Eosinophils, their progenitors and T helper cells in allergic airway inflammation

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At the cover: Newly produced eosinophils from murine lung tissue stained with MBP (red) and BrdU (brown) in OVA sensitised/exposed mice. Photo by You Lu.

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To my beloved family

and

friends

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ORIGINAL PAPERS

The thesis is based on the following papers:

I. You Lu, Margareta Sjöstrand, Carina Malmhäll, Madeleine Rådinger, Jeurink Prescilla, Jan Lötvall and Apostolos Bossios
New production of Eosinophils and the Corresponding Th1/Th2 Balance in the Lungs after Allergen Exposure in BALB/c and C57BL/6 Mice.
Scand J Immunol. 2010; 71(3):176-85.

II. You Lu, Carina Malmhäll, Margareta Sjöstrand, Madeleine Rådinger, Serena E O'Neil, Jan Lötvall and Apostolos Bossios
Expansion of $CD4^+CD25^+$ and $CD25^-$ T-Bet, GATA-3, Foxp3 and RORγt Cells in Allergic Inflammation, Local Lung Distribution and Chemokine Gene Expression.
PLoS One. 2011; 6(5):e19889.

III. You Lu, Margareta Sjöstrand, Madeleine Rådinger, Carina Malmhäll, Jan Lötvall and Apostolos Bossios.
IL-33 regulates lung *in situ* eosinophilopoiesis by affecting their *in situ* proliferation, survival and migration.
In manuscript.

IV. You Lu, Carina Malmhäll, Margareta Sjöstrand, Madeleine Rådinger, Bo Lundbäck, Jan Lötvall and Apostolos Bossios.
Expression of the major trafficking related molecules in circulating eosinophil progenitors and mature eosinophils in asthma patients.
In manuscript.

Abstract

Introduction: Asthma is a heterogeneous chronic lung disease associated with pronounced inflammatory changes in the airways. Eosinophilic inflammation is the trait that is best linked to symptoms and treatment responses in allergic asthma. In addition to eosinophils, T helper (Th) cells of different subsets; Th1, Th2, Th17 and T regulatory (Treg) cells, play an essential role in orchestrating allergic inflammation. Recent studies suggest that they can even affect each other´s development and function. Although the role of eosinophils and Th cells has been studied extensively, the balance of different Th cells during eosinophilic inflammation and the corresponding local lung eosinophilopoiesis has still not been elucidated.

Aim: The aim of the present thesis was to evaluate eosinophilic inflammation and the corresponding T helper cells response during allergic airway inflammation.

Methods: A classical OVA-induced allergic airway inflammation model on two commonly used mouse strains, C57BL/6 and BALB/c, was used initially to evaluate the lung eosinophilia and the corresponding Th1/Th2 balance after allergen exposure. Next, the balance of the different Th cells and the role of IL-33 in the lung during *in situ* lung eosinophilopoiesis were evaluated using the above OVA model in C57BL/6 mice. Finally, evaluation of circulating mature and progenitor eosinophils and their expression of traffick related molecules were assessed in patients with stable asthma.

Results: Allergen exposure induced a different distribution of eosinophils in the lung between the two mouse strains, with no difference in eosinophil production or Th1/Th2 balance. In C57BL/6 mice, allergen exposure led to a local expansion of all Th cells, with a dominant of Th2 cells. These Th cells showed a different local cell distribution, probably due to the different local inflammatory milieu. Allergen exposure induced lung IL-33 expression. IL-33 receptor, ST2, was expressed in all eosinophil progenitors, decreased in immature eosinophils and not expressed in mature eosinophils. ST2 was also expressed in about 60% of Th2 cells. Local blockage of IL-33 during allergen exposure impaired the number of progenitor and immature eosinophils, but not mature eosinophils or Th2 cells. Evaluation of the underlying mechanisms revealed that IL-33 enhances proliferation of lung eosinophil progenitors, protects them from induced apoptosis, and cooperates with eotaxin-1 and -2 to induce their migration. Expression of ST2 was confirmed in circulating human Th2 cells and eosinophils, both mature and progenitor, arguing for their capacity to migrate. Indeed, the last study showed that patients with stable asthma and high, but normal, blood eosinophilia had increased sputum eosinophils and increased circulating eosinophil progenitors compared to the healthy controls. Both mature and progenitor eosinophils expressed selectin PSGL-1 and integrins VLA-4 and Mac-1, although with different patterns. Mature eosinophils showed increased expression of CCR3. However, $CCR3^+$ eosinophil progenitors were more activated (increased expression of CD69 and CD25) compared to $CCR3^+$ mature eosinophils.

Conclusions: This thesis shows that allergic inflammation promotes a different local lung inflammatory milieu, resulting in both eosinophils and T helper cells distributing differently. Th2 cells dominate among other Th cells. Lung Th2 cells and lung eosinophils undergoing maturation express ST2, a receptor for a novel cytokine IL-33, released locally during airway allergic inflammation. This suggests a common link as IL-33 regulates lung *in situ* eosinophilopoiesis by affecting eosinophil progenitor proliferation, apoptosis and migration. Indeed, patients with stable asthma showed an increased number of circulating eosinophil progenitors expressing all molecules required for migration to the lung.

Keywords: eosinophils, eosinophil progenitors, T helper cells, Th2 cells, IL-33, migration, asthma

ABBREVIATIONS

AHR	Airway Hyperresponsiveness
APC	Allophycocyanin
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
BM	Bone Marrow
BrdU	5-Bromo-2'- DeoxyUridine
BSA	Bovine Serum Albumin
7-AAD	7-AminoActinomycin D
CCL11	Chemokine (C-C motif) ligand 11/eotaxin-1
CCL24	Chemokine (C-C motif) ligand 24/eotaxin-2
CCR3	C-C chemokine Receptor 3
CFSE	Carboxyfluorescein diacetate Succinimidyl Ester
ELISA	Enzyme-Linked ImmunoSorbent Assay
FACS	Fluorescence-Activated Cell Sorter
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
Foxp3	Forkhead box protein p3
GATA-3	GATA-binding protein 3
HBSS	Hanks Balanced Salt Solution
ICC	ImmunoCytoChemistry
IHC	ImmunoHistoChemistry
i.n.	intranasal
i.p.	intraperitoneal
IPA	Ingenuity Pathways Analysis
LCM	Laser Capture Microdissection
Mac-1	Macrophage adhesion molecule-1
MBP	Major Basic Protein
MFI	Mean Fluorescence Intensity
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PerCP	Peridinin Chlorophyll Protein Complex
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cy7
PSGL-1	P-selectin Glycoprotein Ligand-1
real-time RT-PCR	real-time Reverse Transcription Polymerase Chain Reaction
rmIL-5	recombinant murine interleukin-5
RORγt	Retinoic acid receptor-related Orphan nuclear Receptor gamma t
Sca-1	Stem cell antigen -1
SSC	Side Scatter
ST2	IL-33 receptor, also known as Interleukin 1 receptor-like 1
T-bet	T-box expressed in T helper 1 cells
VLA-4	Very Late Adhesion molecule 4
WBC	White Blood Cells

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INTRODUCTION

Over the last 40 years, a sharp increase in the global prevalence, morbidity, mortality, and economic burden has been associated with asthma [1]. Asthma is a heterogeneous disease with several clinical subtypes and wide spectrum, ranging from mild, episodic, wheezy breathlessness to chronic, intractable, corticosteroid dependent chronic airway narrowing [2]. Allergic asthma is characterized by chronic airway inflammation involving resident cells (epithelial, fibroblasts, smooth muscle cells and endothelial cells) and an abundance of inflammatory cells, such as lymphocytes, mast cells, neutrophils and dendritic cells. Eosinophilic infiltration is the most striking feature of allergic asthma, with the release of mediators that trigger bronchoconstriction, mucus secretion and remodeling [3].

Asthma phenotypes

Eosinophils are considered important in the characterization of specific "asthma phenotypes" [3]. Characterization of asthma phenotypes has recently become a research target, as it is now recognized that asthma is a heterogeneous disorder. Although different types of asthma have long been recognized, *i.e.* allergic and non-allergic, there has not been a cellular or molecular basis for these. Trying to understand the underlying asthma pathophysiology has resulted in new phenotypes being proposed. Wenzel [4] has proposed at least four distinct phenotypes based on the presence or absence of the main inflammatory cells: eosinophilic, neutrophilic, and mixed inflammatory and paucigranulocytic. Furthermore, Haldar *et al*. [5] using a cluster analysis technique, has identified four other distinct phenotypes/clusters; a) a group with well-controlled symptoms/minimal persistent airway inflammation, b) a group with early-onset atopic asthma/ severe symptoms, persistent airway inflammation, and markedly variable airflow obstruction, c) a group of predominantly females who have late-onset asthma with marked symptoms, but minimal eosinophilic inflammation, many of whom are obese and finally d) a predominantly male group with late-onset asthma characterized by persistent eosinophilic inflammation in the absence of symptoms. In both approaches, the presence and absence of eosinophils and inflammation is vital for the characterization.

Eosinophils

The role of eosinophils in allergic asthma

Eosinophilic inflammation is the trait that is best linked to symptoms and treatment response in allergic asthma. Eosinophilia in sputum, bronchial biopsies and blood has been found to be related to asthma severity, asthma

symptoms and risk of exacerbations [6]. In asthma, eosinophils are multifunctional leukocytes playing a dual role as both effectors and immunoregulatory cells. Effector eosinophils induce damage to the airway mucosa and the associated nerves by releasing cytotoxic granules and lipid mediators, which may cause bronchoconstriction. In addition, eosinophils demonstrate numerous immune regulatory functions, such as the production of cytokines and chemokines that leads to the exacerbation of inflammation, mucus hypersecretion, and lung remodeling [7-11]. During the initiation of the Type 2 immune response, eosinophils may be one of the primary sources of IL-4, which can recruit T cells to the lungs during the development of asthma [12, 13]. Furthermore, growing evidence suggests that eosinophils can serve as antigen presenting cells [14]. The development of eosinophils is governed by several transcription factors, including GATA-1, PU.1 and C/CBP, as well as an array of cytokines, in particular GM-CSF, IL-3, IL-9 and especially IL-5 [7, 15, 16]. Among others, IL-5 is reported to be more specific and efficient at promoting the development of eosinophil lineage [17, 18].

Effector functions of eosinophils

Eosinophils contain numerous basic and cytotoxic granule proteins that are released upon activation. They also produce numerous enzymes and lipid mediators, which are implicated in the effector functions of eosinophils. Granule proteins increase vascular permeability and stimulate mucus production, resulting in tissue oedema and airway obstruction. The primary granules such as major basic protein (MBP), eosinophilic cationic protein (ECP), and eosinophil peroxidase (EPO) appear during the promyelocytic stage of eosinophil development and are toxic for the respiratory epithelium. Considerable evidence suggests a link between these eosinophil granule proteins and human diseases. MBP-mediated mast cell degranulation triggers the release of leukotrienes and histamine, which in turn leads to bronchoconstriction. In addition, MBP directly alters the smooth muscle contraction response by dysregulating vagal muscarinic receptor function. MBP has been localized to damaged sites of bronchial epithelium in patients of asthma, with its concentration correlated to the severity of bronchial hyperreactivity. Furthermore, instillation of human MBP and EPO provokes bronchoconstriction, and MBP increases airway responsiveness to inhaled methacholine [11]. In addition, the release of pro-inflammatory mediators, such as leukotreine B4 (LTB4), the cysteinyl leukotrienes (LTC4, LTD4 and LTE4) and prostaglandin (PG) D2 have been shown to further regulate eosinophil accumulation and migration [9, 19-21].

Immunoregulatory role of eosinophils

Accumulating evidence suggests that eosinophils can perform various immune regulatory functions through the presentation of antigens, as well as the production and release of numerous cytokines and chemokines. Eosinophils

possess the ability to internalize, process, and present antigenic peptides within the context of surface expressed MHC II [9]. In addition, they also have the capacity to provide co-stimulatory signals to T cells and physically interact with $CD4^+$ T cells [10]. Eosinophils can produce and release numerous cytokines and chemokines, such as IL-3 and GM-CSF, which are able to act on eosinophils themselves, and TGF-α, TGF-β1, osteopontin and metalloproteinases (MMPs), which can affect tissue cells. Additonally, IL-4, TNF-α and chemokines, such as MIP-1α and RANTES (CCL5), may modulate the functions of other immune cells. Eosinophil recruitment into the sites of Th2-type inflammation was thought to be a result of the activation of the adaptive immune responses that produced IL-5 and eotaxins (human: CCL11, CCL24 and CCL26 (eotaxin-3); mouse: CCL11 and CCL24) [7]. However, increasing evidence revealed that an early influx of eosinophils into sites of inflammation precedes that of lymphocytes. The mechanisms of how eosinophils modulate Th2 responses are not fully understood. The deficiency in eosinophils in attenuated airway production of chemokines and genes, suggests that eosinophils may be involved in priming the tissue environment for the effective mobilization of Th2 cell [11].

The role of IL-5 in allergic airway inflammation

Interleukin-5 (IL-5) has been proposed to be a novel therapeutic target in allergic inflammation, such as asthma, as it can increase proliferation and differentiation of bone marrow progenitor cells into mature eosinophils. It has also been shown that IL-5 modulates various functions of eosinophils, including eosinophil cellular adhesion, chemotaxis, degranulation, cytotoxicity, mediator release, prolonged survival and activation. However, there is some conflicting data regarding anti-IL-5 antibody therapy in both animal models of allergic inflammation and asthma patients. Anti-IL-5 treatment abolished airway eosinophilia with no effect on Airway hyperresponsiveness (AHR) in an animal model of established airway inflammation [22]. Anti-IL-5 (mepolizumab) applied to patients with mild asthma reduced blood and sputum eosinophils without significant improvement in symptoms of asthma [23, 24]. Another anti-IL-5 (reslizumab) used in severe asthma showed a reduction of blood eosinophils, but no effect on clinical parameters [25]. Repeated treatment of mepolizumab demonstrated only 55% reduction in bronchial mucosa eosinophils [26], and a large scale clinical trial of it showed no effect in improving symptoms of asthma in patients with moderate persistent asthma [27]. The residual eosinophils in the tissues suggests that the survival and function may not depend on IL-5, as eosinophils downregulate their IL-5-receptor-α (IL-5Rα) expression, and that tissue eosinophils may survive in the absence of IL-5 [28]. Recently, anti-human IL-5Rα mAb therpy was issued due to its potential for eliminating eosinophils localized in the inflammatory tissues by antibody-depent cell-mediated cytotoxicity [29]. Intravenous administration of MEDI-563 (anti-human IL-5Rα

mAb) resulted in marked reduction of blood eosinophils within 24hrs in patients with mild atopic asthma [30].

Many studies suggest that eotaxins play an important role in eosinophil recruitment from blood microvessels, while IL-5 stimulates the release of eosinophils from the bone marrow (BM) into the circulation. The inflammatory process in response to airway allergen exposure can be divided into several distinct steps, one of which is the activation of the bone marrow resulting in both the release of eosinophils into the circulation and the production of new eosinophils, which are also released into the blood and migrate to the site of inflammation [17]. Eosinophils are generated in the BM from $CD34^+$ progenitor cells under the influence of IL-5. Both human and animal studies have shown that allergen exposure activates BM to produce more eosinophils, as well as the up regulation of the IL-5Rα in $CD34^+$ progenitor cells [31, 32]. Expression of IL-5Rα can characterise both mature (granulocytes: $CD45^+$/IL-$5Ra^+$), as well progenitor eosinophils (mononuclear cells: $CD45^+$/IL-$5Ra^+$/$CD34^+$) in humans [18]. We have shown that eosinophil maturation can occur not only in the BM, but locally in the airway tissues during inflammation [33, 35].

Eosinophil progenitors

Hematopoietic stem cells (HSCs) are pluripotent stem cells that give rise to all blood cell types from myeloid and lymphoid lineages, which have the limitless capacity for self-renewal. When these stem cells proliferate, some remain as HSCs and some become progenitor cells with limited replication. The majority of the hematopoietic activity takes place in the BM. CD34 antigen is one of the most important markers for hematopoietic progenitors, the expression of which is mainly on primitive progenitor cells of all lineages, while it is also expressed on endothelial cells to act as a ligand for CD62L and plays a role in adhesion. The expression of CD34 decreases with cell maturation, with expression being lost on mature cells within the hematopoietic system [36].

Definition of eosinophil progenitor cells

Eosinophil progenitors differentiate from common myeloid progenitors (CMP) in response to IL-3, IL-5 and GM-CSF. IL-5 stimulates the release of eosinophils from the bone marrow into the peripheral circulation. The committed eosinophil progenitors are previously defined as $CD34^+$ cells that co-express IL-5Rα on their surface [37]. In humans, it has been shown that there is an increasing number of $CD34^+$/IL-$5R\alpha^+$ and $CD34^+$ cells expressing CCR3 in the bone marrow in allergic asthma patients compared to controls [38]. Mori *et al.* [18] identified the human eosinophil committed progenitors as an IL-$5R\alpha^+$ fraction (IL-$5R\alpha^+CD34^+CD38^+CD45RA^-$IL-$3R\alpha^+$) of conventional human common myeloid progenitors. Mature eosinophils are defined to be the IL-$5R\alpha^+$ fraction

of polymophonuclear cells (PMN). In mice, stem cell antigen-1 (Sca-1) is also a marker that defines hematopoietic stem cells. $CD34^+$ cells co-expressing Sca-1 represent progenitors at an early stage of differentiation [39]. We have previously found that $CD34^+CCR3^+$ eosinophil progenitor cells increased in BM, blood and in bronchoalveolar lavage fluid (BALF). Furthermore, $CD34^+Sca\text{-}1^+CCR3^+$ eosinophil progenitor cells increased in BALF after allergen exposure [35].

The role of eosinophil progenitor cells in allergic airway inflammation

Eosinophil progenitors are increased in the peripheral blood in patients with allergic rhinitis, nasal polyps and asthma compared to controls [32]. Increasing expression of IL-5Rα mRNA in $CD34^+$ cells was found in bronchial mucosa from atopic patients with asthma, and the $CD34^+$IL-5Rα $mRNA^+$ cells were found to be correlated to FEV1, suggesting that eosinophil progenitors may contribute to the clinical symptoms in asthma [40]. In allergen challenged mice, there are expansion of eosinophil progenitors ($CD34^+$ and $CD34^+$IL-5Rα^+) in the BM, blood and airway during allergic airway inflammation [33, 41, 42]. These eosinophil progenitors correlated with the induction of AHR [41]. IL-5 responsive eosinophil colony forming units could be grown from progenitor cells harvest in BALF and in lung tissue from allergen challenged mice [33]. All above suggests that there are two distinct mechanisms by which eosinophil progenitor may contribute to allergic airway inflammation. The first, is the imitation of hematopoietic maturation that give rise to mature eosinophils within the BM. The second, is the trafficking of the progenitor through the peripheral circulation and migration into the allergic tissue where they undergo the hematopoietic maturation under the control of specific local mediators. The latter process was termed as *in situ* hematopoiesis [33, 42]. Otsuka *et al.* [43] suggested that eosinophil progenitors might traffick from the BM to the airways where they undergo *in situ* hematopoiesis, with evidence that circulating eosinophil progenitors during allergic season were decreased in blood compared to before and after season. Additionally, Menzies-Gow *et al.* [44] showed a decline in eosinophil progenitors and an increase in mature eosinophils in the bronchial mucosa of asthmatics 24hrs after inhalation of IL-5. Robinson *et al.* [40] found that eosinophil progenitors can expand in the local tissue of allergic patients due to an increased number of $CD34^+$IL-5Rα $mRNA^+$ cells in the bronchial mucosa of asthmatics. We have previously found that there were an increased number of $CD34^+$ progenitors and eosinophil progenitors in the nasal mucosa during pollen season in allergic rhinitis [45]. In a mice model, $CD34^+CCR3^+$ eosinophil progenitors were shown to undergo *in situ* proliferation locally in the lung tissue after allergen exposure [35]. Furthermore, an *in vitro* study revealed that upregulation in CCR3 expression was associated with an increased migration by BM $CD34^+$ cells towards CCL11, suggesting that CCR3 may assist the mobilization of progenitor cells from BM in response to allergen [46].

The migration of eosinophils in allergic asthma

As eosinophils are produced in the BM, migration to the lung during allergic airway inflammation represents a major part of the inflammation process. Blood eosinophils from asthma patients have a number of phenotypic alterations, particularly in relation to their adhesive properties [57]. Transmigration of the eosinophil through the vascular endothelium is a multistep process; rolling, tethering, firm adhesion and transendothelial migration [58, 59], which is regulated by the coordinated interaction between networks involving chemokine and cytokine signaling as well as eosinophil adhesion molecules.

Adhesion molecules include selectins and integrins and their counter-ligands expressed on the endothelium [9].

Chemokines are thought to primarily regulate the migration pattern of eosinophils and promote the activation and function of eosinophils [50] Chemokines are a group of small proteins that possess the ability to induce cell migration or chemotaxis in numerous cell types. Their activity is regulated through the binding to members of the 7- transmembrane, G protein-coupled receptor superfamily [47].

Among the cytokines and chemokines implicated in leukocyte recruitment, only IL-5 and the eotaxins selectively regulate eosinophil trafficking [60].

The role of eotaxins/CCR3 in allergic airway inflammation

Eotaxins (CCL11, CCL24 in both mice and humans and CCL26 only in humans) have generated considerable interest because of their lineage-specific effect on eosinophils, as opposed to other cell types [61]. In a mice study, pulmonary eosinophilia and airway eosinophils were significantly reduced in the absence of CCL11 and CCL24 [48]. Local administration of anti-eotaxins (CCL11 and CCL24) reduced newly produced eosinophils and $CD34^+$ eosinophils in the airway, but with no effect on either BM or blood eosinophils, suggesting that both CCL11 and CCL24 target young cells and are mainly important in the local airways in response to allergen exposure [49]. Following allergen challenge in the human lung, CCL11 is induced early (6 hours) and correlates with early eosinophil recruitment, while CCL24 correlates with eosinophil accumulation at 24 hours [50]. CCR3 is a transmembrane G protein-coupled receptor, expressed primarily on eosinophils. The eotaxins signal works exclusively through the CCR3 receptor, which is the primary chemokine receptor responsible for the eosinophil recruitment to inflamed tissues [9]. Eosinophil recruitment to the airways is greatly reduced in the airway inflammation of CCR3 deficient mice compared to allergen challenged wild type mice [51]. AHR and airway remodelling were prevented after CCR3 antagonist treatment following the establishment of allergic airway inflammation in mice [35, 52]. Studies with

neutralizing antibodies demonstrate that other chemokines such as CCL5, MCP-5, and MIP1α are also important in eosinophil tissue recruitment [50, 53]. Eosinophils highly express CCR3 receptors, which bind eotaxins, as well as CCL5, and MCP-3, making the CCR3 receptor an attractive therapeutic target in inhibiting eosinophil activated chemokines [54-56].

T cells in allergic airway inflammation

Excluding eosinophils, T cells are the other critical mediator in the allergic airway inflammation seen in asthma. Allergen-specific T cells are generated in regional lymph nodes and then recruited into the airway following the chemoattractants produced by the asthmatic lung. Mice specifically deficient $CD4^+$ T helper cells (Th cells), were shown to be unreliable to develop allergic responses, emphasizing the importance of these cells in allergic disease [62].

Effector T cell subsets

In an antigen draining lymph node, naive $CD4^+$ T cells activated by their interaction with antigen-presenting cells can differentiate into several effector T cell subsets, such as Th1, Th2, interleukin 17 (IL-17)-producing Th17 and regulatory T cells (Treg cells). The development of these subsets is dictated by their specific transcription factors, T-bet, GATA-3, RORγt and Foxp3, respectively [63-65]. Functionally, Th1 cells can clear intracellular pathogens and mediate autoimmune tissue inflammation, while Th2 cells are required for the clearance of extracellular pathogens i.e. parasites, with an exaggerated Th2 response inducing asthma, allergy and atopy. In contrast, Th17 cells seem to be involved in controlling both intracellular and extracellular pathogens and in orchestrating autoimmune tissue inflammation [66, 67]. As Th1, Th2 and Th17 cells can induce inflammation, the maintenance of immune homeostasis and prevention of immunopathology requires regulatory mechanisms to control these effector T cells by Treg cells. Two different formats of Treg cells are exist, the naturally occurring Treg cells ($CD4^+CD25^+Foxp3^+$) and IL-10-producing Treg type (Tr1) cells [68]. Naturally occurring Treg cells, which are generated in the thymus, inhibit effector T cells and are crucial in the maintenance of peripheral tolerance [69]. In addition, emerging data suggests that $Foxp3^+$ T cells can also be generated in peripheral immune compartments [70]. These Th cells have specific effector and regulatory functions and recruit different cell types at the site of inflammation by the cytokines they produce. For Th1 cells, these cytokines include interferon-γ (IFN-γ) and IL-12, while for Th2 cells, these include IL-4, IL-13 and IL-5. Th17 cells produce IL-17A and Treg cells produce IL-10 and TGF-β. It must be cautioned that there may be some plasticity in these responses [71].

Furthermore, it has now been reported and proposed that the above T cell phenotypes show plasticity and are simultaneously affecting each others differentiation and function in a competitive manner [71-74]. Recent reports suggest an inter-regulation among them. Foxp3 can directly interact with GATA-3 to inhibit GATA-3-mediated trans-activation of IL-5, which is one of its target genes [75]. RORγt can indirectly interact with Foxp3 by binding to, and acting together with, Runx1 during IL-17 transcription. This interaction was necessary for the negative effect of Foxp3 on Th17 differentiation [76].

Th2 cells in allergic asthma

The main effector cells involved in the pathogenesis of asthma are the Th2 cells, which mediate the inflammatory process, in addition to other mechanisms, by releasing a range of cytokines and chemokines to amplify the Th2 response [77].

By releasing IL-4 and IL-13, allergen-specific Th2 cells induce a class of switching B cells that result in IgE production. Cross-linking of IgE bound to FcεRI on the effector mast cells and basophils results in the release of histamine, lipid mediators, chemokines and cytokines to amplify the Th2 response and exacerbate the inflammation [78]. IgE is involved in the exacerbation of allergic diseases, the induction of goblet cell metaplasia and mucus hypersecretion in prolonged cases of allergic asthma [79]. Th2-derived cytokines are associated with pathogenesis of IgE and eosinophilia. IL-5 can be produced both by Th2 cells and eosinophils [80]. Many studies indicating that allergic asthma cannot develop without IL-13, which is correlated with AHR [81]. IL-13 upregulates chemokines, such as CCL11, CCL24, CCL17 and CCL22, which are important for immune cell infiltration into the lung tissue [79].

However, recent studies suggest that eosinophils themselves can also regulate T effector cell inflammatory recruitment in the lung during allergic airway inflammation [13], revealing a more complex interaction and arguing for a possible common upstream inflammatory regulator. Potentially paradigm-shifting findings have been reported on the relationship between eosinophils and T cells in allergic airway diseases, while how they influence each other in the pathogenesis of this disease remain ambiguous.

Trafficking of effector T subsets

Chemokines play a major role in T cell trafficking in allergic and asthmatic inflammation [62]. T cell trafficking is a tightly regulated and complex process that involves expression of different adhesion molecules and chemokines. These signals allow T cells to migrate into the tissue, where once in the tissue, further signals generated from chemokine gradients can guide T cells into specific microcompartments. Thus, the signals that determine the migration and homing of T cells into the lung are crucial for effective immune function. For example,

Th1 reported chemokines such as CCL17 and CCL2 [47, 77]. CCL8 was recently reported by Islam *et al*. [82] as a potent chemoattractant for GATA-3 and IL-5$^+$ Th2 cells in skin allergy. Furthermore, the chemokine CCL19 is a chemoattractant for Treg, as CCL20 is the chemokine for Th17 cells [77, 83, 84].

IL-33 in allergic airway inflammation

Another newly described chemoattractant for Th2 cells is interleukin-33 (IL-33), which has been shown both *in vitro* and *in vivo,* indicating that IL-33 may play a role in Th2 cell mobilization in humans [85], however the mechanism is not known. Both human and mouse Th2 cells are selectively attracted to IL-33, indicating that IL-33 can both recruit and activate Th2 cells. IL-33 has also been reported to enhance chemokinetic activity, similar to IL-5, while not a direct chemoattractant for human eosinophils [86].

IL-33 is a nuclear bound cytokine that is assumed to act as an "alarmin" and upregulated in response to cell damage. IL-33 is the ligand for the orphan Th2 associated receptor ST2 and able to influence Th2 function *in vitro* and *in vivo*. IL-33 can also promote the eosinophilic inflammation after allergen exposure, suggesting that it could be a regulator for both Th2 cells and eosinophils [87].

IL-33 is found to be released in large amounts from airway structural cells; epithelial [88], smooth muscle [89] and vascular endothelial cells, in both patients with asthma, as well as in mouse models of airway inflammation [90]. IL-33 and its receptor are part of the IL-1 family and their interactions promote a variety of actions from a number of different cell types. IL-33 exerts its cytokine-like activity via its heterodimeric receptor consisting of ST2 [91] and the ubiquitously expressed IL-1R accessory protein (IL-1RAcP) [92]. The IL-33/ST2 axis is thought to be intimately involved in the promotion and maintenance of allergic inflammation via a number of cell types inducing Th2 cells, mast cells, basophils and structural cells. Animal models show that IL-33 induces eosinophilia, induces the release of Th2 type cytokines and increases IgE levels *in vivo*. It can affect eosinophils and Th2 cells, as well as basophils, mast cells, NK and NKT cells, CD34$^+$ precursor cells and newly identified nuocyte cells *in vitro* [93]. In addition, a recent study showed that in an animal model, IL-33 directly stimulates eosinophil differentiation from CD117$^+$ progenitors in an IL-5-dependent manner in the BM [94]. Furthermore, another *in vivo* study showed that systemic blockage of IL-33 resulted in reduced numbers of eosinophils and lymphocytes, as well as Th2 cytokines, in BALF [95], suggesting a direct role for IL-33 in both cell types. However, the study did not ascertain which cell population was the primary target. The data above suggests that IL-33 could be a common link for both eosinophils and Th2 cells.

Mouse models of allergic airway inflammation

Human biology can be partly displayed by mouse models, particularly in allergic airway inflammation. The mouse model is an important and valuable tool for improving understanding of the mechanisms of allergic diseases, even though it is not a perfect replica of human allergic inflammation. There are several advantages with mouse models, including genetic homogeneity allowing reproducibility, the availability of genetically manipulation, and the variety of specific reagents available for phenotypic and functional analysis of the cellular response. Despite the conservation of these features, differences exist between mice and humans in immune system. Humans are genetically heterogeneous compared with most monogenetic mouse strains. In addition, large differences exist concerning the eosinophil between mice and humans in allergic inflammation [96].

Mouse and human eosinophils

Structurally, eosinophil granules between mouse and human are different. The size of them is bigger in humans, and the eosinophil-associated ribonucleases are divergent between mouse and humans. Human eosinophils include Charcot-Leyden crystal protein, which is not detectable in mouse eosinophils. Biologically, in related to some immune response, mouse eosinophils have some limitation and different responses compared to human eosinophils. It is not known whether the property of mouse eosinophils caused the outcomes from the asthma mouse model. Anyway, it should keep in mind that the species divergence as well as the difficulties in applying mouse models to human disease. Therefore, the most appropriate way to study complex human disease is to complement human studies with mouse models [11, 97].

AIMS OF THE STUDY

The overall aim of this thesis was to study the eosinophilic lung inflammation and the corresponding T helper cell response during allergic airway inflammation.

The thesis was addressed by investigating the following specific aims;

- To utilize a classical OVA-induced allergic airway inflammation mouse model to investigate if lung eosinophilia and the corresponding Th1/Th2 balance differs in BALB/c and C57BL/6 mice, as they have both been used as *in vivo* models of skewed Th2 and Th1 inflammatory response respectively.

- To study whether other T helper cells besides Th2, *i.e.* Th1, Th17 and Treg cells, are involved in allergic airway inflammation, and to determine their relative presence.

- To assess if the IL-33 receptor is expressed on eosinophil progenitors and the role of IL-33 in lung eosinophilic inflammation *in situ* during allergic airway inflammation.

- To investigate if circulating human eosinophil progenitors in the blood have the capacity to migrate in patients with asthma.

METHODOLOGY

In vivo studies (I-III)

Mice strains (I-III)

Mice studies were approved by the Animal Ethics Committee in Gothenburg, Sweden (no. 442-2008; 376-2009). Male BALB/c (I) and C57BL/6 (I-III) mice, which were 5-6 weeks old, were purchased from Taconic (Ry, Denmark). All mice were kept under conventional and pathogen-free animal housing conditions and provided with food and water *ad libitum*.

Allergen sensitisation (I-III)

Mice were sensitised twice, with an interval of five days, by the intraperitoneal (i.p.) injection of 0.5 ml of 8 µg chicken ovalbumin (OVA) (Sigma-Aldrich®, St Louis, MO, USA) bound to 4 mg aluminum hydroxide $Al(OH)_3$ (Sigma-Aldrich®) in phosphate buffered saline (PBS). OVA is a conventional allergen and the one most often used in different animal models. Aluminum hydroxide has been shown to specifically enhance the Th2 responses [98].

Allergen exposure (I-III)

The animals underwent repeated allergen exposure for five days inducing an acute allergen exposure. Sensitised mice exposed to allergen elicit a series of responses, including a Th2 response, followed by a sustained eosinophilic inflammation and altered airway function [99, 100].

Allergen exposure was administrated intranasally (i.n.) to mice, which is an effective and non-invasive technique used for the delivery of allergens, drugs, gene therapy, immunotherapy and pathogens to the upper and lower respiratory tracts. Compared to the aerosol inhalation method, intranasal administration allows control of a defined dose of the allergen into the airway. In addition, structural changes to the allergen, which can be induced by aerosolisation, are avoided. Both routes of allergen administration showed similar eosinophilic inflammation in the airways. By using intranasal administration, about one-third of the relative amount of allergen can reach the trachea, bronchi and lungs with intranasal exposure, as proven by the distribution of instilled Evans blue dye [31].

Allergen exposure was administered eight days after the second sensitization. The animals were briefly anesthetized using isoflurane and exposed intranasally to 100 µg OVA in 25 µl PBS on five consecutive days (Figure 1), while the control group was exposed to PBS.

Figure 1. Allergen sensitisation and exposure protocol (papers I-III).

In vivo labeling of newly produced inflammatory cells (I-III)

A thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) was used to label newly produced inflammatory cells during the allergen exposure period. BrdU is incorporated into the DNA during the S-phase of the cell cycle by replacing thymidine [101].

In paper I, all mice were administered 4 mg BrdU (Roche Diagnostics Scandinavia AB, Bromma, Sweden), with two different administration schedules. In the first schedule (BrdU2*2), BrdU was given in a dose of 1 mg in 25μl PBS by i.p. injection on two occasions, 8 hours apart on days 1 and 3 of allergen exposure. Allergen exposure was performed 1 hour after the first BrdU injection. In the second schedule (BrdU5*1), BrdU was given at a dose of 0.8 mg in 20 μl PBS by i.p. injection once a day just after allergen exposure, on days 1-5 of allergen exposure.
In paper II and III, all mice were given 4 mg BrdU (BrdU Flow Kits, BD Pharmingen™, San Diego, CA) with BrdU5*1.

In vivo treatment with anti-IL-33 (III)

The effects of anti-IL-33 on allergic airway inflammation were investigated with the pretreatment of the OVA-exposed animals with anti-IL-33 monoclonal antibody [102] (clone 396118, R&D Systems). The animals were briefly anesthetised using isoflurane and received an intranasal administration of anti-IL-33 or its isotype control (Rat IgG2A, clone 54447, R&D Systems) in a dose of 20 μg per animal in 25 μl PBS on five consecutive days, one hour prior to OVA exposure.

Sample collection and processing

In paper I-III, samples were collected 24 hours after the final allergen exposure. The mice were deeply anaesthetised by an i.p. injection with a mixture of xylazin (130 mg/kg, Rompun®, Bayer, Germany) and ketamine (670 mg/kg, Ketalar®, Parke-Davis). First, blood was collected by puncturing the right heart ventricle. Second, the mice were tracheostomized and BAL was performed by instilling 0.25 ml of PBS through the tracheal cannula, followed by gentle aspiration and repeated with 0.2 ml PBS. An additional 1 ml PBS was used to wash away lumen inflammatory cells. Third, the pulmonary circulation was perfused with PBS and the lungs were removed from the thoracic cavity to harvest parenchymal inflammatory cells. The left lobe was filled with Tissue-Tek® O.C.T. Compound (Sakura Finetek Europe B.V., 2382 AT, Zoeterwoude, The Netherlands) and PBS containing 20% sucrose (Sigma-Aldrich) and then immediately frozen in liquid nitrogen. The right lobes were stored in Hank balanced salt solution (HBSS) (Sigma-Aldrich) on ice for flow cytometry. At the end, BM cells were harvested by excising one femur, which was cut at the epiphysis and flushed with 3 ml HBSS.

Blood, BALF, Bone marrow (I-III)

In paper I, blood (200 µl) was added to 2 mM EDTA (800 µl) (Sigma-Aldrich) in PBS and red blood cells (RBC) were lysed in 0.1% potassium bicarbonate and 0.83% ammonium chloride for 15 minutes at room temperature (RT). White blood cells (WBC) were resuspended in 1% fetal calf serum (FCS) (Sigma-Aldrich) in PBS.

In paper III, BALF samples from the animals treated with anti-IL-33 or isotype control were centrifuged at 1000 x g for 10 minutes at 4ºC and the cells were resuspended in 0.03% BSA in PBS.

In paper I, BM samples were centrifuged at 1000 x g for 10 minutes at 4ºC and the cells resuspended in PBS for counting. In paper III, BM cells were resuspended in RPMI-1640 with 5% FCS after centrifugation for *in vitro* study.

The cell count for all compartments was performed in a standard hemocytometer and cytospins prepared, either for staining with May-Grünwald-Giemsa for differential counts or for immunocytochemistry (ICC). Cell differentiation was determined by counting 300-500 cells using a light microscope (Zeiss Axioplan 2, Carl Zeiss, Germany). The cells were identified using standard morphological criteria, and BM mature and immature eosinophils were determined by nuclear morphology, cell size and cytoplasmic granulation.

Lung tissue (I-III)

In paper I, the right lobes of the lung were weighed and pushed by a plunger through a cell strainer with a 100 µm nylon mesh (BD Biosciences Erembodegem, Belgium). The strainer was washed with 5 ml of PBS supplemented with 1% FCS and the cell solution was passed through a 40 µm nylon mesh. The cells were washed as above and diluted in PBS supplemented with 1% FCS. The cell count was performed as mentioned above. Lung cells were saved for flow cytometry analysis.

In paper II and III, the apical lobe was stored at -80ºC for cytokine measurement and another three lobes, without any connective tissue, were stored on ice in HBSS for flow cytometry analysis. The three lung lobes were weighed and rinsed in a petri dish before being transferred to a gentleMACS™ C Tube (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) containing 5 ml of HBSS supplemented with 10% FCS, 100 µl Collagenase D solution (final concentration 2 mg/ml) and 20 µl DNase I solution (final concentration 80 U/ml). The mouse lung was dissociated using a gentleMACS Dissociator (Miltenyi Biotec) according to the manufacturer's instructions. Lung cell pellets were collected and washed in PBS supplemented with 10% FCS and the lung cells saved for flow cytometry analysis.

Immunostaining (I, II)

Immunocytochemistry (I)

Immunocytochemistry (ICC) presents a possibility to co-localize multiple antigens within different cell types. Double ICC staining was performed as a sequential method based on two individual staining procedures. Cells were determined by counting 300-500 cells using a light microscope. The immunostaining is described in detail in the paper I and II.

Double staining of MBP together with BrdU (I)

Major basic protein (MBP), an eosinophil granule protein, is expressed early in eosinophil-lineage committed cells, as well as in mature eosinophils. Double staining of MBP, together with nuclear BrdU, allows us to detect newly produced cells at an early stage in the development of the eosinophil lineage.

Immunohistochemistry (I, II)

Immunohistochemistry (IHC) is widely used in basic research to understand the distribution and localization of biomarkers in different parts of a tissue. Double IHC staining was also performed as a sequential method based on two individual staining procedures. The distribution of the cells was determined by using a light

microscope and Digital Camera (Canon power shoot G6, Japan). The immunostaining is described in detail in the paper I and II.

In paper I and II, the left lung lobe was embedded in Tissue-Tek® O.C.T. Compound and placed in a container of 2-methylbutane (Sigma-Aldrich) which was then dipped in liquid nitrogen. Frozen samples were stored at −80°C until sliced into 5 µm sections with a cryostat Leica CM1900 UV-kryostat® (Leica Microsystems Nussloch GmbH, Germany).

Analyse the double staining of MBP with BrdU in IHC (I)

Sections were stained to detect the $BrdU^+$ eosinophils described above. The stained samples were assessed in a blinded fashion under a light microscope at the magnification of x 400. A semi-quantitative grading system was used to score the extent of inflammation in the histological sections. The eosinophilic inflammatory infiltrate of four compartments (perivascular tissue, alveolar lung tissue, central airways and peripheral airways) was analyzed. Inflammation accumulation was graded as 1 for absent or representing very few cells, 2 for an intermediate number of cells or one cell layer, and 3 for 3 or more layers of cells [103].

Double staining of CD4 together with T-bet or GATA-3 (I)

Cells expressing CD4 were identified as T helper lymphocytes, with T-bet and GATA-3 being the specific transcription factors for Th1 and Th2 cells respectively. Double staining of CD4 together with T-bet allows us to detect Th1 cells, while CD4 together with GATA-3 allows us to detect Th2 cells. The stained samples were assessed in a blinded fashion under a light microscope at a magnification of x 1000. To facilitate counting, a graticule was applied to the ocular. $CD4^+T\text{-}bet^+$ and $CD4^+GATA\text{-}3^+$ cells were evaluated on a 4-point scale (0 = 0-2 cells; 1 = 3-6 cells; 3= 7-10 cells; 4 = > 10 cells per field) [104]. Positive cells were counted in four representative fields (a perivascular tissue, an alveolar lung tissue, a central airway and a peripheral airway). The Th1/Th2 balance was expressed as $CD4^+T\text{-}bet^+$ / $CD4^+GATA\text{-}3^+$ ratio [105]. The immunostaining was described in detail in paper I.

Detection of Th1, Th2, Th17 and Treg cells in the lung tissue (II)

Immunostaining of the specific transcription factors, T-bet, GATA-3, RORγt and Foxp3 in the nucleus was used to identify Th1, Th2, Th17 and Treg cells respectively in the lung tissue sections. The immunostaining was described in detail in paper II. The stained samples were assessed in a blinded fashion using a light microscope at a magnification of x400. To facilitate counting, a graticule was applied to the ocular. Eight representative sections of each of the three lung microenvironments (peribronchial, perivascular and alveolar tissue), were

assessed. Positive cells were counted and the data expressed as the number of cells/mm^2 [105].

Flow cytometry analysis (I-III)

Flow cytometry analysis of lung leucocytes ($CD45^+7\text{-}AAD^-$), eosinophils ($CD45^+CCR3^+$), T lymphocytes ($CD45^+CD3^+$), T helper lymphocytes ($CD45^+CD3^+CD4^+$) (II)

Lung cells were pre-treated with 2% mouse serum (DAKO) for 15 minutes to prevent unspecific binding and thereafter stained with the following surface staining antibodies; Fluorescein (FITC) labelled anti-CD45 (clone 30-F11, BD Biosciences), Peridinin Chlorophyll Protein Complex (PerCP) labelled anti-CD3e (clone 145-2C11), Phycoerythrin (PE) labelled anti-CD4 (clone H129.19, BD Biosciences) and 7-aminoactinomycin (7-AAD) (BrdU Flow Kits, BD Pharmingen™, San Diego, CA) or a matching isotype control antibody. The cells were incubated for 30 minutes at 4°C with the antibodies or isotype controls, followed by two washes with washing buffer (PBS + 10% FCS). All flow cytometry analyses were carried out using a BD FACSAria™ Flow Cytometer (BD Biosciences, San Jose, CA) with 150 000 events being acquired per sample and analysed with FlowJo Software® (Tri star Inc, Ashland, OR, USA).

Flow cytometry analysis of proliferating lung $CD45^+CCR3^+BrdU^+$ cells (I)

In order to enumerate and charcaterise eosinophils that are newly produced, BrdU incorporation was used and measured using a muliparametric flow cytometry analysis. Lung cells were collected and treated as described above with the following antibodies: anti-mouse CD45-FITC, CCR3-PE (clone 83101, R&D, Abington, UK) and 7AAD or a matching isotype control antibody. The cells were then fixed and permeabilized with BD Cytofix/Cytoperm™, followed by two washes and stored at 4°C overnight for the intracellular staining with BrdU on the second day. The BrdU staining was performed according to the BrdU Flow Kit Staining Protocol in the instruction manual from BrdU Flow Kits (BD Pharmingen™). BrdU incorporation and intensity was estimated by rMFI (relative Mean Fluorescence Intensity, which equals MFI of monoclonal antibody/ MFI of corresponding isotype control) [106].

Flow cytometric cell cycle analysis of newly produced and proliferating T helper cell subsets (II)

Lung cells were collected and treated as described above for the staining of different T helper cells, Th1 cells ($CD4^+CD25^+$T-bet$^+$), Th2 cells

($CD4^+CD25^+GATA\text{-}3^+$), Th17 cells ($CD4^+CD25^+ROR\gamma t^+$) and Treg cells ($CD4^+CD25^+Foxp3^+$). Cells were stained with the following antibodies; FITC labelled anti-CD4 (clone RM4-5, BD Biosciences) and Alexa Fluor® 700 labelled anti-CD25 (clone PC61, BioLegend, San Diego, CA) or a matching isotype control antibody. Cells were fixed and intracellular staining performed according to the manufacturer's protocol for the Foxp3 staining buffer set (eBioscience™). Intracellular antibodies used included PE labelled anti-T-bet (clone eBio4B10, eBioscience™), PE labelled anti-GATA-3 (clone TWAJ, eBioscience™), PE labelled anti-RORγt (clone AFKJS-9, eBioscience™) and PE labelled anti-Foxp3 (clone FJK-16s, eBioscience™). The subsequent intracellular staining for BrdU and 7-AAD was performed using BrdU Flow Kits according to the manufacturer's instructions. BrdU was used to identify the newly produced cells and 7-AAD was used as a DNA dye to identify the proliferation of newly produced cells *in situ*.

Flow cytometry analysis of eosinophil progenitors and surface ST2 expression (III)

Eosinophil progenitors were identified as $CD45^+CD34^+IL\text{-}5R\alpha^+CCR3^+Sca\text{-}1^+SSC^{low}$ cells, with the expression of the IL-33 receptor identified as ST2 expression. Cells prepared from lung or BM were described as above for surface staining, with the following antibodies: PerCP labelled anti-CD45 (clone 30-F11, BD Biosciences), Alexa Fluor® 700 labelled anti-CD34 (clone RAM34, eBioscience™), PE labelled anti-CD125 (IL-5Rα) (clone T21, BD Biosciences), FITC labelled anti-CCR3 (clone 83101, R&D Systems), APC labelled anti-Ly-6A/E (Sca-1) (clone D7, eBioscience™), and biotinylated rat anti-mouse T1/ST2 (clone DJ8, MD Biosciences) followed by streptavidin PE-Cy7, Alexa Fluor® 488 labelled anti-IL-5Rα (clone T21, BD Biosciences), Alexa Fluor® 647 labelled anti-CD193 (clone 83101, BD Biosciences) and PE-Cy™7 labelled anti- Ly-6A/E (Sca-1) (clone D7, BD Biosciences).

Protein analysis (II)

Multiplex cytometric bead assay (II)

Cell lysate of the apical lobe of the mouse lung was used for cytokine measurements. BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine kit (BD Biosciences) was used to measure IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A and IL-10 in the lung tissue. Data were acquired on a FACS ARIA and samples were analysed using FCAP Array Software (BD Biosciences).

RNA analysis (II)

Preparation of tissue for laser capture microdissection (LCM) (II)

The frozen lung tissue was sectioned at 7µm in a cryostat Leica CM1900 UV-kryostat® and a minimum of 10 sections per animal was prepared for LCM. The frozen sections were placed on Nuclease Free – Membrane Slides NF 1.0 PEN (Carl Zeiss MicroImaginG GmbH, München, Germany) and then stained with Cresyl Violet acetate (Sigma-Aldrich®) according to the instruction manual "RNA extraction from frozen section" (PALM Laboratories, ZEISS). The slides were allowed to air-dry completely before being stored dessicated at -80°C to prevent activation of endogenous RNase in the tissues.

Laser capture microdissection (LCM) (II)

The Carl Zeiss Laser Microdissection and Pressure Catapulting (LMPC) technology (http://PALM-microlaser.com) was used, with the specimen being microdissected by a focused laser beam, and then a defined laser pulse transports the cut piece of the specimen out of the object plane into a collection device; a AdhesiveCap opaque (Carl Zeiss). The lung section to be subjected to LCM was visualised with the PALM- MicroBeam microscope (Carl Zeiss) at a magnification of x 5. Typically, on each tissue section, most of the peribronchial, perivascular and alveolar tissue were obtained and at least 10 sections were used for each mouse.

RNA isolation

LCM cells were collected from three different microenvironments of three OVA sensitised and exposed (OVA/OVA) mice. A whole lung tissue section of 50 µm from OVA sensitized and PBS exposed (OVA/PBS) mouse was used as control. The RNA was extracted using a QIAGEN RNeasy® Micro Kit (QIAGEN) according to manufacturer's protocol for the purification of total RNA from microdissected cryosections (QIAGEN). The RNA quality and quantity was determined using an Agilent Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies, Deutschland GmbH, Waldbronn).

Real-time reverse transcription polymerase chain reaction (real-time RT-PCR)

RNA from three OVA/OVA mice were pooled based on three microenvironments, while RNA from one OVA/PBS mouse was used as a control. Each cDNA reaction was prepared using the SABioscience's RT^2 First Strand Kit and 135 ng of total RNA, according to the manufacturer's protocol. Real-time RT-PCR was carried out using RT^2 Profiler™ PCR Array (PAMM-0011D) plates containing 84 mouse inflammatory cytokines, chemokines and receptor genes from Super array Bioscience Corporation, USA. SABiosciences's

RT^2 SYBR Green qPCR Master Mix was used for the detection and the array plates were run in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad) according to manufacturer's instructions (SABiosciences). The results were monitored using different controls available on the plates. Gene expression levels were measured by the threshold cycle (Ct) and samples showing more than one peak in the melting curve were excluded from the analysis performed using CFX Manager 2.0 software (Bio-Rad). Twenty-four genes were not considered in the analysis, as they did not pass our quality control, leaving 60 genes for further analysis. Data were normalized with five housekeeping genes; [Gusb (Glucuronidase, beta), Hprt1 (Hypoxanthine guanine phosphoribosyl transferase 1), Hsp90ab1 (Heat shock protein 90kDA alpha (cytosolic), class B member 2), Gapdh (Glyceraldehyde-phosphate dehydrogenase) and Actb (Actin, beta cytoplasmic)] which were available on the plates. Finally, fold-changes compared to the control mice were calculated using the manufacturer's software (SABiosciences). A fold-change cutoff ≥3 was used to identify molecules whose expression was differentially regulated.

Bioinformatic Analysis

Genes of interest identified using the RT^2 Profiler™ PCR Array were further analysed by Ingenuity Pathways Analysis (IPA; version 9.0) (Ingenuity® Systems, www.ingenuity.com), specifically in regards to their interactions. The fold change values were compared to the control. IPA utilises the Ingenuity Pathways Analysis Knowledge Base (IPA KB), a manually curated database of protein interactions from the literature, to analyse data. This KB was used to annotate the genes of interest and reveal their associations.

In vitro experiments (III)

In vitro proliferation assay

For the *in vitro* proliferation assay, animals were sensitised and exposed to OVA and lung cells were harvested as described above. Lung $CD45^+$ cells were enriched by MACS positive separation with mouse CD45 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the $CD45^+$ cell fraction was > 95%, as assessed by FACS. Lung $CD45^+$ cells (1×10^6) were cultured at 37ºC and 5% CO_2 in a 96-well plate in 0.2 ml RPMI-1640 culture medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (all obtained from Sigma-Aldrich).

Two different methods were used for the assays: BrdU and CFSE.

For BrdU labelling, lung $CD45^+$ cells (1×10^6) cells were seeded and divided into groups treated with rmIL-5 (50 ng/ml), rmIL-33 (50 ng/ml), rmEotaxin-2

(250 ng/ml) (all cytokines obtained from PeproTech, London, UK), anti-IL-33 (50 µg/ml, R&D Systems), combinations of these agents or left untreated. Cells were then incubated at 37ºC and 5% CO_2 for 48 hours with BrdU being added at the last 5 hours to label the newly produced cells. After collection, the cells were stained for CD34/IL-5Rα/CCR3/Sca-1/BrdU/7-AAD to identify the newly produced eosinophils and the proliferation of newly produced eosinophils *in situ* by FACS analysis.

For CFSE labelling, lung $CD45^+$ cells (1 x 10^6) were labelled with 0.25 µM CFSE (Molecular Probe, Invitrogen) and seeded in two groups; rmEotaxin-2 + rmIL-5 + rmIL-33, and rmEotaxin-2 + rmIL-5 + rmIL-33 + anti-IL-33. The plates were incubated at 37ºC and 5% CO_2 for 60 hours, to assess the proliferation of eosinophils, with the cells stained for CCR3 and analysed by FACS.

In vitro apoptosis assay

Lung $CD45^+$ cells were collected as described above and cultured in comparable conditions. As a positive control, H_2O_2 was used for the induction of apoptosis. The plates were incubated at 37ºC and 5% CO_2 for 40 hours. After collection, the cells were stained for the surface markers, i.e. CD34/ IL-5Rα/CCR3/Sca-1, together with Annexin-V/7-AAD (Annexin V: PE Apoptosis Detection Kit I, BD Pharmingen™) according to the manufacturer's instructions. Samples were analysed by FACS within one hour.

In vitro transmigration assay

For the transmigration assay, BM cells harvested from OVA sensitised and exposed mice were used. Briefly, the migration of BM eosinophils was assessed using 5 µm polycarbonate membrane transwell inserts in 24-well tissue-culture polystyrene plates (Costar, Corning, NY). The inserts were pre-incubated in media (RPMI-1640 containing 5% FCS) for 1 hour at 37ºC. BM cells (1.5 x 10^6) and 50 ng/ml rmIL-5 in 200 µl media were placed in the inserts to ensure eosinophil survival. The inserts were then placed into the wells containing rmIL-5, rmIL-33, rmEotaxin-1, rmEotaxin-2, anti-IL-33 or combinations of these agents or left untreated. The plates were incubated at 37ºC and 5% CO_2 for 2 hours. The cells that had migrated to the lower wells were collected, counted and stained for CD45/CD34/IL5Rα/CCR3/Sca-1/ST2 and analysed by FACS to identify the different maturation stages of the eosinophils expressing the ST2.

Statistical analysis

All data were expressed as mean ± SEM (standard error of the mean). For *in vivo* experiments, differences between groups were calculated using the Mann-Whitney U test for unpaired data. For the *in vitro* experiments, statistical

analyses were performed with a paired Student's t-test [107]. A *P* value ≤0.05 was considered statistically significant.

Human study (III, IV)

Study subjects (III)

Participants were selected from questionnaire responders who attended a detailed clinical examination at Sahlgrenska University Hospital in Gothenburg, Sweden in the West Sweden Asthma Study (WSAS) [108]. Asthma was diagnosed according to clinical symptoms and characteristics measured (Table 1 & 2, see paper III). Two groups of participants were enrolled, with six stable asthmatics out of pollen season and four asthmatics during the pollen season being involved. Blood samples were taken during clinical visits. The study was approved by the Regional Ethical Approval Committee in Gothenburg, Sweden (no. 593-08).

Human blood samples (III)

Peripheral cells from whole blood were stained for FACS analysis within 1 hour of sampling with two staining panels, CD45/CD34/IL-5Rα/ST2 and CD4/GATA-3/ST2. Intracellular staining (GATA-3) was performed using the FoxP3 Staining Buffer Set (eBioscience™) according to the manufacturer's protocol. The detailed antibody information was available in paper III.

Study subjects and screening visit (IV)

Participants were randomly selected from the West Sweden Asthma Study (aged 16-75 years) [108]. Asthma was diagnosed from reports of common symptoms (chest tightness, shortness of breath, cough and nocturnal symptoms), and the bronchial hyperresponsiveness and concentration of methacholine that reduced FEV_1 by 20% (PD20) was below a cumulative dose of 1.94 µg, or a reversibility of more than 12%. The skin-prick test (SPT) was performed using a standard panel of 11 inhalant allergens (birch, mugwort, timothy, horse, dog, cat, cockroach, Cladosporium, Altenaria, *Dermatophagoides. farinae* and *D. pteronyssinus* (ALK, Hørsholm, Denmark).

Asthmatics were divided in two groups based on their eosinophil numbers during screening visit (visit 1, see paper IV); asthma patients with high eosinophilia when blood eosinophils were ≥0.3 x 10^9/L, and asthma patients with low eosinophilia when values were ≤0.2 x 10^9/L. The normal control subjects reported no asthma symptoms, were non-reactive to methacholine or no-reversible, negative SPT and had a low number of blood eosinophils (≤0.2 x 10^9/L). All study subjects were non-smokers (Table 1, see paper IV).

Study visit (IV)

The selected study population; seven subjects with high eosinophilia (Eos-high group), six subjects with low eosinophilia (Eos-low group) and five non-asthmatic healthy controls (Control group) were recruited/invited for a second visit to the clinic (Visit 2, see paper IV) between December and February *i.e.* out of pollen season. In the four weeks prior to the visit, all subjects had not received any influenza vaccination, changed their asthma medication, had any worsening of asthma symptoms, reported any symptoms of infection/cold, any surgery, any antibiotics, any new medication, or any anti-inflammatory medication. During the study visit, spirometry and fractional exhaled nitric oxide (FeNO) measurements were performed. Blood samples and induced sputum were also collected at Visit 2.

Sputum induction and processing of whole sputum was performed according to the European Respiratory Society guidelines [109].

All subjects were volunteers and gave their written consent after being fully informed about the purpose and nature of the studies, which were approved by the Regional Ethical Approval Committee in Gothenburg, Sweden (no. 593-08).

Human blood samples (IV)

Peripheral whole blood cells were stained for flow cytometry within 1 hour of sampling. Total cell counts were got from the Trucount ™ tubes (BD Biosciences) together with antibody against CD45 according to manufacturer's instruction. To detecting eosinophils, eosinophil progenitors and the related trafficking, activation and apoptosis molecules, a variety of antibody panels was used were used:

a) CD45/CD34/IL-5Rα/CD162,

b) CD45/CD34/IL-5Rα /CD49d/CD29,

c) CD45/CD34/IL-5Rα/CD11b/CD18,

d) CD45/CD34/IL-5Rα/CCR3/CD38/ CD45RA/IL-3Rα,

e) CD45/CD34/IL-5Rα/CCR3/CD69/CD25 and

f) CD45/CD34/IL-5Rα/CCR3/CD95/Active Caspase -3.
The detailed antibody information was available in paper IV.

Cells were stained with surface antibodies as described above, followed by BD FACS Lysing Solution to lyse the red blood cells, then the cells were ready for FACS analysis after washing. Intracellular staining of Active Caspase-3 was performed using the Foxp3 Staining Buffer Set (eBioscience™).

Cells were acquired on a BD FACSAria™ Flow Cytometer and analysed with FlowJo Software®. Analysis was performed using the Fluorescence Minus One (FMO) controls strategy leaving one reagent at a time to set gates. $CD34^+$ cells were gated according to ISHAGE guidelines[110]. Gating was based on CD34 and CD45 positivity, combined with forward scatter (FSC) and side scatter (SSC) characteristics of $CD34^+$ progenitors.

Statistical analysis

All data from human studies are expressed as mean values ± SEM. Comparisons between groups were made with an unpaired t-test; $p<0.05$ was considered statistically significant.

RESULTS AND COMMENTS

New eosinophil production and the corresponding Th1/Th2 balance are similar in both BALB/c and C57BL/6 mice, while the distribution of eosinophils is different (I)

Two commonly used mouse strains, BALB/c and C57BL/6, that were reported to be display Th2 and Th1 *in vivo* responses respectively, were used to compare the pattern of allergic inflammation by measuring the differences in OVA induced eosinophilia. It was found that OVA exposure induced similar eosinophilic inflammation in both strains in BM, blood and lung. While there were no differences in total or newly produced eosinophils (MBP^+BrdU^+) between the two strains, C57BL/6 mice had slightly more MBP^+BrdU^- eosinophils in the lungs, suggesting that these cells had been produced before the first allergen exposure. The comparison of the different administrations of BrdU revealed no major differences in the incorporation of BrdU in the cells. This suggests that the influx and proliferation rate of newly produced eosinophils is equal in both strains. However, the distribution patterns are different. Both mouse strains showed a strong accumulation of eosinophils in the perivascular and central airway tissue, while in the alveolar lung tissue and peripheral airways the eosinophil numbers were significantly greater in C57BL/6 mice compared to BALB/c mice (Table 1 and Figure 2). Furthermore, both mouse strains showed no eosinophilic inflammation without allergen sensitization and exposure. Finally, an evaluation of Th1/Th2 balance in the lung revealed no differences between the two mouse strains (Figure 3). Overall, the above findings demonstrate that both BALB/c and C57BL/6 mice acquire an eosinophilic inflammation, but the distribution of the newly produced eosinophils in the lung differs.

Table 1. Grading of lung histology for the newly produced eosinophils in BALB/c and C57BL/6 mice.

Mouse Strain	Perivascular tissue	Alveolar lung tissue	Central airways	Peripherial airways
BALB/c	3.00 ± 0.00	1.97 ± 0.16	3.00 ± 0.0	2.19 ± 0.15
C57BL/6	3.00 ± 0.00	3.00 ± 0.00**	2.8 ± 0.12	2.85 ± 0.10*

Lung sections was sampled 24 hours after the last OVA exposure from BALB/c and C57BL/6 mice which were OVA sensitized and exposed, lung histology used to evaluate MPB^+BrdU^+ newly produced eosinophils. A semi-quantitative grading system was used to score the extent of inflammation in the histological sections in four compartments: perivascular tissue, alveolar lung tissue, central airways and peripheral airways. Inflammation accumulation was

graded as 1 for absent or representing very few cells, 2 for an intermediate number of cells or one cells layer, and 3 for 3 or more layers of cells. The histological sections were graded in a blinded fashion. Data is expressed as mean severity scores ± SEM, n=6 mice/group, $**P$ = 0.006, $*P$ = 0.017.

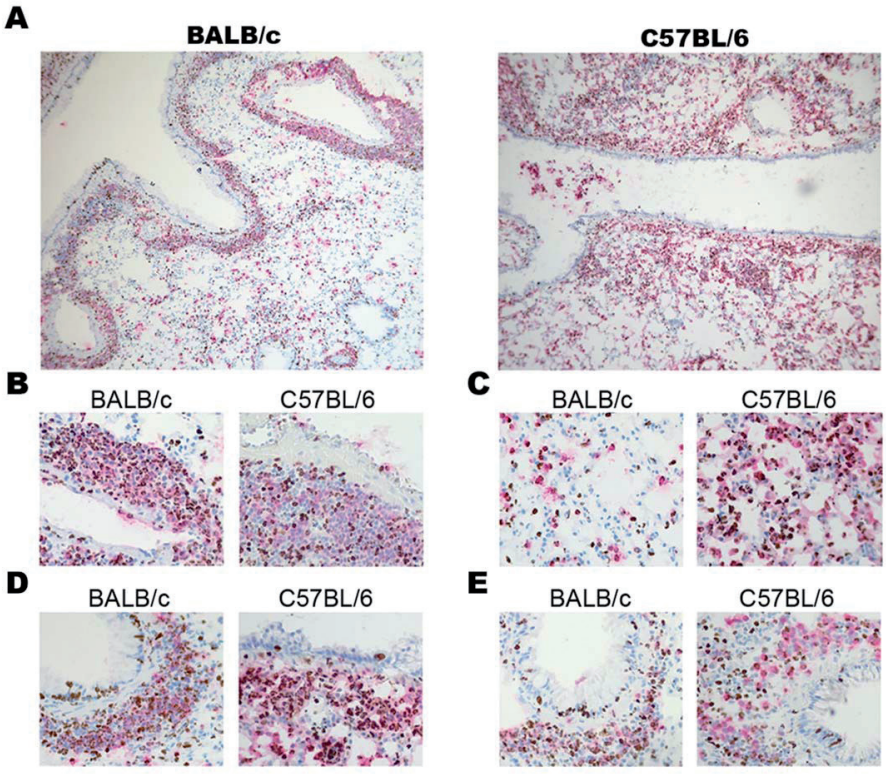

Figure 2. **Newly produced eosinophils (MBP^+BrdU^+) with MBP^+ (red) and $BrdU^+$ (brown) in the lung tissue from OVA/OVA BALB/c and C57BL/6 mice treated with BrdU 2*2.** Representative photomicrographs (x 100 magnification) from A) BALB/c and C57BL/6 mice showed the distribution of the newly produced eosinophils. Higher magnification (x 200) photomicrographs expose eosinophils in B) perivascular tissue, C) alveolar lung tissue, D) central airways and E) peripheral airways.

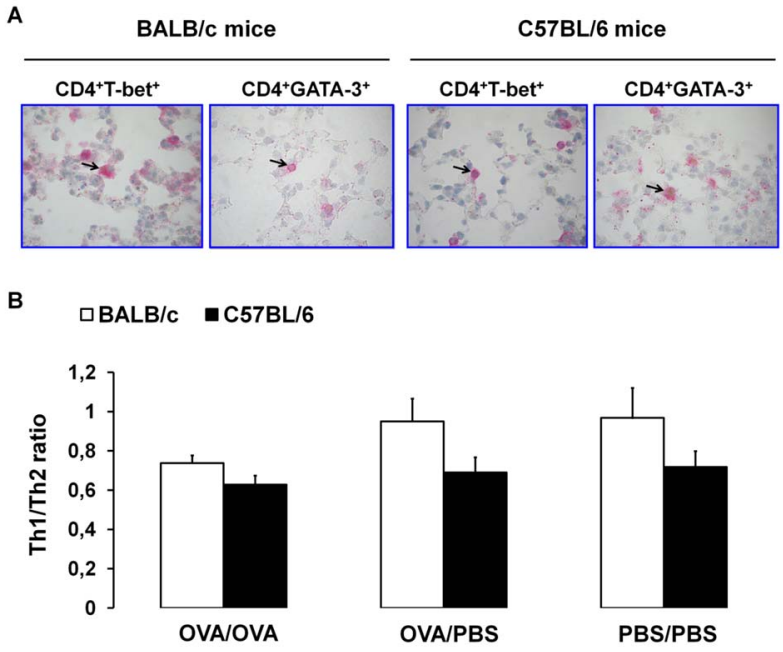

Figure 3. **Th1 ($CD4^+T\text{-}bet^+$) and Th2 ($CD4^+GATA\text{-}3^+$) cells in the lung tissue from PBS or OVA sensitized and exposure BALB/c and C57BL/6 mice treated with BrdU 2*2.** A) Representative photomictographs (x 1000 magnification) from alveolar lung tissue of OVA/OVA BALB/c and C57BL/6 mice. $CD4^+$ shows as a red staining and $T\text{-}bet^+$ or $GATA\text{-}3^+$ shows as a brown nuclear staining. B) Th1/Th2 balance expressed as their ratio in naïve; PBS/PBS, sensitized; OVA/PBS and exposed; OVA/OVA, BALB/c and C57BL/6 mice.

Allergen exposure induced Th1, Th2, Th17 and Treg cell expansion in the lungs, predominantly Th2 cells (II)

The results above led to hypothesise that additional T helper cells could also be involved in eosinophilic inflammation. Therefore, we evaluated the relative presence of all major T helper cell subsets; Th1, Th2, Th17 and Treg cells, during allergic airway inflammation.

Airway exposure to allergen in sensitised mice expands all studied effector T cells in the lungs, including T-bet$^+$, GATA-3$^+$, RORγt$^+$ and Foxp3$^+$ cells , both in those cell populations expressing the IL-2Rα (*i.e.* CD25) and those that do not. Among these, the GATA-3 effector Th2 cell is the one most prominently increased after allergen exposure, followed to a lesser extent by the T-bet$^+$ (Th1) and the RORγt$^+$ (Th17) cells. In contrast, the Foxp3$^+$ (Treg) cells increased in absolute cells numbers in the CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ populations, but decreased compared to the other T effector cells in the CD4$^+$CD25$^+$ fraction. All effector T cells are produced *de novo* and proliferate *in situ* in the lungs, but with different profiles. Th1 and Th2 cells proliferate more extensively than Th17 and Treg cells during allergic inflammation (Figure 4).

The distribution of T cell transcription factors differs in the lung tissue during allergic airway inflammation (II)

As shown by flow cytometry, all of the investigated T effector cells and Treg cells in the lung were increased in numbers, with many in a proliferative state during allergic inflammation. It has been reported that T cells can traffick into the inflamed lung tissue during allergic inflammation, but their micro environmental distribution has not been investigated to date. Therefore, we stained lung tissue from OVA/PBS and OVA/OVA mice for T-bet (Th1), GATA-3 (Th2), RORγt (Th17) and Foxp3 (Treg cells) expressing cells by IHC, and quantified their respective presence in three different areas of the lung, including lung peribronchial tissue, lung perivascular tissue and lung alveolar tissue (Figure 5). We found that allergen exposure results in an accumulation of Th1, Th2 and Treg cells in the peribronchial tissue, as well as an increase in the alveolar lung tissue of Th17 cells. In perivascular tissue, only Th2 and Treg cells increased.

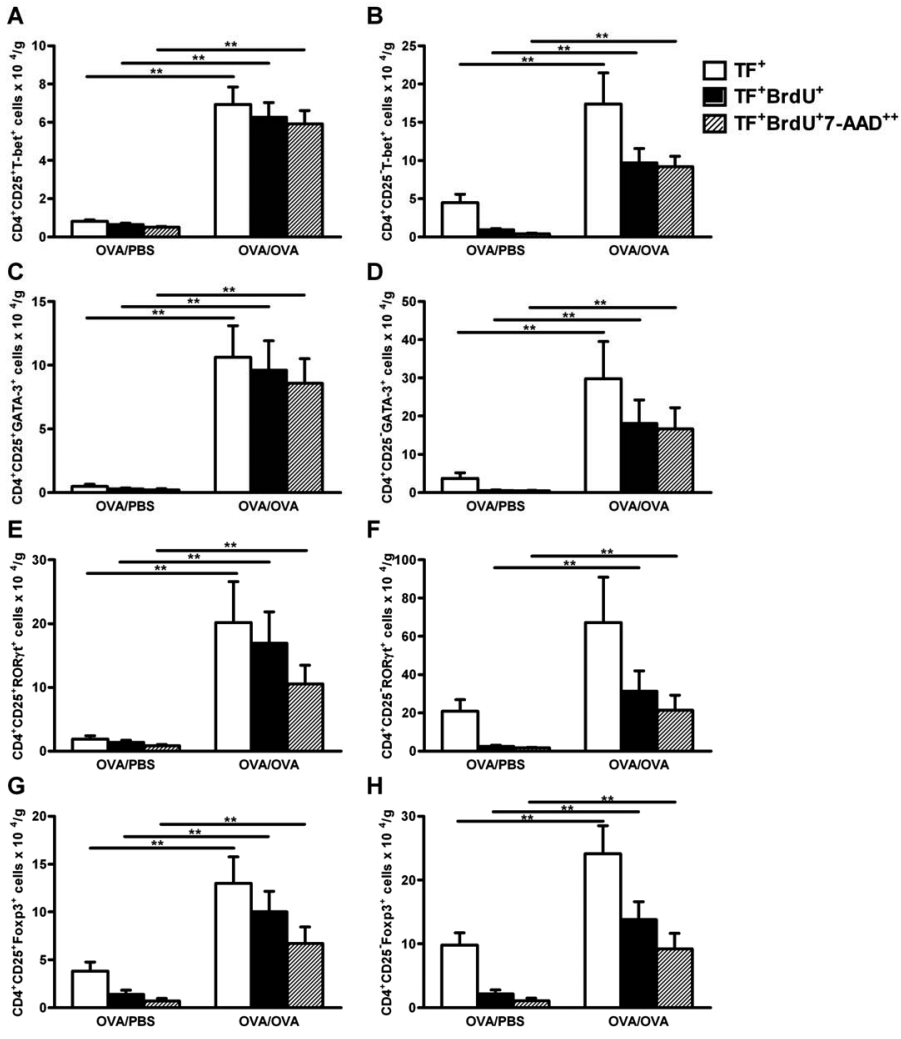

Figure 4. Lung Th1, Th2, Th17 and Treg cells in $CD4^+CD25^+$ and $CD4^+CD25^-$ populations proliferate *de novo* and *in situ* during allergic inflammation.
Number of lung $CD4^+CD25^+$ (A, C, E and G) and $CD4^+CD25^-$ cells (B, D, F, H) that are expressing transcription factors (TF); T-bet (A and B; Th1), GATA-3 (C and D; Th2), RORγt (E and F; Th17) and Foxp3 (G and H; Treg) and have incorporated BrdU (filled column) or have incorporated BrdU and also express a double amount of DNA ($7\text{-}AAD^{++}$; hatched columns) in mice exposed to PBS or OVA. Data are shown as mean number of cells per gram tissue +SEM. ** $p<0.01$.

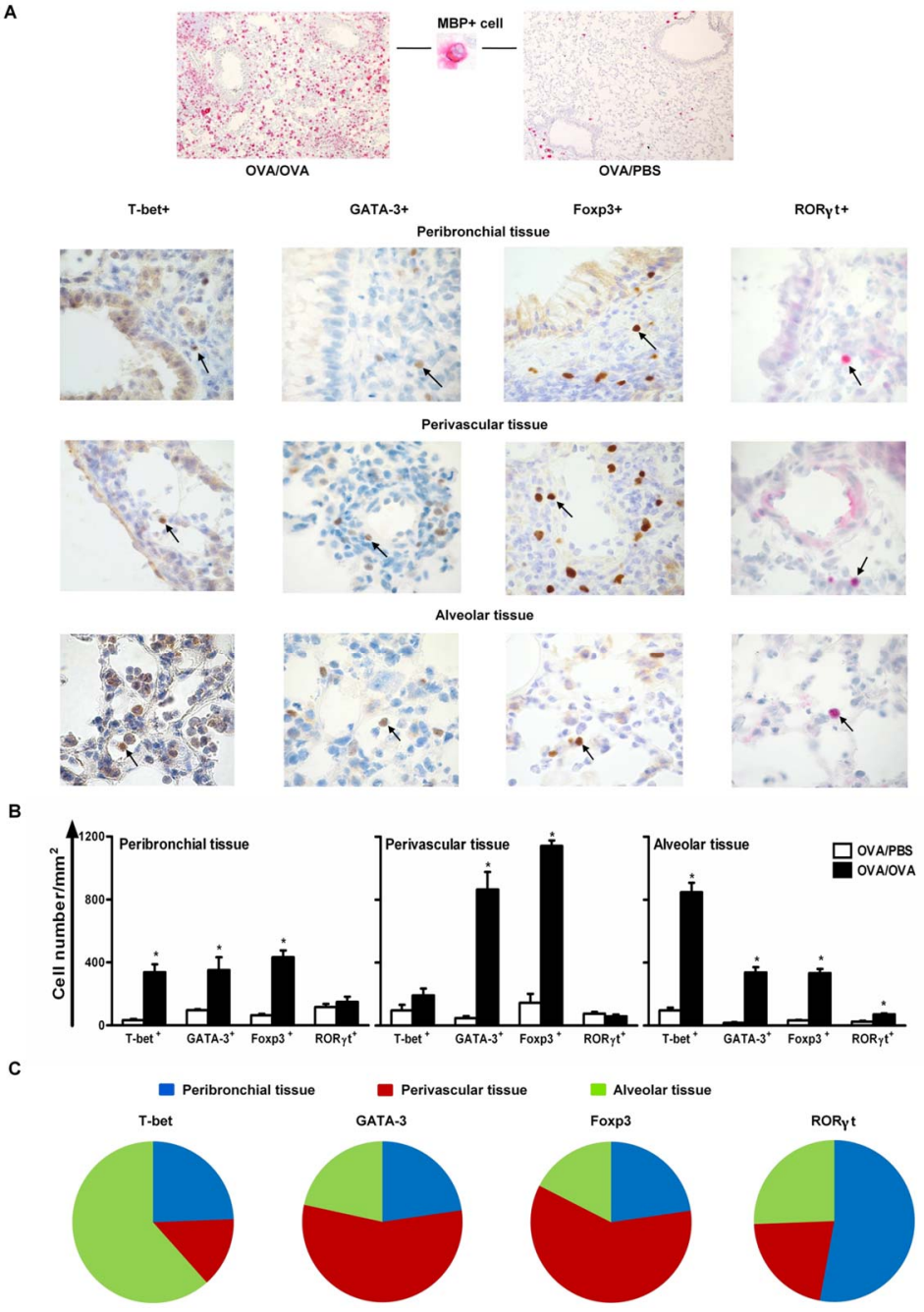

Figure 5. The distribution of T cell transcription factors differs in the lung microenvironments during allergic inflammation. A) Photographs of IHC of MBP^{+} cells (red) in OVA/OVA or OVA/PBS mice; T-bet, GATA-3, Foxp3 (brown) and RORγt (red) in

peribronchial, perivascular and alveolar tissue in OVA/OVA mice. B) Quantification (cells/mm^2) of T-bet, GATA-3, Foxp3 and RORγt expressing cells in different compartments of the lungs, including peribronchial tissue, perivascular tissue and alveolar tissue, after exposure to PBS or OVA. Data shown as mean+SEM, * $p<0.05$. C) Pie chart showing the relative distribution of T-bet, GATA-3, Foxp3 and RORγt expressing cells in the different lung compartments after exposure of mice to OVA.

General inflammatory milieu pattern and the gene expression in peribronchial, perivascular and alveolar lung tissue as a marker of the local inflammatory response (II)

To determine the general inflammatory milieu in the lung, pro and anti-inflammatory cytokines, as well as cytokines relevant to the different T helper cell subsets, were quantified in the lung supernatant. A general inflammatory milieu in the lung was shown to involve cytokines, such as IL-4 (Th2), IFN-γ (Th1), and IL-17(Th17), as well as several pro-inflammatory cytokines including IL-6. The low concentration of IL-10 argues for a relatively low activity of Treg cells.

To further evaluate the local inflammatory microenvironment in the different lung tissue compartments, we used laser capture microdissected peribronchial, perivascular and alveolar tissue from OVA/OVA mice and control mice. The RNA was analysed by a real-time RT-PCR and the expression of 60 different cytokines, chemokines or respective receptor genes was quantified (Figure 6). The effect of allergen exposure in all three tissue compartments was expressed as the fold change compared to the gene expression in OVA/PBS animals (OVA-OVA/OVA-PBS). Allergic inflammation resulted in the expression of a wide range of inflammatory genes in the lung, with differences occurring between the three compartments. Some of the inflammatory genes have tissue specific expression, arguing for the importance of the local microenvironment. Next, we focus on the differences between the three compartments. For analysis, we grouped genes into three categories; genes involved in general inflammation, genes involved in allergic inflammation and genes from chemokines that can affect T effector cell migration, as reported in the literature, followed by the analysis of their relationship using Ingenuity Pathways Analysis. Several chemokine genes supported the accumulation pattern of T effector cells (Figure 7).

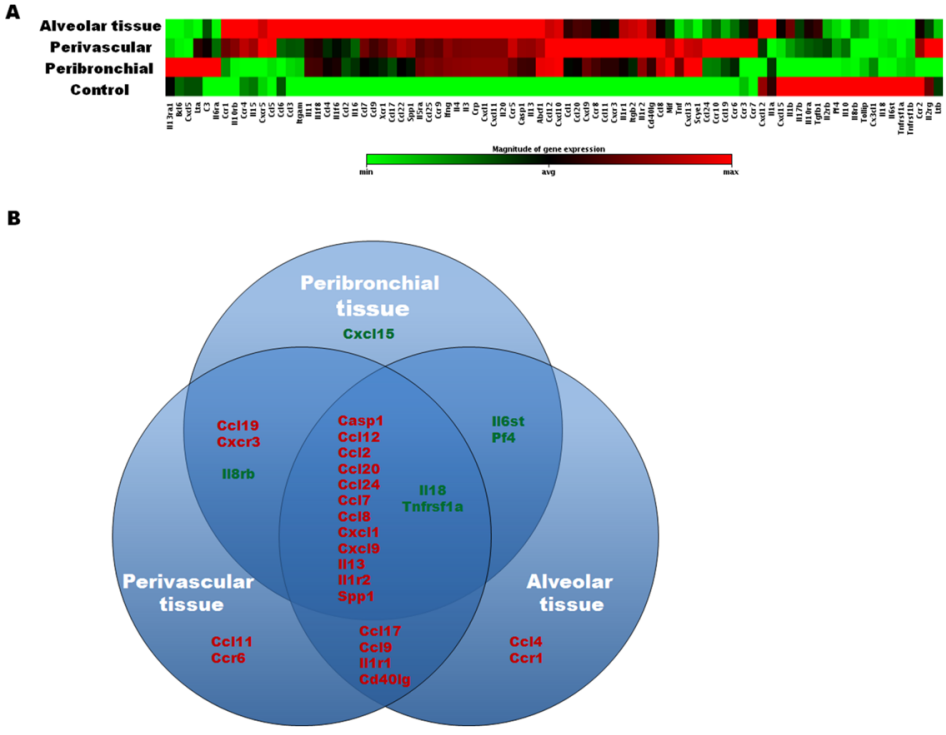

Figure 6. **Expression of inflammatory cytokine, chemokine and receptor genes in the lung after allergen exposure compared to control.** A) The different lung tissue compartments (alveolar, perivascular and peribronchial) were isolated from lung tissue from OVA or PBS exposed mice using the PALM microBeam microscopy. Lung compartment samples were taken from three different animals and then pooled. RNA was extracted and the inflammatory response in each microenvironment was assessed using real-time RT-PCR array of 84 inflammatory cytokines, chemokines and receptors. The expression of these genes in each compartment is visualized as heat map. Red colour indicates up-regulated genes, green colour indicates down-regulated genes, and the dark colour indicates great fold change. B) Venn diagram of genes with fold change ≥ 3 times after allergen exposure in at least one tissue compartment compared to control. Up-regulated genes are shown in red font and down regulated genes are shown in green font.

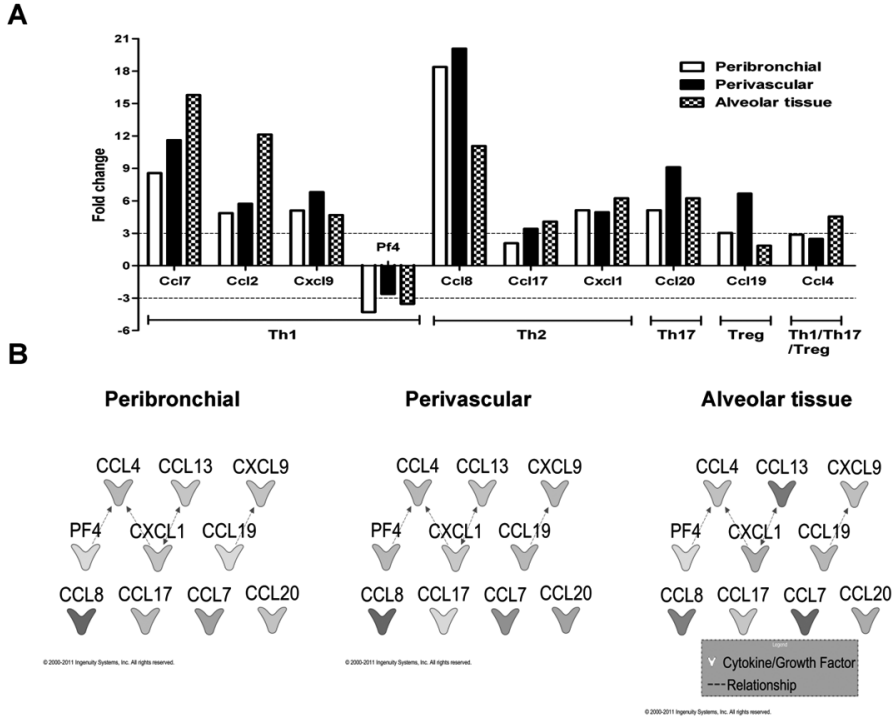

Figure 7. Chemokines related to Th1, Th2, Th17 and Treg cells during allergic inflammation. A) Fold change of genes related to chemoattractants to Th1, Th2, Th17 and Treg cells in peribronchial, perivascular and alveolar tissue. Fold change is calculated by comparing OVA exposed mice to PBS control. 3 fold change threshold indicated by dotted line was considered biological relevant. B) Network of differentially expressed genes in OVA exposed mice compared to PBS exposed controls. A network of chemokines, generated by IPA (3.0 fold change threshold), of OVA/OVA mice compared to OVA/PBS mice. The expression of these genes in three lung microenvironments was examined; peribronchial, perivascular and alveolar tissue. Lines indicate relationships between molecules (dashed line=indirect interaction). Arrows at the end of these lines indicate the direction of the interaction. The exact fold change values of each molecule are shown in Table 1 (see paper II).

IL-33 involved in the regulation *in situ* lung eosinophilopoiesis (III)

The two studies above confirmed the correlation between Th2 cells and eosinophils during allergic airway inflammation. ST2 is expressed on the cell surface of both Th2 cells and eosinophils, suggesting a possible link between them during allergic airway inflammation. However, its expression in eosinophil progenitors and its role during the *in situ* lung eosinophilic inflammation is still

unclear. We provide evidence that lung IL-33 regulates *in situ* eosinophilopoiesis, and eventually eosinophilia, in a direct manner through several mechanisms. This regulation is possible through a combination of the increased amount of IL-33 protein expression during allergic airway inflammation and the different levels of expression of the IL-33R/ST2 in lung eosinophil sub-populations.

We describe, for the first time, five different eosinophilic populations (Table 3, paper III) in the lung, which may represent different stages of eosinophil development. Those populations showed a differential expression of ST2 depending upon their maturation status. Almost all eosinophil progenitors ($CD45^{+}CD34^{+}IL\text{-}5R\alpha^{+}CCR3^{+}Sca\text{-}1^{+}SSC^{low}$) express ST2, with the expression decreasing slightly during the next two stages of immature eosinophils; early ($CD45^{+}CD34^{+}IL\text{-}5R\alpha^{+}CCR3^{+}Sca\text{-}1^{+}SSC^{high}$) and intermediate ($CD45^{+}CD34^{+}IL\text{-}5R\alpha^{+}CCR3^{+}Sca\text{-}1^{-}SSC^{high}$), followed by the main decrease in the last stage, late immature ($CD45^{+}CD34^{-}IL\text{-}5R\alpha^{+}CCR3^{+}Sca\text{-}1^{-}SSC^{high}$), while mature eosinophils ($CD45^{+}CD34^{-}IL\text{-}5R\alpha^{-}CCR3^{+}Sca\text{-}1^{-}SSC^{high}$) essentially express no ST2. We have also shown that up to 60% of Th2 cells ($CD4^{+}GATA\text{-}3^{+}$) express the ST2 receptor (Figure 8). To determine the role of IL-33 in the lung *in situ* eosinophilic inflammation, a blocking antibody was administered intranasally during allergen exposure. Local lung blockage of IL-33 during allergen exposure with anti-IL-33 treatment impaired the number of all eosinophil populations, except the mature eosinophils, but did not have any effect on the number of Th2 cells. This data argues that local lung IL-33 can regulate *in situ* eosinophilopoiesis in a direct manner.

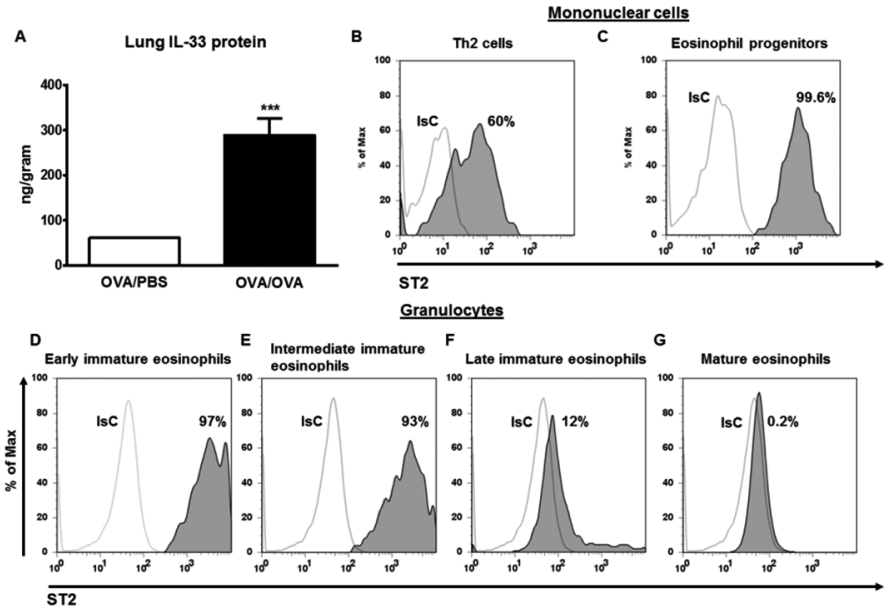

Figure 8. IL-33 protein and ST2 (IL-33 receptor) expression in lung eosinophils during different stages of development and Th2 cells during allergic inflammation.
Concentration of IL-33 A) was assessed using ELISA; Results are expressed as ng/gram lung tissue, in mice exposed to PBS or OVA. Data are shown as mean ± SEM, ***p <0.001. Representative FACS histogram showing the relative expression of ST2 on B) $CD4^+GATA\text{-}3^+$ cells (*i.e.* Th2), C) $CD45^+CD34^+IL\text{-}5R\alpha^+CCR3^+Sca\text{-}1^+$ mononuclear cells (i.e. eosinophil progenitors), D) $CD45^+CD34$+$IL\text{-}5R\alpha^+CCR3^+Sca\text{-}1^+$ granulocytes (i.e. early immature eosinophils), E) $CD45^+CD34^+IL\text{-}5R\alpha^+CCR3^+Sca\text{-}1^-$ granulocytes (i.e. mid-immature eosinophils), F) $CD45^+CD34^-IL\text{-}5R\alpha^+CCR3^+Sca\text{-}1^-$ granulocytes (i.e. late immature eosinophils) and G) $CD45^+CD34^-IL\text{-}5R\alpha^-CCR3^+Sca\text{-}1^-$ granulocytes (i.e. mature eosinophils) in the lung tissue after OVA exposure. Data are shown as %.

IL-33 regulated lung *in situ* eosinophilopoiesis by affecting their *in situ* proliferation, survival and migration (III)

In order to understand the possible mechanisms through which IL-33 regulates *in situ* eosinophilia, further analyses were performed. Several mechanisms affecting the different sub-populations of eosinophils have been revealed. Firstly, as eosinophil progenitors can proliferate *in situ* and undergo further maturation, it was hypothesised that anti-IL-33 may reduce the proliferation of the early stages of eosinophils in the lung. IL-33 participates in the proliferation of eosinophil progenitors both *in vivo* and *in vitro*, partly by cooperation with CCL24. Secondly, it has recently been shown that IL-33 protects from apoptosis

in a cellular model of heart ischemia. We found that IL-33 participates in eosinophil survival, as it can protect both progenitors, as well as late immature eosinophils, from oxidative stress induced apoptosis. Furthermore, another mechanism of decreased eosinophils is the migration. We found that IL-33 is necessary for the migration of eosinophil progenitors, as well as early and intermediate immature eosinophils, in response to CCL11 and CCL24. Both have been found to be expressed in the lung after allergen exposure with a predominance of CCL24.

ST2 expression on human eosinophils and Th2 cells (III)

All the results described above reveal that IL-33 can regulate lung *in situ* eosinophilopoiesis by several mechanisms; proliferation, survival and migration. In addition, the expression of ST2 differed with the maturation status of eosinophils. In order to confirm the observations above in humans, we chose to take fresh blood from human subjects with physician diagnosed asthma participating in two different ongoing studies, originating initially from the WSAS [108].

The expression of ST2 on human eosinophils was evaluated in a small group of six subjects with stable asthma out of pollen season (Table 1, paper III). The expression of ST2 was assessed on three different cell populations representing the different stages of maturation in accordance with our mice models (Table 4, paper III); human eosinophil progenitors $CD45^{+}CD34^{+}IL\text{-}5R\alpha^{+}CCR3^{+}SSC^{low}$, human immature eosinophils $CD45^{+}IL\text{-}5R\alpha^{+}CCR3^{+}SSC^{high}$ and human mature eosinophils $CD45^{+}IL\text{-}5R\alpha^{-}CCR3^{+}SSC^{high}$ by FACS. We found that the expression of ST2 followed the same pattern observed in blood eosinophils in humans.

Furthermore, in a second study of humans, the expression of ST2 on Th2 cells of fresh blood from four different subsets of patients with stable asthma during pollen season was evaluated. Human blood Th2 cells, *i.e.* $CD4^{+}GATA\text{-}3^{+}$ show the same pattern of ST2 expression.

Expression of the major trafficking related molecules in circulating eosinophil progenitors and mature eosinophils in asthma patients (IV)

The above finding showed that human eosinophil progenitors express ST2 through which IL-33 plays a role in their migration in the lung. This led to the next study to evaluate the capacity of circulating human eosinophil progenitors to migrate in patients with asthma. We hypothesised that patients with asthma have more circulating eosinophil progenitors, which express the needed

trafficking related molecules, i.e. selectin, integrin and chemokine receptors and ligands, thereby enabling them to migrate to the lung during allergic airway inflammation.

The selection of the markers for the different stages of eosinophils was based on their specific antigens, CD34 and IL-5Rα, as mentioned by Mori *et al.* [18]. We found that patients with stable asthma and high, but within normal range, blood eosinophils have more circulating early eosinophil progenitors ($CD45^+CD34^+IL\text{-}5R\alpha^+CD38^+CD45RA^-$), eosinophil progenitors ($CD45^+CD34^+IL\text{-}5R\alpha^+SSC^{low}$) and mature eosinophils ($CD45^+IL\text{-}5R\alpha^+SSC^{high}$) compared to healthy individuals (Figure 9). These patients also show an increased number of eosinophils in the airways, suggesting a continuous systemic eosinophilic inflammation from bone marrow up to airways.

The initial steps of eosinophil rolling and tethering are regulated by selectins [111]. CD162 (P-selectin glycoprotein ligand-1; PSGL-1) expressed on eosinophils, which interact with P-selectins and regulate eosinophil tethering to endothelium [111]. Both mature eosinophils and eosinophil progenitors express the major selectin, PSGL-1, giving them the capacity to start migration by rolling and tethering into the endothelium. Interestingly, in the next intermediate stage of migration, even more eosinophil progenitor expressed both selectin and integrin (CD162/CD29). The firm adhesion of eosinophils and transmigration across the vascular epithelium into tissue is regulated by eosinophil adhesion molecules, *i.e.* integrins [9]. The interaction of integrins with adhesion receptors facilitates eosinophil migration into various tissue compartments during inflammation. It has been shown that VLA-4 ($CD49^+CD29^+$) and Mac-1 ($CD18^+CD11b^+$) were involved in eosinophil mobilization from the circulation into the lungs after antigen challenge in mice [112]. Both eosinophil populations express the main integrin, VLA-4, while integrin Mac-1 was mostly expressed by mature eosinophils (Figure 10). However, there were differences in the level of expression of each component of integrins in cell level as expressed by their intensity. The eotaxins, which are chemokines, are important for the mobilization of eosinophils [9]. Thus, the expression of their receptor, CCR3, is another critical component of tissue infiltration. The CCR3 was expressed on all mature cells, while decreased in earlier eosinophil stages, proposing a correlation with maturation (Figure 11). However, more progenitor cells expressing CCR3 were activated compared to mature ones, by analyzing CD69 and CD25, proposing a special role for them (Figure 12). Finally, no difference was observed in their survival potency by measuring CD95 and active caspase-3.

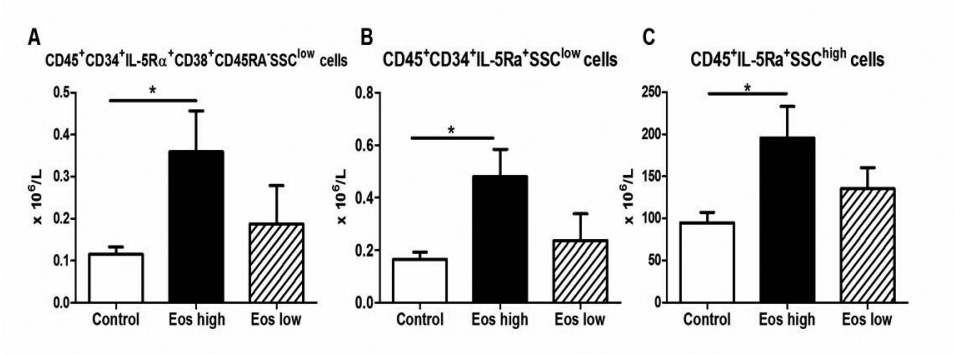

Figure 9. Blood early eosinophil progenitors, eosinophil progenitors and mature eosinophils in patients with asthma and healthy individuals. The number of circulating A) early eosinophil progenitors ($CD45^+CD34^+IL\text{-}5R\alpha^+CD38^+CD45RA^-SSC^{low}$), B) eosinophil progenitors ($CD45^+CD34^+IL\text{-}5R\alpha^+SSC^{low}$) and C) mature eosinophils ($CD45^+IL\text{-}5R\alpha^+SSC^{high}$) of healthy individuals (white column) and of asthma patients with high blood eosinophils (black column) and low blood eosinophils (hashed column). Data is shown as mean ± SEM, $n_{Control}$=5 healthy individuals, $n_{Eos\ high}$=7 asthma patients, $n_{Eos\ low}$=6 asthma patients, * p<0.05.

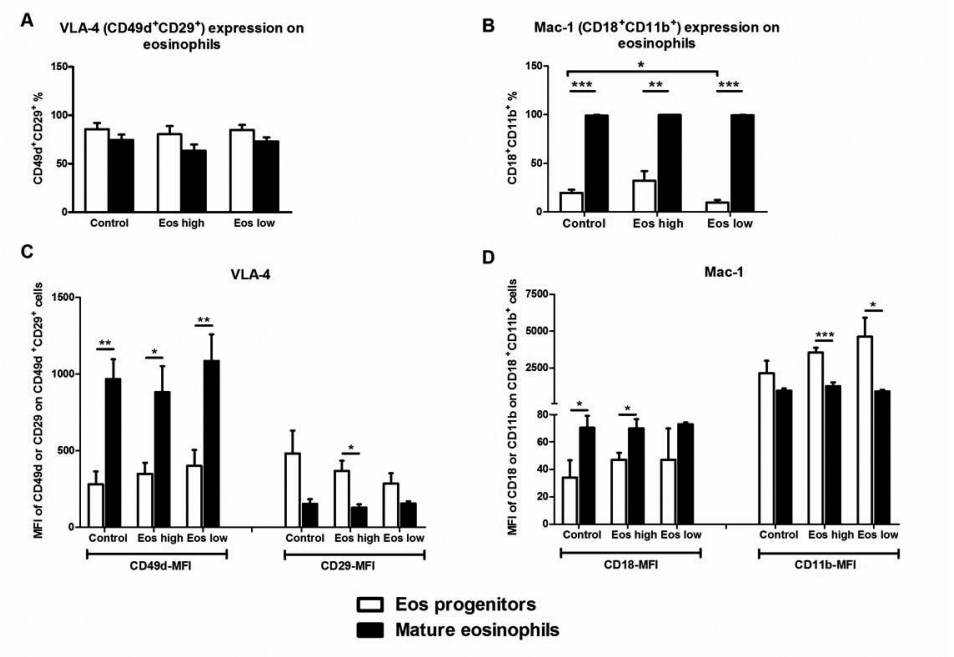

Figure 10. The expression of VLA-4 ($CD49d^+CD29^+$) and Mac-1 ($CD18^+CD11b^+$) on circulating eosinophil progenitors and mature eosinophils in the blood of patients with asthma and healthy individuals.

Frequency of VLA-4 ($CD49d^+CD29^+$) (A) and Mac-1 ($CD18^+CD11b^+$) (B) on circulating eosinophil progenitors (white column) and mature eosinophils (black column) of healthy individuals, asthma patients with high blood eosinophils and low blood eosinophils by flow cytometry.
Intensity of expression as measured by MFI (Mean Fluorescence Intensity) of CD49d and CD29 (C), as well as CD18 and CD11b (D) on circulating eosinophil progenitors (white column) and mature eosinophils (black column) from healthy individuals and from asthma patients with high blood eosinophils and low blood eosinophils by flow cytometry. Data is shown as mean ± SEM, $n_{Control}$=5 healthy individuals, $n_{Eos\ high}$=7 asthma patients, $n_{Eos\ low}$=6 asthma patients, * p<0.05, ** p<0.01, *** p<0.001.

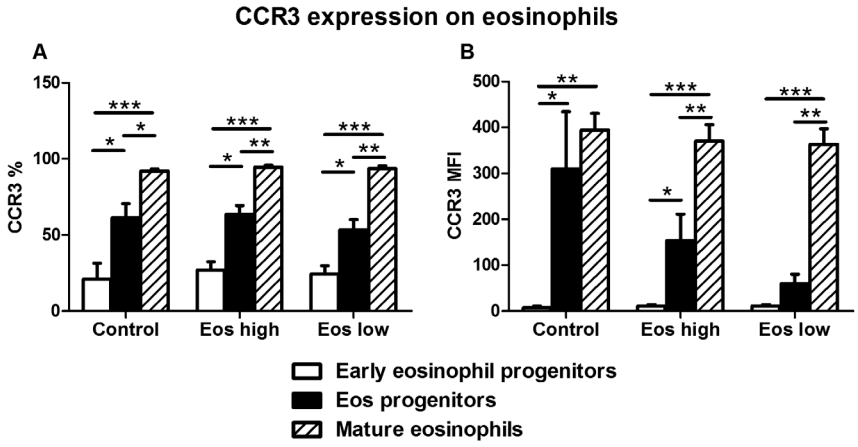

Figure 11. The expression of chemokine receptor CCR3 on circulating early eosinophil progenitors, eosinophil progenitors and mature eosinophils in blood in patients with asthma and healthy individuals. Frequency of CCR3 (A) and the intensity of expression (MFI) of CCR3 (B) on circulating early eosinophil progenitors (white column), eosinophil progenitors (black column) and mature eosinophils (hash column) of healthy individuals and of asthma patients with high or low blood eosinophils by flow cytometry. Data is shown as mean ± SEM, $n_{Control}$=5 healthy individuals, $n_{Eos\ high}$=7 asthmatic patients, $n_{Eos\ low}$=6 asthmatic patients, * p<0.05, ** p<0.01, *** p<0.001.

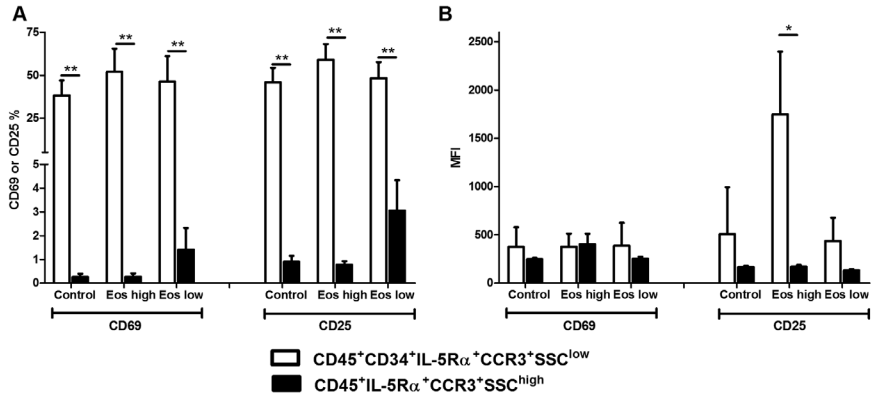

Figure 12. The expression of activation marker (CD69, CD25) on circulating CCR3$^+$ eosinophil progenitors (CD45$^+$CD34$^+$IL-5Rα$^+$CCR3$^+$SSC^{low}) and CCR3$^+$ mature eosinophils (CD45$^+$IL-5Rα$^+$CCR3$^+$SSC^{high}) in blood in patients with asthma and healthy individuals. Frequency of CD69 and CD25 (A) and the intensity of expression (MFI) of CD69 and CD25 (B) on circulating CCR3$^+$ eosinophil progenitors (white column) and CCR3$^+$ mature eosinophils (black column) of healthy individuals and asthma patients with high or low blood eosinophils by flow cytometry. Data is shown as mean ± SEM, $n_{Control}$=5 healthy individuals, $n_{Eos\ high}$=7 asthmatic patients, $n_{Eos\ low}$=6 asthmatic patients, * p<0.05, ** p<0.01.

GENERAL DISCUSSION

Increasing evidence from animal and human studies strongly suggests that allergic asthma is characterised by lung eosinophilia and regulated mainly by Th2 cells. Anti-IL-5 therapeutics was successful to downregulate eosinophils in circulation and the airways in asthma patients. However, the clinical symptoms, such as AHR in patients with asthma were not affected. The human studies suggest that eosinophilic inflammation persists in lung tissue may be sufficient to induce the asthmatic symptoms. This present thesis mainly focused on lung eosinophilic inflammation during allergic airway inflammation.

This thesis showed that systemic eosinophilic inflammation was pronouncedly induced after allergen exposure in mice model. Between the mice model of allergic airway inflammation with BALB/c and C57BL/6, there was no difference in eosinophilic proliferation and the corresponding Th1/Th2 balance in the lung, while the distribution of the newly produced eosinophils differs. Except eosinophils, all detected T helper cells; Th1, Th2, Th17 and Treg cells, were increased, with a greatest expansion of Th2 cells after allergen exposure in the lung of C57BL/6 mice. All of these T helper cells have the ability to proliferate both *de novo* and *in situ* in the lung tissue during allergen exposure, with the higher ability of activated ones. Different distribution of T helper cells was found in peribronchial, perivascular and alveolar tissue in the lung, suggesting the different local inflammatory milieu. In that case, this thesis confirmed that the local inflammatory milieu differed in the evaluated cytokines, chemokines and receptor genes, and may contribute to the expansion and distribution of T helper cells. Furthermore, a novel Th2 cytokine, IL-33, is suggesting to be involved in allergic airway inflammation. This thesis showed that all eosinophil progenitors expressed the receptor of IL-33, which decreased on immature eosinophils, while not present on mature eosinophils. Local blockage of IL-33 during allergen exposure impaired early stages of eosinophils, but not mature eosinophils and Th2 cells. IL-33 has the capacity to regulate lung *in situ* eosinophilopoiesis by affecting their *in situ* proliferation, survival and migration. Moreover, human circulating eosinophils and Th2 cells also expressed the receptor of IL-33. Since IL-33 regulated the eosinophilopoiesis by affecting their migration, we further evaluated the trafficking capacity of eosinophils and their progenitors. Patients with stable asthma and blood eosinophils in the upper level of the normal range had increased sputum eosinophils and circulating eosinophil progenitors compared to the healthy controls. These eosinophils and eosinophil progenitors expressed all major trafficking related molecules, such as PSGL-1, VLA-4 and Mac-1, and CCR3.

In situ proliferation - eosinophils and effector T cell subsets

Eosinophils

Eosinophils initially develop within the bone marrow from $CD34^+$ progenitor cells with recent studies showing that during allergic inflammation, $CD34^+$ eosinophil progenitor cells, but not mature eosinophils, migrate to the sites of allergic inflammation, i.e. the lung, where they undergo further *in situ* maturation and differentiation, i.e. eosinophilopoiesis [32, 35, 113]. In the mouse model, the proliferation rate was not different between two mouse strains and both strains had significantly increased newly produced eosinophils (*i.e.* MBP^+BrdU^+ or $CD45^+CCR3^+BrdU^+$ cells) located locally in the lungs after allergen exposure. The explanation for this could be that the eosinophils proliferated in the bone marrow and then migrated to the lung tissue, or immature eosinophils migrated to the lung tissue and then proliferated under the influence of local cytokines and chemokines during allergic airway inflammation. Rådinger *et al.* [35] found that $CD34^+CCR3^+$ eosinophil-committed progenitors have the ability to undergo *in situ* proliferation during allergic airway inflammation.

The eosinophils *in situ* proliferation by detailing the role of IL-33 was further explored. IL-33 is a relatively newly described cytokine member of the IL-1 family that has gained a lot of attention during the recent years. The receptor of IL-33 has been found to be expressed on both eosinophils and Th2 cells, arguing a possible link between the two cell types. The interaction of IL-33 with its receptor ST2 promotes a variety of actions in the cells. A body of evidence emphasises the central role that IL-33 plays in the development of allergic inflammation. This thesis confirmed and expanded the original eosinophilopoiesis data by using more markers to identify the eosinophil progenitors as $CD45^+CD34^+IL\text{-}5R\alpha^+CCR3^+Sca\text{-}1^+SSC^{low}$. This population belongs to the CD45 dim part, as mentioned by the ISHAGE guidelines and other studies [46, 110, 114]. By analysing the above markers on the mature eosinophils, *i.e.* $CD45^+CCR3^+$ granulocytes, it was able to define four different populations in mice depending upon the expression of early hematopoietic markers Sca-1 and CD34, as well as the expression of IL-5Rα and granularity (SSC). So based on the maturation stages, $CD45^+CCR3^+IL\text{-}5R\alpha^+Sca\text{-}1^+CD34^+SSC^{high}$ was proposed as early immature eosinophils, with the loss of Sca-1 expression identifying intermediate immature eosinophils and the loss of both Sca-1 and CD34 identifying the late immature eosinophils. The eventual loss of the expression of IL-5Rα defines mature eosinophils. The expression of ST2 has a decreased pattern during the maturation, suggesting that IL-33 was involved in the maturation of eosinophils. The cooperative effect of both CCL24 and IL-33 argues for a special role of IL-33 together with eotaxin during the early stages of eosinophil differentiation and maturation. The outcomes of this

thesis is in accordance with previous reports which showed that BM $CD117^+$ cells generate eosinophils under the influence of IL-33 [94], as well with a recent study describing a method for the large scale expansion of eosinophils by IL-33 [115]. Interestingly, despite lung Th2 cells expressing ST2, the blockage of IL-33 had no effect on their number. In an earlier study, IL-33 was blocked systematically [95] with a reduction of lymphocytes observed in BALF. This discrepancy could be due to the different routes of administration. It has also been described that IL-33 blockage decreases Th2-like cytokines, such as IL-5 and IL-13. However, it cannot exclude in our model that the Th2 cells showed decreased function after IL-33 blockage. In that case, there would have an additive effect, as we have shown the direct effect of IL-33 in eosinophilopoiesis.

In addition, our finding shows that circulating human eosinophils have a similar pattern to mice in the expression of ST2 during the maturation of eosinophils and Th2 cells in patients with stable asthma, arguing the similar role of IL-33 in asthmatic patients. The identification of human eosinophil progenitors, immature and mature eosinophils was based on the markers we used in our mouse study based mainly on the expression of CCR3.

T helper cell subsets

With the exception of eosinophils, T helper cells of different subsets, such as Th1, Th2, Th17 and Treg cells, play a critical role in orchestrating allergic inflammation. In addition, recent studies have shown that these T helper cells can affect each other in a sophisticated network during inflammation. Although the role of T helper cells has been extensively studied these years, the balance of them during local lung eosinophilopoiesis is still not clear. During the past two decades, the Th1/Th2 paradigm of a balance required to regulate the development of allergy has prevailed, and these two cell types have been implicated in the pathogenesis of different inflammatory diseases, including asthma. However, recent evidence suggests that the dichotomous view of T cell differentiation must be broadened to several other T cells, including Th17 [116] and Treg cells [117-119]**.** We found that all T effector cells were increased after allergen exposure, however, predominantly Th2 cells. Interestingly, both the $CD4^+CD25^+$ and $CD4^+CD25^-$ populations expressed the specific transcription factors; T-bet, GATA-3, RORγt and Foxp3, for Th1, Th2, Th17 and Treg cells respectively. The relative expression is quite small in the $CD4^+CD25^-$ population, as a majority of cells are negative for any of the transcription factors in control mice. Additional T helper subsets are being suggested, including Th9 [120], Tr1 [59], Th3 [121] and Th22 cells [122], however these new subsets were not investigated in the current study. Some of T cell subsets may indeed contribute to the fraction of $CD4^+CD25^+$ and $CD4^+CD25^-$ that do not express any of the currently studied transcription factors. Clearly, some of these cells may also be involved in the

inflammatory process in this model of allergic airway inflammation and warrant investigation in future studies. All of the effector T cell and Treg cell subsets increase their proliferation *de novo* and *in situ* in the lung during allergen exposure, to a greater extent in the $CD4^+CD25^+$ population compared to the $CD4^+CD25^-$ population. The greatest proliferative ability was also found in Th2 cells. Thus, the lung itself is a microenvironment in which all of the studied T-lymphocytes can proliferate. Several mechanisms for the suppression of T effector cells have been proposed. Recent observations have suggested that there could be interaction between different T cell subsets or with other inflammatory cells, with or without direct cell-to-cell contact [123-126].

Local inflammatory microenvironments in the lung during allergic airway inflammation

A different distribution of newly produced eosinophils was found between two mouse strains after allergen exposure. Our data also shows that allergen exposure results in an increased accumulation and distribution of T-bet, GATA-3, RORγt and Foxp3 in the lung tissue of C57BL/6 mice. These differences argue for the different homing process of these cells to the different microenvironment or different mediators, which is a unique contribution of the current study to the literature.

The lung tissue represents a highly complicated and sophisticated network of many different cells that can react differently to a variety of stimuli, resulting in unique local inflammatory milieus of cytokines, chemokines and mediators in different microenvironments. The inflammation itself can affect different subsets of Th cells and eosinophils that accumulate in different tissue. For example, it has been shown that Nitric Oxide (NO) production from human airway epithelium can affect the Th1/Th2 balance [127]. Another possible mechanism could be that the different T-helper cells that have accumulated in the lungs further drive the inflammation by releasing further cytokines. Lordan *et al*. [128] have shown that the addition of Th2 cytokines, in an already established allergic environment, have the potential to sustain further airway inflammation.

A large variation in the local expression of CCL24, which represents a major chemoattractant for eosinophils, can possibly explain the results of the high accumulation of eosinophils in perivascular tissue [129]. This chemokine may also have cytokine properties, as we have shown that it can play a role in eosinophilopoiesis locally in the lung [35]. Another example is the expression of osteopontin (Spp1), which recently has been proposed to enhance Th2 responses and is related to human asthma severity [130]. Indeed we have described extensive chemokine networks expressed in all investigated tissues, but with uneven expression in the different compartments. The pattern of their expression can, at

least, partly explain our immunohistochemistry results. Thus, Th1 reported chemokines, such as CCL17 and CCL2 [47, 62], were primarily expressed in alveolar tissues, where T-bet cells are primarily located. CCL8 was quite recently reported by Islam *et al*. [82] as a potent chemoattractant for GATA-3, IL-5^+ Th2 cells in skin allergy, which is in line with our finding of the expression of this chemokine in, primarily, the perivascular milieu, where GATA-3 cells were accumulated. Furthermore, the chemokine CCL19 may explain the microenvironmental localisation of Treg, while the chemokine CCL20 may not explain Th17 cells distribution [62, 83, 84]. By Ingenuity Pathways Analysis, we have documented extensive interactions between the chemokines and cytokines, suggesting that a small difference in expression of one may affect the action of the other, and can thus influence the whole inflammatory network in the microenvironment. This becomes clearer when some chemokines, such as CCL4, can influence the traffick of more than one of the studied Th cells. The interaction between the different chemokines is complicated, since we observed a downregulation of platelet factor 4 (PF4), which is related to Th1 responses [131], even though other Th1 chemokines were increased in the same environment.

Our finding confirmed that there are different local inflammatory milieus in the lung tissue compartments during allergic airway inflammation. This includes both immune cells, such as Th cells and eosinophils, as well as local inflammatory mediators, *i.e.* cytokines and chemokines. The chemokine genes expression are mainly contribute to different T helper cells and eosinophil distribution and the pattern of their expression can at least partly explain our immunohistochemistry results.

Eosinophil migration

In vitro mouse eosinophil migration

In the mouse study, this thesis showed that IL-33 participates in the regulation of eosinophil progenitors and immature eosinophil migration by affecting their response to CCL11 or CCL24. It has also been reported that both CCL11 and CCL24 are involved in recruiting eosinophils to the lung tissue [48]. We found CCL11 and CCL24 attracted the late immature eosinophils, but not the eosinophils in earlier stages. CCL24 and IL-33 together were more effective at migration of eosinophil progenitors, while CCL11 and IL-33 were more effective in intermediate immature eosinophils. Suzukawa e*t al.* [86] also reported that IL-33 is not a direct chemoattractant for eosinophils, in accordance with our findings. However, they report the induction of a sharp change in the response in human eosinophils and dose-dependently enhanced chemokinetic activity, similar to IL-5.

Migration of eosinophil progenitor in allergic asthma patients

As IL-33 regulates eosinophil progenitors in mice, mainly through the migration, what is the migration capacity of human eosinophil progenitors in asthma? Especially we found that human eosinophil progenitors also express IL-33R, ST2. It has been shown earlier that allergen exposure increases $CD34^{+}IL\text{-}5Ra^{+}$ cells in both bone marrow, as well in airways, indicating that eosinophil progenitors can migrate to the airways and may differentiate *in situ* [38, 132], proposing an ongoing recruitment of pro-inflammatory cells to sites of allergic inflammation where those cells can undergo an "*in situ*" maturation. However, it is still unclear if this is the case in stable asthma.

In this study, we identified the human eosinophil progenitors and mature eosinophils based on the research from Mori *et al.* [18]. Our data expanded the above observations by showing that even in participants with stable asthma, and with blood eosinophils within normal values, there is an increased number of circulating eosinophil progenitors, proposing continuous bone marrow activation. Importantly, we have showed that both mature eosinophils and progenitor eosinophils express all the major trafficking related molecules, although in a different manner. This might be important and should be considered in the planning of new therapeutic approaches, such as those that block eosinophil specific adhesion molecules [97]. Expression of PSGL-1 suggests that eosinophil progenitors have a similar capacity of mature eosinophils to transit across endothelial and epithelial barriers. Indeed, mice with P-selectin gene-deleted showed reduced pulmonary eosinophilia, proposing a valuable therapeutic target [133].

Some studies have investigated the role of VLA-4 and Mac-1 during the allergic inflammation. However, in the studies above, outcomes include eosinophils with no distinction between mature and progenitor. We found that the same percentage of both mature eosinophils and eosinophil progenitors express the VLA-4. As VLA-4 is expressed constitutively, our data proposes that it is expressed early in their differentiation. However, each component of VLA-4, *i.e.* CD49d or CD29, showed opposite patterns of expression per cell as shown by intensity measurements. Those differences in expression might play a role when new therapeutic approaches with blocking antibodies are planned [134-137]. Almost all mature eosinophils express Mac-1, in comparison to the 30-40% of eosinophil progenitors expressing Mac-1. This difference can reflect a different activation stage or a response to a mature eosinophil specific stimulant.

Among the chemokines implicated in the leukocyte recruitment, eotaxins acts as an eosinophil-selective chemoattractant. CCR3 antagonists selectively inhibit eosinophil influx into the lung elicited by chemokine or allergenic challenge in mice [138]. Our finding shows that the expression is low on early eosinophil progenitors, increases further on progenitors and increases even further on

mature eosinophils. Our findings propose a link between CCR3 expression and maturation status. Shinagawa *et al.* [139] reported that eosinophils survive longer under the influence of CCR3-reactive chemokines, arguing that survival capacity of CCR3. They also suggested those eosinophils infiltrated into the tissue may survive even longer if they encounter survival factors at local inflammatory sites. If this is the case, the increased expression of CCR3 during the maturation of eosinophils revealed that the survival period of mature eosinophils is longer than eosinophil progenitors, and even longer than early eosinophil progenitors. Of course, it must be kept in mind that eosinophil progenitors mature *in situ* in the lung. However, when evaluating apoptosis in progenitor eosinophils expressing CCR3 and in mature eosinophils expressing CCR3, no difference was found in either active caspase-3 or CD95 expression, arguing that the main factor is probably, as described by Shinagawa *et al.* [139], the local inflammatory milieu. $CCR3^+$ progenitor and mature eosinophils revealed a higher activation status compared to the mature eosinophils, with more progenitor cells expressing CD69 and CD25. Eosinophils expressed the activation markers CD69, CD25, which correlated with the severity of disease and elevated serum IL-5 in active Churg-Strauss syndrome (CSS) [140], suggesting that it can correlate with disease activity. Importantly, a recent study showed that the early activation receptor CD69, is an intrinsic modulator of immune allergic processes through negative regulation of allergen induced T cell effector response. It mainly regulates the Th2 and Th17 cytokine production in the BALF and lung in allergic airway inflammation [141], proposing a regulatory role for CD69. In that case, eosinophil progenitors that express CCR3 and CD69 may represent a specific regulatory population that try to downregulate the allergic inflammation.

Eosinophil apoptosis

Moreover, even though IL-33 does not directly effect the spontaneous apoptosis of eosinophils, it protects eosinophil progenitors and late immature eosinophils from the oxidative stress induced apoptosis. Furthermore, it is known that an increased oxidative stress in the lung during allergic airway inflammation represents an important component of inflammation [142]. Thus, our data suggests that during allergen exposure, overproduction of IL-33 protects eosinophils from the induced oxidative stress, further promoting the eosinophilic inflammation. However, the complexity of the system must be considered, as it has been shown that IL-33 can be efficiently cleaved by caspase-3 and -7 at physiological concentrations within apoptotic cells, suggesting a regulatory mechanism for the pro-inflammatory activity of IL-33 [143].

In summary, this thesis, using both mice and human material, shows that eosinophils and T helper cells are distributed differently in lung tissue, probably due to a distinct local inflammatory milieu. Even if all T helper cells participate, Th2 cells dominate. A novel cytokine, IL-33, is released in the lung during allergic inflammation, with Th2 cells and eosinophils undergoing maturation expressing its receptor, ST2. IL-33 regulates lung *in situ* eosinophilopoiesis by affecting eosinophil progenitor *in situ* proliferation, apoptosis and migration. Finally, this thesis confirmed that patients with asthma showed an increased number of circulating eosinophils, even if they have a stable asthma with eosinophilia within normal ranges, where progenitor and mature eosinophils also express all the required molecules that allow them to migrate to the lung during airway inflammation.

CONCLUSIONS

There is a difference in the distribution of eosinophils in the lung between the C57BL/6 and BALB/c mice representing *in vivo* models of Th1 and Th2 response, but no difference in eosinophil production or Th1/Th2 balance.

Allergen exposure affects all T effector cells in lung, with a predominance of Th2 cells, but with different local cell distribution, probably due to a distinct local inflammatory milieu.

Lung eosinophil progenitors express ST2 during different stages of maturation. IL-33 has the capacity to regulate lung *in situ* eosinophilopoiesis by affecting eosinophil progenitor cells *in situ* proliferation, survival and migration.

Patients with stable asthma and high eosinophilia, but within normal ranges, showed a systematic eosinophilic inflammation with elevated sputum eosinophils and increased blood eosinophil progenitors, expressing all major trafficking related molecules (PSGL-1, VLA-4 and Mac-1, and CCR3). Eosinophil progenitors prompted to migrate to the airways showed an increased activation status compared to mature eosinophils.

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