INTERACTIONS BETWEEN MAST CELLS AND SMOOTH MUSCLE IN ASTHMA MODELS

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ABSTRACT

Allergic asthma is the major phenotype of asthma and is characterized by specific responses to allergens as well as airway hyperresponsiveness (AHR), inflammation and remodelling. Mast cells and airway smooth muscle cells (ASM) play an important role in the allergic airway response and there are several indications that a cross-talk exists between these two cell types contributing to asthma pathogenesis. However, these interactions and the mechanisms behind them are not fully understood. The overall aim of this thesis was to further elucidate the interactions between mast cells and ASM in different animal models of allergic asthma.

When responses between wild-type and mast cell-deficient mice, with and without mast cell engraftment were compared, we found that AHR was both mast cell dependent and independent using the same chronic mouse model of asthma. Although pulmonary mast cell number, distribution and possibly prostanoid secretion influenced the severity and localisation of AHR and levels of specific-IgE, inflammatory cell infiltration and remodelling was unaffected. However, the mere presence of mast cells had an effect on the levels of interleukin 17 and 33 (IL-17 and IL-33), two cytokines that are associated with asthma.

By studying airway responses in a guinea pig model of allergic asthma, we found that the early allergic reaction caused an altered airway resistance more similar to the response in humans than that seen in mice. This could be explained by the greater number and different distribution of mast cells, as well as the secretion of histamine and cysteinyl-leukotrienes from mast cells, suggesting that there are advantages to using guinea pigs compared to mice in asthma research.

We continued by studying how IL-33 affected the early allergic reaction in a mouse model of allergic sensitisation. We found that intranasal IL-33 in sensitised mice increased the smooth muscle contraction in isolated airways from these mice when challenged with allergen ex vivo. The increase was mast cell and IL1RL1 (the receptor for IL-33) dependent and was mediated via an enhanced secretion of serotonin. The direct effects of IL-33 on mast cells to increase their production, storage and mediator release were confirmed in mast cell cultures. Finally, we observed that IL-33 increased airway resistance, in a mast cell- and serotonin-dependent fashion following allergen challenge in vivo.

To further investigate how IL-33 influenced other features of allergic asthma, we studied the effects of IL-33 combined with an allergenic antigen on AHR, inflammation and remodelling in two different mouse models of asthma. No differences were observed when using a mast cell-independent protocol of sensitisation and challenge. However, using a protocol that has been shown to induce mast cell-dependent AHR and inflammation in sensitised and challenged mice, IL-33 combined with antigen elicited synergistic deleterious effects on AHR, inflammation and remodelling.

The studies in this thesis emphasise the importance of choosing the appropriate animal model when studying allergic asthma. The findings herein support that pulmonary mast cell number and distribution affects lung function in the disease state. Finally, we have shown that release of IL-33 is increased during allergic inflammatory conditions, and the level of the increase is affected by mast cells. IL-33 worsens several characteristic features of allergic asthma including the early allergic reaction, AHR, inflammation and remodelling, for some features in a mast cell-dependent manner. In conclusion, we have highlighted the importance of IL-33 in conjunction with mast cells for asthma development and severity and have identified a potential new target for asthma therapy.
LIST OF PUBLICATIONS

I. Fuchs B, Sjöberg L, Möller Westerberg C, Ekoff M, Swedin L, Dahlén SE, Adner M, Nilsson GP
   **Mast cell engraftment of the peripheral lung enhances airway hyperresponsiveness in a mouse asthma model**

II. Riley JP, Fuchs B, Sjöberg L, Nilsson GP, Karlsson L, Dahlén SE, Rao NL, Adner M
    **Mast cell mediators cause early allergic bronchoconstriction in guinea-pigs in vivo: a model of relevance to asthma**
    *Clin Sci (Lond)*. 2013;125(11):533-42

III. Sjöberg L, Gregory JA, Dahlén SE, Nilsson GP, Adner M
    **Interleukin 33 causes airway smooth muscle contraction via an enhanced secretion of serotonin from mast cells in the early allergic reaction**
    *Manuscript*

IV. Sjöberg L, Zoltowska A, Gregory JA, Lei Y, Adner M, Nilsson GP
    **Interleukin 33 elicits synergistic effects with antigen on airway hyperresponsiveness, inflammation and remodeling in a mouse model of asthma**
    *Manuscript*
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<tbody>
<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
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<tr>
<td>alum, AlOH₃</td>
<td>Aluminium hydroxide</td>
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<td>ASM</td>
<td>Airway smooth muscle</td>
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<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
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<td>BMNC</td>
<td>Bone marrow-derived mast cells</td>
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<tr>
<td>Col1a1</td>
<td>Collagen, type I, alpha 1</td>
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<td>Col3a1</td>
<td>Collagen, type III, alpha 1</td>
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<td>Col5a1</td>
<td>Collagen, type V, alpha 1</td>
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<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>CXCL</td>
<td>C-X-C motif chemokine</td>
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<tr>
<td>CysLT</td>
<td>Cysteinyl-leukotrienes</td>
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<tr>
<td>DNP</td>
<td>Dinitrophenyl</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>FEV₁</td>
<td>Forced expiratory volume in one second</td>
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<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1RAcP</td>
<td>IL-1 receptor accessory protein</td>
</tr>
<tr>
<td>IL1RL1</td>
<td>Interleukin 1 receptor-like 1</td>
</tr>
<tr>
<td>ILC2</td>
<td>Group 2 innate lymphoid cells</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
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<tr>
<td>OVA</td>
<td>Ovalbumin</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>T₉</td>
<td>T helper</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TP</td>
<td>Thromboxane receptor</td>
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<tr>
<td>Tph-1</td>
<td>Tryptophan hydroxylase 1</td>
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<tr>
<td>TSLC</td>
<td>Tumor suppressor in lung cancer</td>
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<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
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1 INTRODUCTION

1.1 ASTHMA

Asthma is a chronic inflammatory airway disease that currently affects more than 300 million individuals worldwide [1]. It is one of the most common chronic diseases and the global incidence continues to increase. Historically, the increase was assumed to occur predominantly in developed countries [2, 3], but recent data has suggested that asthma prevalence is increasing in developing countries as well [4].

Individuals with asthma suffer from episodic airflow obstructions that are reversible either spontaneously or by treatment [1]. These airflow obstructions results in asthma symptoms, such as recurrent episodes of wheezing, chest tightness, breathlessness and coughing, particularly at night or in the early morning. Airflow obstructions are a consequence of smooth muscle contraction, mucus secretion, airway oedema, airway wall thickening or a combination of these occurrences. Characteristic pathophysiological features of asthma are airway hyperresponsiveness (AHR), inflammation and remodelling.

The origin of asthma remains a mystery. However, several causative factors have been proposed. Numerous polymorphisms in different genes, such as IL33, have been linked to asthma development [5, 6]. In addition, environmental factors, such as the timing of infectious exposures during development, have been shown to increase the risk of developing asthma later in life [7, 8]. The most likely scenario is that the origin consists of interplay between both genetic and environmental factors. Once an individual has developed asthma, several factors can act as asthma triggers, such as allergens, infections and smoke, which can cause exacerbations of the disease symptoms [9].

Asthma was long considered to be a single disease that was allergy-mediated, T\(_{H2}\)-driven, eosinophilic and corticosteroid responsive [10]. Today, asthma is known to consist of several different phenotypes that are based on the underlying biological mechanisms as well as clinical and physiological features, including the response to treatment.

At present, there are several different therapeutics available for asthma treatment. However, there are no therapeutics that prevent or cure the disease. The Global Initiative for Asthma consortium currently recommends that the aim of treatment should be to achieve and maintain control of the disease during sustained periods [1]. Assessment of asthma control includes two parts: assessment of clinical manifestations, such as symptoms and lung function, and assessment of future risks, such as exacerbations and the side-effects of treatment. Asthma therapeutics can be divided into controllers, such as inhaled glucocorticosteroids, which are used on a daily basis to achieve asthma control, and relievers, such as inhaled β\(_2\)-agonists, which are used as needed to reverse bronchoconstriction. There is a five-step program to follow when considering an asthma treatment regime today. Step 1 includes reliever medications alone and steps 2-5 also include controller medications. The treatment protocol is augmented until control has been achieved. If the asthma is still severely uncontrolled following step 4 treatment, oral glucocorticosteroids can in some cases be prescribed in
step 5, despite the severe side-effects associated with their use. Even though there are various controller medications to choose from today, such as leukotriene modifiers and anti-IgE treatment, there are still some individuals that suffer from uncontrolled (severe) asthma following step 5 of treatment. The underlying mechanisms for the different phenotypes of asthma, which may in some cases account for the occurrence of uncontrolled asthma in certain individuals, are now being further characterised. As a consequence of this, there is a possibility that cases of severe asthma might become more readily treatable as new medications based on a previously unknown target are developed.

1.2 AIRWAY SMOOTH MUSCLE

Airway smooth muscle (ASM) cells are mononuclear, non-striated structural cells of mesenchymal origin [11, 12]. ASM cells can be found in the walls of hollow organs and blood vessels and their contraction is involuntary. In the airways, these cells circumscribe both the upper and lower airways, which make it possible for the airways to constrict. In response to various stimuli such as neurotransmitters, inflammatory mediators and exogenous substances, ASM constricts or relaxes in order to regulate and facilitate ventilation. ASM cells were, for long, thought to act as passive responders, mediating contraction or relaxation. However, ASM cells are now appreciated to have an important function as immunomodulatory cells [13]. The ASM secretes a variety of cytokines, chemokines, cell adhesion molecules and growth factors that have the possibility to modulate the response of other cell types in the vicinity. A critical role of ASM, mediating airway narrowing following contraction in asthma, has long been accepted [14]. In addition, ASM cells are now also suggested to be involved in AHR, inflammation as well as remodelling [15].

1.3 MAST CELLS

The mast cell is a cell of the immune system, known mostly for its involvement in allergic reactions [16]. It has also been suggested to have implications in other diseases, such as rheumatoid arthritis, multiple sclerosis, heart disease and cancer. More recently, protective effects that mast cells have as part of our immune system has been brought into focus. Mast cells are part of the innate immune system, responding both to bacterial and viral infections and they even possess the ability to neutralise snake venom. Mast cells originate from hematopoietic progenitors that circulate in the blood and lymphatic system [17]. When their progenitors migrate into a tissue they mature under the influence of the tissue-specific microenvironment. Different phenotypes exist and mast cells are most commonly divided into mucosal (MC\textsubscript{T}} or connective tissue (MC\textsubscript{TC}) mast cells. However, a recent study has shown that site-specific subpopulations of these phenotypes exist in the human lung [18]. Mast cells are tissue-resident cells that predominantly populate sites opposed to the external environment such as the lung, skin and gastrointestinal tract [19]. Mast cells are often located near blood vessels and nerves where they can secrete mediators that act either locally or systemically [20]. The mast cell can respond to a wide range of physical, biological and chemical stimuli with the most well-known interaction being IgE-receptor crosslinking when bound to a specific allergen [21]. The response from a mast cell, when activated, can be divided into three types of responses which include: degranulation (rapid onset), release of lipid mediators (within minutes) or release of cytokines, chemokines and growth factors
(within hours). When mast cells are activated they respond in different ways to different stimuli.

Mast cells or their mediators have been shown to be involved in the pathogenesis of different phenotypes of asthma, with the most well characterised being allergic asthma. The immune system of people that suffer from allergic asthma responds aberrantly to otherwise harmless substances such as pollen. When a person with allergic asthma inhales an allergen, crosslinking of allergen-specific IgE bound to its receptor FceRI causes mast cells activation [22]. Following activation, mediators released from mast cells affect different phases of the allergic reaction. The early allergic reaction happens within minutes and causes airway smooth muscle contraction, vasodilatation, increased airway permeability and epithelial mucus secretion leading to airflow obstructions. Within hours, inflammatory cells are recruited to the lung and in some individuals the late allergic reaction also occurs with reappearance of airflow obstructions. Due to repeated allergen exposure during a substantial time period, mast cell activation is not only important for the early allergic reaction, but also for the chronic inflammation, AHR, and remodelling.

1.4 AIRWAY HYPERRESPONSIVENESS

The definition of AHR is the excessive contractile response of the airways following exposure to substances that normally have little or no effect in healthy individuals [1]. In humans, airway narrowing is usually assessed by the measurement of forced expiratory volume in one second (FEV₁).

AHR is a clinical feature of asthma, which can be assessed during bronchial provocation tests using direct or indirect stimuli [23]. Direct stimuli, including methacholine and histamine, induce contraction by direct interaction with receptors on ASM. Indirect stimuli, such as mannitol, adenosine or allergens, act on inflammatory cells present in airways from persons with asthma, which in turn release mediators that induce smooth muscle contraction. These indirect stimuli have been shown to activate mast cells or increase levels of mast cell mediators in the body following stimulation [24, 25].

It has been suggested that the components that cause AHR can be divided into two categories, persistent or variable [23, 26]. The persistent component of AHR is suggested to be composed of structural changes of the airways, such as basement membrane thickening, smooth muscle hypertrophy/hyperplasia or collagen deposition. These changes lead to increased airway wall thickening and are best assessed when using direct stimuli. Airway remodelling has the possibility to lead to both a stronger contraction to the same stimuli (involvement of ASM) but also to an increased baseline airway narrowing. On the other hand, the variable component is thought to be related to inflammatory events that vary over time and is best assessed using indirect stimuli. The persistent component appears to be less affected by environmental exposure and less responsive to inhaled glucocorticosteroids, which might reflect the chronic nature of the disease. This classification is almost certainly an oversimplification and interplay between the different categories likely exists. It has also been suggested that the cause of AHR can be hyperreactive muscle cells that contract more readily to the same
stimuli which could be induced by different substances, such as IL-13 [27]. ASM from individuals with asthma have shown to be hyperresponsive when studied in vitro [26]. It is clear that the mechanisms that underlie AHR are complex and the understanding of them remains incomplete.

1.5 AIRWAY INFLAMMATION

Inflammatory processes in the body exist to protect us from dangerous stimuli, such as pathogens and toxins and are an aspect of wound healing. Inflammation can also lead to tissue damage, which is why inflammation is a strictly regulated process. In the case of asthma the immune system responds aberrantly to non-dangerous particles, such as allergens or other environmental stimuli [22]. The inflammatory response in asthma can be divided into two categories, acute and chronic.

Acute inflammation appears after exacerbations [22]. In response to a trigger signal, tissue resident cells, such as mast cells, dendritic cells and macrophages become activated and induce an immune response. Various mediators that are released recruit other immune cells, such as lymphocytes, eosinophils and neutrophils, as well as facilitate their migration into the tissue by upregulating adhesion molecules on vascular endothelial cells. The mediators also activate and influence the biology of innate and adaptive immune cells. Several inflammatory cell types release IL-13, which is thought to affect ASM contractility [27]. Inflammation also affects structural cells, such as goblet cells that secrete mucus into the airways and thus participate in the protective response against contaminants entering the lung. Additionally, structural cells release mediators that influence inflammatory cells [15], thus creating a feedback loop between the immune and the structural cells. The quantity and type of acute inflammation can be assessed using non-invasive methods, such as in induced sputum, following provocation tests or during natural periods of exacerbations [28]. These periods can, for example, be during the pollen season for individuals suffering from allergic asthma with a pollen allergy. The acute inflammation is thought to mainly influence the variable part of AHR.

When exposure to the trigger is persistent over a sustained period of time, chronic inflammation appears [22]. The inflammatory pattern during chronic inflammation might be different and less variable than acute inflammation and can be sustained without triggering events [22, 28]. Chronic inflammation is associated with airway damage and remodelling, since the repair processes are constantly ongoing. Biopsies from patients with well controlled asthma that are not suffering from exacerbations can provide a picture of the current state of chronic inflammation. Chronic inflammation is thought to affect the persistent phase of AHR, since it is associated with remodelling.

Different phenotypes of asthma display diverse patterns of lung inflammation, in which the type of inflammatory cells that are present and site of inflammation differ [10]. Allergic asthma displays a typical T\textsubscript{H}2-type inflammation, with the presence of cytokines, such as IL-4, IL-5 and IL-13, and a predominant eosinophilic infiltration. Other phenotypes of asthma may display more of a T\textsubscript{H}17-type inflammation, with increased levels of IL-8 and IL-17 and neutrophil infiltration.
1.6 AIRWAY REMODELLING

Airway remodelling refers to structural changes of the airways, such as an increased number of goblet cells, areas of tissue damage and repair within the epithelium, increased matrix deposition and hypertrophy/hyperplasia of ASM [22, 29]. These changes result in the occurrence of mucus plugs within the airway lumen and increased airway wall thickness. Airway remodelling can lead to persistent airway narrowing and ongoing increased airflow obstructions that are not reversible with treatment. Airway remodelling has also been shown in studies of fatal asthma in which abnormal alveolar attachment and decreased elastic fibre content were present in the periphery, which could increase lung elastance and promote airway closure [30, 31].

Airway remodelling has long been suggested to appear due to chronic inflammation induced by recurrent tissue damage and repair, and also in response to prolonged exposure to inflammatory mediators. As an example, TGF-β has been shown to activate sub-epithelial mesenchymal cells, which then proliferate and release matrix components [32]. Recently, it has also been shown that remodelling can occur as a consequence of repeated ASM contraction, even in the absence of inflammation [33]. ASM hyperplasia/hypertrophy appears in the airways of asthmatics [34]. In addition to the thickening of the airway wall, it has also been suggested that an increased muscle mass could lead to enhanced ASM contraction[35]. These changes result in increased persistent AHR.

1.7 INTERACTIONS BETWEEN MAST CELLS AND AIRWAY SMOOTH MUSCLE CELLS

Both mast cells and ASM each produce and secrete mediators that have a role in asthma pathology [36]. They also form mediators that have the potential to directly affect each other. Mast cells release many mediators that have the potential to act as bronchoconstrictors, such as histamine, prostaglandin D2 and leukotriene C4. Tryptase has been shown to induce bronchoconstriction in sheep and to enhance contraction to histamine and induce proliferation of human ASM cells in vitro. Mast cells secrete cytokines, such as IL-13, that have been shown to increase contractility to acetylcholine [37]. IL-4 and TNF-α induce AHR and pharmacological interference with TNF-α reduces AHR in patients with asthma [36]. Tryptase, activin A, TNF-α, PDGF and TGF-β, all possible mast cell mediators, have been shown to induce ASM cell proliferation [38]. ASM cells on the other hand release chemokines, such as CXCL10 and stem cell factor, that act predominantly to attract mast cells and promote their growth [37, 39]. Mast cells have also been found to adhere to ASM cells via TSCLC1 [36]. Lately, several studies have shown that mast cell numbers within the smooth muscle cell layer are increased in association with airway obstruction in people suffering from asthma [40-42]. The significance of this phenomenon is not yet known. However, since both mast cells and ASM cells have been shown, by themselves, to be significant cells in asthma pathogenesis, the interaction between these cell types could be of great importance.
1.8 INTERLEUKIN 33

IL-33 belongs to the interleukin 1 family of cytokines and was first described in 2005 [43]. Two year prior, it had been characterised as a nuclear factor expressed in lymph node-associated endothelial cells and was then called NF-HEV [44]. IL-33 is expressed in many different cell types, but it is primarily found in structural cells, such as epithelial cells and ASM [45, 46]. IL-33 is released following necrosis or cell damage, and is thought to act as a molecule that signals danger [47, 48]. IL-33 also appears to be released in the absence of cell damage, for example inhalation of the allergen Alternaria alternata causes increased levels of IL-33 in BALF without elevation of lactate dehydrogenase which is used as a measure of cell damage [49]. Full-length IL-33 is biologically active, but while cleavage by apoptotic caspases has been shown to inactivate the biological activity of IL-33 [48, 50], cleavage by neutrophil-derived proteases has been shown to amplify its bioactivity [51]. IL-33 binds to its receptor IL1RL1 (also termed ST2) and signals via this receptor along with its co-receptor IL-1RAcP [52]. The IL1RL1 receptor exists in both a transmembrane as well as a soluble form and the soluble form has been suggested to inhibit IL-33 activity by binding free IL-33 which in turn prevents its binding to the transmembrane receptor [53]. Recently, it has also been suggested that an additional receptor component is involved in the signalling process since a recombinant soluble receptor did not succeed in inhibiting IL-33-induced cytokine production in some of the tested cell types [54]. As a result of receptor signalling, downstream activation of signalling molecules, such as NFκB [43] is initiated. Numerous cell types, such as T\(_H\)2 cells, mast cells and eosinophils have been reported to be activated by IL-33 [55]. Activation by IL-33 was initially described to result in the production of T\(_H\)2-type cytokines, but it has also been shown that T\(_H\)1-type cytokines can be produced following cellular activation by IL-33 [56].

Polymorphisms in the genes for IL-33, its receptor or in the downstream signalling proteins have, during recent years, been associated with asthma development and disease severity in several genome wide association studies [5, 6, 57]. In individuals suffering from asthma the epithelial barrier has been reported to be impaired and epithelial damage is common in the airways [58, 59]. This means that the underlying tissue is more readily accessible to invading foreign particles, such as allergens. Several common allergens, such as house dust mite, Aspergillus fumigatus and Alternaria alternata have been shown to induce the release of IL-33 [60-62]. This might constitute one explanation as to why atopy does not always lead to the development of allergic asthma [63]. Increased expression and elevated levels of IL-33 in bronchoalveolar lavage fluid (BALF) has been described in patients with asthma [64]. In addition, the soluble form of IL1RL1 in serum and the expression of the transmembrane form on fibrocytes have been shown to be increased in association with asthma exacerbations [65, 66].

IL-33 levels have also been linked to airway remodelling in humans. Expression of IL-33 is increased in endobronchial biopsies from children with severe therapy-resistant asthma and is correlated with thickening of the reticular basement membrane [67]. In addition, IL-33 administration to mice has been shown to induce AHR, assessed following administration of methacholine [68].
Several different cell types of the immune system that are present in the lung during asthmatic conditions have been shown to respond to IL-33 [55]. Macrophages have the possibility both to respond to IL-33 and to produce it. Macrophages increase the expression of IL-33 in response to TLR3 and TLR4 agonists. Macrophages, basophils, group 2 innate lymphoid cells (ILC2), dendritic cells and Th2 cells have all been shown to respond to IL-33 leading to an enhanced Th2-type immunity. Eosinophils release inflammatory mediators, such as CXCL8, in response to IL-33. Mast cells have also been shown to be activated by IL-33 followed by the release of different cytokines, such as IL-5, IL-13 and CXCL8 [69, 70]. In addition, IL-33 promotes maturation, survival and adhesion to fibronectin of mast cells [69] as well as increasing their tryptase expression [71]. Thus, IL-33 has been linked to ASM cells and mast cells, both of which have been shown to be associated with AHR, inflammation and remodelling, all characteristic features of asthma.

1.9 ANIMAL MODELS

Asthma is a complex disease and only when performing studies on human subjects with asthma can all the aspects of the disease be considered as a whole [72]. However, for obvious ethical and practical reasons, experiments using human subjects are limited in scope and detailed studies to understand disease mechanisms can be difficult or impossible to perform. Basic mechanisms can be modelled and investigated in various in vitro systems, but the use of animal models is the only approach available for studies of in vivo processes in an intact organism [64].

Very few animals naturally and spontaneously develop a syndrome similar to asthma [72]. Therefore an artificial asthmatic reaction must be generated in animals, typically through the induction of allergic asthma. Mice are the most commonly used animal in asthma research today due to readily available genetically modified animals and the widespread availability of experimental reagents, such as recombinant proteins and tools to study gene and protein expression. Although, considerable differences exist between humans and mice concerning airway physiology, such as airway anatomy and the pharmacological response to drugs, the general biology and molecular mechanisms are similar between the two species [73]. Indeed, using animal models have provided novel mechanistic insights into different aspects of allergic asthma [74].

The guinea pig has also been used for many years in asthma research, but these studies have largely been focused on the pharmacological aspects of the disease, in which the guinea pig is a superior model compared to mice. Reasons for this are primarily due to the anatomical and physiological likenesses between humans and guinea pigs compared to the mouse, especially with respect to airway branching, pulmonary circulation, smooth muscle distribution and pharmacological reactions as well as mast cell localization and secretion [75]. However, due to the paucity of genetically modified guinea pigs strains and pharmacological tools available for this animal, as well as, the less well described inflammatory and immune systems compared to mice, guinea pigs have not been as attractive as a model for researchers attempting to understand asthma pathogenesis and progression.
Ideally, when using animal models to study the course of any given disease, one should have an understanding of the disease etiology [73]. In asthma studies using human subjects, little has been revealed about the initiation of the disease, since in most cases individuals being studied have already developed asthma. As such, it has been challenging to develop relevant animal models of asthma that serve as a true representation of the disease. Instead, progress has been made in recapitulating aspects of the disease, such as lung inflammation, AHR and remodelling. Considering that asthma is now emerging as a complex disease consisting of multiple phenotypes [10], animal models that mimic specific phenotypes or features of the disease may assist in furthering our knowledge of different asthma mechanisms. Perhaps, an appropriate approach going forward is to identify, in humans, molecules that are relevant to asthma pathogenesis, and to study their effects in relevant animal models [72]. Naturally, discoveries in animal models must be interpreted with caution and must always be confirmed in humans.
2 AIMS OF THE THESIS

The overall aim of the thesis was to investigate and describe interactions taking place between mast cells and airway smooth muscle cells during the establishment and subsequent disease progression in asthma models.

Specific aims:

**Paper I:** To investigate how the number and distribution of pulmonary mast cells can influence airway hyperresponsiveness and inflammation in a mouse model of chronic allergic asthma.

**Paper II:** To elucidate which mast cell mediators are being released during the early allergic reaction that cause airflow obstructions *in vivo* in a guinea pig model of allergic asthma.

**Paper III:** To examine how IL-33 can affect the early allergic reaction and the resultant smooth muscle contractions and airflow obstructions in a mouse model of allergic sensitisation.

**Paper IV:** To investigate how IL-33 can influence common features of asthma, such as AHR, inflammation and remodelling, in two distinct mouse models of allergic asthma.
3 METHODOLOGY

This is a summary of the different methods used in the studies of this thesis. Detailed descriptions of the methods used herein are included in the materials and methods sections of the publications and manuscripts included in this thesis.

Animals
Mice used in this thesis were C57BL/6, BALB/c, genetically mast cell-deficient C57BL/6-Kit<sup>W-sh/W-sh</sup> and IL-33 receptor-deficient BALB/c-T1/St2-deficient mice. All animal handling and experimentation was conducted in accordance with ethical permits approved by the Regional Committee of Animal Experimentation Ethics (Stockholm, Sweden).

Experiments from study II using Dunkin–Hartley guinea pigs were performed in the United States of America and were conducted in accordance with local Janssen Institutional Animal Care and Use Committee (IACUC) guidelines under an approved animal use protocol.

Mast cell cultures
To generate bone marrow-derived mast cells (BMMC), bone-marrow cells from C57BL/6 mice were differentiated for approximately five weeks in vitro. The cells were then cultured in the presence of IL-3 as well as stem cell factor (SCF) in some experiments. The cells were used in experiment when more than 95% stained positive with toluidine blue.

Mast cell engraftment
For mast cell engraftment, mast cell-deficient C57BL/6-Kit<sup>W-sh/W-sh</sup> mice were injected intravenously with in vitro differentiated BMMC (5 x 10<sup>6</sup> cells/mouse). The mice were used in experiments 12 weeks following the engraftment.

Protocols to induce different models of allergic asthma combined with interventions
A variety of protocols were used in the different studies. In study I (Fig 1), diclofenac sodium was given 1 h before each OVA-challenge and 1 h before the methacholine challenge via intraperitoneal injection, and as an intravenous injection at the start of the anaesthesia. The protocols for study II (Fig 2), study III (Fig 3) and study IV (Fig 4) are also depicted below.

<table>
<thead>
<tr>
<th>Antigen sensitization</th>
<th>Antigen challenge</th>
<th>Endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>10µg OVA / 1mg alum i.p.</td>
<td>50 µg OVA i.n.</td>
<td>i.p. and i.n.</td>
</tr>
<tr>
<td>Day</td>
<td>1</td>
<td>11</td>
</tr>
</tbody>
</table>

Figure 1. The experimental protocol used in study I to induce a model of allergic asthma in mice.
Figure 2. The experimental protocol used to induce a model of allergic asthma in guinea pigs in study II. The pharmacological treatments were intraperitoneal injections of pyrilamine, montelukast and indomethacin.

Figure 3. The experimental protocol used to induce allergic sensitisation in mice, and time points for cytokine administrations in study III.

Figure 4. The experimental protocols used to induce a model of allergic asthma in mice in study IV. Time points when IL-33 was administered are indicated in grey. a) The classical OVA/alum protocol used on BALB/c mice. b) The mast cell-dependent protocol used on C57BL/6 mice.
Measurements of airway smooth muscle contraction in isolated airways
To measure airway smooth muscle contraction \textit{ex vivo}, isolated airways from OVA-sensitised mice were placed between two mounting hooks in a myograph. The force applied to one of the hooks when the tissue contracted was recorded. A typical recording is shown below (Fig 5). To confirm tissue viability, the tissue was exposed to KCl three times. To measure contraction of smooth muscle during the early allergic reaction, the tissue was exposed to OVA in the presence or absence of ketanserin. To measure the maximal contraction of the tissue in response to serotonin, the primary mediator causing smooth muscle contraction during the early allergic reaction, increasing doses of serotonin were added to the bath. Finally, to measure maximal contraction, the tissue was exposed to increasing doses of carbachol in the presence of indomethacin.

![Figure 5](image)

\textit{Figure 5.} A typical trace of a recording of the force (mN) generated by the tissue during smooth muscle contraction of isolated airways \textit{ex vivo}.

\textit{In vitro} treatment of mast cells
Sensitised mast cells were cultured with or without IL-33 for four days. The cells were then activated by IgE-receptor crosslinking or lysed followed by collection of cell free supernatant. The cell pellet was also collected.

\textit{Ex vivo} Treatment of isolated airways
Airways from OVA-sensitised mice were cultured with OVA for 30 minutes \textit{ex vivo} to activate mast cells. Subsequently, the cells in the tissue were lysed with Triton X-100.

Measurements of $\beta$-hexosaminidase ($\beta$-hex) release
Levels of $\beta$-hex were measured in supernatants from cells and isolated airways. Supernatants were incubated with 4-Nitrophenyl N-acetyl-$\beta$-D-glucosaminide, the reactions were then stopped with glycine and the absorbance of the supernatants was read at 405nm.

\textit{In vivo} lung mechanic measurements
In this thesis we used an invasive method to measure lung mechanics in anesthetised, tracheotomised and ventilated animals. This method uses a forced-oscillation technique to convert physiological responses into changes in different test signals, such as pressure, volume and flow signals. Two different mathematical models have been used to characterise the complex relationships between these test parameters. In studies I and II, where we measured lung mechanics in mice and guinea pigs we applied the single compartment model, where test signals are assessed following forced oscillation with a single frequency. Using this model, lung resistance which reflects both narrowing of conducting airways and changes in the tissue, as well as compliance which reflects
mainly changes in the peripheral tissue, can be calculated. In study I, III and IV we applied the constant phase model, where forced oscillations of three prime wave frequencies are used. Using this model, physiological events occurring in the conducting airways can be discriminated from events occurring in the peripheral tissue. The parameters that were assessed from these measurements were Newtonian resistance \( (Rn) \), reflecting narrowing of the conducting airways, tissue damping \( (G) \) which reflects energy dissipation in the lung and tissue resistance \( (H) \), which reflects energy storage in the tissue (tissue stiffness).

In study I, II and IV, lung mechanics were measured following administration of increasing doses of methacholine to assess AHR. In study II and III lung mechanics were measured during the early allergic reaction. To induce an early allergic reaction OVA was administered to OVA-sensitised animals. Methacholine and OVA were administered either as an aerosol or by intravenous injections.

**Real-Time Quantitative PCR**
To measure gene expression of \( \text{Tph-1} \) in mast cells and of \( \text{Col1a1, Col3a1 and Col5a1} \) in lung tissue, total RNA was extracted from the cell pellet or tissue. cDNA was synthesised from total RNA, followed by an amplification with specific primer pairs and was analysed using real-time quantitative PCR.

**Histology**
Thin sections (5µm) from paraffin embedded lung tissue and isolated airways were stained with specific stains to detect different structures within the tissue. Mast cells, inflammatory cells and collagen were visualised in this manner. Smooth muscle cells and different mast cell phenotypes were stained using immunohistochemistry, in which specific proteins within the cell were detected using specific antibodies. Quantification was performed by counting the cells, semi-quantitative grading or by measurement of stained area using a software program.

**Differential cell counts**
Cells from BALF were plated on glass slides followed by staining and subsequent quantification of different inflammatory cell populations.

**Enzyme linked immunosorbent assay (ELISA)**
The levels of serotonin were measured in supernatants from cells and isolated airways using ELISA. OVA-specific IgE in serum and IL-17A levels in lung homogenate were also measured using ELISA.

**Multiplex assay**
Quantification of cytokines and chemokines in lung homogenates was performed using a multiplex technique, Luminex.

**Statistical analysis**
D’Agostino and Pearson omnibus normality test was used to test if the data came from a Gaussian distribution. ANOVA (one- or two-way) or Kruskal-Wallis followed by Bonferroni’s post correction were used to compare differences between three or more groups. A t-test or the Mann-Whitney test were used when comparing differences.
between two groups. Fisher’s exact test or Chi-square tests for trends were used to analyse qualitative data. A P-value of < 0.05 was considered statistically significant.
4 RESULTS AND DISCUSSION
4.1 MAST CELL ENGRAFTMENT OF THE PERIPHERAL LUNG ENHANCES AIRWAY HYPERRESPONSIVENESS IN A MOUSE ASTHMA MODEL (STUDY I)

Inflammation and AHR are characteristic features of asthma [1]. Mast cells play an important role in the allergic asthmatic response and increased mast cell numbers in different compartments within the lung have been associated with enhanced disease symptoms [40, 76-78]. Since the role of mast cells in asthma are not fully understood, we wanted to investigate how mast cell numbers and distribution can influence AHR and inflammation in a mouse model of chronic allergic asthma.

To do this, we used C57BL/6 mice (wt) with endogenous mast cell numbers and distribution, mast cell deficient Kit\textsuperscript{W-sh/W-sh} mice (Wsh) that are completely devoid of mast cells, and mast cell-deficient mice that we engrafted with bone-marrow derived mast cells (Wsh+MC) differentiated \textit{in vitro}. When we compared the number and distribution of pulmonary mast cells between the different groups, we could confirm that mast cells were absent in Wsh mice (Fig 6a). In wt mice there were few mast cells, and these cells were located within the perivascular space adjacent to the central airway or within the submucosa around the central airway itself (Fig 6a-b), which is consistent with previous findings [79]. Furthermore, Wsh+MC mice had more than a 15-fold increase in the total number of mast cells compared to wt mice and the majority of the cells were located in the parenchyma and in the perivascular space. Thus, our results support other studies that have shown that reconstitution of Wsh mice with mast cells leads to an increased number and a more peripheral distribution of mast cells [80-82].

![Figure 6. Pulmonary mast cell numbers. a) Total mast cell numbers/lung section. wt, C57BL/6 mice; Wsh, mast cell deficient Kit\textsuperscript{W-sh/W-sh} mice; Wsh+MC, Wsh mice engrafted with mast cells. Results are expressed as mean ± SEM, or mean. * P<0.05. b) Proportion of mast cells in different compartments of the lung in mice that received either PBS or OVA intranasally.](image-url)
A phenomenon that was seen in Wsh+MC mice, but not in wt mice, was a shift towards a more peripheral distribution of mast cells in OVA/alum sensitised mice that received intranasal OVA challenges compared to those that received intranasal PBS. The absence of OVA-induced mast cell redistribution in wt mice in our study agrees with previous findings in C57BL/6 mice, despite a previously observed OVA-induced mast cell increase in BALB/c mice [83]. Although the mechanism behind the redistribution of mast cells in our study is not known, redistribution of pulmonary mast cells, as we observed in Wsh+MC mice following OVA challenge, has also been described previously in individuals suffering from asthma [40, 84, 85]. With respect to mast cell-specific protease expression as assessed by immunohistochemistry, we did not observe any differences between the groups, suggesting that the phenotype is not altered following engraftment. We have therefore confirmed that the number of pulmonary mast cells increases in Wsh+MC mice and that these mast cells populate the parenchyma following engraftment, a location in which they are normally not found in wt mice. In addition to this we have detailed the number of mast cells in different compartments of the lung before and after OVA challenge. The findings regarding the redistribution of pulmonary mast cells following OVA challenges could have important implications when studying mast cell relocation in individuals suffering from asthma.

Lung mechanics measurements were performed in vivo to establish if any of the experimental groups showed a different pattern of AHR following administration of intravenous methacholine. The assessment of total lung resistance measurements indicated that resistance was increased in all experimental groups that had received OVA challenges compared to those that had received PBS. In fact, the resistance was increased to the same extent in both wt and Wsh mice, supporting results from previous studies showing that sensitisation with alum does not cause a mast cell-dependent AHR [86, 87]. However, following methacholine administration, Wsh+MC mice showed a further increase in total lung resistance compared to both wt and Wsh mice, suggesting that the increased number or relocation of mast cells influences AHR in this protocol. In order to distinguish between responses in the conducting airways from those in the peripheral parts of the lung, the constant phase model of lung mechanics was applied. Similar results as those observed in total lung resistance were seen when studying resistance of the conducting airways (Newtonian resistance; \(R_n\)). The most prominent increase in \(R_n\) was seen in Wsh+MC mice and we were also able to detect an increase in conducting airway resistance in Wsh mice compared to wt mice (Fig 7a). The mechanism behind this increase was investigated by studying responses in mice treated with the non-selective cyclooxygenase inhibitor diclofenac, which prevents prostanoid production. Wt mice treated with diclofenac had an increased resistance in conducting airways compared to untreated wt mice, showing that the removal of prostanoid production in the airways leads to increased resistance. These data suggest that protective prostanoids are present in the airways of wt mice following challenge with OVA and support previous studies showing that a release of prostanoids provides a partial protection from the development of AHR [88-90]. Since mast cell engraftment in Wsh mice does not lead to mast cell reconstitution of the most proximal airways, such as the trachea [80], it is possible that the proximal mast cells that are only present in wt mice possess a different pattern of prostanoid release, generating a dampening effect on AHR. There is also a possibility that the absence of mast cells during development in Wsh mice could cause other cells to alter their pattern of prostanoid
expression. This hypothesis is supported by a previous study showing that treatment with diclofenac during the OVA-challenge phase does not affect AHR. On the other hand, if diclofenac is only administered before methacholine provocation, the AHR is enhanced [90], suggesting a constitutive release of prostanoids that is not affected by OVA challenge. The enhanced resistance that we observed in Wsh+MC mice compared to Wsh mice, could be explained by the increased number of mast cells in the smaller airways of these mice. Hence, mast cell number, distribution, and importantly also prostanoid release, seems to influence AHR in the conducting airways.

**Figure 7.** Assessment of AHR following administrations of intravenous methacholine, in OVA/alum-sensitised mice. *a*) Newtonian resistance \((R_n)\), resistance in the conducting airways, *b*) tissue damping \((G)\) and *c*) tissue elastance \((H)\). wt, C57BL/6 mice; Wsh, mast cell deficient KitW-sh/W-sh mice; Wsh+MC, Wsh mice engrafted with mast cells. Results are expressed as mean ± SEM. *P < 0.05.

In addition, assessment of the peripheral lung mechanics (tissue damping; \(G\) and tissue elastance; \(H\)) showed that peripheral AHR was most pronounced in Wsh+MC mice (Fig 7b-c). Both wt and Wsh mice had an increase in \(G\) suggesting that this effect is mast cell independent. However, Wsh+MC mice displayed an even further increase in tissue damping, implying that mast cells can affect some aspects of peripheral resistance. Furthermore, the only OVA-challenged experimental group with an increased tissue elastance, compared to the PBS challenged group, were the Wsh+MC mice. The increased tissue elastance in this group could be blocked with diclofenac, suggesting that this effect could be mediated by PGD\(_2\) or TXA\(_2\), mast cell-derived prostanoids that have previously been shown to be involved in the pathogenesis of
asthma [91]. The stronger effects on AHR measured in the periphery, compared to those measured in the conducting airways in Wsh+MC mice, suggest that the numerous parenchymal mast cells present in this group are responsible for this effect. Taken together, these results show that mast cell numbers, distribution and prostanoid release can affect both the degree and localisation of AHR. Our findings also indicate that AHR can be both mast cell independent as well as dependent within the same mouse model of allergic asthma.

To determine the level of allergic sensitisation in the different groups, OVA-specific IgE was measured. Both wt and Wsh mice, challenged with OVA, had increased levels of OVA-specific IgE, further supporting previous findings that induction of specific IgE occurs independent of mast cells [86]. However, OVA-specific IgE was further increased in Wsh+MC mice, showing that although the induction of specific IgE is not mast cell dependent, the levels of specific IgE can be influenced by mast cells. The possibility exists that the increased number of mast cells in the lymph nodes following engraftment of mast cells to Wsh mice, as shown previously [80], could explain the upregulation of OVA-specific IgE, since physical contact between mast cells and B-cells promotes maturation of B-cells into plasma cells [92]. Furthermore, administration of IgE has been shown to upregulate the expression of the IgE receptors on mast cells and basophils, causing an increased release of cell mediators following IgE-receptor crosslinking [93, 94]. As increased OVA-specific IgE levels were associated with an enhanced AHR in Wsh+MC mice, this offers an additional explanation as to how mast cells can contribute to the enhancement of AHR in these mice. In conclusion, OVA-specific IgE levels seem, in the same way with AHR, to be both mast cell dependent and independent in the same chronic mouse model of allergic asthma.

Because the protocol used in this study has been previously shown to induce chronic inflammation and remodelling [95], which are both characteristic features of asthma, we wanted to study if these factors also were affected by mast cell numbers and distribution in our model. There was a similar inflammatory cell infiltration in BALF and in lung tissue following OVA challenge in all groups of mice, further supporting previous studies showing that sensitisation with alum does not lead to a mast cell dependent inflammatory response [86, 87]. This finding also indicates that inflammation does not influence AHR in this protocol, a phenomenon that also holds true in individuals suffering from asthma [96, 97]. Neither collagen deposition nor ASM thickness circumscribing the airways were increased in any of the OVA challenged groups compared to PBS challenged groups, showing that the protocol used herein did not induce remodelling, irrespective of the presence of mast cells. The lack of remodelling in our study likely reflects our use of C57BL/6 mice in lieu of BALB/c mice that have previously been shown to develop signs of remodelling [95], which may be a limitation of this protocol. These findings show that the inflammation induced in this model is mast cell independent and that remodelling does not occur irrespective of the presence of mast cells. Thus, this further supports the notion that AHR can be induced independently of airway inflammation or structural changes.

To evaluate the inflammation induced in our model further, we also measured the levels of inflammatory cytokines in lung homogenate. The levels of CXCL1, CXCL10 and IL-5 were increased in all OVA-challenged groups compared to PBS challenged
groups, indicating a mixed granulocytic type of inflammation, which reflects the chronic challenge protocol used in this study. Irrespective of the presence of mast cells, there was no difference between the experimental groups, indicating that the levels of these cytokines were not affected by mast cells. The levels of IL-17 and IL-33, cytokines that have been associated with asthma [98, 99], were, however, mast cell dependent. IL-17 was increased in wt and Wsh+MC mice challenged with OVA compared to PBS challenged mice (Fig 8a). The level of IL-17 was, however, lower in the Wsh+MC group compared to the wt group, which could be explained by the higher level of IL-23 in the wt group, a cytokine that has been associated with promotion of IL-17 production [100]. Since intranasal delivery primarily deposits OVA in the upper airways [101] and the wt group is the only group that has mast cells located in the proximal airways, it might be expected that this group has higher levels of IL-23 and IL-17. IL-33 was increased in all OVA challenged groups compared to those that were challenged with PBS, and further increased in wt and Wsh+MC mice compared to Wsh mice (Fig 8b). Despite the fact that the levels of IL-17 and IL-33 could be linked to the presence of mast cell, these cytokines could not be associated with the development of AHR in this model.

**Figure 8.** Levels of inflammatory cytokines measured in lung homogenate from OVA/alum-sensitised mice. a) Levels of IL-17. b) Levels of IL-33. wt, C57BL/6 mice; Wsh, mast cell deficient KitWsh/Wsh mice; Wsh+MC, Wsh mice engrafted with mast cells. Individual values and group means are shown. *P < 0.05 comparing allergen challenged groups and #P < 0.05 comparing OVA challenged groups to the corresponding group that were challenge with PBS.

In this study we have described that both AHR and specific IgE levels can be mast cell dependent as well as independent in the same mouse model of chronic allergic asthma. We have further demonstrated that mast cell engraftment causes an increase in the number of pulmonary mast cells, especially at peripheral locations, and this is associated with an increase in AHR that was strongest in the parameters associated with peripheral lung mechanics. Mast cell number, distribution and prostanoid release were associated with AHR at different locations of the lung. Cellular inflammation was mast cell independent and not associated with AHR, which corresponds to findings in individuals with asthma. Although levels of IL-17 and IL-33 were mast cell dependent, they were not linked to the severity of AHR. Since elevated numbers of mast cells at
different pulmonary locations have been correlated to decline in lung function in asthma sufferers [84], this model might offer an advantage compared to other existing models since it both morphologically and functionally more closely resembles finding in humans.

In an effort to further explore the role of mast cell number and distribution on AHR and inflammation, we attempted to engraft C57BL/6 mice with BMMC. Since Wsh mice have additional abnormalities aside from mast cell deficiency, such as increased neutrophil, basophil and platelet numbers [102], engraftment of C57BL/6 mice would create a model that has the advantages of the Wsh+MC model described above, but that lacks the abnormalities found in Wsh mice. However, MC engraftment of OVA/alum-sensitised C57BL/6 mice did not result in increased pulmonary mast cell numbers compared to those not engrafted, irrespective of OVA challenge. This suggests that the signalling pathway that recruits BMMC to the lung in Wsh mice, following injection, does not exist in C57BL/6 mice. In addition, we also tried to develop the model by administering the BMMC via intranasal instillation, to achieve a local engraftment in the lung and thus reducing the abnormal mast cell numbers in other tissues as can occur following intravenous injections [80, 81]. However, administration of BMMC intranasally in Wsh mice did not result in any mast cells entering the lung tissue, as assessed by histology. Although it has been shown previously that eosinophils have the capacity to enter the lung tissue following intratracheal instillation [103-105], these cells are much more mobile compared to mast cells, which tend to be static tissue resident cells and it is not clear if mast cells have the capacity to enter the lung tissue through the epithelial barrier.
4.2 MAST CELL MEDIATORS CAUSE EARLY ALLERGIC BRONCHOCONSTRICTION IN GUINEA-PIGS *IN VIVO*: A MODEL OF RELEVANCE TO ASTHMA (STUDY II)

The early allergic reaction is an important feature to consider when studying allergic asthma. This reaction is initiated in sensitised individuals following exposure to an allergen. This is followed by degranulation of mast cells and the release of mediators that induce airway smooth muscle contraction and epithelial mucus secretion with the concomitant acute airflow obstructions. Mast cell mediators of a different kind compared to those found to be responsible for smooth muscle constriction during the early allergic reaction in humans, are found in the commonly used mouse models of allergic asthma. The early allergic reaction in guinea pigs, however, has been shown to be mediated via the same mast cell mediators as in humans *ex vivo*. We therefore chose to study the mast cell mediators involved in the early allergic reaction in a guinea pig model of allergic asthma *in vivo*.

In this study, using the same method to measure lung mechanics as in study I, we observed a prominent increase in lung resistance following intravenous administration of OVA in sensitised guinea pigs (Fig 9a). This phenomenon has previously been shown to occur *in vivo*, using non-invasive plethysmography [106–109]. Importantly, sensitised and challenged guinea pigs also displayed AHR following administration of increasing doses of aerosolised methacholine, as well as, lung inflammation as assessed by histology. The presence of AHR and inflammation demonstrates that the model used in this study, in addition to the development of the early allergic reaction, also presents other features of allergic asthma.

![Figure 9](image-url)

**Figure 9.** Traces showing mean airway resistance. a) Airway resistance measured during the early allergic reaction following administration of aerosolized OVA and assessment of AHR following aerosolized administration of methacholine (0.3 mg/ml). b) Airway resistance measured during the early allergic reaction following administration of OVA, with or without the H₁ receptor antagonist pyrilamine, given via intraperitoneal injection. c) Airway resistance measured during the early allergic reaction following administration of OVA, with or without the CysLT₁ receptor antagonist montelukast, given via intraperitoneal injections.

However, the kinetics of the responses we observed following OVA and methacholine challenges were different. While methacholine administration immediately induced an increase in lung resistance that rapidly diminished, the response following OVA
administration did not reach maximum resistance until after three minutes and then slowly declined during the following 20 minutes. This shows that methacholine acting directly on ASM quickly induces a transitory contraction compared to the OVA response, in which the release of mast cell mediators induces a more delayed, but longer lasting ASM contraction.

To investigate the mast cell mediators involved in the early allergic reaction in this model we used inhibitors of: histamine (pyrilamine, histamine H₁ receptor antagonist), cysteinyl-leukotrienes (CysLT) (montelukast, CysLT₁ receptor antagonist) and prostanoids (indomethacin, non-selective COX inhibitor). No single treatment with any of the inhibitors had any significant effect on the amplitude of lung resistance during the early allergic reaction. Thus, no single mast cell mediator alone is responsible for the early allergic reaction in vivo. This finding is supported by a previous study where contractions in isolated guinea pig lung parenchyma were measured ex vivo [110]. However, inhibition of the histamine response did however delay the time to peak and there was a trend showing that inhibition of the CysLT response decreased the time to peak (Fig b-c). These data confirm previous results showing that histamine is responsible for the initial response and that CysLT are responsible for the sustained response during the early allergic reaction [110-113]. Interestingly, double inhibition of both histamine and CysLT significantly reduced lung resistance during the early allergic reaction (Fig 10a), suggesting that these substances are the primary mediators causing airflow obstructions during the early allergic reaction in guinea pigs. These mediators have also been shown to be the most important mast cell mediators leading to airflow obstructions following allergen challenges in humans suffering from allergic asthma [114-116]. Only treatments that included inhibition of histamine delayed the time to peak response and this was found to be delayed to the same extent as histamine inhibition alone, indicating that there are no synergistic effects on this component of the early allergic reaction when the treatments are combined.

**Figure 10.** Airway resistance measured during the early allergic reaction following OVA administration, expressed as area under the curve. a) Dual treatment with pyrilamine and montelukast. b) Dual treatment with pyrilamine and indomethacin. c) Triple treatment with pyrilamine, indomethacin and montelukast. Results are expressed as mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001.

Dual inhibition of histamine and prostenoids led to a tendency of reduced lung resistance (Fig 10b), but a triple inhibition of all three mediators did not show any significant reduction of lung resistance compared to the dual inhibition of histamine
and CysLT (Fig 10c), showing that prostanoids appear to have a minimal role on the early allergic reaction in this model. These findings are in contrast with a previous study performed in the isolated, perfused and ventilated guinea pig lung ex vivo, which showed that prostanoids are important for smooth muscle constriction during the early allergic reaction [117]. The conflicting findings between the two studies could be attributed to dissimilarities between responses ex vivo and in vivo. The different responses could also be a consequence of the differences in protocols. In our study, we challenged the airways of the sensitised guinea pigs with OVA, which could cause an airway inflammation consisting of a more complex release of contractile and relaxant prostanoids [117-119], during the early allergic reaction. It is possible that the replacement of indomethacin with a specific TP-receptor antagonist for the triple inhibition experiments, would generate a further reduction in lung resistance. This notion is supported by a recently published study showing that the TP receptor mediates a part of the guinea pig smooth muscle contraction during the early allergic reaction ex vivo [120]. Nevertheless, these results demonstrate that the primary mediators in vivo that cause increased lung resistance during the early allergic reaction in a guinea pig model of allergic asthma are histamine and CysLT. The same mediators are responsible for airflow obstructions during the early allergic reaction in individuals suffering from allergic asthma, suggesting that guinea pigs are suitable to use as experimental subjects when studying asthmatic responses.

Since the early allergic reaction is triggered by mast cells that encounter an allergen, followed by an immediate release of mediators, the low number and proximal distribution of pulmonary mast cells in mice are a disadvantage of using mouse models when studying the early allergic reaction [79]. In the guinea pigs used in this study, mast cells were located in the submucosa along both large and small airways as well as in the perivascular space and parenchyma (Table 1). This observed mast cell distribution is more similar to that seen in humans compared to mice [18]. The response in mice, when measuring resistance in the airways during the early allergic reaction, is weaker, more transient and mediated through release of mast cell serotonin [121, 122]. Our data further support that the difference in magnitude and appearance of the early allergic reaction in guinea pigs compared to mice, are related to mast cell numbers and distribution, in combination with a different mediator release.

Table 1. Number of mast cells (MC) in different anatomical compartments of the guinea pig lung. Data are expressed as mean±SEM (n=5). *MC/mm².

<table>
<thead>
<tr>
<th>Ø (mm)</th>
<th>Airways</th>
<th>Blood vessels</th>
<th>Parenchyma</th>
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<tbody>
<tr>
<td></td>
<td>&gt;0.5</td>
<td>0.2-0.5</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>MC/mm²</td>
<td>14.6±1.9</td>
<td>5.2±1.1</td>
<td>6.2±1.1</td>
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We have shown that the increased lung resistance during the early allergic reaction in guinea pigs in vivo is essentially mediated via histamine and CysLT, the same primary mediators as in humans. In addition, mast cell numbers and distribution in the lungs of guinea pigs are more similar to humans, which might underlie why the early allergic response is stronger and more sustained compared to the response in mice. These data
suggests that this model mimics the early allergic reaction taking place in individuals suffering from asthma more accurately, and emphasises the importance of the guinea pig model in asthma and allergy research. H₁ receptor antagonists have shown restricted effects in the treatment of asthma and it has been reported that a combination of CysLT₁ and H₁ receptor antagonists, were more effective in treating asthma compared to a CysLT₁ receptor antagonist alone [123, 124]. Our study further corroborate that a combination treatment should be used to reduce airflow obstructions during the early allergic reaction in individuals suffering from allergic asthma.

Although we have, in this study, shown that guinea pigs are more suitable to use as a model compared to mice when studying the early allergic reaction, there are still some limitations of this model. The lack of suitable reagents, such as recombinant proteins and tools to measure or regulate gene and protein expression in guinea pigs limits the extent to which this animal is used in research today. The multitude of genetically modified mouse strains are another advantage this species holds over the guinea pig in the study of disease etiology and progression. For these reasons we decided to continue our investigation into the mechanisms involved in the early allergic reaction using mouse models of allergic asthma.
4.3 INTERLEUKIN 33 CAUSES AIRWAY SMOOTH MUSCLE CONTRACTION VIA AN ENHANCED SECRETION OF SEROTONIN FROM MAST CELLS IN THE EARLY ALLERGIC REACTION (STUDY III)

The cytokines TSLP, IL-25 and IL-33 are known to modulate immune responses at mucosal sites and have all been associated with asthma [125]. All three cytokines have been shown to be endogenously released following allergen exposure in mice, and mast cells have the potential to respond to at least TSLP and IL-33 [61, 69, 70]. During the early allergic reaction, following inhalation of allergens in individuals suffering from allergic asthma, release of mast cell mediators causes airflow obstructions that can be life-threatening. Since the effect of TSLP, IL-25 and IL-33 on the early allergic reaction is unknown, we wanted to study this effect in a mouse model of allergic sensitisation.

To measure smooth muscle contraction during the early allergic reaction, isolated airways from OVA/alum-sensitised BALB/c and C57BL/6 mice were isolated and challenged with OVA *ex vivo*. Of the three cytokines investigated, IL-33 was the only cytokine that increased the airway smooth muscle contraction when delivered intranasally (Fig 11a), and it was shown to do so in a dose-dependent manner. This increase was abolished in airways taken from mice lacking the IL-33 receptor (IL1RL1) (Fig 11b). The smooth muscle response was completely eliminated in airways from mast cell-deficient mice (sash), and in airways treated *ex vivo* with ketanserin, a 5-HT$_{2A}$ receptor antagonist, irrespective of IL-33 administration.

![Figure 11. Smooth muscle contraction and levels of secreted serotonin from isolated airways, measured during the early allergic reaction *ex vivo*. a) Smooth muscle contraction in airways from sensitized mice that received intranasal instillations of three different inflammatory cytokines. b) Smooth muscle contraction in airways from sensitized mice that received either PBS (white bars) or IL-33 (grey bars). a-b) Results are expressed as mean ± SEM. * P<0.05 comparing the IL-33 group to its control group. c) Secreted serotonin levels, measured in supernatants pre and post OVA challenge. * P<0.05, comparing responses between PBS and IL-33 groups pre and post OVA activation. # P<0.05, comparing levels of measured substances pre and post OVA activation within the same group.](image-url)
These data support previous findings showing that the smooth muscle contraction during the early allergic reaction in mice is mediated through release of mast cell serotonin [122, 126, 127].

Serotonin can mediate contraction directly through interaction with 5-HT2A receptors on smooth muscle cells [128] or indirectly by binding to 5-HT2A receptors on epithelium or neurons followed by release of acetylcholine, which mediates the contraction [122, 126, 127]. Thus, the effect of IL-33 could be mediated through activation of several different pathways of the structural cells and neurons. One important signalling molecule that is known to be of importance for airway responses and to increase the maximal response of the smooth muscle contraction is IL-13 [27] that can also be secreted from IL-33 stimulated mast [129]. However, in our study there was no difference in smooth muscle cell contraction comparing airways from mice that received PBS to those that received IL-33, following administration of either serotonin or carbachol to the airways ex vivo, indicating that IL-33 does not affect the response of the structural cells per se. Instead, the results suggest that the increased smooth muscle contraction could be due to an enhanced secretion of serotonin from mast cells, something that was later confirmed when measuring serotonin levels in supernatants, following OVA challenge of airways from IL-33 or PBS treated mice ex vivo (Fig 11c). The levels of β-hexosaminidase (β-hex) were also increased in the supernatant, indicating a general enhanced secretion of mast cell granular contents.

Since IL-33 has previously been reported to increase mast cell survival, maturation and adhesion to fibronectin [69, 70], we next examined if the increased responses during the early allergic reaction could be caused by an increased number of mast cells in the airways. We could not, however, find any differences in mast cell numbers between the groups, thus rebutting that theory. When the mast cell number and airway smooth muscle contraction data were combined from both groups of OVA-sensitized mice that were given either PBS or IL-33, there was a correlation between mast cell numbers in the airways and smooth muscle contraction, further supporting that mast cells are the source of serotonin. However, when the two groups were separated, the strong correlation observed in airways from control mice, was absent in airways from mice given IL-33, which supports the notion that the increased secretion of serotonin from the airways is not due to an increased number of mast cells, but instead that the tissue mast cells secrete more serotonin per cell.

We next examined if the levels of OVA-specific IgE in serum were altered after IL-33 administration since it has previously been shown that intraperitoneal administration of IL-33 increases the levels of total IgE in mice [43, 130]. However, we could not observe any differences in OVA-specific IgE levels between the groups. The difference in our findings compared to those reported previously could be explained by different routes of IL-33 administration or rather that specific IgE levels do not always correlates to total IgE levels. In any event, the observed enhanced secretion of serotonin in this study was not caused by increased levels of OVA-specific IgE. We did observe the appearance of a cellular infiltration, composed primarily of eosinophils, in the airways of mice that had received IL-33, but not in control mice. This further supports that IL-33 induces cytokine production leading to Th2-type inflammation [43] and recruitment
of eosinophils to the airways [68]. These findings suggest that IL-33 acts locally causing airway inflammation that could affect secretion of serotonin from mast cells.

To further investigate if IL-33 directly or indirectly induces the increased secretion of mast cell serotonin, we made use of mast cell culture to study these effects in a system where the influence of other cell types is eliminated. Bone marrow-derived mast cells (BMMC) were differentiated from bone marrow cells during the course of five weeks. Thereafter, the cells were cultured with IL-33 for four consecutive days, and on the first and last of those days they were also sensitized with anti-DNP IgE. Twenty-four hours after the final addition of IL-33 and IgE, the cells were washed and placed in buffer, followed by adding DNP to the buffer to incite mast cell activation. Both serotonin and β-hex levels measured in the supernatant were increased following activation of cells cultured with IL-33 (Fig 12a). The levels of the same mediators were also increased in the supernatants after the cells had been lysed (Fig 12b), demonstrating that total granular content was also increased when the cells had been cultured with IL-33. These data support previous findings showing that IL-33 causes granular accumulation of tryptase in BMMC, following co-culture for seven days [71]. When we analysed mRNA levels from BMMC we could also observe an increased expression of *tryptophan hydroxylase 1* (*Tph-1*) (Fig 12c), the gene for the enzyme involved in the rate-limiting step of serotonin synthesis, suggesting that an increased production of serotonin by mast cells occurs when cultured with IL-33. Together, these data show that IL-33 directly acts on sensitised mast cells to enhance their production, storage and release of serotonin following activation by IgE-receptor crosslinking.

![Figure 12](image)

**Figure 12.** a-b) Serotonin levels measured in the supernatant of BMMC culture a) Serotonin levels following IgE-receptor crosslinking. b) Total levels of serotonin measured following cell lysis. c) Relative quantitation of gene expression of *Tph-1* in BMMC. Results are expressed as mean ± SEM. * P<0.05, comparing results between IL-33 and PBS treated cells.

To verify the described effect *in vivo*, we investigated if IL-33 could affect the airflow obstructions caused by allergen challenge, in sensitized C57BL/6 mice. To do this we used an invasive method to measure lung mechanics (the same method as in the first study of this thesis), following intravenous OVA administration in OVA-sensitised mice. Newtonian resistance (*Rn*), a measure of the resistance in the conducting airways, displayed an increased resistance with a peak maximum within minutes in OVA- but not in sham-sensitized mice (Fig 13a), supporting previous findings [122], as well as our own *ex vivo* data. IL-33 administration increased *Rn* further, but the response was abolished in sash mice and in mice that were injected with ketanserin intraperitonally.
30 minutes prior to anesthetisation, suggesting that the increased airflow obstructions in vivo are also mediated through mast cell-derived serotonin (Fig 13b). Although less robust, the same pattern appeared when studying the peripheral tissue parameters, where IL-33 caused a tendency to increase both $G$ and $H$ in OVA-sensitized mice compared to those that received PBS, and was significantly increased compared to sham-sensitized and sash mice. Since mice lack mast cells in the parenchyma, the modest effect seen peripherally could indirectly be caused by parenchymal distortion arising from narrowing of the conducting airways [131]. It is possible that the increased resistance of the airways in mice that received IL-33 could also be caused by a systemic release of mast cell serotonin, but this seems unlikely since IL-33 in our study was administered locally to the airways.

Figure 13. Resistance of the conducting airways, measured during the early allergic reaction in mice in vivo. a) A representative trace from one mouse in each group. b) Peak Newtonian resistance measured for each group. Results are expressed as mean ± SEM. * P<0.05, compared to the OVA-IL-33 group.

To avoid systemic effects it would be preferably to administer OVA locally to the airways as an aerosol. This approach has been used with house dust mite as an allergen in BALB/c mice with a methodology similar as ours to measure airway resistance [132]. The response was, however, not shown to be mediated via serotonin in that study and in C57BL/6 mice the allergen-induced increase in airway resistance was less pronounced, which are the reasons why we chose to administered OVA intravenously in our study. We also measured the levels of serotonin in BALF in an attempt to determine if BALF serotonin levels correlate to the airway resistance. However, in addition to mast cells, other cell types also release serotonin[133], and the background levels in BALF were thus too high, making it impossible to detect any changes between the groups following allergen challenge. A recently published study showed increased levels of serotonin in BALF, 24 hours after the last allergen challenge in a mouse model of allergic asthma, indicating a constitutively increased release of serotonin [134]. The enhanced features of allergic asthma were associated with release of serotonin from platelets, not mast cells. Since the mice in our study were only sensitised, never challenged via the airways, it is not surprising that we could not detect similar differences in BALF serotonin levels between sham- and OVA-sensitised mice. However, following OVA challenge during lung mechanics measurements, serotonin
release from mast cells most likely mediates smooth muscle contraction via a local increase of serotonin levels, leaving the overall serotonin concentration found in BALF unchanged. In conclusion, IL-33 increases resistance of the conducting airways following allergen challenge, in a mast cell- and serotonin-dependent manner. This shows that IL-33 increases airflow obstructions during the early allergic reaction \textit{in vivo} in a manner similar to the increases in smooth muscle contraction we observed \textit{ex vivo}.

In this study we have demonstrated that IL-33 increases the production, storage and release of serotonin following activation of mast cells by IgE-receptor crosslinking. During the early allergic reaction this leads to enhanced smooth muscle contractions \textit{ex vivo} and increased airflow obstructions \textit{in vivo}. IL-33 is constitutively released by different cell types, but during inflammation and following tissue damage, which can occur due to several factors, such as exposure to proteolytic allergens, viruses, pollutants and mechanical tension, the release is increased [29, 125]. Polymorphisms in the genes for IL-33, IL1RL1 or in the downstream signalling proteins have been associated with asthma development and disease severity [6, 57]. In sensitised individuals that have an aberrantly increased release of IL-33, allergen exposure might lead to more severe asthma exacerbations. Persistent allergen exposure with a chronic mast cell activation has the possibility to aggravate airway inflammation, remodelling and hyperresponsiveness, all characteristic features of chronic allergic asthma [22], suggesting that the findings in this study might also have more long-term consequences.

In this study we have described a new mechanism by which IL-33 has the capacity to worsen asthma exacerbations during the early allergic reaction. New therapeutics targeting IL-33 or related molecules could offer a new strategy for asthma treatment and prevention.
4.4 INTERLEUKIN 33 ELICITS SYNERGISTIC EFFECTS WITH ANTIGEN ON AIRWAY HYPERRESPONSIVENESS, INFLAMMATION AND REMODELING IN A MOUSE MODEL OF ASTHMA (STUDY IV)

In the first study of this thesis we saw that the levels of IL-33 in the lung were upregulated in a mouse model of allergic asthma, and that this upregulation was enhanced by the presence of mast cells. In the third study we described a new mechanism by which IL-33 worsens airflow obstructions, in a mast cell-dependent fashion during the early allergic reaction in a mouse model of allergic sensitisation. Therefore, in this final study we wanted to investigate if IL-33 could also affect other characteristic features of allergic asthma, such as AHR, inflammation and remodelling. Since we sought to investigate the mast cell involvement in these features, we chose to use two different mouse models of allergic asthma. The classical OVA model, where mice are sensitized with OVA/alum, which has been proven to induce mast cell-independent AHR and inflammation [86, 87], and a mast cell-dependent model, where mice are sensitised to OVA without alum, which has shown to induce mast cell-dependent AHR and inflammation [87, 135, 136]. We used BALB/c mice for sensitisation with OVA/alum, since this protocol does not induce a significant AHR in C57BL/6 mice [137]. On the other hand, C57BL/6 mice were selected for sensitisation with OVA without alum, since this protocol does not induce a mast cell-dependent AHR and inflammation in BALB/c mice [135].

We used the same method as in previous studies to measure lung mechanics in mice in vivo. AHR was assessed following administration of increasing doses of aerosolised methacholine. For each sensitisation protocol, four groups of mice were included: sham-sensitised mice that received intranasal PBS (termed control) or IL-33 (termed IL-33), and OVA-sensitised mice with or without alum that received intranasal PBS (termed OVA) or IL-33 (termed OVA+IL-33) (See methodology section for detailed description of the protocols). Assessment of resistance of the conducting airways, as well as the two tissue parameters, showed that AHR was induced in both models in the OVA and IL-33 groups (Fig 14a-c). Intranasal administration of IL-33 has previously been shown to induce AHR in BALB/c mice, independent of T- and B-cells, suggesting that this response is independent of the adaptive immune system [68]. In the OVA+IL-33 groups, a synergistic increase of AHR was observed in all parameters in C57BL/6 mice sensitised without alum, while no increases were observed in BALB/c mice sensitised with alum. The difference that we observed between the two protocols could be due to the use of different mouse strains, and to further explore this, the sensitisation protocol without alum should also be assessed in BALB/c mice. Since the synergistic increase was observed in the OVA model that has been shown to induce mast cell-dependent AHR, the synergistic increase we observed is potentially dependent on mast cells. Preliminary results using the same protocol in mast cell-deficient sash mice show that the AHR is diminished in all parameters in the OVA group, supporting that AHR induced by using this protocol is influenced by mast cells. Furthermore, OVA and IL-33 together in OVA+IL-33 mice produced a synergistic increase in the tissue parameters in this model. However, this feature was absent when measuring the resistance of the conducting airways, suggesting that in this model, mast cells influence
AHR in the conducting airways but not in the peripheral parts of the lung. Since mast cells are only located in the central airway of mice, it stands to reason that whatever effect IL-33 has on the mast cell response, the subsequent effects on lung mechanics would be more prominent when studying conducting airway resistance compared to peripheral tissue changes in this model.

**Figure 14.** AHR was assessed following administration of aerosolised methacholine in sham- or OVA-sensitized C57BL/6 mice that received intranasal instillations of IL-33 or PBS. a) Newtonian resistance ($R_n$), resistance in the conducting airways, b) tissue damping ($G$) and c) tissue elastance ($H$). Results are expressed as mean ± SEM. * P < 0.05, comparing PBS PBS to OVA PBS or PBS IL-33. # P < 0.05, comparing OVA IL-33 to OVA PBS or PBS IL-33.

Although mast cells may play one important role in the lung, other cell types, such as T_{H}2 cells, basophils, eosinophils, dendritic cells and ILC2 also contribute to lung function in important ways. These cells appear in the lung during allergic inflammation and have all been shown, by themselves or in combination with other cell types, in response to IL-33, to produce T_{H}2 cytokines, such as IL-13, that is known to induce AHR [27, 43, 138-143]. As such, one or more of these cell types could potentially mediate the synergistic increase in AHR that we observed in the OVA+IL-33 group, in C57BL/6 mice sensitized without alum.

OVA sensitisation increased the levels of OVA-specific IgE in both strains, irrespective of IL-33 administration, although not to a significant level in the OVA group of C57BL/6 mice. There was, however, a further increase in OVA-specific IgE only in the OVA+IL-33 group compared to the OVA group in C57BL/6 mice, sensitised without alum. It has previously been shown that administration of IL-33 increases total IgE levels in mice [43, 130], also suggesting that an increase in specific IgE could be possible. The increased levels of OVA-specific IgE in the OVA+IL-33 group suggests that the increased AHR observed in this group might be influenced by mast cells or basophils since IgE increases both the expression of FcεRI on the cell surface of these cells, as well as the release of mediators following crosslinking of FcεRI [93, 94]. To further investigate if the increased levels of OVA-specific IgE are due to strain differences, the same protocol of sensitisation without alum that we used herein with C57BL/6 mice, should also be applied to BALB/c mice.
The number of inflammatory cells in BALF was increased in both the OVA and IL-33 groups compared to the control group, in both strains, although this result was not significant in the OVA group in C57BL/6 mice. This finding is in agreement with a previous study showing that IL-33 administration enhances levels of inflammatory cells in BALF in BALB/c mice [68]. However, the OVA+IL-33 group only had increased numbers of inflammatory cells compared to the OVA and IL-33 groups, in C57BL/6 mice sensitised without alum. Sensitisation with alum is known to induce strong T_{H2}-type inflammation in different strains of mice [144], and the same protocol that we used in this study has previously been shown to induce inflammatory cells in BALF to the same degree in both BALB/c and C57BL/6 mice [137]. Since BALB/c mice already display a T_{H2}-prone phenotype [145], administration of IL-33, which is a T_{H2}-inducing cytokine, might not enhance inflammation further. On the other hand, it has been shown that C57BL/6 mice, which display a T_{H1}-prone phenotype, are less predisposed to sensitisation compared to BALB/c mice when using a sensitisation protocol without alum [135, 146]. Therefore, we hypothesise that the T_{H2}-prone cytokine IL-33, has a larger impact on airway inflammation in a protocol and strain that is less susceptible to develop T_{H2} inflammation, such as the protocol used in C57BL/6 mice in our study.

Since we saw a synergistic increase of both AHR and the number of inflammatory cells in BALF in the OVA+IL-33 group in C57BL/6 mice sensitised without alum, we continued to further study the responses in these mice. Lung inflammation was induced in both the OVA and IL-33 group (Fig 15a). Moreover, lung inflammation was dramatically increased in the OVA+IL-33 group, in which the number and density of inflammatory cells were enhanced both around the airways and in the lung tissue.

**Figure 15.** Pulmonary inflammation. a) Representative images of lung tissue from sham- or OVA-sensitised C57BL/6 mice that received intranasal PBS or IL-33. b) Differential cell counts in BALF. Results are expressed as mean ± SEM. * P < 0.05, comparing OVA PBS or PBS IL-33 to PBS PBS. # P < 0.05, comparing OVA IL-33 to OVA PBS or PBS IL-33.

The assessment of differential cell counts in BALF showed that all cell types studied were upregulated in both the OVA and IL-33 group, with the exception of macrophages in the OVA group (Fig 15b). These results confirmed previous findings showing that
intranasal administration of IL-33 recruits eosinophils to the airways, and that this sensitisation protocol induces a mixed inflammatory response [68, 135]. However, eosinophils were the only cell type that was further increased in the OVA+IL-33 group. A recently published study also showed a steroid-resistant increase in numbers of eosinophils in BALF following OVA/alum-sensitisation and challenge combined with IL-33 administration in C57BL/6 mice, which was associated with ILC2 and endogenously released TSLP [147]. However, we could not detect any increase in TSLP levels in the lung (gene expression analysis; data not shown), suggesting that a different mechanism is in place when mice are sensitised without alum, as was done in our study. The number of pulmonary mast cells was not different between any of the groups. Previous results have shown that IL-33 increases mast cell survival, maturation and adhesion in vitro [69, 70], but to our knowledge this finding has never been shown in vivo. The increased inflammatory response in the OVA+IL-33 group is thus not mediated through an increased mast cell number. However, as we did show in the third study of this thesis, IL-33 can increase the synthesis and thereby the release of mast cell mediators during the early allergic reaction, creating the possibility that the enhanced effects that we see in this study can be mediated through an enhanced release of mast cell mediators.

Airway remodelling was assessed by measuring the thickness of the smooth muscle layer around the central airway and expression levels in the lung of Col1a1, Col3a1 and Col5a1, three collagen genes that have been associated with reticular basement membrane fibrosis, a condition which appears around the airways in individuals suffering from asthma [148]. The levels of Col1a1 and Col3a1 were significantly different, showing a trend to be increased in the OVA+IL-33 group, compared to the other groups (Fig 16a-b). Col5a1 levels were not different between the groups. A previous study described increased levels of collagen in the lungs of BALB/c mice following intranasal IL-33 administration given over the course of two weeks [67]. However, an increase following IL-33 administration was not observed in our study, where IL-33 was administered during a shorter time period. Instead, there seems to be a synergistic increase in collagen gene expression following administration of both OVA and IL-33.

![Figure 16](image)

**Figure 16.** Assessment of structural airway remodelling. **a-b)** Relative quantitation (RQ) of mRNA levels from Col1a1 and Col3a1 measured in lung tissue. PBS (light grey bars) and IL-33 (dark grey bars). Results are expressed as mean ± SEM. **c)** Thickness of the airway smooth muscle layer around the central airway evaluated by blinded scoring using a scale from 0 to 2. # P < 0.05.
The thickness of the smooth muscle layer around the central airway was not increased in either the OVA or IL-33 group (Fig 16c). However, the airway smooth muscle layer thickness was increased in the OVA+IL-33 group, compared to both the OVA and IL-33 groups, again showing that even with respect to smooth muscle thickness there was a synergistic increase following administration of both OVA and IL-33. Hence, we have for the first time, to our knowledge, described a synergistic effect of antigen and IL-33 administration on airway remodelling.

The importance of endogenously released IL-33 for the induction of asthma features in mouse models following allergen provocations, have been shown in several studies. An impaired response was observed in mice deficient in the IL-33 receptor or treated with either the soluble IL-33 receptor or with anti-IL-33 [60, 61, 67, 149-151]. The importance of endogenously released IL-33 in different OVA models of allergic asthma has been the subject of controversy within the field [152]. It is possible that the differences seen in allergic airway responses induced by OVA are due to differences in the protocols used. In this study we have shown that exogenously administered IL-33, in combination with an allergenic antigen, synergistically increases these features of asthma. IL-33 is released following tissue damage or during inflammatory conditions, which are common phenomena in the airways of asthma sufferers, suggesting that individuals with asthma that have an aberrantly high production of IL-33 or its related molecules, might suffer from severer asthma symptoms.

We have in this study shown that IL-33, in combination with an allergenic antigen, synergistically increases several characteristic features of asthma, such as AHR, inflammation and remodelling, possibly due to mast cell activity. This study serves to broaden the understanding of how IL-33 acts to modulate airway function in the allergic setting and in combination with studies I and III, as well as, studies by other authors, further supports the notion that IL-33 should be considered as a novel target for allergic asthma therapy.
5 CONCLUSIONS

The main conclusions from this thesis are:

- The induction of AHR can be both mast cell dependent as well as independent in the same model of allergic asthma.

- Mast cell engraftment of Kit<sup>W-sh/W-sh</sup> mice creates a mouse model with mast cells distributed throughout the lung, as seen in humans, and which is also associated with an increased AHR throughout the lung.

- Mast cells can affect the release of prostanoids in the airways which can result in both protective and harmful effects on AHR.

- These studies further support the belief that increases in AHR do not necessarily correlate with airway inflammation.

- The early allergic reaction in guinea pigs is similar to that seen in humans, suggesting that this animal is a better model to use compared to mice when studying allergic asthmatic responses.

- The increased levels of pulmonary IL-33 following induction of allergic airway inflammation can be regulated by mast cells.

- IL-33 increases airway smooth muscle contraction and worsens airflow obstructions by an enhanced release of mast cell serotonin during the early allergic reaction.

- Administration of IL-33 combined with allergic responses synergistically exacerbates several characteristic features of asthma, such as AHR, inflammation and remodelling.
6 GENERAL DISCUSSION

In this thesis we investigated interactions taking place between mast cells and smooth muscle cells in the airways in different models of allergic asthma.

To investigate how mast cells influence different aspects of allergic asthma we used mast cell-deficient Kit<sup>W<sub>sh</sub>/W<sub>sh</sub></sup> mice (sash) in our studies. Sash mice have a mutation in the regulatory elements upstream of the c-kit gene, resulting in a marked reduction of KIT receptors on the mast cell surface [153]. Since KIT is the receptor for the primary mast cell growth and survival factor, stem cell factor, these mice lack mature mast cells. However, other cell types, such as haematopoietic stem cells [154], also express KIT, resulting in other abnormalities in addition to mast cell deficiency in sash mice. As such, comparing data between wild-type mice and sash mice might result in differences that are independent of mast cells. Engraftment of sash mice with in vitro differentiated BMMC from wild-type mice offers the possibility to assess the role of mast cells on aspects of allergic asthma in mice with the same genotype. However, mast cell engraftment leads to an aberrant repopulation of mast cells in different organs [80, 81]. Of particular importance in the context of airway research is that the trachea is not engrafted with mast cells in this model, though as we have shown in our first study, the mast cell number is markedly increased in the parenchyma following engraftment. To avoid the disadvantages associated with c-kit mutant mice, several groups have recently developed mast cell-deficient mice that have a normal expression of KIT [155-159]. The mast cell depletion in these mice has been connected to mast cell specific or associated proteins and the mast cell deficiency can be either constitutive or inducible. Mice with an inducible mast cell deficiency have the advantage of normal mast cell populations during development, and the possibility to induce a mast cell deficiency at different time-points throughout disease development. The ultimate model for mast cell deficiency would also offer a possibility to selectively deplete mast cells in specific tissues. Although these mice do not have the disadvantages of KIT deficient mice, other disadvantages have been observed, such as incomplete mast cell depletion or altered basophil numbers [160]. Since these mice have only recently been described, more abnormalities might be reported in the future. Thus, there seems to be no mouse strain available today whose sole abnormality is mast cell deficiency. When studying the role of mast cells in different diseases the optimal approach would be to validate findings through the use of different types of mast cell deficient mice in an attempt to minimize the risk of obtaining results that are caused by factors other than mast cell deficiency.

One must also consider that biological events rarely depend on only one cell type. This can be thought of as nature’s way of securing the functionality of critical biological processes through redundancy. Thus, using an experimental model that lacks one cell type might not reveal the true contribution of that cell type to the parameter being studied. Even in light of these considerations, in our first study we could describe that the AHR induced in a mouse model of allergic asthma was both dependent and independent of mast cells. This was achieved by using mice that completely lacked mast cells, mice with a low number of mast cells and mice with a marked increase in their number of mast cells. In our fourth study we found that certain aspects of AHR
could be dependent on mast cells. Interestingly, in our third study we found that the IL-33-induced increase in the early allergic reaction appeared to be entirely dependent on the presence of mast cells.

The allergen that we used in our studies to induce allergic sensitisation was OVA. OVA might not be considered as an appropriate allergen in the sense that it is not one of the allergens causing airway obstructions in individuals with allergic asthma. Because of that, many laboratories have during the recent years started to use other perhaps more relevant allergens, such as mites or fungi extract to induce allergic airway inflammation. The reason why we chose to use OVA in study three was that to our knowledge no other allergen has been shown to induce smooth muscle contraction of sensitised airways ex vivo. In a pilot experiment before the study was initiated we used a house dust mite model that we have developed in the lab to study AHR, but the airways from these mice did not constrict when challenged with house dust mite ex vivo. In the fourth study we continued to use OVA since we wanted to utilize a model that has been shown to induce mast cell dependent AHR and inflammation, which to our knowledge has only been shown using OVA.

There are myriad different OVA protocols to use in order to induce an allergic airway inflammation in mice. The different protocols have shown different results when studying various responses in mast cells. Alum as an adjuvant to OVA seems to induce a strong Th2 response in different strains of mice, and it also seems to circumvent the role of the mast cell in the induction of allergic airway inflammation [86, 144]. Protocols without alum have shown to induce a mast cell-dependent AHR and inflammation, but only in certain strains of mice [81, 135, 136]. As a consequence, it is important to choose an appropriate protocol when studying mast cell responses. It might be more crucial to choose a protocol which leads to mast cell involvement when studying processes that can still be carried out by other cells even in the absence of mast cells. Moreover, when studying cell specific responses, it may be more important to use a protocol that has been shown to have features specific to the cell of interest, compared to using an antigen that may be considered more relevant in terms of human exposure. Nevertheless, one can still question the usefulness of OVA protocols when studying allergic asthma. However, asthma does not consist of a single phenotype and different features of asthma are affected by different mechanisms [10]. One has to keep in mind that an experimental model is only that, a model, and to date there are no models that mimics every aspect of the different phenotypes of asthma. Therefore, researchers must choose the model that best reflects the aspects of asthma that they wish to understand.

In all four of our studies we used the forced-oscillation technique to measure lung mechanics in anesthetised and tracheotomised mice. This method is more precise compared to non-invasive techniques, such as plethysmography, which can be affected by other parameters such as upper airway resistance [161, 162]. In study I, III and IV we applied the constant phase model which enabled us to study effects in the conducting airways compared to effects in the peripheral lung tissue. This is of importance since the main aim of this thesis was to investigate interactions between mast cells and smooth muscle cells, and in mice these cell types are only present in the central airways. Of course mast cells can also interact with one another and secrete
substances that can affect other parts of the lung, but smooth muscle contraction and effects upon it would mainly result in changes of Newtonian resistance ($R_n$). The studies in this thesis have shown that $R_n$ following methacholine provocations is influenced by the number of mast cells and prostanoid production in the conducting airways, as well as by IL-33 administrations. In addition, $R_n$ is increased in a mast cell dependent fashion during the early allergic reaction in mice that received IL-33.

As we show in paper II, guinea pigs have several advantages over mice as an animal model for studies of allergic asthma. However, as mentioned previously the lack of appropriate tools for this model hinders its widespread use. Currently, a major focus of our laboratory is to find and develop new tools as well as develop new asthma protocols to be able to take better advantage of the guinea pig as an experimental asthma model.

The increased smooth muscle contraction and airway resistance following IL-33 administrations measured during the early allergic reaction in study III have a somewhat different appearance compared to that seen in humans. The main mast cell mediators that are responsible for smooth muscle contraction in humans, as well as in guinea pigs, are histamine and cyste-leukotriens, while in mice it is serotonin. One could argue that our findings regarding how IL-33 affects the early allergic reaction in mice are not of relevance to human patients with allergic asthma, since their response are mediated through different mediators. Mast cells and allergen-specific IgE are, however, the primary components driving this response in both species [22] and we have, in our study, shown that IL-33 increases the release of mast cell granular contents in general. Humans have an abundance of mast cells in their lungs, with an increasing number residing in the periphery [18]. Since mice have only a limited number of peripherally located mast cells, with the majority located in the central airway, it is tempting to speculate that the effects of IL-33 that we observed on the early allergic reaction in mice would be even more pronounced in humans. Recently, it has been shown that remodelling of the airways can be induced solely by the mechanical force applied to the tissue during smooth muscle cell contraction [33]. This implies that the results from this study could also have long-term implications with respect to airway remodelling. Polymorphisms in the genes for IL-33, IL1RL1 and in the downstream signalling proteins have been associated with asthma development [6], but recently also with the number of hospitalizations in young children, which are commonly caused by acute exacerbations [57]. Our study showing that IL-33 aggravates allergic airway obstructions, which are known to cause acute exacerbations, provides a possible underlying mechanism to explain this association, if the IL-33-related polymorphisms result in an increased IL-33 signalling.

The studies involved in this thesis have only focused on interactions taking place between mast cells and airway smooth muscle cells in models of allergic asthma. Other forms of asthma pathogenesis that also include a mast cell component, such as exercise induced asthma [163] [10], are likely to also be related to the findings herein. In study III, we could see that IL-33 increased the production and total content of serotonin in mast cells, which would lead to an increased mediator release regardless of the type of activation.
Interestingly, during recent years many new three-dimensional cell models have been developed to resemble the outer wall of the airway [164, 165]. These models usually include fibroblasts embedded in some type of collagen matrix, as well as airlifted epithelial cells in a milieu that resembles that of the airway. These models can of course never be used to study systemic effects, but a model that also includes mast cells and smooth muscle cells would offer a possibility to study the functions and interactions taking place between these cell types using human cells. This kind of model would be advantageous to other in vitro systems, such as single or co-cultures of different cell types, since the cells in the three-dimensional model would be situated within an airway architecture. This approach would also be advantageous compared to using isolated airways since the user can chose which cell types to include, and there is a possibility to manipulate the cells before or after they are included in the model.

In three out of four studies included in this thesis we have observed effects on or of IL-33. In the first study, the levels of endogenous IL-33 were shown to be mast cell dependent in a model of allergic asthma. In the third study, IL-33 increased airway smooth muscle contraction and worsened the airflow obstructions by an enhanced release of mast cell serotonin, during the early allergic reaction, in a model of allergic sensitisation. In the fourth study, AHR, inflammation and remodelling were synergistically increased by IL-33 combined with allergen in a mouse model of allergic asthma, possibly due to mast cell involvement. Thus, IL-33 seems to have the potential to have an important role in the pathogenesis and progression of allergic asthma, and its effects seem, at least in some cases, to be mediated through mast cells. It remains to be seen if a therapeutic targeting IL-33 will improve the treatment regime of allergic asthma.


I den första studien undersökte vi hur antalet mastceller och deras position i olika delar av lungan kan påverka inflammationen och överkänsligheten i luftvägarna. För att göra detta så användes tre olika typer av möss, normala möss, möss som saknar mastceller och möss som initialt saknade mastceller men som vi gav tillbaks sådana till. De olika typerna av möss gjorde att vi kunde studera hur reactionerna skiljde sig mellan möss som helt saknade mastceller, de som hade ett fåtal mastceller lokaliserade i de stora luftvägarna och de som hade ett stort antal mastceller utspridda över hela lungan. Vi kunde se att överkänsligheten i luftvägarna kan uppstå oberoende av närvaro av mastceller, men den kan också förvärras avsevärt när antalet mastceller stiger på olika platser i lungan. Den berodde också på vilka ämnen som mastcellerna släpper ut. Inflammationen påverkades till viss del av närvaron av mastceller, men den följde inte samma mönster som överkänsligheten i luftvägarna, vilket tillför ytterligare bevis utöver tidigare studier för att dessa fenomen är oberoende av varandra. Mängden av ett frisläppt inflammatoriskt ämne, kallat IL-33, visade sig också bero på närvaron av mastceller.
I den andra studien ville vi undersöka om marsvin kan vara ett bättre alternativ än möss när man ska studera allergiska astmareaktioner. Vi kunde se att mastcellernas antal och lokalisering i lungan hos marsvin mer liknade de hos människan. Allergiska luftvägsförträngningar hos marsvin var också starkare än hos möss, vilket gjorde att de blev lättare att mäta. Slutligen kunde vi också se att de ämnen som frisläpps från mastceller och som skapar muskelkontraktion i luftvägarna hos marsvin till stor del är samma som de hos människan, men väldigt olika de hos möss. I denna studie använde vi en ny metod för att specifikt mäta luftvägsförträngningar hos marsvin som visar tecken på astmasymptom. Resultaten visar att det skulle vara fördelaktigt att använda marsvin istället för möss när man studerar allergisk astma.

I den tredje studien ville vi undersöka hur några specifika inflammatoriska ämnen som tidigare har kopplats samman med astma påverkar de akuta luftvägsförträngningarna. Trots att vi i den föregående studien visade att marsvin är en bättre djurmodell när man vill studera denna typ av reaktioner, så valde vi i detta fall att återigen använda möss eftersom möjligheten att använda djur med en viss del av arvsmassan borttagen endast finns hos dessa. Vi kunde visa att ett av de inflammatoriska ämnena, IL-33, bidrog till att öka muskelkontraktionen i isolerade luftvägar från möss efter att de hade utsatts för ett allergen. Vi kunde också se att muskelkontraktionen uppstod efter att mastcellerna hade släppt ut ett ämne i luftvägarna som kallas serotonin. För att visa att IL-33 direkt binder till och påverkar mastceller så utsatte vi sådana i odling för IL-33 och kunde då också se en ökad utsöndring av serotonin när de utsattes för ett allergen. Vi kunde också uppmärka att mastcellerna producerade och förvarade mer serotonin inom sig. Slutligen så kunde vi också se att de allergiska luftvägsförträngningarna hos möss ökade om de fått IL-33. Sammantaget så visar detta att IL-33 har möjligheten att förvärra de akuta luftvägsförträngningar som personer med allergisk astma upplever efter att de kommer i kontakt med ett allergen. Resultaten från denna studie föreslår att ett läkemedel som motverkar IL-33’s funktion skulle kunna hjälpa personer med astma.

Eftersom den tredje studien visade att IL-33 påverkar de akuta luftvägsförträngningarna, så ville vi i den fjärde och sista studien undersöka hur IL-33 påverkar andra händelser som senare leder till luftvägsförträngningar. Vi undersökte hur inflammationen, överkänsligheten i luftvägarna och strukturförändringarna påverkades av IL-33 i två olika djurmodeller av allergisk astma. I den ena modellen så kunde vi se att alla de tre parametrarna som vi mätte kraftigt förvärrades av att de allergiska mössen fått IL-33, medan inga av parametrarna påverkades i den andra modellen. Eftersom vi i denna studie använde två olika modeller som gav olika resultat, så måste ytterligare studier genomföras innan några tydliga slutsatser kan dras. Det finns många olika typer av celler i lungan som kan vara orsaken till den försämring som vi ser i den ena modellen. För att utreda mastcellens betydelse behöver möss som saknar mastceller även undersökas.

Sammanfattningsvis visar resultaten från alla fyra studierna att antalet mastceller på olika platser i lungan påverkar olika astmasymptom men också att det är viktigt att våga för- mot nackdelar när man väljer vilken typ av djurmodell som man ska använda. De visar även att IL-33 kan påverka de akuta luftvägsförträngningarna hos en person med allergisk astma. Slutligen så visar de också att IL-33 kan bidra till senare uppkomna luftvägsförträngningar som uppståt utan tydlig orsak så som direkt exponering av ett...
allergen. Detta betyder att nya läkemedel som motverkar effekten av IL-33 eventuellt skulle kunna ge en effektivare behandling för patienter med allergisk astma.
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9 REFERENCES


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