ALLERGIC AND NON-ALLERGIC ASTHMA: TRANSLATIONAL RESEARCH IN IMMUNOLOGY

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ABSTRACT

Asthma is a chronic heterogeneous respiratory disease affecting more than 300 million people globally. The different phenotypes, including allergic and non-allergic asthma, are driven by different underlying mechanisms illustrating the complexity of the disease. Allergic asthmatics often exhibit elevated levels of type 2 (T2) cytokines including interleukin (IL)-4, IL-5 and IL-13 which typically coincide with increased numbers of blood and airway eosinophils. This group generally responds well to treatments. However, more research is needed to develop personalized treatments targeting inflammatory drivers in asthmatics that are unresponsive to currently available treatments, which is more common in non-allergic asthmatics.

Type 2 innate lymphoid cells (ILC2s) are a major source of T2 cytokines and are implicated in both allergic and non-allergic eosinophilic asthma. ILC2s can respond to the cytokine IL-33 that functions as an alarm signal upon tissue damage or cell injury. We identified bone marrow ILC2s to be IL-33-responsive cell types contributing to IL-5-dependent eosinophilic inflammation induced by the protease allergen papain (**Paper I**). In the second study (**Paper II**), we concluded that bone marrow ILC2s might have a critical role at the onset of bone marrow eosinophilia in response to the allergen house dust mite. We also identified IL-33-responsive eosinophils and T helper cells in this study. Targeting ILC2s therapeutically via the IL-33/ST2 axis might be promising in eosinophilic diseases such as asthma. In addition, different pathways are required for pro-inflammatory properties of ILC2s that can be modulated. We demonstrated that airway exposure of IL-33 induced mechanistic target of rapamycin complex 1 (mTORC1) activity in bone marrow ILC2s and that the mTORC1 pathway was crucial for IL-5 production

in experiments using the mTORC1 inhibitor rapamycin (**Paper III**). Other drivers in inflammatory diseases are microRNAs (miRNAs). These short non-coding RNAs exert immune-modulatory effects and can act pro-inflammatory and anti-inflammatory. We identified distinct miRNAs in lung and bone marrow that might drive responses caused by IL-33 (**Paper IV**). There is a need for new stable biomarkers and identification of disease drivers in non-allergic asthma which can be either T2 low or T2 high. We identified unique proteomic profiles in bronchial lavage supernatants from allergic and non-allergic asthmatics (**Paper V**). Proteins distinguishing the two asthma groups, were for example shown to be involved in ciliogenesis, mucociliary clearance and complement activation.

With the ultimate goal of identifying new therapeutic targets, these studies increase our understanding of different inflammatory mechanisms contributing to the ongoing inflammation that might cause airway disease.

Keywords: ILC2s, eosinophils, IL-33, miRNAs, proteomics

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Allergisk och icke-allergisk astma: Translationell immunologisk forskning

Över 300 miljoner människor i världen lider av astma som är en kronisk inflammatorisk lungsjukdom och har en negativ inverkan på livskvaliteten. Innan puberteten är astma vanligast hos pojkar medan efter puberteten är det fler kvinnor än män som blir diagnostiserade med astma. Det är vanligt att astma beror på allergi om sjukdomen utvecklas tidigt i livet, medan icke-allergisk astma blir vanligare med stigande ålder. Faktum är att icke-allergisk astma av svår karaktär främst drabbar kvinnor senare i livet. Utöver uppdelningen mellan allergisk eller icke-allergisk astma finns det flera undergrupper. Det är många immunceller och signalmolekyler som bidrar till inflammation i luftvägarna. Både allergiska astmatiker och icke-allergiska astmatiker kan ha höga nivåer av den vita blodkroppstypen eosinofiler i kroppen. Ett exempel på en astma-undergrupp är icke-allergisk eosinofil astma. Eosinofiler finns för att skydda kroppen mot bakterier och parasiter, men hos ett stort antal astmatiker bidrar just denna celltyp till inflammation och vävnadsskada i lungan. Det finns en stark länk mellan högt antal eosinofiler och svår astma. Idag finns det läkemedel som slår ut eosinofilerna, dock svarar inte alla individer med astma tillräckligt bra på dessa mediciner och nya behandlingar behöver utvecklas.

Eosinofiler bildas i benmärgen liksom många av våra celler. De är särskilt beroende av signalmolekylen interleukin-5 (IL-5) som bildas av eosinofiler, T-hjälparceller av typ 2 (T_H2) eller medfödda lymfoida celler av typ 2 (ILC2). ILC2-cellen är en relativt nyupptäckt cell i vårt immunförsvar som reagerar snabbt på främmande ämnen genom att producera signalmolekyler i en högre koncentration som bidrar till eosinofil-inflammation jämfört med T_H2-celler. Vi har studerat ILC2-celler från möss i **delarbete I – III** med särskilt fokus på cellerna i benmärgen, eftersom eosinofiler rekryteras från benmärgen till luftvägarna och på så sätt bidrar till inflammationen som driver astma.

IL-33 är ytterligare en signalmolekyl som kan aktivera många immunceller, inklusive ILC2-celler, och bidra till ökat antal eosinofiler. Längs med luftvägarna finns luftvägsepitelet som består av epitelceller och formar en barriär mot skadliga partiklar såsom virus. Exempelvis kan allergener göra att IL-33 frisätts från denna barriär och påverka flera olika typer av celler i immunförsvaret. I **delarbete I – IV** är vi intresserade av att studera hur IL-33 bidrar till inflammationen med höga nivåer av eosinofiler. IL-33 kan driva

inflammationen i både allergisk och icke-allergisk astma och just nu pågår det kliniska läkemedelsstudier där man undersöker om blockering av IL-33 kan hjälpa astmapatienter. I **delarbete I** och **II** visar vi att ILC2-cellerna i benmärgen svarar på IL-33 i möss som exponeras för allergener. Exempelvis verkar det som att ILC2-celler som reagerar på IL-33 är särskilt viktiga i början av eosinofil inflammation som svar på exponering av allergiframkallande kvalster. I **delarbete III** visar vi att en specifik cellsignaleringsväg i ILC2-celler från benmärgen är kritisk för bildandet av IL-5 och utveckling av eosinofiler efter stimulering med IL-33. I **delarbete IV** har vi studerat hur IL-33 påverkar icke-kodande RNA-molekyler, som kallas mikroRNA (miRNA), i lungan och benmärgen. Dessa miRNA kan reglera uttryck av proteiner i kroppens celler och många miRNA har visat sig bidra till inflammation. Genom att identifiera kritiska miRNA som bidrar till inflammation i luftvägarna skulle potentiella nya läkemedel mot astma kunna tas fram som riktar sig mot dessa RNA-molekyler.

I **delarbete V** studerade vi hur uttrycket av olika proteiner skiljde sig åt mellan allergiska astmatiker och icke-allergiska astmatiker genom att mäta proteinnivåer i prover från lungan. Vi kunde identifiera flera proteiner vars uttryck var olika mellan astmagrupperna. Skillnaderna i proteinuttryck kan potentiellt bidra till de olika sjukdomsbilderna hos de två patientgrupperna. Dessa fynd behöver valideras och det hade varit värdefullt att undersöka detta vidare hos fler individer med astma.

Sammanfattningsvis bidrar studierna i denna avhandling till kunskapsläget kring hur olika mekanismer i immunförsvaret av allergisk och icke-allergisk karaktär driver astma. Resultaten stärker bilden av den inflammations-bidragande roll som ILC2-celler har och visar att de dels påverkar immunceller i benmärgen och därigenom kan bidra till sjukdom i lungan. Det kan vara värdefullt att identifiera nya miRNA-molekyler och proteiner som bidrar till astma för att hitta nya måltavlor för läkemedel och hjälpa de astmapatienter som inte svarar på dagens behandlingar.

LIST OF PAPERS

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

I. **Boberg E**[*], Johansson K[*], Malmhäll C, Calvén J, Weidner J and Rådinger M.
Interplay between the IL-33/ST2 axis and bone marrow ILC2s in protease allergen-induced IL-5-dependent eosinophilia.
Front Immunol. 2020;11:1058.

[*] *Equal contributions*

II. **Boberg E**, Johansson K, Malmhäll C, Weidner J and Rådinger M.
House dust mite induces bone marrow IL-33-responsive ILC2s and T_H cells.
Int J Mol Sci. 2020;21(11).

III. **Boberg E**, Weidner J, Malmhäll C, Calvén J, Corciulo C and Rådinger M.
Rapamycin dampens inflammatory properties of bone marrow ILC2s in IL-33-induced eosinophilic airway inflammation.
In revision.

IV. **Boberg E**, Johansson K, Weidner J, Malmhäll C, Calvén J, Lässer C and Rådinger M.
Altered bone marrow and lung microRNAs in interleukin-33-induced eosinophilic airway inflammation.
In manuscript.

V. **Boberg E**, Lässer C, Johansson K, Malmhäll C, Mincheva R, Ekerljung L, Olsson H and Rådinger M.
Proteomic profiles in non-allergic and allergic asthma: A clinical study on bronchial lavage samples using LC-MS/MS.
In manuscript.

CONTENT

ABBREVIATIONS

BAL	Bronchoalveolar lavage
BL	Bronchial lavage
CCL	Chemokine (C-C motif) ligand
CCR3	C-C chemokine receptor type 3
cDNA	Complementary DNA
ELISA	Enzyme-linked immunosorbent assay
FeNO	Fractional exhaled nitric oxide
FMO	Fluorescence minus one
GATA	GATA-binding protein
GO	Gene ontology
HDM	House dust mite
ICS	Inhaled corticosteroids
IgE	Immunoglobulin E
IL	Interleukin
ILC2s	Type 2 innate lymphoid cell
LABA	Long acting beta agonist
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
miR/miRNA	microRNA
MS	Mass spectrometer
mTORC1	Mechanistic target of rapamycin complex 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NGS	Next generation sequencing
nLC	Nano-liquid chromatography
OVA	Ovalbumin
PBS	Phosphate buffered saline
PCA	Principal component analysis
qPCR	Quantitative real-time polymerase chain reaction
Rag	Recombination-activating gene
RISC	RNA-induced silencing complex
SABA	Short-acting beta agonists
sST2	Soluble form of ST2
T2	Type 2
T_H	T helper cell
TMT	Tandem mass tag
Treg	T regulatory cell
TSLP	Thymic stromal lymphopoietin
WSAS	West Sweden Asthma Study
WT	Wild type

1 Introduction

Asthma is a respiratory disease that affects over 300 million people in the world [1, 2]. This thesis focuses on different immune mechanisms in asthma, a disease that previously was considered as a single disease entity. Asthma is now seen as a more complex disease with different underlying mechanisms. Ideally, a person with asthma should be offered a tailored, i.e. personalized, treatment. However, there are individuals with asthma that are poor responders to the current treatments that are available today. Therefore, research that focuses on the immune system and the inflammatory pathways that drive the asthma pathogenesis is needed with the long-term goal to develop novel personalized medicines.

The work in this thesis focuses on pro-inflammatory properties of type 2 innate lymphoid cells (ILC2s) in response to the cytokine interleukin (IL)-33 that promote eosinophilic inflammation in asthma. Moreover, the role of IL-33-induced gene-regulatory microRNAs (miRNAs, miRs), molecules that can modulate inflammatory responses in many diseases including asthma, is studied. Furthermore, unique proteomic profiles in bronchial lavage (BL) fluid characterizing allergic and non-allergic asthmatics that may drive asthma pathogenesis in the two asthma phenotypes are described.

1.1 Asthma – A heterogeneous respiratory disease

Asthma is a complex disease where genetics and environmental exposures are important components. Together these factors alter the immune system. The immune system protects the body from harmful parasites, bacteria, fungi and viruses, but the immune system can also be overactive, release cells and inflammatory signals that initiate inflammation and cause diseases including asthma. These molecular and cellular mechanisms in asthma pathogenesis need to be studied in more detail. Research has been carried out to identify disease drivers of asthma and gain more knowledge around the groups of asthmatics that are poor responders or even unresponsive to the available treatments and are in need of new medicines. More knowledge is also required to better understand why some individuals develop severe asthma late in life and some at young age. Today, advances in the research technology have given us useful tools to identify asthma endotypes, i.e., disease drivers and underlying mechanism(s) have been identified. High-throughput omics methods could be one way forward to identify new asthma endotypes. This includes transcriptomics and proteomics, which have been used in **Paper IV**

and **Paper V**, respectively. In contrast to endotype, a clinical phenotype provides the information about observable characteristics such as late-onset asthma or allergic asthma. Phenotypes and endotypes will be discussed in more detail below. Identification of new asthma endotypes are needed to develop new personalized treatments with the ultimate goal to find the right drug for the right patient.

1.1.1 Epidemiology

Worldwide, asthma has the highest prevalence of all chronic respiratory diseases affecting more than 300 million people [1, 2]. There is a large variation in asthma prevalence globally that ranges from 1 – 18%, and the prevalence in Sweden is estimated to approximately 8% [2-4]. There is a limited number of studies that includes representative samples of the general population in countries that allow evaluation of asthma prevalence trends [5]. However, asthma prevalence tendencies have been extensively studied in children and one comprehensive report showed that prevalence of current wheeze, a symptom of asthma, varied between 3 – 32% in different countries for children 13 – 14 years of age and 4 – 35% for children aged 6 – 7 years [6]. The countries with generally low prevalence of asthma included Albania, Indonesia, Georgia, China and Taiwan whereas countries with the highest prevalence included westernized countries such as New Zealand, Australia, the UK and the USA. A recent study that included adolescents and children demonstrated that the prevalence and severity of asthma symptoms exhibited high diversity and was dependent on age, country, income, and region [7]. Furthermore, data indicate that asthma prevalence might have reached a plateau in parts of Europe and Australia, whereas asthma prevalence in most parts of the world are expected to continue to increase [5, 8, 9]. The prevalence of asthma increased in western Sweden between 2008 and 2016 [10]. Furthermore, between the 1990s and the 2000s, a 54% increase in asthma medication use, with a five-fold increase in the use of inhaled corticosteroids (ICS) in adults, was shown in a study from one of Europe´s largest population-based asthma cohorts, the West Sweden Asthma Study (WSAS) [11]. Asthma shows high morbidity, affects life-quality and has a large burden on a society level [2, 12]. The goal is, therefore, to reach good symptom control which can be achieved with efficacious medicines, increased adherence and, thereby, minimized risk of mortality. The prevalence of severe asthma, i.e., asthma that is poorly controlled despite treatment, is estimated to be 5 – 10% among all individuals with asthma [13, 14]. In western Sweden the number is estimated to approximately 3% and a similar prevalence has been seen in the Netherlands [15, 16]. Identification of new therapeutic targets that will lead to the development of novel medicines are needed for this group of severe asthmatics.

1.1.2 Disease characteristics

Asthma is a life-long chronic inflammatory disease and is characterized by respiratory symptoms such as shortness of breath, wheeze, chest tightness and/or cough together with airflow limitation that may later become persistent [17]. Asthma is often associated with bronchial hyperresponsiveness and inflammation of the airways, but is not needed or sufficient for diagnosis [17]. To confirm diagnosis, history of respiratory symptoms is assessed, followed by spirometry testing to determine variable expiratory airflow limitation [2]. A correct diagnosis is essential to avoid unnecessary treatments or over-treatment but also to avoid diagnostic error. Asthma symptoms can vary over time, since different triggers such as allergens, infections or weather changes can worsen symptoms and airflow limitation. The worsening in symptoms can cause asthma exacerbations that are caused by an increase of the existing inflammation and loss of disease control [18]. Viral respiratory infections are the most common triggers of exacerbations, especially caused by rhinovirus [19, 20].

1.1.3 Phenotypes and endotypes

Phenotypes are defined as an observable clinical presentation, e.g. allergic asthma. Several asthma phenotypes, have been identified through, for example, clustering analysis [21, 22]. Asthma onset often occurs early in life and is most common in boys [2, 23-26]. Allergic asthma, often with eosinophilic airway inflammation, is the most common asthma phenotype and has the highest incidence from early age, with an estimated number of 80% affecting children, and a steady decreased number by increased age with data suggesting that 50% of adult asthma has an allergic component [27-29]. After puberty there is a shift with higher incidence in girls [30, 31]. Non-allergic asthma can be either eosinophilic, neutrophilic or paucigranulocytic and is a less common phenotype that usually develops later in life, tends to be more severe and affects more women [2, 28]. However, late-onset asthma can also be allergic. Other phenotypes include asthma with persistent airflow limitation and obesity-related asthma [32, 33]. The different asthma phenotypes emphasize the complexity of the disease with differences in severity. It also increases the understanding about the patients that do not respond well or are unresponsive to treatments. An airway inflammation that is monitored and caused by type two (T2) inflammation and eosinophilia is generally better targeted by steroid treatment than T2 low asthma [34, 35]. It is important to clinically characterize an asthmatic individual in terms of predicting treatment response. Inflammatory cells, such as eosinophils and neutrophils, are markers of airway inflammation and can be measured in, for example, sputum [36].

A distinct mechanistic pathway causing inflammation and disease is referred to as an asthma endotype. Defining asthma endotypes is important for a personalized medicine approach and development of new medicines. Clinical phenotyping in combination of immunological characterization is needed to improve our understanding of the pathogenesis of asthma.

1.1.3.1 ALLERGIC ASTHMA

Allergic asthma typically has a T2 high endotype which is the most common and studied endotype [27, 35]. In allergic asthma, the allergen is presented by antigen-presenting cells such as dendritic cells to naïve T cells which can differentiate to T helper type 2 (T_H2) cells. Both adaptive T_H2 cells and ILC2s produce the type 2 (T2) cytokines IL-4, IL-5 and IL-13, which stimulate T2 inflammation. IL-5 is key for eosinophil functions, IL-4 induces allergen-specific immunoglobulin E (IgE) antibodies from plasma cells that triggers mast cell degranulation and IL-13 affects airway smooth muscle contractility [37, 38]. Furthermore, T2 inflammation can be characterized by high eosinophil numbers in sputum and blood [32, 39].

Different allergens and other environmental factors can activate epithelial cells to release the alarmins thymic stromal lymphopoietin (TSLP), IL-33 and IL-25 that activate different cell types including cells that produce T2 cytokines causing a variety of downstream inflammatory effects (Figure 1) [39]. These include IgE production by plasma cells in response to allergens, airway epithelial cell responses, airway remodeling and chemotaxis of mast cells, eosinophils and basophils [39].

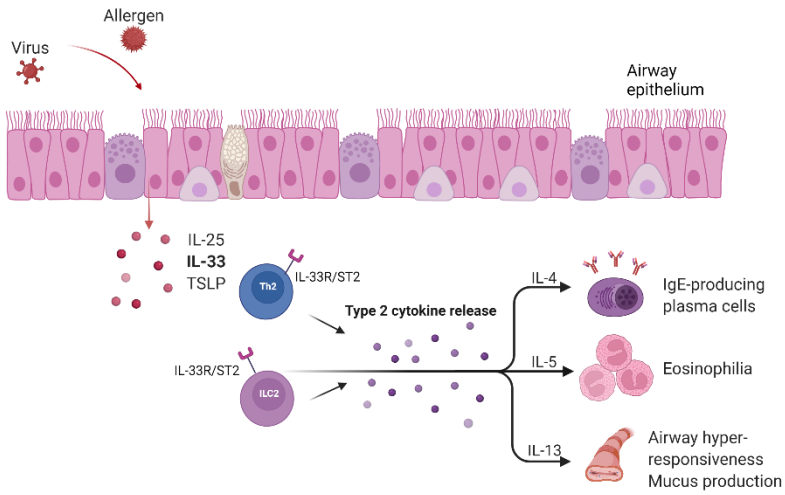

Figure 1. Activation of ILC2s and T_H2 cells by alarmins, such as IL-33, in response to pathogens cause type 2 inflammation in the airways. Figure created with BioRender.com.

Presence of IgE in serum is a hallmark of allergic T2-driven asthma. Other biomarkers associated with mechanisms involved in T2 inflammation are fractional exhaled nitric oxide (FeNO), serum periostin and blood and sputum eosinophils [40, 41] . With that said, raised levels of FeNO and elevated levels of sputum and blood eosinophils were suggested to be better predictors of T2 severe asthma compared to IgE or serum periostin [42]. However, there could be different inflammatory pathways that cause the elevated biomarkers in these asthma patients. Allergic asthmatics generally respond well to treatment with ICS in contrast to non-allergic asthmatics, which often are less responsive to ICS [2].

1.1.3.2 NON-ALLERGIC ASTHMA

Non-allergic asthma means that the patient has asthma that lacks response to allergic sensitization [43, 44]. In contrast to allergic asthma, this phenotype often develops later in life, tends to be more severe and is more common in women [2, 28]. Although, non-allergic asthma exist in children which are less responsive to corticosteroids and display lower levels of IgE [45-47]. The immunopathology varies in non-allergic asthma and can be either T2 low or T2 high [44]. However, many disease features are similar in allergic and non-allergic asthma with high levels of T2 cytokines and eosinophils [44, 48, 49]. Even though eosinophils are present in both sputum and blood of most T2 asthmatics, this cell type may not drive the disease in all of the patients [50].

In a clinical study evaluating efficacy, safety and patient characteristics in patients with severe eosinophilic asthma and treated with the monoclonal antibody mepolizumab, approximately 50% of the patients were non-allergic [51]. These findings highlight that other molecular mechanisms drive T2 responses and eosinophilic inflammation in contrast to allergen-driven T_H2 responses seen in allergic asthma.

As described in the allergic asthma section the alarmins IL-25, IL-33 and TSLP are important in T2 inflammation (Figure 1). IL-33 activates a number of pro-inflammatory cells including mast cells, basophils, ILC2s, T_H2 cells and eosinophils that express the IL-33 receptor, ST2, which in turn contributes to the ongoing airway inflammation [52]. ILC2s are potent sources of T2 cytokines and have been shown to produce more IL-5 and IL-13 compared to T_H2 cells [53]. The effect of T2 cytokines released from ILC2s might explain persistent eosinophilia observed in severe non-allergic asthmatics [54]. The functions of ILC2s are described in more detail in section 1.2.3. Moreover, eosinophilic inflammation can be driven by T_H2 cells in an innate-like fashion independent of T cell antigen receptor [55].

Non-T2 or T2 low asthma could be either neutrophilic or paucigranulocytic, meaning that both eosinophilic and neutrophilic inflammation is absent. The inflammation in asthmatics with a non-T2 neutrophilic inflammation has been associated with activation of T_H17 and T_H1 cells and cytokines from T_H17 cells have been shown to be more resistant to steroids [50, 56]. Increased numbers of neutrophils in sputum could also be caused from airway infections [50]. Other factors that may cause non-T2 or T2 low asthma are mast cell activation and infiltration in airway smooth muscle that cause bronchial obstruction, airway remodeling and airway hyperresponsiveness [50, 57]. The variation of inflammatory cells and cytokines consist of several endotypes that might need different treatments. There is an ongoing debate whether neutrophils are drivers or markers of non-T2 or T2 low asthma. Overall, more research is needed in this area since non-T2 or T2 low asthma is less responsive to steroids and no biologics or other specific treatments are currently available [2, 49, 50].

Asthmatics that initially display a non-T2 endotype might have an underlying T2 inflammation and the biomarkers of T2 might be suppressed from high doses of steroids [2, 35]. Many studies are cross-sectional with influence from steroid treatment and it is, therefore, important that several measurements are performed to more accurately define non-T2 or T2 low asthma and, if possible, remove steroid treatment [50]. A thorough clinical characterization of the patient is key to offer optimal treatment for the patient.

1.1.4 Treatments

The most commonly used type of asthma treatment that decreases the airway inflammation are ICS. Bronchodilators are also combined with other asthma treatments or used separately as symptoms appear or on a daily basis. Many asthmatics respond well to ICS and bronchodilators with good symptom control and reduced risk of exacerbations [2]. Short-acting beta agonists (SABA) and long acting beta agonists (LABA) are commonly used bronchodilators that induce relaxation of the airways. As-needed therapy with ICS plus LABA have been shown to be superior, in terms of asthma control, compared to as-needed treatment using SABA [58]. A group of asthmatics are unresponsive to ICS and most often these patients are prescribed oral corticosteroids to improve symptom control [2, 59]. However, there are side effects associated with oral steroids, such as diabetes and osteoporosis [60]. Another treatment option for this group of patients are biologic drugs. Currently six biologic therapies are approved for severe asthma [61]. Drugs that target IL-5 or its receptor (mepolizumab, reslizumab and benralizumab), can be prescribed to individuals with severe eosinophilic asthma irrespective of allergy [61]. Another drug that targets T2 inflammation is dupilumab, an anti-IL-4 receptor antibody that

inhibits IL-4-mediated and IL-13-mediated inflammatory processes. Dupilumab reduced number of severe exacerbations and improved asthma control with decreased levels of eosinophils and total serum IgE, making this drug a good option for allergic asthmatics with normal or elevated levels of eosinophils [61, 62]. Dupilumab is also approved for patients with chronic rhinosinusitis and atopic dermatitis and could be an adequate treatment alternative if one of these comorbidities exist in patients with severe asthma [61]. Another treatment option for patients with severe allergic asthma is omalizumab that targets IgE [61].

In December 2021, the first anti-alarmin drug tezepelumab, an anti-TSLP antibody was approved by United States Food and Drug Administration, as an add-on maintenance treatment in patients with severe asthma. No asthma phenotyping or specific biomarkers are required to prescribe tezepelumab and is an option for T2 low asthmatics as the number of exacerbations was decreased regardless of asthma phenotype [61, 63]. Additionally, patients on this treatment showed improved asthma control, lung function and quality of life [63]. Today, clinically applicable biomarkers for T2 low asthma are lacking and there is a medical need to identify new biomarkers and therapeutic targets in severe non-eosinophilic asthma [64]. Alarmins drive T2 high responses, but may also be critical regulators in T2 low asthma [63, 64]. Randomized controlled trials with moderate to severe asthmatics have shown efficacy for the antibody itepekimab which targets the alarmin IL-33 [65]. Patients with moderate to severe asthma exhibited better asthma control and improved lung function [65]. IL-33 mediates both T2 and non-T2 responses and targeting IL-33 or ST2 might have a good effect in different phenotypes of asthma [65, 66]. Although itepekimab reduced eosinophil levels, it does not seem to be as efficient as anti-IL-5 therapies [65] . Anti-ST2 therapies are currently in clinical trials and data show that an anti-ST2 antibody reduced asthma exacerbations in different phenotypes of asthma including those with low eosinophil counts [66]. It is imperative to select the right treatment for a patient suffering from severe asthma. The full clinical profile alongside comorbidities needs to be taken into consideration where, for example, eosinophil levels, allergy status, time of asthma onset, nasal polyps or rhinitis are important clinical factors [61]. The patient perspectives in terms of potential side effects and ease of drug administration are also important, since this may affect treatment adherence and clinical outcome. In addition, research around new therapeutic targets and stable biomarkers to monitor the response are needed, since there still are asthma patients that suffer from severe asthma despite existing therapies [65].

1.2 Leukocytes and IL-33

Leukocytes, also known as white blood cells, are crucial components in the immune system. However, leukocytes can also be drivers in inflammatory diseases, such as asthma. Eosinophils, ILC2s and T_H cells will be introduced in this section. These cell types can respond to the alarmin cytokine IL-33. The role of IL-33 in asthma is also included in this section.

1.2.1 Eosinophils

In 1879, the Nobel Prize winning Paul Ehrlich published methods for differential blood cell counting by staining blood films and described the eosinophil [67]. Although, a number of researchers likely observed eosinophils before Ehrlich [67]. Eosinophils are terminally differentiated granulocytes with critical functions protecting from parasitic, bacterial or viral infections [68]. However, eosinophils contribute to tissue damage and disease pathogenesis in a number of diseases including asthma, affecting for example airway remodeling by releasing toxic inflammatory mediators [69-72]. Eosinophils develop from $CD34^+$ progenitor cells in the bone marrow in presence of eosinophilopoietic growth factors IL-3, granulocyte-macrophage colony-stimulating factor and IL-5 [73-75]. IL-5 is essential for eosinophil development with roles in terminal differentiation, proliferation, survival and cell migration [76-78]. As IL-5-deficient mice have homeostatic levels of eosinophils it is suggested that other factors are important for eosinophilopoiesis, i.e., production of eosinophils [79, 80]. IL-5-producing cells in the bone marrow includes ILC2s, $CD4^+$ T cells and $CD34^+$ progenitor cells [81-84]. Furthermore, systemic levels of IL-5 might regulate eosinophil lineage in the bone marrow [85]. One study reported that asthma patients treated with the anti-IL-5 antibody, mepolizumab, had 70% lower levels of mature eosinophils and unchanged levels of eosinophil progenitors in the bone marrow compared to patients given placebo [86]. Other factors regulating eosinophilopoiesis are transcription factors, including GATA binding protein (GATA)-1 and GATA-2, which have been shown to regulate differentiation [87]. Eosinophils express the C-C chemokine receptor type 3 (CCR3) which binds to three subtypes of eotaxins selective for eosinophils [88]. In this way, eosinophils migrate from the bone marrow to the airways via the bloodstream when eotaxins are expressed and released in the airways (Figure 2) [89]. To summarize, many factors including cytokines, transcription factors and growth factors regulate the eosinophilopoiesis.

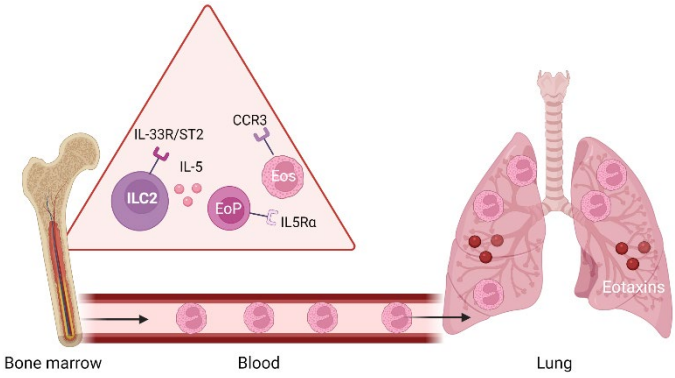

Figure 2. Eosinophils develop from progenitor cells in the bone marrow in presence of IL-5. Eosinophils expressing CCR3 can be recruited to the airways via the bloodstream during airway inflammation when eotaxins are present in the airways. Figure created with BioRender.com

Eosinophils are key inflammatory cells in asthma, contributing to airway dysfunction [89]. Elevated levels of eosinophils in sputum correlated with asthma severity as well as the risk of asthma exacerbations [90, 91]. In addition, lung function has been predicted by blood eosinophil status [90, 92, 93]. Allergic asthmatics with early asthma onset usually display elevated eosinophil levels with a classical T2 high phenotype. This group responds well to steroid treatment. However, a subgroup of asthmatics that have a non-allergic asthma phenotype with high eosinophil levels, are poor responders to steroids and, therefore, need other treatments [2]. There are currently three biological treatments approved for eosinophilic asthma targeting IL-5 or its receptor [61]. The effects on the levels of blood and sputum eosinophils and eosinophil progenitors varies between the different drugs [94]. The anti-IL-5 monoclonal antibody, mepolizumab, suppressed eosinophils levels in the circulation but not in the sputum of patients with severe prednisone-dependent asthma [95]. Moreover, there were no effects on the prednisone dose among these patients. In contrast, decreased airway eosinophilia was observed in patients with prednisolone-dependent asthma taking benralizumab that targets the IL-5 receptor and was associated with better asthma control, increased lung function and reduction in oral corticosteroid use [96]. Many patients with severe eosinophilic asthma respond well to these treatments with decreased number of exacerbations [97]. However, there are treated asthmatics with elevated eosinophil levels who are non-responders or respond poorly to anti-IL-5 treatment [68]. This indicates that the underlying mechanism(s), i.e., asthma

endotype is different. Future research is needed to identify the complex mechanism(s) behind asthma endotypes of eosinophilic asthma.

1.2.2 The alarmin interleukin-33

IL-33 is part of the IL-1 family, has a role in host defence, protecting us from parasitic and viral infections, but can act pro-inflammatory in several diseases [98]. IL-33 functions as an alarm signal upon tissue damage or cell injury in allergic conditions and chronic inflammatory diseases including asthma [99]. This alarmin cytokine activates several immune cells and contributes to inflammatory processes including eosinophilia. IL-33 binds to ST2, on key cells in inflammation and allergy such as ILC2s, conventional T cells, T regulatory cells (Tregs), macrophages, mast cells, basophils, neutrophils, natural killer cells and eosinophils [52, 98, 100-102]. ST2, also known as Il1rl1, was discovered in 1989 and gained interest in immunology research after it was discovered that ST2 was expressed on T_H2 cells and not T_H1 cells [103-105]. Almost twenty years later the ST2 ligand IL-33 was discovered in a study which identified that signaling via IL-33/ST2 axis activated NF-κB and mitogen-activated protein kinases (MAPKs) and stimulated cytokine production from T_H2 cells [106]. IL-33 is expressed in epithelial cells, endothelial cells and fibroblast-like cells during homeostatic conditions as well as inflammation [99]. Other sources of IL-33 are immune cells such as macrophages, mast cells and dendritic cells that are able to express IL-33 upon activation including allergen exposures from, for instance, house dust mite (HDM) [106-110].

The IL-33 protein is cleaved and inactivated by caspase-1, an important mechanism to avoid immune activation since the full length active form exerts its biological effect via ST2 and the co-receptor, IL-1 receptor accessory protein which induces MyD88-dependent inflammatory signaling [111]. Moreover, the soluble form of ST2 (sST2) negatively regulates the activity of IL-33 by its binding together with soluble interleukin-1 receptor accessory protein [112].

Mechanistic studies investigating the role of the IL-33/ST2 pathway in asthma use a variety of *in vivo* models. Exogenous IL-33 or other triggers, including allergens such as HDM, alternaria or papain, that induce IL-33 release of the airway epithelium and/or IL-33 production by other cell types with downstream inflammatory effects are often used in these models [113, 114]. Mice lacking IL-33 or ST2 exhibit lower blood eosinophil levels indicating that IL-33 is needed for basal eosinophil homeostasis [115]. Reduced levels of T2 cytokines and decreased eosinophilia have been observed in mice deficient in

ST2 or treated with neutralizing anti-IL-33 antibodies [116-119]. Moreover, IL-33 administered together with ovalbumin (OVA) in OVA-sensitized mice led to inflammation with increased eosinophils, ILC2s and T2 cytokines compared IL-33 only or OVA-sensitized and challenged with OVA [120]. IL-33 can induce eosinophilopoiesis in the bone marrow and the periphery and bone marrow ILC2s produce IL-5 in response to IL-33 contributing to eosinophilopoiesis [82, 115].

Emerging evidence suggests a crosstalk between the three epithelium-derived cytokines IL-33, TSLP and IL-25 which may worsen T2 inflammation [121, 122]. For instance, mice challenged with alternaria exhibited decreased IL-33 levels in bronchoalveolar lavage (BAL) when treated with anti-TSLP antibodies [123]. Furthermore, elevated levels of T2 cytokines driving eosinophilic inflammation, including IL-5 and IL-13, were detected in supernatants of isolated lung ILC2 that are stimulated with TSLP and IL-33 compared to IL-33 only [123]. Interestingly, ST2-deficient mice exposed to a combination of three allergens exhibit increased levels of TSLP in ILC2-mediated inflammation of asthma and highlight the complexity targeting alarmins [124].

IL-33 plays a part in both T2 asthma and non-T2 asthma [125]. Several genome wide association studies have identified both IL-33 and its receptor as major susceptibility genes for developing allergic diseases and asthma [126-129]. Moreover, elevated levels of IL-33 in BAL from asthmatics with severe uncontrolled asthma or moderate asthma compared to asthmatics with milder asthma or healthy controls have been observed [130, 131]. Higher messenger RNA (mRNA) levels of IL-33 in endobronchial biopsies of asthmatics and particularly severe asthmatics compared to healthy controls have been reported as well as IL-33 expression on both gene and protein level in airway smooth muscle cells [132]. Elevated levels of sST2 have been observed in serum from atopic asthmatics compared to healthy controls and the increase of sST2 positively correlated with the severity of asthma exacerbations [133]. Higher serum sST2 levels might also be used to predict exacerbations in the general asthma population irrespective of atopy [134]. The IL-33/ST2 axis is indeed a potential therapeutic target in patients with severe asthma, uncontrolled asthma and other IL-33-mediated diseases. Clinical trials investigating the IL-33/ST2 axis as a drug target are ongoing [65, 66]. Asthmatics that were administered a monoclonal antibody targeting IL-33 exhibited lower blood eosinophilia and improved lung function [65, 135].

1.2.3 Type 2 innate lymphoid cells

In 2010, ILC2s derived from mouse and human adipose tissue were discovered [136]. These cell types were described as lineage-negative innate lymphocytes which produced T2 cytokines and were referred as natural helper cells [136]. It was reported that the cells expressed the IL-33 receptor and responded to IL-33 and helminth infection by producing IL-13, leading to goblet cell hyperplasia, a critical mechanism during helminth infections [136]. The same year ILC2s in other organs including mesenteric lymph nodes, bone marrow and lung were identified and the cells were also referred as nuocytes [137, 138].

ILCs develop from the common lymphocyte progenitor in the bone marrow and progress into ILC precursors that generate three subpopulations ILC1s, ILC2s and ILC3s [139, 140]. The ILC subsets mirror the $CD4^+$ T cells in terms of transcription factors and cytokine profiles: T_H1/ILC1 (T-bet; INF-γ, TNF-α), T_H2/ILC2 (GATA-3: IL-4, IL-5, IL-13) and T_H17/ILC3 (RORγt: IL-17, IL-22) [141]. Similarly, to Tregs, ILCs have been shown to produce IL-10 with immunosuppressive functions. Regulatory ILCs seem to produce IL-10 irrespective of FOXP3 expression [142]. Recently, it was demonstrated that IL-10^+ ILC2s dampened T_H2 responses and restored the epithelial barrier integrity in patients receiving allergen immunotherapy [143].

As part of the innate immune system, ILC2s respond quickly to environmental insults where, in contrast to T cells, ILC2s do not require clonal expansion for an efficient immune response [144]. ILC2s can be activated and release the T2 cytokines IL-4, IL-5 and IL-13, in response to the alarmin cytokines IL-25, TSLP or IL-33 induced by allergens, viruses or other environmental factors [145, 146]. IL-5 is essential for eosinophilia whereas IL-4 and IL-13 induces several asthma features including mucus production, IgE mediated responses and airway remodelling [146]. These classical allergen-responses were initially hypothesized to only be induced by T_H2 cells [136]. Even though ILC2s can function without the adaptive immune system, which have been demonstrated in mice lacking adaptive immune cells, inflammatory properties of ILC2s can be amplified when interacting with adaptive immune cells [147]. For example, soluble factors such as IL-5 from murine lung ILC2s were shown to induce an early antibody response by B cells [148]. The interactions between ILC2s and T cells are described in more detail in section 1.2.4.

ILC2s play a critical role in regulating inflammation and maintaining tissue homeostasis, however, ILC2s are also involved in the pathogenesis of several inflammatory diseases including asthma and allergy [146, 149, 150]. The research field has expanded in the last decade with ILC2s as potential therapeutic targets

[151-153]. Extensive research has been done in both translational murine and human studies. A higher number of sputum and blood ILC2s, being the predominant source of T2 cytokines, have been observed in patients with severe asthma compared to mild asthma [53]. Higher levels of ILC2s in BAL which coincided with elevated levels of IL-33 have been reported in asthma patients compared to healthy controls [119]. Moreover, allergen challenge in mild to moderate asthmatics with high eosinophil levels resulted in increased numbers of airway ILC2s and lower levels of blood ILC2s, suggesting that ILC2s may be recruited from the blood to site of inflammation [154]. The same study reported several genes involved in T2 inflammation to be activated in ILC2s from the airways, whereas this was not observed in blood ILC2s [154]. Air-liquid interface co-culture system with human bronchial epithelial cells and ILC2s revealed that ILC2s disrupted epithelial barrier function which was further induced by IL-33 [155]. In addition, neutralization of IL-13 prevented this disruption and it was also demonstrated in an *in vivo* ILC2-dependent model driven by IL-33 that IL-13 production by ILC2s contributed to epithelial barrier breakdown [155]. IL-33 is a potent activator of ILC2s in different organs such as lung, skin and bone marrow, possessing pro-inflammatory properties [141]. IL-33-activated lung ILC2s were critical in development of airway hyperreactivity and eosinophilia in response to respiratory syncytial virus [156]. Furthermore, rhinovirus infection of bronchial epithelial cells induced IL-33 that activated human ILC2s [157]. Allergen challenge with the fungal alternaria can stimulate IL-33-responsive lung ILC2s to produce IL-5 [85]. Airway exposure of alternaria induced bone marrow progenitor ILC2s to migrate to the lungs, an IL-33-dependent mechanism [113]. Moreover, bone marrow ILC2s were found to be producers of IL-5 and important for eosinophilopoiesis during IL-33-induced inflammation [82].

It has been reported that ILC2s can be regulated by sex hormones like oestrogen and testosterone [158-162]. Current data suggest that androgens, with testosterone as an example, might play a protective role in T2 inflammation with effects on, for example, ILC2 expansion during IL-33-induced eosinophilic inflammation [158]. Furthermore, higher levels of circulating ILC2s have been observed in women with moderate to severe atopic asthma compared to men with moderate to severe atopic asthma [159]. Conversely, other studies have reported no differences in circulating or airway ILC2s numbers between men and women with asthma [162, 163]. More studies including clinically well-characterized asthmatics are needed to investigate potential ILC2 gender differences and its clinical relevance for asthma and other inflammatory diseases.

The local tissue environment can generate other subsets of ILCs which is referred as plasticity. For example, circulating human ILC2s were found to co-express the T_H1 cytokine interferon-γ which coincided with increased T-bet expression [164]. Moreover, ILC2-to-ILC3 plasticity may contribute to the pathogenesis of chronic diseases associated with IL-17 [165]. Lung ILC2s expressing ST2 have been shown to produce IL-17 in response to IL-33 [166]. ILCs are generally considered as tissue-resident cells demonstrated in parabiosis experiments, i.e., when the biological systems of two living organisms are combined using surgery [167, 168]. However, plastic ILC2s are able to adapt to the surrounding environment and may migrate to site of inflammation [169]. ILCs are heterogeneous cells and the functional role of how plasticity of ILCs contribute to chronic inflammation and allergy needs to be addressed in prospective studies [169].

1.2.4 IL-33-responsive T cells

In contrast to ILCs, T cells are part of the adaptive immune system and can recognize foreign antigens derived from viruses, bacteria and allergens with their specific T cell antigen receptor [170]. Both T_H cells and Tregs can respond to IL-33 via ST2 that has been shown to take part in the regulation of T_H2 cells and T2 responses. Blockage of IL-33 signaling causes impaired T_H2 effector functions with lower levels of IgE, type 2 cytokines and eosinophils in allergen models of asthma [171-173]. Despite the strong association of ST2 and functions of T_H2 cells, T_H1 cells was shown to transiently express ST2 upon differentiation in response to viral infection [174]. IL-33 induces production of T2 cytokines by T_H2 cells and can also work as a chemoattractant for T_H2 cells [101, 175, 176]. Furthermore, IL-5 production by T cells was induced in mice exposed to IL-33, which worsened an allergen-induced airway inflammation [117].

There is substantial evidence that ILCs can modulate T_H2 responses [177]. One study reported an impaired allergic inflammation mediated by T_H2 in ILC2-deficient mice [178]. ILC2-derived IL-13 was crucial for dendritic cells to migrate from lung to the lymph node to prime naïve T cells into T_H2 cells [178]. Interestingly, the same study showed that activation of ILC2s and T_H2 differentiation in response to papain was IL-33-dependent. Mouse studies have shown that ILCs express major histocompatibility complex (MHC) class II and might act as antigen-presenting cells [177]. Another study demonstrated that MHC class II were expressed on ILC2s in lymph nodes and interacts with antigen-specific T cells to enhance T2 response against a roundworm by $CD4^+$ T cell activation and expansion [179]. Furthermore, the same study showed that human ILC2s express MHC class II in response to HDM and that antigen

presentation to T cells initiated cytokine expression. In a mouse model of asthma using papain, depletion of lung ILC2s during allergen re-challenge caused impaired T_H2 cell recruitment to the lungs which was dependent on ILC2-derived IL-13 [180]. Interestingly, it has also been shown that ILC2 induction require T cell activation in a murine HDM-induced asthma model of airway inflammation [181].

1.3 Regulation of gene expression by microRNAs

Non-coding miRNAs are short (~22 nucleotides) RNAs that may inhibit gene expression via post-transcriptional regulation of mRNA translation [182]. The first miRNA was discovered approximately 30 years ago and the research interest in non-coding RNAs, including miRNAs has increased as these molecules can modulate several cellular processes including cell proliferation, differentiation and migration [183-185]. Moreover, miRNAs can regulate inflammatory responses and are involved in immune regulation of several diseases, including allergy and asthma [186-188]. Recently, specific miRNAs have also been identified as potential therapeutic targets.

1.3.1 Biogenesis and regulation by microRNAs

The biogenesis of miRNAs require several steps starting in the cell's nucleus where they are transcribed, mainly by RNA polymerase II, into structured primary miRNAs, known as pri-miRNAs, with hairpin structures (Figure 3) [185, 189, 190]. The enzymes Drosha and the protein DiGeorge syndrome critical region 8 further process pri-miRNAs into precursor miRNAs, i.e., pre-miRNAs, that consist of approximately 60 – 70 nucleotide stem-loop structures. Following export from the nucleus to the cytoplasm by Exportin-5, cleavage of pre-miRNAs occurs by the RNase enzyme Dicer and the Dicer-binding protein TRBP into approximately 22 nucleotide miRNA duplexes with a -5' and -3' overhang on each end. The guide strand is loaded into the protein Argonaute forming an RNA-induced silencing complex (RISC) whereas the other strand, referred to as the passenger strand, is degraded. Within the silencing complex the mature miRNA can interact with a target mRNA resulting in either degradation of the mRNA or translational repression. Most often, the miRNA binds to a complementary seed sequence of approximately 8 nucleotides in the -3' untranslated region of their target mRNA, but it could also bind to the -5' untranslated region or coding sequences [191, 192]. Indeed, miRNA regulation is complex as one miRNA can target several mRNAs and several miRNAs can target one mRNA[185]. The miRNAs can also be exported in miRISC or loaded into secretory vesicles that may affect numerous cell types.

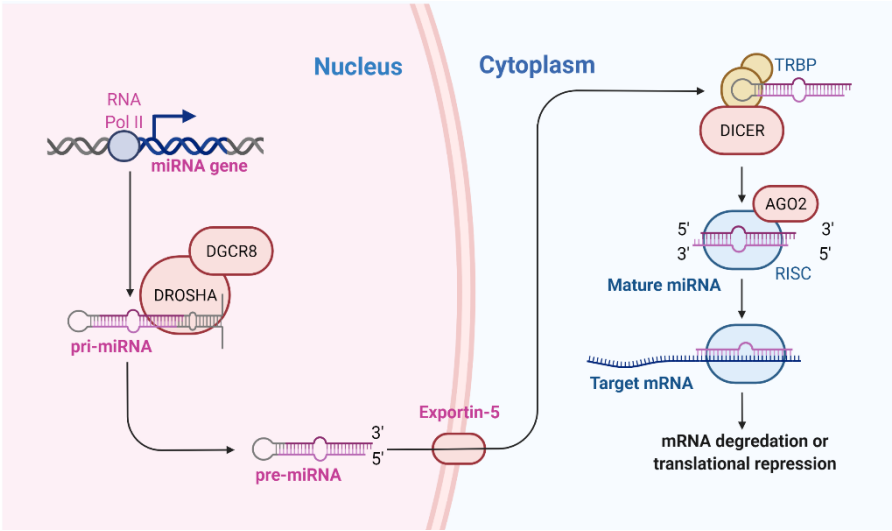

Figure 3. miRNA biogenesis and mechanisms of action. Figure created with BioRender.com

1.3.2 microRNAs in allergy and asthma

Pro-inflammatory and anti-inflammatory properties are exhibited by miRNAs and identifying the contribution of specific miRNA(s) to the mechanism(s) driving inflammatory diseases could make the targeting of miRNAs promising as potential therapeutics or biomarkers [193].

Many miRNAs have been proposed to be involved in the pathogenesis of asthma and allergic disease. A thoroughly studied miRNA in allergy and asthma, with implications in innate and adaptive immunity, is miR-155 which regulates many cell types involved in inflammatory processes [194-198]. Mice that are deficient in miR-155 are protected from eosinophilic inflammation in the lung in both allergic and non-allergic airway inflammation models driven by T_H2 and ILC2s responses [199, 200]. miR-155 has also been extensively studied in humans. For example, miR-155 in plasma was increased in severe asthmatics compared to individuals with milder asthma and healthy controls [201]. Interestingly, reduced levels of miR-155 was observed in breath condensates from asthmatics compared to healthy controls highlighting the complexity of miRNAs [202]. In addition, miR-155 expression was higher in circulating $CD4^+$ T cells in asthmatics compared to individuals with allergic rhinitis and non-allergic controls [203]. The same study showed that miR-155 expression positively correlated with T2 cytokine levels [203]. Furthermore, miRNA

expression in serum, including miR-155, has been shown to correlate with clinical parameters in individuals with different subgroups of asthma [194].

Due to the role of eosinophils in asthma and allergy, investigations into miRNA regulation of eosinophilic inflammation have been performed. For example, inhibition of miR-126 with an antagomiR, i.e., a molecule that inhibit the miR, in mice exposed to HDM resulted in decreased airway hyperreactivity and T_H2 responses with decreased eosinophilic inflammation [204]. During allergic airway inflammation miR-1 regulated eosinophil trafficking and has therapeutic potential not only in in asthmatic patients but also in patients with chronic rhinosinusitis [205]. Moreover, inhibition of miR-21 resulted in decreased eosinophil levels in a mouse model of asthma and miR-21 has been involved in both inflammatory properties of ILC2s and T cells in other inflammatory diseases [206, 207]. Several eosinophil enriched miRNAs have been identified and were suggested to be involved in eosinophil development [208-210]. For example, miR-21 was shown to be crucial for eosinophil progenitor cell growth and miR-21 knock out mice exhibited lower eosinophil colony-forming unit capacity in the bone marrow [209]. In addition, serum miR-21 might serve as a potential biomarker in diagnosis of eosinophilic asthma [211]. Increased eosinophil levels have been associated with miR-223 and loss of miR-223 led to defects in eosinophil development [210, 212, 213]. Moreover, increased levels of serum miR-223 correlated with blood eosinophils from non-allergic asthmatics and the expression levels were higher in non-allergic asthmatics compared to allergic asthmatics [214]. Another miRNA that is enriched in circulating eosinophils is miR-185 and it was shown that a miRNA profile containing this miRNA was able to distinguishing asthmatics from healthy individuals suggesting that it may serve as a potential asthma biomarker [215].

There is currently limited data on miRNAs that might regulate IL-33-induced eosinophilic inflammation. One study reported that murine lung ILC2s deficient in miR-17~92, a cluster of miRNAs that exhibit immune-modulatory effects on lymphocytes, demonstrated insufficient cytokine production in response to IL-33, TSLP and IL-7 [216]. Moreover, the same study reported reduced levels of T2 cytokines by lung ILC2s and reduced eosinophilia in mice deficient in miR-17~92 that were challenged with the allergen papain which can induce IL-33 responses [216]. Another study, demonstrated that IL-33-exposed mice deficient in miR-155 exhibited decreased eosinophilic inflammation with impaired pro-inflammatory properties of lung ILC2s [199]. In summary, studies in murine models of asthma and in humans show the effects that miRNAs possess on immunity.

1.3.2.1 Clinical perspectives for miRNAs

There are currently no approved miRNA-based drugs however, several miRNA mimics and antagomiRs tested for cancer treatment and other diseases, excluding asthma, are currently in clinical trials [217]. One miRNA can target a large number of mRNAs and can potentially regulate an entire biological pathway making them interesting from a therapeutic perspective. However, being pleiotropic regulators, off-target effects might occur which is a concern when it comes to treating patients [218]. The use of miRNA mimics and antagomiRs in, for example, murine models of asthma or *ex vivo* studies on human cells or tissues have been useful to assess the anti-inflammatory effects and to identify pathways that are either regulated by miRNAs or that regulate miRNAs [188, 217]. One challenge for the clinical application of miRNAs as treatment is to deliver miRNAs to specific tissues. By developing new drug delivery systems in combination with optimized mimics and antagomiRs, this may lead to new miRNA-based therapeutics.

1.4 Asthma and proteomics

In recent years, high-throughput proteomics, such as mass spectrometry, have advanced in identifying proteins in clinical samples by for example mass spectrometry and several thousands of proteins can be detected in one sample [219, 220]. There is currently a need for new stable biomarkers for diagnosis of asthma phenotypes and endotypes. Quantitative proteomics of human samples have been used in many research areas including the respiratory field with the goal of identifying successful biomarkers and disease drivers. Other methods that are often used to quantify proteins are western blot and enzyme-linked immunosorbent assay (ELISA). Indeed, proteomics seem to be a promising method to investigate and characterize disease drivers in different phenotypes in asthma [50, 221, 222]. Biological fluids such as plasma, sputum, nasal lavage fluid and BAL are used for comparison of protein expression between healthy controls and asthma groups [223-227]. Also cells can be used, for example, proteomic characterization of T cells in blood from asthmatic patients and healthy controls revealed differentially expressed proteins [228]. Moreover, increased levels of a specific calcium binding protein were identified in sputum from severe uncontrolled asthmatics with neutrophilic inflammation compared to controlled asthmatics [223]. In addition, increased levels of a fatty acid binding protein were observed in sputum from allergic asthmatics and linked to inflammation and airway remodeling [225]. There is a limited number of studies on the proteomic profiles in human BL samples in diseases including asthma, possibly due to high complexity of both samples and the disease itself.

Generally, there is a lack of proteomic studies on well-characterized asthmatics with different phenotypes such as allergic asthma and non-allergic asthma [229].

Quantitative proteomics, including tandem mass tag (TMT)-based techniques, are also applied in asthma mouse models and potential therapeutic targets have been identified [230-232]. Moreover, one study analyzing proteomics by liquid chromatography-mass spectrometry (LC-MS) reported proteins in BAL to be differently expressed in mice with neutrophilic asthma compared to eosinophilic asthma [233]. There are challenges concerning proteomics of human samples in contrast to mice with less variability factors. The biological variability can indeed be high and tissue samples, from e.g. patients, often exhibit higher variability than for example cell culture samples. Ideally, individuals in the same group have similar proteomic profiles and the compared groups should be well separated, i.e., a low biomarker level in non-allergic asthma versus high in allergic asthma.

A substantial number of studies have identified asthma susceptibility genes [234, 235]. The gained knowledge on protein expression is valuable as proteins are the functional molecule in the body in contrast to genes that needs to be transcribed and translated into proteins. Moreover, a gene can by RNA splicing and gene arrangements code for several proteins [236]. However, the proteome is dynamic and is constantly changing and only a snapshot is obtained of the proteomic profiles when the actual sampling occurred.

2 Aim

The aim of this thesis was to determine disease drivers with potential roles in allergic and non-allergic asthma. This included *in vivo* studies investigating pro-inflammatory properties of bone marrow ILC2s and miRNAs during eosinophilic inflammation as well as studying the proteome in allergic and non-allergic asthmatics.

Specific aims were to:

Paper I: Determine the role of bone marrow ILC2s *in vivo* in the regulation of allergen-induced and IL-33-induced eosinophilia using wild type (WT) mice and genetically modified mice.

Paper II: Determine if ILC2s, T_H cells and mature eosinophils in the bone marrow are responsive to IL-33 in mice exposed to airway challenges of HDM.

Paper III: Determine if the mechanistic target of rapamycin complex 1 (mTORC1) pathway is induced in bone marrow ILC2s *in vivo* in response to IL-33. Further, examine if mTORC1 inhibition affects the pro-inflammatory properties of bone marrow ILC2s and IL-33-induced eosinophilia.

Paper IV: Determine miRNA expression patterns in the bone marrow and lung in mice exposed to IL-33 and determine if IL-33-induced candidate miRNAs in the bone marrow are affected by mTORC1 inhibition.

Paper V: Determine the proteomic profiles in BL supernatants from allergic and non-allergic asthmatics to identify differences in protein expression between the groups that may have a role in asthma pathogenesis.

3 Methods

The following section describes and discuss the methods applied in this thesis. More detailed information about the methodology is provided in each paper.

3.1 Mouse models of airway inflammation

Asthma is a heterogeneous disease with different underlying molecular mechanisms. Mouse models of asthma have been extensively used to study the airway inflammation and has been valuable in the research field [237]. Molecularly, the genomes of mice and human are conserved and data show approximately that 80% of mouse genes have one identifiable orthologue in the human genome [238]. Recently, a comprehensive atlas comprising immune differences between humans, non-human primates and mice was developed using mass cytometry [239]. Cell type frequencies differed between species and, for example, mice had 10 times more B cells in blood compared to humans and 10 times fewer blood neutrophils compared to the other primates [239]. Moreover, unique cell phenotypes were identified in the different species. It is critical to understand immune differences of humans in relation to other species to increase the success rate when identifying new targets to potentially develop new drugs.

Mouse models of asthma using WT mice or genetically manipulated mice are the major choice of *in vivo* asthma models and will most likely continue to dominate. However, it is important to be aware of its limitations. For example, mice do not spontaneously develop asthma in contrast to guinea pigs which have been widely used as an asthma model [237, 240]. However, mouse models have been useful to study inflammation, airway hyperreactivity and airway remodelling in a variety of asthma mouse models [241, 242]. No models of asthma will fully represent the complexity of the human disease but will continue to provide us with new important knowledge about the disease mechanisms [237].

Mouse models in this thesis was used to study the ongoing inflammation with main focus on IL-33 and ILC2 cells. Both allergen-induced models and non-allergic models have been used and are described below (Figure 4). Age- and sex-matched mice were used in all experiments. In **Paper I** we used genetically modified mice in addition to C57BL/6 WT mice that were used in **Paper I – IV**. All animal experiments were approved by the Gothenburg County Regional Ethical Committee (permit numbers 126/14 and 2459/19).

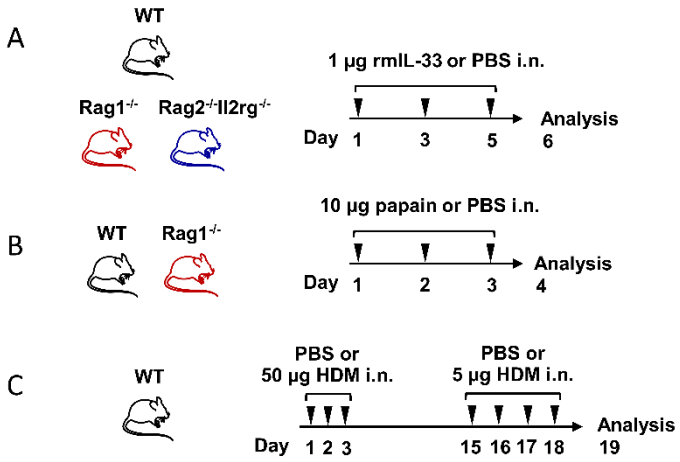

Figure 4. Mouse models of allergic and non-allergic airway inflammation used in this thesis. **(A)** IL-33-induced inflammation (Paper I, III and IV). **(B)** Papain-induced inflammation (Paper I) and **(C)** HDM-induced inflammation (Paper II). i.n.: intranasal, WT: wild type, rm: recombinant mouse.

3.1.1 IL-33-induced eosinophilic inflammation

The alarmin cytokine IL-33 induces T2 responses with robust eosinophilic response and is often used as a non-allergic mouse model of asthma [243]. IL-33 was given directly via the intranasal route to mimic natural exposures (Figure 4A). WT mice and genetically modified mice including recombination-activating gene-1 (Rag1)-deficient mice that lacks mature T and B cells and Rag2$^{-/-}$ interleukin-2 receptor subunit gamma (Il2rg)$^{-/-}$ mice that lacks all lymphocytes were exposed to IL-33 in **Paper I, III** and **IV**. In **Paper III** and **IV** mice were treated with the mTORC1 inhibitor rapamycin before intranasal challenges of rmIL-33.

3.1.2 Allergen-induced inflammation

In this thesis, two allergen-induced mouse models have been used. In **Paper I** we exposed mice with intranasal doses of the protease allergen papain (Figure 4B). Allergy to papain can occur in both occupational and non-occupational settings [244, 245]. As papain can induce IL-33 release from the airway epithelium, it is a suitable allergen to use to study IL-33-induced inflammation indirectly. We challenged both WT mice and Rag1$^{-/-}$ mice with papain in this study. In **Paper II**, the clinically relevant aeroallergen HDM was used where mice were first sensitized to HDM and weeks later challenged with 1:10 of the sensitization dose (Figure 4C). Intranasal exposures of HDM proteases in mice

mimics respiratory allergies fairly well. HDM is a suitable allergen in mouse models since many asthmatics have early and late asthmatic responses after challenges with HDM [243-245]. Furthermore, the HDM allergen Der p 1 belongs to the cysteine protease papain-like protein family [246] . Both HDM and papain trigger T2 responses and are frequently used in models of asthma [116, 247]. In **Paper I,** anti-IL-5 antibodies were given intraperitoneally to determine if the papain-induced eosinophilia was IL-5-dependent.

3.2 Sample collection

3.2.1 Blood and BAL collection from mice

Blood was collected by puncturing the heart. Serum was used for mediator analysis. Following blood sampling, BAL fluid was obtained by instilling 0.25 mL of cold phosphate buffered saline (PBS) through the tracheal cannula followed by a gentle aspiration. This procedure was thereafter repeated and the BAL fluid was immediately placed on ice. The BAL cells were used for cytospins and the supernatants for mediator analysis.

3.2.2 Lung tissue

Lung tissue was collected for miRNA experiments in **Paper IV**. Following BAL sampling, the apical lobe was immediately placed in RNAlater stabilization solution and stored at 4°C followed by -80°C storage until further processing.

3.2.3 Bone marrow

Femurs were cut at the epiphysis and then flushed with 5 mL of wash buffer (2% fetal bovine in 1xPBS) through a 100 µm cell strainer to obtain bone marrow cells used for cytospins, flow cytometry and *ex vivo* experiments. RNA from bone marrow cells was obtained by flushing femur or tibia, depending on experiments, with 1 – 2 mL RNAprotect cell reagent while filtered. The cells were centrifuged and resuspended in QIAzol™ lysis reagent for RNA processing.

3.2.4 RNA isolation

Lung tissue that had been stored in RNAlater was washed with 1xPBS to remove residual RNAlater before being homogenized in QIAzol™ lysis reagent using gentleMACS™ M tubes with the gentleMACS™ Dissociator. Total RNA from either lung homogenate or bone marrow was isolated using the miRNeasy mini or micro kits according to the manufacturer's instructions.

3.3 Cell analysis

3.3.1 Differential cell count

This method was used in **Paper I – IV** to analyze the proportions of white blood cells with focus on eosinophils in BAL and bone marrow (Figure 5). Approximately, 10,000 – 50,000 cells were collected on cytospins stained with Hemacolor Rapid® stain. The eosinophil granules turn red to red-brown using this staining method and are easily identified. Around 300 – 400 cells were counted using a Zeiss Axioplan 2 microscope. Differential cell count is an easy method that takes a couple of minutes providing valuable information about the inflammation in the tissue. Differential cell counts are expressed per volume or as percentage of total cells.

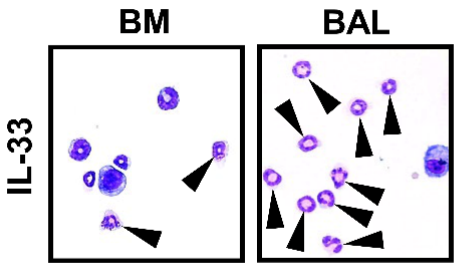

Figure 5. Cytospins of bone marrow and BAL cells with arrows showing the eosinophils in WT mice challenged with IL-33. Figure from Boberg *et al*. Front Immunol. 2020.

3.3.2 Flow cytometry

Cell analysis by flow cytometry was used in **Paper I – IV** for surface staining and intracellular staining of bone marrow cells. Flow cytometry is a powerful technology which provides a well-established method and is widely used to rapidly identify and quantify cells in solutions [248]. In addition to cell analysis, flow cytometry can be used for analysis of for example nanoparticles or extracellular vesicles [249].

In this thesis, cells were stained with fluorochrome-conjugated antibodies and processed on a BD FACSVerse™ flow cytometer running FACSuite™ software and analyzed with FlowJo Software®. The gating of surface markers and the intracellular marker IL-5 was carried out using the Fluorescence Minus One (FMO) approach controlling for the level of fluorescent spread in the flow cytometry panels. A FMO control includes cells that are stained with all fluorochromes except the one of interest.

Detailed description regarding the work flow and information about the antibodies can be found in each paper. Antibody panels for identification of ILC2s, T_H cells, eosinophil progenitors and mature eosinophils are provided in Table 1. Flow cytometry is widely used and an essential method for detection of ILC2s due to the relatively low numbers of ILC2s in various tissues and the multiple markers that are required to identify them.

Table 1. Antibody panels used in flow cytometry

Cell type	Marker
Eosinophil progenitor	$SSC^{lo}CD45^{+}CD34^{+}IL5R\alpha^{+}$
Mature eosinophil	$SSC^{hi}CD45^{+}CD34^{-}IL5R\alpha^{lo}CCR3^{+}Siglec\text{-}F^{+}$
ILC2	$SSC^{lo}CD45^{+}Lin^{-}(CD3^{-}CD45R/B220^{-}CD11b^{-}TER\text{-}119^{-}$ $Ly\text{-}G6/Gr1^{-}CD11c^{-}CD19^{-}NK\text{-}1.1^{-}FceR1^{-})$ $CD127^{+}CD25^{+}ST2^{+}$
T_H	$SSC^{lo}CD45^{+}CD3^{+}CD4^{+}$

ILC2: Type 2 innate lymphoid cell, T_H: T helper cell

3.4 Quantification of mediators

Quantification of cytokines and chemokines in BAL, serum and bone marrow culture supernatants from mice was carried out using the sensitive method ELISA. Commercially available DuoSet™ ELISA kits from R&D Systems was used (Mouse chemokine ligand (CCL)24/Eotaxin-2/MPIF-2, Mouse IL-5 and Mouse IL-33 DuoSet™) according to manufacturer's instructions.

Concentrations of the twelve cytokines in the clinical study, **Paper V**, were measured in cell-free BL samples by the bead-based immunoassay LEGENDplex™ human T_H cytokine panel according to manufacturer's instructions. The samples were acquired on a BD FACSVerse™ flow cytometer running BD FACSuite™ and analyzed by BioLegend's LEGENDplex™ Data Analysis Software. Bead-based assays offer the advantage of analyzing several mediators in a small sample volume compared to the traditional ELISA method. However, both methods are sensitive to measure mediators at low levels.

3.5 microRNA analysis

3.5.1 RNA yield and integrity

In **Paper IV**, RNA concentrations and integrity were determined by chip-based capillary electrophoresis using Agilent 2100 Bioanalyzer and Agilent RNA 6000 Pico Chip. The Agilent 2100 Expert Software was used for analysis of the Bioanalyzer electropherograms. In some experiments, concentrations of RNA were determined using a NanoDrop™ photospectrometer.

3.5.2 Transcriptomic analysis

In **Paper IV**, RNA from murine bone marrow and lung was analyzed by microRNA microarray using a mouse Oligo chip 4plex. This analysis was performed at TATAA Biocenter (Gothenburg, Sweden). The intensity of each miRNA was measured by the 3D-Gene Scanner 3000 (Toray industries Inc.). The procedures were carried out according to manufacturer's instructions. In some experiments found in **Paper IV**, RNA from bone marrow samples were analyzed using the Nanostring nCounter Analysis System at Lund University, Sweden, according to manufacturer's protocol. The normalization procedures of the generated miRNA data from microarray and NanoString technology are described in more detail in **Paper IV.**

A number of miRNA assays are currently available with different technologies varying in, for example, accuracy, efficiency, cost and time-consumption [250]. Fold change data from microarray platforms are comparable with fold change data from NanoString technology. In addition, there are correlations between quantitative real-time polymerase chain reaction (qPCR) analyses and nCounter analyses including relative expression levels and fold change. Similarly, next generation sequencing (NGS) has been compared to NanoString and microarray where NanoString identified the highest number of detected miRNAs between the three technologies and microarray the lowest number [251]. High reproducibility using the NanoString technology has previously been shown and this technique does not require technical replicates [252]. High reproducibility was observed between the other two platforms and significant levels of shared detection were observed between all platforms [251]. It was suggested that the normalization method differed between the platforms which could account for potential differences [251]. In contrast to NGS, NanoString does not require an amplification step whereas no hybridization is needed for NGS compared to NanoString and microarray. Another study concluded that the microRNAs that were commonly detectable using both microarray and NanoString techniques displayed similar detection levels of microRNA transcripts [253]. The number of samples and amount of starting

material should be taken into account considering the choice of the different techniques. For example, the NanoString technology require as little as 100 ng and the miRNA profiling is performed with high precision and no validation of the results is required, which is a big advantage. In contrast to NGS, both microarray and NanoString technology uses selected target sequences. However, NGS is time-consuming and associated with higher costs.

3.5.3 Quantitative PCR

For validation of microarray data, miRNA expression was determined by qPCR which is a rapid and sensitive method and the gold standard for quantifying gene expression. In **Paper IV**, RNA was converted to complementary DNA (cDNA) through reverse transcription using the miRCURY® LNA® RT kit (Qiagen) and the control UniSp6 RNA Spike-in was added into each reaction in this step. qPCR analysis was performed on a Bio-Rad CFX96 Real-Time PCR detection system using diluted cDNA, LNA primer sets for various miRNAs including RNU5g as a control, and SYBR green Master mix kits as described by manufacturer (Exiqon). The miRNA expression was normalized to the reference gene RNU5g and fold change was calculated from controls.

3.6 Clinical study

3.6.1 Asthmatic cohort

In **Paper V**, women with non-allergic asthma, allergic asthma and healthy controls were recruited from WSAS cohort or via external advertisement. WSAS is a large population-based cohort focusing on asthma and allergic diseases in west Sweden [4, 11, 15]. All participants were clinically characterized with phenotyping including skin prick tests, lung function tests and differential cell counts. A detailed questionnaire which included questions about asthma, rhinitis, respiratory symptoms and asthma medications was filled out by all participants [4]. Allergic asthmatics with two or more positive skin prick tests (≥3 mm) were included in the study. A standardized panel of the 10 allergens, fungi Cladosporium herbarum and Alternaria alternate, house dust mites D. pteronyssinus and D. farinae, cat, dog, horse, timothy grass, birch and mugwort were used for the skin prick test. The allergic asthmatics had a physician-diagnosed current asthma with early asthma onset at the age of 20 or younger. The non-allergic asthma group were diagnosed by a physician with disease onset from school age or older and had negative skin prick tests. All asthmatics had mild to moderate asthma and were treated with ICS. The healthy control group had no allergies nor respiratory diseases. All participants were under 65

years of age with no autoimmune diseases, cancer or other respiratory diseases. The participants underwent bronchoscopies within two weeks after the clinical examination and additional samples were taken the day of the bronchoscopy to make sure no current inflammation was observed. This included blood samples measuring C-reactive protein levels and leukocyte counts. Study participants provided written informed consent to the study and the independent ethics committee, the regional ethical review board in Gothenburg, Sweden approved the study (permit number 228-14).

3.6.2 Bronchial lavage

In **Paper V**, proteins in BL supernatants, obtained during bronchoscopies at Sahlgrenska University Hospital, were analyzed from the study participants included in the clinical study. There is currently limited data on proteomics specifically from BL samples and the study material is therefore unique and holds valuable information about the disease. In general, analysis of BAL fluid has provided the field with valuable information about the asthma pathogenesis, however, the sampling of the airways, especially the distal airways is rather invasive with risk of potential complications [254, 255].

In brief, 20 mL of sterile free PBS was instilled into the segmental bronchi followed by another two 20 mL washes. The BL fluid was immediately stored on ice awaiting further processing. The material from the first wash was not used for analysis in this study. Protease inhibitor was added to the cell-free BL supernatant and protein concentrations were analyzed using Micro BCA™ Protein Assay Kit.

3.6.3 Proteomics

The proteomic profiles were determined in cell-free BL supernatants from allergic asthmatics, non-allergic asthmatics and healthy controls by quantitative proteomics in **Paper V**. This work was performed at the Proteomics Core Facility at Sahlgrenska Academy University of Gothenburg and more details about the procedure can be found in **Paper V**.

Global and targeted quantitative proteomics is a promising technique for biomarker discovery and to investigate disease drivers in diseases such as asthma [221, 222]. The technology and instruments are constantly advancing and it is today possible to discover several thousands of proteins in a single sample.

Relative quantification using the isobaric labeling method TMT was applied in **Paper V**, enabling comparisons of the abundance of peptides and corresponding proteins in many samples. Identical peptides from different

samples can be separated upon fragmentation in the mass spectrometer since each peptide variant yields a unique reporter ion which is used for quantification. In brief, equal amounts of proteins per sample, i.e. 50 µg, were digested by trypsin into peptides which were thereafter labeled with a unique tag containing different isotopes but has identical mass and molecular structure. One sample is a reference pool containing aliquots from each samples included in the study enabling relative quantification between samples. Each tagged sample are thereafter combined into one sample. The pooled sample was fractionated prior nano-liquid chromatography (nLC) mass spectrometry (MS) in our study due to high complexity of the samples which enables the chance of identifying low-abundant proteins hence it reduces complexity of the sample and allows for higher amounts of starting material. The peptides are analyzed by nLC coupled to a (MS). Tagged peptides with identical mass elute simultaneously in the liquid chromatography to the first MS following additional fragmentation in the second MS where tags are cleaved which generates different masses of the tagged peptides. Thereby, the relative intensity of one peptide in different samples is obtained.

Peptides that are unique for a certain protein are considered for quantification. It is a strength that all samples can be combined and analyzed together since it eliminates variations that may occur if the samples were to be run separately. In **Paper V**, TMT 11-plex was used allowing comparisons up to eleven samples in one experiment. The peptides can be identified and relatively quantified using fragmentation databases and applying thresholds for false discovery rates of both peptides and proteins. In **Paper V**, comparisons between study groups were carried out analyzing ratios of the relatively quantified proteins. The TMT technology is widely used and holds many advantages with low technical variability and high sensitivity. It is also cost-efficient and time-saving.

3.7 Bioinformatics and statistical analysis

For the *in vivo* studies (**Paper I – IV**), the non-parametric Mann-Whitney U test was used for comparison between groups. If statistical analysis was performed for more than two study groups, the Kruskal-Wallis test was firstly used to compare the study groups, which was followed by Mann-Whitney U test for statistical analysis between two independent groups. Paired Student's t-tests was used for statistical analysis in the *in vitro* experiments (**Paper I – III**). In the clinical study (**Paper V**), unpaired Student's t-test was used to for statistical analysis, comparing the average in protein expression of two independent study groups. A p-value below 0.05 was considered as statistically

significant. The fold change criteria were ≥1.5 or ≤-1.5 for the comparison between the study groups. Proteins that were statistically significant and met the fold change criteria were considered for further analysis. All statistical analysis in the studies in this thesis were performed using GraphPad Prism and Excel software.

In **Paper V**, graphical visualization was performed using hierarchical clustering which means that samples cluster if shared similarities are found. A heatmap was used to visualize the data matrix. Both hierarchical clustering and principal component analysis (PCA) are unsupervised methods. This makes them suitable for exploratory data analysis. PCA was used to visualize the difference and/or similarities between study groups. Clustering and PCA analysis was performed with the software Qlucore Omics Explorer (Qlucore, Lund, Sweden).

There are many platforms available for pathway analysis of omics data, either free online or as commercial software. In **Paper IV** and **Paper V**, miRNA and protein data was analyzed, respectively. Pathway analysis was performed for differentially expressed miRNAs and proteins to predict if the components in the data set were associated with, for example, specific biological processes, signaling pathways or cellular compartments. Generally, a pathway is enriched when several components from the data set are associated with that pathway. In this thesis the freely available platforms miRSystem, DAVID and Enrichr were used.

4 Results and discussion

The main findings of **Paper I – V** are summarized and discussed in this section.

4.1 Paper I – IL-33-responsive bone marrow ILC2s *in vivo* during papain-induced eosinophilia

Eosinophils are key inflammatory cells in allergy and asthma. However, underlying mechanisms contributing to eosinophilopoiesis are not fully understood. Eosinophils develop in the bone marrow and can migrate to the airways in response to allergens or other disease triggers in presence of chemotactic signals [256]. IL-33 plays a role in eosinophilopoiesis and bone marrow ILC2s produce IL-5, contributing to eosinophil expansion [82, 85, 115]. In **Paper I**, the role of bone marrow ILC2s during allergen-induced and IL-33-induced eosinophilia was investigated. To study the role of ILC2s, WT mice, mice that lack mature T and B cells but have ILC2s ($Rag1^{-/-}$ mice) and mice that lack all lymphocytes ($Rag2^{-/-}Il2rg^{-/-}$) were used.

An acute allergen-induced inflammation model with the protease papain was used in this study. Papain exposures in WT mice and $Rag1^{-/-}$ mice resulted in higher levels of IL-33 in BAL fluid compared to control mice given saline (Figure 6A). Moreover, increased numbers of eosinophils in the bone marrow and airways (Figure 6B and 6C) with elevated levels of eotaxin-2/CCL24 were seen in papain-exposed WT mice and $Rag1^{-/-}$ mice. Experiments using anti-IL-5 antibodies in mice before allergen exposures revealed that the inflammation model was IL-5-dependent, exhibited by decreased levels of eosinophils in both bone marrow and BAL (Figure 6D and 6E). Direct administration of IL-33 in $Rag1^{-/-}$ mice resulted in similar inflammation profile as in WT mice with increased numbers of bone marrow and BAL eosinophils which coincided with elevated levels of eotaxin-2/CCL24 in BAL compared to control mice. Altogether, $Rag1^{-/-}$ mice show similar inflammation as WT mice which suggests that the adaptive immune system is dispensable in both allergen-induced and IL-33-induced eosinophilic inflammation in the studied models.

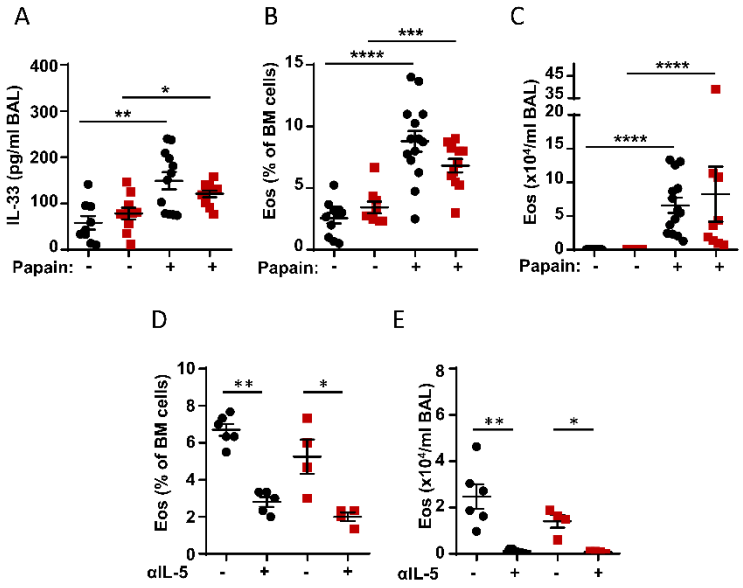

Figure 6. Wild type mice (black) and Rag1$^{-/-}$ mice (red) respond similarly to airway challenges of papain with increased levels of **(A)** IL-33 in BAL and **(B)** eosinophils in bone marrow and **(C)** BAL. Mice treated with anti-IL-5 antibodies before airway challenge of papain had reduced levels of eosinophils in **(D)** bone marrow and **(E)** BAL. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data are displayed as the mean ± SEM. Figures from Boberg *et al*. Front Immunol. 2020.

Assessment of bone marrow ILC2s revealed increased numbers of ILC2s in Rag1$^{-/-}$ mice compared to WT mice at baseline. Rag1$^{-/-}$ mice exhibited higher levels of serum IL-5 and CCL24 post IL-33-challenge compared to WT. A possible explanation could be that the mice lack inhibitory signals from the adaptive immune system, which could to be addressed in future studies. For example, induced Tregs are able to suppress cytokine production by ILC2s [257]. Moreover, the higher number of ILC2s in Rag1$^{-/-}$ mice compared to WT mice might contribute to the higher IL-5 levels in serum from Rag1$^{-/-}$ mice. *Ex vivo* restimulation with IL-33 caused increased levels of IL-5 in bone marrow cultures from WT mice and Rag1$^{-/-}$ mice. It has previously been reported that lung ILC2s in IL-33-challanged or papain-challenged mice respond more robust to a second challenge of IL-33 or papain, months after the first challenge, with increased cytokine production compared to naïve ILC2s [258]. This memory response in ILC2s, that share gene signatures with memory T cells, might be the reason to the increased IL-5 production observed in our study after *ex vivo* restimulation with IL-33 [258]. Furthermore, increased number of bone marrow ILC2s was observed in WT mice exposed to either IL-33 or papain compared to control mice given saline. An increased number of bone

marrow ILC2s after IL-33 exposures has been observed in previous studies [259-261]. The increase in ILC2 numbers might explain the higher systemic levels of IL-5 in IL-33-challenged WT mice compared to control mice that were observed the present study. Furthermore, elevated levels of bone marrow ILC2s may explain the increase in mature eosinophils in the bone marrow of both IL-33-challenged mice and papain-challenged mice.

To determine if IL-33-driven eosinophilia could occur in absence of bone marrow ILC2s $Rag2^{-/-}Il2rg^{-/-}$ mice were challenged with IL-33. $Rag2^{-/-}Il2rg^{-/-}$ mice exhibited lower numbers of eosinophil progenitors and mature eosinophils in the bone marrow compared to WT mice at baseline. Furthermore, IL-33 did not cause airway nor bone marrow eosinophilia in $Rag2^{-/-}Il2rg^{-/-}$ mice and IL-5 was not detected in either serum nor in IL-33-stimulated bone marrow cultures. A previous study reported impaired airway eosinophilia and mucus hypersecretion in $Rag2^{-/-}Il2rg^{-/-}$ mice exposed to papain and adoptive transfer of ILC2s reconstituted the symptoms [114]. Collectively, pro-inflammatory functions of ILC2s seem to be important for eosinophils during both homeostasis and during inflammation, specifically during terminal differentiation and maturation of eosinophils.

Several immune cells express IL-33 receptor ST2, including ILC2s and eosinophils [52]. In the present study**,** bone marrow ILC2s of both WT mice and $Rag1^{-/-}$ mice responded similarly to both IL-33 and papain with increased ST2 expression. Higher ST2 expression on bone marrow ILC2s in response to IL-33 during eosinophilic inflammation has been observed previously [82, 259, 260]. Furthermore, we performed a kinetic study analyzing eosinophil infiltration and ST2 expression on ILC2s after 3 h, 6 h, 24 h, 48 h and 72 h after a single dose of papain. The ST2 expression peaked after 24 h which coincided with elevated levels of IL-33 in BAL. After 48 h, the ST2 expression on ILC2s was lower whereas an increased number of bone marrow eosinophils was observed. IL-5 is most likely produced by ILC2s after IL-33 binds to its receptor contributing to the increased number of eosinophils. However, no $IL\text{-}5^{+}$ ILC2s were detectable at any studied time point.

An increased ST2 expression was observed on mature eosinophils in the bone marrow in mice challenged with papain compared to WT mice. Increased ST2 expression on sputum and blood eosinophils have been observed in allergic asthmatics after allergen challenge [262]. Interestingly, decreased numbers of $ST2^{+}$ eosinophils in the bone marrow were observed in mice treated with anti-IL-5 antibodies before papain-challenge. These findings suggest a direct link between ST2 expression on eosinophils and IL-5 highlighting a role of IL-33 in allergen-induced eosinophilia. However, future studies are needed to

address whether IL-5 may affect ST2 expression on eosinophils with a direct or indirect mechanism.

4.2 Paper II – The role of bone marrow ILC2s *in vivo* during house dust mite-induced eosinophilic inflammation

Studies examining IL-33-responsive cell types in the bone marrow during allergic eosinophilic airway inflammation are needed. Therefore, we aimed to determine whether ILC2s, eosinophils and T_H cells were IL-33-responsive cell types locally in the bone marrow in mice sensitized or sensitized and challenged intranasally to the common aeroallergen HDM (HDM/PBS and HDM/HDM, respectively) in **Paper II**. HDM proteases trigger IL-33 release from the airway epithelium initiating inflammatory responses downstream in the T2 cascade [173, 263].

Airway eosinophilia was only observed in HDM/HDM mice which coincided with increased levels of eotaxin-2/CCL24 suggesting that eosinophils were recruited to the airways. Analysis of bone marrow eosinophils revealed increased numbers of eosinophil progenitors in the bone marrow of sensitized mice whereas elevated levels of mature eosinophils were seen in HDM/HDM mice only. These findings indicate that airway exposures of HDM induced eosinophilopoiesis in the bone marrow. In **Paper I**, we identified an increased expression of ST2 on mature eosinophils in mice exposed to airway challenges with the allergen protease papain [264]. In **Paper II**, IL-33-responsive eosinophils in mice exposed to HDM were found with an increased number of $ST2^+$ eosinophils compared to control mice given saline. Eosinophils can produce IL-5 in an autocrine fashion and increased number of $ST2^+$ eosinophils may lead to increased production of IL-5 and, thereby, regulating eosinophilic inflammation [84, 94, 265, 266]. Eosinophil progenitors in the bone marrow can produce IL-5 in response to IL-33, however, future studies are warranted to determine the effects from the increase in $ST2^+$ eosinophils during HDM-induced inflammation [82].

In **Paper I**, IL-33-responsive bone marrow ILC2s induced by papain were identified [264]. In **Paper II**, we further investigated whether ILC2s were IL-33-responsive to airway challenges of HDM in addition to papain. Higher ST2 expression was observed on ILC2s in HDM-sensitized mice compared to control mice and mice that were challenged with HDM in addition to the HDM sensitization. The ST2 expression on bone marrow ILC2s in HDM/PBS mice and HDM/HDM mice positively correlated with increased numbers of

eosinophil progenitors (Figure 7). Collectively, these findings indicate a role for IL-33-repsonsive ILC2s in the bone marrow at onset of allergen-induced eosinophilia. Interestingly, a decreased number of bone marrow ILC2s was found in HDM-sensitized mice which could be due to ILC2 migration to the airways. ILC2 migration from the bone marrow and other tissues in response to allergen challenge has previously been reported [267].

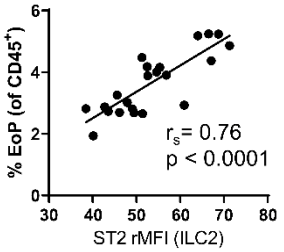

Figure 7. The ST2 expression on ILC2s in the bone marrow positively correlated with the number of eosinophil progenitors in mice exposed to house dust mite indicating a pro-inflammatory role of ILC2s at the onset of allergen-induced eosinophilia. r_s: Spearman correlation coefficient. Figure from Boberg *et al*. Int J Mol Sci. 2020.

Several immune cells express ST2 including conventional T cells [176]. IL-33-responsive T_H cells have been studied in models of asthma but their responsiveness to IL-33 in the bone marrow during allergic airway inflammation remains unclear. In this study, bone marrow T_H cells were examined in mice exposed to HDM. Analysis by flow cytometry revealed an increase in $ST2^+$ T_H cells in mice sensitized to HDM compared to control mice and no further increase was found in mice challenged to HDM in addition to sensitization. Moreover, in contrast to bone marrow ILC2s, no correlation was found between eosinophil numbers and ST2 expression by T_H cells. Mice directly challenged with IL-33 intranasally responded by upregulating ST2 on bone marrow T_H cells in a previous study [82]. However, that study did not identify T_H cells as a source of IL-5.

In this study we examined the capacity of ILC2s and T_H cells to produce IL-5 *ex vivo*. Bone marrow cells from control mice and HDM/HDM mice were stimulated with IL-33, HDM or the combination of the two. Increased IL-5 production by ILC2s was observed in cultures stimulated with IL-33 but not HDM. Interestingly, in cultures stimulated with both IL-33 and HDM exhibited higher IL-5 production compared to IL-33 only suggesting a possible synergistic effect of HDM and IL-33. One explanation could be that other cell types in the bone marrow responded to HDM by secreting IL-33. For example, macrophages, dendritic cells and mast cells may produce IL-33 and, thus, be potential sources of IL-33 in the bone marrow intensifying the IL-5 production

by ILC2s [106-108, 268]. Macrophages and dendritic cells produced IL-33 in response to HDM and could be sources of IL-33 in our *ex vivo* experiments [109, 110]. Moreover, the IL-5 responses were similar comparing PBS/PBS mice and HMD/HDM mice. This was in contrast to our previous results in **Paper I** where an increase of IL-5 producing ILC2s was observed if mice were exposed to IL-33 *in vivo* before IL-33 stimulation *ex vivo* [264]. T_H cells did not produce IL-5 in any of the conditions suggesting that bone marrow ILC2s are an important source of IL-5 locally in the bone marrow and, thus, regulators of eosinophilic inflammation. The functionality of IL-33-responsive T_H cells in allergen-driven and IL-33-driven eosinophilic inflammation needs to be investigated further in prospective studies. In this study we analyzed all T_H cells and not T_H2 cells specifically. ILC2s are the innate counterpart of T_H2 cells in terms of function and ST2 is highly expressed on T_H2 compared to T_H1 cells that transiently express ST2 when activated [269]. Indeed, a follow up study examining T_H2 cells exclusively is needed in order to rule out their responsiveness to IL-33 indirect via HDM exposures. To summarize, the findings support that ILC2s contributed to eosinophilopoiesis locally in the bone marrow in response to airway challenges of HDM.

4.3 Paper III – Rapamycin dampens inflammatory properties of bone marrow ILC2s *in vivo*

The pro-inflammatory properties of ILC2s are controlled by regulators in the local microenvironment including activating or suppressing cytokines, cell to cell interactions and sex hormones [270, 271]. Depending on the microenvironment, ILCs exhibit tissue-specific properties [272]. IL-33 is an activator of ILC2s and induces cytokine production and proliferation, often by activation of the NF-κB and MAPK signaling pathways [271]. The MAPK signaling pathway has been shown to regulate functions of the mTORC1 signaling pathway [273]. New pathways involved in regulating ILC2s in T2 immunity have been identified in the recent years. For example, the programmed cell death protein-1 was shown to downregulate effector functions of pulmonary ILC2s during IL-33-induced inflammation and holds therapeutic potential [274]. In addition, the transcription factor signal transducer and activator of transcription 3 was shown to be critical for ILC2 effector functions in both papain-induced and IL-33-induced airway inflammation [275]. Identification of new pathways and gain more in-depth knowledge around already identified pathways that activate and regulate ILC2s might lead to new therapeutic targets. Moreover, expression of ST2 on ILC2s varies between organs with higher expression in the lung compared to the bone marrow [272]. In **Paper III**, the aim was to determine whether the mTORC1 pathway was

induced in bone marrow ILC2s specifically by IL-33 and whether the IL-5 production by ILC2s was affected by mTORC1 inhibition.

In vitro, IL-33-stimulation of bone marrow cultures induced phosphorylation of the mTOR target rpS6 which was completely inhibited by the mTORC1 inhibitor rapamycin. *In vivo,* increased numbers of $pRPS6^+$ ILC2s in mice exposed to IL-33 compared to control mice given saline were observed. A reduction of $pRPS6^+$ ILC2s was seen in mice given with rapamycin before intranasal IL-33-challenge compared to mice given IL-33 only. This is the first study that shows on a single cell level, that IL-33 induced mTORC1 activity in bone marrow ILC2s. These findings are in line with previous observations with a decreased activity of pRPS6 in sorted lung ILC2s, stimulated with IL-33 and rapamycin *in vitro* compared to stimulation with IL-33 only [175]. In contrast to ILC2s, an induction of mTORC1 was not observed in T_H cells after IL-33 stimulation in the present study. The mTORC1 induction in response to IL-33 may be unique for ILC2s but T_H cells in the bone marrow might respond at another time point compared to ILC2s. The kinetics and, potentially the mechanism(s), for the difference in IL-33-response could be addressed in prospective studies.

Assessment of the eosinophilic inflammation was carried out by differential cell count and flow cytometry. A reduction of airway eosinophils and mature eosinophils in the bone marrow which coincided with decreased levels of eotaxin-2/CCL24 in the airways was observed in mice given rapamycin and IL-33 compared to IL-33 only. The chemokine receptor CCR3 is expressed on eosinophils and is a chemokine receptor that binds eotaxins [77]. The CCR3 expression was reduced in mice given rapamycin in addition to IL-33 compared to IL-33 only. The rapamycin treatment did likely affect the recruitment of eosinophils to the airways in response to IL-33. Moreover, IL-33-stimulation induced IL-5 production by bone marrow ILC2s, whereas rapamycin treatment reduced the number of $IL\text{-}5^+$ ILC2s. In addition to inducing maturation of eosinophils, IL-5 also promote eosinophil migration [276]. Rapamycin treatment may affect both terminal eosinophil maturation and recruitment of eosinophils during IL-33-induced inflammation. However, the eosinophil progenitor numbers remained the same in all treatment groups. Our findings are in line with previous studies demonstrating decreased numbers of airway eosinophils in mice treated with rapamycin and IL-33 or allergens [175, 277, 278]. Importantly, there might be a time-dependence of the anti-inflammatory rapamycin effects. One study reported that rapamycin decreased inflammation when administered together with HDM but worsened the inflammation when administered after an established allergen-induced inflammation by HDM [279].

To our knowledge, the present study is the first to investigate whether rapamycin affected ST2 expression on T_H cells, ILC2s and eosinophils in the bone marrow. IL-33 induced ST2 expression in all cell types and these findings are in line with a previous study [82]. In contrast to ILC2s and eosinophils, we found decreased ST2 expression on T_H cells in mice given rapamycin and IL-33 compared to IL-33 only. However, rapamycin treatment might affect ST2 expression on eosinophils and ILC2s at other time points. In summary, the mTORC1 pathway regulated ILC2s locally in the bone marrow with anti-inflammatory effects on eosinophilia. Asthma is not considered as a single disease entity and our findings provide insights how the mTORC1 signaling pathway regulated cell types in the bone marrow with potential effects on airway inflammation.

4.4 Paper IV – Distinct microRNAs in lung and bone marrow *in vivo* during IL-33-induced eosinophilic inflammation

Pro-inflammatory properties that affect cellular processes such as cytokine production, cell proliferation and cell migration and may have a critical role in allergy and asthma are exhibited by miRNAs [184-186, 188]. In **Paper IV**, the aim was to determine miRNA expression patterns in lung and bone marrow in IL-33-induced eosinophilic inflammation. In addition, we explored how differently expressed candidate miRNAs in the bone marrow in IL-33-induced eosinophilia were affected by mTORC1 inhibition.

To determine differently expressed miRNAs during IL-33-induced eosinophilic inflammation, RNA from bone marrow and lung of IL-33-exposed mice (n=4) and control mice given saline (n=4) was used for miRNA microarray analysis. In the bone marrow, 129 miRNAs and 34 miRNAs were downregulated and upregulated, respectively, in IL-33-exposed mice compared to control mice. In the lung, 62 upregulated miRNAs were found in IL-33-exposed mice compared to saline mice but no significantly downregulated miRNAs were identified in response to IL-33. A possible explanation to these findings could be that cells containing these miRNAs migrated from the bone marrow to the lung resulting in many downregulated miRNAs in the bone marrow with increased, i.e. upregulated, numbers of miRNAs in the lung. Another explanation could be the local inflammation of the airways induced by IL-33 potentially leading to higher levels of pro-inflammatory miRNAs regulating the inflammation. A miRNA previously reported to be upregulated in lung ILC2s and a critical regulator of airway inflammation including IL-33-induced inflammation is miRNA-155 [199, 200]. In

the current study, miR-155 was found to be upregulated in lung cells of IL-33-exposed mice and thereby supporting previous findings of miR-155 regulating airway inflammation. Another miRNA upregulated in the lung was miR-21 which was also found to be downregulated in the bone marrow in mice exposed to IL-33. During allergic airway inflammation, miR-21 was shown to be induced and inhibition of miR-21 have been associated with reduced eosinophil levels [206, 280]. Thus, it would be interesting to conduct follow-up studies on miR-21 and its potential role in IL-33-induced inflammation.

Pathway analysis of differentially expressed miRNAs in both lung and bone marrow showed associations with the mTOR signaling pathway. Decreased cytokine production and eosinophil levels after mTOR inhibition have previously been shown in murine models of airway inflammation induced by IL-33 or allergens [175, 277, 278, 281]. In the bone marrow, miR-150 was found to be one of the most downregulated miRNAs after IL-33 challenge. This was also confirmed by qPCR analysis. Previously, miR-150 has been reported to negatively regulate mTOR signaling and affects cell proliferation and cell differentiation [282-284]. Moreover, in **Paper III**, decreased IL-5 production by ILC2s was seen after rapamycin treatment, i.e. mTORC1 inhibition, and IL-33-stimulation compared to IL-33-stimulation only. We next investigated the effect from rapamycin treatment on miR-150 expression in bone marrow cells from mice exposed to IL-33. The miRNA expression was determined by the NanoString technology with samples from three different experiment days. Rapamycin did not have a clear effect on the miR-150 expression in the present study. However, more studies are needed to determine whether mTOR inhibition has an impact on miR-150 expression. Potentially a higher mTOR inhibition is required and/or the inhibition of the mTOR complex 2 may be required to have an effect on miR-150 levels. Importantly, a significant reduction of miR-150 expression was observed in IL-33-exposed mice compared to control mice receiving saline which validate previous findings of miR-150 expression from microarray and qPCR. In this study, a correlation between the number of T_H cells and miR-150 expression was found. It would be interesting to do follow-up studies to assess potential effects on miR-150 regulation in T_H cells in IL-33-induced inflammation.

Several miRNAs have been identified as potential therapeutic targets due to the regulatory role in different diseases including asthma. In the present study, candidate miRNAs that might regulate inflammatory responses driven by IL-33 were identified. A top candidate was miR-150 to proceed with in in prospective mechanistic *in vivo* studies. For example, miR-150 mimics could be used to investigate whether higher miR-150 levels could alleviate

eosinophilia and if the mTOR signaling pathway is involved in this potential mechanism.

4.5 Paper V – Allergic and non-allergic asthmatics display unique protein profiles in bronchial lavage fluid

In **Paper V**, the aim was to identify the proteomic profiles in BL supernatants from allergic asthmatics and non-allergic asthmatics and determine whether the proteomic profiles were distinguishable from each other. New stable biomarkers are needed, especially for non-allergic asthma, to identify disease drivers causing a specific asthma endotype. By using quantitative proteomics as a screening method it also generates new hypotheses for future asthma studies. Based on our findings, it is possible that TMT-based proteomics can be used on complex patient samples, such as BL supernatants. Indeed, quantitative proteomics is a promising method to characterize disease drivers in asthma [50, 222, 229].

Non-allergic asthma is more common in women and often develops later in life in contrast to allergic asthma [2, 285]. This study included women which limits the possibility to generalize the findings to the whole asthma patient population including men as the proteomic profiles potentially may differ. In total, 4883 proteins were identified. Out of these, a total of 3854 proteins were quantified and 2962 proteins were quantified in all BL samples included in the comparison between study groups. Gene Ontology (GO) terms associated with the proteome in BL supernatants from all participants were associated with for example antigen processing and presentation via MHC class I and MHC class II, cell-cell adhesion, T cell receptor signaling pathway or complement activation.

Hierarchical cluster analysis of the three study groups revealed that the majority of the significantly altered proteins were downregulated in the asthma groups compared to healthy controls. In the present study, the focus was to compare the proteomic profiles between the asthma groups. Eighty-seven proteins were identified as downregulated in non-allergic asthmatics compared to allergic asthmatics and 12 proteins were upregulated. The downregulated proteins were associated with GO terms such as cilium morphogenesis, lung epithelial cell differentiation or cell-cell adhesion. Ciliary dysfunction in the epithelium is a signature in respiratory disorders including asthma. Interestingly, epithelial ciliary dysfunction has been reported to be associated with neutrophilic asthma [286]. The upregulated proteins in non-allergic

asthmatics were associated with GO terms including response to glucocorticoid or receptor-mediated endocytosis.

Top proteins with higher expression in non-allergic asthmatics compared to allergic asthmatics were shown to be involved in the complement system, mucus function, muscle contraction or cellular adhesion. One upregulated protein in non-allergic asthmatics compared to allergic asthmatics was Mucin 5AC (MUC5AC). MUC5AC has a protective role against pathogens in the airways under normal physiological conditions regulating mucus function and increased levels contributed to asthma pathogenesis and mucus dysfunction [287, 288]. In addition, higher levels of MUC5AC may modify properties of mucus and contribute to worsening of chronic obstructive pulmonary disease [289]. Proteins involved in the complement regulation of the immune system including Serpin Family G Member 1 and Complement Factor H Related 2 were found to be upregulated in non-allergic asthmatics compared to allergic asthmatics. The complement proteins might contribute to asthma pathogenesis and are, therefore, of interest to investigate further [290]. Moreover, multiple proteins with lower protein expression in non-allergic asthmatics compared to allergic asthmatics were shown to be involved in the cilia function and mucociliary clearance. Mucociliary clearance is an important mechanism and dysfunction contributes to respiratory diseases and could be a mechanism causing airway disease that is different between non-allergic asthmatics and allergic asthmatics [291, 292].

Among the 2962 proteins quantified in all BL samples, less than 100 proteins had a statistically significant difference when comparing the proteomic profiles in allergic asthmatics and non-allergic asthmatics. This low number demonstrates how high natural variability in human biology, complexity of BL samples and relatively low number of study subjects may affect the interpretation [293]. The BL sample matrix often contains lipids and other contaminators that may affect the LC-MS/MS analysis. Interesting findings from this study need to be validated by another method for example western blot.

In summary, this study provides new knowledge of the proteome from BL supernatants of clinically characterized asthmatics with different phenotypes including allergic and non-allergic asthmatics. We identified candidate proteins distinguishing the two asthma groups which require further validation.

5 Conclusion

Main conclusions from **Paper I – V** were:

Paper I: This study identified bone marrow ILC2s to be IL-33-responsive cells contributing to the IL-5-dependent eosinophilic inflammation in response to intranasal challenges with the protease allergen papain. Targeting ILC2s therapeutically via the IL-33/ST2 axis might be promising in eosinophilic diseases such as asthma.

Paper II: This study further demonstrated that bone marrow ILC2s drive eosinophilic inflammation in the bone marrow. More specifically, bone marrow ILC2s might have a critical role at the onset of HDM-induced bone marrow eosinophilia. T_H cells and eosinophils were also found to be IL-33-responsive cell types in the bone marrow. Targeting the IL-33/ST2 axis may have an anti-inflammatory effect mediated by several IL-33-responsive cell types in the bone marrow.

Paper III: Airway exposure of IL-33 resulted in increased mTORC1 activity in bone marrow ILC2s. The mTORC1 inhibitor, rapamycin, decreased the mTORC1 activity which coincided with decreased eosinophilic inflammation potentially caused by inhibition of IL-5-producing ILC2s.

Paper IV: Unique miRNA expression patterns were observed in lung and bone marrow in mice during IL-33-induced inflammation. Exposure of IL-33 reduced levels of miR-150 in the bone marrow, a miRNA suggested to negatively regulate mTOR. Thus, miR-150 might be a promising candidate miRNA to alleviate IL-33-induced eosinophilic inflammation.

Paper V: Unique proteomic profiles in BL samples from allergic and non-allergic asthmatics were discovered. Candidate proteins with functions in cilia, mucus and the complement system, that distinguished the two asthma phenotypes require further validation. Quantitative proteomics is a promising approach to characterize disease drivers in asthma and identify new stable biomarkers.

6 Future perspectives

Despite research breakthroughs in the last decade including targeted therapies such as biologic treatments to treat severe asthmatics, there are still asthma patients with unmet needs. Moreover, new therapeutic targets need to be identified as it is suggested that asthma prevalence will increase in most parts of the world [5, 8, 9]. With that said, it will be exciting to see what the future holds. New and powerful technologies have been developed in recent years and it is now possible to study the entire immune system at once [294]. The healthy human immune system is diverse and vary between individuals. Asthma is a complex heterogeneous disease with immune cells infiltrating to the lungs from other organs via the bloodstream. Therefore, it is clinically relevant to study the immune system as a complete system. Cells and different molecules such as cytokines and miRNAs work together to regulate immune responses throughout our bodies.

A primary component of the immune system is the bone marrow. Haematopoietic stem cells in the bone marrow produce immune cells throughout an individual's life. Last year, researchers found that 6 – 7 weeks early in the second trimester of pregnancy many blood and immune cells differentiated into specific cell types including eosinophils and neutrophils important for protection against bacterial infections [295]. This is the first time all known blood and immune cells were identified in the developing bone marrow which was achieved with single cell RNA technology. Even stromal cells with function of supporting hematopoiesis were identified. This is a promising example of the knowledge that can be generated with new experimental techniques. Ongoing research aims to characterize and understand the healthy immune system, to identify disease drivers in immune-mediated diseases. That knowledge could potentially be obtained with the new technologies and methods in the nearest future. Regulatory mechanisms in the bone marrow that control inflammation throughout the body are important to study in inflammatory diseases such as asthma. In this thesis, **Paper I – IV** involve the bone marrow compartment and its role *in vivo*. Eosinophils are hallmark of allergic diseases and asthma that can migrate from the bone marrow to the airways [77]. We show that eosinophils themselves respond to IL-33 by upregulating ST2 in allergen models of asthma using papain or HDM, in **Paper I** and **Paper II**, respectively. In **Paper I**, we showed that the ST2 expression was dependent on IL-5 suggesting a link between ST2 and IL-5 levels. It would be interesting to explore this link further in prospective studies and its impact on inflammation. Furthermore, bone marrow ILC2s and T_H cells responded to intranasal allergen challenges and our data suggest that IL-33-responsive

ILC2s might be more critical at the onset of the HDM-induced eosinophilic inflammation. There might be local sources of IL-33 in the bone marrow contributing to the observed increased ST2 expression on the cells in the allergen-induced models. Moreover, IL-33 that is released from the airway epithelium could reach the bone marrow via the circulation contributing to IL-33-mediated inflammation. The source of IL-33 in the bone marrow would need to be investigated in future translational *in vivo* studies.

ILC2s are crucial players in T2 inflammation and are seen as potential therapeutic targets with their effector functions that control immune responses in inflammatory diseases. It will be interesting to see whether therapeutics targeting ILC2s could dampen inflammation or even cure inflammatory diseases including asthma. The few numbers of ILC2s in tissues make mechanistic studies challenging. In addition, the heterogeneity that ILC2s holds is another challenge. However, new techniques and state of the art technology will most likely overcome these challenges. Single-cell RNA sequencing has been, and still is, revolutionary in understanding the complex immune system. This technique would indeed be helpful understanding the heterogeneity of ILC2s in different tissues and diseases [296]. Part from sequencing methods, advancements within mass cytometry have been remarkable. More than 50 cellular markers can be used simultaneously on millions of individual cells using cytometry by time of flight technology which is a version of flow cytometry that uses metals instead of fluorochromes.

Genetically modified animals have been useful in the research field of asthma and have rendered increased knowledge regarding molecules, signaling pathways and receptors involved in asthma [297]. Breakthrough discoveries of new cell types including ILC2s were discovered in mice without mature B and T cells [136]. In **Paper I**, we studied the immune response in mice that lack mature T and B cells and in mice that lack all lymphocytes including ILC2s. We could conclude that despite a deficient adaptive immune system, the mice responded similarly to the protease allergen papain with increased eosinophil levels compared to WT mice. In mice that lacked all lymphocytes, addressing the impact of ILC2s during IL-33-induced inflammation, no eosinophilia was present suggesting that ILC2s played a crucial role in IL-33-induced eosinophilic inflammation. Needless to say, the immune system is different in rodents and humans and despite promising data generated from genetically modified mice, not all findings can be directly translated to human pathophysiology. Thus, mice that have a human component engrafted and referred to as humanized mice, have been developed to overcome this translation hurdle [297]. Humanized mice have been utilized in asthma research and holds promising potential [298, 299]. As an example, a recent study reported a

role of leukocyte-associated immunoglobulin-like receptor 1 in regulating human ILC2s and induction of airway hyperreactivity in a humanized mouse model [299].

Another disruptive and Nobel Prize awarded breakthrough is the CRISPR/Cas9 gene editing system that has revolutionized the research field in recent years with increased precision during gene therapy [300]. A decade ago, it was demonstrated that CRISPR could be reprogrammed and, thereby, modified the DNA of eukaryotes [301]. The CRISPR/Cas9 technology was used to deplete a long non-coding RNA, AK085865, in mice which were exposed to the allergen Der f1. Mice that were genetically modified showed a decreased inflammation with fewer eosinophils and alternatively activated macrophages that could promote differentiation of ILC progenitors to ILC2s compared to WT mice [302]. The CRISPR/Cas9 technology has also opened up the opportunity to genetically modify ALI cultures which are normally challenging to transfect or genetically modify [303]. A recent study showed that by targeting miR-141 with CRISPR/Cas9, IL-13-induced mucus was reduced in ALI-cultured human bronchial epithelial cells [304]. Indeed, clinical gene editing using the CRISPR/Cas9 technology might be a promising approach for future research in areas including asthma.

In **Paper IV**, we identified unique IL-33-induced miRNA expression profiles in murine lung and bone marrow using the technologies microarray and NanoString. For example, we found that miRNA-150 is a promising miRNA to investigate further and might be a miRNA regulating eosinophilic inflammation in asthma. There is no doubt that miRNAs can regulate ongoing inflammatory processes *in vivo*. However, the question is whether miRNAs will be successful therapeutic targets for future treatments and/or biomarkers used to diagnose and monitor asthma. A number of miRNAs correlated with clinical characteristics in asthmatics, including miR-155, miR-223 and miR-21, that might be promising candidates to investigate further. A major challenge is to enable proper drug delivery of the miRNAs to specific tissues and new drug delivery systems and technologies, in combination with optimized mimics and antagomiRs, might lead to a future with novel miRNA-based therapeutics for asthmatics that are poor or non-responders to available treatments.

In **Paper V**, we used the state of the art TMT-based LC-MS/MS to determine proteomic profiles in BL fluid from allergic and non-allergic asthmatics. We identified proteins, distinguishing the asthma groups, that for example were involved in mucus function, mucociliary clearance and the complement system. Nearly 5000 proteins were identified in our study and quantitative

proteomics is a promising technique to characterize disease drivers in asthma [50, 222, 229]. However, BL sampling is invasive and not ideal from a biomarker perspective that would be clinically useful in the future. However, NAL or induced sputum which might reflect disease of the airways, would be more optimal from a patient perspective due to its less invasive sampling. It would be interesting to characterize the proteomic profiles in NAL and sputum samples from allergic and non-allergic asthmatics and compare the results with BL samples to gain a more complete understanding of how the differences and similarities in the sample types reflect the asthma pathogenesis in the airways.

There is a need to identify new asthma endotypes and stable biomarkers, especially for T2 low asthma with no current clinical biomarkers. Potentially, the term T2 low and T2 high asthma might be an over-simplification. T2 high asthma is heterogeneous with different underlying mechanisms and more knowledge is needed to meet patients' unmet needs in this patient subgroup. For example, not all patients with eosinophilic asthma achieve good symptom control with biologic treatments targeting IL-5. In addition to IL-5, type 2 inflammation is induced by several inflammatory signals and type 2 signals are initiated by upstream mediators including the alarmins TSLP, IL-33 and IL-25. The recently approved anti-TSLP therapeutic, tezepelumab, was shown to be beneficial in different asthma phenotypes which is a major advantage [61, 63]. Therapeutics targeting IL-33 or ST2 are currently in clinical trials and have so far shown promising results including improved lung functions and decreased eosinophil levels [65, 135]. Future clinical trials and research on the alarmins will increase the understanding of asthma disease heterogeneity and severity with the overall goal to offer the right drug for the right patient to increase quality of life and help patients with poor disease control.

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