

# T cell subsets in Asthma and Allergy

– role of glucocorticoids,  
plasticity and microRNA-155

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Cover illustration: CD4<sup>+</sup> T cell from an allergic asthmatic individual co-expressing transcription factor GATA-3 (green) and FOXP3 (red). Nucleus stained blue and merged image in mixed colors. The image was acquired at the Centre for Cellular Imaging, Sahlgrenska Academy at University of Gothenburg. Photo by Carina Malmhäll.

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*”Att människor blir äldre, det visste jag,  
men att det skulle drabba mig var jag inte beredd på”*

*Sven-Bertil Bärnarp*

*Till min familj och mina vänner*



# **T cell subsets in Asthma and Allergy**

- role of glucocorticoids, plasticity and microRNA-155

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## **ABSTRACT**

The focus of this thesis is the different subsets of CD4<sup>+</sup> T cells involvement in asthma and allergy. It encloses studies of asthma and allergy in both humans and mice. The work of the three papers has been performed during a time where the field has moved from a paradigm of separate entities of the studied T cell subsets to more flexibility and plasticity in these cells due to the microenvironment. Furthermore, the field of ribonucleic acid (RNA) has grown to include new RNAs such as microRNA (miRNA), demonstrating high impact on the microenvironment.

The aims of this thesis were to determine in: Paper I. Glucocorticoid treatment during natural pollen exposure and the effects it poses on T regulatory (Treg), T helper 1 (Th) and Th2 cells in the nasal mucosa of allergic rhinitis patients. Paper II. Plasticity in circulating Treg, Th1, Th2 and Th17 cells and the relationship to eosinophilia in asthmatic individuals. Paper III. miRNA-155 affecting T cell dependent allergen induced eosinophilic airway inflammation.

The results demonstrates that glucocorticoid treatment during pollen exposure affected the number of Treg and Th2 cells as well as the balance between the subsets investigated at site of inflammation. Furthermore, T cells co-expressing several regulatory transcription factors were found in asthmatics as well as in healthy controls. Finally, miRNA-155 deficiency reduced the number of airway eosinophils, Th2, Th17 and Treg cells after allergen challenge in a mouse model of allergic airway inflammation, while the transcription factor PU.1 was upregulated. Adoptive transfer of allergen specific CD4<sup>+</sup> T cells resulted in a similar degree of airway eosinophilia in miR-155 KO and WT mice.

We conclude that nasal glucocorticoids attenuate the allergic inflammation by maintaining the local relationship between Th1 and Th2 cells as well as between Treg and Th2 cells. Furthermore, T cells ability to co-express

several regulatory transcription factors in both asthmatics and healthy controls indicates plasticity *in vivo*. Finally, miRNA-155 contributes to the regulation of allergic airway inflammation by modulating Th2 responses, via the transcription factor PU.1.

Taken together these studies support that T cell shows flexibility and plasticity which can be affected by treatment, allergen exposure and miRNA expression and thus are in important regulators of asthma and allergy. Increasing the understanding of these processes may hopefully result in more specific future treatments.

**Keywords:** Asthma, Allergy, T regulatory cells, Th1, Th2, Th17, FOXP3, T-bet, GATA-3, ROR $\gamma$ t, glucocorticoids, plasticity, microRNA-155, PU.1

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# SAMMANFATTNING PÅ SVENSKA

Astma och allergi är vanliga sjukdomar världen över med ca 300 miljoner människor som lider av astma och ca 10-40 % som lider av allergier. Astma är en heterogen kronisk inflammatorisk sjukdom som kan bero på allergi, men som också kan ha andra underliggande orsaker. Kombinationen astma och allergi är dock vanlig, ca 70 % av alla människor med astma har också allergisk rinit. Klassificering av astma med olika fenotyper (synliga karaktärsdrag) som till exempel med eller utan eosinofil inflammation, har visat sig användbara men inte fullständiga för att förutse svar på behandling. Den vanligast förekommande behandlingen är kortison, dvs. glucocorticoider. Eosinofil inflammation och andra fenotyper omfattar troligen undergrupper av sjukdom med distinkta molekylära mekanismer s.k. endotyper dvs. subtyper av ett tillstånd, definierat av en distinkt funktionell eller patofysiologisk mekanism. För att förstå bakomliggande mekanismer för sjukdom är det viktigt att subgrupper inte bara karaktäriseras baserat på kliniska fenotyper utan också på immunologiska karakteristika.

T-celler har en central roll i nästan alla kroppens immunsvaret pga. deras reglerande förmåga. De s.k. CD4<sup>+</sup> T-cellerna omfattar T-regulatoriska (Treg) celler, vars huvudsakliga uppgift är att dämpa immunsvaret mot kroppsegna antigen men också mot främmande antigen när immunsvaret blir farligt för organismen. T-effektor celler s.k. T-hjälpar (Th) celler ingår också i CD4<sup>+</sup> T-cellerna, vars huvudsakliga uppgift är att skydda mot patogener. Alla Th-celler producerar sina specifika proteiner s.k. cytokiner som förändrar mikromiljön och attraherar andra inflammatoriska celler. Th2-cellerna är de som starkast sammanknippas med allergi, då produktion av cytokinerna interleukin (IL)-4 och IL-13 förmår B-cellerna att producera IgE, ett immunoglobulin som känner igen allergen. Th2-cellerna producerar även IL-5 som är mycket viktigt för eosinofilers överlevnad och förökning. Det är dock viktigt för organismen att uppnå balans för att skydda mot patogener men inte döda organismen. Man har tidigare ansett att dessa olika T-celler var linjespecifika dvs. antog en viss profil och att denna sedan var slutgiltig. Numera börjar man inse att det inte är helt så enkelt utan att dessa T-celler kan förändras beroende på mikromiljön och uppvisar plasticitet. Mikromiljön påverkas inte bara av proteiner utan även av korta sekvenser ribonukleinsyra (RNA) s.k. mikroRNA (miRNA). Enligt den klassiska dogman kodar deoxyribonukleinsyra (DNA) för alla gener i organismen som sedan transkriberas till budbärarRNA (mRNA) för proteinsyntes. miRNA har nu visat sig ha en reglerande funktion genom att kunna inhibera denna proteinsyntes.

Målen med denna avhandling var att utforska:

- I. Effekten av behandling med en glucocorticoid i samband med naturlig pollen exponering på antalet Treg-, Th1- och Th2-celler i nässlemhinnan hos patienter med allergisk rinit.
- II. Om plasticitet hos Treg-, Th1-, Th2- och Th17-celler förekommer *in vivo* och om det finns någon relation till eosinofil inflammation i blodet hos astmatiker.
- III. Om miRNA-155 påverkar den T cells beroende allergen inducerade eosinofila luftvägsinflammationen, studerat genom användandet av en experimentell djurmodell.

Resultaten visade att:

- I. Behandling med en glucocorticoid i samband med pollen exponering minskade framförallt antalet Th2 celler men även Treg celler. Det medförde att balansen mellan Treg/Th2 och Th1/Th2 bibehölls lokalt i den inflammatoriska vävnaden.
- II. T celler som samtidigt uttryckte flera signaturspecifika transkriptionsfaktorer hittades hos både astmatiker och hos friska kontrollindivider.
- III. Brist på miR-155 ledde till minskat antal eosinofiler, Th2-, Th17- och Treg-celler i luftvägarna efter allergen exponering medan transkriptionsfaktorn PU.1, som har en negativ effekt på Th2 cytokinproduktion, var förhöjd. När allergen specifika CD4<sup>+</sup> T-celler gavs till möss före allergen exponering resulterade det i liknande eosinofil inflammation i luftvägarna, både för möss som saknade miR-155 som för vanliga möss.

Slutsatsen är att behandling med en glucocorticoid lindrar den allergiska inflammationen genom att bevara den lokala balansen mellan Th1/Th2-celler och mellan Treg/Th2-celler. Dessutom, uttrycker cirkulerande T-celler flera signaturspecifika transkriptionsfaktorer vilket indikerar plasticitet *in vivo*. Slutligen, bidrar miR-155 till regleringen av allergisk luftvägsinflammation genom att modulera Th2-svaret, via transkriptionsfaktorn PU.1. Sammantaget styrker dessa studier att T-celler uppvisar flexibilitet och plasticitet som kan påverkas av behandling, allergen exponering och miRNA uttryck och är därför viktiga regulatorer i astma och allergi. Att ytterligare öka förståelsen för dessa processer kan förhoppningsvis resultera i mer specifika terapier framöver.



# LIST OF PAPERS

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

- I. **Malmhäll C**, Bossios A, Pullerits T, Lötval J. Effects of pollen and nasal glucocorticoid on FOXP3<sup>+</sup>, GATA-3<sup>+</sup> and T-bet<sup>+</sup> cells in allergic rhinitis.  
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- II. **Malmhäll C**, Bossios A, Rådinger M, Sjöstrand M, Lu Y, Lundbäck B, Lötval J. Immunophenotyping of circulating T helper cells argues for multiple functions and plasticity of T cells in vivo in humans - possible role in asthma.  
*PLoS One*. 2012;7(6):e40012.
- III. **Malmhäll C**, Alawieh S, Lu Y, Sjöstrand M, Bossios A, Eldh M, Rådinger M. MicroRNA-155 is essential for T<sub>H</sub>2-mediated allergen-induced eosinophilic inflammation in the lung.  
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### **Additional publications not included in this thesis:**

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# CONTENT

<b>ABBREVIATIONS .....</b>	<b>1</b>
<b>1 INTRODUCTION .....</b>	<b>1</b>
1.1 Asthma and Allergy .....	1
1.1.1 Prevalence .....	1
1.1.2 Characteristics .....	1
1.1.3 Allergen sensitization and exposure .....	2
1.1.4 Phenotypes and endotypes .....	3
1.1.5 Glucocorticoids .....	4
1.2 T cell subsets .....	5
1.2.1 T regulatory cells .....	5
1.2.2 T helper 1 cells .....	7
1.2.3 T helper 2 cells .....	9
1.2.4 T helper 17 cells .....	11
1.2.5 T cell plasticity .....	12
1.3 RNA .....	13
1.3.1 MicroRNA .....	13
1.3.2 MicroRNA-155 .....	15
<b>2 AIM.....</b>	<b>17</b>
<b>3 PATIENTS AND METHODS.....</b>	<b>18</b>
3.1 Study designs .....	18
3.1.1 Paper I.....	18
3.1.2 Paper II.....	19
3.1.3 Paper III.....	20
3.2 Clinical parameters.....	21
3.2.1 Structured interviews (II) .....	21
3.2.2 Skin prick test (I, II) .....	21
3.2.3 Lung function, reversibility and methacholine test (II).....	21
3.2.4 Exhaled nitric oxide (II) .....	22

3.3	Sample collection and preparation .....	22
3.3.1	Nasal biopsies (I).....	22
3.3.2	Nasal lavage (II) .....	22
3.3.3	Induced sputum (II).....	23
3.3.4	Blood (II, III).....	23
3.3.5	BALF (III).....	24
3.3.6	Lung tissue (III).....	24
3.3.7	Lung single cell (III) .....	24
3.3.8	Lung homogenate (III) .....	25
3.3.9	Spleen and Peribronchial lymphnodes (III).....	25
3.3.10	Bone marrow (III) .....	25
3.3.11	Total RNA isolation (III).....	25
3.4	Analysis.....	26
3.4.1	Immunohistochemistry (I).....	26
3.4.2	Differential cell count (II, III) .....	26
3.4.3	Lung histology (III).....	26
3.4.4	Flow Cytometry (II, III) .....	26
3.4.5	<i>In vitro</i> activation (II, III).....	28
3.4.6	Confocal microscopy (II) .....	28
3.4.7	Cytokines and Chemokines (III) .....	29
3.4.8	RNA (III).....	29
3.5	Adoptive transfer (III).....	30
3.6	Statistical analysis .....	31
<b>4</b>	<b>RESULTS AND DISCUSSION.....</b>	<b>32</b>
4.1	T cell subsets in the nasal mucosa of patients with allergic rhinitis (I).....	32
4.2	Effect of a natural grass-pollen season and glucocorticoid treatment on mucosal T cell subsets (I) .....	33
4.3	T cells subsets possibility to distinguish specific asthma endotypes (II) .....	35

4.4 Co-expression of master transcription factors in T cell subsets indicating ability of plasticity (II) .....	36
4.5 MicroRNA-155 regulates airway eosinophilia (III).....	39
4.6 MicroRNA-155 plays a role for the development of airway T cell subsets (III) .....	39
4.7 MicroRNA-155 modulates allergic inflammation by influencing allergen-mediated Th2 responses (III) .....	40
4.8 MicroRNA-155 targets PU.1 in a model of allergen induced airway inflammation (III) .....	41
<b>5 CONCLUSIONS.....</b>	<b>43</b>
<b>6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES.....</b>	<b>44</b>
<b>ACKNOWLEDGEMENT.....</b>	<b>47</b>
<b>REFERENCES .....</b>	<b>49</b>

# ABBREVIATIONS

3'UTR	3'Untranslated Region
7AAD	7-Amino-Actinomycin-D
AHR	Airway hyper responsiveness
AR	Allergic rhinitis
BALF	Bronchoalveolar lavage fluid
BHR	Bronchial hyper responsiveness
BIC	B-cell integration cluster
CBA	Cytometric bead array
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
c-Maf	Musculo aponeurotic fibrosarcoma oncogene homolog
CTLA	Cytotoxic T-lymphocyte antigen
DNase	Deoxyribonuclease
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
F(ab') <sub>2</sub>	Paired fragment antigen-binding, part of an antibody lacking the fragment crystallizable region (Fc)
FCS	Fetal calf serum
FeNO	Fraction of exhaled nitric oxide
FEV <sub>1</sub>	Forced expiratory volume in 1 second
FOXP	Forkhead box protein
FP	Fluticasone propionate
GATA	GATA binding protein
HDM	House dust mite
i.n.	Intranasal
i.p.	Intraperitoneal
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin



ILC	Innate lymphoid cells
KO	Knock-out
LNA	Locked nucleic acid
MCH	Methacholine
miR-155	microRNA-155
miRNA	Micro RNA
mRNA	Messenger RNA
OVA	Ovalbumin
PBLN	Peribronchial lymph node
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD20	Provocative dose resulting in a 20% reduction in FEV <sub>1</sub>
PU.1	Synonym sfpi1, spleen focus forming virus proviral integration oncogene
qPCR	Quantitative real-time polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
ROR	Retinoic acid-related orphan receptor
RT	Reverse transcription or room temperature
SHIP	SH2 domain containing inositol-5-phosphatase 1
snRNA	Small nuclear RNA
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
T-bet	T-box transcription factor
TCR	T cell receptor
TGF	Transforming growth factor
Th cells	T helper cells
TNF	Tumor necrosis factor
Treg	T regulatory
WSAS	West Sweden Asthma Study
WT	Wild type



# 1 INTRODUCTION

The focus of this thesis is the different subsets of CD4<sup>+</sup> T cells involvement in asthma and allergy. It encloses studies of asthma and allergy in both humans and mice. The work of the three papers has been performed during a time where the field has moved from a paradigm of separate entities of the studied T cell subsets to more flexibility and plasticity in these cells due to the microenvironment. Furthermore, the field of ribonucleic acid (RNA) has grown to include new RNAs such as microRNA (miRNA), demonstrating high impact on the microenvironment.

## 1.1 Asthma and Allergy

### 1.1.1 Prevalence

Asthma is one of the major non communicable diseases affecting some 300 million people worldwide [1, 2]. The prevalence in Western Europe and North America is decreasing or may have reached a plateau in adults as reported recently in the West Sweden Asthma Study (WSAS) [1, 3] but still with increasing prevalence elsewhere resulting in both health problems for the individual and economic burden for the society [1]. The role of allergens in the development of asthma is well established, although some uncertainties remain [4]. The association between allergy and asthma is strong as about 70% of asthmatics also have allergic rhinitis (AR) [5]. The prevalence of allergic diseases is still increasing in both developed and developing countries affecting 10-40% of people worldwide [6].

### 1.1.2 Characteristics

Asthma is a life-long chronic inflammatory disorder of the airways associated with increased bronchial hyper responsiveness (BHR) and airflow obstruction, which leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing [1]. BHR is an abnormal reaction by bronchoconstriction when the asthmatic individual is subjected to irritants at levels that healthy individuals do not react [7]. The presence of BHR is often determined using direct challenge test using methacholine (MCH) or histamine. These challenge tests are not specific to asthma and the reaction varies for an individual with asthma depending on exacerbations and exposure to irritants such as allergens [8]. In asthma the bronchoconstriction is often spontaneously reversible or by treatment. Airway hyper responsiveness (AHR) is an integrative marker of more than a single

mechanism, including smooth muscle phenotype and airway inflammation. Normally, inflammation is a beneficial response to eliminate microbes and promoting tissue restoration. However, the chronic inflammation in the airway mucosa is considered to play a fundamental role in the asthma pathogenesis.

In allergic disease the immune response is dysregulated resulting in a chronic condition involving an abnormal reaction to an ordinarily harmless substance called allergen. AR is generally considered the most common allergic disease and a risk factor for asthma. AR is defined by the presence of nasal congestion, anterior and posterior rhinorrhea, sneezing and nasal itching secondary to immunoglobulin E (IgE)-mediated inflammation of the nasal mucosa [6].

Major characteristic features of allergic airway inflammation includes activation of T cells, eosinophilia, macrophages and mast cells, increased levels of IgE, epithelial shedding, and goblet cell hyperplasia and plasma exudation [9]. Allergic asthma is a multifaceted disease that is suggested to be actively controlled by T cells [10]. The focus in the present thesis is T cells involved in the pathogenesis of asthma and allergy.

### **1.1.3 Allergen sensitization and exposure**

The allergic disease progress can be divided into two phases. In the first sensitization phase susceptible individuals becomes sensitized to an allergen. This phase is then followed by an effector phase which is induced when the sensitized individual subsequently is exposed to the allergen and clinical symptoms occur. The development of symptoms can then be classified as an early phase, within one hour after allergen exposure and a late phase, occurring several hours after exposure. In humans as well as in the mouse the exposure of sensitized humans/mice to allergen causes a series of responses, including infiltration of immune cells to the airways and alterations in airway function.

During the sensitization phase, antigen presenting cells such as dendritic cells (DCs), phagocytes and presents the invading allergen to T cells resulting in priming of allergen-specific CD4<sup>+</sup> T helper 2 (Th2) cells and in production of Th2 cytokines. These cytokines induce Ig class switching in B-cells and they start producing IgE antibodies specific for the allergen. IgE sensitizes mast cells and basophils by binding of the high affinity receptor for IgE, FcεRI, expressed on their surface. Crosslinking of allergen-specific IgE-FcεRI complexes results in the early phase by degranulation of mast cells/basophils

releasing allergic mediators such as histamine, prostaglandins, leukotrienes, chemokines and cytokines. IgE also binds FcεRI on DCs and monocytes and the low-affinity receptor for IgE FcεRII on B-cells. The increased uptake of allergen by APCs and the presentation of allergen-derived peptides to specific CD4<sup>+</sup> T cells drive the late phase allergic reaction [11]. Additionally, allergic mediators attract circulating basophils, neutrophils, eosinophils and other cells to migrate into the tissue. The recruited immune cells secrete mediators of their own that sustain inflammation, result in tissue damage and recruit even more immune cells.

To mimic the sensitization and effector phase in our animal model an adjuvant of aluminum hydroxide is bound to the allergen to enhance the immune response [12-14].

### 1.1.4 Phenotypes and endotypes

The term “phenotype” has been defined as “the visible characteristics of an organism that result from the interaction between the genetic makeup and the environment” [15]. Asthma is a heterogeneous disease; patients express different phenotypes and have differences in severity, natural history, and response to treatment [15-17]. At least four different phenotypes based on their inflammatory cell composition, has been suggested: Eosinophilic, neutrophilic, mixed inflammatory and paucigranulocytic [15]. In addition to these phenotypes another four phenotypes has been suggested: a) well-controlled/minimal persistent airway inflammation, b) early-onset atopic asthma/severe symptoms/persistent airway inflammation/variable airway obstruction, c) predominantly females with late-onset asthma /minimal eosinophilic inflammation with symptoms /often obese, and d) predominately males with late-onset asthma /eosinophilic inflammation with no symptoms [17]. In both classifications, a characteristic feature is the presence and absence of eosinophilic inflammation.

Furthermore, asthma classification into phenotypes eosinophilic and non-eosinophilic has proved useful for predicting treatment response. Careful phenotype characterization of patient subpopulations is required to make improvement in the field of heterogeneous diseases such as asthma, and the clusters of phenotypes are likely to encompass subgroups of disease with distinct molecular mechanisms; endotypes [18]. An “endotype” of disease is defined as “a subtype of a condition, which is defined by a distinct functional or patho-physiological mechanism”[19]. Asthma demonstrates differences in their clinical expression, which suggests that the disease have different underlying mechanisms in different patients [19]. Maybe asthmatics can be

divided into several subgroups, each with characteristic asthma symptoms that arise from distinct pathological processes [20].

To further understand mechanism of disease, careful consideration needs to be applied to the patient selection and their phenotype characteristics, which may reflect a specific disease process in a disease endotype. Asthma subgroups will need to be characterized not only using clinical phenotyping, but also by immunological characterization using modern immunophenotyping tools.

### 1.1.5 Glucocorticoids

Glucocorticoids are a class of steroid hormone also natural occurring as cortisol in the body. Local administration of glucocorticoids is used extensively in the treatment of asthma and AR, and is considered to be the most efficient safe anti-inflammatory treatment available [21]. In general, treatment with different glucocorticoids have had similar efficacy, but there are small differences in potency, e.g. fluticasone propionate (FP) has double the potency of beclomethasone [22, 23]. The effect of glucocorticoids is during both early and late phase response by the decrease of mediator levels and the inhibition of inflammatory cell influx. The most consistent attenuation has been demonstrated with eosinophils even at low dose, while the reduction of T cells required higher doses of glucocorticoids [24].

Glucocorticoids exert their effects by binding to cytosolic glucocorticoid receptor (cGR) of target cells. The activated GR complex in turn upregulates the expression of anti-inflammatory proteins in the nucleus and represses the expression of pro-inflammatory proteins. In addition, GR can modulate, positively or negatively, directly or indirectly, the activity of other transcription factors [25, 26]. Glucocorticoids have been demonstrated to inhibit T-box transcription factor (T-bet), the master regulatory transcription factor for Th1 cells, its transcriptional activity both at messenger RNA (mRNA) and protein level suggesting to promote a shift toward Th2 differentiation [27]. Glucocorticoid treatment has also been demonstrated to upregulate the master regulatory transcription factor of T regulatory (Treg) cells Forkhead box protein 3 (FOXP3), its mRNA expression in a human *in vivo* study, suggesting increased suppressive capacity by either increased numbers of Treg cells or more effective suppressive capacity of the Treg cells [28] thus indicating a new role for glucocorticoids. This led us to investigate if the number of FOXP3<sup>+</sup>, T-bet<sup>+</sup> and GATA binding protein 3 (GATA-3) positive cells were affected also in the nasal mucosa by local glucocorticoid treatment.

## 1.2 T cell subsets

T cells play a central role in the control of virtually any immune response, including the allergic airway inflammation, due to their regulatory capacity. The CD4<sup>+</sup> T cells include Treg cells whose main function is to suppress the immune responses toward auto antigens, as well as exogenous antigens when they induce immune responses that can become dangerous for the host and also several subtypes of T effector cells named T helper cells (Th) which main function is to protect from pathogens. The Th1 and Th2 cells were first identified in both mice and humans, displaying different function and cytokine secretion pattern [29, 30]. The Th1/Th2 paradigm was maintained until some years ago, when a third subset, the Th17 cell was identified [31]. Recently additional subsets have been proposed such as Th9 [32], Th22 [33-35] and T follicular helper cells [36]. Lineage-specifying T cell transcription factors are defined by their sufficiency and necessity to establish cell identity, coordinate cellular differentiation, and maintain developmentally established transcriptional programs. To understand exactly how Th cells respond to immune challenges would be of great interest for future treatment of a variety of diseases.

### 1.2.1 T regulatory cells

Tissue inflammation, prevention of immunopathology, maintenance of immune homeostasis requires tight regulatory mechanisms to control exaggerated responses by T effector cells. The most prominent function of Treg cells is maintaining self-tolerance and immune homeostasis [37, 38]. The CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells and interleukin (IL)-10-producing Treg type 1 (Tr1) cells are crucial in regulating effector T cell functions [39, 40]. Naturally occurring Treg cells (nTreg), which are generated in the thymus and express the transcription factor FOXP3, inhibit effector T cells and are crucial in the maintenance of peripheral tolerance [41]. In addition, FOXP3<sup>+</sup> T cells can also be generated in the periphery, called induced Treg cells (iTreg) [42-44]. The suppressive function of the CD4<sup>+</sup>CD25<sup>+</sup> Treg cell was demonstrated when depletion of these cells resulted in multiorgan autoimmune disorders [45]. Later on, Treg cells were shown to suppress both Th1 and Th2 responses *in vitro* [46-48].

The IL-2 receptor  $\alpha$  chain (CD25) was the first surface marker on CD4<sup>+</sup> T cells which was proposed as a Treg marker [45]. CD25 is constitutively expressed on nTreg cells but CD25 is also up-regulated on T effector cells upon activation. At present, the most specific marker for Treg cells is FOXP3, a transcription factor that is essential for Treg development and

function [41, 49-51]. FOXP3 is recognized as the master regulator for Treg function controlling the expression of a wide array of genes including cytokines and surface molecules [52] and it has been demonstrated that a continuous expression of FOXP3 is needed to actively maintain the differentiated state [53]. However, even FOXP3 can be transiently up-regulated in T effector cells upon activation [54, 55] and arguments both pro and con exist for the possibility that this transient expression of relatively low amounts of FOXP3 observed in human T cells coincides with suppressor capacity at that very point in time [54-57].

Cytotoxic T-lymphocyte Antigen 4 (CTLA-4) is co-stimulatory receptors constitutively expressed on nTreg cells but are also up-regulated on T effector cells following activation [58]. The constitutive expression of CTLA-4 on Treg cells contributes to their suppressive function due to its superior affinity to CD80/CD86 (proteins expressed on APCs), outcompetes the activating receptor CD28 and thus inhibits the activating signal for T effector cells [59].

A potent immunoregulatory cytokine produced by Treg cells, with anti-inflammatory functions is IL-10. IL-10 has been reported to decrease the expression of MHC class II and costimulatory molecules on DCs, thus keeping them in a tolerogenic state [60]. IL-10 also regulates the activation and function of mast cells [61], as well as cytokine production by eosinophils [62], and has been shown to directly suppress T-cell proliferation [63].

Another immunoregulatory cytokine is transforming growth factor- $\beta$  (TGF- $\beta$ ) a crucial factor involved in the generation of iTreg cells as CD4<sup>+</sup>CD25<sup>-</sup> T cells can acquire a Treg phenotype in the presence of TGF- $\beta$  [44, 64, 65]. A crucial co-factor, which synergizes with TGF- $\beta$ , is IL-2 as neutralization of IL-2 during iTreg induction abolishes the development of suppressive function [66]. However, TGF- $\beta$  in the presence of proinflammatory cytokines, such as IL-1 $\beta$  and IL-6, can divert the induction of Treg cells toward the induction of Th17 cells [67]. Similarly can high T cell receptor (TCR) stimulation inhibit T cells to upregulate FOXP3 during differentiation towards Treg cells and instead they acquire the capacity to make tumor necrosis factor (TNF) and interferon-gamma (IFN- $\gamma$ ), as well as IL-17 and IL-9 [68].

Finally, Treg cells have been shown to play a major role in allergen specific immunotherapy (SIT) by suppression of both Th1 and Th2 cytokine responses [69]. Increasing Treg numbers and activity to maintain immune homeostasis during allergy and asthma could be a goal for future treatment.



## Treg cells in disease

Genetic mutations in the FOXP3 gene has been shown to result in a severe and fatal immune disorder known as Immune dysregulation, Polyendocrinopathy, Enteropathy X-linked (IPEX) syndrome in humans and in analogous disease in mice called Scurfy [70]. IPEX is characterized by an allergic phenotype with dermatitis and hyper-IgE syndrome and an autoimmune phenotype with enteropathy, type I diabetes, thyroiditis, hemolytic anemia and thrombocytopenia. This disease led to the finding of the importance of the transcription factor FOXP3 as a master regulator of Treg cells.

Treg numbers and activity have been found to be increased or decreased in different diseases compared to healthy controls. High numbers of CD4<sup>+</sup> Treg cells has been demonstrated in solid tumors and are associated with poorer prognosis in both humans and mice [71]. Increased Treg activity has also been demonstrated in infectious diseases such as retroviral, mycobacterial and parasitic infections [72]. On the other hand, in autoimmune disease such as multiple sclerosis, rheumatoid arthritis and type 1 diabetes [73] Treg cells are less frequent compared to healthy controls. Recent studies have also indicated that Treg cells may be involved in controlling metabolic diseases, including atherosclerosis [74] and obesity [75] displaying reduced Treg numbers.

Furthermore, in allergy, decreased numbers of Treg cells have been associated with maintained clinical active allergy to cow milk while children with increased number of Treg cells became tolerant with age [76]. In a chronic murine model, accumulation of FOXP3<sup>+</sup> Treg cells in local draining lymph nodes of the lung correlated with spontaneous resolution of chronic asthma [77]. In patients with asthma and atopic dermatitis Treg numbers was negatively correlated with IgE, eosinophilia, and IFN- $\gamma$  levels, and the FOXP3<sup>+</sup>/CD4<sup>+</sup> ratio was decreased in comparison to healthy subjects [78].

### 1.2.2 T helper 1 cells

Regulation of an efficient immune response to eliminate dangerous microbes depends on a balance of diverse Th cell subsets [79]. This is achieved by polarization of naive CD4<sup>+</sup> Th cells into effector Th cell subsets depending on the priming cytokine milieu. Th1 cells can be generated by exposure to cytokines primarily IL-12 and IFN- $\gamma$  and their role is to defend against viruses, intracellular bacteria and protozoan pathogens by activating CD8 cytotoxic T cells, Natural Killer cells and phagocytes such as macrophages [80].

IFN- $\gamma$  and IL-12 signaling promotes the expression of T-bet, a transcription factor belonging to the T-box family. T-bet is rapidly and specifically induced in developing Th1 cells and is critical for initiating Th1 development. Therefore, T-bet is considered the master regulator of Th1 differentiation [81].

IL-12 also strongly activates Signal Transducer and Activator of Transcription (STAT) 4 which can directly induce IFN- $\gamma$  in activated CD4<sup>+</sup> T cells and initiate a positive feedback-loop through T-bet to produce more IFN- $\gamma$  [82, 83]. Other cytokines such as IL-18 seems to play an important role in Th1 responses when synergizing with IL-12 to induce IFN- $\gamma$  [84, 85]. However, IL-18 seems to be able to also promote Th2 cytokine production in T cells and NK cells in synergy with IL-2 and in basophils and mast cells in synergy with IL-3 [86].

### **Th1 in disease**

In addition to their protective functions against pathogens, Th1 cells can also cause tissue damage and cause unwanted inflammatory disease and self-reactivity and thus contribute to the development of immune-mediated disorders such as inflammatory bowel disease [87], Crohn's disease, sarcoidosis, atherosclerosis [88] and graft-versus-host disease [89]. Furthermore, Th1 cells have also been implicated in autoimmune disorders such as insulin-dependent diabetes [90] and rheumatoid arthritis [91]. In chronic obstructive pulmonary disease predominate Th cells are Th1 together with Th17 [92].

Th1 cells are suggested to be inhibitory of asthmatic airway inflammation and IL-12 and IFN- $\gamma$  seems to have the capacity to inhibit antigen-induced AHR in animal models possibly through the inhibition of Th2 cytokine responses [93, 94]. Additionally, T-bet knockout (KO) mice develop spontaneous AHR and increased airway eosinophilia, including a T-bet-directed function for IL-13 in the asthmatic airway [80, 95]. In a study treating asthmatic children with inhaled corticosteroids, a T-bet polymorphism was associated with responsiveness to treatment, supporting a protective role of Th1 cells in asthma [96]. However, Th1 cell-induced AHR and inflammation has also been reported in murine experimental models [97, 98]. Finally, decreased T-bet expression by lung CD4<sup>+</sup> T cells has been demonstrated in patients with asthma, as well as several different T-bet polymorphisms associated with allergic asthma [99, 100]. Increasing Th1 activation might be a way to go for future asthma treatment in some patient groups.

### 1.2.3 T helper 2 cells

Similarly to Th1 cells, Th2 cells can also be involved in immune-mediated disorders, but are also important in the humoral defense against extracellular pathogens. [101-103]. Abnormal increase of the Th2 response often leads to chronic inflammatory airway diseases, such as atopic asthma and allergy [104-106]. The cytokine most potent and essential for Th2 cell differentiation is IL-4 [107-109]. Th2 cells themselves produce and release IL-4, IL-5, IL-9, and IL-13 [30] and are thereby contributing to the accumulation of effector cells to the site of inflammation [110-112].

Key characteristics of allergic airway inflammation are Th2 cells driven recruitment of eosinophils through IL-5 and mast cells through IL-9. In addition, IL-4, IL-9 and IL-13 are directly acting on epithelial cells inducing mucus production, goblet cell metaplasia and AHR and on smooth muscle cells through IL-4 and IL-13 [113-115].

Th2 cytokines also effect T cells, macrophages and promotes B-cells IgE synthesis [113]. IL-4 contributes to Th2 cell differentiation by activating STAT6 [116-118] and one of the mechanisms for STAT6 is to induce high levels of the transcription factor GATA binding protein 3 (GATA-3) [119]. GATA-3 expression is sufficient and necessary for Th2 differentiation [120-124]. Therefore, GATA-3 is regarded as the master regulator for Th2 differentiation. TCR signaling can also induce GATA-3 expression leading to IL-4-independent endogenously produced early IL-4 production which seems to be required for priming of CD4<sup>+</sup> T cells to develop into high-rate IL-4-producing (Th2) cells [125]. In addition to being involved in gene transcription of Th2 cytokines GATA-3 also inhibits Th1 cytokine production [120, 126].

Similar to GATA-3, another transcription factor musculo aponeurotic fibrosarcoma oncogene homolog (c-Maf) is preferentially expressed in Th2 cells [127] and selectively regulates IL-4 expression by signaling via the IL-4/IL-4 receptor/STAT6 pathway [128]. In contrast to GATA-3 and c-Maf, PU.1 obstructs the Th2 phenotype by antagonizing GATA-3 activity resulting in low IL-4 expression. [129, 130].

In addition to previously mentioned Th2 cytokines there is IL-25, an IL-17-related cytokine produced by Th2 cells, also known as IL-17E [131-133]. Interestingly, IL-25 is also produced by lung epithelial cells in response to allergens and suggested to serve as an initiation factor as well as an amplification factor for Th2 responses [114]. IL-25 can as well as IL-4 and

IL-13 induce the production of chemokines such as eotaxins, the strongest chemotactic agents for eosinophils and their progenitors [134-138]. There are three family members in humans, eotaxin-1/chemokine ligand (CCL) 11, eotaxin-2/CCL24 and eotaxin-3/CCL26, but only two in mice, eotaxin-1/CCL11 and eotaxin-2/CCL24. Eotaxins has been shown to be produced by a number of cell types including macrophages, monocytes, basophils, eosinophils and lymphocytes [139-143]. In addition, many lung structural cells such as epithelial cells, airway smooth muscle cells, vascular endothelial cells and fibroblasts have the capacity to produce eotaxins [144-147]. All three eotaxins act through the eotaxin-receptor CCR3 present primarily on eosinophils in both humans and mice, but also on a subset of Th2 cells and mast cells in humans [148-150].

Furthermore, Th2 cytokines and especially IL-13 contributes to remodeling effects in the asthmatic airway [151, 152]. Recent data demonstrates that Th2 cytokines regulates periostin, an extracellular matrixprotein, promoting increased remodeling and accelerating inflammation by enhancing chemokine production and thereby eosinophil recruitment [153, 154].

## **Th 2 cells in disease**

The classical Th2 cytokines initiate and maintain key pathophysiological features of asthma and allergy: IL-4 being important for allergic sensitization and IgE production, and IL-5 is crucial for eosinophil survival; IL-13 has pleiotropic effects in the lungs, including a central role in the development of AHR and tissue remodeling [155].

A disease strongly associated with atopic disease is eosinophilic esophagitis where Th2 cells play an important role by inducing eosinophilic inflammation [156]. Th2 polarization has also been demonstrated in systemic sclerosis with reports of high levels of Th2 cytokines in serum [157]. Lymphocytic form of hypereosinophilic syndromes [158] including eosinophilic granulomatosis with polyangiitis also known as Churg–Strauss [159] are other examples of diseases driven by a strong Th2 immune response. IL-4 has also been implicated in autoimmune disorder like Sjögren’s syndrome by its involvement in proliferation and differentiation of B and T cells, but in this context leading to class switching to pathogenic IgG1 autoantibodies [160]. Chronic graft-versus-host disease was initially considered a Th2-mediated disease but the immune mechanisms seems to be more complex involving several other cells and mediators [161]. Suppression of Th2 activation could therefore be a goal of therapy in not only allergic disease.

## 1.2.4 T helper 17 cells

Similar to Th1 and Th2 cells, Th17 cells are differentiated by specific cytokines. The development of Th17 cells seems to include three overlapping steps of differentiation, amplification, and stabilization. Naïve CD4<sup>+</sup> T cells differentiate in response to TGF- $\beta$  plus IL-6 whereas IL-21 produced by developing Th17 cells mediates amplification and IL-23 is needed for expansion and stabilizes previously differentiated Th17 cells [162-165].

Th17 cells received their name from their capability to produce IL-17 and not Th1 and Th2 cytokines. IL-17A is the prototypic member of the IL-17 family of cytokines and includes additionally five members IL-17B, -C, -D, -E (also known as IL-25) and -F [166-169]. Th17 cells themselves produce IL-17A IL-17F [170, 171], IL-21 and IL-22 [172-176]. Th17 cytokines are capable to mobilizing innate immunity [165], promotes tissue inflammation and are key cytokines in neutrophil migration [177-179].

Both maintenance of cytokine production in Th17 cells *in vitro* and Th17 cell-mediated inflammation *in vivo* requires the induction of retinoic acid-related orphan receptor- $\gamma$ t (ROR $\gamma$ t) [180, 181]. Thus, ROR $\gamma$ t is regarded as the key lineage defining transcription factor for Th17 cells [180, 182]. However, essential for ROR $\gamma$ t is activation of STAT3 [183, 184].

In addition, Th17 cells have been shown to promote B cell IgG2a and IgG3 synthesis [185] and to play a critical role in forming ectopic lymphoid follicles in target organs, a hallmark of several autoimmune inflammatory diseases. [186].

### Th17 cells in disease

Th17 cells are important in the immune response to extracellular pathogens and play critical role in autoimmunity. IL-17 expression has been associated with diseases such as multiple sclerosis, rheumatoid arthritis, psoriasis, inflammatory bowel disease and chronic obstructive pulmonary disease [187-190], as well as in allergic responses.

In several different murine models of allergy and asthma the contribution of Th17 cells have been demonstrated. IL-17A-mediated neutrophilia contributes to impaired regulation of Th2 responses resulting in AHR and enhanced airway inflammation observed in T-bet KO mice. [191-193]. In murine models of allergic asthma and models of LPS promoted sensitization an IL-17-dependent AHR develops [194]. Lung Th17 cell numbers are also elevated in house dust mite (HDM) allergy model in mice [195]. Thus, IL-17 cytokines can contribute to the enhancement of Th2 cell responses and in the

development of asthma but seemed to have inhibitory effect on already established allergic disease [178, 196, 197].

In humans, IL-17 and ROR $\gamma$ t has been shown to be elevated in peripheral blood mononuclear cells (PBMC) and in sputum of asthmatic subjects [198], shown to correlate with AHR [199] and associated with severe asthma [200]. Thus, Th17 cells may potentially be implicated in subtypes of asthma and allergy.

### 1.2.5 T cell plasticity

Naive T cells travel to T-cell areas of secondary lymphoid organs in search of antigen presented by DCs [201, 202]. Upon activation, they rapidly proliferate, generating T effector cells that can migrate to B-cell areas or to inflamed tissues [203-206]. A small number of primed T cells persist as circulating memory cells that can, upon secondary challenge, confer protection and resulting in enhanced response [207-209].

Each T cell subset maintains its cytokine expression and represses opposing T cell cytokine expression by the regulation of master transcription factors [210], chromatin remodeling and epigenetic modifications of specific cytokine gene loci [211, 212]. These epigenetic modifications are inherited by daughter cells as the cell divides and persists in memory cells thus explaining the commitment of T cell lineages [211].

Recently, the classical T cell lineage commitment has been challenged [213] by accumulating evidence from animal models and *in vitro* studies to suggest that Th cell subsets are not irreversibly differentiated but can exhibit plasticity by changing cytokine production, transcription factor expression or by expressing multiple transcription factors [213-217]. It has been reported that Treg cells can be converted into Th17 cells under Th17 polarizing conditions, and that Th17 cells can become Th1 cells in the presence of IL-12 [218-220]. Th17 cells co-expressing both ROR $\gamma$ t and T-bet are highly pathogenic in inducing experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis [221]. Furthermore, Th1 and Th2 cells have been demonstrated to be able to be converted under proper conditions promoting the opposite Th cell [222]. In addition, Th2 memory cells have been converted into functional Treg cells [223] or Treg cells acquiring GATA-3 to be able to accumulate at inflamed sites and to maintain high levels of FOXP3 expression in various polarized or inflammatory settings [224].

This plasticity of T cell differentiation seems to play a role in modulating inflammatory disease [213, 225]. It is likely advantageous for Th cells to be able to convert from one type to another to efficiently fight against ongoing immunological insults. This accumulating evidence from animal models and *in vitro* studies [226-229] raised the question of whether circulating T cells could co-express master regulatory transcription factors in healthy humans and/or asthmatics.

## 1.3 RNA

RNA is involved in the expression of all genes. RNA is a single stranded polymer composed of four different nucleotides; adenine, guanine, cytosine and uracil. RNA can be divided into coding and non-coding RNA. The coding RNAs include mRNA and it is translated into protein by the ribosome, thereby acting as template for protein synthesis and comprises ~1-5% of the total RNA in a cell. The remaining ~95% is made up of non-coding RNA with ribosomal RNA (rRNA) (~80%), transfer RNA (tRNA) and small RNA, such as miRNA. Noncoding RNAs are participants in gene regulation of gene expression that is regulated in every step for complex organism development and appropriate responses to cell stress and environmental stimuli.

### 1.3.1 MicroRNA

miRNA are small non-coding RNA molecules, which are endogenously expressed and conserved among species [230]. They function as posttranscriptional modulators of gene expression resulting in reduced protein production [231, 232]. In fact, a single miRNA can regulate many biological functions by suppressing hundreds of target mRNAs by binding to complimentary sequences at the 3'untranslated regions (UTRs) of target mRNAs called the 'seed' [233]. This group of small gene regulating RNAs was first discovered in nematodes in 1993 [230], and more than 17,000 mature miRNAs in 140 species have been identified [234].

The biogenesis of miRNA starts in the nucleus where it is transcribed by RNase polymerase II as long primary miRNAs (pri-miRNAs) [235]. Pri-miRNAs are then processed by the RNase III enzyme Drosha and Pasha/DiGeorge syndrome critical region 8 (DGCR8) creating ~70 nucleotide stem-loop structures called pre-cursor miRNAs (pre-miRNAs) [236, 237]. Pre-miRNAs are then transported out of the nucleus to the cytoplasm by Exportin-5, where it is cleaved by the enzyme Dicer into ~22 nucleotide miRNA duplexes [238-240]. The miRNA duplex is then unwound

by the helicase activity, and subsequently one of the mature miRNA strands is assembled into the RNA-induced silencing complex (RISC) which includes Argonaute protein. This stabilizes the miRNA, whereas the passenger strand is short lived. However, in some cases the passenger strand can also become functional [241]. The miRNA-RISC complex then binds to the target mRNA, repressing translation or degrading the mRNA [242-246]. Like protein-coding genes, miRNAs can be regulated at the transcriptional level, downstream of signaling pathways that activate or inhibit transcription factors and chromatin remodeling. Furthermore, regulation of mature miRNA turnover in the immune system has also been demonstrated [247].

miRNAs play a role in a large diversity of biological processes including development, homeostasis, metabolism, cell proliferation, cell differentiation and angiogenesis. The effect of an individual miRNA on a target's protein level tends to be subtle, usually less than 2-fold, indicating that for most interactions miRNAs acts as fine-scale tuners to protein output [248, 249]. Organisms are therefore usually able to compensate for loss-of-function mutations, but there are miRNA-target interactions that involve multiple sites for a given target resulting in much stronger repression [250, 251] or that different miRNAs work together to target a given mRNA, so their combined repressive effect exceeds the individual contributions. On the other hand, by repressing negative regulators in a pathway, a miRNA can also increase signal output [252]. Cross-regulation between signaling proteins and miRNAs can also occur by participating in regulatory loops controlling cellular responses [253]. However, abnormal expression of miRNAs can result in changed post-transcriptional regulation of mRNAs, which can lead to a diversity of diseases such as cancer and inflammatory diseases [254, 255].

### **miRNA in allergy**

The role of miRNA in murine models of allergic airway inflammation has been demonstrated as miR-21 regulates Th2 polarization by regulation of IL-12p35 [256]. In addition, miR-126 and miR-145 has been shown to be of interest in both innate and adaptive immune responses in a HDM mouse model of allergic airway inflammation showing that antagonism of these miRNAs results in significantly suppressed Th2 effector functions [257, 258]. These studies associate miRNAs in the pathogenesis of allergic inflammation.



### 1.3.2 MicroRNA-155

miR-155 is known as a multifunctional miRNA, expressed both in mice and humans, that has been implicated in carcinogenesis as well as in innate and acquired immunity [259, 260]. miR-155 amplifies signaling and is required for robust innate and adaptive immune responses thus important for normal immune function [261]. miR-155 is transiently regulated in many cell types of the immune system demonstrated by activation of T cells, B cells, DCs and macrophages leading to increased expression [232, 254, 255]. miR-155 expression in myeloid cells promotes expression of pro-inflammatory cytokines, suggested to be regulated by suppressor of signaling 1 (SOCS1) and SH2 domain-containing inositol phosphatase-1 (SHIP1), both negative regulators of the pro-inflammatory pathways [253, 262]. Furthermore, macrophages inhibited in miR-155 expression seem to be tolerant to endotoxin while overexpression leads to hypersensitivity [262, 263]. However, LPS treatment *in vivo* did not affect miR-155 expression in the lung [264].

In general, miR-155 KO mice seem to have a weak immune response to immunization and infection [261, 265]. Additionally, miR-155 deficient CD4<sup>+</sup> T cells were found to produce more IL-4 and less IFN- $\gamma$  upon TCR stimulation under neutral conditions *in vitro* and it was suggested that miR-155 KO mice were skewed towards a Th2 response and miR-155 was important for Th1 function. Furthermore, TCR stimulation *in vitro* of Th2 cells from these mice had increased c-Maf expression, a direct target of miR-155 [261]. In Treg cells, FOXP3 induce miR-155 expression, leading to repression of unwanted genes such as SOCS1, demonstrating regulatory effects of miR-155 on Treg development and homeostasis, but not affecting suppressor function [266, 267]. In addition, miR-155 deficiency resulted in reduced expression of CD103 on Treg cells which might affect their recruitment to sites of infection [268].

#### miR-155 in disease

Furthermore, in animal models of autoimmune disease a role for miR-155 has been demonstrated: miR-155 KO mice were shown to be resistant to EAE due to compromised inflammatory T-cell development and myeloid DC function [269]. miR-155 deficiency dampen T cell-mediated induction of colitis in a model of inflammatory bowel disease and miR-155 KO mice were resistant to antigen-specific Th17 cell responses in a model of collagen-induced arthritis [270, 271].

In terms of allergic diseases it is interesting to note that the *B-cell Integration Cluster (BIC)/miR-155* gene is located within a region on chromosome 21q2 which is associated with pollen sensitivity and asthma and atopic dermatitis susceptibility [272]. Furthermore, recent studies demonstrate miR-155 overexpression in the nasal mucosa of AR patients and in the skin of atopic dermatitis patients, suggesting a role for this miRNA in the pathogenesis of allergic diseases [273, 274].

## 2 AIM

### **The overall aims of this thesis are to investigate**

- I. Glucocorticoid treatment and natural pollen exposure and the effects it poses on T cell subsets in the nasal mucosa of AR patients.
- II. Plasticity in circulating T cell subsets and the relationship to eosinophilia in asthmatic individuals.
- III. miRNA-155 affecting T cell dependent allergen induced eosinophilic airway inflammation.

### **Specific questions for each paper are**

#### Paper I:

- Whether the amount of Treg, Th1 and Th2 cells is altered in the nasal mucosa of patients with AR.
- Whether a natural grass-pollen season as well as concomitant glucocorticoid treatment in the nasal mucosa of AR patients affects the amount of and the balance between Treg, Th1 and Th2 cells.

#### Paper II:

- Whether a specific asthma endotype could be distinguished by the immunological profile of circulating Treg, Th1, Th2 and Th17 cells.
- Whether circulating Treg, Th1, Th2 and Th17 cells express more than one master transcription factor, indicating an ability of plasticity.

#### Paper III:

- Whether miR-155 regulates allergen induced T cell dependent airway eosinophilia and if so through eosinophilopoiesis or recruitment
- Whether miR-155 plays a role for the development of airway Treg, Th1, Th2 and Th17 cells.
- Whether miR-155 modulates allergic inflammation by influencing allergen-mediated Th2 responses.
- Whether a specific target of miR-155 can be determined in a model of allergen induced airway inflammation.

## 3 PATIENTS AND METHODS

The methods used in this thesis are described below. Roman letters indicates referred paper. For additional information see the material and methods section in paper I-III.

### 3.1 Study designs

#### 3.1.1 Paper I

The Ethics Review Committee of Clinical Research Studies at the University of Tartu, Estonia approved the study and all patients gave their written informed consent.

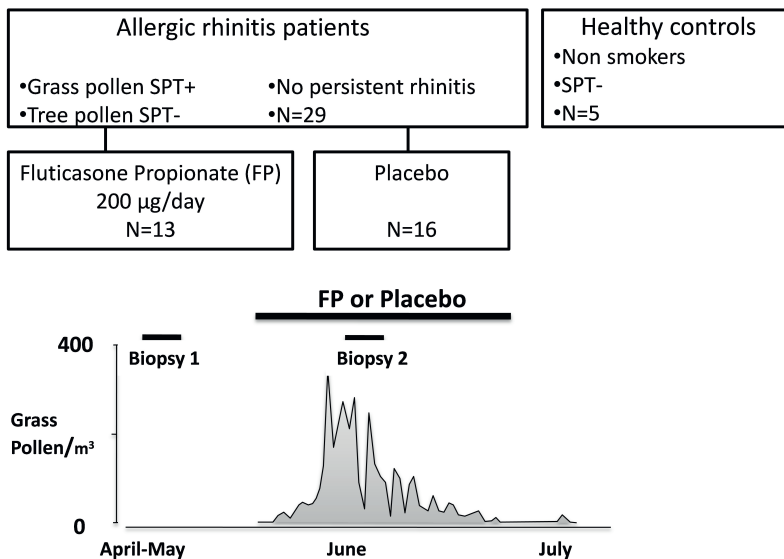


Figure 1. Study design paper I. Nasal biopsies from AR patients were obtained on two occasions, prior to and at the peak of the grass-pollen season. AR patients received either FP treatment or placebo.

Patients included in the study were 15-48 years old. They had a diagnosed grass-pollen induced AR during at least two previous years. Allergy to grass pollen was confirmed by skin prick tests (SPT). Exclusion criteria included perennial rhinitis and a positive SPT response to tree pollens. Grass-pollen sensitized AR patients were treated with either a nasal glucocorticoid (FP,

200 $\mu$ g/day) or respective placebo for 50 days throughout the grass-pollen season, starting approximately 2 weeks before the expected beginning of the season (figure 1). Healthy controls had no history of allergic disease as confirmed by negative SPT [275].

### 3.1.2 Paper II

Ethical approval for the study was granted by the Regional Ethical Approval Committee in Gothenburg, Sweden (no.593-08). All subjects gave their written informed consent.

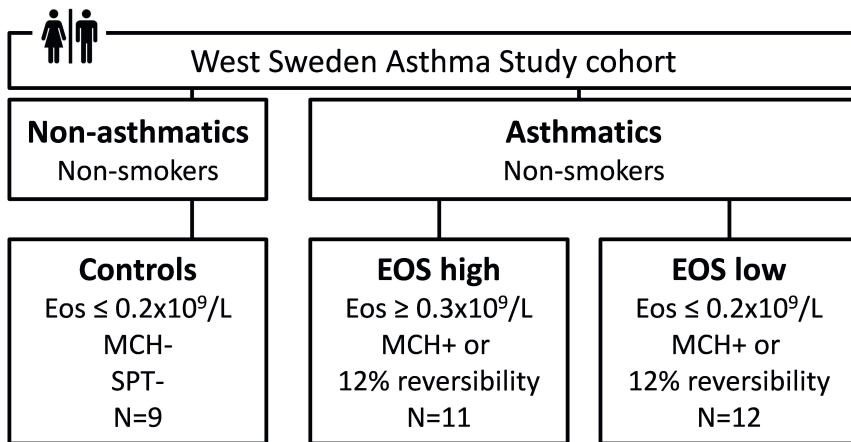


Figure 2. Study design paper II. Study participants from the WSAS study were divided into groups based on MCH reactivity, SPT status and blood eosinophil levels.

Study participants were selected from questionnaire respondents in the asthma cohort study WSAS who attended a detailed clinical examination at the Krefting Research Centre, Gothenburg, Sweden and for whom clinical data was available. Participants included in the study were 27-57 years old. Inclusion criteria were asthma diagnosed from reports of common symptoms and a PD20 (provocative dose resulting in a 20% reduction in forced expiratory volume in 1 second (FEV<sub>1</sub>)) for MCH below a cumulative dose of 1.96mg or FEV<sub>1</sub> reversibility greater than 12%. Asthmatics were considered to have high numbers of eosinophils if blood eosinophils were above 0.3x10<sup>9</sup>/L and low numbers of eosinophils if values were below 0.2x10<sup>9</sup>/L. Healthy controls did not report asthma symptoms, were non-reactive to MCH or non-reversible, were SPT negative and had blood eosinophil count below 0.2x10<sup>9</sup>/L (figure 2). Four weeks preceding the participation in the study

none of the subjects had received any vaccination, changed their asthma medication, had any worsening of asthma symptoms, reported any symptoms of infection or cold, had any surgery, had any antibiotics, had any new medication, or had any anti-inflammatory medication i.e. non-steroidal anti-inflammatory drugs (NSAIDs).

### 3.1.3 Paper III

The Animal Ethics Committee at University of Gothenburg, Gothenburg, Sweden approved the study (no. 323-2011). Gene knockout mice lacking *bic/miR-155* function/reporter allele (B6.Cg-*MiR155tm1.1Rsky/J*) and control C57BL/6J mice were purchased from Jackson Laboratories, Bar Harbor, ME.

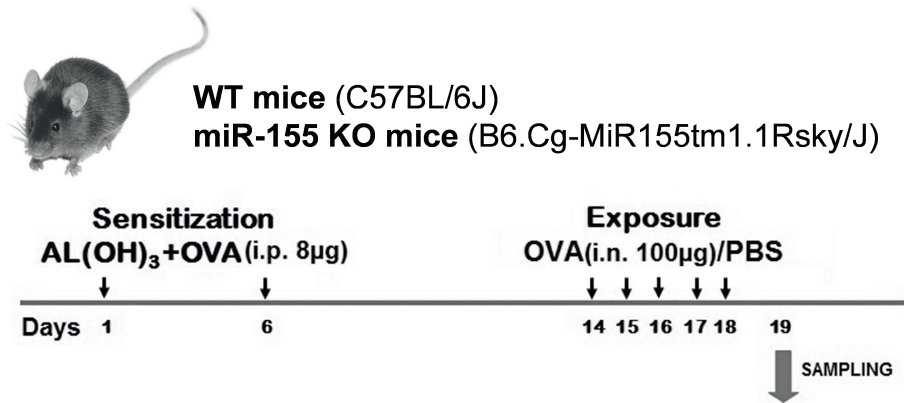


Figure 3. Standard protocol for the model of allergen induced airway inflammation. WT and miR-155 KO mice were allergen sensitized and exposed (OVA/OVA) or allergen sensitized and PBS exposed (OVA/PBS) or kept naïve.

Age- and sex-matched mice were used in all experiments and were used at 6-10 weeks old. Mice were kept in pathogen free conditions with food and water *ad libitum*. The animals were briefly anaesthetized using isoflurane when exposed. The standard protocol to sensitize and expose the mice were performed by two intraperitoneal (i.p.) injections of the allergen ovalbumin (OVA) bound to aluminum hydroxide in phosphate buffered saline (PBS), followed by five intranasal (i.n.) installations of allergen or PBS as a control (figure 3). In some experiments miR-155 KO mice received i.n. instillation of eotaxin-2 (5µg in 25µl PBS 0.1% BSA) one hour prior to allergen challenge. In adoptive transfer experiments naïve wild type (WT) and miR-155 KO mice received allergen-specific CD4<sup>+</sup> T cells i.p. 24 hours prior to allergen challenge (described in more detail below and in fig E1 paper III).

## 3.2 Clinical parameters

### 3.2.1 Structured interviews (II)

Structured interviews were conducted by trained nurses at the clinical visit. Questions on airway symptoms and diseases, rhinitis and allergies were included. Additional questions were type of medication and frequency of usage and smoking history.

### 3.2.2 Skin prick test (I, II)

SPT were used to test for sensitization to airborne allergens. Histamine (10mg/ml) and diluent with no allergen was used as a positive and negative control, respectively. The allergens were applied according to standardized methods and a wheal and flare reaction of larger than 3mm was considered positive [276]. Study participants were asked to avoid usage of anti-histamines for at least 72 hours before the test.

#### Paper I

AR patients were tested for 6 allergens: HDM *Dermatophagoides (D.) pteronyssinus*, cat, birch and grass-pollens from *Lólium perénne* (perennial ryegrass), *Festúca praténsis* (meadow fescue) and *Phleum preténse* (timothy grass). Most important was to test if patients were sensitized to birch as this was one of the exclusion criteria's. To exclude sensitization in control subjects, they were tested with a standard panel consisting of the 10 most common aeroallergens.

#### Paper II

A standardized panel was used consisting of the following 11 allergens: HDMs *D. pteronyssinus* and *D. farinae*, fungi *Alternaria alternate* and *Cladosporium herbarium*, cockroach *Blatella germanica*, dog, cat, horse, *Phleum preténse* (timothy grass), mugwort and birch.

### 3.2.3 Lung function, reversibility and methacholine test (II)

Lung function was performed using a Masterscope Spirometer (Jaeger, Hoechberg, Germany). FEV<sub>1</sub>% predicted was calculated using the ECCS reference equation [277]. Study participants were asked to avoid usage of long-acting and short-acting bronchodilators for 24 and 8 hours, respectively, before the test.

Reactivity to MCH was determined using Spira equipment (Spira Respiratory Care Center Ltd.) following a shortened protocol. The highest cumulative dose was 1.96mg. The cumulative dose where a 20% decrease in FEV<sub>1</sub> was reached, was calculated using the formula:  $PD20 = A + ((20-B)*(C-A))/(D-B)$ , where A = administered dose MCH prior to 20% decrease in FEV<sub>1</sub>, B = % decrease in FEV<sub>1</sub> after A, C = administered dose MCH causing a minimum of 20% decrease in FEV<sub>1</sub> and D = % decrease in FEV<sub>1</sub> after C.

Reversibility test was performed at the same visit as MCH challenges, meaning that not all subjects were tested in an optimal way. In cases where the subject first underwent a MCH challenge, the subjects were given 4x0.1mg of salbutamol (Ventoline®) followed by two capsules of 4µg ipratropium bromide (Atrovent®) with the reversibility spirometry measured 30 minutes after. In cases where no MCH was given, the subject was administered 4x0.1mg of Ventoline and spirometry was performed after 15 minutes.

### **3.2.4 Exhaled nitric oxide (II)**

Fraction of exhaled nitric oxide (FeNO) was performed to identify inflammation in different parts of the lung. The study participants performed two exhalations, measured using a NIOX (Aerocrine AB) at a flow rate of 50ml/s. Values given in paper II are an average of the two measurements.

## **3.3 Sample collection and preparation**

### **3.3.1 Nasal biopsies (I)**

Samples from patients were obtained on two occasions during the study. The pre-season samples were taken 1 to 2 months before initiation of treatment and the in-season samples during the peak of the grass-pollen season. Samples from healthy controls were obtained on one occasion prior to the grass-pollen season (figure 1). Nasal biopsies were immediately embedded in Tissue-Tek O.C.T. compound and snap-frozen in liquid nitrogen. Frozen samples were stored at -80°C.

### **3.3.2 Nasal lavage (II)**

Nasal lavage was collected from all participants who gave their consent. With the head tilted back and pharynx closed, 5ml of 10% saline was instilled into the left nostril. Immediately after instillation the head was tilted forward and the fluid passively collected. The nasal lavage samples were centrifuged,



cells resuspended and cytospin preparations for differential cell count were prepared.

### **3.3.3 Induced sputum (II)**

Sputum induction and processing of whole sputum was performed according to the European Respiratory Society guidelines [278] with minor modifications.

A pre-bronchodilator FEV<sub>1</sub> was performed to ensure that the participant could perform the test safely. Administration of one dose of 0.4 mg inhaled salbutamol was followed, 10 min after, by a post-bronchodilator FEV<sub>1</sub>. Induced sputum was not performed if FEV<sub>1</sub> was less than 1.5L or 50% of expected value.

Induced sputum was performed by inhalation of a fixed concentration of 4% sterile saline solution for 7 min using an ultrasonic nebuliser. The nebulization was stopped if symptoms of obstruction occurred and the participant received a bronchodilator if needed. The specimen was obtained by coughing and spitting followed by a FEV<sub>1</sub> measure. In the case of a fall of FEV<sub>1</sub> of >20% compared with the post-bronchodilator value the test was stopped and if needed the participant received a bronchodilator. If FEV<sub>1</sub><20%, the procedure was repeated.

The specimen consisting of sputum and saliva were kept cold and examined within 2 hours, using a modified method described by Pizzichini et al [279]. Sputum plugs were selected using a microscope, weighted and then mixed with 4 volumes x sputum weight of Sputolysin (6.5mM dithiothreitol in 100mM phosphate buffer, pH 7.0). Samples were incubated for 15 min during gentle mixing in room temperature (RT). PBS was added in an equal volume to the homogenized sputum and then passed through a 70µm cell strainer. The cell suspension was centrifuged and the cell pellet was resuspended in PBS. Cell counts were performed using a standard hemocytometer and cytospin preparations for differential cell counts were prepared.

### **3.3.4 Blood (II, III)**

#### **Paper II**

Peripheral blood was collected into ethylenediaminetetraacetic acid tubes (EDTA; BD Vacutainer® K2E). Peripheral whole blood cells were stained for flow cytometry analysis within 1 hour of sampling. PBMCs obtained from whole blood were used for *in vitro* activation.

### **Paper III**

The animals were deeply anesthetized with a mixture of xylazin (130mg/kg, Rompun®) and ketamine (670mg/kg, Ketalar®). Blood were collected from animals by puncturing the right heart ventricle. One part blood was centrifuged and serum saved. A second part blood was added immediately to 4 parts of 2mM EDTA in PBS. Red blood cells were lysed in 5ml lysing solution (0.1% potassium bicarbonate and 0.83% ammonium chloride in distilled water) for 15 min at RT. White blood cells were washed and resuspended in 1% fetal calf serum (FCS) in PBS. Cell counts were performed using a standard hemocytometer and cytopsin preparations for differential cell counts were prepared.

#### **3.3.5 BALF (III)**

Following blood collection, the mice were tracheotomised and bronchoalveolar lavage was performed by instilling 0.25ml PBS through the tracheal cannula, followed by gentle aspiration and a second lavage with 0.20ml PBS. The bronchoalveolar lavage fluid (BALF) was centrifuged and supernatant and cells separated. Cell counts were performed using a standard hemocytometer and cytopsin preparations for differential cell counts were prepared. Cell-free supernatant was kept at -80°C until further analysis.

#### **3.3.6 Lung tissue (III)**

After BALF recovery, an additional 1ml of PBS was used to wash away airway lumen inflammatory cells, before the lungs were perfused and removed to detect/harvest parenchymal inflammatory lung cells. The left lobe was in some experiments filled with 1ml of OCT compound and PBS solution containing 20% sucrose and immediately frozen in liquid nitrogen, stored in -80°C until further processed for lung histology. The left lobe were in some experiments frozen without being embedded in OCT compound and later used for lung homogenate. The apical lobe was placed in RNAlater and after 24 hours at 4°C, stored at -80°C until further processed for RNA isolation. Three lobes, without any connective tissue, were stored on ice in Hanks balanced salt solution (HBSS) until further processed for single cell suspension.

#### **3.3.7 Lung single cell (III)**

The right lung lobes were weighted and rinsed in a Petri dish before being transferred to a GentleMACS™ C Tube containing 5ml of HBSS supplemented with 10% FCS, 100µl collagenase D solution (final concentration 2mg/ml) and 20µl deoxyribonuclease (DNase) I solution (final

concentration 80U/ml). The mouse lung was dissociated using a GentleMACS™ Dissociator according to the manufacturer's instructions. Lung single cells were washed in PBS supplemented with 10% FCS. Cell counts were performed using a standard hemocytometer and cytopspin preparations for differential cell counts were prepared. Single cells were further used for flow cytometry analysis.

### **3.3.8 Lung homogenate (III)**

Left lobe was used for measurement of cytokines and chemokines in lung homogenates. Briefly, each lobe was weighted and then put in a GentleMACS™ C tube containing 1ml PBS supplemented with 1 dissolved tablet of protease inhibitor, Complete-Mini. Lobes were then sliced using the GentleMACS™ Dissociator. Tissues were further disrupted by mixing with a pipette and then centrifuged. Supernatants were collected and kept at -80°C until cytokine or chemokine measurements.

### **3.3.9 Spleen and Peribronchial lymphnodes (III)**

Spleen and peribronchial lymph node (PBLN) were removed and single cell suspension of splenocytes and PBLN cells were obtained by passing exudate through cell strainers. Samples were washed and cell counts were performed using a standard hemocytometer and cytopspin preparations for differential cell counts were prepared. Single cells were further processed for *in vitro* assays.

### **3.3.10 Bone marrow (III)**

Bone marrow was harvested by excising one femur which was cut at the epiphysis and flushed with 2ml of PBS and the cells were collected in tubes through a 70µm cell strainer. Bone marrow samples were washed and cell counts were performed using a standard hemocytometer and cytopspin preparations for differential cell counts were prepared.

### **3.3.11 Total RNA isolation (III)**

After termination of the experiment, lung, spleen and PBLN tissue was immediately placed in RNAlater and after 24 hours at 4°C samples were kept in -80°C until further processing. Tissues were then homogenized and lysed with QIAzol Lysis Reagent in GentleMACS™ M tubes using a GentleMACS™ Dissociator. Total RNA including miRNA was isolated with the Qiagen miRNeasy mini kit and contaminating DNA was removed by DNase treatment of the samples with Turbo DNA-free™ kit, according to manufacturer's protocol.

## **3.4 Analysis**

### **3.4.1 Immunohistochemistry (I)**

Immunostaining of tissue sections from human nasal mucosa was used to evaluate the number of Th1, Th2 and Treg cells using antibodies to detect cells expressing transcription factors T-bet (mouse anti-human T-bet, clone 4B10), GATA-3 (mouse anti-human GATA-3, clone HG3-31) and FOXP3 (mouse anti-human FOXP3, clone 236A/E7) respectively. Biotinylated Rabbit F(ab')<sub>2</sub> anti-mouse Ig followed by ExtrAvidin-Peroxidase was used as a secondary steps. Bound antibodies were visualized by means of the Liquid DAB Substrate-Chromogen System and Mayers Hematoxylin was used for counterstaining. All slides were coded and assessed in a blinded manner. Cells were counted using a Zeiss Axioplan microscope, the tissue area was measured with the image analysis software Beta 4.0.3 of ScionImage (Scion Corporation) and data expressed as cells/mm<sup>2</sup>.

### **3.4.2 Differential cell count (II, III)**

Cells were stained with May-Grünwald and Giemsa and differential cell counts were established by counting 300-500 cells using a Zeiss Axioplan microscope. The results of the differential counts from induced sputum are expressed as a percentage of the total number of non-squamous cells. Differential cell counts from animal samples are expressed as percentage of total cells, per gram tissue weight or per volume.

### **3.4.3 Lung histology (III)**

Mouse lung tissue sections of 5µm were cut using a Leica CM1900 UV-cryostat®. Hematoxylin-eosin staining was performed for evaluation of inflammatory cells. Periodic Acid Schiff (PAS) was used for detection of mucus producing cells and quantified by counting 10 high power fields in each slide using a Zeiss Axioplan microscope.

### **3.4.4 Flow Cytometry (II, III)**

Flow Cytometry is an excellent method to analyze protein expression at single-cell level. However, instrument capacity and availability of antibodies for the target of interest sets the limit. Gating of transcription factors and surface markers were determined using control samples by the Fluorescence Minus One (FMO) approach i.e. controls containing all markers except the one of interest were used to set gates. All flow cytometric analyses (400,000-500,000 events per sample) were performed using a BD FACSAria™ Flow

Cytometer running BD FACS Diva™ v6.0 Software and analyzed with FlowJo Software®.

## Paper II

Peripheral whole blood cells were stained for flow cytometry within 1 hour of sampling. The total cell count was obtained using Trucount™ tubes together with antibodies detecting CD45/CD3/CD4 or CD45/CD3/CD4/CD14/CD19 according to manufacturer's protocol. Additionally, the following antibody panels were used:

- a) CD4/CD25/T-bet/GATA-3/ROR $\gamma$ t/FOXP3 w/wo CD3
- b) CD45RO/CD45RA/CD3/TCR $\alpha\beta$ /TCR $\gamma\delta$ /CD4/CD8
- c) CD45/CD56/CD16/CD19/CD14
- d) CD4/CD25/Ki-67/T-bet/GATA-3/ROR $\gamma$ t
- e) CD4/CD25/Ki-67/T-bet/ROR $\gamma$ t/FOXP3

All monoclonal antibodies were purchased from BD Biosciences, eBioscience or Invitrogen (detailed in Table S3, paper II). In some samples, the nucleic acid dye 7-Amino-Actinomycin D (7AAD) was used to select viable cells. Red blood cells were lysed using BD FACSTM Lysing solution according to manufacturer's protocol. After washing in PBS supplemented with 1% FCS, cells were run immediately or subjected to further intracellular staining after fixation with Fix/Perm solution according to the manufacturer's protocol for the Foxp3 staining buffer set (eBioscience™).

## Paper III

For detection of surface antigens; CD3-Fluorescein (FITC; clone 145-2C11), CD4-phycoerythrin (PE; clone H129.19), CD4-Alexa Fluor®700 (clone RM4-5), CD25-allophycocyanin (APC; clone PC61), were used. After fixation with Fix/Perm solution according to manufacturer's protocol for the Foxp3 staining buffer set, intracellular staining proceeded with the following antibodies: T-bet-Peridinin-Chlorophyll-Protein-Complex-Cyanine 5.5 (PerCP-Cy5.5; clone eBio4B10), GATA-3-PE (clone TWAJ), ROR $\gamma$ t-APC (clone AFKJS-9) and FOXP3-Alexa Fluor®488, FOXP3-PE (clone FJK-16s). Viable lung cells were identified as 7-AAD<sup>-</sup> cells in a gated population based on forward and side scatter (FSC-SSC) profiles. T cells were then identified as CD3<sup>+</sup> or CD4<sup>hi</sup> cells from a mononuclear cell gate and within these gates the subsequent T cell subsets were identified as Th1 cells (CD4<sup>+</sup>T-bet<sup>+</sup>), Th2 cells (CD4<sup>+</sup>GATA-3<sup>+</sup>), Th17 cells (CD4<sup>+</sup>ROR $\gamma$ t<sup>+</sup>) and Treg cells (CD4<sup>+</sup>FOXP3<sup>+</sup>).

### 3.4.5 *In vitro* activation (II, III)

#### Paper II

T cell activation is triggered by interaction of the TCR by antigens associated with the cognate major histocompatibility complex (MHC) molecules followed by a second signal from co-stimulatory molecules such as CD28. This activation is simulated by the following method. Human PBMCs were isolated by density centrifugation using Ficoll-Paque™ and cultured in serum-free AIM-V® + AlbuMAX® medium alone or in plates pre-coated with anti-CD3/anti-CD28 antibodies (1 µg each/mL) for 48 hours, before flow cytometry staining using panel A i.e. CD3/CD4/CD25/T-bet/GATA-3/RORγt/FOXP3. Cell viability was analyzed using 7AAD exclusion in the FSC-SSC gate. Cell viability was ~96% (range 96.3–99.1%) in cells harvested from medium alone and ~88% (range 88.4–95.8%) in cells harvested from anti-CD3/anti-CD28 coated wells.

#### Paper III

Single cell suspension of splenocytes and PBLN cells from allergen sensitized and exposed treated WT and miR-155 KO mice were processed for further enrichment of untouched splenic CD4<sup>+</sup> T cells was performed using magnetic separation according to manufacturer's protocol (CD4<sup>+</sup> T cell Isolation kit II, MACS®). Lymph node cells and spleen CD4<sup>+</sup> T cells were cultured separately *in vitro* at a density of 5x10<sup>6</sup>/ml in complete media (RPMI 1640 medium, 10% FCS, 1% penicillin/streptomycin, 1% sodium pyruvate, 1% L-glutamine) in the presence or absence of 200 µg/ml OVA for 6 days. Cell-free supernatants were harvested and stored at -80°C until analysis.

### 3.4.6 Confocal microscopy (II)

CD4<sup>+</sup> T cells were enriched from PBMCs according to the manufacturer's protocol (Human CD4 T Lymphocyte Enrichment Set, BD IMag™) and stained with the following combinations: a) T-bet/GATA-3, b) FOXP3/GATA-3, c) T-bet/RORγt and d) FOXP3/RORγt. The detection signal was enhanced using fluorochrome conjugated secondary antibodies (detailed in Table S3, paper II). Cytospins were made after completion of the staining procedure and mounted with ProLong® Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI). Images were captured with a Zeiss LSM 510 META microscope using Zeiss LSM 510 software. Confocal images were made by Z sectioning at scale 0.38 µm.

### 3.4.7 Cytokines and Chemokines (III)

For quantification of cytokines and chemokines in lung homogenates, BALF, serum and cell free supernatants we used enzyme-linked immunosorbent assay (ELISA) or BD™ Cytometric bead array (CBA). Both methods are very sensitive and pictogram levels of released mediators can be detected.

#### ELISA

Eotaxin-1/CCL11, eotaxin-2/CCL24, and periostin levels in cell-free BALF and eotaxin-1/CCL11, eotaxin-2/CCL24, monocyte chemoattractant protein 1 (MCP-1)/CCL2, and thymus and activation-regulated chemokine (TARC)/CCL17 levels in lung were quantified using commercially available ELISA kits from R&D Systems (Mouse CCL11/Eotaxin DuoSet, Mouse CCL24/Eotaxin-2/MIPF-2, Mouse MCP-1/CCL2, Mouse TARC/CCL17 DuoSet, and Mouse Periostin/OSF-2 DuoSet) according to manufacturer's protocol.

#### CBA

IL-2, IL-4, IL-6, IFN- $\gamma$ , TNF, IL-17A, IL-10 in *in vitro* cell-free supernatants were quantified using Mouse Th1/Th2/Th17 Cytokine kit. Mouse Flex set was used to quantify IL-5 and IL-13 in *in vitro* cell-free supernatants and in BALF. Monocyte induced by gamma interferon (MIG)/ chemokine receptor (CXCR) 9, macrophage inflammatory protein 1 alpha (MIP-1 $\alpha$ )/CCL3 and regulated on activation normal T cell expressed and secreted (RANTES)/CCL5 was quantified in lung homogenate supernatants. All measurements were performed using commercially available CBA kits from BD™ according to manufacturer's protocol. CBA data were acquired on a BD FACSAria™ Flow Cytometer running BD FACS Diva™ v6.0 Software and analyzed using FCAP Array™ Software.

### 3.4.8 RNA (III)

#### RNA yield and integrity

Total RNA yield and integrity was analyzed using chip-based capillary electrophoresis (Agilent 2100 Bioanalyzer) with the total RNA 6000 Nano Kit, according to manufacturer's protocol. Bioanalyzer electropherograms were analyzed using Agilent 2100 Expert B.02.07 software that includes data collection, presentation and interpretation functions. An RNA integrity number (RIN) for assigning integrity values to RNA measurements is provided by the software [280]. All samples used had a RIN value above 7.

## **mRNA**

Quantitative real-time polymerase chain reaction (qPCR) was used for determination of target mRNAs of c-Maf, CTLA4, GATA-3, PU.1 (synonym *sfp1*, spleen focus forming virus proviral integration oncogene), SHIP1, SMAD1 (homologs of both the drosophila protein mothers against decapentaplegic (MAD) and the *C. elegans* protein SMA), SOCS1, STAT3, STAT6, TGFB1, TNF super family member 9 (TNFSF9) in PBLN tissue.

First strand cDNA synthesis was performed using 1.5µg total RNA per cDNA reaction and RT<sup>2</sup> First strand kit (Qiagen) according to manufacturer's protocol. Quantitative real-time PCR was performed on a CFX-96™ Real Time PCR Detection System by adding the cDNA and RT<sup>2</sup> SYBR® Green qPCR Master mix to a Mouse Custom Array CAPM11696 (SABiosciences, Qiagen) as described by manufacturer. The array was pre-set with primers for the target mRNAs mentioned above and reference genes β2microglobulin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin. Each target mRNA was normalized to all reference genes and fold regulation in miR-155 KO allergen-challenged mice was calculated from WT allergen-challenged mice using CFX Manager™ 3.0 software.

## **miRNA**

Locked nucleic acid (LNA) primer sets for miR-155 were used to determine miR-155 expression in lung and spleen tissue. Reverse transcription (RT) of miRNA was performed in replicates using 40ng of total RNA per cDNA reaction using the Universal cDNA Synthesis kit (Exiqon) according to manufacturer's protocol. qPCR was performed on a CFX-96™ Real Time PCR Detection System using diluted RT product, LNA™ Primer sets for miR-155 and U6 snRNA and SYBR® Green Master mix kit as described by manufacturer (Exiqon). miR-155 expression was normalized to U6snRNA and fold regulation in allergen-challenged WT mice was calculated from PBS exposed WT mice CFX Manager™ 3.0 software.

## **3.5 Adoptive transfer (III)**

Splenocytes from OVA sensitized WT mice were used to generate OVA-specific T cells. CD4<sup>+</sup> T cells were enriched by magnetic separation as described above and cultured for 4 days at 37°C in the presence of OVA (200µg/ml). After washing and resuspension in PBS, CD4<sup>+</sup> T cells (4x10<sup>6</sup> cells per mouse) were then transferred into naïve recipient WT and miR-155 KO mice by i.p. injection. Twenty-four hours after the cell transfer, mice were exposed to OVA (100µg) once daily for a total of 5 days. Lung tissue



and BALF were isolated 24 hours after the final allergen challenge and airway eosinophilia was assessed by differential cell counts.

## **3.6 Statistical analysis**

### **Paper I**

Non-parametric descriptive and analytical statistics were employed. The Wilcoxon signed rank test was used to analyze pollen-season induced changes within a patient group, while the Mann-Whitney U-test was employed for comparison of these changes ( $\Delta$  change) between the two patient groups as well as to compare the control group with the patient group at baseline. A p-value of less than 0.05 was considered statistically significant in all analyses.

### **Paper II**

Data were tested for adherence to a normal distribution with the Kolmogorov-Smirnov test (with Dallal-Wilkinson-Lilliefors corrected P value). If a normal distribution of sample means could be assumed, unpaired test or paired t-test was used; otherwise the Kruskal-Wallis test followed by the Mann-Whitney U test were applied. Correlations were performed using Pearson correlation test. Values of  $p < 0.05$  were considered statistically significant.

### **Paper III**

Statistical analysis was performed using the unpaired Student t-test or the Mann-Whitney U test, where appropriate. A P value  $< 0.05$  was considered statistically significant.

## 4 RESULTS AND DISCUSSION

In this section, the main findings will be presented and discussed. For more detailed information please see respective paper.

### 4.1 T cell subsets in the nasal mucosa of patients with allergic rhinitis (I)

We evaluated the presence of Th1, Th2 and Treg cells based on their expression of respective master regulatory transcription factors T-bet, GATA-3 and FOXP3. Nasal biopsies were obtained and numbers of respective T cell subtype were determined in samples from symptom-free AR patients, pre pollen season and healthy non-allergic controls. A new finding at the time of this study was that we could demonstrate presence of FOXP3<sup>+</sup> cells in the nasal mucosa of both healthy individuals and AR patients. However, all three T cell types were found in the nasal mucosa in both groups but to different extent. FOXP3<sup>+</sup> and GATA-3<sup>+</sup> cells, but not T-bet<sup>+</sup> cells, were significantly increased in the symptom-free AR patient group in comparison to the control group.

Our data indicates a minimal persistent inflammation outside pollen season. Minimal persistent inflammation has been described as a subclinical inflammatory state in AR patients driven by very low doses of allergen, causing inflammatory cell infiltration in the nasal mucosa and other markers of inflammation without causing allergic symptoms. This minimal persistent inflammation may contribute to hyper reactivity and increased susceptibility to development of clinical symptoms [281]. Possibly, this inflammation might be controlled by the suppressive Treg cells and might be the reason that the AR patients consider themselves symptom-free.

In contrast to our data, a recent Norwegian study demonstrated that grass or birch pollen allergic patients and non-allergic controls had similar quantities of FOXP3<sup>+</sup> cells outside pollen season [282] indicating AR patients a heterogeneous group. To confirm the function of either of these Treg cells it is necessary to isolate T cells from the tissue and examine the suppressive capacity of Treg cells as well as the responsiveness of effector T cells to suppressive signals. Thus, it can only be proposed with this type of descriptive data.

## 4.2 Effect of a natural grass–pollen season and glucocorticoid treatment on mucosal T cell subsets (I)

It is well known that allergic individuals respond to allergens by upregulating a Th2 response and that this response can be attenuated by glucocorticoids [283]. In addition, a previous study demonstrates increased FOXP3 expression and circulating Treg cells numbers in asthmatics by glucocorticoid treatment [28] indicating a new role for glucocorticoids. In our study we evaluated the upregulation of GATA-3<sup>+</sup> Th2 cells as well as the change in T-bet<sup>+</sup> Th1 cells and FOXP3<sup>+</sup> Treg cells in response to a natural occurring pollen exposure by giving AR patients placebo treatment. We further compared this group to an AR group receiving treatment with an intranasal glucocorticoid, FP. Since it has previously been shown that these three T cell subtypes have the possibility to alter each other's function with the release of their specific cytokines into the microenvironment we investigated the internal relationship by counting number of cells in the nasal mucosa.

Our results confirmed a Th2 response in the nasal mucosa in the placebo treated AR group demonstrated by a significant upregulation of GATA-3<sup>+</sup> cells during pollen season. This was not accompanied by an upregulation of neither FOXP3<sup>+</sup> nor T-bet<sup>+</sup> cells. This suggests an insufficient inhibitory response in sensitized individuals. Similar result was reported recently and included increased expression of STAT6 [284], but also contrasting result with increased numbers of Treg cells after experimental pollen exposure has been reported [282]. It has been suggested that the suppressive activity of Treg cells on T effector cells can be dependent on the allergen concentration and type of allergen, with different thresholds for different allergens and individuals correlated with their sensitization/atopy status [285]. However, glucocorticoid treatment effectively attenuated the increase of GATA-3<sup>+</sup> cells but leads to reduced numbers of FOXP3<sup>+</sup> cells while T-bet<sup>+</sup> cells were unaffected.

By analyzing the ratios between T-bet<sup>+</sup>/GATA-3<sup>+</sup> and FOXP3<sup>+</sup>/GATA-3<sup>+</sup> respectively we evaluated the effect of glucocorticoid treatment on the balance between the subsets that might regulate the degree of inflammation [286, 287]. The overall effect of glucocorticoid treatment is improved ratio despite a supposedly negative effect of Treg cell reduction. However, the levels of healthy controls are not reached. It is clear from our data that the inhibitory effect of a local glucocorticoid on Th2 cells is relatively more significant than the induction of either suppressive Treg or Th1 cells,

demonstrating an anti-inflammatory effect rather than an immune regulatory effect, for the clinical benefit of this group of drugs.

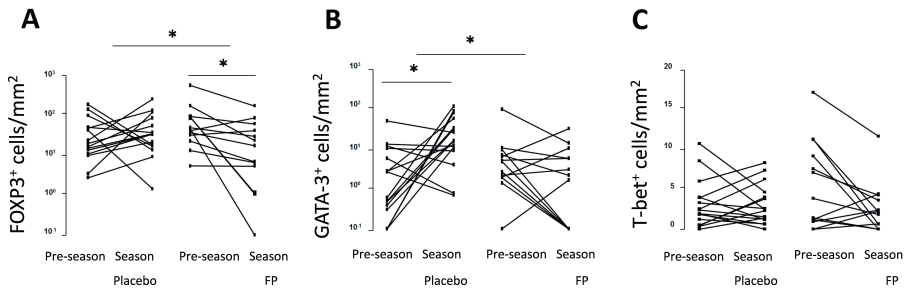


Figure 4. The number of FOXP3<sup>+</sup> (A), GATA-3<sup>+</sup> (B) and T-bet<sup>+</sup> (C) cells/mm<sup>2</sup> was counted in nasal biopsy samples taken prior to and during the pollen season. Grass-pollen sensitized AR patients were treated with either a placebo (n = 16) or FP (200µg/day; n = 13) throughout the grass-pollen season (\*P < 0.05).

Our results demonstrating a negative effect of glucocorticoid treatment on Treg cells has been discussed and more recent studies has reported both similar results [288] and opposing results of increased numbers of Treg cells [289]. These controversial findings of Treg cell activation in different tissues and during different courses of glucocorticoid treatment underline the importance of further studies to increase our understanding of the anti-inflammatory effects of glucocorticoids. However, the AR patient group is probably not a homogenous group and this can be seen in our data as well. Taking a closer look at the individual data in figure 4, one can clearly see individually different response to pollen exposure and to glucocorticoid treatment. Our dataset is unfortunately too small to evaluate subgroups of patients but it would be interesting to perform such a study.

The third T effector cell, Th17, discussed in this thesis was never evaluated in this study. However, there are other studies evaluating the effect of glucocorticoid treatment in both human samples and in a murine model. Oral glucocorticoid treatment demonstrated reduced levels of IL-17 in bronchial biopsy specimens [290] whereas inhaled glucocorticoid treatment was not able to suppress IL-17-driven airway neutrophilic inflammation in patients [291, 292]. The latter was confirmed by a study in mice which showed that glucocorticoid treatment were not able to attenuate the neutrophil influx to the airway or reduce the AHR in RORγt transgenic mice after allergen challenge but was efficient in GATA-3 transgenic mice by attenuating eosinophil influx and AHR [293]. Furthermore, in a study comparing persistent AR, intermittent AR and controls only the expression of GATA-3

mRNA was upregulated in the nasal mucosa by pollen exposure in AR groups in comparison to controls and neither T-bet, FOXP3 nor RORC mRNA [294]. Recently, even double positive FOXP3<sup>+</sup>IL-17<sup>+</sup> T cells have been demonstrated to be localized in the nasal mucosa of patients with both AR and nasal polyps, but not in patients with AR alone suggesting that these cells play a role in remodeling [295].

Finally, glucocorticoid treatment exerts their inhibitory effect not only on Th2 cells but have been demonstrated to exert inhibitory effect on several other cell types involved in AR as reviewed by Okano [296].

### 4.3 T cells subsets possibility to distinguish specific asthma endotypes (II)

Our hypothesis was that patients with a specific asthma endotype could be distinguished by the immunological profile of their circulating CD4<sup>+</sup>T cells. We therefore compared the absolute numbers of circulating Th1, Th2, Th17 and Treg cells based on their expression of respective master regulatory transcription factors T-bet, GATA-3, ROR $\gamma$ t and FOXP3. All participants, both asthmatics and healthy subjects, were selected from an epidemiologically based asthma cohort study, WSAS. The asthmatics displayed similar clinical disease profiles, but with distinct differences in the number of circulating eosinophils, in the study referred to as EOS high and EOS low group respectively.

When comparing these two groups of well-controlled asthmatics to healthy controls no obvious significant differences were revealed indicating an unsuccessful approach. However, a sub analysis of each subset revealed that T cells expressing only FOXP3 and none of the other three transcription factors, was increased only in EOS high asthmatics compared to controls. Increased numbers of airway Treg cells has previously been shown in moderate/severe asthmatics [297]. Furthermore, non-specific *in vitro* stimulation of PBMCs without addition of any exogenous polarizing cytokines resulted in increased single expressing or multi expressing GATA-3<sup>+</sup> cells in asthmatics compared to controls but no clear distinction between the two groups of asthmatics could be made. However, a successful definition of endotypes should link key pathogenic mechanisms with the asthma phenotype. We therefore asked ourselves if the different T cell subsets in the fresh samples might correlate to main features of asthma indicating clinical importance. Our result demonstrated correlation between GATA-3<sup>+</sup> and

ROR $\gamma$ t<sup>+</sup> cells with FEV<sub>1</sub> reversibility and FeNO, respectively. When dividing the asthmatics into subtypes the association to these main features of asthma was still significant in the EOS high group but not in the EOS low group, indicating two different asthma subgroups.

Our study is one approach to endotype asthma, based on CD4<sup>+</sup> T cell subsets in peripheral blood samples. The usage of blood is non-invasive, but our method of flow cytometry on fresh blood requires considerable effort and is not likely to be the method of choice for a large study or to be used for diagnostic purposes. However, successful endotyping of asthma based on disease mechanisms could be advantageous in clinical studies and finally contribute to individualized therapy [19, 298]. In a heterogeneous disease syndrome as asthma this has already been demonstrated in the example of anti-IL-5 therapy, which at first was considered a drawback, but when appropriate patient selection were made it has proven successful in a specific endotype such as in the IL-5-mediated eosinophilia persisting despite moderate doses of glucocorticoids [299, 300]. Another example is anti-IL-13 therapy to asthmatics with high serum periostin levels [301].

## 4.4 Co-expression of master transcription factors in T cell subsets indicating ability of plasticity (II)

The phenomenon of plasticity is defined as a cell type, while retaining its identity, acquires new functional properties and transcriptional output, which together can define a new cellular subtype. Plasticity and flexibility in T cell subsets has become evident by several experimental [224, 226-229] and *in vitro* studies [213-217] and has been suggested to play a role in modulating inflammatory disease. We therefore aimed to investigate if the same phenomenon occurred in circulating T cells in healthy humans and/or asthmatics. In our approach we labeled fresh blood cells within one hour of sampling, without any form of artificial manipulation, to detect co-expression of transcription factors known to be key regulators of Th1, Th2, Th17 and Treg cells. We found that circulating T cells can be classified into at least two main categories: those that express only one of the master regulatory transcription factors: T-bet, GATA-3, FOXP3 or ROR $\gamma$ t or those that co-express two or more of these transcription factors. This finding indicates ability for plasticity *in vivo* in both asthmatics and healthy individuals.

Evidence based on *in vitro* studies demonstrated that progenitor cells unable to proliferate remained uncommitted and bipotent, expressing both T-bet and

GATA-3 mRNA in response to polarizing signals for maturation [302]. To investigate whether circulating T cells co-expressing transcription factors were able to proliferate we included the expression of Ki-67, a marker for proliferation. Interestingly, our data suggests that actively proliferating cells co-express multiple transcription factors *in vivo* to a great extent, at least in healthy control subjects.

Upon CD3/CD28-activation *in vitro*, we observed a substantial increase in the number of T cells expressing multiple transcription factors leaving only a very small population of single expressing cells. This strongly argues a capacity for T cells, to upregulate additional transcription factors upon further stimulation. If T cells co-expressing transcription factors are undergoing lineage commitment or whether an already committed cell can express multiple transcription factors remains unanswered by our study.

Demonstrating the finding of T cell subsets capability to co-express several master transcription factors we were curious to investigate the site of inflammation i.e. the lung. For this question we used a murine model of allergic airway inflammation. In single cell suspension of lung tissue from naïve, sensitized/PBS exposed (OVA/PBS) and sensitized/allergen exposed (OVA/OVA) mice, we investigated the relative numbers of CD4<sup>+</sup> T cells based on their expression and co-expression of respective master regulatory transcription factor T-bet, GATA-3, RORγt and FOXP3. We found that the different subsets could also be found in the lung tissue and that there is a distinct shift in the expression due to exposure to allergen (figure 5, unpublished data).

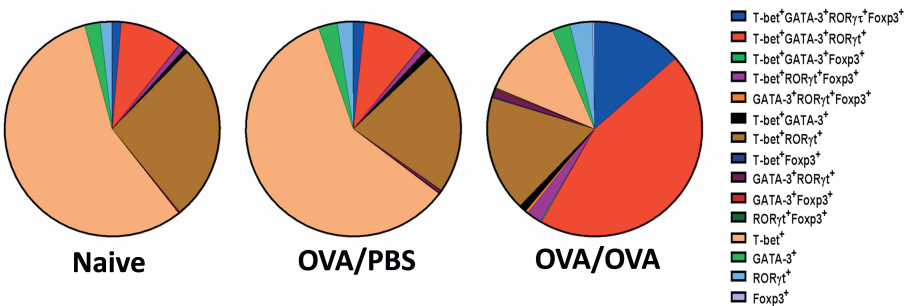


Figure 5. Allergen exposures induce a distinct shift in transcription factor expression pattern in the lung *in vivo*. Fresh lung tissue was collected from naïve, OVA/PBS and OVA/OVA WT mice. CD4<sup>+</sup> T cells divided into subtypes expressing one, two, three or four transcription factors based on mean values ( $n = 4-6$ /group).

If the co-expressing T cell subsets have different functions has not been investigated in our study, but data from other studies suggests that they might have multiple functions. In a model of non-allergic chronic lung inflammation, lung tissue CD4<sup>+</sup>IL-17<sup>+</sup>IL-13<sup>+</sup>IL-4<sup>+</sup> cells co-expressed ROR $\gamma$ t and GATA-3 after local or systemic immunization with inflammatory dendritic cells [303]. Th17/Th1 [304] and Th17/Th2 cells [305] seem to have a more aggressive and pathogenic phenotype than conventional Th17, Th2, or Th1 phenotypes. Both experimental and clinical observations suggest Th17, more than Th2 cells, to play a pathogenic role [306]. Recent studies report the existence of Th17/Th2 cells that secrete both IL-4 and IL-17, co-express the transcription factors GATA-3 and RORC, in asthmatic patients [228, 307].

Several studies have investigated the co-expression of other key regulatory transcription factors in FOXP3<sup>+</sup>Treg cells. FOXP3<sup>+</sup>IL-17<sup>+</sup> cells expressing ROR $\gamma$ t still showed suppressive function *in vitro* [308]. T-bet induction in Treg cells, after infection, facilitated these cells to suppress Th1 inflammation [309, 310]. Expression of GATA-3 in Treg cells reduced the induction of T-bet and ROR $\gamma$ t under inflammatory settings by stabilizing FOXP3 and CD25 expression [224]. Furthermore, the microenvironment has an important role for the balance of co-expression. T cells receiving a TGF- $\beta$  signal can develop into either Treg cells or Th17 cells depending on the absence or presence of pro-inflammatory cytokines respectively [229].

The master regulatory transcription factors has been proven to be crucial for the respective T cell subtype but the cell identity probably depends on the integration of signals from a network of transcription factors, where those that respond to environmental signals also play a significant role. The cytokine signaling is sensed through STAT family transcription factors and their recruitment of p300, a histone acetyltransferase [311, 312] predictive of tissue-specific regulatory activity [313]. In this way, signal transduction can be linked to chromatin biology and epigenetic regulation. Even if the real frequency with which T cells alter their cytokine production *in vivo* is still not fully known, these findings argue for a high plasticity of T cells in both cytokines and transcription factors expression. To study tissue resident cells in humans, both control subjects, as well as asthmatics, would be of great interest in understanding the role in disease progression. Significantly, the importance of this flexibility suggests a possibility to intervene therapeutically in immune mediated diseases.



## 4.5 MicroRNA–155 regulates airway eosinophilia (III)

miR-155 has been shown to be upregulated in the nasal mucosa of AR patients exposed to allergen [273]. To investigate a role for miR-155 in the regulation of allergic inflammation *in vivo*, we used miR-155 KO mice sensitized and exposed to OVA. Allergen-challenged miR-155 KO mice had reduced number of inflammatory cells, especially eosinophils, in BALF and lung compared with WT mice. The miR-155 KO mice also demonstrated decreased mucus hypersecretion. These features were accompanied by a more than 4-fold upregulation of miR-155 expression in the lung of WT mice after allergen challenge. Eosinophilia is a hallmark of allergic airway inflammation so we questioned if the reduced eosinophilia in the miR-155 KO mice was due to reduced eosinophilopoiesis or if the recruitment to the site of inflammation i.e. the airways was disrupted. When evaluating the eosinophil levels in bone marrow, spleen, and blood we found no effect of miR-155 deficiency on allergen-induced bone marrow or spleen eosinophilia but a slight but significant reduction in allergen-induced blood eosinophilia in miR-155 KO mice when compared with that seen in allergen-induced WT mice.

Furthermore, miR-155 deficiency resulted in altered allergen-induced chemokine expression in the airways. Eotaxins especially has proven potent chemoattractants for eosinophils [137, 149] and eotaxin-2/CCL24 was significantly decreased in allergen-challenged miR-155 KO mice compared to WT mice. By i.n. instillation of eotaxin-2/CCL24 before allergen challenge we could demonstrate partial restoration of airway eosinophilia in miR-155 KO mice. Our data suggest that miR-155 has a significant role in airway eosinophilia by regulating the recruitment of eosinophils to the site of inflammation without affecting bone marrow eosinophilopoiesis.

## 4.6 MicroRNA–155 plays a role for the development of airway T cell subsets (III)

T cells rapidly upregulate miR-155 expression upon stimulation [314] and deficiency in miR-155 has been shown to lead to impaired immune response due to defective T cell immunity [261]. Our data revealed that miR-155 deficiency results in impaired numbers of activated CD4<sup>+</sup>CD25<sup>+</sup> T cells and defective inflammatory T-cell development in the lung after allergen

challenge even though no significant differences were found in the relative numbers of CD4<sup>+</sup> T cells between WT and miR-155 KO mice. Homeostasis of the immune system requires the generation of different T-cell subtypes, which is dysregulated during disease [315]. We therefore compared the relative numbers of lung Th1, Th2, Th17 and Treg cells based on their expression of respective master regulatory transcription factors T-bet, GATA-3, ROR $\gamma$ t and FOXP3 in naive, PBS-exposed, and allergen-exposed WT and miR-155 KO mice. In all conditions tested, there was no significant difference in the relative numbers of Th1 cells in WT and miR-155 KO mice. Th17 and Treg cells demonstrated an initial reduction in naïve miR-155 deficient mice but not after sensitization. However, after allergen challenge, miR-155 deficiency resulted in reduced numbers of Th2, Th17, and Treg cells compared with those seen in WT mice. These data suggest a role for miR-155 in the development of T cells of the Th2, Th17, and Treg lineages, which might have important consequences in allergic inflammation.

While the effect on Th2 cells by miR-155 has not previously been demonstrated, modulating effect on Th17 and Treg cells have been shown in non-allergic models. miR-155 has been found significantly up-regulated in the IL-17 producing T cells in rheumatoid arthritis [316] and has been described to promote the development of inflammatory T cells including Th17 cells in EAE [269]. Additionally, reduced numbers of Treg cells under steady-state conditions and during inflammation have been demonstrated in miR-155 deficient mice [266].

## **4.7 MicroRNA–155 modulates allergic inflammation by influencing allergen-mediated Th2 responses (III)**

In our study, using a well characterized OVA model of allergic airway inflammation, we clearly demonstrate a modulatory role for miR-155 in regulating eosinophilic inflammation, mucus hypersecretion and T cell development. The overall Th2 response seems to be attenuated due to miR-155 deficiency further demonstrated by lack of increased levels of eotaxin-2/CCL24, IL-13 and periostin in the airways in response to allergen-challenge. IL-13 has been shown to regulate eotaxin production and extracellular matrix proteins, including periostin. Both IL-13 and periostin have direct profibrotic and remodeling effects which contributes to additional characteristic feature in the allergic lung [151-154, 317]. It is well known that IL-4, IL-5, and IL-13 play important roles in the pathophysiology of allergic inflammation. We demonstrate that PBLN cells from miR-155 KO mice had

reduced capacity to release Th2 cytokines when restimulated *in vitro* with allergen. Finally, adoptive transfer of WT OVA-specific CD4<sup>+</sup> T cells into naïve WT and naïve miR-155 KO mice restored allergen-induced airway eosinophilia in the two mouse strains to a similar degree.

Taken together these findings indicate that miR-155 is associated with Th2-mediated leukocyte infiltration and activation locally in the airways after allergen challenge, arguing that the impaired allergy parameters in miR-155 KO mice are due to a defect in Th2 cells.

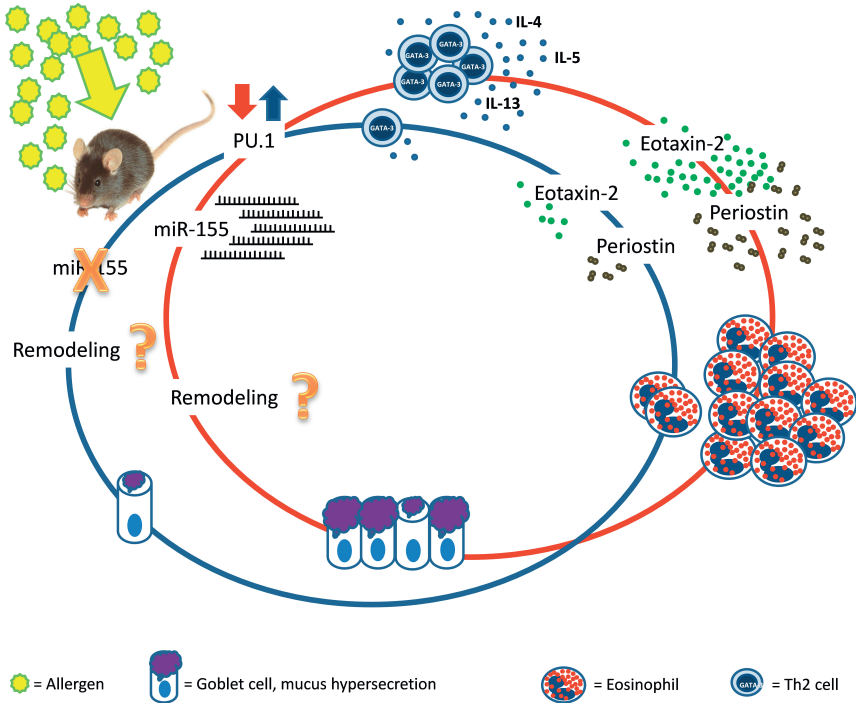
## 4.8 MicroRNA-155 targets PU.1 in a model of allergen induced airway inflammation (III)

miR-155 has been shown to be a multifunctional miRNA implicated in carcinogenesis, as well as in innate and acquired immunity [259]. However, contradictory data demonstrates, upregulation of miR-155 at site of inflammation upon allergen-challenge caused by inhibition of the target CTLA-4 [274] but also that miR-155 deficiency results in a Th2 profile due to inability to target the transcription factor c-Maf [261]. Another interesting target was SOCS1, where miR-155 deficiency leads to attenuated Th1 differentiation *in vitro*. [318]. Therefore, we explored a number of mRNAs previously found to be directly or indirectly targeted by miR-155.

We found an increased expression of the transcription factor PU.1 in PBLN tissue after allergen challenge in miR-155 KO mice compared to WT mice. PU.1 is a direct target of miR-155 [319] and has been demonstrated to negatively regulate Th2 cytokine production [130]. PU.1 functions partially by binding directly to GATA-3 and thus interfering with GATA-3 DNA binding [130]. Although we could not detect decreased mRNA expression of GATA-3 as a result of an increased PU.1 expression in miR-155 KO, we suggest that PU.1 could negatively regulate GATA-3 without affecting GATA-3 mRNA expression through decreased GATA-3 DNA binding activity. This decreased DNA binding activity would alter the Th2 cytokine loci and thus regulate the Th2 phenotype as observed in the allergen-challenged miR-155 KO mice. These data suggest that PU.1 might be an important transcription factor contributing to the reduced Th2 cytokine profile in allergen-challenged miR-155 KO mice.

PU.1 is not only targeted by miR-155, but has also been demonstrated to be indirectly inhibited by miR-126 in a HDM model of allergic airway

inflammation [257]. miR-155 deficiency or blockage of miR-126 leads to increased PU.1 expression resulting in reduced effector functions of Th2 cells and eosinophilic inflammation in different models of allergic airway inflammation.



*Figure 6. The role of miR-155 in allergic airway inflammation. Red circle: miR-155 targets PU.1 leading to increased GATA-3 and Th2 cells. Th2 cells secrete cytokines IL-4, IL-5 and IL-13 which in turn induce eotaxin-2/CCL24 and periostin production leading to influx of eosinophils and goblet cell hyperplasia and mucus hypersecretion. Blue circle: In miR-155 deficient mice PU.1 is upregulated interfering with GATA-3 binding, leading to reduced number of Th2 cells. Reduced levels of Th2 cytokines results in reduced levels of eotaxin-2/CCL24 and periostin followed by reduced number of eosinophils and less mucus hypersecretion. The long-term effect on remodeling has not been investigated in this thesis.*

## 5 CONCLUSIONS

### Paper I

FOXP3<sup>+</sup> and GATA-3<sup>+</sup> cells, but not T-bet<sup>+</sup> cells, were significantly increased in the symptom-free AR patient group in comparison to the control group. Our data indicates a minimal persistent inflammation outside pollen season.

Nasal glucocorticoids attenuate the allergic inflammation induced by natural pollen exposure partly by reducing the number of Th2 cells, and not by local upregulation of Treg cells. The local relationship between Th1 and Th2 cells as well as between Treg and Th2 cells is maintained by nasal glucocorticoid treatment.

### Paper II

No clear distinction between the two groups of well-controlled asthmatics could be distinguished by the immunological profile of circulating Treg, Th1, Th2 and Th17 cells.

Circulating CD4<sup>+</sup> T cells can express a single or several of the master regulatory transcription factors: T-bet, GATA-3, FOXP3, or ROR $\gamma$ t. This finding indicates ability for plasticity *in vivo* in both asthmatics and healthy individuals. However, whether co-expression is a transition state between different subsets or stable co-expression has not been confirmed.

### Paper III

miR-155 plays a significant role in airway eosinophilia by regulating the recruitment of eosinophils to the site of inflammation without affecting the bone marrow eosinophilopoiesis.

miR-155 plays a role in the development of airway Treg, Th2 and Th17 cells, but not Th1 cells in allergic inflammation.

miR-155 is associated with Th2-mediated leukocyte infiltration and activation locally in the airways after allergen challenge, arguing that the impaired allergy parameters in miR-155 KO mice are due to a defect in Th2 cells.

miR-155 contributes to the regulation of allergic airway inflammation by modulating Th2 responses through the transcription factor PU.1.

## 6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Taken together the studies in this thesis supports that T cell subsets in asthma and allergy influenced by the microenvironment shows flexibility and plasticity which can be affected by treatment, allergen exposure and miRNA expression and thus are important regulators of these diseases. Increasing the understanding of these processes may hopefully result in more specific future treatments. However, several interesting topics arise and some are discussed below.

### **Glucocorticoids and plasticity**

Mechanisms of glucocorticoid resistance in asthmatic patients might include alteration of glucocorticoid receptor activities as cofactors that mediate transcriptional activation include steroid receptor co-activators such as histone acetyltransferase p300. As mentioned previously p300 has been demonstrated to be recruited by STAT family transcription factors after sensing cytokine signaling [311, 312]. The different family members then induce master regulatory transcription factors. It is possible that T cell plasticity interferes with or is affected by glucocorticoids.

### **Glucocorticoids and miRNA**

The impact of glucocorticoids on miRNA expression has not been studied to any great extent. One recent study demonstrates no difference between airway biopsies of mild asthmatics before and after treatment with inhaled glucocorticoid when analyzing 227 miRNAs [320]. Another study describes alterations of airway epithelial cell miRNA levels in both steroid-naive and more severe asthma, with inhaled glucocorticoid demonstrating modest effect on miRNA levels. IL-13 was linked to these alterations, especially to repression of miR-34/449 family members that have established roles in airway epithelial cell differentiation [321]. It is likely that miRNAs have an important role in asthma and allergic diseases. However, more experimental studies and studies in human patients are needed to understand the biological impact during disease and the response to treatment.

### **Plasticity and miRNA**

We hypothesized that miR-155 might contribute to T cell plasticity in our allergen induced airway inflammation model. When quantifying the different CD4<sup>+</sup> T cell subsets in the lung, previously mentioned in section 4.4, we found reduced numbers of T cells co-expressing several transcription factors

in miR-155 KO mice in comparison to WT mice after allergen challenge (unpublished data). Plasticity in CD4<sup>+</sup> T cells seems to, at least in part, be dependent on miR-155. However, this finding needs to be addressed further.

### **Circulating miRNA, vesicles or ribonucleoproteins**

Circulating miRNAs are being developed as biomarkers for cancer and other diseases. They appear quite stable and have been demonstrated to be distributed both in vesicles, such as exosomes or bound to ribonucleoprotein complexes [322]. In a systemic investigation of plasma and serum obtained from healthy controls, different miRNAs appeared to be distributed differently. miR-155 was not found among the circulating miRNAs while miR-221 appeared to be distributed in both vesicles and bound to ribonucleoprotein complexes and miR-146a only bound to ribonucleoprotein complexes [322]. Whether in vesicles or in ribonucleoprotein complex, it is becoming clear that miRNAs can be selectively and actively secreted by one cell to be delivered into another recipient cells where they have the potential to modulate the transcriptome [323]. Understanding how this form of intercellular communication is regulated and whether it is selective or not may provide more insights into the overall biological impact of circulating miRNAs.

### **Therapy to target miRNAs**

Inhibitors to miRNA that have demonstrated effective in animal models *in vivo* are chemically modified anti-miRNAs, such as cholesterol-conjugated 'antagomirs', LNA oligonucleotides, and peptide nucleic acids (PNAs) [324]. In a recent study performed on CD4<sup>+</sup> T cells obtained from AR patients this was functionally tested by targeting two complementary miRNAs, miR-223 and miR-139-3p to alter the release of Th2 cytokines [325]. Furthermore, inhibition of microRNAs in preclinical models of asthma, cystic fibrosis, and idiopathic pulmonary fibrosis has shown therapeutic promise. In animals, inhibitors of miRNAs directly delivered to the airway, at doses suitable for nebulizers or hand-held inhalers, up-regulate expression of cohorts of genes containing complementary "seed" sequences within their mRNA UTRs [326].

### **Innate lymphoid cells resembling T cells**

In addition to the different T cell subtypes, discussed in this thesis, at least three groups of innate lymphoid cells (ILCs) have been found. With the identification and characterization of new ILC subsets it becomes apparent that ILCs are a complex family of innate cells that have a diverse range of functions in innate immunity. Following infection or inflammation, ILCs rapidly provide critical early cytokine production prior to the development of adaptive Th cell subsets. ILCs and T cell subsets share many functional

attributes that can be explained, at least partially, by their common signals for generation, differentiation, and activation [327]. Briefly, innate lymphoid cells group 1 express T-bet and produce IFN- $\gamma$  and TNF and comprise NK cells and ILC1. Innate lymphoid cells group 2 also called nuocytes or innate helper cells express GATA-3 and are a source of Th2 cytokines, suggested to provide a missing link between the innate and adaptive Th2 response [328-330]. In mice, these cells are found in many different tissues in the absence of immune challenge, and they are particularly found in the mesenteric lymph nodes, spleen, gut and liver, as well as in the lungs [331]. Innate lymphoid cells group 3 comprises ILC3 and lymphoid tissue-inducer cells. ILC3 produce IL-17 and/or IL-22 and express ROR $\gamma$ t [327]. New emerging data suggest a role for particularly ILC2 in the pathophysiology of asthma and allergy. Future studies delineating the role of innate lymphoid cells and T cell dependent responses could provide better understanding of the biological impact during disease and the response to treatment.



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