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Mast cells and their progenitors in respiratory diseases

Understanding their connection to lung function and airway inflammation

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Abstract

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Mast cells are rare immune cells involved in allergic diseases, including asthma. These cells are derived from mast cell progenitors (MCps) that migrate to the peripheral tissues via the blood in response to allergic or non-allergic stimuli. The main purpose of this thesis was to investigate the role of mast cells and MCps in the lung function decline observed in mouse models of airway inflammation. We also investigated the MCp frequency during natural allergen exposure using patient samples.

Our aim in paper I was to investigate the effect of age and weight on lung function parameters in naïve mice using a pulmonary function test (PFT). We showed that age and weight positively correlated with lung function and successfully used the PFT to monitor asthma outcomes and distinguish between treated and untreated experimental asthma.

In paper II, we investigated the specificity of a basophil-deficient mouse model that relies on the deletion of the mast cell protease 8 (mMCP-8), a classical basophil marker. We found that lung mast cells expressed mMCP-8, and deleting this protease reduced lung mast cells in mice with allergic airway inflammation.

Mast cells express ST2 and thus can be activated by interleukin-33 (IL-33). Hence, in paper III, we used $Cpa3^{cre/+}$ mast cell-deficient mice to investigate the role of mast cells in airway inflammation induced by intranasal IL-33 administration. We identified a new mechanism in which mast cells participate in T-cells mobilization into the alveolar space via the CXCL1/CXCR2 axis.

We have previously described increased circulating MCps in subjects with reduced lung function. However, if and how MCps change upon allergen exposure is unknown. Therefore, in paper IV, we investigated the frequency of blood MCps in birch pollen-sensitized asthma patients in and out of the birch pollen season. We demonstrated that in allergic asthma patients, circulating MCps were increased during natural pollen exposure and were associated with more asthma symptoms and less asthma control.

This thesis involves both basic and translational research and provides new insights into the role of mast cells and their progenitors in type 2 inflammation.

Keywords: Mast cells, mast cell progenitors, pulmonary function test, allergic airway inflammation, experimental asthma model

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List of Papers

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

I. **Alvarado-Vazquez PA**, Mendez-Enriquez E, Hallgren J. Use of spirometry-like measurements to monitor house dust mite-induced experimental asthma in mice. Allergy. 2021 Jul;76(7): 2204-2207.

II. **Alvarado-Vazquez PA***, Cardenas EI*, Das A, Hallgren J. Depletion of Mcpt8-expressing cells reduces lung mast cells in mice with experimental asthma. Allergy. 2022 Nov 27 * equal contribution.

III. **Alvarado-Vazquez PA**, Mendez-Enriquez E, Pähn L, and Hallgren J. Mast cell-derived CXCL1 promotes bronchoalveolar $CXCR2^{+}$ T-cells in mice with IL-33-induced airway inflammation. Manuscript.

IV. **Alvarado-Vazquez PA,** Mendez-Enriquez E, Salomonsson M, Waern I, Janson C, Wernersson S, Malinovschi A, and Hallgren J. Circulating mast cell progenitors increase in frequency during natural birch pollen exposure in allergic asthma patients. Manuscript.

Contents

Abbreviations

5-HT	Serotonin
ACQ	Asthma control questionnaire
ACT	Asthma control test
AHR	Airway hyperresponsiveness
ASM	Airway smooth muscle
BAL	Bronchoalveolar lavage
BMCPs	Basophil and mast cell progenitors
CPA3	Carboxypeptidase A3
CTMC	Connective tissue type mast cells
DT	Diphtheria toxin
DTA	Diphtheria toxin A gene
DTR	Diphtheria toxin receptor
FeNO	Fractional Exhaled Nitric Oxide
FEV_1	Forced expiratory volume in one second
FOT	Forced oscillatory technique
FVC	Forced vital capacity
HDM	House dust mite
IL	Interleukin
Lin	Lineage
MCps	Mast cell progenitors
mMCP-8	Mouse mast cell protease-8
OVA	Ovalbumin
PEF	Peak of expiratory flow
PFT	Pulmonary function test
SCF	Stem cell factor
TV	Tidal volume

Introduction

According to the world health organization, asthma is a major noncommunicable disease, affecting around 300 million people worldwide. Characteristics of asthma include airway inflammation and obstruction, as well as hyperresponsiveness to a variety of stimuli. The symptoms that patients with asthma experience, such as chest tightness and difficulty breathing, have detrimental effects on their quality of life. Therefore, it is essential to monitor lung function and asthma control in these patients, which can be accomplished using spirometry or by conducting surveys, respectively.

Asthma pathophysiology is complex and involves the participation of various immune cells, including mast cells. Mast cells reside in all vascularized tissues of the body and upon IgE- or non-IgE-mediated activation release inflammatory mediators that are instrumental in asthma symptom development. Mast cells are derived from mast cell progenitors (MCps) that leave the bone marrow, enter the circulation, and reach peripheral tissues to complete their maturation. In patients with asthma, mast cells accumulate in the lung and are associated with disease severity. However, the role of mast cells and their progenitors in asthma and other airway diseases is poorly understood.

This thesis focuses on the participation of mast cells and their progenitors in lung function decline as well as immune cell infiltration to the airways induced in mouse models of type 2 inflammation. Moreover, the association of blood circulating MCps with asthma symptoms was studied in a cohort of allergic asthmatic patients. The validation of lung function parameters and the specificity of mast cell protease 8 (mMCP-8)-driven cell deletion in mice with experimental asthma induced by house dust mite (HDM) were investigated in **paper I** and **II**, respectively. Furthermore, the participation of mast cells in a mouse model of airway inflammation induced by interleukin (IL)-33 was studied in **paper III**. The possible association between MCps in circulation and asthma symptoms was investigated in a cohort of allergic asthma patients during allergen exposure in **paper IV**.

Background

Mast cells

In 1879, Paul Ehrlich used aniline dyes to stain mast cells in connective tissues and coined the term "mast cells". Since then, the study of mast cells has extended across multiple disciplines[1]. Mast cells reside in peripheral tissues, they produce and release mediators such as histamine, serotonin (5-HT), proteases, leukotrienes, cytokines, and chemokines that have diverse biological activity. C-kit, the stem cell factor (SCF) receptor, and Fc epsilon receptor I (FcεRI) are classical mast cell markers. However, mast cells can respond to multiple stimuli via a plethora of receptors that recognize cytokines, neuropeptides, complement proteins, among others. Mast cells are active players during host defense against helminths and bacteria and contribute to snake and bee venom detoxification[2-4]. Still, they are mostly known for their detrimental role in asthma and allergies, where mast cell activation and accumulation in peripheral tissues are associated with disease severity[5].

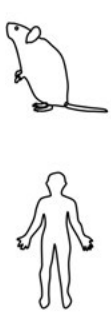
Mast cells are commonly classified into two groups: connective tissue mast cells (CTMC) and mucosal mast cells (MMCs) in mice, which in humans correspond to mast cells expressing tryptase and chymase (MC_{TC}) and mast cells that express tryptase but not chymase (MC_T), respectively (Figure 1). These two phenotypes are defined based on granule content, origin, and localization within the tissues. Nonetheless, transcriptome analysis suggest complex mast cell heterogenicity across tissues[6]. CTMCs are situated close to venules or nerve endings in the skin, peritoneal cavity, tongue, and other connective tissues. CTMCs store the β-chymase mMCP-4 (mouse mast cell protease-4), mMCP-5, an α-chymase with elastase-like activity, the tryptases mMCP-6 and -7, carboxypeptidase A3 (CPA3), and contain heparin[7-9]. In contrast, mucosal mast cells (MMCs) store the β-chymases mMCP-1 and -2 and contain chondroitin sulfate. MMCs also express the tryptases mMCP-6 and -7, and CPA3[7-9]. In humans, mast cells store CPA3, one alpha chymase, alpha and beta tryptases, and express a transmembrane gamma tryptase[10]. Mast cells expressing tryptase, chymase, and CPA3 are denominated MC_{TC} and are mainly found in the skin. In mucosal spaces, mast cells mainly express tryptase alone, and they are classified as MC_T[11] (Figure 1).

Mast cell origin

In contrast with granulocytes, such as basophils, which circulate in the bloodstream in their mature form, mast cells circulate as immature cells that infiltrate peripheral tissues and mature according to their environmental cues.

In the 70s, Kitamura et al. showed that bone marrow cells could restore mast cells in the gastrointestinal tract but only partially in the skin[12]. It was later reported that yolk sac and fetal liver cells could form mast cell colonies when injected into the skin[13]. Recent studies using fate mapping systems, which track transcription factors at different embryonic stages, showed that mast cells indeed arise during fetal development from erythromyeloid progenitors found in the yolk sac[14,15]. In later hematopoiesis waves, mast cells get supplemented by progenitor cells found in the fetal liver or hematopoietic stem cells from the aorta-gonad-mesonephros endothelium[14-16].

In adult mice, mast cells in mucosal tissues are replenished from hematopoietic stem cells found in the bone marrow, while in connective tissues, mast cells can originate in situ[17,18]. Figure 1 summarizes the characteristics of human and mouse mast cells.



CTMCs		MMCs	
Chymases:	mMCP-4	Chymases:	mMCP-1, 2
Tryptases:	mMCP-6, 7	Tryptases:	mMCP-6, 7
Elastase:	mMCP-5	Carboxypeptidase:	CPA3
Carboxypeptidase:	CPA3	Proteoglycan :	Chondroitin sulfate
Proteoglycan :	Heparin		

MC_{TC}		MC_T	
Chymase:	CMA1	Chymase:	-
Tryptases:	α, β, γ	Tryptases:	α, β, γ
Carboxypeptidase:	CPA3	Carboxypeptidase:	-
Proteoglycan :	Chondroitin sulfate and Heparin	Proteoglycan :	Chondroitin sulfate and Heparin

Figure 1. Differences between mouse and human mast cells. In mice mast cells are denominated CTMC in connective tissues and are classified as MMCs in mucosal tissues. In humans, mast cells expressing tryptase and chymase are called MC_{TC}, while mast cells that express tryptase but not chymase are denominated MC_T.

Mast cell progenitors in mice

MCps are rare hypo-granular cells first described in fetal mouse blood as c-kit$^+$ Thy-1$^+$ cells[19]. In the presence of IL-3 and SCF, these MCps developed into functional mast cells. Moreover, intravenous injection of c-kit$^+$ Thy-1$^+$ cells could reconstitute mast cell-deficient mice[19]. Others, subsequently reported that bone marrow MCps expressing transcripts for mast cell-related proteases, gave rise to mast cells in vitro, or were able to reconstitute mast

cell-deficient mice[20-22]. A committed MCp population was later identified in adult mouse blood, in both BALB/c and C57BL/6 mice, and defined as lineage-negative c-kit$^+$ ST2$^+$ FcεRI$^+$ integrin β7$^+$ CD16/32$^+$ cells using flow cytometry[23]. However, C57BL/6 mice displayed a smaller proportion of FcεRI expressing MCps than the BALB/c strain[23]. In naïve BALB/c mice, committed MCps represented around 0.0045% of the mononuclear fraction, and when cultured in a myeloerythroid cytokine cocktail, gave rise to mast cells[23]. Similar populations could also be found in steady states in other peripheral tissues, i.e., the peritoneum and lung[24]. Indeed, in the lungs of naïve mice, MCps can be distinguished from mature mast cells by their high expression of integrin β7 and low side scatter. In contrast, mature mast cells express low integrin β7 levels and are more granulated and thus appear higher on the side scatter axis[24]. Integrins facilitate the MCp recruitment to the lungs during inflammatory conditions and modulate MCp homing to the periphery[25]. Interactions between α4-β1 or α4-β7 integrins with vascular cell adhesion molecule-1 (VCAM-1) mediate the entry of MCps into the lungs in a model of allergic airway inflammation[26]. This process is dependent on lung CXCR2 expression, which is necessary to increase VCAM-1 expression in the lung endothelium[27]. The lack of CD4$^+$ T-cells, CD11c$^+$ cells, stromal CCR2, and IL-9 also impairs the MCp recruitment into the lungs during allergic airway inflammation, although to different degrees[28-30].

During allergic airway inflammation, MCps are expanded and can be identified in the lungs as CD45$^+$ Lineage (Lin)$^-$ (CD3$^-$ CD4$^-$ CD8b$^-$ CD11b$^-$ CD19$^-$ B220$^-$ TER119$^-$ Gr1$^-$) c-kit$^+$ ST2$^+$ FcεRI$^+$ integrin β7$^+$ CD16/32$^+$ cells using flow cytometry (Figure 2)[31]. In contrast, mature mast cells exhibit low integrin β7 expression[24,31]. Additionally, a population of mast cells with intermediate levels of integrin β7 (induced mast cells) can be observed in the lungs of mice with allergic airway inflammation but not in naïve mice[31]. Thus, three mast cell populations can be found in the lungs of mice during allergic airway inflammation (Figure 2).

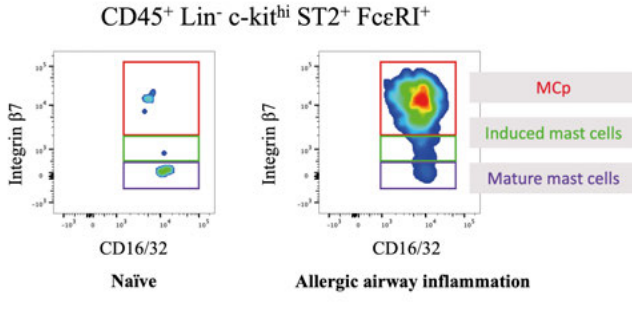


Figure 2. Lung mast cell populations in a naïve mouse and a mouse with allergic airway inflammation induced by house dust mite (HDM, see experimental set up for details).

Bipotent mast cell and basophil progenitors

Mast cells and basophils are immune cells with distinctive characteristics, but they both are active players during IgE-mediated responses (Table 1). Common progenitors that give rise to both mast cells and basophils (BMCPs) have been described in the spleen of C57BL/6 mice [22]. Single-cell studies have also revealed that BMCPs in the bone marrow of C57BL/6 mice can give rise to mast cells and basophils in culture[32]. Hamey et al. reported a single-cell map for the branching of BMCP and showed that these cells express Mcpt8, a basophil-related protease[33]. Additionally, Derakhshan et al. reported that an integrin $\beta7^{hi}$ mast cell population in the lungs expressed transcripts encoding for the Mcpt8 gene[34]. The Mcpt8 gene encodes the mouse mast cell protease-8 (mMCP-8), a basophil marker[35]. The protease mMCP-8 cleaves macrophage inflammatory protein-3 alpha and platelet-derived growth factor-B in vitro, but its function during inflammation is not clearly known[36]. Researchers have taken advantage of this protease to design basophil-deficient models and explore the role of basophils during sepsis or nematode infections in mice[37-39]. Although mature mast cells express negligible levels of mMCP-8, the expression of Mcpt8 transcripts in BMCPs could impact the generation of mature mast cell populations in Mcpt8-deficient mice.

Table 1. Differences between mast cells and basophils[40]

	Mast cells	**Basophils**
Localization	Tissue resident	Blood circulating
Maturation	Peripheral tissues	Bone marrow
Life span	Months	Days
Surface receptors	c-kit$^+$ FcεRI$^+$	c-kit$^-$ FcεRI$^+$

Human mast cell progenitors

The ontogeny of mast cells is poorly studied in humans, but progenitors can be found in the bone marrow[41-43] and fetal tissues[44,45]. These cells are mainly derived from the CD34$^+$ fraction that can also give rise to other lineages, i.e., basophils, eosinophils, or monocytes.

In 2016 Dahlin et al. described a committed MCp in human peripheral blood as CD4$^-$ CD8$^-$ CD19$^-$ CD14$^-$ CD34^{hi} CD117$^+$ FcεRI$^+$ using flow cytometry[46]. The blood MCps expressed classical mast cell genes encoding for tryptase (Tpsab1/Tpsb2), CPA3 (Cpa3), and serglycin (Srgn)[46]. Furthermore, MCps represented 0.005% of all the mononuclear cells, expressed integrin β7, and, when cultured in a myeloerythroid cytokine cocktail, gave rise to mast cells[46]. In a separate study, Dahlin et al. reported that human MCps are present in the

blood of patients treated with an anti-CD117 antibody and can survive without SCF/KIT signaling in vitro[47]. Dahlin et al. also reported that individuals with reduced lung function had an increased frequency of blood MCps [46]. Similarly, Salomonsson et al. showed that a higher frequency of circulating MCp correlated with lower lung function in a cohort of young individuals[48].

Activation of human peripheral blood MCps by IgE crosslinking induces expression of the activation markers CD63 and LAMP-1[49]. Furthermore, immature mast cells bound to IgE have been reported in human fetal tissues, suggesting that these cells can be activated in utero[50]. Others have also reported that in patients with chronic rhinosinusitis an immature mast cell population that expresses integrin β7 and $CD117^+$ is enriched in nasal polyps[51]. Murphy et al. reported a $CD34^+$ $CD117^+$ $Fc\varepsilon RI^+$ population in sputum samples of atopic patients[52], which suggests mobilization of MCp into the peripheral tissues.

Asthma

Asthma is a chronic and heterogeneous lung inflammatory disease affecting about 300 million patients worldwide and around 8% of the Swedish population[53,54]. The main clinical characteristics of asthma are respiratory symptoms such as shortness of breath and wheezing as well as airway obstruction and hyperresponsiveness to diverse stimuli that can severely limit the daily activities of asthma patients[55].

Mast cells in asthma

In asthma patients, an accumulation of mast cells has been reported at important lung sites. For example, immunohistochemical analysis revealed an increased number of mast cells infiltrating airway smooth muscle (ASM), epithelium, and the alveolar parenchyma of patients with asthma[56-58]. Others have also shown that the number of mast cells in the ASM correlates with asthma severity[5]. In contrast, an increased number of MC_{TC} correlated with better lung function in patients with severe asthma, suggesting a complex role of mast cells in asthma pathobiology[59].

IgE-mediated mast cell activation via the FcεRI is central in allergic asthma. In atopic patients, exposure to innocuous stimuli can result in the production of IgE antibodies. These allergen-specific IgE antibodies have a high affinity for the FcεRI that exists on the surface of mast cells and basophils. FcεRI comprises of an alpha chain that binds to IgE, a membrane-spanning beta chain that amplifies the signal, and two intracellular gamma chains that mediate the downstream signaling. Subsequent allergen exposures trigger

crosslinking and aggregation of IgE-FcεRI complexes. The activation of mast cells via IgE induces the release of mediators that can be observed in the bronchoalveolar lavage (BAL) of sensitized asthma patients[60-62]. These inflammatory products affect smooth muscle constriction, tissue vasodilation, production of mucus, and tissue permeability that trigger eye, nose, or respiratory symptoms common during allergen exposure.

Asthma endotypes

Asthma is a heterogeneous disease with different clinical and pathobiological characteristics. Multiple asthma phenotypes have been described using cluster analysis of patients with distinct traits, disease onset, asthma triggers, or associated diseases[63,64]. Early-onset atopic asthma is a phenotype commonly seen in children. Boys have an increased prevalence of asthma than girls, and after puberty, asthma is most prevalent in women[65,66]. Late-onset asthma is observed in adulthood and can be eosinophilic or be associated with other factors like smoking, obesity, or advanced age[67-69]. The underlying mechanism driving airway inflammation is called the endotype. In asthma, two distinct endotypes are recognized type 2 and non-type 2 inflammation[70]. Patients with non-type 2 asthma respond poorly to steroids and present with airway infiltration that is neutrophilic or paucigranulocytic, (eosinophils and neutrophils are not elevated). Triggers of non-type 2 inflammation include exposure to environmental pollution, airborne irritants, infections, obesity, or cigarette smoke. Non-type 2 inflammation is associated with, e.g., the IL-17, IL-6, or interferon γ-mediated pathways[71,72]. The most common type of asthma is type 2 asthma. Type 2 inflammation involves the participation of the cytokines IL-4, IL-5, IL-9, and IL-13 and will be described in a later section.

Lung function and asthma control

The assessment of changes in lung volume provides vital information for monitoring asthma and other respiratory diseases. Spirometry is a technique that is often used to measure the volume and the speed of the airflow during inspiration-exhalation cycles. Tidal volume (TV) is a basic parameter defined as the volume of air exhaled and inhaled during normal breathing. Taking a deep breath and producing a maximal effort that surpasses the TV can produce inspiratory or expiratory reserve volumes. A residual volume will remain in the lungs and is indirectly calculated based on the functional residual capacity and expiratory reserve volume[73]. Lung capacities can be calculated using these lung volumes (Figure 3).

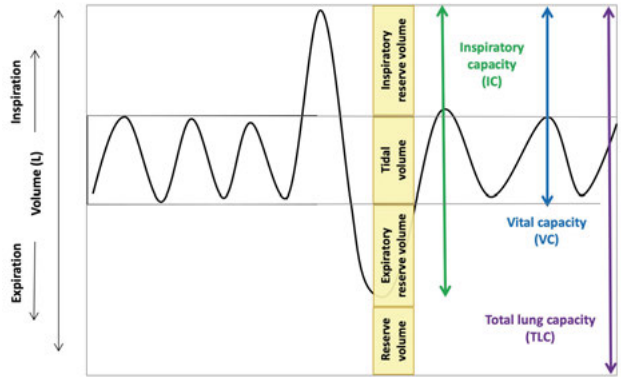


Figure 3. Lung capacities are calculated based on the sum of two or more volumes measured by spirometry. During the spirometry test, patients are asked to take a deep breath followed by a full exhalation, which is used to obtain the value of different lung volumes.

Other relevant lung measurements in asthma are functional indices calculated after a forced expiration in a given time. Some of the most reported parameters include forced expired volume in one second (FEV_1), which represents the volume of air exhaled in 1 second after full inspiration; forced vital capacity (FVC), which represents the volume of gas exhaled at maximal effort; and the peak of expiratory flow (PEF) defined as the maximal speed that the patient achieves after a full expiration. Reference values of lung function parameters are adjusted to the weight, age, sex, and height based on population studies[74].

In adults, the FEV1/FVC ratio, also called the Tiffeneau index, can be used to determine airway obstruction[75]. Airway obstruction can also be investigated using the bronchodilator response. In this test, changes in FEV_1 can be measured after administration of a short-acting β-agonist, e.g., salbutamol that acts on adrenergic receptors preventing the contraction of airway smooth muscles. In this case, airflow limitation is suspected when there is an increase in FEV_1 of more than 200 ml or 12% from the pre-bronchodilator baseline measurements[76,77]. Another key characteristic of asthma patients is the sensitivity of their airways to narrow in the presence of constrictor agents, known as airway hyperresponsiveness (AHR)[78]. In the clinic, AHR is usually assessed after methacholine that directly binds to muscarinic receptors in the smooth muscles and induces airway contraction[79]. During a provocation test, the dose or concentration of methacholine that induces a 20% reduction in FEV_1 compared to baseline is recorded[78].

Other widely used approaches to quantify disease severity are clinical scores, e.g., the asthma control test (ACT) and asthma control questionnaire (ACQ), which are vital in assessing the burden of asthma in patients. The ACT

evaluates the degree of asthma control in the past four weeks, while ACQ evaluates asthma control during the past week[80,81]. In these surveys, patients are asked questions such as: How much time did your asthma prevent you from doing your normal activities? How much shortness of breath did you experience because of your asthma? How often were you woken by your asthma during the night? How often have you used a rescue inhaler or nebulizer medication? The patients score these questions on a 1-5 scale (1 = all the time and 5 = none of the time) in the ACT, and in a scale 0-6 (0 = totally controlled and 6 = maximum impairment) on the ACQ. Scores below 19 points on ACT and higher than 1.5 on the ACQ indicate poor asthma control [80,81]. Together with lung function data, these tests provide helpful insights for monitoring asthma severity.

Type 2 inflammation

Type 2 inflammation can occur after exposure to smoke, pollution, allergens, or infectious agents. These factors disrupt epithelial junctions and can activate pattern recognition receptors or protease-activated receptors, that disrupt the epithelial barrier. Epithelial damage leads to the release of the cytokines IL-33, IL-25, or thymic stromal lymphopoietin (TSLP), also called alarmins[82]. As shown in Figure 4, a plethora of immune cells are involved in type 2 inflammation. Dendritic cells can be activated by these alarmins and present antigens to naïve T-cells to promote their differentiation into T-helper (Th2) cells. The Th2 subset secretes type 2 cytokines and expresses the transcription factor GATA-3. Innate lymphoid immune cells type 2 (ILC2) that reside in the mucosal tissues and mirror the Th2 subset, are also a source of type 2 cytokines. Type 2 cytokines affect multiple cell types, including eosinophils, basophils, and mast cells, which can release type 2 cytokines and different pro-inflammatory mediators perpetuating the inflammatory response[83]. For example, IL-4 and IL-13 promotes class switch recombination in B-cells, increasing IgE antibody production. IL-5 is crucial for eosinophil development and accumulation in lung tissue. IL-9 is important for the accumulation of MCps in the lungs, mucus hypersecretion, and AHR. Similarly, IL-13 acts on goblet cells and ASM, inducing mucus production, and AHR, respectively[82]. Because of their roles in type 2 inflammation, these cytokines are now the target of biological drugs that are used to specifically block their actions[84].

Biomarkers for type 2 inflammation are used to identify patients with this asthma endotype. For example, values of allergen-specific IgE higher than 0.35 kU/L are considered an indication of allergy[85]. Screening of sputum or blood eosinophils is often used to identify patients with type 2 asthma or evaluate their response to therapy[86,87]. The exhaled nitric oxide test (FeNO) is used as a proxy for the measurement of nitric oxide produced by immune cells in the lungs and participates in bronchodilation and vasodilation of the airways. In asthma patients, baseline values >25 parts per billion (ppb) are associated

with a higher exacerbation rate[88]. Other markers, such as sputum periostin, have also been associated with type 2 inflammation[89].

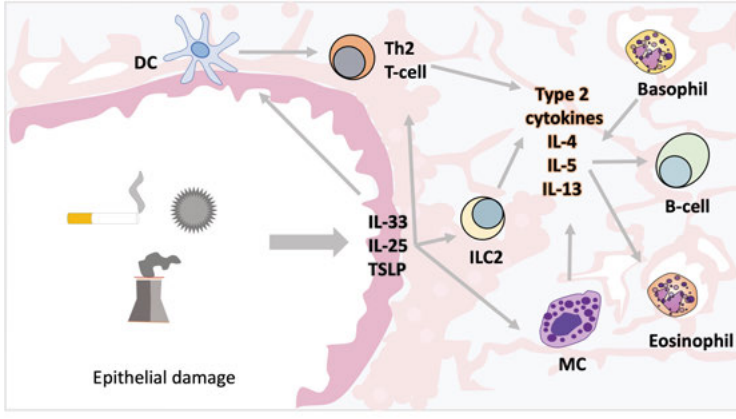


Figure 4. Overview of the major cell types and cytokines that participate in type 2 inflammation.

Asthma treatment

The treatment of asthma is aimed at controlling the symptoms of the disease. Thus, by minimizing exacerbations and side effects, the quality of life of patients can be improved. Common drugs used in asthma treatment are inhaled corticosteroids (ICS), which suppress the inflammation, and long or short-acting β-agonists (LABA and SABA, respectively), which act as bronchodilators[90]. These therapies can be taken as needed or as long-term treatment, depending on the frequency and severity of the asthma symptoms[90,91]. Treatment recommendations for patients with moderate to severe asthma symptoms include add-on therapies like oral corticosteroids, which are effective anti-inflammatory agents that interfere with proinflammatory signal pathways by binding to nuclear steroid or glucocorticoid receptors. However, chronic use of glucocorticoids is not recommended due to the risk of osteoporosis, diabetes, and infections[92].

Currently, six monoclonal antibodies are approved for treating severe asthma [93]. Omalizumab blocks FcεRI-IgE interactions by binding to the Fc portion of IgE. Dupilumab binds to the IL-4Rα, blocking the signaling of both IL-4 and IL-13. Mepolizumab and Reslizumab neutralize IL-5. Benralizumab blocks IL-5Rα and it has been reported to induce antibody-mediated cytotoxicity in eosinophils and basophils. Tezepelumab binds to TSLP and is the most recent biological therapy approved for patients with severe asthma[93]. Emerging therapies targeting alarmins upstream of the inflammatory response are now the focus of the intense investigation. Blocking the IL-33 has also been reported

to improve asthma control and quality of life in patients with moderate to severe asthma and reduce exacerbations in patients with chronic obstructive pulmonary disease[94,95]. Additionally, blocking ST2 antibodies reduces asthma exacerbations in severe asthma patients[96].

Mouse models of airway inflammation

Mice are often used to model many human diseases, and asthma is no exception. The mouse as a model offers various advantages since the murine immune system is well characterized, and there are many transgenic strains available. Furthermore, mice are easy to maintain, handle, and breed. However, there are aspects of the mouse models of airway inflammation that do not fully resemble the human condition.

For instance, there are differences in pulmonary anatomy between both species, i.e., in mice, airways represent 11% of the lung, while in humans, it is only 5.7%. Moreover, the branching of mouse airways is monopodial and has 13-17 airway generations, while human airways have dichotomous branching, and between 17-21 airway ramifications[97]. Another important difference between mice and humans is that mice do not suffer from asthma spontaneously. Most of the existing asthma models in mice focus on inducing the disease by sensitizing and challenging the mice with different antigens. These models capture pivotal characteristics of human allergic asthma, i.e., increased IgE levels, increased type 2 cytokines, and AHR[98].

House dust mite model

Dermatophagoides are common species of house dust mites that cause allergies in humans. Lyophilized preparations of whole-body extracts are commonly used to induce allergic airway inflammation in mice[98]. These extracts contain active compounds that trigger immune responses. For example, extracts from *Dermatophagoides pteronyssinus* contain the peptidases Derp1, 3, and 9. The Derp1 peptidase increases barrier permeability by cleaving epithelial junctional proteins. Other peptidases, i.e., Derp 3 and 9, induce activation of protease-activated receptor 2 in lung epithelium leading to immune cell recruitment into the lungs[99]. Although no model is perfect, the HDM model of allergic airway inflammation reproduces features found in allergic asthma, such as increased numbers of eosinophils in the lung and BAL, increased infiltration of mast cells into the airway smooth muscle or epithelium, AHR to methacholine and elevated levels of specific and total IgE[31,98].

Interleukin-33 model

IL-33 has also been employed to induce airway inflammation in mouse models by mirroring the release of alarmins after epithelial damage. IL-33 binds to the ST2 receptor present in many immune cells. Upon IL-33 binding, ST2 forms a complex with the adaptor protein IRAcP protein necessary for the IL-33/ST2 signaling. The ST2/IL33 signaling pathway involves the response of MYD88, IRAK1, IRAK4, and TRAF6. This activates transcription factors, such as NF-κB and mitogen-activated protein kinases, resulting in the proliferation, survival, and cytokine production of several immune cells[100]. In mice, intranasal IL-33 administration induces type 2 inflammation, AHR, and eosinophilia[101]. Furthermore, four consecutive intranasal IL-33 administrations induce MCp recruitment into the lungs in an ST2-depenndent manner (Figure 5)[102].

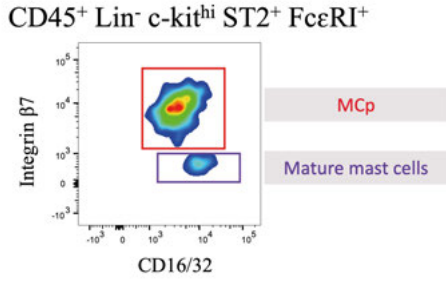


Figure 5. Flow cytometry plot of lung mast cell populations in mice that received four daily intranasal IL-33 administrations (3 μg/mouse, see experimental set up for details).

Mast cell-deficient mice

Mast cell-deficient mice have been used to explore the role of mast cells in models of allergic airway inflammation. Kit mutant mice were first used as mast cell-deficient models. In the $WBB6F_1$/- $Kit^{W/W\text{-}v}$ mice, a spontaneous kit mutation resulted in a lack of melanocytes and tissue mast cells, but they also displayed anemia and a reduction in basophils and T-cells[12]. The C57BL/6-$Kit^{W-sh/W-sh}$ mice bear an inversion mutation that affects the transcriptional regulatory elements of the c-kit gene. These mice have abnormal numbers of basophils and neutrophils and display a significant reduction of mast cells in peripheral tissues[103]. The genetic background of the strains is important since mast cells are necessary for intact AHR development in C57BL/6-$Kit^{W-sh/W-sh}$ but not in BALB/c-$Kit^{W-sh/W-sh}$ mice in a model of airway inflammation induced by ovalbumin (OVA)[104,105].

There are also kit-independent models that were engineered to target mast cell proteases, e.g., mMCP-5 or CPA3, to produce mast cell ablation. Induced mast cell depletion can be achieved by breeding the diphtheria toxin receptor (DTR) floxed mouse with a mouse strain that expresses cre-recombinase under the Mcpt5 promoter or a promoter fragment of the Cpa3 gene[106]. However, diphtheria toxin (DT) injections are needed to deplete the DTR-expressing cells.

In contrast, constitutive mast cell ablation is achieved by, e.g., breeding $Rosa26^{DTA/+}$ mice with $Mcpt5^{cre/+}$ mice. In these mice, mast cell ablation is due to the cre-mediated removal of the floxed-stop cassette upstream of the diphtheria toxin fragment A gene (DTA) gene[106]. Another mouse strain used to constitutively deplete mast cells is the Cre-master or $CPA3^{cre/+}$ mouse. In this strain, Cpa3-expressing cells are ablated due to the genotoxic effect of cre-recombinase, which is expressed under the Cpa3 promoter[8]. The $CPA3^{cre/+}$ mouse strain lacks CTMC, MMCs, and displays a reduction in spleen basophils[8]. Using this mouse strain, we have shown that HDM-induced AHR was decreased by around 50% in $Cpa3^{cre/+}$ mice. Furthermore, we showed that mast cells release 5-HT in response to methacholine, which enhances bronchoconstriction[31].

Lung function measurements in mice

Measuring lung function is crucial to successfully monitor disease progression as well as response to treatments in experimental models of asthma. In mice, lung function can be measured by noninvasive and invasive methods. Noninvasive methods provide information about changes in respiratory patterns in real-time in conscious mice. An example of this is whole-body plethysmography, which involves placing a mouse in a sealed chamber and monitoring changes in air pressure as the mouse breathes in and out[107]. This allows the measurement of parameters related to lung function, e.g., tidal volume, respiratory rate, or minute ventilation[107]. Noninvasive methods allow the assessment of respiratory patterns over time, but they only provide indirect measurements of the lung volumes[108].

In contrast, invasive techniques allow for more precise and direct measurements of lung function. However, they have the disadvantage of being terminal procedures that cannot be used to follow up disease progression in the same mouse[108]. During invasive lung function measurements, mice are anesthetized and connected to a ventilator that exposes the airway to controlled air volume, oscillatory waveforms, or negative pressure. For example, lung function parameters for airway resistance, tissue damping, and tissue elastance can be measured using a broadband forced oscillatory technique in intubated mice. These parameters have been used to monitor changes in tissue mechanics in models of experimental fibrosis or emphysema[109,110]. In experimental models

of asthma, the use of single-frequency forced oscillations can be used to detect airway resistance after the administration of methacholine[109,110]. Another invasive technique performed in tracheotomized, anesthetized mice is the pulmonary function test (PFT). This technique utilizes negative pressure-driven forced expiration to introduce air into the lungs and produce flow-volume loops used to calculate spirometry-like parameters (Figure 6). Some of the parameters that can be calculated are PEF, FEV in 0.1 milliseconds (FEV 0.1), and FVC, among others. The PFT has been reported for the evaluation of lung function in mouse models of emphysema or fibrosis[109-111]. However, PFT is less used to detect changes in lung function in experimental asthma models. Only two studies have reported its use for this purpose. One study used PFT to evaluate lung function in two different experimental asthma models but did not detect changes in spirometry-like parameters[109]. In another study, Davos et. al. reported a small decrease in FVC in mice with experimental asthma induced by HDM[110].

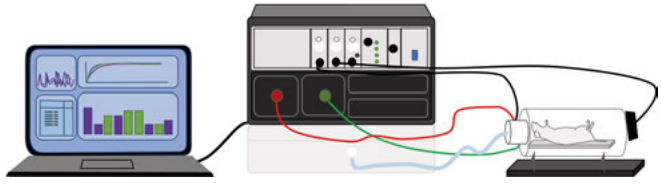


Figure 6. Pulmonary function test (PFT). During PFT measurements, anesthetized mice are connected through the trachea to a ventilator that applies air pressure creating pressure-volume curves to calculate spirometry-like parameters.

Like in humans, the values of lung parameters in mice are also affected by biological variables. For example, changes in lung function associated with strain, sex, and mice growth were reported using whole-body plethysmography[112]. Furthermore, compliance and resistance are increased with age in mice C57BL/6 mice up to 30 months old using the forced oscillatory technique[113]. Therefore, it is vital to control these parameters in order to obtain reproducible and accurate measurements.

Impact of IL-33 on mast cells and T-cells

The IL-33/ST2 axis has been widely studied in the context of asthma. In genome-wide association studies, polymorphisms in the Il1rl1(ST2) gene were linked to childhood-onset asthma susceptibility[114], and IL-33 levels correlated inversely with low values of FEV_1 in patients with asthma[115,116]. Recently, clinical trials have shown that blocking the IL-33/ST2 axis is an effective therapeutic intervention in asthma and chronic obstructive pulmonary disease [94-96].

IL-33 is constitutively expressed by epithelial and endothelial cells but can be produced by macrophages, dendritic cells, or mast cells, under inflammation [117,118]. In mast cells, IL-33 induces survival, proliferation, and release of cytokines, i.e., IL-6 and IL-13 and chemokines CCL1-4, and CXCL1[119,120]. Mast cell proteases, in turn, affect the activity of IL-33. Both mast cell tryptase and chymase cleaved the full-length IL-33 and produce shorter forms that are 2-30 times more active[121]. These mature forms induced higher secretion of IL-5 and IL-13 in ILC2s [121]. However, human chymase also degrades IL-33, which suggests an important modulatory role of these serine proteases in the inflammatory response[122]. IL-33 can be used to induce AHR in a process independently of adaptive immunity using the RAG-2 deficient mice[101]. In mouse models of airway inflammation, expression of IL-33 or ST2 were necessary for the complete development of AHR and eosinophilia[123,124]. Other reports, suggested that intranasal IL-33 exacerbated the AHR induced by OVA in wildtype but not in mast cell-deficient $Kit^{W-sh/W-sh}$ mutant mice[125]. In contrast, $Kit^{W-sh/W-sh}$ mice engrafted with ST2 deficient bone marrow derived mast cells displayed decreased PGE_2 and exacerbated HDM-induced AHR[126]. These results suggest a complex role for mast cells to regulate AHR, which may be influenced by various factors including the mast cell activation state and the nature of the stimulus.

IL-33 can also induce the activation, chemotaxis, and expansion of $ST2^+$ T-cells [127-129]. In fact, the expression of ST2 was first reported to be an exclusive marker for effector Th2 cells[130,131]. Later, in vitro studies suggested that Th2 cells upregulate ST2 in a process that is GATA-3 dependent[128]. In the context of airway inflammation, impairment of the IL-33 signaling decreases the secretion of type 2 cytokines by Th2 cells[132]. Another T-cell subset that is affected by IL-33 are regulatory T-cells (Tregs). Tregs are characterized by the transcription factor FoxP3 and are involved in limiting inflammatory responses[133]. In naïve mouse lungs, a portion of Tregs is ST2 positive, however, influenza, allergic inflammation, and intranasal IL-33 administrations can induce the expansion of $FoxP3^+$ $ST2^+$ Tregs[134-136]. IL-33 also increases the expression of CD25, the IL-2 receptor, which is an important factor in Treg function[135]. The effects that IL-33 elicits on Tregs are diverse. Exposure to IL-33 induced the release of amphiregulin, which participates in lung tissue healing[137]. On the other hand, the Treg phenotype can be altered by IL-33. For example, Chen et al. reported that Tregs from mice exposed to intranasal IL-33 keep FoxP3 but also expressed GATA-3 and released IL-5 and IL-13 but not TGF-β[134]. The interactions between IL-33, mast cells and T-cells are complex and context-dependent, and thus, more research is needed to fully understand their role in asthma and other respiratory diseases.

CXCL1 in inflammation

The recruitment of immune cells into the inflamed tissue is vital for initiating and maintaining the immune response. In patients with asthma or chronic obstructive pulmonary disease, CXCL1 levels are increased in the sputum [138,139]. CXCL1 is a member of the chemokine family that participates in innate immune responses and can be released by hematopoietic cells such as macrophages, epithelial cells, mast cells, and basophils under inflammatory conditions. CXCL1 binds to CXCR2 expressed on immune cells and can also bind to glycosaminoglycans found in the lung endothelium. CXCL1 is known for its chemotactic effect on neutrophils. However, other immune cells, i.e., monocytes and T-cells also show detectable CXCR2 expression[140,141]. Furthermore, CXCL1 has been shown to induce the mobilization of T-cells in a transwell assay[141]. Additionally, specific deletion of CXCR2 in T-cells reduces their infiltration into zones in the brain rich in CXCL1 in an experimental autoimmune encephalomyelitis mouse model[142].

Pre-clinical models of inflammation have shed light on the role of mast cells and CXCL1 in inflammatory responses. For example, intradermal IL-33 injections induce ear swelling in wildtype mice partially due to a mast cell component since the $Kit^{W-sh/W-sh}$ mice showed reduced ear thickness[143]. Further, intraperitoneal lipopolysaccharide administration induced CXCL1 release by both mast cells and macrophages, leading to neutrophil infiltration[144]. Early neutrophil infiltration to the peritoneal wall was severely reduced in Mcpt5-$cre^{+}iDTR^{+}$mice that lack CTMCs but not in clodronate liposome-treated mice that lack macrophages[144]. Suggesting that mast cells are responsible for early CXCL1 release in the peritoneum in response to lipopolysaccharide.

Present investigation

Aims

The main aims of this thesis were to investigate the involvement of mast cells and their progenitors in loss of lung function and inflammation in mouse models of allergic and non-allergic airway inflammation. We also investigated the possible association of circulating MCps and asthma symptoms in patients with allergic asthma during allergen exposure.

The specific aims were:

Paper I
To determine whether lung function parameters correlate with weight or age in naïve BALB/c mice and whether the PFT could be used to distinguish treated and untreated mice with experimental asthma.

Paper II
To investigate if Mcpt8-driven cre-mediated cell depletion affects lung mast cell populations in mice with allergic airway inflammation induced by HDM.

Paper III
To gain deeper insight into the role of mast cells and their progenitors in the reduced lung function and airway inflammation induced by intranasal IL-33 administrations.

Paper IV
To investigate the frequency of circulating MCps in samples from allergic asthma patients collected during pollen season and out of the season.

Experimental setup

Ethical considerations

All mouse experiments were approved and performed in conformity with the Uppsala animal ethics committee (5.8.18-05248/2018; 5.8.18-21870/2022). The studies involving human samples were approved by Uppsala Regional Ethics Review Board (Dnr 2017/535).

Mice

In **paper I**, the experiments were performed with BALB/c mice breed in house and originally purchased from Bommice (Ry, Denmark) or Taconic (BALB/cBomTac). In **paper II**, we used B6.129S4-Mcpt8tm1(cre)Lky/J mice on BALB/c background ($Mcpt8^{Cre/+}$; also known as Basoph8) obtained from Jackson Laboratories and B6.129P2 Gt (ROSA)26Sortm1(DTA)Lky/J mice ($Rosa26^{DTA/+}$; also known as R DTA mice) provided by Prof. Axel Roers and Prof. David Voehringer. In **paper III**, the experiments were performed in BALB/c mice, $Cpa3^{Cre/+}$, and Il1rl1 (ST2) $^{-/-}$ mice on BALB/c background originally provided by Prof. Hans-Reimer Rodewald and Andrew McKenzie, respectively.

Mouse models of allergic airway inflammation

Allergic airway inflammation was induced by intranasal instillation of *Dermatophagoides pteronyssinus* extract (50 μg/50 μl, 25 μl per nostril) under isoflurane anesthesia. The intranasal administrations were performed on days 0, 3, 6, 9, 12, 15, and 18 (Figure 7A). In **paper I** samples were collected one (day 19) after the last HDM administration, and in **paper II** the samples were collected on day 19 or eight days after the last HDM administration on day 26. In **paper I**, dexamethasone or vehicle (0.9% sterile saline) was administered intraperitoneally on days 9, 12, 15, and 18 before HDM instillation at a dose of 3 mg/kg.

In paper III, airway inflammation was induced by intranasal administration of mature mouse IL-33 (3 μg/40 μl, 20 μl per nostril). Bovine serum albumin (0.1% in 1x PBS, 20 μl per nostril) was used as a vehicle. IL-33 was administrated intranasally for four consecutive days, and sample collection on lung function analysis was performed 30 min after the last intranasal administration (Figure 7B).

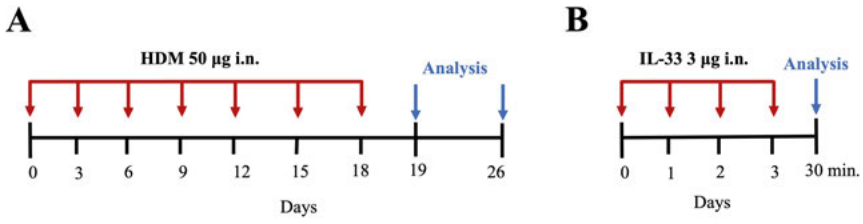


Figure 7. Experimental mouse models utilized in this thesis. (A) Airway inflammation was induced by intranasal (i.n.) administration of a house dust mite extract (HDM) or (B) interleukin-33 (IL-33).

Measurement of spirometry-like parameters and AHR in mice

For lung function analyses, mice were anesthetized using 100 mg/kg ketamine, 20 mg/kg xylazine, and 3mg/kg acepromazine intraperitoneally. Tracheotomy was performed to connect the mice airways to the Buxco PFT or Buxco Fine Point RC system. The Buxco PFT system was used to obtain spirometry-like parameters in anesthetized mice that were ventilated at 150 breaths per minute. Changes in airway resistance were measured after equilibration in mice exposed to 10 µl of PBS or 5, 10, and 20 mg/kg of aerosolized methacholine using the Buxco Fine Point RC system. All the lung function analyses were terminal.

Bronchoalveolar lavage and lung sampling

The BAL fluid was obtained from euthanized mice (isoflurane overdose) by flushing the airways with 1 ml of cold PBS. BAL fluid was centrifuged (400 g, 5 min), and the supernatants were removed and stored at -20°C until analysis. The cell pellet was dissolved, and BAL cells were analyzed by flow cytometry. The lungs were collected after cardiac perfusion of cold 1x PBS (10 mL) to remove blood cells. Single-cell suspensions from the lung were obtained by mechanical and enzymatical digestion using a gentle MACS Octo Dissociator and a mouse lung dissociation kit. Cell debris was removed by centrifuging the samples with 44% Percoll at 400 g for 20 min. After the lysis of red blood cells, the samples were counted in a hemocytometer chamber using trypan-blue exclusion. Samples were stained with fluorescently labeled antibodies to detect different immune cell populations by flow cytometry.

Identification of immune cells by flow cytometry

Lung and BAL samples were stained for 30 min on ice and then washed twice with 2% fetal calf serum in 1x PBS before their analysis in a LSR Fortessa cytometer (BD Biosciences) or a Beckman Coulter LS Cytoflex. The data were analyzed using FlowJo software (TreeStar Inc). Lung mast cell populations were identified as $CD45^+$ $CD3^-$ $CD4^-$ $CD8b^-$ $CD11b^-$ $CD19^-$ $B220^-$ $TER119^-$ $Gr1^-$ $c\text{-}kit^{hi}$ $ST2^+$ $Fc\varepsilon RI^+$ $CD16/32^+$ cells. Based on their integrin β7

expression, they were defined as MCps expressing high levels of integrin β7, induced mast cells expressing intermediate levels of integrin β7, and mature mast cells expressing low levels of integrin β7. In paper **II and III**, basophils were also identified in the same samples as $CD45^+$ $CD3^-$ $CD4^-$ $CD8b^-$ $CD19^-$ $B220^-$ $TER119^-$ $Gr1^-$ $c\text{-}kit^-$ $Fc\varepsilon RI^+$ $CD49b^+$ cells (Figure 8).

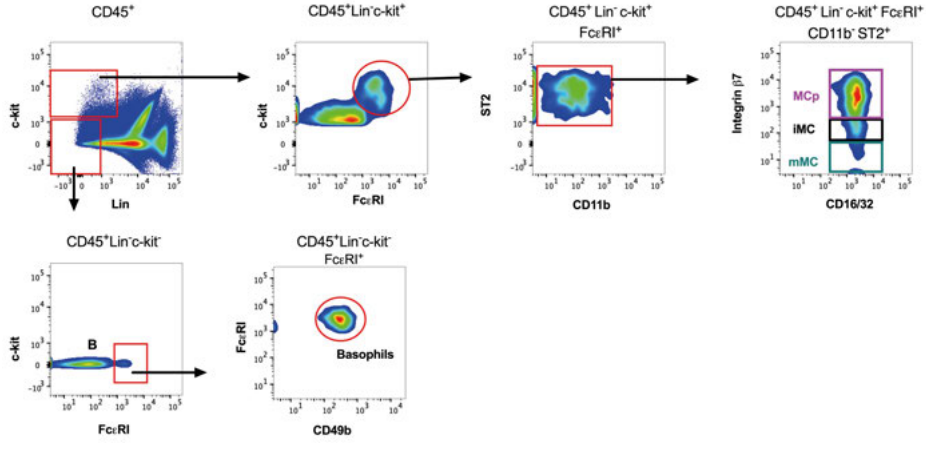


Figure 8. Gating strategy for lung mast cell populations and basophils in mice with airway inflammation using flow cytometry.

In papers **I-III** eosinophils ($CD45^+SiglecF^+$ $CD11c^{-/lo}$), neutrophils ($CD45^+$ $SiglecF^-$ $CD11c^{-/lo}$ $CD11b^+$ $Ly6G^+$), alveolar macrophages ($CD45^+$ $Siglec\text{-}F^+$ $CD11c^+$), $CD4^+$ and $CD8^+$ T-cells ($CD45^+$ $SiglecF^-$ $CD11c^{-/lo}$ $CD11b^-$ $Ly6G^-$ $CD3^+$) were identified in BAL (Figure 9).

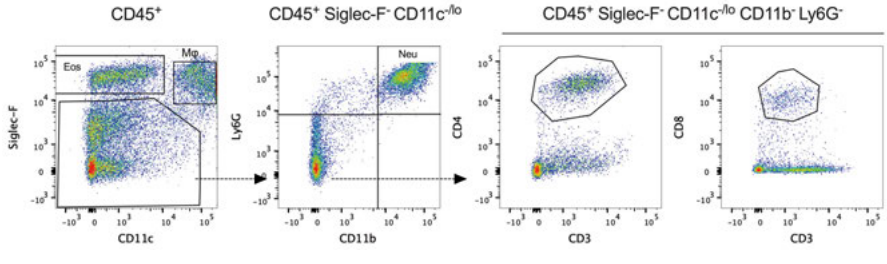


Figure 9. Gating strategy for eosinophils (Eos), alveolar macrophages (Mφ), neutrophils (Neu), $CD4^+$ and $CD8^+$ T-cells in BAL using flow cytometry.

Analyses of human samples

The studies involving human samples were approved by the Uppsala Regional Ethics Review Board (Dnr 2017/535). This study included 33 patients with asthma diagnoses that were recruited via the lung clinic at the Uppsala university Hospital. Only patients with positive birch pollen-specific IgE (>0.35

kU/L) were included in the study. Blood samples were collected during pollen season in May and out of the pollen season in November-January (Nov.). Spirometry parameters, ACT and ACQ scores, as well as the degree of eye, nose or general symptoms were recorded at each visit.

Quantification of human MCps in blood by flow cytometry

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using Ficoll-Paque Premium. PBMCs were stored in freezing media (10% dimethyl sulfoxide in fetal calf serum) at -80°C until analysis. The vital frozen PBMCs were thawed and stained with fluorescently labeled antibodies and analyzed by flow cytometry in Fortessa. MCps were identified as $CD4^{-}$ $CD8^{-}$ $CD19^{-}$ $CD14^{-}$ $CD34^{hi}$ $CD117^{+}$ $Fc\varepsilon RI^{+}$ cells.

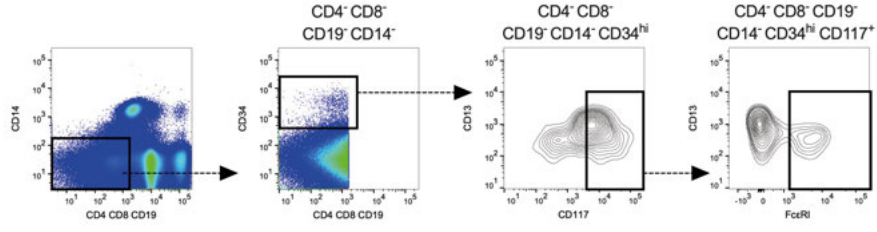


Figure 10. Gating strategy for human MCps.

Statistical analyses

Ordinal data and other non-parametric paired data were analyzed using Wilcoxon signed test. Unpaired or paired student's t-test was used when comparing two groups, and correlation analyses were determined by Spearman's rank correlation test. One-way analysis of variance with Tukey's post-hoc test was performed when comparing three or more groups. Two-way analysis of variance followed by Bonferroni's multiple comparison test was used to analyze AHR data. A p-value of <0.05 was used to determine the statistical significance.

Results, discussion, and future perspectives

Paper I

Use of spirometry-like measurements to monitor house dust mite-induced experimental asthma in mice

Results and discussion

Spirometry-like parameters are essential to monitoring lung function changes in asthma patients. Therefore, evaluating lung function in mouse models of experimental asthma is crucial in enhancing our understanding of asthma pathology and identifying new therapeutic interventions. In the clinic, reference values for spirometry parameters are based on population studies that examine spirometry in male or female individuals across different weights and heights. Nonetheless, spirometry-like parameters have not been validated for their use in mouse models of experimental asthma.

In **Paper I**, we evaluated the effect of weight and age on lung function parameters in naïve mice using PFT. We found that in naïve mice, weight and age correlated positively with FEV 0.1 and PEF. Earlier studies have reported lower PEF in BALB/c mice compared to C57BL/6 mice, and higher PEF in male mice compared to female mice using whole-body plethysmography, a less invasive and less accurate techinque[112]. We evaluated lung function parameters in male and female mice of the same weight, and found that thirty-week-old females had increased FEV 0.1 and PEF values compared to ten-week old males of the same weight. These results suggest that weight, age, and sex of mice have a significant effect on spirometry-like parameters.

Next, we tested whether PFT can distinguish mice with experimental asthma from control mice. We assessed spirometry-like parameters in a model of allergic airway inflammation induced by HDM[31]. We found that HDM-exposed mice displayed a 30% reduction of FEV 0.1, and around 10% reduction in PEF, compared to PBS-exposed control mice. Previously, others have reported no changes in PEF or FEV 0.1 and a slight reduction of FVC using PFT in HDM-, OVA-, or chemical-induced experimental asthma models[109,110]. In our study, we used five times more HDM extract, and it is possible that the strong airway inflammation induced in our model produced a marked decline in lung function. Additionally, we showed that the number of smooth muscle cells in mice exposed to HDM was increased, which is in line with clinical reports showing the thickening of ASM in asthma patients[5].

We also evaluated whether dexamethasone reversed the decline in lung function induced by HDM. The systemic administration of dexamethasone in HDM-exposed mice restored lung function to levels of PBS-exposed mice and reduced the number of BAL eosinophils. Similarly, previous reports showed

that systemic dexamethasone reduced the number of BAL eosinophils and prevented the development of AHR in a model of experimental asthma induced by OVA[145]. Since mast cells are increased in models of allergic airway inflammation, we investigated whether the lung mast cell populations change in mice with reduced lung function induced by HDM. We identified $CD45^+$ Lin^- $c\text{-}kit^+$ $Fc\varepsilon RI^+$ $ST2^+$ $CD16/32^+$ lung mast cells and classified them based on their integrin β7 expression as MCp ($\beta7^{hi}$), induced mast cells ($\beta7^{int}$), and mature mast cells ($\beta7^{low}$) using flow cytometry as previously reported[31]. Although the anti-FcεRI antibody we used has been shown to cross-react with other Fc receptors that also express the gamma chain (FcγRI and IV)[146], we obtained similar quantifications by staining with an anti-IgE antibody. In mice with HDM-induced allergic airway inflammation, the treatment with dexamethasone reduced 60% of lung MCps and approximately 10% of mature mast cells. These results are in line with clinical data showing decreased mast cells in airway epithelium and bronchi of asthma patients that are treated with corticosteroids[147]. Furthermore, pre-clinical studies have shown that dexamethasone does not induce mast cell apoptosis but reduces the levels of SCF[148]. Indeed, in the same study, the topical application of a corticosteroid decreased the number of skin mast cells which could be restored by local SCF administration[148]. These results suggest that the effect of dexamethasone on mast cells could be mediated by the reduction of their survival signals in the lung.
Dexamethasone can also produce downregulation of surface FcεRI expression in bone marrow-derived mast cells[149]. Likewise, in our study, dexamethasone reduced the FcεRI expression on MCps and mMCP-1 levels in HDM-exposed mice. In a previous study, we reported a high frequency of circulating MCps in individuals with reduced FEV_1 and PEF[48]. Thus, we performed a similar analysis and found that the MCp frequency of untreated and HMD-exposed mice correlated negatively with FEV 0.1 and PEF. We conclude that it is vital to control the age-, sex-, and weight of mice when performing PFT measurements. We also observed that PFT could be used to evaluate lung function and response to treatment in an experimental asthma model. Our results also suggest that higher MCp numbers are associated with low lung function in the HDM model of experimental asthma.

Future perspectives

Further studies could use PFT to investigate the effect of different treatments, e.g., bronchodilators and biologics, on lung function in experimental asthma models. It is also interesting to study the effect of asthma-associated diseases on lung function. For example, PFT could be used to test if there are any differences in spirometry-like parameters between mice fed with a high-fat diet compared to mice fed under a normal diet regimen. Or whether spirometry-like parameters are different in obese mice compared to normal-weight mice in a model of experimental asthma. Other studies have shown that a high-fat

diet induces AHR to methacholine in mice[150]. However, the evaluation of spirometry-like parameters in mice fed with a high-fat diet and subjected to an experimental asthma protocol could give new insights into the connection between these two diseases.

In our study, we observed fewer lung mast cells in HDM-exposed mice treated with dexamethasone. This could be due to reduced SCF levels, as previously reported. Thus, it could be interesting to measure SCF levels using an ELISA in lung homogenates of vehicle and dexamethasone treated mice exposed to HDM. We also observed reduced FcεRI expression on MCps in HDM-exposed mice that received dexamethasone. What is the molecular mechanism behind the suppressive effects of dexamethasone on FcεRI? Yamaguchi et al. reported that dexamethasone induced a decrease in surface FcεRI expression but no changes at mRNA level in mouse mast cells, suggesting that a post-translational mechanism could be involved. One possibility is that the receptor is being internalized, and thus less surface expression is detected. This could be investigated by examining the surface expression of FcεRI on MCps in lung sections from HDM-exposed mice that received dexamethasone or vehicle. Using confocal microscopy MCps could be identified using fluorescently labeled antibodies against integrin β7 and mMCP-6. The localization of FcεRI in these cells could be examined using an anti-FcεRI together with a dye that labels lipids in the membrane.

Paper II

Depletion of Mcpt8-expressing cells reduces lung mast cells in mice with experimental asthma

Results and discussion

Mast cells and basophils are closely related but distinctive immune cells that participate in type 2 immune responses. Deletion of cells expressing mMCP-8, a classical basophil marker, is widely used to study the function of these cells. However, recent data showed Mcpt8 mRNA expression in lung mast cells in a model of allergic airway inflammation[34]. Thus, in **Paper II,** we studied if Mcpt8-driven cell deletion affected the lung mast cell populations (MCps, induced, and mature mast cells) observed during acute lung inflammation induced by HDM[31].

We first investigated the protein expression of mMCP-8 in lung mast cells in HDM-exposed mice. We used the Mcpt8$^{Cre/+}$ mice, which express a yellow fluorescent protein (YFP) in a cre-dependent manner, and observed YFP-mMCP-8 expression in 96% of lung basophils and 37, 10, and 4 % of MCps, induced, and mature mast cells, respectively. Others have shown a lack of YFP-mMCP-8 in mature mast cells in the peritoneum of the Mcpt8$^{Cre/+}$ mice[39].

In addition, we screened the YFP expression in eosinophils, neutrophils, $CD4^+$ and $CD8^+$ T-cells, and alveolar macrophages in BAL from HDM-exposed mice and did not detect any YFP signal. These results suggest that the expression of mMCP-8 is not restricted to basophils, but also lung mast cells express this protease during allergic airway inflammation.

Next, we investigated the effect of Mcpt8 deletion on lung mast cell populations in the HMD model of experimental asthma. We generated Mcpt8-deficient mice by crossing the $Mcpt8^{Cre/+}$ mice with the $Rosa26^{DTA/+}$ strain. In these mice, DTA expression is restricted to Mcpt8-cre-positive cells resulting in its ablation[151]. During acute HDM-induced airway inflammation, $Mcpt8^{Cre/+}$ $Rosa26^{DTA/+}$ mice had 96 and 40 % reduction of lung basophils and MCps, respectively. Furthermore, a tendency for reduced induced mast cells was observed in these mice. We kept a group of HDM-exposed mice after the induction of acute airway inflammation and collected lung tissue eight days after the last HDM administration. We quantified the number of lung mast cells using mMCP-6 as a mast cell marker. In the lungs of $Mcpt8^{Cre/+}$ $Rosa26^{DTA/+}$ mice, around 28% of mMCP-6^+ cells were lacking. Others have reported no changes in CTMCs in a lung infection model with *N. brasiliensis* or a sepsis model induced cecal ligation and puncture [38,39]. Similarly, Pellefigues et al., used an inducible model in which the DTR expression is restricted to Mcpt8-cre positive cells and showed no changes in peritoneal mast cells after DT injection[37]. We also observed that $Mcpt8^{Cre/+}$ $Rosa26^{+/+}$ mice carrying only one allele for the Cre-recombinase displayed a 3-fold increase of lung basophils compared to $Mcpt8^{+/+}$ $Rosa26^{+/+}$ wildtype littermates during acute inflammation induced by HDM. Similar results were observed in $Mcpt8^{Cre/+}$ mice, suggesting that breeding with the $Rosa26^{DTA/+}$ strain was not the problem. We did not observe differences in blood basophils of naïve mice in any of the strains, nor did we find differences in other immune populations in BAL.

The results of this study suggest that expression of mMCP-8 is not restricted to basophils, but lung mast cells also express this protease during allergic airway inflammation. We also found that the deletion of Mcpt8 cells in the $Mcpt8^{Cre/+}$ $Rosa26^{DTA/+}$ mice exposed to HDM reduces the number of MMCs in the lung. Furthermore, we found that $Mcpt8^{Cre/+}$ mice overrepresent basophils in inflammatory models. Therefore, we conclude that appropriate controls, i.e., $Rosa26^{DTA/+}$ or wildtype littermates, should be included when using the $Mcpt8^{Cre/+}$ $Rosa26^{+/+}$ strain.

Future perspectives

The mechanism behind the increased basophil influx in the lungs of mice carrying a cre-recombinase allele remains to be investigated. Off-target effects of the cre-lox system are usually linked to cre-mediated toxicity[152], so it is unexpected that our observations suggest an expansion of the cre-expressing cells.

Internal Ribosome Entry Sites (IRES) like the one present in the Mcpt8$^{cre/+}$ construct are usually used to enhance the translation of target genes by recruiting ribosomes directly into the mRNA. Thus, it is possible that this enhancer could also induce the expression of other genes linked to cell proliferation. This could be investigated in mice exposed to HDM by separating mMCP-8 positive and negative MCps using fluorescence activated cell sorting (FACS). Cell proliferation can be assessed in these cells using carboxyfluorescein diacetate succinimidyl ester. This fluorescent compound is incorporated into the cell, and as the cells divide, the intensity of the compound decreases, which can be analyzed by flow cytometry.
Several other questions about the nature of the mMCP-8$^+$ MCps remain to be answered. Does mMCP-8$^+$ MCp give rise preferentially to basophils or mast cells in culture? This could be investigated by FACS sorting mMCP-8$^+$ MCps and placing them in a myeloerythroid cocktail that supports the generation of both mast cells and basophils. Moreover, it would be interesting to investigate the localization of mMCP-8$^+$ MCps in the lungs of mice with allergic airway inflammation induced by HDM. This can be investigated using immunofluorescence and confocal microscopy in lung sections. Based on our data MCps could be identified with an anti-integrin β7 antibody together with a specific mast cell marker such as mMCP-6. To detect the expression of mMCP-8$^+$ the endogenous YFP, an anti-mMCP-8, or anti-YFP antibody could be used.

Paper III

Mast cell-derived CXCL1 promotes bronchoalveolar CXCR2$^+$ T-cells in mice with IL-33-induced airway inflammation

Results and discussion

Increased IL-33 levels in the lungs can be induced by epithelial damage caused by, e.g., pathogens, smoke from cigarettes, or pollution. Since IL-33 is an emerging target for airway diseases[94-96], in **paper III,** we investigated the role of mast cells in the airway inflammation and reduced lung function induced by this cytokine.
Airway inflammation was induced by intranasal IL-33 administration (four consecutive days). We found that IL-33 reduced the spirometry-like parameters FEV 0.1, PEF, FVC, and IC and increased BAL eosinophils, CD4$^+$ and CD8$^+$ T-cells, in an ST2-dependent manner. However, spirometry-like parameters (FEV 0.1, PEF, and FVC) after IL-33 administrations did not differ between Cpa3$^{cre/+}$ mast cell-deficient mice and Cpa3$^{+/+}$ wildtype littermate mice. This suggests that other ST2-responsive cells could be mediating the decline in spirometry-like parameters induced by IL-33. Moreover, the number of lung MCps and basophils but not mature mast cells increased after IL-33, which is in line with previous publications from our lab[102]. Furthermore, in

Cpa3$^{cre/+}$ mice exposed to IL-33, there was about 50% and 70% reduction in MCps and basophils, respectively.

Increased AHR after multiple intranasal IL-33 administrations has been previously reported[101]. Similarly, we also observed AHR in mice that received intranasal IL-33. We also investigated AHR in Cpa3$^{cre/+}$ mice and found a 10% reduction in the AHR induced by IL-33 in Cpa3$^{cre/+}$ mice compared to Cpa3$^{+/+}$ wildtype littermates. Others have reported a mast cell-dependent effect on AHR exacerbation induced by IL-33 in an OVA model of allergic airway inflammation[125]. Additionally, in a previous, study we reported that Cpa3$^{cre/+}$ mice with allergic airway inflammation induced by HDM had 50% less AHR[31]. These differences might be due to the model used, while HDM administrations induce a prominent MCp accumulation, in the IL-33 model the MCp accumulation is modest.

IL-13-producing cells such as ILCs and the mast cell protease mMCP-1 are associated with airway smooth muscle contraction in response to IL-33 stimulation[153,154]. In our model, the levels of BAL mMCP-1 and lung IL-33 in the lung were reduced in Cpa3$^{cre/+}$ mice, and a tendency for less 5-HT in BAL was also detected in these mice, and no changes in IL-13 were observed. These results suggest that mast cells contribute to the release of IL-33, and mMCP-1, and have a partial role in the AHR induced by IL-33. We also found a 50% reduction in CD4$^+$ and CD8$^+$ BAL T-cells in Cpa3$^{cre/+}$ mice following IL-33 administrations. Furthermore, IL-33 administrations induced the expression ST2 in both CD4$^+$ and CD8$^+$ in T-cells, and increased the expression of GATA-3, CD25, and FoxP3 in CD4$^+$ T-cells. These results are in line with previous investigations[128,134,135].

Next, we investigated the factors that could be involved in the reduction of T-cells in IL-33-exposed Cpa3$^{cre/+}$ mice. An initial screening showed that CXCL1 was reduced in the lungs of Cpa3$^{cre/+}$ mice. Furthermore, IL-33 induced CXCL1 levels in BAL that were seven times higher than in the lung. CXCL1, through binding to its receptor (CXCR2) regulates the mobilization of neutrophils into inflamed areas. However, CXCR2 can also be expressed in other cell lineages[140]. Thus, we also characterized the expression of the CXCR2 in T-cells and found that around 2-3% of T-cells in the lung and around 7-8% of T-cells in the BAL expressed the receptor after IL-33 exposure. These results are in line with reports showing CXCR2 expression and chemotaxis of T-cells exposed to CXCL1 in vitro[141]. Others have also shown that specific deletion of CXCR2 in T-cells reduces its mobilization into zones of increased CXCL1 in a mouse model of encephalomyelitis[142].

We also investigated if mast cells were a source of CXCL1 during IL-33 exposure. Mast cell derived-CXCL1 is implicated in the mobilization of neutrophils in response to IL-33 or LPS[143,144]. Moreover, the induction of CXCL1 after IL-33 has been documented in human mast cells at mRNA and protein levels[120]. In our study, we observed that lung-derived mast cells released CXCL1 after IL-33 incubation. In the lungs of IL-33-exposed mice, CXCL1$^+$

mast cells were localized close to the epithelium. We also observed $CXCL1^+$ basophils in the parenchyma of mice that received IL-33 intranasally.
In summary, this study suggest that airway inflammation induced by IL-33 induces the release of CXCL1 from mast cells, which contributes to T-cell mobilization into the bronchoalveolar space.

Future perspectives

Future studies in this project could investigate whether T-cell CXCR2 expression is directly induced by IL-33. This could be investigated in ST2-deficient mice exposed to intranasal IL-33. The expression of CXCR2 could be compared between control wildtype littermates and ST2 deficient mice exposed to IL-33. In vitro experiments can also be used to investigate this. For example, naïve $CD4^+$ T-cells could be isolated from mouse spleen or lymph nodes and polarized to Th2 using IL-4 and IL-2. The cultured cells could be enriched for $ST2^+$ using FACS or magnetic beads and incubated with vehicle or different IL-33 concentrations. This system could be used to test the expression of CXCR2 and the intracellular cytokine expression in these Th2 cells using flow cytometry. It would also be interesting to investigate which cytokines are expressed by $CXCR2^+$ T-cells in vivo. This could be achieved by sorting $CXCR2^+$ and $CXCR2^-$ T-cells from the lungs of mice that received IL-33 and exposed them to phorbol myristate acetate and ionomycin. These compounds activate T-cells and stimulate the release of diverse cytokines. The cytokines released in the supernatant could be determined in the $CXCR2^+$ and $CXCR2^-$ T-cells by ELISA or multiplex.
Previous studies have shown that intranasal CXCL1 increased BAL neutrophils[155]. However, the effect of intranasal CXCL1 on BAL T-cells has not been studied. This could be investigated by collecting BAL from mice that received vehicle or intranasal CXCL1 during four consecutive days (like in the IL-33 model). The number of T-cells and their CXCR2 expression could be quantified by flow cytometry in the BAL samples. These experiments could clarify if CXCL1 directly induces the mobilization of T-cells into the BAL.
Testing the contribution of mast cell-derived CXCL1 in the airway inflammation induced by IL-33 could be explored using conditional CXCL1 deletion in mast cells. This could be achieved by generating a mouse strain that expresses loxP sites flanking the CXCL1 gene through CRISPR/Cas9 technology and then breeding it with the Mcpt5-cre or Mcpt1-cre mice to generate the deletion of CXCL1 in CTMC or MMCs, respectively.

Paper IV

Circulating mast cell progenitors increase in frequency during natural birch pollen exposure in allergic asthma patients

Results and discussion

Patients with allergic asthma have increased symptoms due to seasonal allergen exposure, which disrupts their daily activities and decreases their quality of life. Enrichment of MCps in the blood of individuals with reduced lung function has been previously reported[48]. Thus, in **paper IV** we investigated whether the frequency of circulating MCps in patients with allergic asthma was modified during natural allergen exposure.

Samples from allergic asthma patients were collected during pollen season (May) and compared to paired samples taken out of pollen season (Nov.). We quantified MCps in peripheral blood using flow cytometry as previously reported[48]. We found that the frequency of circulating MCps was increased during birch pollen season in May compared to out of the pollen season in Nov. This is the first time that the frequency of MCps was quantified during and out pollen season. In experimental asthma models, MCp recruitment into the lungs precedes an increase of mature mast cells during allergen exposure[26,27,30,31]. Others have shown that in humans, mast cells in nasal mucosal, eye conjunctiva, and bronchial epithelium are increased during allergen exposure, which could be due to the recruitment of MCps to these sites[156-158].

FcεRI is a cardinal marker of mast cells, and in our study, the expression of FcεRI in MCps correlated positively with birch pollen-specific IgE. A relationship between FcεRI and IgE has been previously reported[159-161]. Moreover, treatment with an anti-IgE antibody lowers IgE levels and induces reduced expression of FcεRI in mast cells and basophils[160,161]. Interestingly, we found that during May, the surface FcεRI expression in MCps was lower than in Nov. Similarly, others have reported that basophils from allergic patients exhibit reduced FcεRI expression during pollen exposure[162]. We speculate that the birch pollen allergen might interfere with the detection of the FcεRI due to steric hindrance. However, the exact mechanism behind this remains to be investigated.

We also investigated the MCp frequency in patients that reported low asthma control or more asthma symptoms during the pollen season. Asthma control surveys, i.e., ACQ and ACT, provide information about the limitations that patients experience due to asthma symptoms. The patients in our cohort reported more asthma symptoms and higher ACQ scores corresponding to low asthma control during May compared to Nov. Furthermore, when we investigated the MCp frequency in these patients we found that during May, the MCps numbers were increased in patients that reported moderate to severe asthma symptoms or reported low asthma control (>1.5 ACQ scores). Similarly, the ACT scores, were decreased during May compared to Nov., and the

MCp frequency was higher in patients that reported ACT scores ≤19, corresponding to low asthma control. Furthermore, the changes in ACT correlated with the change in MCps. These results suggest that patients with low asthma control and asthma symptoms have a higher MCp frequency during pollen exposure. When the patients were grouped by sex, we observed that women were the group most affected by asthma symptoms and exhibited increased MCps in blood during pollen season. We have reported that MCp frequency is higher among women than men[48]. However, in the current study, a similar pattern was observed among men.

An increase in allergy-related symptoms that affect the eyes or nose during pollen exposure is widely reported in the literature[163,164]. Therefore, we investigated the MCp frequency in patients that reported eye, nose, or general symptoms (fatigue, difficulty concentrating, and reduced physical activity) during May and Nov. We found that patients that reported nose or eye symptoms (regardless of the severity) and moderate to severe general symptoms had increased MCps during the May. It has been reported that patients with allergic rhinitis are commonly also affected by asthma[165]. In our cohort, patients that reported rhinitis during May had a trend for higher blood MCps frequency compared to patients that did not report rhinitis during May. Thus, is possible that there was an overlap in the allergy symptoms of these patients.

We previously reported a negative correlation between blood MCp frequency and FEV_1 and PEF in a cohort of young individuals (25 years old on average)[48]. In contrast, our population consisted of an older population (48 years-old on average), and we did not find any associations between MCp frequency and different spirometry outcomes.

In summary, the findings in this study suggest that natural pollen exposure triggers an increase in blood MCps in allergic asthma patients. Furthermore, we uncover an association between allergy symptoms and blood MCps during natural pollen exposure in patients with allergic asthma.

Future perspectives

Further studies could investigate if the MCp frequency is altered, for example, by exposure to outdoor pollutants. This could be investigated by quantifying blood MCps from samples of asthma patients that live in urban or industrial areas with high pollution and comparing them to samples from asthma patients that live in zones with low pollution. Additionally, a group of healthy individuals could also be included in the study to assess the magnitude of the effect of air pollution on MCps. Another possibility is to study the timeline for the increase of MCps during allergen exposure. This could be explored in a controlled environment by collecting blood samples at different time points before and after a single allergen exposure of sensitized asthma patients. MCps could be quantified in these blood samples, and bronchial biopsies could be collected at the last time point to determine if a rise in blood MCps precede an increased number of tissue mast cells after a single allergen exposure. It

would also be interesting to study the mechanism driving the changes in the surface expression of FcεRI observed in MCps during May. One possibility is that the allergen is inducing steric hindrance, and thus, fewer antibody molecules can bind to FcεRI. This could be investigated using blood samples from birch pollen-allergic asthma patients. The samples can be taken during Nov. since MCps display an increased FcεRI signal than in May, and the birch pollen allergen is not present. The samples of these patients can be incubated with or without Bet v1, which is the major birch pollen allergen. Next, the levels of FcεRI on MCps can be measured by flow cytometry using an anti-FcεRI antibody.

Further studies could also investigate if there are any differences in the transcriptome of MCps from paired blood samples taken during May and Nov. This could be achieved by isolating MCps from PBMCs directly into an RNA lysis buffer solution using FACS. The gene expression of human MCps can be investigated using a microarray analysis. These studies can help to identify the differentially expressed genes between the two samples and gain a better understating of the specific molecular pathways that are affected by seasonal changes. Another future perspective is to investigate the potential alterations in the activation markers of MCps during pollen season, in contrast to periods outside of the season. This could be investigated by staining the activation markers CD63 and LAMP-1 on MCps from samples taken during pollen or non-pollen season. Such a study could provide invaluable insights into the activation of MCps in response to environmental triggers.

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