would probably give similar effects as using the anti IL-5 or IL-13, as shown in several mouse studies [235, 405, 406]. As IL-25 is dominantly studied in asthma, the role of this cytokine has not been brought up in COPD. Although, since IL-25 seems to regulate the typical Th2

immune response involving eosinophils yet is linked to fibrosis and remodelling. Due to this, we could possibly speculate that COPD subtype involving eosinophilia could be associated with occurrence of IL-25, as it can be initiated by viral infections [386] it can possibly be involved in the exacerbation of COPD lungs.

Exacerbations apparently involve many interconnected events and we have to look at what type of acute condition this is and what we are dealing with, to date. Considering that the various phenotypes of asthma and COPD need personalised treatment might also propose future therapy for treating exacerbations to be designed in a personalised manner. As discussed in previous sections, another option would be to tackle the exaggerated immune response that occurs at exacerbation, despite if the baseline disease involves the eosinophilic COPD or neutrophilic corticosteroid resistant asthma [407, 408]. One proposed action would be to target the pro-inflammatory cytokines, and as studied in the last paper; IL-1β actions at exacerbation. After all, it is the exaggerated response that keeps the vicious cycle going. If the general overreacted response could be dampened irrespective of the type of inflammation, being Th1; Th2 or Th17, would the less damaged tissue also lower inflammation.

This thesis has discussed the importance of the epithelium at viral infection, where elevated levels of the Th2 upstream cytokines as well as deficient interferon might be important reasons to why patients experience such exaggerated reaction when having a cold. There seems to be an important interplay of protective properties of both the functions of interferon regulation as well as epithelial cell integrity that together provide the anti-viral defence. Once the barrier functions properly, the exaggerated immune cell recruitment might be reduced and mediator release as well as cell death occurring in the lumen or lung tissue, will lead to less damage. The delayed wound healing of the bronchial epithelium is discussed and studies pointing toward the important first line of defence and its cell integrity as well as secretion [409-411].

Further, importance of the inflammation clearance must not be forgotten. The balance of apoptosis and cell proliferation might influence more than we think. Homeostasis of the epithelium is the key, for successful repair and minimal damage [412].

Future Perspectives

The results from HBECs treated with **RES and CPZ** presented in paper I highlights the importance of treating the dual issues of obstructive lung diseases, thus the inflammation and the bronchoconstriction. The strategy is tested and should be possible to optimise and improve. The concept of dual function can further improve and by performing further SAR studies and eventually finding one optimal molecule giving the most bronchorelaxing properties as well as inhibiting the TSLP or other Th2 or Th2-upstream cytokine expression while keeping the interferon production intact. A second step would be to further test the newly obtained capsasepinoids in an animal model expressing the clinically relevant symptoms as in human respiratory exacerbations.

Already tested effects in humans are the cholesterol-lowering drugs **Simvastatin**, as we have used in paper II. The safety of the drug is already tested and now by taking advantage of its pleotropic anti-inflammatory effects in primary HBECs we have studied the inhibitory property among cytokines such as TSLP, TNFα, CXCL8 and IFNβ. As we have used several different types of the small-inhibitory molecules in paper I, the follow-up of different statins might be one approach towards finding the most optimal statin-compound with least anti-interferon but high anti-TSLP effects. HBECs have already been used in a pilot study, in our laboratory, to test various concentrations of other statin compounds such as Pitavastatin, Pravastatin, Rosuvastatin, Fluvastatin and Lovastatin. Further using the molecular backbone of the best fitted statin compound, regarding its anti-inflammatory properties, can be further used and modified by exchanging various functional groups in order to finally get Statin-alike compounds, and eventually be able to test those substances in an *in vivo* model of exacerbation.

The presented **animal model of asthma exacerbation** was designed aiming to produce a translational and relevant model, to use for further studies providing intervention and more mechanistic observations. Then, we would be able to elucidate other mechanisms involved in asthma exacerbations and the relationship of TSLP, IL-33 and IL-25 in viral-induced asthma exacerbation. The experimental asthma induced in mice by HDM-challenges is based on allergic inflammation actually using the clinically relevant allergen, and through clinically relevant administration route, thus intranasal inhalation. We have in addition mirrored some important features of the human disease in this model. Primarily, this model

can be used for further investigation involving knockout animals, as we initiated in the last paper of this thesis. Thus, mechanistic studies could involve both by using genetically modified mice, biological blocking substances, and small-molecular inhibitors. Secondarily, this model will have use in studying the role of pattern recognition receptors in anti-viral response to respiratory viral infections in asthma, as we have pointed out the epithelium, owning the key-regulatory role in exacerbation.

Epithelial cells posses the key-regulatory role in both inflammation as well as responses induced at viral infection. Therefore, we purpose more focus on the epithelial derived Th2 upstream cytokines in the search for potential drug targets. We aimed to inhibit the TSLP production from primary HBECs in the first two papers, looking into two different transcription factors regulating the expression of TSLP by using inhibitory substances. Further we developed asthma exacerbation models to be able to study both TSLP as well as other Th2 upstream cytokines.

Populärvetenskaplig sammanfattning (Svenska) – Exacerbation

Exacerbation – överdrivet immunsvar?

Astma och KOL är båda två lungsjukdomar med kronisk inflammation i luftvägarna som gemensamma nämnare. Patienter med astma eller KOL lever dagligen med en mer eller mindre ständigt närvarande inflammation i luftvägarna. Tidvis upplever patienterna en tillfällig försämring av sin sjukdom, vilket även kallas för exacerbation. Dessa tillfälliga försämringstillstånd kan leda till en kraftig sänkning av lungfunktionen och patienterna upplever oftast andnöd, tryckkänsla över bröstet och ibland även omfattande hosta.

Exacerbation kan framkallas av olika saker men oftast är det en infektion som ligger bakom. Majoriteten av dessa infektioner beror på förkylningsvirus. Även andra utlösare av exacerbationer kan förekomma, så som pollen, kemikalier, cigarettrök och allergen. Studier har även visat att frekvensen av inträffade exacerbationer är relaterat till försämrad lungfunktion på lång sikt. Därmed är behovet stort för behandling av exacerbationer och framförallt att stoppa förloppet av den destruktiva utvecklingen av en alltmer försämrad lungfunktion.

Dagens behandling och framtiden

I dagsläget existerar ingen behandling av exacerbationer hos astmatiker eller KOL-patienter. Läkemedel som verkar luftrörsvidgande och anti-inflammatoriskt används för att dämpa tillfälligt besvärande symptom men garanterar in förebyggandet av nya exacerbationer.

Det pågår intensiv forskning angående luftvägsinfektioner, men även kring behandling av astma och KOL. Trots detta finns det fortfarande många kunskapsluckor vad gäller infektions-inducerad exacerbation inom dessa sjukdomsområden. En viktig anledning till att det ännu inte existerar optimal behandling är för att de underliggande mekanismerna ännu ej är helt kända. Stor orsak till detta beror på att de djurmodeller som tidigare använts ej är relevanta modeller och speglar inte sjukdomsscenariot som existerar i lungorna hos människor. Då djurmodeller använts har oftast astma eller KOL studerats i

samband med potentiell framtagning av läkemedel, men få studier använder virus för att studera de synergistiska effekterna av både inflammation och infektion.

Mekanismer som styr exacerbation

Trots att man känner till vad som triggar igång exacerbation och vilka stimuli som är de mest förekommande, så vet vi i dagsläget inte de exakta molekylära mekanismerna i detalj. För att studera de olika förloppen; innan- och under en pågående exacerbation har vi använt oss av olika modeller, som finns presenterade i denna avhandling.

Epitelceller från luftvägarna som donerats från astmatiker och KOL patienter har används för att studera hur och när olika protein har sin samverkan under en virus infektion. De donerade cellerna behåller, så gott som, sin vanliga funktion inklusive de sjukdomsdrag som yttrade sig i lungan hos patienten. Därmed finns möjligheten att studera exacerbation och cellers svar på virusinfektion i cellkulturen, där mekanismerna kan observeras ur perspektivet så som patienten skulle svarat vid en exacerbationen. Då har man möjlighet att studera receptorer gener och proteiner som kan spela den avgörande rollen vid en exacerbation och som utgör skillnaden mellan en frisk lunga jämfört med en kroniskt inflammerad lunga så som hos en astmatiker eller KOL-patient.

För att dessutom ta kunskapen vidare till ett helt fungerande system med flera interagerande organ i kroppen, har musmodeller används i denna avhandling. Med allergen-provokation och i kombination med virus-härmande molekyler blev det möjligt att inducera astma exacerbation och då studera hur vissa nyckel-protein samverkar och regleras i lungorna. I en hel lunga har man dessutom möjligheten att observera interaktion mellan strukturella celler och vita blodkropparna, vilket bidrar med en mer komplex nivå. Detta har varit en viktig utveckling från cellkulturen till att komma vidare och studera astma exacerbation på individ-nivå.

Translationell forskning

Att använda sig av cell- och vävnadsmaterial och utveckla modeller som är så nära verkligheten som möjligt är en mycket viktig komponent i forsknings-sammanhang. Samtidigt som det är viktigt att även kunna studera isolerade system, så som att använda specifika celler, för att komma till botten med vilken reaktion som styrs av vad.

Translationell forskning har varit ett stort fokus i avhandlingen, och att just skapa dessa förhållanden, där både isolerade celler men även djurmodeller används, för att studera inflammatoriska proteiner och vad som är den drivande mekanismen i en exacerbation.

Därtill har även läkemedel använts i cellkulturerna för att utöka förståelsen med farmakologisk aspekt, samt välkända läkemedel, så som anti-inflammatoriska steroider som har använts inom astma och KOL sedan länge. Men även kontroversiell användning av kolesterol-sänkande läkemedel har applicerats i anti-inflammatoriskt syfte. Dessutom har nytillverkade substanser testats i cellkulturerna för att testa deras anti-inflammatoriska verkan efter att ha observerat luftrörsvidgande effekter. Detta försök till att designa och tillverka framtida läkemedel med dubbel funktion; där ett både luftrörsvidgande och inflammations-sänkande medel skapas, skulle vara en utmärkt behandlingsmetod i både astma och KOL, men framförallt vid de akuta scenarion så som vid exacerbation.

Strukturella celler och nyckel-protein

Nyckel-protein som nyligen hittats i förhöjda nivåer hos både astmatiker och KOL-patienter är bland annat TSLP, som främst produceras i de strukturella cellerna som klär insidan av luftrören i lungan och kallas epitelceller. Då epitelcellerna dessutom utgör den första kontakten med den yttre miljön, kommer de i kontakt med viruspartiklar och är därmed de första cellerna som initierar ett immunsvar vid en infektion.

Substanserna som testades i cell kulturerna sänkte uttrycker av just TSLP, som ansetts vara en brygga mellan virus och astma-utveckling i samband med infektioner. Utöver TSLP finns även andra viktiga reglerande protein så som IL-33 och IL-25, som utsöndras från epitelcellerna och som tillsammans med TSLP startar patologiska mekanismerna i lungan hos astmatiker och KOL patienter.

IL-1β

Femte och sista studien i avhandlingen innebar att studera astma exacerbation i en musmodell med avsaknad av en viktig gen som kallas IL-1β. Vanligtvis styr den inflammationen i autoimmuna sjukdomar så som gikt eller reumatism, men som även reglerar kroppens svar på infektioner med bland annat initiering av feber.

Utöver dessa funktioner visade vi sambandet mellan IL-1β och de epitel-utsöndrade proteinerna. Vid inducerad exacerbation i mössen där IL-1β närvarade, utsöndrades TSLP och IL-33 i förhöjda nivåer i lungvävnaden. Däremot, hade TSLP och IL-33 inte alls påverkats i de möss som hade avsaknad av fungerande IL-1β.

Popularna nauka – (Bosanski) Exacerbation – epizodni napadi

Astma i 'hronična opstruktivna plućna bolest' (COPD) su bolesti kojima nema lijeka, i mogu da se pretvore u vrlo ozbiljna oboljenja, tako zvana pogoršanja ili kao na engleskom jeziku 'Exacerbation'. Sam naziv exacerbation podrazumijeva, da ova obolenja konstantno sadrže inflamaciju i cijelo vrijeme postoji rizik da se stanje pacienata naglo pogorša, koje uzrokuje otežano disanje. To znaći, da stezanje mišićni ćelija oko disajnih puteva u plućima i dodatna inflamacija koja se stvara ujedno u plućnim ćelijama, smanjuje protok zraka u disajnim putevima. Takođe, i velika količina šlajma se stvara u slućaju napada astme ili COPD tako da dovodi pacijenta u kritično stanje. Za ove pretjerane reakcije pluća, ni dan danas nema lijeka. Postoje ljekovi za astmu ali ne smanjuju epizodne napade, koji dovode do kritićnog stanja pacienta. Svaki epizodni napad ostavlja posljedice i negativni trag na disajne puteve.

Razlozi koji utiću na epizodne napade kod ovih bolesti su razlićiti, ali u većini slućajeva je infekcija prouzrokovana bakteriama ili virusima. Svaka obićna prehlada znaći omogućuje napade i pretjerane reakcije pluća, koje je teško kontrolisati i izlijećiti. Prehlada zna i duže da traje u poređenju sa zdravim osobama, takođe i kašalj se zadržava duže vremena poslje prehlade u ovoj grupi pacienata.

Ova tema i doktorska disertacija istražuje nove mogućnosti sa ciljom da se pronađu novi potencijalni lijekovi da bi se smanjili ili skroz zaustavili epizodni napadi. Radeći na disertaciji u periodu stažiranja, došli smo do nekih saznanja i za razvijanjem boljih modela i poboljšanjem dosdašnjih modela.

Da bi se pronašao trag i riješenje uvezi molekularni reakcija u detalj, koje se odigravaju u vrijeme akutnih napada, izvršeni su eksperimenti nad ćelijama donirane od astmatićara i pacienata s obstruktivnom plućnom bolesti, izazvane uzrokom pušenja cigareta. Takođe su rađeni eksperimenti na miševima upotrebljavajuci ih kao modele i uključujući provociranje astme i simtoma prehlade, davajući sintetićke preparate.

Rad s modelima plućnih ćelija i miševa opisanim u ovoj doktorskoj disertaciji, pronađeni su važni molekuli koji su u povišenim količinama kod pacienata sa hroničnom bolesti, poredeći sa zdravim ljudima. Ovi molekuli su zvani TSLP, IL-33 i IL-25 koji vode reakcije pretjerane imunske odbrane, i mogli bi služiti kao potencijalna meta prema kojima bi se proizvodio novi alternativni lijek, također bi sprječavali akutne napade pacienata sa astmom i hroničnom upalom zvanom COPD. Pored toga, studiran je naizad molekul zvani IL-1β, koji je pronađen u povišenim količinama u krvi i plućima u ovim grupama pacienata. U ovoj knjizi smo pronašli da IL-1β upravlja i utjeće na prisutnost molekua: TSLP, IL-33 i IL-25.

Konaćno smo uspjeli da blokiramo izraz TSLP u plućnim ćelijama sa substancama sintetićko napravljenim, takođe koristeći se sa već poznatim substancama koje se u današnji dan upotrebljavaju u klinikama u vezi sasvim drugih bolesti.

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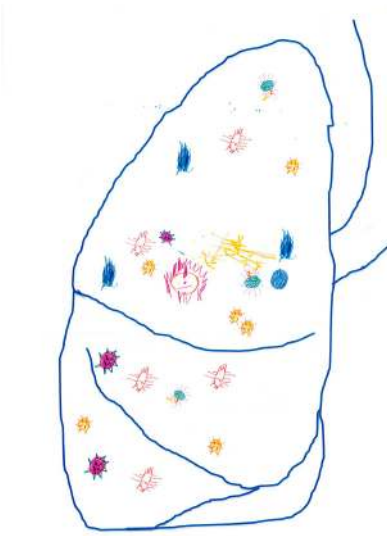
Respiratory viral infections, such as the common cold, affect patients with asthma and chronic obstructive pulmonary disease (COPD) more severely compared to healthy individuals. These acute episodes of shortening of breath are called exacerbation and are burden both on patients and society due to lack of efficient treatment. The aim of this thesis was to develop translational models which mirror human disease. Furthermore, we used our models to study some of the most important immunoregulatory proteins/mediators in exacerbation as potential targets for more efficient therapy.



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