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# A STUDY ON THE ROLE OF T HELPER CELLS IN ALLERGIC INFLAMMATION

Julian Mario Stark


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# A study on the role of T helper cells in allergic inflammation

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

By


## **Julian Mario Stark**


*Principal Supervisor:*
Associate Professor Jonathan Coquet
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

*Co-supervisor:*
Professor Gunilla Karlsson Hedestam
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

*Opponent:*
Professor Clare Lloyd
Imperial College London
Faculty of Medicine
National Heart & Lung Institute

*Examination Board:*
Professor Maria Jenmalm
Linköping University
Department of Biomedical and Clinical Sciences
Division of Inflammation and Infection

Associate Professor Colm Nestor
Linköping University
Department of Biomedical and Clinical Sciences
Division of Children's and Women's Health

Professor Francesca Chiodi
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

*For Karl Stark 1921-2013, Inventor & grandfather*

# ABSTRACT

Asthma is a chronic inflammatory disease of the airways affecting hundreds of millions of people worldwide. Central to the development of asthma and allergic airway inflammation are CD4 T cells, which upon activation through their T cell antigen receptor, differentiate into T helper (Th) cells with the potential to secrete distinct sets of cytokines. These cytokines have the ability to potentiate or regulate inflammation in all the organs of the body. In asthma, Th2 cells that produce IL-4, IL-5 and IL-13, have been heavily implicated in promoting pathogenesis in the lung. However, other T helper cell subsets, such as IL-17-producing Th17 cells may also play a pathogenic role.

**Paper I** established that peroxisome proliferator activated receptor gamma (PPAR-γ) was an important factor for the differentiation of IL-5- and IL-13-producing Th2 cells in a mouse model of allergic airway inflammation with the allergen, house dust mite (HDM). Mice lacking PPAR-γ in T cells did not develop Th2 cell-associated pathologies, including airway eosinophilia and goblet cell metaplasia. Further, these mice were unable to mount a protective immune response to infection with *Heligmosomoides polygyrus*. Mechanistically, PPAR-γ appeared to promote the expression of the IL-33 receptor on Th2 cells, which enhanced their pathogenic functions. **Paper II** used single-cell RNA sequencing (scRNA-seq) to resolve distinct subsets of T helper cells in the HDM model of asthma. Th2 cells were found to differentially express over 100 genes including known Th2 cell genes such as *Gata3*, *Il13*, *Il1rl1* and *Pparg* (described in paper I) as well as many other genes not previously reported to have a specific role in promoting Th2 cell function. Th2 cells were enriched for genes associated with lipid metabolism, which when blocked, impaired Th2 cell-driven inflammation in the lung tissue and airways. scRNA-seq also resolved a population of Th cells responding to type-I interferons. **Paper III** established a novel mouse model using dog allergen extract instillations to induce airway inflammation, which was characterized by a mixed Th2/Th17 response. In characterizing this model it demonstrated that dog allergen extracts can induce high levels of airway eosinophilia and neutrophilia, airway hyperresponsiveness, goblet cell metaplasia and increased serum levels of IgE. scRNA-seq of T helper cells from the airways of dog allergen-exposed mice resolved several distinct subsets, providing a gene expression signature for responding Th17 cells and providing a largely overlapping gene signature for Th2 cells as that which was elucidated in paper II. Sublingual immunotherapy using recombinant dog allergens proved successful in ameliorating airway eosinophilia and the Th2 cell response to dog allergen challenge, but did not affect the Th17 cell response.

The work presented in this thesis improves our understanding of the factors governing T helper cell differentiation and function in the context of allergic airway inflammation. It showcases that scRNA-seq is a powerful tool to study diverse T helper cell populations and sheds light on genes and cellular processes of relevance to specific subsets. Finally, the novel mouse model of dog allergies presents a tool with which to study airway inflammation driven both by Th2 and Th17 cells as well as an approach to treat dog allergies.

# LIST OF SCIENTIFIC PAPERS

I. Chen, T., Tibbitt, C.A., Feng, X., Stark, J.M., Rohrbeck, L., Rausch, L., Sedimbi, S.K., Karlsson, M.C.I., Lambrecht, B.N., Karlsson Hedestam, G.B., Hendriks, R.W., Chambers, B.J., Nylén, S., and Coquet, J.M. (2017). PPAR-gamma promotes type 2 immune responses in allergy and nematode infection. Sci Immunol 2.

II. Tibbitt, C.A.*, Stark, J.M.*, Martens, L., Ma, J., Mold, J.E., Deswarte, K., Oliynyk, G., Feng, X., Lambrecht, B.N., De Bleser, P., Nylén, S. Hammad H., Arsenian Henriksson, M., Saeys, Y., and Coquet, J.M. (2019). Single-Cell RNA Sequencing of the T Helper Cell Response to House Dust Mites Defines a Distinct Gene Expression Signature in Airway Th2 Cells. Immunity 51, 169-184 e165.

III. Stark, J.M., Liu, J., Tibbitt, C.A., Ma, J., Wintersand, A., Adner, M., Grönlund, H., Gafvelin, G., and Coquet, J.M. Dog allergens induce a dual neutrophilic and eosinophilic infiltrate hallmarked by vigorous TH2 and TH17 cell responses, Manuscript

*equal contribution

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II. Stark, J.M., Tibbitt, C.A., and Coquet, J.M. (2019). The metabolic requirements of Th2 cell differentiation. Frontiers in immunology 10, 2318.

III. Khoenkhoen, S., Erikson, E., Ádori, M., Stark, J.M., Scholz, J.L., Cancro, M.P., Pedersen, G.K., and Karlsson Hedestam, G.B. (2019). TACI expression and plasma cell differentiation are impaired in the absence of functional Iκ BNS. Immunology and cell biology 97, 485-497.

IV. Centa, M., Prokopec, K.E., Garimella, M.G., Habir, K., Hofste, L., Stark, J.M., Dahdah, A., Tibbitt, C.A., Polyzos, K.A., and Gisterå, A., Johansson D.K., Maeda N.N., Hansson G.K., Ketelhuth D.F.J., Coquet J.M., Binder C.J., Karlsson M.C.I., Malin S. (2018). Acute loss of apolipoprotein E triggers an autoimmune response that accelerates atherosclerosis. Arteriosclerosis, thrombosis, and vascular biology 38, e145-e158.

V. Ádori, M., Pedersen, G.K., Ádori, C., Erikson, E., Khoenkhoen, S., Stark, J.M., Choi, J.H., Dosenovic, P., Karlsson, M.C., Beutler, B., and Karlsson Hedestam, G.B. (2018). Altered Marginal Zone B Cell Selection in the Absence of IκBNS. The Journal of Immunology 200, 775-787.

VI. Soldemo, M., Àdori, M., Stark, J.M., Feng, Y., Tran, K., Wilson, R., Yang, L., Guenaga, J., Wyatt, R.T., and Karlsson Hedestam, G.B. (2017). Glutaraldehyde cross-linking of HIV-1 env trimers skews the antibody subclass response in mice. Frontiers in immunology 8, 1654.

VII. Pedersen, G.K., Ádori, M., Stark, J.M., Khoenkhoen, S., Arnold, C., Beutler, B., and Karlsson Hedestam, G.B. (2016). Heterozygous mutation in IκBNS leads to reduced levels of natural IgM antibodies and impaired responses to T-independent type 2 antigens. Frontiers in immunology 7, 65.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 15d-PGJ2 | 15dD12,14-PGJ2 |
| 2-DG | 2-deoxy-D-glucose |
| 3'-UTR | three prime untranslated region |
| AHR | airway hyperresponsiveness |
| AIDS | acquired immune deficiency syndrome |
| AIT | allergen specific immunotherapy |
| APC | antigen presenting cell |
| ATAC-seq | assay for transposase-accessible chromatin using sequencing |
| BAL | bronchoalveolar lavage |
| DC | dendritic cells |
| DN | double negative |
| DP | double positive |
| FACS | fluorescence-activated cell sorting |
| GC | germinal center |
| G-CSF | granulocyte colony-stimulating factor |
| GO | gene ontology |
| GSEA | gene set enrichment analysis |
| GWAS | genome-wide association study |
| HLA | human leukocyte antigen |
| HDM | house dust mite |
| ICOS | inducible costimulator |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| IL-33R | interleukin 33 receptor |
| ILC | innate lymphoid cells |
| JAK | Janus kinase |
| medLN | mediastinal lymph node |
| mesLN | mesenteric lymph node |
| MHC | major histocompatibility complex |
| mRNA | messenger RNA |
| mTOR | mammalian target of rapamycin |
| mTORc | mammalian target of rapamycin complex |
| nTreg | natural regulatory T cell |
| PBS | phosphate-buffered saline |
| PMA | phorbol 12-myristate 13-acetate |
| PPAR-γ | peroxisome proliferator-activated receptor gamma |
| RAPTOR | regulatory associated protein of mTOR |
| RICTOR | rapamycin-insensitive companion mTOR |
| RORγt | retinoic acid receptor-related orphan receptor gamma |
| scRNA-seq | single-cell RNA sequencing |
| SLIT | sublingual immunotherapy |
| SNP | single nucleotide polymorphism |

| | |
|---|---|
| STAT | signal transducer and activator of transcripiton |
| TCA | tricarbolic acid |
| TCR | T cell receptor |
| Tfh cell | T follicular helper cell |
| Th cell | T helper cell |
| TLR | Toll-like receptor |
| Treg cell | regulatory T cell |
| TSLP | thymic stromal lymphopoietin |
| t-SNE | t-distributed stochastic neighbor embedding |
| VAT | visceral adipose tissue |
| WT | wilde type |

# 1 INTRODUCTION

## 1.1 ASTHMA AND ALLERGIC DISEASE

More than one billion people are currently affected by allergy and the incidence of allergic diseases is rising rapidly, with 4 billion people estimated to be affected by 2050 (Akdis and Agache, 2014). One popular explanation for the increase of allergic diseases and asthma is the so called “hygiene hypothesis” that postulates that a decrease in exposure to infectious organisms have led to the current allergy epidemic (Strachan, 1989). Over the last few years, this has come to encompass changes in diet and an altered microbiome as well, which help to shape our overall microbial composition (Lambrecht and Hammad, 2017). Over 300 million people worldwide suffer from asthma, a chronic inflammatory disease of the airways. Yearly, more than 400,000 people are estimated to die from asthma (Network, 2018). Asthma is characterized by airway hyperresponsiveness (AHR), airflow obstruction, mucus overproduction and airway remodeling. These physiological changes can lead to shortness of breath, wheezing and chest tightness.

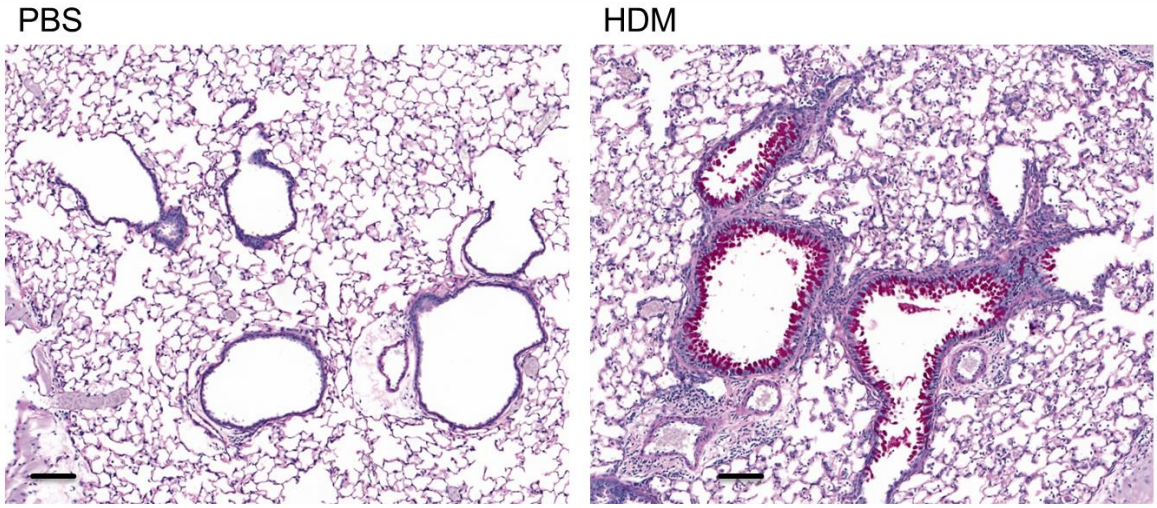


**Figure 1.** Periodic acid-Schiff stain of lung sections of mice administered either PBS or house dust mite (HDM) extract. Shows inflammation, wall thickening and goblet cell metaplasia in the lung of HDM-exposed mice. (Bar = 100µm)

Many different cell types from both the innate and adaptive immune system as well as epithelial cells are involved in the development and pathology of asthma (Lambrecht and Hammad, 2012). Chronic inflammation and associated tissue healing induces airway remodeling, typified by increased thickness of the basement membrane and lung smooth muscle layer, and goblet cell metaplasia (See Fig. 1) (Vignola et al., 2001). The risk to develop asthma has been shown to have a strong genetic component, with heritability estimated to be as high as 60% based on twin studies (Duffy et al., 1990). Further risk factors for the development of asthma include exposure to air pollution and cigarette smoke, obesity, infections with respiratory syncytial virus early in life, and exposure to a smaller range of microorganisms (Burke et al., 2012; Camargo et al., 1999; Clark et al., 2010; Ege et al., 2011; Szabo et al., 2013). Young children lacking a diverse gut microbiota are also at a higher risk of developing asthma later in life (Abrahamsson et al., 2014; Sjögren et al., 2009). Acute

asthma exacerbations can be caused by a range of triggers including allergens, irritants (such as smoke or dust), cold air, exercise and respiratory virus infections (Jackson et al., 2011).

### 1.1.1 Classifications of asthma

In 1947, Rackemann proposed that asthma should be segregated into 'intrinsic' and 'extrinsic' phenotypes (Rackemann, 1947). 'Intrinsic' asthma was associated with a late onset of age and associated with a range of co-morbidities including polypoid sinusitis, while 'extrinsic' asthma was associated with rhinitis and bronchiolitis and presented in childhood. Later, these terms extrinsic and intrinsic were replaced by 'allergic' and 'non-allergic' respectively, as it became increasingly appreciated that elevated allergen-specific IgE was at the heart of 'allergic asthma' (Novak and Bieber, 2003). Now, it is appreciated that some asthmatics may fall into a third 'mixed' group, and that all three forms of asthma entail airway inflammation and bronchoconstriction. Hallmarks of allergic asthma include elevated serum levels of allergen-specific IgE (Romanet-Manent et al., 2002), enhanced production of Th2 type cytokines (interleukin (IL)-4, IL-5, IL-6, IL-9, IL-10 and IL-13) (Novak and Bieber, 2003; Yasruel et al., 1997), elevated numbers of cells expressing the high-affinity receptor for IgE (FεRI) (Humbert et al., 1996), and recruitment of eosinophils (Conti and DiGioacchino, 2001). Non-allergic asthma has a later onset, a more severe clinical course, no evidence of IgE-mediated allergy but also enhanced Th2 type cytokines in the lung parenchyma and airways (Humbert et al., 1999; Novak and Bieber, 2003). More recent work suggests to further separate patient groups based on mono- and polysensitization to known allergens and comorbidities (Anto et al., 2017).

### 1.1.2 Allergens

Allergens are often harmless, environmental antigens, which can lead to sensitization and induction of a humoral and cellular immune response. They are commonly proteins and glycoproteins and sources can be furry animals, arthropods (for example house dust mites (HDM)) or plants. Nomenclature of allergens employ abbreviations of the Latin names following the Linnean system and sequential numbers, e.g. Ara h 1 is the major peanut (*Arachis hypogaea*) allergen. Why only a small number of proteins in the environment is able to elicit an allergic response is not fully understood, but several allergens have been found to share common properties. Many allergen proteins, such as the HDM allergen Der p 1 exhibit protease activity which enhances the potential of antigen presentation by degradation of epithelial barriers, causes the release of damage associated molecular patterns and by directly stimulating epithelial cells to produce proinflammatory cytokines such interleukin- (IL-) 33 (Jacquet, 2011). Others bind directly to receptors of the innate immune system, thereby inducing an immune response. The HDM allergen Der p 2 for example has been shown to directly activate TLR2 and TLR4 (Chiou and Lin, 2009; Hammad et al., 2009), whereas the cat allergen Fel d 1 enhances TLR2 and TLR4 signaling not through direct binding but by

interacting with lipopolysaccharide (LPS). The dog allergen Can f 6 has also been shown to bind LPS (Herre et al., 2013).

HDM allergens are very prominent in society. Dust mites are so prevalent that one study found more than 10 µg per gram of carpet dust in close to 80% of houses surveyed (Hannaway and Roundy, 1997). In addition to the allergenic properties of HDM, mites also contain their own microbiota, which may function as natural adjuvants (Valerio et al., 2005). *Dermatophagoides pteronysinnus* and *D. farinae* are the main species linked to allergies and the most prevalent with 87% and 47% occurrence, respectively (Colloff, 2010). An early study revealed that the majority of allergic asthmatics had high levels of IgE to this allergen, suggestive of a causative link between HDM and the development of asthma (Sporik et al., 1990). However, it has come to be observed that sensitization to HDM is very common in society (Calderón et al., 2015). Frequent cleaning and good ventilation can reduce the concentration of HDM in homes, however the positive effects of such interventions on improving asthma symptoms are not very strong and have been subject of debate (Gøtzsche and Johansen, 2008; Pingitore and Pinter, 2013). Which could in parts be due to exposure to HDM in public places (Tovey et al., 2013). Several HDM allergens show cross reactivity to other invertebrates, such as Der p 10 to the shrimp allergen Pen a 1 (Shanti et al., 1993; Sidenius et al., 2001).

Many allergens from furry animals such as the dog allergens Can f 1, 2, 4 and 6, the mouse allergen Mus m 1 or the bovine allergen Bos d 2, belong to the lipocalin family. Members of this protein family have been shown to bind to human dendritic cells and to promote T helper (Th) 2 cell responses (Klaver et al., 2020). Structural similarities between lipocalins from different species are thought to be the cause of multi-sensitization commonly found in allergic patients (Nilsson et al., 2014b). Other pet allergens belong to the serum albumin, kallikrein, latherin and secretoglobin protein families (Curin and Hilger, 2017). Can f 1, a lipocalin, and Can f 5, a prostatic kallikrein, are major dog allergens to which up to 70% of dog allergic patients are sensitized (Curin and Hilger, 2017; Mattsson et al., 2009). Can f 2 and Can f 4 are also lipocalins and Can f 3 is a serum albumin. These are considered minor dog allergens with sensitization rates between 23 and 35% of dog-allergic individuals (Curin and Hilger, 2017) .Cross-reactivity is not only seen between different types of aeroallergens but also between aeroallergens and food allergens, such as birch-apple or mite-shrimp. This is mostly seen in adults following sensitization with cross-reactive aeroallergens (Popescu, 2015).

## 1.2 TREATMENT OF ASTHMA

Common treatments for asthma are bronchodilators and corticosteroids, which ameliorate airway constriction and inflammation. Patients with mild asthma can often be treated with inhaled adrenergic β2 receptor agonists (bronchodilator) (Alangari, 2014). While treatment is effective and safe in most patients, these drugs can cause severe side effects and an estimated 10% of patients do not respond to common treatment options (Dahl, 2006; Olin and Wechsler, 2014). While only a minority of patients show a poor response to treatment, the

costs of treating these patients are incredibly high (Breekveldt-Postma et al., 2008). It is increasingly appreciated that these common treatments may perform better in certain forms of asthma over others. For instance, childhood-onset asthma appears to be quite responsive to standard therapies, while a greater proportion of people with adult-onset asthma appear insensitive to these therapies (Lang et al., 2008; Wenzel, 2012).

### 1.2.1 Corticosteroid treatment

Corticosteroids have been used in asthma treatment since 1956 (Christie et al., 1956). Corticosteroids ameliorate asthmatic disease by exerting an anti-inflammatory effect on a range of target cells, including eosinophils, T cells, mast cells and epithelial cells (Barnes and Adcock, 2003). Corticosteroid treatment reduces the amount of airway infiltrating inflammatory cells and reduces AHR (Barnes, 1996). Corticosteroids bind to the glucocorticoid receptor (GR) in the cytoplasm, triggering the release of chaperone proteins and exposing nuclear translocation signals (Grad and Picard, 2007). In the nucleus GR binds to glucocorticoid response elements in DNA which leads to the regulation of gene expression and down regulation in transcription mediated by activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) (Barnes, 2001). Corticosteroids also promote the expression of anti-inflammatory genes encoding IL-10 (John et al., 1998) and β2-adrenoceptors (Baraniuk et al., 1997). Steroid resistant airway inflammation has been associated with Th17 cells, antimicrobial activated airway macrophages and reduced IL-10 production (Goleva et al., 2008; Hawrylowicz et al., 2002; McKinley et al., 2008). Neutrophils are less sensitive to the effects of corticosteroids, including resistance to corticosteroid induced apoptosis (Liles et al., 1995). The induction of anti-inflammatory genes in neutrophils by corticosteroids is counteracted by the simultaneous induction of pro-inflammatory genes (Ronchetti et al., 2018). Thus, it is perhaps not surprising that neutrophilic asthma has been associated with steroid resistance (Poon et al., 2012).

## 1.3 THE ROLE OF THE IMMUNE SYSTEM IN ALLERGIC DISEASE

Airway epithelial cells are among the first cells to come in contact with inhaled allergens. They express various pattern recognition receptors, such as Toll-like receptor (TLR4), which can be triggered by allergens (Salazar and Ghaemmaghami, 2013). After activation, epithelial cells secrete multiple cytokines and chemokines, including IL-25, IL-33, thymic stromal lymphopoietin (TSLP) and granulocyte-macrophage colony stimulating factor (GM-CSF), which trigger receptors on various innate cells including dendritic cells (DC) (Lambrecht and Hammad, 2012). Pulmonary DC, which encounter and acquire allergens upon inhalation also express pattern recognition receptors and can act as antigen presenting cells. Additional DC are recruited to the airway through signals from the epithelium upon allergen exposure (Pichavant et al., 2005; Plantinga et al., 2013). Antigen-loaded DC migrate to the lung draining lymph node where they affect the differentiation of CD4 T helper cells into different subsets (Lambrecht and Hammad, 2009; Maazi et al., 2013; Otero et al., 2010). Figure 2 gives a schematic overview over the development of an immune response to allergen exposure in the airways.

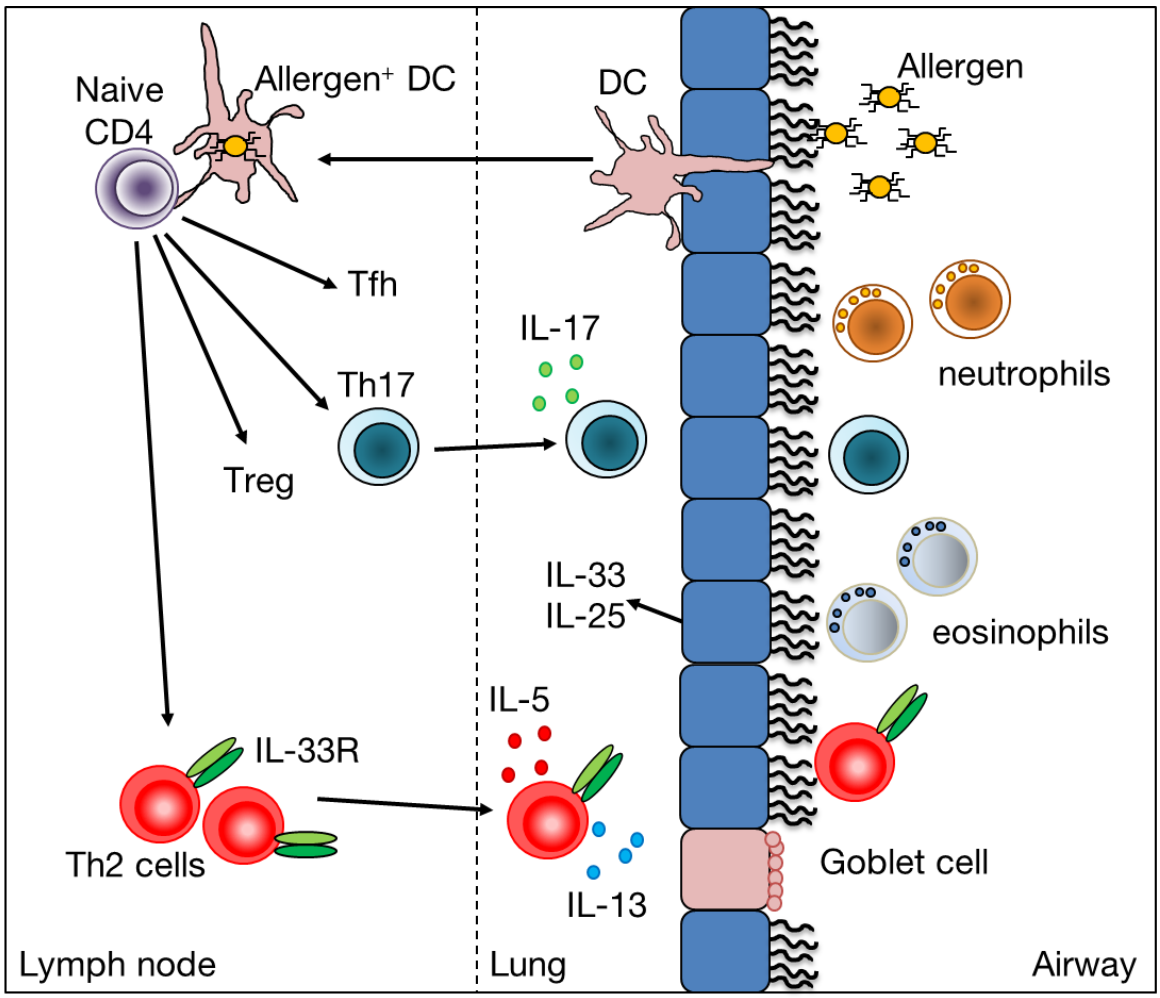


**Figure 2.** Allergens inhaled in the airways are taken up by DC and transported to the draining lymph node where the DC presents antigens to lymph node T cells. CD4 T cells are activated upon recognition of presented antigen through the TCR and develop into several distinct subpopulations. Th2 cells, the main subset implicated in asthma and other Th cell subsets migrate to the lungs after receiving migration cues. Th cells receive further instructions through cytokines released by lung epithelial cells such as IL-33 and IL-25, which are induced through protease activity of the allergen or by activation of pattern recognition receptors. The production of Th2 effector cytokines leads to the recruitment of eosinophils, goblet cell metaplasia and to enhanced mucus production. IL-17 producing Th17 cells are a feature of certain types of asthma and these cells recruit neutrophils to the airways. Modified from (Coquet et al., 2015b).

### 1.3.1 T cells

T lymphocytes, expressing a T cell antigen receptor (TCR) have been heavily implicated in the development of asthma and other allergies (Betts and Kemeny, 2009; Corrigan and Kay, 1992). Conventional T cells recognize peptides presented in the context of major histocompatibility molecules (MHC), which are known as human leukocyte antigens (HLA) in humans. Two major forms of MHC molecules present peptide antigens to T cells; MHC-I and MHC-II, which present antigens to $CD8^+$ T and $CD4^+$ T cells, respectively (Germain, 2002).

T cell development first requires the migration of lymphoid progenitor cells from the bone marrow to the thymus. At this stage lymphoid precursors do not express CD3 molecule family members, which are co-receptors for the TCR, nor the antigens CD4 and CD8. Thus, they were termed triple negative cells (Godfrey et al., 1993), a nomenclature subsequently revised to double negative (DN, CD4-CD8-) cells. Double negative cell differentiation proceeds through DN1 to DN4 based on the expression of the IL-2 receptor alpha subunit (CD25) and CD44. During this early stage, a pre-TCR alpha chain is expressed in combination with a successfully rearranged TCR beta chain, forming the pre-TCR. In a process known as beta-selection in the thymic cortex, signaling through the pre-TCR induces proliferation, expression of both CD4 and CD8 (double positive, DP), the rearrangement of the $\alpha$-chain and suppresses further rearrangement of the $\beta$-chain (Krangel, 2009; Murphy and Weaver, 2016). In the process of positive selection, DP cells are thereafter tested for their reactivity to MHC-I and MHC-II molecules expressed on cortical epithelial cells. T cells successfully recognizing peptide in the context of MHC-II in T cells, lose their expression of CD8 becoming CD4 T cells, while those selected on MHC-I lose CD4 and become CD8 T cells. Only a fraction of double positive T cells make it through positive selection (Germain, 2002; Taniuchi, 2018). The CD4 lineage is solidified through signals received through the TCR inducing the expression of the transcription factors GATA3 and ThPOK (He et al., 2009), while CD8 T cells rely on the expression of Runx3 to consolidate their fate. Following positive selection, cells upregulate expression of CCR7 leading to their migration from the cortex to the medulla (Ueno et al., 2004). While positive selection produces T cells able to bind antigen, negative selection is required to prevent the development of autoreactive T cells in order to protect the body from potentially devastating effects. Cells with a high specificity for self-antigens are either removed by apoptosis or appear to differentiate into natural regulatory T cells (nTreg) (Josefowicz and Rudensky, 2009), considered the suppressive cells of the immune system.

Naïve T cells leave the thymus to patrol the periphery. They are activated upon encountering a cognate peptide antigen presented by professional antigen presenting cells (APC), such as DC. Full T cell activation requires the recognition of a peptide antigen in the context of MHC-I or MHC-II as well as co-stimulatory signals through receptors including CD28 and CD27, which act to facilitate TCR-mediated signals, promote proliferation, survival and impact on the transcriptional profile of cells (Chen and Flies, 2013; Coquet et al., 2015a; Tripathi and Lahesmaa, 2014). Upon activation, CD8 T cells differentiate into cytotoxic T cells and mediate killing of virus-infected or cancerous cells in a peptide-specific manner. CD4 T cells differentiate into T helper cells, which provide help to CD8 T cells and B cells, and have a profound ability to potentiate and regulate immune responses via the secretion of cytokines.

T helper cells have been found to differentiate into subsets with distinct cytokine-producing potentials and functions. Their differentiation is defined by so-called ‘master regulators’ of transcription and typically, on signals received through cytokine receptors, which activate the signal transducer and activator of transcription (STAT) family members, janus kinases

(JAKs) and Smad proteins (Murphy and Weaver, 2016). Figure 3 provides an overview of the differentiation of several known Th cell subsets. Naïve CD4 T cells exposed to IL-12 differentiate into Th1 cells under the control of STAT1, STAT4 and T-bet and produce interferon gamma (IFN-γ). Th2 cells differentiate following signals through the IL-4 receptor, which induces activation of STAT6 and the transcription factor, GATA 3. The cytokines produced by Th2 cells are IL-4, IL-5 and IL-13. Differentiation of Th17 cells requires TGF-β and IL-6, which trigger the expression of STAT3 and retinoic acid receptor-related orphan receptor gamma (RORγt). Th17 cells also benefit from IL-21 and IL-23 and secrete IL-17. Follicular T helper (Tfh) cells produce IL-21 and require costimulatory molecules such as CD40 and ICOS to induce expression of Bcl6 and STAT3. And regulatory T cells (Treg) can be induced from naïve CD4 T cells by TGF-β, which induces activation of Smad3 and expression of Foxp3. Treg cells ameliorate inflammation through the expression of IL-10 and TGF-β (Murphy and Weaver, 2016). Research is ongoing to define distinct novel subsets as well as subpopulations within established subsets.

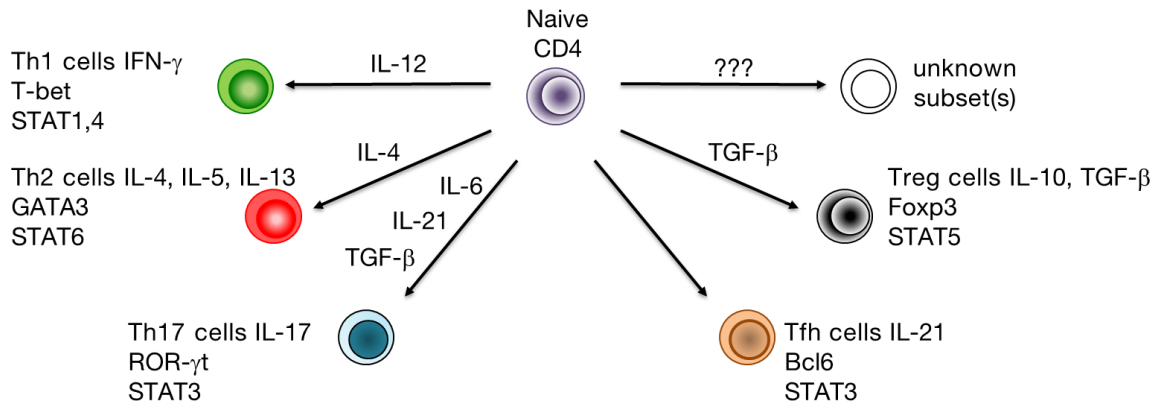


**Figure 3.** Naïve CD4 T cells differentiate into functionally-distinct subsets after activation through their TCR. Differentiation into a specific subset is determined by cytokine signals during T cell priming, which leads to the expression of subset specific master regulators and the secretion of specific cytokines that mediate effector function.

T cells are incredibly important for the control of viruses, cancers, bacteria, and increasingly implicated in the promoting organismal health. The importance of CD4 T cells to our general health can be seen in the setting of acquired immune deficiency syndrome (AIDS), where HIV infects and depletes CD4 T cells (Fanales-Belasio et al., 2010). Intriguingly, there is also a growing appreciation that CD4 and CD8 T cells play a role in inflammatory disorders such as inflammatory bowel disease, and in autoimmune diseases and allergies. In psoriasis for example, Genome wide association studies (GWAS) have repeatedly shown a strong association for single nucleotide polymorphisms (SNPs) in HLA-C, implicating a role for CD8 T cells in disease (Feng et al., 2009; Strange et al., 2010). SNPs at the HLA-D locus, encoding for MHC-II, have been shown to be involved in the pathogenesis of asthma (Li et al., 2010; Michel et al., 2010; Moffatt et al., 2010). These findings have been validated repeatedly in clinical and preclinical studies, such that T helper cells are seen as a central player in the development of asthma.

### 1.3.2 Th2 cells

Th2 cells were first described by Mosmann and Coffman over thirty years ago (Mosmann et al., 1986). They are characterized by the expression of effector cytokines IL-4, IL-5 and IL-13 and their development is controlled by the lineage defining transcription factors GATA3 and STAT6 (Kaplan et al., 1996a; Walker and McKenzie, 2018; Zheng and Flavell, 1997). Th2 cells support the protective immune response against large parasites and venoms (Galli et al., 2016; Paul and Zhu, 2010), but are also central in several allergic disorders including asthma (Lambrecht and Hammad, 2015). Naïve T helper cells exposed to IL-4 activate STAT6 through signaling via the IL-4 receptor (IL-4R) and JAK1/3 (Kaplan et al., 1996a). STAT6 then induces expression of the "master regulator" of Th2 cell differentiation, GATA3. Conditional deletion of GATA3 prevents differentiation of Th2 cells (Pai et al., 2004; Zhu et al., 2004) whereas forced expression induces committed Th1 cells or cells lacking STAT6 to produce IL-4 (Lee et al., 2000; Ouyang et al., 2000). GATA3 expression induces changes in the chromatin landscape across the *Il4/Il13/Rad50/Il5* locus leading to IL-4 production, which creates a positive feedback loop stabilizing Th2 cell identity (Ansel et al., 2006). GATA3 also promotes expression of characteristic surface markers of Th2 cells such as *Il1rl1* (ST2 subunit of IL-33R) (Nawijn et al., 2001) and the chemokine receptor *Ccr8* (Wei et al., 2011). Aside from the factors described here, many others could play a role in the differentiation and function of Th2 cells. This is a field of ongoing research.

### 1.3.3 Th2 cells – the main protagonist in asthma

Th2 cells are most intimately linked with the development of asthma. Repeatedly, GWAS analysis has identified not only SNPs in HLA-D as a major determinant of the risk of developing asthma, but also several canonical Th2 cell-associated genes including *GATA3, IL4, IL5, IL1RL1, IL18R1, RORA, IL33* (Li et al., 2010; Michel et al., 2010; Moffatt et al., 2010). Furthermore, following bronchoalveolar lavage of patients with asthma, clear increases in Th2 cells and their associated cytokines have been observed (Parronchi et al., 1991; Robinson et al., 1992). These clinical associations have been corroborated by many studies in preclinical models of allergy and asthma (Gavett et al., 1994; Gavett et al., 1995; Nakajima et al., 1992). IL-4 has been shown in mouse models to be required for the B cell switch to IgE antibody production (Del Prete et al., 1988). IL-13 is required for AHR in Th2 cell driven models of asthma (Wills-Karp et al., 1998). Blocking of IL-13 in mouse models of allergic airway inflammation reduced AHR, mucus production and airway eosinophilia (Grünig et al., 1998). Trials blocking IL-13 or signaling downstream of IL-4 and IL-13 in patients with severe asthma have successfully improved lung function of patients (Corren et al., 2011; Wenzel et al., 2013), demonstrating the causative role of Th2 cells in asthmatic disease. IL-5 appears to primarily support differentiation, proliferation and survival of eosinophils (Yamaguchi et al., 1988a; Yamaguchi et al., 1988b) and a meta-analysis of IL-5 blockade in humans shows a reduction in exacerbations by 40% (Cabon et al., 2017).

### 1.3.4 Other T helper cell subsets:

Though Th2 cells are the main T helper cell subset implicated in allergy, roles for Th1, Th17, Treg, Tfh, and CD8 T cells have also been postulated, either in combination with Th2 cells or in types of asthma lacking Th2 cells.

### 1.3.5 Th1 cells

Th1 cells are characterized by their secretion of IFN-γ as well as IL-2 and tumor necrosis factor (TNF) and are protective against intracellular pathogens (Romagnani, 1999). Th1 cells require IL-12 signaling to differentiate, which signals through STAT-1 and STAT-4 to induce T-bet and IFN-γ production (Jacobson et al., 1995; Kaplan et al., 1996b; Szabo et al., 2000). The secretion of IFN-γ by Th1 cells induces the production IL-12 by antigen presenting cells, thereby creating a positive feedback loop and inhibiting Th2 cell differentiation (Abbas et al., 1996). Th1 cells and IFN-γ are known to counteract the differentiation of Th2 cells and may be protective in the context of asthma since administration of IL-12 or IL-18 ameliorates Th2 cell-driven symptoms (Gavett et al., 1995; Hofstra et al., 1998). However, studies have revealed a more complex and less beneficial role of Th1 cells in the context of asthma. Th1 cells are able to induce AHR (Cui et al., 2005) and allergen-specific Th1 cells fail to control Th2 responses and lead to high levels of airway inflammation (Hansen et al., 1999). This is supported by the detection of increased levels of IFN-γ in the airways of asthmatic patients (Krug et al., 1996).

### 1.3.6 Th17 cells

Th17 cells secrete IL-17, IL-17F and IL-22 (Alcorn et al., 2010). They develop from naïve CD4 T helper cells stimulated with TGF-β, IL-6, IL-1β and IL-23 which leads to the activation of STAT-3 and expression of RORγt (Bettelli et al., 2006; Korn et al., 2009). Binding of IL-17 to IL-17R on epithelial cells induces secretion of the neutrophil chemokine CXCL8 (Korn et al., 2009). In asthma, IL-17 levels in patient sputum correlate with increased neutrophil numbers and increased AHR following methacholine challenge (Alcorn et al., 2010; Barczyk et al., 2003). IL-17 stimulation of epithelial cells further leads to increased expression of Mucin 5AC and goblet cell metaplasia (Fujisawa et al., 2009; Hashimoto et al., 2005) and has a direct effect on smooth muscle cells, inducing hyperresponsiveness (Chiba et al., 2017; Kudo et al., 2012). Cells producing both Th2 and Th17 cytokines have been reported in atopic asthma patients and in mouse model using OVA, leading to a diverse influx of inflammatory cells (Wang et al., 2010). It has also been proposed that diesel exhaust particles may induce IL-17 production in the HDM model of asthma in mice (Brandt et al., 2013).

### 1.3.7 Th9 cells – A variant of Th2 cells?

IL-9 has been found in the airways of patients with atopic asthma and is upregulated upon allergen exposure (Erpenbeck et al., 2003). IL-9 producing Th9 cells were originally thought to be a subset of Th2 cells, given its positive association to allergic disease. Studies have also

suggested that Th9 cells may represent a functionally-distinct T helper cell subset (Schmitt and Bopp, 2017; Veldhoen et al., 2008), however recent analyses have suggested once again that IL-9 production is specific to cells expressing high levels of IL-5 and IL-13 (Micossé et al., 2019). The differentiation of IL-9-secreting cells requires both IL-4 and TGF-β (Schmitt et al., 1994). Transfer of *in vitro* generated Th9 cells to naïve mice induces airway eosinophilia, increased numbers of mast cells and raised serum IgE levels (Jones et al., 2012). IL-9 signaling through the IL-9R affects a range of cells and processes relevant to the development of asthma. It suppresses Th1 cell immune responses (Wu et al., 2008), promotes mast cell proliferation and airway remodeling (Kearley et al., 2011) and enhances survival, differentiation and expression of the IL-5 receptor on eosinophils (Gounni et al., 2000). IL-9 may also be produced by other T cell subsets, mast cells and ILC2 (Koch et al., 2017).

### 1.3.8 Follicular helper T cells

Follicular helper T (Tfh) cells are essential for the formation of germinal centers (GC) and affinity maturation of antibodies produced by B cells (Crotty, 2014). The transcription factor Bcl6 is the master regulator of Tfh cell differentiation (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009) and the cytokine IL-21 is a hallmark of Tfh cells (Chtanova et al., 2004). IL-6 induces transient expression of Bcl6 by signaling through the IL-6 receptor (Nurieva et al., 2009) and is thought to be important for Tfh cell differentiation. Development of Tfh cells further requires inducible costimulator (ICOS) signaling during priming by DC. This induces Bcl6, which in turn induces CXCR5 (Choi et al., 2011). This first stage of Tfh cell development is not sufficient for full Tfh effector function, since Tfh cells in the absence of additional antigen presenting cells besides DC fail to produce the key cytokine IL-21 (Goenka et al., 2011). Expression of the chemokine receptor CXCR5 leads to colocalization with B cells, which provide ICOS ligand and present antigen to the Tfh cell (Crotty, 2014). Down regulation of the Epstein-Barr virus-induced G protein coupled receptor 2 (EBI2) on B and Tfh cells facilitates their localization to the germinal center (Hannedouche et al., 2011). IL-21 secretion by Tfh cells in the GC promotes proliferation of B cells, affinity maturation and differentiation to memory B cells (Zotos et al., 2010). Higher frequencies of Tfh cells produce IL-4 in the lymph node compared to Th2 cells (King and Mohrs, 2009; Reinhardt et al., 2009). IL-21 is required for the development of Th2 but not for Th17 cell responses (Fröhlich et al., 2007; Sonderegger et al., 2008). Mice sensitized and challenged with HDM develop IL-21-producing Tfh cells in the mediastinal lymph node but $CXCR5^{-}$ $IL\text{-}21^{+}$ cells are also present in the lungs, which promote the Th2 cell response to inhaled allergens (Coquet et al., 2015b). IL-21 has also been shown to inhibit suppressive Treg cell responses by promoting apoptosis of Treg cells (Tortola et al., 2019). It has been a point of contention whether IL-4-producing Tfh cells are precursors of Th2 effector cells as some studies have suggested (Ballesteros-Tato et al., 2016).

### 1.3.9 Regulatory T cells

Regulatory T (Treg) cells play an important role in dampening immune responses through the secretion of IL-10, CTLA-4 and TGFβ (Rudensky, 2011; Sakaguchi et al., 2008). The

transcription factor Foxp3 is the master regulator of Treg cell development (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). CD4 T cells can commit to the Treg cell lineage early in their development in the thymus or can be induced from naïve CD4 T cells in the periphery (Feuerer et al., 2009). Mice lacking Treg cells only survive for a few months before succumbing to autoimmune disease (Brunkow et al., 2001). Adoptive transfer experiments have shown that regulatory T (Treg) cells are able to suppress asthma through secretion of the cytokines IL-10 and TGF-β (Lewkowich et al., 2005). Treg cells also constitutively express the inhibitory receptor CTLA-4 (Sansom and Walker, 2006). Several SNPs in the *CTLA-4* gene have been identified to be associated with serum IgE levels and reduced lung function (Munthe-Kaas et al., 2004). Some studies have reported on elevated Treg cell numbers in the lungs of asthmatic patients (Smyth et al., 2010). Whereas others have shown Treg cell numbers in the blood of severe asthmatic patients to be reduced and their suppressive capabilities impaired (Mamessier et al., 2008). A subset of Treg cells expressing TIGIT has been identified that is able to suppress Th1 and Th17 cells but not Th2 cells (Joller et al., 2014).

### 1.3.10 CD8 T cells

CD8 T cells are activated upon antigen recognition in the context of MHC-I to become cytotoxic effector cells that eliminate infected cells and cancers (Murphy and Weaver, 2016; Zinkernagel and Doherty, 1974). Subsets of CD8 T cells expressing similar cytokine profiles as Th1, Th2, Th9 and Th17 cells have been described. They are known as Tc1, Tc2, Tc9 and Tc17 and are characterized by the expression of IFN-γ, IL-4, IL-9 and IL-17 respectively (Baraldo et al., 2016; Betts and Kemeny, 2009; Srenathan et al., 2016). While the role of CD4 T cells in asthma has been extensively studied, less is known about CD8 T cells in the context of allergic lung inflammation. While some studies have suggested a protective role (Noble et al., 2016), others have claimed that CD8 T cell involvement is irrelevant to asthma (Ali et al., 2007), and yet others have found CD8 T cells to promote disease exacerbations (Dakhama et al., 2013). The discrepancy in results could be due to the presence of different CD8 T cell subsets having different impacts on disease progression. Tc1 cells, protecting from viral infections, and IL-10 secreting CD8 cells, ameliorating pathogenic inflammation, are thought to be beneficial in the context of asthma. Tc2 and Tc9 cells on the other hand are considered detrimental, with IL-13 contributing to AHR and IL-9 and IL-4 supporting Th2 cell-driven inflammation (Baraldo et al., 2016).

### 1.3.11 B cells

B cells play an important role in the immune system by producing antibodies that bind to antigens with high specificity, leading to, depending on the antigen, neutralization or phagocytosis of pathogens and toxins. Binding of antibody to antigen can also lead to the activation of other effector cells mediated through Fc receptors. In the course of an immune response antibodies not only mature to gain higher specificity but B cells also undergo class switching in order to produce different classes of antibodies that are useful in different settings (Murphy and Weaver, 2016). IgE is the central antibody class relevant for allergic

disease. Mast cells and basophils are activated by crosslinking of IgE on the high affinity IgE receptor FcεRI (Gould and Sutton, 2008; Lambrecht and Hammad, 2015). Blocking IgE with the antibody omalizumab reduces the number of FcεRI and IgE expressing cells, as well as numbers of eosinophils, B cells and T cells at the site of inflammation in asthmatic patients (Holgate et al., 2005).

Aside from their ability to produce antibodies, B cells also act as antigen presenting cells and are able to amplify Th2 cell responses in the HDM model (Dullaers et al., 2017; Wypych et al., 2018). IL-10-producing regulatory B cells have also been postulated to play a role (Floudas et al., 2016). Regulatory B cells from patients with allergic asthma produce less IL-10 in response to LPS stimulation and have a reduced ability to induce IL-10 production in CD4 T cells (van der Vlugt et al., 2014).

### 1.3.12 Mast cells

Mast cells are thought to have originally evolved as a defense against parasitic worm infections but play an important role in the pathology of asthma. They can be activated by cross-linked IgE, or by complement components C3a and C5a. Activation triggers the release of cytosolic granules containing cytokines (IL-4, IL-5, IL-6, IL-13), histamine and serotonin, proteases and lipid mediators, such as leukotrienes, prostaglandins, sphingolipids and platelet-activating factor (Kubo, 2017). They also stimulate T cells by secretion of tumor necrosis factor (Nakae et al., 2006). However mast cells are not exclusively pathogenic in the context of allergic airway inflammation. IL-33 stimulated mast cells promote Treg cell expansion by the secretion of IL-2 and mice lacking mast cells have increased levels of airway inflammation after administration of papain (Morita et al., 2015).

### 1.3.13 Basophils

Basophils perform similar roles as mast cells, but basophils are short-lived compared to the longer life span of mast cells (Kiernan, 1979; Kubo, 2017; Ohnmacht and Voehringer, 2009). Basophils, as well as Th2 cells and eosinophils, express the chemokine receptor CCR3. They are recruited to the site of inflammation from the peripheral blood by the chemokine eotaxin (CCL11) (Uguccioni et al., 1997). Basophils are activated through the FcεRI, C3aR and or C5aR similar to mast cells, followed by the release of granules containing a similar range of effector molecules as mast cells (Falcone et al., 2006). Secretion of IL-4 by basophils is required for ILC2-driven airway eosinophilia in response to protease allergens (Motomura et al., 2014). Basophils support type 2 responses and are able to aggravate airway inflammation (Wakahara et al., 2013) and it has been proposed that they are required for Th2 cell priming (Perrigoue et al., 2009). More recent studies however have demonstrated that they are not essential for the development of airway inflammation in the context of OVA administration (Ohnmacht et al., 2010) or for Th2 cell priming in a model using HDM allergens (Hammad et al., 2010).

### 1.3.14 Eosinophils

Eosinophils are granulocytes that play a central role in host defense against multicellular parasites and in many types of asthma (McBrien and Menzies-Gow, 2017). They differentiate in the bone marrow, mediated by the cytokines IL-3, IL-5 and GM-CSF (Her et al., 1991) and are recruited to sites of inflammation by IL-5, IL-13, histamine, prostaglandin $D_2$ and eotaxins (Fulkerson and Rothenberg, 2013). Eosinophils secrete cytokines promoting Th2 cell responses, including IL-4 and release toxic granular proteins (Kita, 2013). IL-5 promotes release of granule proteins from eosinophils (Kita et al., 1992). This release can be gradual or immediate, through lysis of the cell (McBrien and Menzies-Gow, 2017). While eosinophils differentiate predominantly in the bone marrow, studies have shown precursors to be present in the blood of atopic patients and in bronchial biopsies of atopic asthma patients (Robinson et al., 1999; Sehmi et al., 1997). Eosinophil numbers correlate with severity of asthma and higher frequency of exacerbations (Bousquet et al., 1990; Price et al., 2015). Eosinophils secrete mediators promoting AHR, including IL-13 which can also induce goblet cell metaplasia (McBrien and Menzies-Gow, 2017). Antibody treatment against IL-5 significantly reduces the level of eosinophilia in asthmatic patients (Leckie et al., 2000).

### 1.3.15 Neutrophils

Neutrophils are short lived cells and among the earliest responders to any infected or wounded site. Up to $10^{11}$ neutrophils are produced in the bone marrow each day and they are the most abundant circulating leukocytes in humans (Summers et al., 2010). The central regulator of neutrophil development is granulocyte colony stimulating factor (G-CSF), controlling commitment to the myeloid lineage, proliferation and release of mature cells from the bone marrow (Lord et al., 1989; Richards et al., 2003). Neutrophils are easy to activate since they express every TLR except for TLR3 (Hayashi et al., 2003). The lifespan of neutrophils extends dramatically upon activation (Colotta et al., 1992). Neutrophils release a wide range of cytokines, including IL-1β, IL-18, IFN-γ, IL-4, and BAFF (Mantovani et al., 2011). They are also able to release intracellular and nuclear components, such as DNA, histones and enzymes from granules such as elastase. These structures are called neutrophil extracellular traps (Lee et al., 2017). Neutrophils are also able to produce and release reactive oxygen species. The abilities to both release potent inflammatory cytokines and cytotoxic substances taken together explain the severity of neutrophilic asthma (Ciepiela et al., 2015). Epithelial cells during wound healing release cytokines that enhance neutrophil recruitment and survival, with more infiltrating, longer lived neutrophils causing more tissue damage and healing this creates a positive feedback loop (Uddin et al., 2013). Even though neutrophils are associated with severe asthma, a recent study has shown that presence of neutrophils can limit ILC2 mediated airway inflammation by negatively regulating expression of G-CSF (Patel et al., 2019).

### 1.3.16 Innate lymphoid cells

Innate lymphoid cells (ILC) lack TCRs but have been found to acquire functions akin to several T helper cell subsets. ILC1, ILC2, ILC3 and natural killer cells mirror Th1, Th2, Th17 cells and cytotoxic CD8 cells respectively. ILC1 are specialized producers of IFN-γ, ILC2 produce IL-4, IL-5 and IL-13, ILC3 produce IL-17 and IL-22 while NK cells are innate killers. As their names suggest, they are generally thought to act as a first line of defense prior to the initiation of adaptive immunity, and in some settings may also promote T cell responses (Halim et al., 2018). Increasingly, ILC2 are also implicated in the regulation of metabolic and tissue homeostasis in adipose tissue and in the gut (Lloyd and Snelgrove, 2018; Moro et al., 2010; Vivier et al., 2018). Similar to Th2 cells, ILC2 respond to cytokines produced by the epithelium such as IL-25, IL-33 and TSLP (Ricardo-Gonzalez et al., 2018) and to prostaglandin $D_2$ (Xue et al., 2014). ILC2 have been shown to be a major source of IL-5 and IL-13 in mouse models using HDM or OVA (Wolterink et al., 2012), and there is evidence that ILC2 are increased in the blood and lungs of asthmatic individuals(Liu et al., 2015). Protease allergens, such as papain, can induce AHR, mucus production and lung inflammation independently of an adaptive immune response, as was shown in *Rag2*$^{-/-}$ mice lacking B and T cells. This inflammatory response is mediated by IL-33 and ILC2 (Kubo, 2017; Oboki et al., 2010). Secretion of IL-13 by ILC2 disrupts tight junctions in the lung epithelia of both mice and humans. Epithelial barrier leakiness allows for higher penetration of allergens and microbes promoting inflammation and allergic responses (Sugita et al., 2018).

### 1.3.17 Allergen specific immunotherapy

To date the only long-term cure for allergic disease is allergen-specific immunotherapy (AIT). The goal of AIT is to induce tolerance by administering low doses of allergens to patients over an extended period of time. The proposed mechanism is the shift of allergen-specific effector T cells towards a regulatory phenotype, thereby suppressing DC-mediated activation of effector Th cell development, suppressing T helper cells directly, shifting the antibody balance from IgE towards IgG4 and suppressing eosinophil, mast cell and basophil activation (Akdis and Akdis, 2011). IgG4 has several properties that make it anti-inflammatory, it has a lower affinity for certain Fcγ receptors and it does not activate the complement system (Aalberse and Schuurman, 2002; Meiler et al., 2008; Van der Neut Kolfschoten et al., 2007). There are several ways to administer AIT; either by subcutaneous immunotherapy (SCIT) or by sublingual immunotherapy (SLIT) or even intralymphatic delivery (Martínez-Gómez et al., 2009). While SCIT has been shown to be more effective, the risk for inducing systemic anaphylaxis is lower in SLIT (Dhami et al., 2017; James and Bernstein, 2017). AIT trials have had mixed success for different allergens. For example AIT trials for bee venom using T cell epitope peptides (Müller et al., 1998) and peanut allergies were successful (Fleischer et al., 2013), whereas other allergies have proven to be more difficult to treat with AIT. A recent review of 17 AIT trials for dog allergies reported on poor and conflicting results (Smith and Coop, 2016). AIT has also been tested for prophylactic

treatment of allergies. An AIT trial to prevent further sensitization in children with sensitization to one HDM allergen was successful (Pajno et al., 2001) whereas a trial targeting high-risk children has not been successful (Holt et al., 2013).

Many AIT regimens rely on the use of natural extracts which have the advantage of being relatively easy to produce and contain known as well as unknown allergens, but depending on the supplier, allergen source and batch there can be significant differences between concentration of specific allergens (Wintersand et al., 2019). A few studies have been conducted using recombinant allergen proteins, which have the advantage of being less prone to contaminations and allow for more consistent allergen concentrations. This has been successful in a mouse model for cat allergies and for allergies against HDM (Haspeslagh et al., 2019; Neimert-Andersson et al., 2008).

## 1.4 T HELPER CELLS METABOLISM

T helper cells require both energy and specific metabolites for biosynthesis pathways in order to proliferate and differentiate into functional effector cells (O'Neill et al., 2016). Naïve T cells use predominantly energy efficient metabolic pathways, that do not yield many side products such as the tricarboxylic acid (TCA) cycle and beta oxidation of fatty acids (MacIver et al., 2013). Naïve T cells however already accumulate untranslated mRNAs for genes required for glycolysis and fatty acid synthesis in order to be able to rapidly switch on these pathways upon activation (Ricciardi et al., 2018). Recently activated T cells turn on glycolysis as their main metabolic pathway. While glycolysis is not as energy-efficient, it provides the cells with acetyl-CoA for the TCA cycle, NADH and other intermediates to fuel biosynthesis of amino acids, nucleotides and fatty acids (Michalek et al., 2011). Differentiation and proliferation of T helper cells also require *de novo* synthesis of fatty acids (Lochner et al., 2015).

Metabolism, cell growth and proliferation is centrally controlled by the kinase mammalian target of rapamycin (mTOR) (Saxton and Sabatini, 2017). Effector T helper cell subsets are dependent on mTOR complex1 (mTORc1), including the scaffold protein RAPTOR (regulatory associated protein of mTOR) (Yang et al., 2013). As well as mTORc1, Th2 cells also require mTORc2, containing the scaffold protein RICTOR (rapamycin-insensitive companion of mammalian target of rapamycin), as mTORc2 inhibits SOCS5 (suppressor of cytokine signaling-5), an inhibitor of STAT6 activation (Delgoffe et al., 2011; Seki et al., 2002). Other targets of mTORc2, such as SGK1 and the GTPase RhoA have also been shown to promote Th2 cell function (Heikamp et al., 2014; Yang et al., 2016).

Metabolism does not only provide cells with energy and the required metabolites for proliferation and the production of effector molecules, it also directly exerts control over effector functions. Active glycolysis is required for IFN-γ production. The glycolysis enzyme GAPDH binds to the 3'UTR of *Ifng* mRNA while glycolysis is inactive and thereby suppresses translation (Chang et al., 2013). *De novo* fatty acid synthesis is required for the development of Th17 cells and inhibition of the enzyme acetyl-CoA carboxylase 1 promotes

differentiation to Treg cells instead (Berod et al., 2014). CD5L shifts the lipidome of Th17 cells towards polyunsaturated fatty acids and thereby controls the availability of lipid ligands for the master regulator of Th17 cells, RORγt (Wang et al., 2015). The link between immune cell function and metabolism is of course not restricted to T cells and has been a recently growing field of interest for the study of various immune cells in different contexts (O'Neill et al., 2016).

## 1.5 ANIMAL MODELS

Mice do not spontaneously develop asthma, but administration of allergens and allergen extracts can be used to induce symptoms of allergic airway disease. For a long time, the model antigen ovalbumin (OVA) was used in combination with alum to induce OVA-specific Th2 cell-mediated responses. Mice sensitized to OVA were subsequently challenged through the airways with OVA and this was sufficient to induce several traits of allergic inflammation including airway eosinophilia, mucus secretion and AHR (Kumar et al., 2008). However, lacking in this approach were allergens themselves, which have enzymatic activity and activate the airway epithelium. In the past decade especially, a focus on using natural allergens has predominated studies of allergic airway inflammation. Several preclinical animal models using natural extracts or recombinant proteins of clinically relevant allergen sources have been developed, such as mouse models of house dust mite, cat or fungal allergies (Cates et al., 2004; Havaux et al., 2005; Neimert-Andersson et al., 2008), as well as models for lupine and peanut food allergies (Andreassen et al., 2018; Burton et al., 2017). These have shown that the nature of the allergen can greatly affect the outcome of allergy (Lambrecht and Hammad, 2015; Nials and Uddin, 2008). Models using allergens including the protease papain, have been instrumental for studying the role of ILC in allergic airway inflammation (Halim et al., 2012; Halim et al., 2014). The advantage of mouse models is the availability of a large number of gene-targeted strains and the relative ease with which mice can be housed, bred and scaled up. Guinea pigs have also been used as animal models of asthma for over 60 years (Noelpp and Noelpp-Eschenhagen, 1952). Guinea pigs develop an early and a late asthmatic reaction and the lung is the primary target of anaphylaxis. However, less inbred strains and species specific reagents are available in guinea pigs compared to mice (Ricciardolo et al., 2008; Shin et al., 2009). Larger animals including horses and monkeys naturally develop allergic sensitivities, but are expensive and more difficult to handle and therefor are used less frequently than smaller laboratory animals (Shin et al., 2009).

# 2 AIMS

The aim of my thesis was to better understand the factors regulating T helper cell differentiation in the context of allergic inflammation.

**Paper I** investigated the role of PPAR-gamma in Th2 cell-mediated immunity using mice in which *Pparg* was specifically absent in T cells.

**Paper II** assessed the plasticity and differentiation of T helper cells in the HDM model using single cell RNA-seq.

**Paper III** developed a mouse model for allergic inflammation using dog allergens and explored the use of sublingual immunotherapy with recombinant dog allergens as a treatment option.

# 3 RESULTS AND DISCUSSION

## 3.1 PAPER 1

The incidence of asthma and allergic disease is increasing worldwide, requiring a better understanding of the underlying immune response (Moorman et al., 2012). A strong type 2 immune response is essential for the protection from worm infection but is also a hallmark of many allergies. Type 2 responses are mediated by Th2 cells and ILC2 through the production of IL-4, IL-5 and IL-13 (Artis and Spits, 2015; Paul and Zhu, 2010). Central to the differentiation of Th2 cells is induction of the master regulator GATA-3 by signal transducer and activator of transcription 6 (STAT-6) (Paul and Zhu, 2010; Zheng and Flavell, 1997). GATA-3 not only promotes proliferation and expression of the Th2 cell effector cytokines, but also expression of the IL-33 receptor (IL-33R) composed of a common IL-1RAP chain and ST2 (Nawijn et al., 2001).

We found that *Pparg* was upregulated in gene expression profiling of whole T helper cells from the lungs of mice after intranasal HDM administration. Several target genes of PPAR-γ also showed higher expression levels when compared to PBS-administered mice (Coquet et al., 2015b; Schneider et al., 2014). Cells from the lungs of mice after HDM sensitization and challenge were purified and divided into Th2 and Th17 cells based on the surrogate surface markers ST2 and CCR6 respectively, in order to determine which subset may express *Pparg* (Fig. 4A-D). *Pparg* was significantly more highly expressed in the sorted Th2 ($ST2^+$) cells, where *Il5* mRNA was also detected at high levels (Fig. 4B, 4C). Cells sorted from mesenteric lymph nodes (mesLN) of mice infected with the nematode *Heligmosomoides polygyrus* showed a similar pattern of expression (Fig. 4E-I), as did *in vitro*-generated T helper cell subsets. *In vitro*-generated Th2 cells from mice lacking one allele of *Gata3* had significantly lower *Pparg* mRNA levels compared to cells from wild-type (WT) mice. This showed that PPAR-γ expression was specific for Th2 cells in multiple contexts.

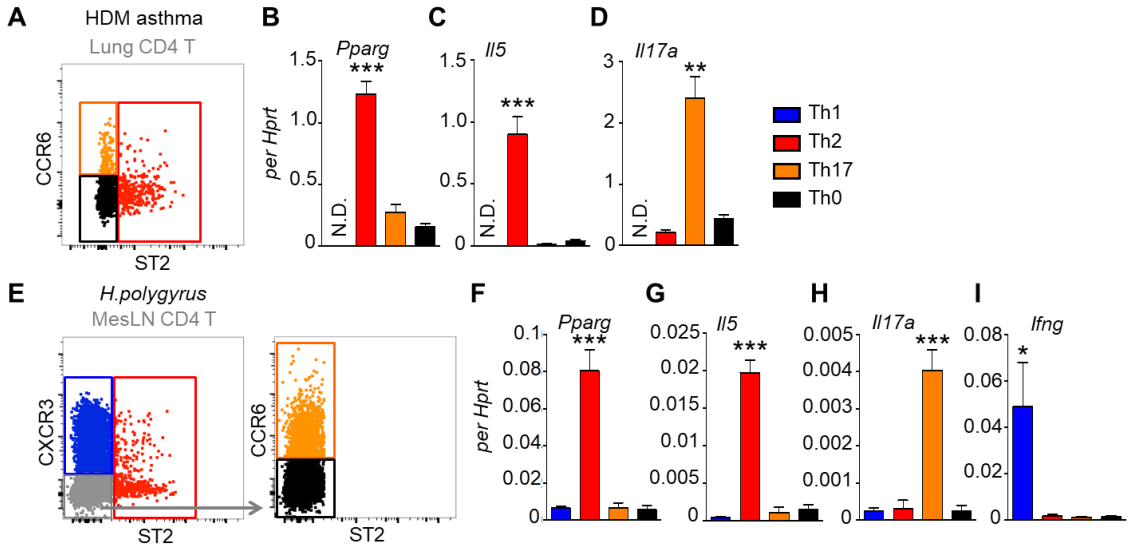


**Figure 4.** mRNA levels of key cytokines and *Pparg* in T helper cell subsets **A-D** sorting strategy for Th0, Th2 and Th17 and expression levels of *Pparg*, *Il5* and *Il17a* in cells from the lung of HDM administered mice. **E-I** sorting strategy for Th0, Th1, Th2 and Th17 and expression levels of Pparg, *Il5*, *Il17a* and *Ifng* in cells from the mesLN 15 days after infection with *H. polygyrus*.

We crossed mice with LoxP sites engineered into the *Pparg* gene with mice expressing the *Lck-Cre* transgene in order to explore the role of PPAR-γ in Th2 cell differentiation and function. Mice in which the *Pparg* gene was targeted for deletion in T cells will be referred to as $Pparg^{Lck\Delta}$ and WT littermate control mice lacking the *Lck-Cre* transgene as $Pparg^{+}$. Bronchoalveolar lavage (BAL) from $Pparg^{+}$ and $Pparg^{Lck\Delta}$ revealed similar levels of alveolar macrophages (Fig. 5A). *In vitro*-differentiated Th2 cells from $Pparg^{Lck\Delta}$ had a 90% reduction in expression of *Pparg* mRNA compared to $Pparg^{+}$ demonstrating almost complete deletion of *Pparg* in the CD4 T cell lineage (Fig. 5B).

Mice were sensitized and challenged with HDM extracts to explore the role of PPAR-γ *in vivo*. Airway eosinophilia (Fig. 5C, 5D) and the number of airway-infiltrating T cells were reduced in $Pparg^{Lck\Delta}$ compared to $Pparg^{+}$ mice after HDM instillations, whereas the number of alveolar macrophages and neutrophils was not affected. $Pparg^{Lck\Delta}$ mice lacked mucus-secreting goblet cells in the airways, as determined by periodic acid-Schiff staining of lung sections, while $Pparg^{+}$ mice showed goblet cell metaplasia after HDM sensitization and challenge. Lungs and mediastinal lymph nodes (medLN) had comparable numbers of $CD4^{+}CD44^{+}$ in $Pparg^{+}$ and $Pparg^{Lck\Delta}$ mice after HDM administration. However, these effector cells expressed lower levels of IL-5 and IL-13 in the lung in $Pparg^{Lck\Delta}$ mice, while expression of IL-4 and IL-17 was not affected by the lack of *Pparg* expression in those cells. Production of Th2 cell cytokines in the medLN was similar between $Pparg^{Lck\Delta}$ and $Pparg^{+}$ mice. This suggested that PPAR-γ was important for the development of $IL\text{-}5^{+}IL\text{-}13^{+}$ Th2 cell responses in the inflamed lung but not for the initial priming in the lymph node.

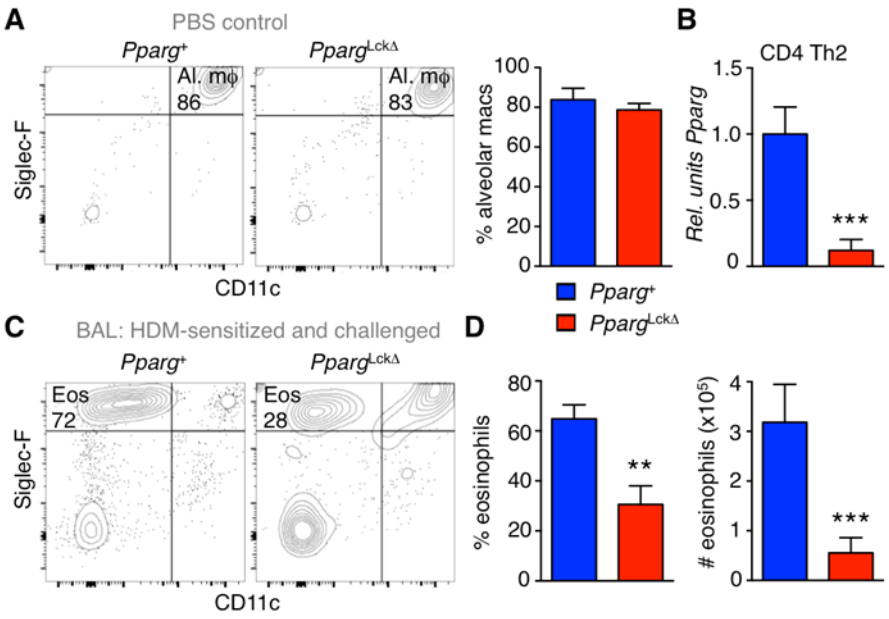


**Figure 5.** The response of *Pparg*$^{+}$ and *Pparg*$^{LckD}$ mice to instillations of HDM **A**, representative plots of CD11c versus Siglec-F expression in PBS treated (*Pparg*$^{+}$ n=3) and (*Pparg*$^{LckD}$ n=4). and frequency of alveolar macrophages. **B**, relative expression of Pparg mRNA naïve CD4 T cells activated and cultured in Th2 cell differtiation conditions (*Pparg*$^{+}$ n=6; *Pparg*$^{LckD}$ n=8) **C**, representative plots of CD11c versus Siglec-F expression in mice after administration of HDM, Eos (eosinophils CD11c$^{-}$ Siglec-F$^{+}$) (*Pparg*$^{+}$ n=3) and (*Pparg*$^{LckD}$ n=4). **D,** frequency and total number of eosinophils shown in D.

*H. polygyrus*-infected mice were used to explore whether lack of PPAR-γ had a similar effect on Th2 cell responses in parasite infections. Both *Pparg*$^{+}$ and *Pparg*$^{LckΔ}$ mice had increased numbers of total lymphocytes and effector CD4 T cells in the mesLN fifteen days after infection. Similar to what was observed in the HDM model, mice lacking *Pparg* expression in CD4 T cells had lower frequencies of IL-5$^{+}$IL-13$^{+}$ cells in the mesLN and lower frequencies of eosinophils. *H. polygyrus*-infected *Pparg*$^{LckΔ}$ mice did not have reduced numbers of IL-4$^{+}$ or IL-17$^{+}$ T helper cells when compared to *Pparg*$^{+}$ mice. *Pparg*$^{LckΔ}$ mice had reduced serum levels of IgE after worm infection, further indicating an impaired type 2 immune response. Worm burden after primary infection with *H. polygyrus* was similar between *Pparg*$^{+}$ and *Pparg*$^{LckΔ}$ mice, but when mice were dewormed two weeks after the first infection and reinfected one week later, *Pparg*$^{LckΔ}$ mice had a significantly higher worm burden. Thus, *Pparg* was required for the differentiation of IL-5$^{+}$IL-13$^{+}$ Th2 cells and was required for protective immunity to *H. polygyrus*.

The use of PPAR-γ agonists allowed us to explore the effect of PPAR-γ on Th2 cell effector functions. The prostaglandin derivative 15dΔ12,14-PGJ$_2$ (15d-PGJ$_2$) was added to Th2 cell differentiation cultures. We did not observe an increase in IL-5 or IL-13 production, but expression of *Il1rl1* (the gene encoding for ST2) was upregulated in cells exposed to 15d-

$PGJ_2$, and this was confirmed at the protein level by flow cytometry. A similar increase in ST2 expression could be induced with the synthetic PPAR-γ agonist pioglitazone (PIO). Both 15d-$PGJ_2$ and PIO could not increase the frequency of ST2 expression in Th2 cell differentiation cultures on cells from *Pparg*$^{LckΔ}$ mice. The addition of PPAR-γ agonists could therefore be a way to increase the expression of ST2 on *in vitro*-differentiated Th2 cells, whose low expression *in vitro* has been recognized as a shortcoming of Th2 differentiation assays (Guo et al., 2009; Meisel et al., 2001).

Next, we investigated whether lack of *Pparg* expression would lead to reduced ST2 levels *in vivo*. Effector CD4 T cells from the lungs of HDM-administered *Pparg*$^{LckΔ}$ mice expressed less ST2 compared to *Pparg*$^{+}$ mice. As described for IL-5 and IL-13 above, this difference could not be observed in effector cells from the medLN, further confirming the role of PPAR-γ in the development of a pathogenic Th2 cell response in the lung. Effector CD4 T cells from the mesLN and Peyer's patches of *H. polygyrus*-infected *Pparg*$^{LckΔ}$ mice similarly showed less ST2 expression than cells from *Pparg*$^{+}$ mice. Chimeric mice were created by injecting lethally irradiated congenic CD45.1$^{+}$ mice 1:1 with bone marrow cells from WT (CD45.1$^{+}$CD45.2$^{+}$) and *Pparg*$^{LckΔ}$ (CD45.2$^{+}$) mice, in order to explore whether the requirement of PPAR-γ for Th2 cell responses was cell-intrinsic. When these mice were sensitized and challenged with HDM eight weeks after radiation and cell transfer, only a small proportion of *Pparg*$^{LckΔ}$ effector CD4 T cells produced IL-5 and IL-13 in the lung compared to WT effectors. ST2 expression was also much lower in effector CD4 T cells from the *Pparg*$^{LckΔ}$ fraction. In order to control for the effect of the *Lck-Cre* transgene alone, HDM was administered to *Lck-Cre*$^{+}$*Pparg*$^{+/+}$ and *Lck-Cre*$^{-}$*Pparg*$^{+/+}$ mice. There was no difference in levels of airway infiltrating eosinophils or expression of ST2 on effector CD4 T cells.

We sorted CD4$^{+}$CD44$^{+}$ST2$^{+}$ cells from lungs of *Pparg*$^{+}$ and *Pparg*$^{LckΔ}$ mice and performed whole-genome expression arrays, in order to better understand which genes may be regulated by PPAR-γ. Expression of master transcriptional regulators, such as *Foxp3*, *Gata3*, *Bcl6*, *Tbx21* and *Rorc*, did not differ between both groups of mice. Analysis showed 29 genes to be differentially expressed by at least twofold between cells from *Pparg*$^{+}$ and *Pparg*$^{LckΔ}$ mice. These included genes known for their function in Th2 cell responses like *Il5* and *Il13* as well as known PPAR-γ target genes such as *Chi3l3*. The expression of several genes involved in metabolic processes such as carbohydrate synthesis (*Galnt3*), metabolite transport (*Slc7a8*) or lipid storage (*Plin2*) was increased in cells from *Pparg*$^{+}$ mice while *Tagap1* and *Rras2*, encoding for two small GTPases, were reduced. Increased expression of *Galnt3* and *Plin2* from *in vitro*-generated Th2 cells could be observed when 15d-$PGJ_2$ was added. PPAR-γ potentially supports Th2 cell function in the lung by promoting the expression of metabolic regulators.

Visceral adipose tissue (VAT) contains populations of both CD4 T cells and ILC2 that have been proposed to play a role in the regulation of glucose metabolism (Cipolletta et al., 2012; Molofsky et al., 2013). *Pparg* mRNA could be detected in both CD4 T cells as well as ILC2

from the VAT albeit at lower levels than found in VAT macrophages. ILC2 from the VAT of *Pparg*$^{LckΔ}$ mice did not have reduced levels of *Pparg* expression and were found at the same frequency as in the VAT of *Pparg*$^{+}$ mice. Treg cells in the VAT have been described to express high levels of ST2 (Cipolletta et al., 2012). ST2 expressing CD4 T helper cells from both conventional Foxp3$^{-}$ CD4 T helper cells and Treg cells from the VAT of *Pparg*$^{LckΔ}$ mice were significantly reduced. CD4 T helper cells from the VAT of *Pparg*$^{+}$ and *Pparg*$^{LckΔ}$ mice were restimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin to assess the influence of PPAR-γ on cytokine production in these cells. Cells from *Pparg*$^{LckΔ}$ mice had a decrease in IL-5$^{+}$ frequency but an increase in IFN-γ$^{+}$ cells compare to cells from *Pparg*$^{+}$ mice. IL-4, IL-5 and IL-10 have previously been described to promote glucose sensitivity and to mediate positive metabolic effects (Cipolletta et al., 2012; Wu et al., 2011). Contrary to that, we observed that *Pparg*$^{LckΔ}$ mice gained less fat when aging and showed better control of glucose levels after overnight fasting. These findings indicate a potentially detrimental role of ST2$^{+}$ Treg and Th2 cells for glucose sensitivity in aging animals, in line with recent studies on the effect of fat-resident Treg cells on age-associated insulin resistance (Bapat et al., 2015).

Paper I described an important role of PPAR-γ in the full effector differentiation of IL-5- and IL-13-expressing Th2 cells both in HDM-induced allergic airway inflammation and infection with the intestinal parasite *H. polygyrus*. PPAR-γ seemed to promote the expression of the IL-33 receptor, which has been described to play an important role in the acquisition of Th2 cell-specific functions , including secretion of IL-5 and IL-13 (Van Dyken et al., 2016). Furthermore PPAR-γ potentially influenced Th2 cell metabolism by regulating various metabolic genes.

## 3.2 PAPER 2

T helper cells play a central role in the development of asthma and allergic airway disease, with several subsets, such as Th2, Th9 and Th17 cells contributing to different types of disease (Koch et al., 2017; Lambrecht and Hammad, 2015). Single cell-RNA sequencing (scRNA-seq) allows for unbiased examination of gene expression at the single-cell level (Picelli et al., 2013), making it an excellent tool to study diverse populations of cells, which are difficult to sort by surface markers or which exhibit considerable plasticity, like T helper cells. In recent years several studies have employed scRNA-seq to study T helper cell responses in mouse models of malaria infection and multiple sclerosis (Gaublomme et al., 2015; Lönnberg et al., 2017).

We used the HDM model to induce allergic airway inflammation in mice and purified single $CD3^+CD4^+CD44^+$ from the BAL. The BAL was chosen since cytokine-secreting cells are found in higher frequencies in this compartment, compared with the lung tissue and the medLN. Single cells were sorted into 384-well plates in two independent experiments and single-cell RNA sequencing (scRNA-seq) was performed using the SMART-Seq2 platform (Picelli et al., 2013). Out of the overall 12,000 genes that could be detected, 1,971 were variably expressed and six distinct clusters of T helper cells could be identified using unsupervised hierarchical clustering and visualization with t-distributed stochastic neighbor embedding (t-SNE) (Fig. 6A). Clusters were assigned to different types of T helper cells based on the overexpression of previously described key marker genes (Fig. 6B). We could thus identify clusters as Th2 (*Il1rl1*, *Gata3*, *Il13*,*Il5*) (Fig. 6C), Th1 (*Cxcr3*, *Ccl5*, *Ms4a4b*) and Treg (*Foxp3*, *Ctla4*, *Il10*) cells. No cluster could be identified as Th17 cells and expression of *Il17a* was weak, despite the detection of IL-$17^+$ T helper cells after PMA stimulation. *Pparg* was highly enriched in Th2 cells, in line with Paper I and others describing PPAR-γ as having an important role for Th2 cell development and function (Angela et al., 2016; Chen et al., 2017; Nobs et al., 2017). We furthermore identified several genes as enriched in the Th2 cell cluster that have previously not been linked to Th2 cell biology, such as *Igfbp7*, *Plac8*, *Gclc* and many more (Fig. 6C). Thus scRNA-seq could be used to distinguish distinct clusters of T helper cells from the airways of mice administered HDM.

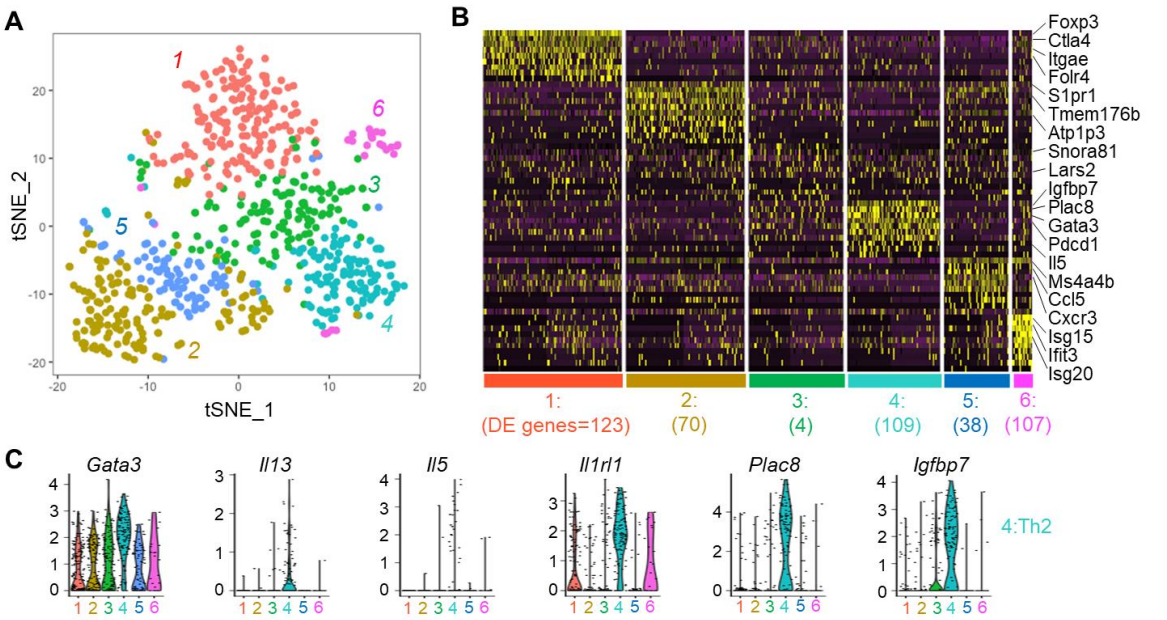


**Figure 6.** 695 single T helper sorted from the airways of mice exposed to HDM and analyzed by SMART-Seq2 **A**, t-SNE representation 695 single T helper cells. **B**, Heatmap of the ten most differentially expressed (DE) genes in each cluster (the number in brackets indicates the total number of DE genes per cluster based on adjusted p value). **C** Violin plots of key genes for the Th2 cell cluster.

One of the clusters was found to be enriched for genes linked to the type-I interferon (IFN) response, including *Ifit3*, *Isg15*, *Isg20*, *Mx1*, *Stat1* and *Stat2*. There was however no preferential expression of the type-I IFN receptors, indicating that cells in this cluster were not predisposed for a type-I IFN response. This response could be triggered by toll-like receptor (TLR) ligands in the allergen extract (Hammad et al., 2009). To test this, wild-type (WT), *Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ mice were sensitized and challenged with HDM and total lung CD4$^{+}$ cells were sorted. Expression of *Isg20* and *Mx1* was assessed by qPCR. WT mice administered PBS and *Myd88*$^{-/-}$ mice administered HDM expressed low levels of both genes compared to WT mice exposed to HDM. *Tlr4*$^{-/-}$ mice had an intermediate phenotype, indicating that HDM possibly induces the production of type-I IFNs through TLR4 and other pattern recognition receptors. When mice were injected with an IFN-α receptor blocking antibody, *Mx1* gene expression in both sorted CD4$^{+}$CD44$^{+}$CD25$^{-}$ST2$^{+}$ or CD4$^{+}$CD44$^{+}$CD25$^{-}$ ST2$^{-}$ cells was reduced.

After showing that Th2 cells could be identified and studied by scRNA-seq in the airways on day 15, we used scRNA-Seq to study their differentiation at earlier time points. The production of Th2 cell cytokines was analyzed on day 0, 8, 10 and 15 in medLN and lung cells in order to determine the best time to purify cells for sequencing. Th2 cells could be detected by cytokine expression in small numbers in the lymph node at day 8 and increasing at day 10. Th2 cells became evident in the lung at day 10 and expanded further at day 15. ST2 was chosen as a marker to enrich Th2 cells for scRNA-seq since a large percentage of Th2 cytokine producing cells on day 10 in the medLN also expressed ST2. This was necessary since only a small proportion of T helper cells in the lung and medLN are Th2 cells. We sorted 200 CD4$^{+}$CD44$^{+}$CD25$^{-}$ST2$^{+}$ cells from medLN and 82 from lung on day 10. An additional 50 cells each of naïve CD4$^{+}$ T and Treg cells from the medLN on day 0 were

purified. Six clusters and an additional seventh cluster containing proliferating cells could be identified from the single cell data set. It was evident that the transcriptional signature of cell clusters was associated with their tissue of origin and time-point. Treg cells could be identified in cells from naïve and day 10 medLN and from day 10 lung whereas naïve $CD4^+$ and Th2 cells were mostly found in single clusters. Cells with a type-I IFN signature again formed a separate cluster, containing both Treg and non-Treg cells, mostly from cells purified from the lung at day 10. Similar to Th2 cells from the airways on day 15, medLN Th2 cells on day 10 were enriched for *Il4*, *Pparg*, *Igfbp7*, *Pdcd1* and *Vdr*. However, they were not enriched for *Il13*, in line with previous studies showing that IL-13 expression is only acquired after migration to the lung (Liang et al., 2012; Van Dyken et al., 2016). While stimulation of day 10 cells from the medLN was able to stimulate expression of IL-13, this might only be produced to a small extent under physiological conditions.

We combined all single-cell data sets to probe Th2 cell differentiation over time. Clusters containing naïve, day 10 Th2 cells and day 15 Th2 cells were used for trajectory analysis by Scorpius (Cannoodt et al., 2016). Trajectory was defined from naïve cells passing through day 10 to day 15 Th2 cells and four gene expression modules could be identified. The first one contained genes whose expression was increased early in Th2 cell development and then maintained to day 15 such as *Gata3*and *Nfkb1*. The second module consisted of genes mainly expressed on day 15 Th2 cells (*Ctla2a*, *Ahnak*, *Rbpj*, *Bhlhe40*). The third and fourth modules were of genes expressed in naïve cells that were maintained at low levels (*Pfn1*, *Gimap3*) or not expressed at later time points (*Lef1*, *Igfbp4*). *Il4* expression was higher in Th2 cells on day 10 than on day 15, implicating a stronger role for IL-4 in the lymph node than in the lung tissue. Another feature of day 10 cells was the expression of a set of genes associated with Tfh cells (*Il6st*, *Tox*, *Cd200*, *Slamf6*, *Rilpl2*, *Plekho1*) (Choi et al., 2015). However some canonical Tfh cell-associated genes, such as *Bcl6* and *Cxcr5* were not enriched in day 10 Th2 cells. Flow cytometry confirmed the high expression level of Tfh cell-associated markers CD200, CD352, and ICOS and the low expression levels of CXCR5 and BCL6 of day 10 Th2 cells.

Several genes found to be enriched in Th2 cells by scRNA-seq were also confirmed by flow cytometric analysis of protein expression. Th2 cells from the lung and airways expressed CD200R1 and RANKL more frequently and had higher levels of PD-1 when compared to other cell subsets. Cells from the medLN showed a similar pattern, except for RANKL which was expressed on both Th2 and non-Th2 cells in similar frequencies. Cells were restimulated with PMA and cytokine expression was measured. IL-6 expression coincided with the expression of IL-5 and IL-13 but not with other cytokines. In the *H. polygyrus* model, CD200R1 and IL-6 were also found to be enriched in Th2 cells from the mesLN whereas PD-1 and RANKL were not preferentially expressed on Th2 cells. We thus confirmed several targets found in the scRNA-seq dataset and showed organ- and model-specific expression patterns.

While scRNA-seq is an excellent tool to capture the gene expression of a single cell at the moment of analysis, it is limited in providing insights into the history of a cell and does not reveal other potential capabilities in gene expression. Analyzing chromatin status can reveal such features. Thus, we purified naïve cells from the medLN and Th2, non-Th2 and Treg cells from airway and lung combined of mice sensitized and challenged with HDM and performed assay for transposase-accessible chromatin using sequencing (ATAC-seq) (Buenrostro et al., 2015). Intervene (Khan and Mathelier, 2017) was used to gain a global understanding of the DNA accessibility. Over 50% out of 43,092 called peaks were specifically enriched in a single subset. Th2 cells showed accessibility at several regions across the *Il4-Il13-Rad50* locus (Ansel et al., 2006), whereas non-Th2 and Treg cells were more accessible at the *Ifng* and *Foxp3* loci respectively (Feng et al., 2014; Shih et al., 2016). Several genes identified by scRNA-seq as highly expressed in day 15 Th2 cells were found to be more accessible in sorted Th2 cells, including *Vdr*, *Il6*, *Plac8*, *Cd200r1* and *Igfbp7*. ATAC-seq further allowed us to explore transcription factor (TF) binding sequences enriched in specific subsets using HOMER (Heinz et al., 2010). Th2 and Treg cell subsets were both enriched for motifs related to nuclear factor $_{\kappa}$B (NF-$_{\kappa}$B), AP-1, STAT and GATA3 activity. Th2 cells were specifically enriched for binding motifs for PU.1, IRFs, CEBP, Mef2d and PPAR-γ, whereas Treg cells were enriched for RORγt and Nur77 motifs.

By using Gene Ontology (GO) analysis (Consortium, 2017), molecular processes enriched in Th2 cells from the airways of HDM administered mice could be identified, including processes related to apoptosis, leukocyte differentiation, cytokine production as well as cellular metabolism to be enriched in Th2 cells. The Seahorse XF analyzer was used to probe the metabolic profile of *in vitro*-generated T helper cell subsets. It was necessary to use *in vitro*-differentiated cells, since these metabolic assays require a high number of cells which makes sorting cells from allergen-exposed mice not feasible. Th2 cells were the most glycolytic and had the highest rate of oxygen consumption compared to Th1and Treg cells. Previous studies (Chang et al., 2013) have described a role of glycolysis directly promoting the production of IFN-γ. To test the requirement for glycolysis in Th2 cells, cells were differentiated *in vitro* in medium containing glucose and glycolysis was either perturbed by the addition of 2-deoxy-D-glucose (2-DG) or by switching cells onto galactose for the last day of culture, which forces cells to use oxidative metabolism instead of glycolysis. Either intervention was able to greatly reduce IL-13 production compared to cells cultured in glucose. Gene set enrichment analysis (GSEA) of Th2 cells from the BAL of mice administered HDM did not identify genes involved with glucose metabolism to be especially enriched. Th2 cells were however enriched for the expression of genes involved in fatty acid oxidation and fatty acid synthesis.

Fatty acid metabolism has been shown to be critical for ILC2 function (Wilhelm et al., 2016). The metabolic inhibitors 2-DG (blocks glycolysis), etomoxir (inhibits fatty acid oxidation) and orlistat (blocks fatty acid synthesis and uptake) were administered daily, starting on day 7 (Fig. 7A)(Kridel et al., 2004; Padwal and Majumdar, 2007; Qu et al., 2016). Inhibition of any of these pathways led to a reduction in airway eosinophilia and numbers of airway-infiltrating

T cells when compared to control mice administered DMSO (Fig.7 A). Periodic acid-Schiff staining of lung sections and qPCR for *Muc5ac* mRNA showed that each drug was able to reduce the amount of mucus production and goblet cell metaplasia after HDM administration. Blocking of glycolysis had the strongest effect, reducing frequencies of Th1 and Th2 cells in the medLN and increasing the percentage of Treg cells in the airways (Fig. 7B). Impacting fatty acid metabolism had a strong effect in reducing Th2 frequencies, both in the airways and the lymph node, but not as strong as administration of 2-DG (Fig.7B). Total IgE levels were also impacted by blocking metabolic pathways, whereas allergen-specific IgG1 levels were not affected.

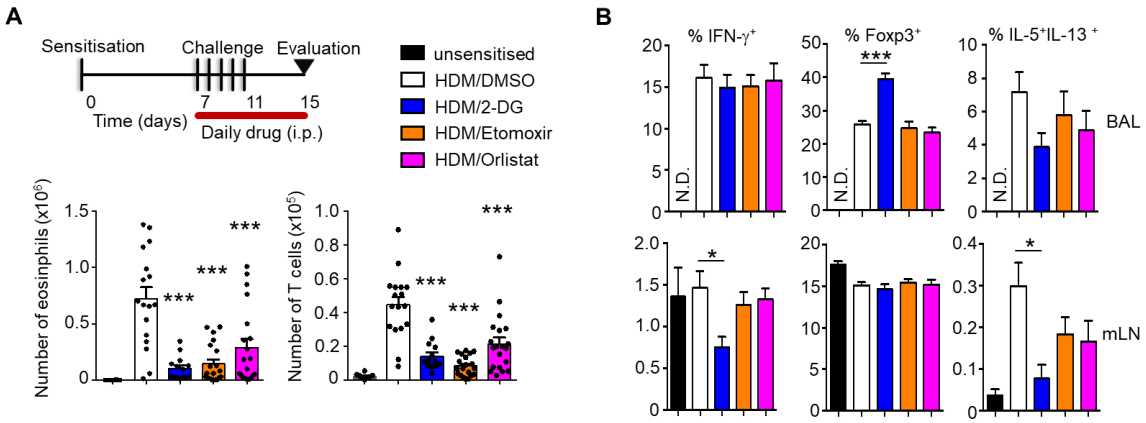


**Figure 7.** The effect of metabolic inhibitors on airway inflammation induced by HDM **A**, Regimen of drug injections and HDM administrations, graphs of eosinophil and T cell numbers from the airways. **B**. Frequency of $CD4^+$ cells from the BAL and the medLN expressing IFN-g, Foxp3 and dual expression of IL-5 and IL-13.

In all, paper II used scRNA-seq to resolve the transcriptional profiles of naïve $CD4^+$ T, Th1, Th2 and Treg cells as well as a population of responsive to type-I interferons from the airways of mice administered HDM. Expression of many genes was enriched in Th2 cells, including *Il6*, *Cd200r1* and *Plac8*, which was further supported by analysis of chromatin accessibility. scRNA-seq performed at different time points revealed gene expression patterns during the development of Th2 cells. GSEA showed Th2 cells to be enriched for fatty acid metabolism and experiments with metabolic inhibitors could demonstrate that Th2 cell responses require both glycolysis and fatty acid metabolism.

## 3.3 PAPER 3

The global incidence of asthma and allergic disease is rising (Moorman et al., 2012). Up to twelve percent of US citizens have been found to be sensitized to dog allergens and exposure to dogs has been estimated to cause over a million asthma attacks in sensitized patients per year in the US alone (Gergen et al., 2018). The only cure for allergic disease is allergen-specific immunotherapy, where allergen is administered to patients in low doses over a long period of time with the goal of inducing tolerance (Akdis and Akdis, 2011). AIT for dog allergies has been attempted in several clinical trials with mixed results (Smith and Coop, 2016). Unlike for many other sources of allergens like cat, house dust mite or peanuts, there is to this date no established mouse model for dog allergies (Burton et al., 2017; Cates et al., 2004; Neimert-Andersson et al., 2008). Such a model would be an important step towards achieving a better understanding of allergic responses to dog allergens and the development of therapies specific to dog allergy. We therefore aimed to establish a mouse model using inhaled dog allergen extracts, to characterize the immune response and to test sublingual administration of a recombinant allergen protein.

We used a mix of dog dander and epithelial extracts for intranasal instillations in a similar regimen as was used in paper I and II (Fig. 8A). Mice sensitized and challenged with dog allergen extracts showed levels of airway infiltrating total cells (Fig. 8A), eosinophils (Fig. 8C) and B cells comparable to the HDM model. Mice administered either PBS, HDM or dog allergen extracts had comparable numbers of alveolar macrophages (Fig. 8D). Administration of either HDM or dog allergen extracts induced goblet cell metaplasia. Dog allergen extracts however led to higher levels of T cells in the airways and a moderate increase of airway infiltrating neutrophils (Fig. 8A). Administration of dog allergen extracts also led to airway hyperresponsiveness when compared to PBS-administered mice. Inhalation of dog allergen extracts therefore induced similar levels of airway inflammation as HDM when used in a fifteen day model.

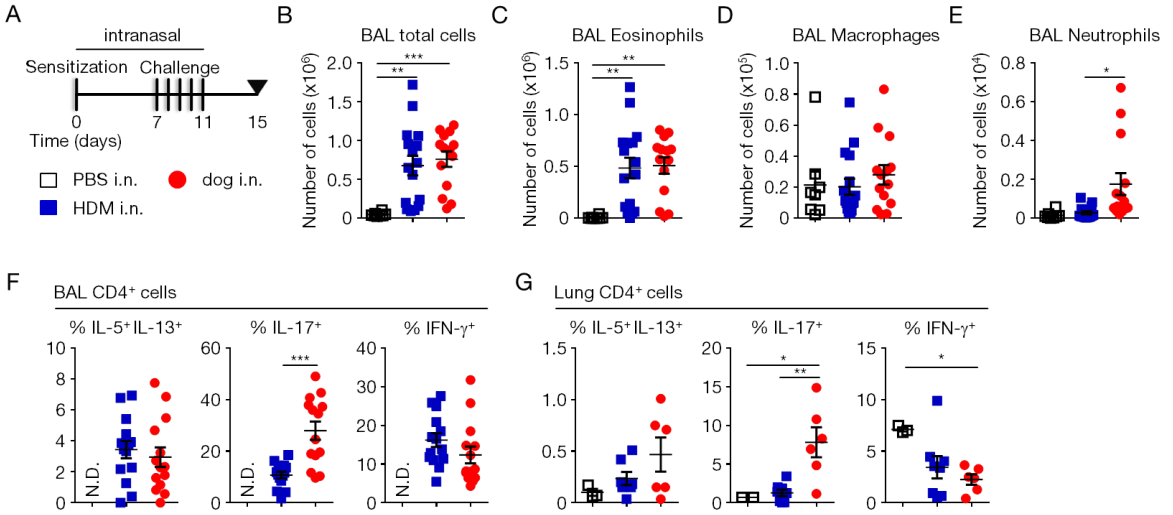


**Figure 8.** Airway inflammation induced by dog allergen extracts **A**, Schematic of allergen instillation regimen. **B-E** Graphs of cell numbers in the BAL **B**, total cells. **C**, eosinophils. **D**, macrophages. **E**, neutrophils. **F-G** percentage of IL-5$^+$IL-13$^+$, IL-17$^+$ or IFN-g$^+$ T helper cells from **F**, the BAL. **G**, the lung.

*Rag1* knockout mice, lacking mature B and T cells, did not develop airway inflammation when exposed to dog allergen extracts which indicated that the observed airway inflammation was due to an adaptive immune response. Frequencies of T helper cells from the BAL of mice either administered HDM or dog allergen extracts producing Th2 cytokines or IFN-γ were similar, but mice exposed to dog allergen extracts showed significantly higher levels of IL-17 producing cells (Fig. 8F). The frequency of IL-17$^+$ CD4 T cells was also higher in the lung of mice exposed to dog allergens (Fig. 8G). Since high levels of endotoxin in allergen extracts have been associated with stronger Th17 responses in mouse models (Zhao et al., 2017), we tested the endotoxin content of the HDM and the dog allergen extracts. Both dander and epithelial extract were found to contain two orders of magnitude more endotoxin than the HDM extract. Th2 cells promote B cell class switching to IgG1 and IgE and serum from mice sensitized and challenged with dog allergen extracts had higher levels of allergen-specific IgG1 and total levels of IgE when compared to mice administered PBS. Th17 cells promote the recruitment of neutrophils to sites of inflammation. The allergen administration regimen was modified by one additional instillation on day 15, three hours before sacrificing the mice since neutrophils are recruited rapidly to sites of inflammation. Mice challenged with this additional dose of dog allergen extracts showed a strong increase of airway infiltrating neutrophils when compared to HDM-administered mice.

Lymph node cell cultures were used to assess specificity of cytokine production. Cells from mice administered either PBS alone, HDM or dog allergen extracts were stimulated with allergen extracts or recombinant Can f 1 or Can f 2 and cytokine production was quantified by a cytometric bead assay on the supernatant after two days of culture. Cells from mice challenged with either HDM or dog allergen extracts could be induced to produce IL-13, IL-5 and IL-10 when stimulated with the respective allergen extract. Only stimulation of cells from mice exposed to dog allergens could induce production of IL-17 and IFN-γ. Dog allergen extracts induced low levels of cytokine production in cells from mice administered PBS or HDM, indicating involvement of an innate source of cytokine production. Both

recombinant allergens could stimulate cells from dog allergen exposed mice to produce IFN-γ, but only Can f 1 could induce production of IL-5, IL-13 and IL-10.

Th17 cells in allergy have been associated with adult-onset and steroid-resistant asthma (Domvri et al., 2018). We treated mice with dexamethasone to test whether corticosteroids could ameliorate the airway inflammation caused by dog allergen extracts. Mice received daily injections of dexamethasone starting on the first day of allergen challenge and one final dose of allergen extracts was administered three hours before sacrifice. Corticosteroid treated mice had lower levels of airway infiltrating eosinophils, B cells and T cells but the number of neutrophils in the airways was not significantly reduced. Numbers of both Th2- and Th17-cytokine producing cells were reduced by dexamethasone injections.

scRNA-Seq was used to further investigate the diversity of the T helper cell response to dog allergen extracts. $CD3^+$ $CD4^+$ cells from the BAL of mice sensitized and challenged with dog allergen extracts were sorted and analyzed using the droplet-based microfluidic system, Chromium (10X Genomics). We performed unsupervised hierarchical clustering and visualization using the Seurat pipeline and were able to identify 8 distinct clusters. Several clusters based on the expression of known marker genes, namely Th2, Th17, Treg and naïve cells were pinpointed. One cluster was enriched for genes associated with recently described CD4 CTL such as *Ccl5*, *Gzmk*, *Ly6c2*, *Nkg7* and *Tbx21*. Notably this cluster was not specifically enriched for *Ifng*, which was expressed in many cell clusters. The Th17 cell cluster was enriched not only for known Th17 cell-associated genes such as *Il17a*, *Ccr6* and *Rorc* but also for several genes like *Aqp3*, *Ramp1* and *Tmem176a* which have only recently been linked to Th17 cell biology (Drujont et al., 2016; Mikami et al., 2012; Zhou et al., 2016). The Th2 cell cluster was found to be enriched for several genes such as *Plac8*, *Zcchc10*, *Cd200r1* and *Il6* that were also found in Th2 cells in the HDM model in paper II. Further confirming our findings from paper II was also GO analysis showing that Th2 cells were enriched for genes involved with the regulation of apoptosis and lipid metabolism. We observed some gene expression overlap of *Ifng*, *Il13* and *Foxp3* with the Th17 cluster and confirmed the existence of IL-$5^+$ IL-$13^+$ IL-$17^+$ triple positive and IL-$17^+$ Foxp$3^+$ or IL-$17^+$ IFN-$\gamma^+$ double positive T helper cells by FACS. Thus scRNA-seq confirmed that dog allergen extracts cause a diverse response of T helper cells subsets including Th2 and Th17 cells.

The analysis of TCR gene usage showed a diverse clonal response to dog allergen extracts. Correlation analysis showed the most clonal overlap between clusters 1-5 with a high frequency of clones shared between the Treg cluster and the other effector cell populations including Th2 and Th17 cells. This could be due to the generation of induced Treg cells (iTreg) from naïve cells or to the transdifferentiation of Treg cells into other subsets. Naïve cells had the least clonal overlap followed by the cluster showing characteristics of CD4 CTL indicating a distinct differentiation pathway for these cells. Thus TCR analysis reveals patterns of shared and restricted clonality between subsets of T helper cells infiltrating the lung after dog allergen extract administration.

We applied the dog allergen model to test prophylactic SLIT treatment using a recombinant protein called “necklace”, comprising Can f 1, Can f 2, Can f 4 and Can f 6 (Nilsson et al., 2014a). Four weeks with three sublingual administrations of Necklace per week prior to allergen sensitization and challenge (Fig. 9A) was sufficient to reduce the number of airway infiltrating total cells (Fig. 9B), eosinophils (Fig. 9C), increase the number of Treg cells in the lung (Fig. 9E) and reduce the frequency of Th2-cytokine producing cells in the airways (Fig. 9F). SLIT however could not reduce the proportion of IL-17 producing T helper cells and even increased the percentage of IFN-$\gamma^+$ cells (Fig. 9F). Necklace SLIT did not reduce airway neutrophilia. Mice treated with Necklace showed reduced airway hyperresponsiveness and serum levels of total IgE were reduced while levels of dog allergen-specific IgG1 were increased.

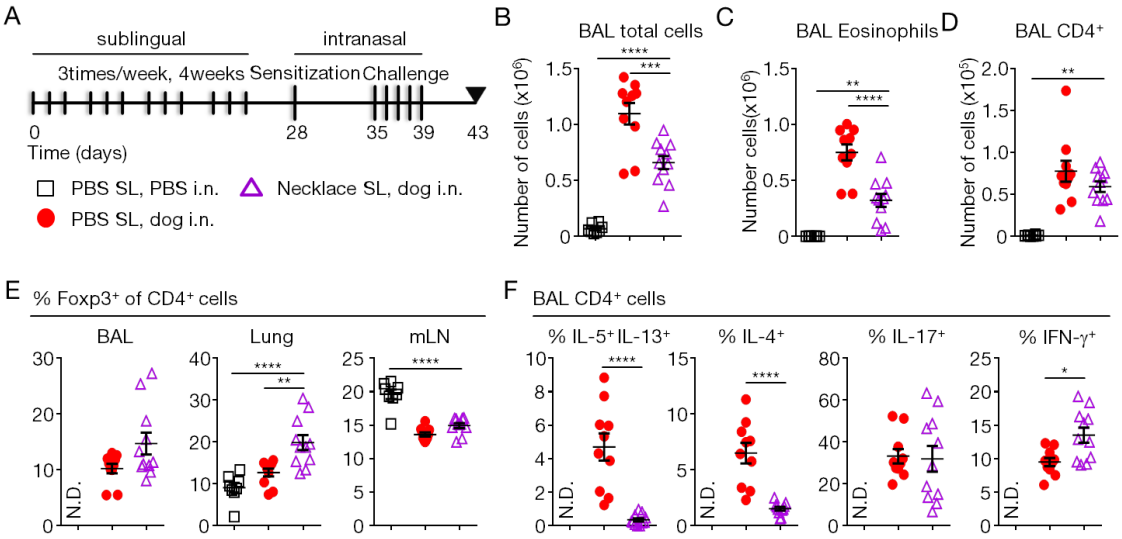


**Figure 9**. Sublingual immunotherapy with recombinant dog allergen **A**, schematic of four weeks of SLIT followed by intranasal allergen extract administrations. **B-D** Graphs of cell numbers in the BAL **B**, total cells. **C**, eosinophils. D, $CD4^+$ T helper cells. **E**, percentage of $Foxp3^+$ of $CD4^+$ cells from the BAL, lung and medLN. **F,** percentage of $IL\text{-}5^+IL\text{-}13^+$, $IL\text{-}4^+$, $IL\text{-}17^+$ or $IFN\text{-}g^+$ T helper cells from the BAL.

Paper III showed that repeat inhalations of dog allergen extracts lead to airway inflammation in mice. This model produced a mixed Th2/Th17 cell response reminiscent of neutrophilic and adult-onset asthma. This study further demonstrated the potential use for SLIT using recombinant dog allergen proteins for treating dog allergen-induced airway inflammation.

## 3.4 FINAL REFLECTIONS AND FUTURE PERSPECTIVES

This thesis provides insights into the diversity of T helper cells involved in allergic airway disease. The major findings are.

- PPAR-γ expression is required for Th2 cells to properly differentiate into IL-5- and IL-13-producing cells in inflamed lung tissue and promotes the expression of the IL-33 receptor on Th2 cells.
- scRNA-seq of T helper cells in the HDM model of allergic airway inflammation reveals a population of T helper cells responding to type-I interferons, a distinct gene expression signature in Th2 cells and shows that Th2 cells in the airways are enriched for lipid metabolism genes. The use of metabolic inhibitors confirms that glucose and lipid metabolism is important for Th2 cell-mediated responses.
- A novel mouse model for allergies to dogs shows a mixed Th2/Th17 cell response. scRNA-seq provides insights into the distinct gene expression profile of Th17, Th2 and other T helper cell subsets in this model, including data showing shared clonality between Th2, Th17 and Treg cells. Amelioration of airway inflammation through SLIT demonstrates the potential use of recombinant dog allergen protein in AIT.

Cell metabolism is important not only for energy generation and to enable cell replication, but also directly controls effector functions of immune cells. Papers I and II expand our knowledge of the metabolic processes governing Th2 cell responses. Since the publication of paper I, several others have reported on the central role of PPAR-γ for Th2 cell biology (Henriksson et al., 2019; Nobs et al., 2017). A recent study has shown that ILC2 in the context of allergic airway inflammation store lipid droplets in a process controlled by PPAR-γ. This lipid storage and ILC2 function could be impaired by switching the mice to a ketogenic diet (Karagiannis et al., 2020). Plin2, a key mediator of lipid droplet formation, was down regulated in T helper cells lacking the *Pparg* gene, as shown in paper I, suggesting that PPAR-γ may serve a similar function in Th2 cells. Thus, with corroborating studies of a role for PPAR-γ in type 2 immunity, further consideration must be given to the impact that environmental factors may have on the development of allergy. PPAR-γ agonists are found in many foods and are common environmental pollutants (Carter et al., 2009; Grün and Blumberg, 2007). For instance, phthalates in polyvinyl chloride (PVC) plastics have known PPAR-γ agonist activity (Hurst and Waxman, 2003). This suggests that studies analyzing the impact of environmental PPAR-γ agonists on the development and severity of asthma might be worthwhile.

Paper II shows that the production of Th2 cell cytokines is dependent on active glycolysis, similar to what has been described for the production of IFN-γ (Chang et al., 2013). Whether the effect on Th2 cells is also mediated through translational control by GAPDH or other glycolysis enzymes remains to be elucidated. While metabolic inhibitors and extreme dietary changes seem to have beneficial effects in mouse models of allergic airway inflammation, it remains to be seen whether those results can be replicated in patients and whether the

potential side effects on the rest of the immune system are worth the results. Targeting specific molecular processes could be a more realistic approach than inhibiting a whole pathway, but this will require further research. While still a fairly broad target, manipulation of mTORc2 signaling could be a more refined approach to affect Th2 cell metabolism and function. To find even more specific targets, studies more deeply investigating the metabolome of Th2 cells will be needed.

One of the strengths of scRNA-seq is the ability to uncover novel target genes for several subsets of T helper cells at the same time. Paper II clearly identifies a gene expression profile for Th2 cells and pinpoints many genes that were not previously associated with Th2 cells. This gene list was largely confirmed by scRNA-seq data from the dog allergen extract model in paper III, where target genes and cellular processes found to be enriched in Th2 cells in the HDM model were also found to be enriched in this completely different allergen model. A recent study has shown genes, such as *Bhlhe40*, described in paper II to be of relevance in Th2 cells in a helminth infection model (Jarjour et al., 2020). These datasets now allow for the comparison of Th2 cells between different organs and disease models. Whether these target genes present an opportunity for novel therapies will require both gene expression data from allergic patients as well as mechanistic studies in mice.

A population of T helper cells responding to type-I interferons as described in paper II has recently been reported in diverse tissues and contexts such as *Alternaria alternata* extract-induced allergic airway inflammation (Gowthaman et al., 2019) or kidney infiltrates of lupus nephritis (Arazi et al., 2019). Interestingly we did not detect this population in the single-cell data set from the dog allergen-extract exposed mice, indicating that these different allergen extracts induce different microenvironments in the airways of exposed animals. Future studies will need to explore the function of these cells and their relevance for human pathologies. This could include studies employing conditional knockout mice lacking IFNAR1/2 receptors in order to determine the impact of type-I IFNs on Th2 cells.

The mouse model of allergic airway inflammation induced by dog allergen extracts presented in paper III is a new model in which scientists can test Th2/Th17 cell-driven airway inflammation and therapies specific to dog allergy. Since analysis of allergen extracts showed high levels of endotoxin in the dog allergen extracts, we will need to explore the role of bacterial compounds in the extract further. We are aiming to test the effects of treating the dog allergen extracts with proteolytic enzymes prior to sensitizing and challenging mice, in order to elucidate the role of protein allergens and non-protein components in the extract.

In this paper, we also show that prophylactic SLIT using recombinant dog allergen Can f 1, f 2, f 4 and f 6 can be used to reduce airway inflammation induced by dog allergen extracts. Our results are in line with other studies showing that use of recombinant cat and HDM allergens can be used for AIT in mouse models (Haspeslagh et al., 2019; Senti et al., 2012). The use of recombinant proteins opposed to natural extracts for AIT has the advantage of being less prone to contaminations and fluctuations in concentrations of allergen proteins, which can differ greatly between suppliers and batches of natural extracts (Wintersand et al.,

2019). Recombinant proteins further offer the possibility to modify the protein in ways to improve/alter the immune response (Senti et al., 2012) or to combine several allergen proteins in one recombinant protein as was done with “necklace” used in paper III. The next step will be to test whether SLIT using necklace can also be used to ameliorate disease after inflammation has already been established. While SLIT was able to reduce the Th2 cell response to dog allergen exposure, it did not reduce airway neutrophilia and frequency of Th17 cells. A paradox of our experiments was that we did not discern much IL-17 production in response to Can f 1 in restimulation cultures despite Can f 1 inducing Th2 and Th1 cytokine production. Yet scRNA-Seq analysis showed high concordance in TCR usage between Th2 and Th17 cells and considerable *Ifng* expression by Th17 cells. It is possible that other allergens in the dog extracts are responsible for inducing Th17 cell differentiation and cytokine production, although this needs to be elucidated. These could be added to a future version of the “necklace” protein in an attempt to reduce the Th17 cell response.

In conclusion, the work presented in this thesis sheds light on factors governing the differentiation and function of Th2 cells and the role of metabolism for Th2 cells. It showcases the power of scRNA-seq to uncover novel cell subsets and novel genes of interest for known populations. It further presents a novel mouse model using dog allergen extracts to induce a mixed Th2/Th17 cell response and demonstrates that prophylactic SLIT using recombinant dog allergen can ameliorate airway inflammation in mice. These findings improve on our understanding of T helper cell biology in the context of allergic airway inflammation and have implications for the development of future therapies.

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