ILC2s and miRNA regulation in allergy and asthma

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Cover illustration: H&E-stained lung section of a microRNA-155 deficient mouse following chronic allergen inhalation. Photo by Kristina Johansson.
Have courage and be kind

Cinderella
ILC2s and miRNA regulation in allergy and asthma

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Abstract

Asthma is a common respiratory disease that is characterized by chronic inflammation of the airways. In most asthmatic subjects, the immune response is driven by pro-inflammatory type 2 cytokines (interleukin (IL)-4, IL-5 and IL-13) that correlate with hypersensitivity to environmental allergens and increased numbers of eosinophils in the airways and blood. In 2010, type 2 innate lymphoid cells (ILC2s) were identified as a novel type 2 cytokine-producing cell population. They were later found to promote allergen-related immune responses in the airway mucosa, where the alarmin cytokine IL-33 is an important driver.

Understanding the molecular mechanisms that cause excessive immune activation is an important area of asthma research. Gene regulatory microRNAs (miRNAs) are emerging as promising targets for modulation of type 2 immunity and play important roles in models of allergic asthma. However, miRNA expression in ILC2s and in the airways of human asthmatics is currently understudied. Using samples from asthmatic subjects and experimental mouse models of asthma we identified that miRNA-155 (miR-155) is critical for ILC2-mediated inflammation in mice (Paper I). Lung ILC2s increased miR-155 expression upon IL-33-mediated activation in vitro and miR-155 deficient ILC2s demonstrated decreased IL-13 production and lowered proliferative capacity to IL-33 administration in vivo. Importantly, this was accompanied by a severe reduction of airway eosinophils. We identified that miR-155 is differentially expressed in airways of subjects with allergic asthma compared to healthy controls (Paper II). Furthermore, induced sputum isolated from allergic asthmatics in and out of pollen season revealed that the level of miR-155 in sputum lymphocytes varied with the season. In Paper III, we identified a previously unrecognized role of ILC2s locally in murine bone marrow. We found that IL-5-producing ILC2s contribute to the development and overproduction of eosinophils that promoted airway inflammation. Finally, we identified distinct differences in miRNA expression by examining miRNA profiles in airway macrophages isolated from bronchial lavage of asthmatic and healthy individuals (Paper IV).
Taken together, these studies demonstrate that miRNAs play important roles in airway immunity; miR-155 is necessary for the pro-inflammatory function of ILC2s, miR-155 expression is altered in airway lymphocytes from asthmatic subjects and a distinct miRNA signature is present in asthmatic airway macrophages. We also demonstrated that ILC2s have additional roles in allergic immunity and support eosinophilic airway inflammation by local reactions in the bone marrow. An increased understanding of the mechanisms that promote chronic type 2 inflammation in various tissues, and in specific cells, is essential for the development of improved prevention and therapy of the disease in the future.

**Keywords**: microRNA, type 2 innate lymphoid cell, IL-33, IL-5, eosinophil

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Det är immuncellens genuppsättning som bestämmer vilken funktion cellen har och den är hårt reglerad via interaktioner mellan celler och utsöndrade proteiner. På molekylär nivå påverkas cellen inte bara av protein utan även av ribonukleinsyra (RNA). MikroRNA består av korta sekvenser RNA som

I det första arbetet i avhandlingen (Paper I) fann vi att ett specifikt mikroRNA, miR-155, är nödvändigt för utveckling av eosinofil inflammation via ILC2-celler i möss. Möss med komplett genuppsättning (vildtyp) utvecklade en kraftfull eosinofil inflammation i luftvägarna vid inhalation av IL-33, vilket korrelerade med en ökning av ILC2-celler i lungan. Däremot var möss som saknade miR-155-sequensen i sitt genom (miR-155 knockout; KO) oförmöga att utveckla eosinofil inflammation som svar på IL-33 och de ökade inte antalet ILC2-celler. Vidare analys visade att IL-33-aktiverade ILC2-celler i lungor från miR-155 KO möss uppvisade en minskad pro-inflammatoryisk funktion och producerade lägre nivåer av cytokinet IL-13 som bidrar till astmasymptom i luftvägarna. I avhandlingens andra arbete (Paper II) fann vi att nivån av miR-155 i upphostningsprov (inducerat sputum) var annorlunda hos allergiska astmatiker jämfört med friska kontrollindivider. Genom att undersöka upphostningsprov från allergiska astmatiker under och efter pollensäsong fann vi även att nivån av miR-155 i lymfocyter varierade beroende på säsong. I vår tredje studie (Paper III) identifierade vi en tidigare okänd roll för ILC2-celler lokalt i benmärg hos möss. Vi fann att IL-5-producerande ILC2-celler bidrog till utveckling och överproduktion av eosinofiler i benmärgen vilket främjade eosinofil inflammation i luftvägarna hos mössen. Slutligen har vi identifierat skillnader i mikroRNA-uttryck hos ILC2-celler jämfört med friska individer genom undersökningar av mikroRNA i makrofager, den vanligaste förekommande immuncellen i luftvägarna (Paper IV).

Sammantaget visar dessa studier att mikroRNA spelar en oerhört viktig roll i immunförsvaret i luftvägarna; miR-155 är nödvändig för pro-inflammatoryiska funktioner hos ILC2-celler, nivån av miR-155 är förändrad i luftvägslymfocyter från astmatiker och en distinkt mikroRNA-signatur finns i makrofager från astmatiska luftvägar. Vi har även visat att ILC2-celler främjar eosinofil luftvägsinflammation genom reaktioner lokalt i benmärgen. Ökad kunskap om mekanismerna som kontrollerar kronisk inflammation i olika vävnader, och i specifika immunceller, är helt avgörande för utveckling av förbättrade behandlingar av astma och andra inflammatoriska sjukdomar i framtiden.
List of papers

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

I. MicroRNA-155 is a critical regulator of type 2 innate lymphoid cells and IL-33 signaling in experimental models of allergic airway inflammation.
   Johansson K, Malmhäll C, Ramos-Ramírez P, Rådinger M.
   *J Allergy and Clin Immunol*. 2017; 139(3):1007-1016.e9

II. Altered miR-155 expression in allergic asthmatic airways.
    Malmhäll C, Johansson K, Winkler C, Alawieh S, Ekerljung L, Rådinger M.

III. Bone marrow type 2 innate lymphoid cells: a local source of interleukin-5 in interleukin-33-driven eosinophilia.
    Johansson K, Malmhäll C, Ramos-Ramírez P, Rådinger M.
    *Immunology*. 2017; 153(2):268-278

IV. MicroRNA signatures in asthmatic and healthy airway macrophages.
    Johansson K, Weidner J, Malmhäll C, McCrae C, Rådinger M.
    *In manuscript*

All published articles were reproduced with permission from the publishers.
List of publications not included in the thesis:

**MicroRNAs in type 2 immunity.**
Johansson K, Weidner J, Rådinger M.
In revision

**Immunmekanismer i icke-allergisk eosinofil astma.**
Johansson K.

**MikroRNA spelar en avgörande roll vid luftvägsinflammation.**
Johansson K.
BestPractice Lungmedicin. 2016; 16:27-31

**Weight gain alters adiponectin receptor 1 expression on adipose tissue-resident Helios+ regulatory T-cells.**
Scand J Immunol. 2016; 83(4):244-254

**Targeting a novel bone degradation pathway in primary bone cancer by inactivation of the collagen receptor uPARAP/Endo180.**
J Pathol. 2016; 238(1):120-133

**Complex determinants in specific members of the mannose receptor family govern collagen endocytosis.**
Preface

This thesis is submitted for the degree of Doctor of Medicine at the University of Gothenburg. The research described was conducted under the supervision of Associate Professor Madeleine Rådinger and co-supervisor Dr. Carina Malmhäll at Krefting Research Centre, University of Gothenburg, between April 2014 and January 2018.

The thesis is based on four original articles and is presented in six chapters: 1 Introduction, 2 Aim, 3 Methods, 4 Results and discussion, 5 Conclusion and 6 Future perspectives.
## Content

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# Abbreviations

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<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<td>BIC</td>
<td>B cell integration cluster</td>
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<tr>
<td>BL</td>
<td>Bronchial lavage</td>
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<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>CBA</td>
<td>Cytometric Bead Array</td>
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<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<td>DGCR8</td>
<td>DiGeorge syndrome critical region 8</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EoP/Eos Prog</td>
<td>Eosinophil progenitor</td>
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<tr>
<td>Eos</td>
<td>Eosinophil</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>FENO</td>
<td>Fraction of exhaled nitric oxide</td>
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<td>FEV₁</td>
<td>Forced expiratory volume in the first second</td>
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<tr>
<td>FMO</td>
<td>Fluorescence minus one</td>
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<td>FOXP3</td>
<td>Forkhead box protein 3</td>
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<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
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<td>GATA-3</td>
<td>GATA-binding protein 3</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>HDM</td>
<td>House dust mite</td>
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<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
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<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<td>IgE</td>
<td>Immunoglobulin E</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
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<td>ILC2</td>
<td>Type 2 innate lymphoid cell</td>
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<tr>
<td>i.n.</td>
<td>Intranasal</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>Lin</td>
<td>Lineage</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<td>miR-155</td>
<td>MicroRNA-155</td>
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<tr>
<td>miRNA</td>
<td>MicroRNA</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>OVA</td>
<td>Ovalbumin</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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PBS  Phosphate buffered saline
PI3K  Phosphoinositide 3-kinase
Prog  Progenitor
qPCR  Quantitative real-time polymerase chain reaction
RAG  Recombination-activating gene
rMFI  Relative mean fluorescence intensity
RORγT  Retinoic acid-related orphan nuclear receptor gamma t
SOCS1  Suppressor of cytokine signaling 1
SSC  Side scatter
STAT  Signal transducer and activator of transcription
T-bet  T-box transcription factor
TCR  T cell receptor
T₉ cell  T helper cell
T₉2  T helper type 2
TNF-α  Tumor necrosis factor alpha
T.REG cell  T regulatory cell
TSLP  Thymic stromal lymphopoietin
UTR  Untranslated region
WT  Wild type
Chapter 1 Introduction

Asthma is a common respiratory disease that affects more than 300 million people of all ages around the world. It is a large burden that causes reduced quality of life for its sufferers and economic losses to societies [1]. Chronic inflammation of the airways is a defining feature of the disease, which gives rise to bronchial hyperresponsiveness and airflow obstruction, leading to respiratory symptoms such as wheeze, cough, breathlessness and chest tightness [2]. According to the West Sweden Asthma Study at Krefting Research Centre in Gothenburg, and other large population-based studies, the prevalence of asthma is approximately 5-10% in Sweden and other westernized countries [3-8]. Epidemiological data have also demonstrated that asthma is a disease with a high degree of heterogeneity [8]. Indeed, several different phenotypes have been reported based on clinical features such as lung function, symptoms, triggers of exacerbations, and age of disease onset [9]. The existence of disease phenotypes makes asthma management challenging and distinct patient groups respond poorly to current medication, while others are well-treated [9, 10]. Increased awareness of this heterogeneity has raised important questions in the field regarding phenotype stability and effects of medication. Importantly, it has also spiked an interest in finding the underlying cellular and molecular mechanisms of the disease. A greater understanding of immunological pathways that control asthma pathology should lead to more targeted and personalized therapeutic approaches in the future.

In this thesis, molecular mechanisms of the immune system that mediate inflammation in asthma were studied. The work focuses on pro-inflammatory type 2 innate lymphoid cells (ILC2s) and a class of gene regulatory molecules that modulate inflammatory responses, called microRNAs (miRNAs). The following chapter describes the background to the presented studies.

1.1 The immune system in asthma

The inflammatory response in asthma involves many different specialized immune cells and structural cells of the airways. Their interactions result in mucus overproduction, smooth muscle contraction, airway wall remodeling and airway narrowing, creating repeated periods of breathing difficulties [11]. These reactions are a consequence of type 2 immune responses that are mediated by interleukin (IL)-4, IL-5 and IL-13, which are referred to as type 2 cytokines [12]. A range of different environmental factors can trigger type 2 immunity including parasite, virus, bacterial and fungal infections as well as
allergens and chemical irritants (Fig 1.1) [13]. Different asthma phenotypes display variable susceptibility to these triggers and traditionally, asthma has been divided in two major phenotypes: allergic and non-allergic asthma [14]. Allergic asthma is the most common phenotype in children and is present in approximately 50% of asthmatic adults [11]. These individuals demonstrate hyper-sensitivity to aeroallergens which correlates with elevated circulating immunoglobulin E (IgE) antibodies and increased numbers of eosinophils in sputum and blood [9, 15]. The close association between asthma and allergic disease highlights the importance of allergen-specific CD4+ T cells in asthma. Early studies of asthmatic patients identified an increased number of airway-infiltrating CD4+ T cells that produced type 2 cytokines and correlated with the degree of eosinophilia [16].

**Figure 1.1.** Type 2 immune responses are triggered by a variety of microbial or non-microbial environmental factors such as parasites, viruses, bacteria, fungi, allergens and chemical irritants. The illustration was adapted from Johansson et al 2018, in revision.

Non-allergic asthma typically develops later in life, is more common in women and associated with obesity [17, 18]. This phenotype often displays more severe symptoms and is difficult to treat [19]. In accordance with this view, a recent study from the West Sweden Asthma Study cohort demonstrated a negative correlation between atopy and severity of asthma symptoms [8]. Innate immune pathways and danger-associated molecular signals are likely
more important in non-allergic asthmatic responses as there is no apparent involvement of CD4+ T helper type 2 (T\textsubscript{H}2) cells and B cells that mediate IgE reactivity to allergens. However, regardless of allergy, airway inflammation in asthma is often eosinophilic in nature [20]. In fact, the presence of eosinophils is a stronger distinguishing factor of asthma subgroups than are allergies (atopy or total IgE) [9, 21]. From detailed studies in mice, we now know that type 2-dependent eosinophilic inflammation can be induced without the involvement of the adaptive immune system [22, 23]. However, immune mechanisms of asthma other than type 2 inflammation are less explored and there are asthmatic patients without any signs of airway or blood eosinophilia [9]. Cluster analysis based on gene expression profiles in airway epithelial brushings were able to divide asthmatics in T\textsubscript{H}2\textsuperscript{hi} and T\textsubscript{H}2\textsuperscript{lo} subgroups defined by the presence (T\textsubscript{H}2\textsuperscript{hi}) or absence (T\textsubscript{H}2\textsuperscript{lo}) of type 2 cytokines and eosinophilia [21]. Corticosteroids, the standard therapy of asthma, have proved more effective in individuals with evident type 2 inflammation and elevated eosinophils as in T\textsubscript{H}2\textsuperscript{hi} asthma [24, 25], while the T\textsubscript{H}2\textsuperscript{lo} subgroup showed resistance to corticosteroids [21, 26]. The lack of effective control of T\textsubscript{H}2\textsuperscript{lo} asthma is a considerable problem that requires further investigations of disease mechanisms.

**Glossary**

*Allergic sensitization* is a result of a process where an antigen-presenting cell digests and presents an allergen to a naïve CD4+ T cell that becomes a T\textsubscript{H}2 cell. The T\textsubscript{H}2 cell interacts with B cells to promote production of allergen-specific IgE, which binds to mast cells, priming them for the next encounter with the allergen.

*Innate immunity* mediates direct and unspecific responses to pathogens through danger-associated signals. ILCs are part of the innate immune system.

*Adaptive immunity* educates cells to recognize specific pathogens, creating immunological memory. It leads to enhanced responses to subsequent encounters with the pathogen. CD4+ T cells and B cells are part of the adaptive immune system.

*Recombination-activating gene (RAG)* is restricted to lymphocytes and encodes RAG1 and RAG2 proteins. Their function is essential for the development of functional T and B cells.

**1.1.1 Type 2 innate lymphoid cells**

The discovery of innate lymphoid cells (ILCs) changed our view of hallmark T cell disorders such as asthma. ILC2s were initially identified as a non-T/non-B cell source of IL-4, IL-5 and IL-13 in RAG2 deficient mice in response to IL-25. In addition to increased type 2 cytokine expression, the mice
demonstrated classically allergen-induced responses previously associated with the effector function of T_{h}2 cells, such as airway eosinophilia, mucus overproduction, airway hyperreactivity and tissue remodeling [22, 27]. Since then, ILC2s have been studied extensively in anti-helminthic and allergic responses, which have advanced our understanding of their importance in type 2 immunity [28-33]. Indeed, it is now known that ILC2-derived IL-13 is critical in early clearance of parasitic helminths [28, 31] and the inability of lymphocyte deficient mice (RAG2^{−/−}IL2rg^{−/−}) to develop eosinophilic inflammation to allergens can be rescued by adoptive transfer of ILC2s [32].

The realization that ILC2s regulate effector functions of other immune cells have highlighted the importance of studying these cells in an intact immune system. For instance, CD4^{+} T cell responses to the protease allergen papain are enhanced by ILC2s [34]. This was demonstrated in a study where IL-13 production by ILC2s activated dendritic cells to migrate to lung draining lymph nodes where they polarized naive CD4^{+} T cells to T_{h}2 cells [34]. Furthermore, direct interactions of ILC2s and CD4^{+} T cells have been found to potentiate expulsion of parasitic worms [35], and B cell proliferation and antibody production are enhanced by ILC2s [34, 36]. Conversely, induction of ILC2s themselves were reported to be dependent on CD4^{+} T cell activation in a model of house dust mite (HDM)-induced airway inflammation [37]. These studies demonstrate that in addition to being an important source of type 2 cytokines, ILC2s amplify type 2 responses through their interaction with other immune cells.

Studies in humans have suggested that ILC2s play a role in the pathogenesis of severe asthma, where increased frequency of the cells have been observed in patients with severe disease [38-41]. Interestingly, a study that compared the relative contribution of type 2 cytokines by ILC2s and CD4^{+} T cells in the airways found that even though CD4^{+} T cells were more abundant than ILC2s in sputum from asthmatic subjects, the ILC2s produced proportionally more cytokines and were the predominant source of IL-5 and IL-13 [38]. Furthermore, circulating IL-13^{+} ILC2s were reported to correlate with asthma control, where the highest levels were found in uncontrolled asthma compared to better controlled asthma groups and healthy subjects [41]. It was also found that ILC2s had higher resistance to glucocorticoid treatment (dexamethasone) compared to T_{h}2 cells, measured by IL-13 production in vitro [41]. This result supports the idea that ILC2s contribute to poor treatment-responses in severe asthma. Importantly, another study of asthmatic patients reported that dexamethasone successfully inhibited type 2 cytokine production in ILC2s isolated from peripheral blood but not in ILC2s that were isolated from bronchoalveolar lavage (BAL) [42]. Recently, a study found that inhaled allergen provocation of allergic asthmatic subjects increased the number of IL-5 and IL-13 producing ILC2s in the airways, which coincided with decreased
number of ILC2s in the circulation [43]. This might suggest that ILC2s are, at least in part, recruited to the airways upon allergen exposure. However, differences in steroid-responsiveness of BAL and blood-derived ILC2s indicated that a distinct ILC2 phenotype is present in the airways. These findings raise important questions regarding tissue residency of ILC2s and if innate memory through epigenetic mechanisms regulate ILC2s of various origin. Currently, the understanding of these mechanisms is limited and requires further study.

ILC2s protect epithelial barriers of the lung, intestine and skin by responding to IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), that are released from the epithelium upon injury or stimulation by environmental factors (Fig. 1.1) [44, 45]. Lymph nodes, adipose tissue, liver and bone marrow are also natural sites for ILC2s [46-50]. Establishment of ILC2s in peripheral tissues occurs during the first weeks of life and is highly influenced by IL-33 [49, 51-53]. While in the tissue, ILC2s are long-lived and can undergo local expansion upon infection [48, 54]. However, airway exposure to the fungal allergen Alternaria alternata (Alternaria) recently showed that ILC2 progenitors left the bone marrow to migrate to the lung in an IL-33-dependent manner in adult mice [49].

Development of ILC2s in the bone marrow has been studied in great detail and involves transcriptional programs that effectively suppress alternative lymphoid faiths (B, T and NK cells). Common lymphoid progenitors progress into common helper ILC precursors that generate ILC1, ILC2 and ILC3 [55-57]. The 1, 2 and 3 ILC subsets mirror the adaptive CD4+ T cell lineages in terms of transcription factor expression and cytokine production: T_H1 (T-bet; IFN-γ, TNF-α), T_H2 (GATA-3; IL-4, IL-5, IL-13) and T_H17 (RORγT; IL-17, IL-22), respectively [58]. Most recently, the ILC equivalent of regulatory T (T_REG) cells was identified in mouse and human intestines. Although the ILCs expressed the regulatory cytokine IL-10 and protected mice from intestinal inflammation, they did not express the T_REG transcription factor FOXP3 [59].

Studies in recent years have found that ILC2s possess substantial plasticity. They can acquire an ILC3s phenotype that co-produce IL-13 and IL-17, and consistent with their dual ILC2/ILC3 function they express both GATA-3 and RORγT. This plasticity has been observed in parasitic and allergic responses in mice [60, 61]. ILC2s have also been found to express T-bet and produce IFN-γ through a process that is regulated by IL-12. [62-65]. This switch to ILC1 was shown to augment virus-induced inflammation and the frequency of ILC1s in patients with chronic obstructive pulmonary disease (COPD) correlated with disease severity and susceptibility to virus-triggered exacerbations [62]. Virus infection in mice was previously found to induce asthma-like airway inflammation via IL-33-mediated activation of ILC2s [66]. However, anti-viral responses of ILC2s in asthma is not completely clear since
virus-induced IFN-γ restricted ILC2 responses *in vivo* in mice and expression of the type 1 interferon (IFN) receptor on human and mouse ILC2s mediated suppression of type 2 cytokine production [67, 68]. Nevertheless, findings in humans support the view that IL-33 promotes pro-inflammatory functions of ILC2s under virus-induced exacerbations in asthmatic patients [69]. The implications of IL-33 in asthma are presented further in the next section.

### 1.1.2 Interleukin-33

IL-33 belongs to the IL-1 family of cytokines and signals through a receptor complex consisting of ST2 and IL-1R accessory protein (IL-1R-AP). The ST2 gene is expressed in a soluble form (sST2) which acts as a decoy receptor for IL-33 [70], and a membrane-bound form that, upon IL-33 binding, activates MyD88-dependent pathways leading to induction of inflammatory mediators in target cells [71, 72]. Proteases regulate the activity of IL-33 and cleavage by intracellular caspases generates two biologically inactive protein products [73, 74]. Inactivation of IL-33 is an important pathway to avoid immune activation under programmed cell death. However, necrotic cell death under pathological conditions involves sudden release of full-length IL-33 that acts as an alarmin with high biological activity. Cleavage of secreted full-length IL-33 by extracellular proteases released during inflammation can further increase the potency of IL-33 [75].

IL-33 and ST2 have been identified as major susceptibility genes for human asthma in several genome-wide association studies [76-80]. In addition, a loss-of-function mutation in the gene encoding IL-33 was recently identified in an Icelandic population that was associated with reduced levels of blood eosinophils and lowered risk of developing asthma [81]. Interruption of IL-33 signaling in mice results in decreased basal levels of eosinophils [82], and allergen-induced eosinophil infiltration and airway hyperresponsiveness are attenuated in IL-33 or ST2 deficient mice [83, 84]. Conversely, administration of IL-33 was demonstrated to exacerbate allergen-induced airway responses [84, 85]. Studies of human asthma have also found a positive correlation between asthma severity and IL-33 levels [39, 86-88]. Interestingly, viral respiratory tract infections are the most common trigger of asthma exacerbations [89], and rhinovirus infection of primary human bronchial epithelial cells were found to be a strong inducer of IL-33 *in vitro* [69]. The same study showed that IL-33 was induced by rhinovirus *in vivo* in asthmatic airways with IL-33 levels that related to severity of exacerbations. Furthermore, supernatants from rhinovirus-infected epithelial cell cultures induced type 2 cytokine production by CD4+ T cells and ILC2s which was suppressed by blocking ST2 [69]. Thus, the authors suggested that IL-33 is a
key component that links viral infections to amplification of type 2 inflammation in asthma.

Differentiated CD4+ T cells, including TH2 cells and TREG cells, both express ST2, and it has been shown that TH2 cells can be activated by IL-33 to produce type 2 cytokines independent of antigen-stimulation via the T cell receptor (TCR) [90, 91]. Furthermore, ST2+ TREG cells were reported to lose their suppressive capacity in response to IL-33 stimulation in vivo, where TREG cells increased expression of GATA-3, ST2 and type 2 cytokines [92]. Plasticity between TREG and TH2 phenotypes were also studied in response to parasite infection, where TREG cells acquired type 2 effector functions in an IL-4-dependent mechanisms which contributed to host defense [93]. However, the role of IL-33 in CD4+ T cell effector functions is not completely clear since earlier studies suggested that ST2 deficient antigen-specific CD4+ T cells aggravated antigen-induced airway inflammation [94]. The relative contribution of IL-33-responsive antigen-specific TH2 cells versus TREG cells in allergic inflammation require further study.

Beyond CD4+ T cells and ILC2s, several different cell types with critical roles in allergy and asthma express ST2, including eosinophils, mast cells, basophils and macrophages. Interestingly, a study of IL-33 signaling in asthmatic subjects found that mast cells and basophils, not ILC2s, were the major cellular targets of IL-33 and producers of type 2 cytokines [95]. However, IL-33-dependent responses in mast cells have been suggested to both enhance and suppress type 2 inflammation in mice [23, 96]. Furthermore, IL-33 may polarize alveolar macrophages to the alternatively activated M2 phenotype that is characteristic in asthma (discussed below). IL-33 stimulation of M2 macrophages promoted airway inflammation by inducing the eosinophil-specific chemokine CCL24/eotaxin-2 and CCL17/TARC which is important in CD4+ T cell recruitment [97]. Although IL-33 was shown previously to activate eosinophils [98], several recent studies examined the role of IL-33 in eosinophil development [82, 99, 100]. One of these studies found that IL-33 signaling in bone marrow precursor cells induced IL-5 receptor expression which controls eosinophil lineage commitment as well as many other important aspects of eosinophils which are presented below.

1.1.3 Eosinophilic inflammation

Eosinophils are terminally differentiated granulocytes that are equipped with preformed toxic proteins and reactive oxygen species that mediate resistance to parasitic helminths and contribute to tissue damage and remodeling in asthmatic airways [101-103]. High levels of eosinophils correlate with increased asthma severity [104, 105], while reduced eosinophils are reported in patients with controlled eosinophilic asthma [106]. This clearly
demonstrates the importance of understanding mechanisms that regulate eosinophilic inflammation in asthmatics.

The high number of eosinophils likely results from a combination of increased production, migration and survival of the cells. Eosinophils develop from CD34+ hematopoietic progenitor cells in the bone marrow under the control of IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [107]. Allergen challenge of the airways promotes bone marrow eosinophilopoiesis in asthmatic subjects as well in murine models of allergic airway inflammation [108-115], which coincides with increased migration of mature eosinophils to the lungs via eotaxins [116, 117].

IL-5 is particularly critical in eosinophil biology and controls several key features such as terminal eosinophil maturation and delayed apoptosis. Eosinophil chemotaxis, endothelial adhesion and mediator secretion are also enhanced by IL-5 [118]. Interestingly, mice deficient in IL-5 or the receptor subunit IL5Rα which is necessary for IL-5 signaling are not completely lacking eosinophils. This suggests that factors other than IL-5 may be involved in, or compensate for, the constitutive generation of eosinophils at basal levels. Recently, Johnston et al found that IL-33 induces eosinophil lineage commitment in murine bone marrow [82]. It was demonstrated that IL-33 administration increased eosinophils in the bone marrow and peripheral blood, however, blockade of IL-5 prevented IL-33-induced eosinophil expansion. Their findings suggest that IL-33 promotes eosinophilic inflammation by increasing IL-5-responsiveness of eosinophil progenitors (EoPs) while at the same time promoting IL-5 production by IL-33-responsive cells. Futhermore, they reported increased levels of IL-5 in IL-33 challenged mice, however, the cellular source of IL-5 was not described in their study.

Both systemic IL-5 and local IL-5 production have been suggested to regulate eosinophil development in the bone marrow. A study of Alternaria-induced allergic airway inflammation in mice demonstrated increased eosinophils in airways and bone marrow together with elevated levels of IL-5 in serum [99]. The authors suggested that Alternaria exposure generated a release of IL-33 in the airways which induced IL-5 production by lung ILC2s that reached the circulation and enabled induction of eosinophils in the bone marrow. Additionally, Nussbaum et al suggested that constitutive IL-5 production by tissue-resident ILC2s were the predominant source of circulating IL-5 which is crucial for eosinophil maintenance under homeostatic conditions [48]. However, earlier studies have suggested that CD4+ T cells and CD34+ progenitors produce IL-5 locally in the bone marrow at homeostasis and in allergen-induced airway inflammation [119-121]. Interestingly, a recent study of mild asthmatic patients found increased activation of CD4+ T cells, but not ILC2s, in the bone marrow in response to airway allergen provocation [43].
Figure 1.2. Eosinophilic airway inflammation is associated with induction of eosinophils in the bone marrow, which are recruited to the airways via eotaxins (CCL24 in mice). IL-33 promotes differentiation of eosinophil progenitors (EoP) into mature eosinophils by inducing IL5Rα expression in the bone marrow, where IL-33-responsive ILC2s might be a source of IL-5.

Collectively, these studies suggest that there is a strong relationship between IL-33 and the development of eosinophils in the bone marrow that may depend
on both local and systemic sources of IL-5. An updated view of IL-5 producing cells in the bone marrow is needed, especially in the light of the recent findings regarding IL-33 signaling in this compartment. It is possible that IL-33 is expressed by bone marrow cells that stimulate IL-5 secretion in cells nearby, such as bone marrow ILC2s (Fig. 1.2).

1.1.4 Macrophages

Alveolar macrophages are found in the airway lumen, in close proximity to the mucosal surface which typically makes them the first line of defense against inhaled particles and pathogens. Their frequency in asthmatic airways have been reported at comparable levels to healthy controls [122], however, the function of alveolar macrophages has been suggested to be altered in asthma. For instance, co-culture experiments have demonstrate that alveolar macrophages from asthmatic subjects induce higher levels of IL-5 in CD4+ T cells compared to macrophages from healthy controls [123]. In addition, alveolar macrophages in children with poorly controlled asthma demonstrated decreased phagocytosis and increased apoptosis compared to healthy cells [124].

Experimental investigations in mice have shown that allergen exposure makes the lung more susceptible to bacterial infections, a process in which alveolar macrophages have been implicated by producing negative regulators of toll-like receptor signaling which impairs neutrophil recruitment and bacterial clearance [125]. Furthermore, GM-CSF is upregulated in the asthmatic epithelium [126], and has been identified as an important factor in the onset and pathogenesis of allergen-induced airway inflammation [127-132]. It facilitates allergic sensitization in experimental models and was reported to inhibit the suppressive capacity of alveolar macrophages [133, 134]. However, after the resolution of allergic airway inflammation, GM-CSF was found to act as a homeostatic regulator that controlled maturation and inflammatory status of alveolar macrophages [135]. It was suggested that reduced production of GM-CSF in the post-allergic lung increased the susceptibility to rhinovirus infection due to insufficient alveolar macrophage maturation. Importantly, anti-viral defense was enhanced by exogenous GM-CSF administration [135]. It is possible that the lack of resolution in asthma, which is associated with increased susceptibility to viral and bacterial infections, contributes to the breakdown of the homeostatic function of alveolar macrophages.

Although inappropriate immune activation to airborne antigens are thought to contribute to the inflammation in asthmatic airways, the role alveolar macrophages in regulation of these responses is not well understood. Depletion of alveolar macrophages prior to allergen challenge augments airway
inflammation and airway hyperreactivity [136-138], which suggests that macrophages have a protective role in these models. A link between lung macrophages and airway remodeling is well-established in asthma and there is evidence that macrophages promote eosinophilic inflammation under experimental conditions [139-142]. Therefore, it is not yet clear whether these cells have a pro-inflammatory or anti-inflammatory role in asthma.

Macrophages are commonly defined based on their cytokine expression profile which is associated with distinct functions of the cells. Two well-established subgroups are the classical M1 and alternative M2 polarized macrophages. The M1 phenotype is involved in the response to intracellular pathogens and is induced by IFN-γ and lipopolysaccharides (LPS) [143]. Phagocytosis of foreign pathogens and apoptotic cells are mediated by M2 macrophages which develop under the influence of type 2 cytokines such as IL-4 and IL-13 [143]. A predominance of M2 macrophages has been described in asthma where they contribute to excessive tissue repair [144]. However, M1 macrophages are also likely to take part in the immune responses in asthma, particularly in aspects concerning virus-triggered exacerbations.

### 1.2 Immune regulatory microRNAs

miRNAs are small noncoding RNAs that mediate sequence-specific repression of target messenger RNAs (mRNAs), inhibiting gene expression at the post-transcriptional level [145]. The first miRNA to be identified was *lin-4* in the round worm *Caenorhabditis elegans* in 1993 [146]. Since then, the field has expanded with more than 1,800 validated miRNAs in humans and over 28,000 total miRNAs have been identified in metazoans [147]. In addition, up to 60% of protein-coding genes are estimated to be regulated by miRNAs which makes them one of the largest classes of regulatory molecules [148].

![Figure 1.3](image)

**Figure 1.3.** A representation of the complexity of miRNA-mediated regulation: One miRNA may regulate several different mRNAs, and several different miRNAs may regulate one mRNA.

miRNAs shape cellular responses in health and disease by regulating fundamental cellular processes such as proliferation, differentiation, migration
and apoptosis [149-151]. A single miRNA may target multiple mRNAs and an individual mRNA may be directly regulated by several different miRNAs (Fig. 1.3). This is highly context-dependent and varies with cell type, tissue or even cell status. Nevertheless, this complexity also represents the strength of miRNAs which often regulate networks of functionally related gene transcripts involved in common biological pathways. It means that altered expression of a single miRNA may change the course of an inflammatory process and affect disease progression [152-154]. This, of course, makes miRNAs highly attractive for therapeutic intervention, where the miRNAs themselves, or downstream gene products, might be targeted.

1.2.1 miRNA biosynthesis

The generation of miRNAs in the cell has been described in great detail (Fig. 1.4) [155, 156]. They are generally transcribed via RNA polymerase II into stem loops with single stranded ends that are called primary miRNAs (pri-miRNAs). Pri-miRNAs are further processed into approximately 60 nucleotide precursor miRNAs (pre-miRNAs) in the nucleus by the enzymes Drosha and DiGeorge syndrome critical region 8 (DGCR8), before transport to the cytoplasm via the exportin-5 complex [157]. In the cytoplasm, pre-miRNAs

![Figure 1.4](image-url)

Figure 1.4. Representation of the major steps in the miRNA biosynthesis pathway. Degradation or translational repression of mRNAs result in reduced protein production. The illustration was adapted from [159].
are bound and cleaved by the enzyme Dicer, creating a miRNA duplex. Subsequent maturation steps via argonaute (AGO) proteins produce a single, approximately 22 nucleotide strand (either -3p or -5p) that is loaded into the RNA-induced silencing complex (RISC) [145]. The unloaded strand was believed to be degraded, but studies have shown that, in some cases, both -3p and -5p miRNAs bind to various cellular targets [158]. The approximately 8 nucleotide, highly conserved seed sequence of the miRNA binds the target mRNA, ultimately leading to degradation or translational inhibition of the transcript. Originally thought to bind solely to the 3’ end of the mRNA strand, miRNAs are now known to also bind within the 5’ end and/or coding regions of the target [160, 161]. Furthermore, in some cases miRNAs have been found to promote translation of targets by binding to elements in the 5’ untranslated region (UTR) of the mRNA, which adds further complexity to miRNA-mediated regulation [162, 163].

### 1.2.2 miRNA regulation of type 2 immunity

Although the study of miRNAs in asthma and allergic inflammation is a relatively young field, it is clear that miRNAs regulate key mechanisms that contribute to the immunopathology of these diseases. For instance, a study of miRNAs in airway epithelium found lower levels of miR-34/449 family miRNAs in asthmatic compared to healthy cells [164]. Interestingly, IL-13 stimulation of healthy epithelial cells induced mucus metaplasia and downregulated miR-34/449, recapitulating the phenotype of asthmatic epithelial cells. Furthermore, several miRNAs have been implicated in eosinophil development [165-168]. One such miRNA that is upregulated during eosinophil differentiation is miR-223 [168]. miR-223 has been found to restrain expansion of EoPs by targeting the insulin-like growth factor 1 receptor, thereby blocking a pathway that stimulates cell proliferation and inhibits apoptosis [168]. miRNAs have also been proposed to regulate macrophage polarization [169], where the expression of miR-124, -342-3p, -378-3p and -511 have been demonstrated to increase following IL-4 and/or IL-13 stimulation [170-173]. Interestingly, miR-124 in murine lung macrophages has also been reported to be induced during allergic inflammation [171]. Furthermore, upregulation of surface markers that are characteristic for the M2 phenotype (CD206, Ym1) and downregulation of characteristic M1 surface markers (CD86, iNOS, TNF) was blocked by a miR-124 inhibitor, suggesting that miR-124 contributes to the control of the M1/M2 balance in macrophages [171].

Studies of CD4+ T cell-specific miRNA expression patterns have identified critical mechanisms in T cell lineage commitment and effector function [174] and deletion of essential components of the miRNA biosynthesis pathway in
CD4+ T cells have a large impact on their functionality [175]. Indeed, CD4+ T cells lacking all miRNAs demonstrate impaired proliferation and are prone to apoptosis, but also become hypersensitive to signals that induce effector T cell differentiation [175-178], suggesting that miRNAs are important in the maintenance of naïve CD4+ T cells.

One of the first studies describing functionality of a miRNA in human asthma pathogenesis identified miR-19a as a promoter of type 2 cytokine production in airway-infiltrating CD4+ T cells [153]. The levels of miR-19a were significantly higher in CD4+ T cells in BAL recovered from asthmatic individuals compared to healthy controls. Furthermore, miR-19a was shown to promote IL-13 production by targeting of the inositol phosphatase PTEN, the signaling inhibitor SOCS1 and tumor necrosis factor alpha-induced protein 3 (TNFAIP3) which encodes A20 [153]. Previous studies of the miR-17–92 cluster, to which miR-19a belongs, have shown that it promotes CD4+ T cell survival and proliferation [179]. However, Simpson et al suggested that upregulation of miR-19a in human asthma could be an indicator and a cause of increased type 2 cytokine production in asthmatic airways.

Several miRNAs have been demonstrated to suppress TH2 activity in vivo [180-182]. For instance, the miR-23–27–24 cluster was recently shown to regulate TH2 cells in mice where two independent reports identified that miR-24 and miR-27 inhibited TH2 differentiation and IL-4 production [181, 182]. miR-27 was shown to repress GATA-3, IKAROS Family Zinc Finger 1 (IKZF1) and Nuclear Factor of Activated T-cells 2 (NFATC2), all of which are upstream mediators of IL-4, while miR-24 directly targeted the 3’UTR of IL-4. Notably, as miR-24 and miR-27 were found to limit IL-4 production in CD4+ T cells, the deletion of these miRNAs promoted TH2 dependent responses in vivo [182].

ILC2s are the most recent addition to the list of type 2 effector cells in which miRNA regulation has been described. Analysis of miRNA profiles of ILC2s and TH2 cells isolated from murine lungs revealed a significant overlap in miRNA expression [183]. Increased levels of miR-21a, miR-98 and miR-155, and decreased levels of let-7c, miR-151 and miR-203 were found in both ILC2s and TH2 cells following in vitro activation. However, a few miRNAs were differentially expressed in ILC2s compared to CD4+ T cells, this list included miR-126a, miR-134, miR-409 and miR-541. Importantly, generation of a mouse model with a selective ILC2-deficiency for the cellular component DGCR8, enabled the study of miRNA deficient ILC2s which revealed that miRNAs are essential for ILC2 homeostasis [183]. Specifically, the miR-17–92 cluster had important functional implications in ILC2s, as cells lacking this family of miRNAs displayed reduced cytokine production and impaired expansion in allergen-induced lung inflammation [183]. The miR-17–92 cluster family member miR-19a was found to negatively regulate A20 and
SOCS1 in ILC2s. These genes were previously reported to repress IL-5 and IL-13 production [153, 184, 185]. Of note, miR-19a regulation of IL-13 production in ILC2s and Th2 cells is mediated via common targets, and the authors suggested that miR-19a might be an attractive therapeutic target for modulation of type 2 inflammation [183].

In summary, increasing evidence supports that individual miRNAs have distinct roles in immune cell functions that are critical to the immunopathology of asthma.

1.2.3 miR-155

miR-155 is likely the most studied miRNA in any mammalian cell and it is highly conserved across species [186]. Well before the discovery of miRNAs, the primary transcript of miR-155, B cell integration cluster (BIC) gene, was identified as a noncoding RNA proto-oncogene in chickens [187, 188]. BIC/miR-155 was later shown to be highly expressed in a variety of human B cell lymphomas [189-191], and miR-155 transgenic mice were shown to develop B cell malignancies [192]. Furthermore, antibody production by B cells and interactions with CD4+ T cells in germinal centers are controlled by miR-155 and play important roles in immune responses in allergy and asthma [193-195]. Analysis of CD4+ T cell functions in the absence of miR-155 revealed normal cell proliferation upon TCR-stimulation (anti-CD3/CD28), but a tendency toward spontaneous Th2 differentiation under neutral conditions in vitro [195, 196]. Additionally, overexpression of miR-155 in CD4+ T cells promote Th1 differentiation in vitro [197], suggesting that miR-155 regulates Th1/Th2 balance.

In contrast to these findings, the first study to investigate miR-155 in allergen-induced airway inflammation found that miR-155 deficient mice displayed diminished Th2 responses and were protected from eosinophilic inflammation [198]. miR-155 was significantly upregulated in the lung tissue but not in peripheral sites, of ovalbumin (OVA)-challenged wild type (WT) mice, and the expression of the transcription factor PU.1 was elevated in lung draining lymph nodes of miR-155 deficient mice. PU.1 is a direct target of miR-155 and suggested to negatively regulate GATA-3 by interfering with its DNA binding activity. Thus, elevated PU.1 in the airways was proposed to contribute to the suppressed Th2 effector functions in miR-155 deficient mice [199]. Other in vivo investigations support that miR-155 is required in Th2 responses, and it was demonstrated in HDM-induced allergic inflammation and helminth infection [180]. This was mediated partially through miR-155 regulation of Sphingosine-1-phosphate receptor 1 (S1pr1), where inhibition of S1pr1 in adoptively transferred CD4+ T cells increased airway eosinophilia, goblet cell hyperplasia and mucus hypersecretion in allergen challenged
recipient mice [180]. The same study identified that miR-146a regulated \( T_{H2} \) responses \textit{in vivo}, where HDM challenge or helminth infection of mice with miR-146a deficient CD4\(^+\) T cells responded with increased airway inflammation. Of note, deletion of miR-146a led to elevated neutrophils which was explained by a mixed \( T_{H1}/T_{H2}/T_{H17} \) response, suggesting that miR-146a regulates CD4\(^+\) T cell differentiation. Thus, the authors proposed that miR-155, but not miR-146a, is a potential therapeutic target of \( T_{H2} \)-mediated inflammation [180]. Importantly, later attempts to suppress allergic airway inflammation using specific antagonists to miR-155 failed to alter airway inflammation, possibly highlighting the importance of the cell-specificity in uptake of miRNA inhibitors [200]. In human studies, decreased levels of miR-155 were measured in exhaled breath condensates from asthmatic subjects compared to healthy controls [201]. Altered expression in the airways indicate that miR-155 is involved in local immune responses, however, further studies of human asthma are required to explain the reduction of miR-155.

Studies of miR-155 in various immune cells have identified several interesting mechanisms. For instance, IL-13 receptor (IL13R\(\alpha1\)) expression in macrophages is a direct target of miR-155, and it was found to suppress STAT6-dependent M2 polarization [202]. Increased miR-155 expression in macrophages was also found to favor the M1 phenotype by targeting negative regulators of M1 polarization [203-205]. Furthermore, miR-155 deficient mast cells demonstrated enhanced degranulation and cytokine release (TNF-\(\alpha\), IL-6 and IL-13) upon IgE-mediated stimulation [206]. In dendritic cells, the expression of the high affinity IgE receptor, FceRI, was recently found to be downregulated by miR-155 in response to toll-like receptor signaling via the transcription factor PU.1 [207].

To conclude, miR-155 is a powerful miRNA with a wide distribution that enables control over multiple mechanisms of critical importance in type 2 immunity. Studies of miR-155 in different immunological settings suggest both pro-inflammatory and immunosuppressive functions, which demonstrates the complexity and context-dependence of miRNA regulation.
Chapter 2  Aim

2  Aim

The aim of this thesis was to identify immunological mechanisms that control inflammatory responses in allergy and asthma. More specifically, pro-inflammatory functions of ILC2s and miRNA-mediated immune regulation were studied using clinical samples from asthmatic subjects and experimental models of allergic inflammation. The specific aims of these studies are presented below.

Paper I

- Determine if miR-155 regulates ILC2 functions under allergic airway inflammation in mice
- Determine if miR-155 is required for development of chronic allergic airway inflammation in mice

Paper II

- Determine if miR-155 and miR-146a are differentially expressed in allergic asthmatic subjects compared to healthy controls

Paper III

- Determine if ILC2s, $T_H$ cells and $CD34^+$ progenitors in murine bone marrow produce IL-5 in response to IL-33 challenge
- Determine if IL-33-driven eosinophilia is dependent on IL-5

Paper IV

- Determine if there are differences in miRNA expression of primary airway macrophages from asthmatic and healthy subjects
3 Methods

The following chapter describes methods and experimental strategies that were employed in this thesis, highlighting some of their strengths and weaknesses. Detailed information about the procedures are provided in the Materials and methods section in indicated papers.

3.1 Mouse models

Mouse models are widely used in asthma and allergy research. With a great availability of specific reagents and genetically modified mice, it is a powerful tool in studies of cellular and molecular responses. Mice recapitulate several pathological features that are crucial in asthma, including airway inflammation, hyperresponsiveness and remodeling [208, 209], but due to fundamental differences in lung anatomy between humans and mice, mouse models can never provide a true representation of the asthmatic disease [209]. Another important aspect is the inability to develop spontaneous respiratory allergy in mice, and in order to study allergic responses they have to be induced by external measures. Mouse models that were used in this thesis are described below. All animal handling and experimentation was approved by the Gothenburg County Regional Ethical Committee.

3.1.1 Antigen-induced inflammation (allergic)

Traditional protocols of allergic inflammation typically use systemic sensitization with the model-antigen OVA together with an adjuvant, and asthma-like airway inflammation is induced by repeated inhalations of OVA. This procedure was employed in Paper I to study OVA-induced airway inflammation in WT and miR-155 knockout (KO) mice; acute airway challenge (Fig. 3.1 A) and prolonged chronic challenge (Fig. 3.1 B) were performed. In ongoing investigations (presented in Results and discussion), we are using the natural aeroallergen house dust mite (HDM) to induce allergic airway inflammation in WT mice (Fig. 3.1 C). In contrast to the OVA models, in Paper I, HDM-induced airway inflammation does not require administration of adjuvants and utilizes a physiological route of sensitization. Indeed, the HDM-model provides a good system to study respiratory allergies since peripheral sensitization models do not mimic how immune cells encounter allergens in human asthma.
Figure 3.1. Mouse models used in this thesis. Acute (A) and chronic (B) OVA-induced airway inflammation (Paper I). C) HDM-induced allergic airway inflammation (preliminary results) and D) IL-33-induced airway inflammation (Paper I, Paper III and preliminary results). I.n., Intranasal; i.p., intraperitoneal; rIL-33, recombinant IL-33.
3.1.2 IL-33-induced inflammation (non-allergic)

Direct administration of recombinant IL-33 to naïve mice has proved powerful in the induction of airway eosinophilia without allergic sensitization [210]. A model of IL-33-induced inflammation (Fig. 3.1 D) was employed in Paper I (WT and miR-155 KO mice), Paper III (WT mice) and in ongoing studies (WT and RAG1 KO mice) presented in Results and discussion. In some experiments (in Paper III), anti-IL-5 and anti-CCL24 antibodies were co-administered with IL-33 to evaluate their involvement in the eosinophilic response.

3.2 Human subjects

Study participants in Paper II and IV were recruited from the West Sweden Asthma Study cohort. Written informed consent was obtained from all subjects included and ethical approval was issued from the Gothenburg County Regional Ethical Committee. The cohort was initiated at Krefting Research Centre in 2008 as a large-scale population-based study focusing on asthma and allergic diseases in west Sweden. Detailed description of the study material is provided in [4], [8] and [211]. Briefly, a postal questionnaire was sent to 30,000 individuals living in the area, aged 16-75 years. Out of 18,087 responders, 2,006 randomly selected subjects and additional subjects reporting ever having asthma or physician-diagnosed asthma underwent clinical phenotyping. This included interviews, lung function tests (FEV₁ and FVC) before and after bronchodilation, test of methacholine responsiveness, skin prick test, FENO measurement and blood (serum and plasma) collection.

Glossary

FEV₁ and FVC are measurements of lung function. FEV₁: The volume of air which can be forcibly exhaled in one second after maximal inhalation. FVC: The total volume of air which can be forcibly exhaled from the lungs after maximal inhalation.

Skin prick test: Assessment of hypersensitivity to allergens by placing a small amount of allergen on the surface of skin, which is penetrated by a needle. A positive reaction to the test occurs when a wheal rises at the punctured site, >3mm in average diameter or the size of, or bigger than, the positive histamine control.

FENO: Assessment of airway inflammation by measurement of nitric oxide in exhaled breath. Nitric oxide is produced by various inflammatory cells.

Paper II included allergic asthmatics and non-allergic healthy control subjects. All were non-smokers over 18 years of age. Asthma was defined by clinical history and positive methacholine challenge: >20% decrease in FEV₁ to <1.94
mg cumulative dose. Allergy was defined by elevated serum IgE and at least one positive skin prick test in a panel consisting of 11 of the most common inhalant allergens. Allergic asthmatics recruited in and out of the pollen season had at least one positive skin prick test against birch or timothy. Reported current use of inhaled corticosteroids (ICS) was mixed in the asthma group, and represents a limitation of the study material since ICS therapy may alter airway inflammation [212].

A strength of **Paper IV** is the clinically well-defined study material. In this study, asthmatic subjects and healthy controls were invited for re-phenotyping which included interviews, physiological tests and blood sampling as described above. All subjects were under the age of 75 years, non-smokers (including ex-smokers for >5 years, <10 pack years) with no autoimmune disease or cancer. Asthmatic subjects had physician-diagnosed asthma, defined by clinical history, reversibility (FEV₁>15%) and positive methacholine challenge (defined above). All asthmatics reported current ICS use. Allergic status was assessed by skin prick test. Age- and sex-matched healthy controls who did not report asthma symptoms were recruited. They were non-reactive to methacholine or non-reversible. Asthmatic and healthy subjects fulfilling inclusion criteria were invited to undergo bronchoscopy within <4 weeks. Individuals who met the criteria but did not want to undergo bronchoscopy were included in the study as “blood-only” subjects. The day of the bronchoscopy additional samples were collected to screen for signs of current inflammation including C-reactive protein (CRP) levels and leukocyte numbers (differential cell count) in peripheral blood and respiratory virus infection by nasal swab.

### 3.2.1 Bronchial lavage

Analysis of BAL fluid has improved our understanding of asthma pathogenesis [213]. However, bronchoscopy is an invasive procedure that requires safety precautions and expert resources which limit the use of the technique in basic research. The study in **Paper IV** was performed in collaboration with researchers at AstraZeneca Gothenburg and clinical researchers at the Lung diagnostic unit at Sahlgrenska University Hospital. Bronchoscopy with bronchial lavage (BL) was collected from 20 asthmatic subject and 10 healthy controls. Usually, instillation of ~60 ml PBS is called BL and instillation of larger volume ~100-300 ml is called BAL. In our study, BL was carried out by flushing 20 ml of sterile pyrogen free PBS (37°C) into the segmental bronchus. The fluid was immediately retrieved and stored on ice until further processing. The procedure was repeated two times. Study participants that demonstrated high responsiveness to methacholine defined by >20% decrease in FEV₁ to <0.53 mg cumulative dose were not considered for bronchoscopy.
3.2.2 Induced sputum

In contrast to BL, induced sputum is a non-invasive method for collection of airway fluid. Although, it is important to note that previous analysis of induced sputum samples and samples collected under bronchoscopy have revealed differences in their cellular composition [214, 215]. Induced sputum had a higher frequency of eosinophils and neutrophils, and lower frequency of lymphocytes and macrophages compared to bronchial washing (instillation of 60 ml PBS) and BAL (instillation of 180 ml PBS) [214].

Induced sputum samples were collected in Paper II in accordance with the European Respiratory Society guidelines with minor modifications [216]. Pre-bronchodilator and post-bronchodilator FEV1 were measured to determine that induced sputum could be performed safely. Induced sputum was not performed if FEV1 was lower than 1.5l or less than 50% of the predicated value. The procedure was done by inhalation of 4% sterile PBS for 7 min and was stopped if symptoms of obstruction occurred and the participant received bronchodilator if needed. Specimens were obtained from coughing and spitting followed by FEV1 measurement. If FEV1 decreased >20% compared to post-bronchodilator value the test was stopped and the participant received bronchodilator if needed. If FEV1 was <20% compared to post-bronchodilator value the procedure was repeated. The induced sputum sample was collected in a petri dish and immediately placed on ice and processed shortly after. Sputum plugs were collected, weighted and mixed with 4x volume plug weight of sputolysin (containing reducing agent DTT). The sample was incubated for 15 min at room temperature with gentle mixing. An equal volume PBS was added to the homogenized sputum solution and passed through a cell strainer (70 uM). Cells were separated by gentle centrifugation and were directly sorted on a FACS Aria Flow cytometer into lymphocyte and monocyte fractions (Paper II). Cell-free sputum supernatants were stored for RNA isolation and analysis of inflammatory mediators (Paper II).

3.3 Cell analysis

3.3.1 Differential cell count of cytospin preparations

Differential cell count analysis was performed on contrast-stained cytospin preparations in Paper I-IV. It is a fast and cost-effective method able to provide a lot of information of inflammatory states. In Paper I and Paper III, we quantified eosinophils, neutrophils, lymphocytes, monocytes and macrophages based on cell morphology in various mouse samples; BAL fluid, bone marrow, single cell-suspensions of lung and peripheral blood. Human eosinophils were quantified in induced sputum samples in Paper II, and eosinophils, neutrophils,
lymphocytes, monocytes and macrophages were enumerated in BL samples in Paper IV. In addition, purity of FACS sorted cells (Paper I, II, III) and macrophages enriched by adherence (Paper IV) were controlled on cytopsin preparations. Detailed information of cellular phenotypes were assessed by flow cytometry, described below.

### 3.3.2 Lung histology

Histological staining was performed on lung sections to evaluate OVA-induced chronic inflammation in Paper I. Hematoxylin and eosin (H&E) was used to visualize infiltration of inflammatory cells and Masson’s trichrome stain was used to assess collagen accumulation. It is important to note that analysis of intact tissues can provide information that might be lost by tissue digestion. For instance, eosinophil numbers were severely reduced in single-cell suspensions of lungs from WT mice following chronic OVA exposure in Paper I. However, increased numbers of eosinophils were quantified in BAL samples from these mice, but a large number of lysed eosinophils were also detected in cytopsin preparations from BAL fluid. Thus, we suspect that eosinophils were highly activated and required sensitive handling in the chronic model.

### 3.3.3 Flow cytometry

Cell analysis by flow cytometry was performed in lung tissue (Paper I) and bone marrow ex vivo and in vitro (Paper III) under inflammatory and homeostatic conditions. Single-cell suspensions were stained with fluorochrome-conjugated antibodies and analyzed on a BD FACSARia™ or BD FACSVerse™ flow cytometer.

The abundance of ILC2s is low in most tissues and the absence of a lineage-specific surface marker made them go unnoticed for many years [217]. Today, we know that ILC2s are a distinct lymphocyte lineage [218] where flow cytometry is the most important tool in their analysis since it requires a combination of several markers. To avoid contamination of other immune cells, an antibody cocktail directed against common lineage markers of lymphocytes (T cells, B cells, NK cells), granulocytes (eosinophils, neutrophils, basophils) and other myeloid cells (dendritic cells, monocytes and macrophages) is included in the analysis. Thus, ILC2s are identified as lineage negative (Lin−) cells that express different cytokine receptors such as CD127 (IL7Rα), CD25 (IL2Rα), ST2 (IL33R), IL-17BR, and other receptors such as Sca-1, ICOS and KLRG1 [218]. ILC2s, CD4+ T cells and eosinophils of various maturation stage were described using cellular markers listed in Tab.
3.1. In the analysis of eosinophils, cellular distribution on side scatter (SSC) was an important factor since it separates highly granulated mature cells (SSC$^{hi}$) from immature granulocytes (SSC$^{int}$) or non-granular cells (SSC$^{lo}$). Cellular markers that were used for cell sorting by flow cytometry is provided in Tab. 3.2.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Cell type</th>
<th>Tissue (mouse)</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/III</td>
<td>ILC2</td>
<td>Lung/Bone marrow</td>
<td>Lin−(CD3−CD45R/B220−CD11b−TER-119−Ly-G6/Gr-1−CD19−NK1.1−FcεRI−) SSC$^{lo}$CD45+CD127+CD25+</td>
</tr>
<tr>
<td>I/III</td>
<td>T$^\text{H}$ cell</td>
<td>Lung/Bone marrow</td>
<td>SSC$^{lo}$CD45+CD3$^+$CD4$^+$</td>
</tr>
<tr>
<td>III</td>
<td>Progenitor</td>
<td>Bone marrow</td>
<td>SSC$^{lo}$CD45+CD34$^+$</td>
</tr>
<tr>
<td>III</td>
<td>Immature Eosinophil</td>
<td>Bone marrow</td>
<td>SSC$^{int}$CD45+CD34$^+$IL5R$\alpha$$^{hi}$ CCR3$^{neg/lo}$</td>
</tr>
<tr>
<td>III</td>
<td>Mature Eosinophil</td>
<td>Bone marrow</td>
<td>SSC$^{hi}$CD45+CD34$^+$IL5R$\alpha$$^{lo}$ CCR3$^{hi}$</td>
</tr>
</tbody>
</table>

Table 3.1. Antibody panels for cellular detection by flow cytometry.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Cell type</th>
<th>Tissue</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/III</td>
<td>ILC2</td>
<td>Lung/Bone marrow (mouse)</td>
<td>Lin−(CD3−CD45R/B220−CD11b−TER-119−Ly-G6/Gr-1−CD19−NK1.1−FcεRI−) SSC$^{lo}$CD45+ICOS$^+$CD25$^+$ST2$^+$</td>
</tr>
<tr>
<td>II</td>
<td>Lymphocyte</td>
<td>Induced sputum (human)</td>
<td>SSC$^{lo}$CD45$^+$</td>
</tr>
<tr>
<td>II</td>
<td>Monocyte</td>
<td>Induced sputum (human)</td>
<td>SSC$^{int}$CD45$^+$</td>
</tr>
</tbody>
</table>

Table 3.2. Antibody panels for cellular sorting by flow cytometry.

3.4 Inflammatory mediators

Enzyme-linked immunosorbent assay (ELISA) was used to quantify proteins in mouse samples (BAL, lung tissue homogenate, serum, and culture supernatants) in Paper I and Paper III. In Paper II, cytokines in cell-free culture supernatants from peripheral blood mononuclear cells (PBMC) were analyzed by ELISA or Cytometric Bead Array (CBA). Cytokine expression in specific cells were measured by intracellular flow cytometry. In these experiments BD
GolgiStop™ was added to inhibit cytokine release from the cells, however, this substance is highly toxic and was added 3h before harvest of in vitro stimulated cultures in Paper III. In addition, ex vivo measurements of intracellular cytokines were performed on lung and bone marrow cells in Paper I and Paper III, respectively. In those experiments, tissues/cells were processed in solutions supplemented with GolgiStop™ to enhance detectability of cytokine-producing cells.

Inflammatory mediators in cell-free culture supernatants from poly(I:C)-stimulated BL macrophages, related to Paper IV (preliminary data), were measured by multiplex immunoassay at Olink Proteomics (Uppsala, Sweden). A panel for detection of 92 inflammatory proteins related to various diseases and cellular processes was used. BL macrophages were rested for 48h before poly(I:C) stimulation based on a previous study of primary airway macrophages which reported a more homogenous cytokine response in rested cells [219].

### 3.5 miRNA analysis

Total RNA, including small miRNAs, was isolated from whole lung tissue and sorted lung ILC2s in Paper I, PBMCs and sputum lymphocytes/monocytes in Paper II, BL macrophages in Paper IV, and IL-33 challenged lungs (preliminary results). RNA was also isolated from cell-free PBMC and sputum supernatants in Paper II. miRNA expression profiles in BL macrophages and murine lung tissue were analyzed by microarray at TATAA Biocenter (Gothenburg, Sweden). Quantitative real-time PCR (qPCR) was used for analysis of miR-155 expression in lung tissue and sorted lung ILC2s in Paper I and for validation of miRNA expression in macrophages in Paper IV. In preliminary studies, miR-155 expression was analyzed in PBMCs by PrimeFlow™ technology, described in [220].

### 3.6 Statistical analysis

All statistical analysis in Paper I-IV and preliminary data presented were performed using GraphPad Prism software. Non-parametric Mann Whitney’s test or Student’s t-test were used in analysis between two independent groups and parametric Student’s t-test was used in analysis of before and after treatments. P-values <0.05 were considered statistically significant.
4 Results and discussion

The main findings of Paper I-IV are summarized in the following chapter. Unpublished data of relevance to the presented studies are also included.

4.1 miR-155 regulates ILC2s

Over the last decade, the importance of ILC2s in type 2 inflammation was extensively studied, however, miRNA-mediated regulation of ILC2s remains completely unexplored. Previous research identified critical roles of miR-155 in lymphocyte function and Th2 immunity [175, 180, 196, 198], thus, the aim of Paper I was to elucidate the contribution of miR-155 in ILC2 responses during allergic airway inflammation. To investigate this, we used miR-155 deficient mice and wild type (WT) mice that were subjected to OVA-induced airway inflammation. Frequencies of ILC2s (Lin−CD45+ CD127+CD25+) in the lungs of OVA-challenged and PBS treated control mice were analyzed by flow cytometry. The number of ILC2s increased in both WT and miR-155 deficient mice as a result of acute OVA exposure. Notably, ILC2 numbers were consistently lower in miR-155 deficient mice compared to WT, and analysis of naïve lungs revealed reduced ILC2s at baseline in miR-155 deficient mice. This might suggest that miR-155 is involved in development and/or maintenance of ILC2s at homeostasis. Interestingly, chronic OVA exposure revealed a severe defect in ILC2 expansion in miR-155 knockout animals, where a barely detectable increase was measured in the lungs. Chronic

![Figure 4.1. A) Airway exposure to IL-33 increases eosinophils (stained pink) in WT but not miR-155 deficient mice. B) miR-155 deficient mice are unable to expand lung ILC2s in response to IL-33. C) Lower frequency of IL-13+ lung ILC2s in miR-155 deficient mice compared to WT mice. [Results from Paper I]
inflammation was established by prolonged airway exposure to OVA for 12 weeks. The level of eosinophilia generated by chronic exposure was lower than the response to acute exposure, but eosinophils in chronically inflamed lungs displayed signs of an activated phenotype and were prone to lyse during tissue processing. For instance, we observed increased ST2 expression on SSC\textsuperscript{hi} cells, a fraction which includes mature eosinophils (data not shown). This might indicate enhanced eosinophil activation by IL-33 in this model of chronic airway inflammation [98, 221]. Indeed, the induction of ILC2s in WT lungs after chronic (and acute) OVA exposure was accompanied by elevated levels of the ILC2-activating cytokine IL-33. In contrast, IL-33 in lungs of miR-155 deficient mice remained at basal levels following acute or chronic OVA exposure. Defective ILC2 responses were previously demonstrated in ST2 or IL-33 deficient mice [29, 31, 222, 223]. Thus, to investigate if impaired IL-33 induction in miR-155 deficient mice was contributing to the inability to mediate ILC2 expansion, we administered IL-33 directly to the airways of naïve mice. This model allowed us to analyze the response in a more ILC2-dependent system, as compared to the antigen-driven OVA models that are highly dependent on CD4\textsuperscript{+} T cells, which are affected by miR-155 deficiency [198]. Interestingly, IL-33-challenged WT mice, but not miR-155 deficient mice, developed eosinophilic inflammation (Fig. 4.1 A) with increased levels of ILC2s in the lung tissue (Fig. 4.1 B). Additionally, miR-155 deficient ILC2s demonstrated reduced proliferation and IL-13 production (Fig. 4.1 C) compared to WT ILC2s. Analysis of ST2 on ILC2s from both mouse strains demonstrated similar expression, suggesting that they were equally susceptible to IL-33 stimulation. This might indicate that defect expansion and cytokine production by ILC2s in response to IL-33 are due to a cell-intrinsic effect of miR-155. To address this question, we sorted ILC2s from WT lungs (Fig. 4.2 A) to analyze miR-155 expression in the cells. We found that IL-33 stimulation resulted in a 10-fold increase of miR-155 in WT ILC2s \textit{in vitro} (Fig. 4.2 B) which coincided with increased receptor expression. Our study in \textit{Paper I} for the first time identifies a miRNA that regulates ILC2s functions. Expression of miR-155 in mouse ILC2s was later confirmed by Singh \textit{et al} [183], however, direct targets of miR-155 have not yet been identified in ILC2s.
4.1.1 miR-155 is required in chronic inflammation

Early studies of miR-155 regulation in CD4+ T cells found that naïve cells were naturally skewed towards a T\(_{H2}\) phenotype in lack of miR-155 [175, 196]. Rodríguez et al, investigated miR-155 deficiency \textit{in vivo} and reported that mice developed lung fibrosis with increasing age (320-350 days at termination), suggesting that miR-155 deficiency aggravates type 2 immune responses of the lung [196]. In contrast, Malmhäll et al found that miR-155 deficient mice were protected from type 2 inflammation and displayed severely impaired T\(_{H2}\) responses locally in the airways during acute allergen-induced inflammation [198]. These findings encouraged us to investigate the requirement of miR-155 in lung immune responses over a longer time period.

In \textit{Paper I}, we set up a model of chronic airway inflammation to evaluate if miR-155 deficient mice demonstrated enhanced or attenuated type 2 responses to prolonged OVA exposure. WT mice developed eosinophilic inflammation and increased expression of miR-155 was measured in whole lung tissue in response to chronic OVA challenge. However, miR-155 deficient mice demonstrated no increase of airway eosinophils. Furthermore, similar to Rodríguez et al, we analyzed accumulation of collagen in the lung tissue by histological staining. We found reduced collagen deposition in miR-155 deficient mice compared to WT mice. Thus, our findings were contrasting the data presented by Rodríguez et al, as no signs of lung fibrosis were found in miR-155 deficient control mice after 12 weeks of PBS exposure, or in naïve 1 year old miR-155 deficient mice (data not shown). Possible differences in the microbial environment may have influenced the outcome of the two studies. Notably, miR-155 has been reported to be involved in responses to bacteria. For instance, LPS-stimulation of macrophages demonstrated increased miR-155 expression [224, 225], and miR-155 deficiency promoted polarization of M2 macrophages [202, 226-228], a phenotype which may contribute to fibrotic responses of the lung [144]. However, the actual reason for this discrepancy, including the involvement of macrophages, has not been addressed.

As previously mentioned, OVA-induced airway inflammation is largely dependent on CD4+ T cells, however, chronic OVA exposure also proved to be a powerful inducer of ILC2s. Studies have found that ILC2s contribute to tissue remodeling by producing amphiregulin, however this was not investigated in our study [229, 230]. Furthermore, the relative contribution of CD4+ T cells and ILC2s was not determined, but our analysis showed no increase in CD25+ CD4+ T cell activation in the lungs after chronic OVA exposure. Our results were similar to Christianson et al who identified that ILC2s, not CD4+ T cells, mediated persistence of chronic inflammation in mice [39]. They identified an important role of IL-33 in this mechanism, which is interesting in chronic inflammation since IL-33 promotes fibrosis [50, 231].
4.1.2 miR-155 is expressed in human blood ILC2s

Ongoing investigations in the group suggest that miR-155 is expressed in human ILC2s isolated from peripheral blood of asthmatic and healthy subjects. A novel approach was applied in these studies and miR-155 expression was analyzed by PrimeFlow™ [220], a method combining *in situ* hybridization technology with flow cytometry. A strong advantage of this strategy is that miRNA expression is visualized in single-cell resolution in a population-context, instead of average level in bulk tissue or sorted cells. Additionally, cells must not be sorted prior to the analysis and miRNA expression can be studied in relation to specific receptor expression, enabling detailed analysis of cellular phenotypes or activation status. Our preliminary findings demonstrate that miR-155 is expressed in human blood ILC2s defined as Lin−CD4−CD127+CD25+ST2+ and/or CRTH2+. *In vitro* stimulation of PBMCs with IL-33 increased ST2 expression on ILC2s, which was further enhanced by addition of the CRTH2 ligand prostaglandin G2 (PDG2). Interestingly, PrimeFlow™ analysis demonstrated that upregulation of ST2 was enriched in the miR-155+ ILC fraction as compared to all ILCs (Fig. 4.3). Continued studies aim to address cytokine production and activation of ILC2s in relation to miR-155 expression in samples from asthmatic and healthy control subjects.

![Figure 4.3](image)

*Figure 4.3. PrimeFlow™ analysis of human blood ILC2s demonstrates that miR-155+ ILCs upregulate ST2 expression upon IL-33/PDG2 stimulation in vitro.* [Malmhäll et al. unpublished data]

4.1.3 miR-155 expression in IL-33-challenged lungs

Analysis of miRNA expression in IL-33-challenged mouse lungs by microarray support our finding that miR-155 controls IL-33-driven responses. miR-155 was identified as one of the top miRNAs that were upregulated in the lungs of challenged mice compared to PBS treated control mice (Fig. 4.4). Another interesting miRNA candidate that increased in response to IL-33 was miR-290a-5p, which was previously identified to target the ST2 gene *Il1rl1* by next generation sequencing from mouse embryonic stem cells [232]. One might speculate that miR-290a-5p negatively regulates IL-33 signaling by decreasing ST2 receptor expression on target cells. However, further study of
miR-290a-5p is required, including validation of expression levels by alternative methods, such as qPCR, as well as studies of specific cells. Interestingly, microarray analysis of miRNAs in the bone marrow of IL-33 or PBS treated mice did not detect miR-155 expression. This might suggest that IL-33-induced responses locally in the bone marrow are miR-155-independent. Indeed, miR-155 deficient mice demonstrate normal induction of eosinophils in the bone marrow in OVA-induced airway inflammation [198] and through direct IL-33 administration in vivo (unpublished data). In addition, bone marrow-derived ILC2s from miR-155 deficient mice and WT mice produce similar levels of IL-5 (Fig. 4.5 A) and IL-13 (Fig. 4.5 B) upon IL-33 stimulation in vitro. Tissue-specific regulation by miR-155 may have important implications in future studies of ILC2s and other tissue-resident immune cells, especially in experimental strategies involving myeloablation and reconstitution with donor bone marrow.

Figure 4.4. Microarray analysis of miRNA expression in whole lungs from IL-33-challenged mice compared to PBS treated controls (n=5/group). [Johansson et al unpublished data]

Figure 4.5. IL-5 (A) and IL-13 (B) production by WT and miR-155 deficient bone marrow ILC2s are similarly induced by IL-33 in vitro (100 ng/ml IL-33, 3h). [Johansson et al unpublished data]
4.2 miR-155 is altered in asthmatic airways

Previous research have linked miR-146a and miR-155 to human asthma [201, 233], but so far, only a limited number of studies have described miRNAs in human asthmatic airways. Therefore, the objective of Paper II was to analyze the expression of miR-146a and miR-155 in asthmatic and healthy airways. Induced sputum and blood were collected from pollen allergic asthmatic subjects and non-allergic healthy controls, recruited from the West Sweden Asthma Study cohort. The asthmatic group displayed elevated levels of eosinophils in sputum and blood. However, in vitro stimulation of PBMCs by anti-CD3/CD28 revealed few or no differences between the groups. The stimulation altered miR-146a and miR-155 expression levels in PBMC cultures in the same manner in asthma and healthy, and analysis of secreted cytokines only found IL-4 to be elevated in the asthmatic group upon TCR stimulation compared to healthy controls. In future studies, it would be interesting to investigate miRNA expression in PBMCs in response to specific TCR stimulation by allergens. Importantly, in Paper II, miRNA expression in allergic asthmatic subjects was altered by exposure to naturally occurring pollen during the spring. Indeed, increased numbers of eosinophils in induced sputum collected during pollen season (Fig. 4.6 A, B) was accompanied by altered expression of miR-155, but not miR-146a, in FACS sorted sputum lymphocytes (Fig. 4.6 C). Differences in miR-155 expression was restricted to lymphocytes as the level in monocytes remained unchanged. Furthermore, miR-155 expression in lymphocytes in induced sputum collected post season demonstrated same levels as in healthy controls (data not shown), suggesting

Figure 4.6. A) Contrast-stained sputum eosinophil. B) Eosinophils in induced sputum are elevated in allergic asthmatic subjects during pollen season. C) miR-155 expression in sputum lymphocytes from allergic asthmatic individuals is lower during pollen season compared to post season. [Results from Paper II]
that miR-155 expression in allergic asthmatics during pollen season was abnormally low. Analysis of selected samples showed that the majority of sputum lymphocytes were CD3+ T cells (data not shown), however, distinct subgroups of cells were not determined. Further studies are needed to describe how the cellular composition varies in induced sputum of allergic asthmatic subjects and healthy controls throughout the year. Additionally, continued analysis should focus on miR-155 and miR-146a expression in specific cell types. Those studies would reveal if altered miR-155 expression is due to variations of the cellular composition in the sputum sample under pollen season or if activation status of one cell type results in altered expression that is measured in the lymphocyte fraction. In summary, the observed differences in miR-155 expression cannot be explained by these data, but altered levels might suggest that miR-155 plays a role in airway immunity to allergens in asthmatic individuals.

4.3 ILC2s produce IL-5 in the bone marrow

Eosinophil expansion is a hallmark of most allergic diseases, but the underlying mechanisms driving this eosinophilia are not well understood. Recently, several studies identified a role for IL-33 in eosinophil development in the bone marrow [82, 99, 100], however, a cellular source of the eosinophil cytokine IL-5 remained unknown. In Paper III, we investigated the possibility that IL-33-responsive cells locally in the bone marrow support eosinophils by producing IL-5. IL-33 administration to the airways of naïve mice resulted in increased eosinophils in BAL, blood and bone marrow, which was accompanied by elevated levels of CCL24. Inhibition of CCL24 by neutralizing antibodies reduced the frequency of BAL eosinophils, indicating that the cells were recruited from the bone marrow in this model. ST2 expression was analyzed on ILC2s, CD34+ progenitors and TH cells, where the latter two were previously described to produce IL-5 in allergen-induced bone marrow eosinophilia [121, 234]. ILC2s and TH cells, but not CD34+ progenitors, increased ST2 expression upon IL-33 challenge. In accordance with Johnston et al [82], we found that ST2 expression on CD34+ progenitors preceded eosinophil lineage commitment (IL5Rα+CD34+). Furthermore, IL-33-induced eosinophilia was highly dependent on IL-5 and a single injection of anti-IL-5 antibodies 1 hour prior to the first IL-33 challenge severely reduced the number of eosinophils in BAL and bone marrow. Ex vivo analysis of intracellular IL-5 in bone marrow cells of IL-33-challenged mice demonstrated increased production in ILC2s and CD34+ progenitors, but not in TH cells. In vitro analysis confirmed that CD34+ cells and ILC2s induced IL-5 production by IL-33 stimulation. Interestingly, ILC2s rapidly induced high
levels of IL-5 (Fig. 4.7 A) which coincided with increased eosinophil differentiation (Fig. 4.7 B). We investigated IL-5 production further in sorted ILC2 cultures (Fig. 4.8 A), where IL-33 stimulation increased the levels of IL-5 in culture supernatants (Fig. 4.8 B). Importantly, when naïve bone marrow cells were cultured with cell-free supernatants from IL-33-stimulated ILC2s the amount of IL5Rα+CD34+ eosinophil progenitors (EoPs) doubled in the cultures (Fig. 4.8 C). This suggests that ILC2-derived factors such as IL-5 promote eosinophil development. However, it cannot be excluded that a part of the upregulation of IL5Rα expression is mediated through a direct effect on CD34+ progenitors by IL-33. Although, kinetic experiments demonstrated that IL5Rα expression (Fig. 4.7 B) correlated with increasing IL-5+ ILC2s (Fig. 4.7 A). The contribution by IL-33 in this system could be addressed by inhibition experiments of IL-33 and comparison of the ability of unstimulated ILC2 media (without IL-33) versus IL-33-stimulated media to promote EoP expansion.

The number of bone marrow ILC2s (Lin-CD45+CD127+CD25+ ST2+) described in Paper III and in ongoing studies (Fig. 4.12 A) demonstrated a wide range, which might indicate heterogeneity in the population. Comparable ILC2 levels were recently reported by Stier et al which identified ILC2s in bone marrow using
similar surface markers [49]. Additionally, a study of human bone marrow by Chen et al also described a large spread in the number of ILC2s [43]. Analysis of additional ILC2 markers in our study revealed that >90% of LinCD45−CD127−CD25+ cells in the bone marrow expressed Sca-1 and ICOS, however, c-Kit was found on <20% and ~30% were KLRG+. Previous studies have suggested that ILC2 precursors in bone marrow are c-Kit+ and KLRG1− while mature ILC2s in the periphery demonstrate variable c-Kit expression but are KLRG1+ [218, 235]. It is possible that IL-5-producing ILC2s in the bone marrow in our study are mature KLRG1+ ILC2s, but it was not confirmed. This might also apply to our study of expanded bone marrow ILC2s, where it is possible that addition of ILC2-stimulating cytokines altered the phenotype. Furthermore, heterogeneity of bone marrow ILC2s might be due to a constitutive turnover of the cells. This could be investigated by incorporation of BrdU to monitor the phenotype of newly produced ILC2s under inflammatory settings. It would also be interesting to study eosinophil development in the bone marrow in absence of ILC2s using ILC2-deficient mice. Such experiments would address the contribution of ILC2 in relation to other cells in the bone marrow. Interestingly, our preliminary results demonstrate that RAG1 deficient mice induce normal levels of eosinophils in the bone marrow in response to IL-33, even in the absence of an adaptive immune system (Fig. 4.9 A). Additionally, ILC2s in the bone marrow of RAG1 deficient mice respond to IL-33 challenge with increased ST2 expression (Fig. 4.9 B) and demonstrate sufficient IL-5 production in vitro (Fig. 4.9 C). These results do not prove that ILC2s are the major source of IL-5, but it support our findings that ILC2s, not CD4+ T cells, contribute to IL-33-induced eosinophil development.

Figure 4.9. Mature eosinophils (A) and ST2 expression on ILC2s (B) increase in bone marrow of RAG1 deficient (KO) mice in response to IL-33 in vivo. C) IL-5 production by WT and RAG1 KO bone marrow ILC2s are similarly induced by IL-33 in vitro (100 ng/ml IL-33, 24h). [Johansson et al unpublished data]
4.3.1 Allergen inhalation induces IL-33 in bone marrow

An important aspect of the IL-33 model is the possibility that activation of bone marrow cells results from systemic levels of IL-33, due to the high dosage administrated (1 μg/intranasal administration day 1, 3 and 5). To address this concern, we investigated the response in the bone marrow following HDM allergen challenge of the airways (protocol in Methods). The number of eosinophils and CCL24 levels increased in BAL of HDM sensitized and exposed mice (HDM/HDM), but no increase of airway eosinophils or CCL24 were detected in HDM sensitized controls (HDM/PBS) or PBS controls (PBS/PBS). Furthermore, airway exposure to HDM resulted in a greater number of eosinophil progenitors (Fig. 4.10 A) and mature eosinophils (Fig. 4.10 B) in the bone marrow. Interestingly, IL-33 levels in the bone marrow increased in HDM treated mice. This was measured by intracellular expression of IL-33 by SSC<sup>lo</sup>CD45<sup>-</sup> cells which might represent a population of non-hematopoietic structural cells in the bone marrow. The amount of IL-33 in

![Figure 4.10. Eosinophil progenitors (Eos Prog) (A) and mature eosinophils (Eos) (B) increase in the bone marrow in HDM-induced airway eosinophilia. [Johansson et al unpublished data]](image)

![Figure 4.11. Increasing levels of IL-33 in a non-hematopoietic population correlate with greater numbers of mature eosinophils in the bone marrow in HDM-induced airway eosinophilia. MFI; mean fluorescence intensity. [Johansson et al unpublished data]](image)
these cells correlated with elevated numbers of mature eosinophils (Fig. 4.11). The source of IL-33 is still a matter of debate in the field [210], and IL-33 is often assigned as an epithelial-derived cytokine, however, it has been established that immune cells also express IL-33 upon activation [142, 236, 237]. Thus, it is possible that hematopoietic cells in the bone marrow also produce IL-33 in allergen challenged mice. Along with increased IL-33 levels in bone marrow, IL-33-responsive ILC2s (Fig. 4.12 A, B) and TH cells (Fig. 4.12 C, D) demonstrated altered cellular numbers and activation status in response to HDM exposure. Interestingly, a decreased number of ILC2s in the bone marrow of HDM sensitized mice (Fig. 4.12 A) might suggest that ILC2s are migrating from the bone marrow upon allergen challenge. This was recently reported in a model of Alternaria-induced airway inflammation [49]. Furthermore, increased ST2 expression on ILC2s (Fig. 4.12 B) coincided with the induction of eosinophil progenitors (Fig. 4.10 A), possibly highlighting the importance of IL-33 early in eosinophil development. However, IL-33-responsive TH cells were only increased in the HDM sensitized and exposed group (Fig. 4.12 D). In summary, our preliminary results support that allergen-induced eosinophil development involves IL-33 in the bone marrow compartment. However, many questions still remain and our investigations of IL-33-responsive bone marrow cells and their contribution in IL-5-driven eosinophilia are being continued.
4.4 Macrophages in asthmatic airways express distinct miRNAs

Macrophages are critical in maintaining immune homeostasis, but studies suggest that their function is altered in asthma [123, 124, 138]. Identification of dysregulated miRNA expression might lead us to genes that control pathological responses. In Paper IV, we sought to determine whether miRNAs were differentially expressed in airway macrophages from asthmatic individuals compared to healthy controls. Study participants, recruited from the West Sweden Asthma Study cohort, underwent bronchoscopy with collection of BL. Macrophages were enriched by adherence to cell culture plates and miRNA expression was analyzed in rested cells by microarray. Ninety-one percent of detected miRNAs were present in macrophages isolated from both asthmatic and healthy airways, and a limited number of miRNAs were uniquely expressed by either of the two groups. The analysis identified significantly lower levels of miR-320a and miR-221-3p in asthmatic macrophages. Validation by qPCR confirmed lower expression of miR-221-3p in asthma, however, miR-320a demonstrated higher levels in macrophages from asthmatics compared to healthy controls. The reason for this discrepancy is unclear since microarray analysis and qPCR validation was performed on the same samples. However, correlation between these methods is influenced by many critical factors such as data filtering, statistical analysis, expression levels and fold-change [23 8]. miR-221-3p and miR-320a demonstrated relatively low fold-change values between healthy and asthma; 1.21 and 1.48 (mean log2), respectively. This might introduce uncertainty in the microarray data analysis.

Interestingly, in silico analysis of miR-320a and miR-221-3p revealed common gene targets in the phosphoinositide 3-kinase (PI3K)-Akt signaling pathway, which might suggest that they are involved in regulation of pro-inflammatory functions in macrophages. PI3K-Akt signaling is a major inflammatory checkpoint and has been implicated in airway pathology, excessive inflammation and impaired glucocorticoid responsiveness in asthma and COPD [239-241]. Furthermore, in anti-viral immunity the major rhinovirus receptor ICAM-1 [242] decreased its expression upon inhibition of PI3K-Akt signaling in human lung epithelial cells [243], which is an interesting finding in relation to rhinovirus-triggered exacerbations in asthma. In fact, in ongoing experiments we are analyzing miRNA expression in stimulated macrophage cultures using poly(I:C) to mimic viral infection. Since virus-triggered exacerbations is a significant problem in asthma, we wanted to investigate if specific miRNAs regulate anti-viral responses in macrophages. BL macrophages from asthmatic individuals and healthy controls were stimulated with 10 μg/ml poly(I:C) for 4h. Cells were harvested for miRNA
analysis by microarray and cell-free culture supernatants were analyzed by multiplex immunoassay (Olink Proteomics, Uppsala, Sweden). Our preliminary data demonstrate that poly(I:C)-stimulation resulted in release of pro-inflammatory mediators by macrophage cultures (Fig. 4.13). Importantly, macrophages from asthmatic subjects, but not healthy subjects, induced asthma-related mediators such as the IL-6-family member Oncostatin M (OSM) and the inflammatory marker S100A12 (EN-RAGE) that were implicated previously in airway remodeling and hyperreactivity in asthma and allergic inflammation [244, 245]. Furthermore, uniquely expressed miRNAs were found after poly(I:C)-stimulation of macrophages from asthmatic compared to healthy subjects. Interestingly, the most highly upregulated miRNA in poly(I:C)-stimulated macrophages in the asthma group was miR-718 (data not shown). A recent study found that miR-718 suppressed pro-inflammatory cytokine production in murine macrophages by directly targeting PTEN in the PI3K-Akt signaling pathway [246]. Of note, miR-718 has not yet been investigated in human cells and in our continued analysis we will not only learn more about the role of miR-718 and its targets, we will also compare previous observations in murine cells to a human setting. Analysis of miR-718, and other interesting miRNAs identified in our study, are being continued in order to uncover inflammatory or anti-viral pathways in macrophages that might contribute to asthma pathology.

Figure 4.13. Inflammatory mediators secreted by macrophages in response to poly(I:C) stimulation (10μg/ml, 4h) compared to unstimulated cells. OSM, EN-RAGE and TWEAK are significantly altered upon stimulation in the asthma group (n=13); TWEAK is significantly altered in the healthy group (n=7) (analyzed by paired t-test). HC, Healthy control; A, Asthma. [Johansson et al unpublished data]
5 Conclusion

The main conclusions from this thesis are:

**Paper I** ILC2s in murine lungs upregulate miR-155 expression upon IL-33 activation, and lack of miR-155 in ILC2s impairs proliferation and cytokine production. Mice deficient in miR-155 exhibit defective IL-33 signaling and are protected from IL-33-dependent airway eosinophilia. This study identifies miR-155 as a novel regulator of ILC2s and IL-33. Targeting of miR-155 may be an attractive therapeutic strategy for regulation of ILC2s in asthma.

**Paper II** miR-155 is differentially expressed in the airways of allergic asthmatic subjects compared to healthy controls. The level of miR-155 in airway-infiltrating lymphocytes in pollen allergic asthmatics varies with natural exposure to pollen. This study identifies human sputum lymphocytes as a potential cellular target of miR-155-mediated regulation.

**Paper III** Airway exposure to IL-33 activates IL-33-responsive ILC2s that produce IL-5 in the bone marrow. Induction of IL-5 by ILC2s coincides with increased eosinophil differentiation and secreted ILC2 factors promote development of bone marrow eosinophils. This study identifies a functional role for ILC2s in an atypical location and opens the possibility that ILC2s promote eosinophilic airway inflammation by supporting eosinophil hematopoiesis locally in the bone marrow. Improved understanding of the mechanisms that regulate eosinophilic inflammation is critical for development of therapies of uncontrolled eosinophilic asthma.

**Paper IV** Primary airway macrophages from asthmatic individuals demonstrate a distinct miRNA expression profile compared to macrophages obtained from healthy airways. Predicted gene targets of miRNAs with altered expression in asthmatic cells modulate inflammatory signaling pathways. This study strengthens previous findings that airway macrophages are part of the immunopathology underlying asthmatic disease.
6 Future perspectives

This thesis investigated the role of ILC2s and miRNAs in inflammatory responses in asthma. The knowledge gained from these studies provides new insights into miRNA-mediated regulation of ILC2s and ILC2s as a source of type 2 cytokines in bone marrow. These studies also provide further evidence for an involvement of miRNAs in human asthma. In this chapter, I reflect on the field related to my studies and areas which, in my opinion, should be addressed in future research.

Over the last decade, a significant interest in the initiating events of type 2 immune responses placed ILC2s in the center of intense research which rapidly expanded our understanding of these cells. Their ability to respond to epithelial-derived danger signals strongly link ILC2s to the early events that occur during allergen exposure as well as the persistence of subsequent inflammatory responses. Indeed, interactions between epithelial cells and ILC2s contribute to the maintenance of chronic asthma [39], where IL-33 is particularly interesting. Many studies of IL-33 have focused on barrier tissues such as the lungs, but our findings suggest that IL-33 has additional roles in the bone marrow. Analysis of ILC2s (Lin-CD45+CD127+CD25+ST2+) in naïve mice show that bone marrow-derived ILC2s exhibit 6-fold higher ST2 expression (rMFI) compared lung-derived ILC2s (unpublished data). It is possible that this is related to differential stages of ILC2 development in bone marrow (i.e. ILC2 precursors) versus the lung, but more importantly, it indicates that IL-33 signaling plays a role in both compartments, perhaps for different reasons. Constantly exposed to the inhaled environment, ILC2s of the airway mucosa are indeed located in a prime position to react to IL-33. The bone marrow on the other hand, is privileged in this respect and the high ST2 expression might suggest that bone marrow ILC2s are more sensitive to IL-33, perhaps even low levels of IL-33 are enough to activate the cells. The consequences of IL-33 signaling in specific cell types, and in various tissues, require further attention. For instance, it seems like pathways that activate ILC2s also target T\textsubscript{REG} cells. ST2\textsuperscript{+} T\textsubscript{REG} cells have been described as T\textsubscript{H}2-biased with GATA-3 expression and IL-5/IL-13 production [92, 247], but surprisingly, they are superior to ST2\textsuperscript{-} T\textsubscript{REG} cells in suppressing CD4\textsuperscript{+} T cell proliferation, independent of IL-33 [247]. Similar to ILC2s, ST2\textsuperscript{+} T\textsubscript{REG} cells preferentially reside in non-lymphoid sites, such as small intestine and colon and especially in the lung [247]. Perhaps immunosuppressive ST2\textsuperscript{+} T\textsubscript{REG} cells co-localize with ILC2s to limit type 2 cytokine production by T\textsubscript{REG}-induced IL-10 and TGF-β [248]. In addition, similar to IL-2 signaling in T\textsubscript{REG} cells...
expression of ST2 on TREG cells might even have a “sponge effect” that limits the access to IL-33 for ILC2s to further dampen activation.

An emerging field with important implications in ILC2 biology is innate immune memory [251]. The understanding that not only cells bearing antigen receptors build immunological memory is challenging current concepts. Innate immune memory is mediated by epigenetic reprogramming, leading to sustained changes in gene expression and, unlike adaptive immune cells, do not involve permanent genetic changes such as mutations or recombination. It was recently found that allergen-experienced ILC2s demonstrated stronger response to secondary challenge compared to naïve ILC2s [252]. Interestingly, ILC2s previously exposed to papain or recombinant IL-33 displayed higher responsiveness even to unrelated allergens [252]. It would be interesting to investigate if ILC2s in bone marrow from allergen exposed mice are primed to react more vigorously to IL-33 stimulation. Inclusion of KLRG1 surface marker expression analysis and BrdU labeling would elucidate if such an effect was mediated by ILC2 precursors or mature ILC2s as well as the life-span of these cells. The fact that ILC2s possess memory-like features highlights the importance of understanding these cells at transcriptional level. As tissue-resident cells, future studies need to focus on their role in various locations under specific conditions, since their microenvironment is likely reflected in their transcriptional programming.

miRNA-mediated gene regulation is an important epigenetic mechanism that implements transient changes in cell physiology. At the time of our first study ([Paper I]), miRNA expression in ILC2s was completely unexplored. We identified that miR-155 regulates ILC2 functions, although, the exact molecular mechanisms remain unclear and further studies are warranted to determine the role of miR-155 and other miRNAs in ILC2 biology. An important area for future investigations is the involvement of miRNAs in the rapid and proportionally large production of type 2 cytokines by ILC2s compared to other lymphocytes [38]. Furthermore, studies of CD4+ T cells have demonstrated that miRNA expression support the robustness of T_{H1} phenotypes [175, 178], and it will be interesting to learn how and which miRNAs contribute to ILC2 development and maintenance of lineage stability. Indeed, recent reports describing flexibility of the ILC2 phenotype indicate a possibility for therapeutic intervention [60, 62, 64, 65], in this regard, the targeting of miRNAs may be an attractive approach. Identification of miRNAs that regulate steroid-induced responses is also an interesting area for future research, and might aid the development of novel therapeutics of steroid-resistant asthma phenotypes. Analysis of miRNA expression profiles in ILC2s and CD4+ T cells in steroid naïve patients compared to steroid-responsive and steroid-insensitive patients would be an important step in this direction.
Continued investigation of miR-155-mediated regulation of ILC2s is needed. For instance, reduced baseline levels of ILC2s in naïve lungs indicate that miR-155 is involved in ILC2 development, maintenance and/or seeding of the lung during the perinatal period. Generation of conditional knockouts with miR-155 deficiency restricted to ILC2s or inducible knockdown of its expression would provide important tools for further investigations of miR-155 in experimental asthma. In the short-term perspective, having access to full body knockout mice, adoptive transfer experiments of WT ILC2s to miR-155 deficient mice would provide some answers, including if miR-155 sufficient ILC2s are able to rescue eosinophilic inflammation in an otherwise miR-155 deficient setting. Additionally, RNA sequencing of WT and miR-155 deficient ILC2s would be needed to identify potential direct targets of miR-155. To this end, it would be interesting to compare mRNA expression of bone marrow and lung-derived ILC2s in miR-155 deficient mice to evaluate if miR-155-mediated regulation is restricted to the airways as previously suggested [198].

There are many “allergy-related” immune cells that are affected by miR-155 deficiency and it would be interesting to examine miR-155-dependence in hematopoietic cells versus non-hematopoietic cells in allergic inflammation. This could be accomplished by restricting miR-155 deficiency to CD45+ leukocytes. Some of the controversies related to the cellular source of IL-33 could perhaps be addresses in such a model since full-body miR-155 knockout mice demonstrated impaired IL-33 induction in our study. Intact miR-155 signaling in structural cells might restore IL-33 levels. Importantly, given the strong correlation between IL-33 and asthma, studies of miR-155 might lead the way to the discovery of pathways with therapeutic potential that control IL-33 activity.

Novel techniques such as PrimeFlow™ that combines miRNA expression analysis with investigations of cellular phenotypes will certainly bring the field forward. Removing the need for additional processing steps such as cellular sorting and RNA isolation makes it an important tool for investigations of limited clinical samples and rare cell populations. Of relevance to Paper II, PrimeFlow™ analysis of induced sputum would be a powerful way to link altered miR-155 expression to specific cells or activation status. A cell type of interest in sputum is TREG cells where FOXP3 was previously demonstrated to drive miR-155 expression [253]. Possible defective immunosuppression in asthmatic airways might be related to TREG phenotypes. Macrophages have also been implicated in loss of tolerance in asthmatic airways. In our continued investigations in Paper IV, dysregulated miRNAs could be functionally evaluated in vitro, focusing on specific receptor expression and inflammatory mediator release in response to inhibition of specific miRNA candidates or their predicted targets. With limited access to BL fluid, it might be feasible to proceed with sputum-derived macrophages.
Finally, the last area I will mention concerns the class of regulatory molecules to which miRNA belongs: noncoding RNA [254]. Only a small fraction of the human genome contains protein-coding information. In fact, most of the transcriptional output is “noncoding” and over the last decades the view of noncoding RNA has gone from ‘transcriptional noise’ to ‘key regulators of biological processes’ [254]. The largest group consists of long noncoding RNAs (lncRNAs). They regulate a wide range of cellular functions, including genome organization and stability, and gene expression by post-transcriptional regulation [255]. Interestingly, lncRNAs can even regulate miRNA activity by complementary binding, preventing miRNAs to act on their target mRNAs [255]. However, there is a significant gap in our understanding of lncRNAs in respiratory diseases. Consisting of >200 nucleotides, lncRNAs are highly cell- and tissue-specific and have great potential as disease biomarkers [256]. But the long-term aim of the study of lncRNAs and miRNAs in our field should be to acquire a deeper understanding of their mechanisms of action to discover key components that control asthma pathology.

To summarize, we need to improve our basic understanding of how RNA biology can be utilized in novel therapeutic approaches of inflammatory diseases. Further translational research is required to assign RNA profiles to distinct effector cells and disease phenotypes. The strong association between ILC2s and chronic type 2 inflammation clearly demonstrates the importance to target these cells in asthma.
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