From the Institute of Environmental Medicine,
the Unit of Experimental Asthma and Allergy Research,
Karolinska Institutet, Stockholm, Sweden

DEFINING THE ROLE OF MAST CELLS IN GUINEA PIG MODELS OF ASTHMA

Jielu Liu

刘洁露

Karolinska Institutet

Stockholm 2022

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2022

ISBN 978-91-8016-560-0
Cover illustration: The guinea pig airways before (left) and after repeated exposures to house dust mite extract (right). Several structural changes can be found: airway narrowing, goblet cell hyperplasia, thickening of basement membrane, subepithelial fibrosis, increased thickness of smooth muscle layer, recruitment of inflammatory cells and mast cell hyperplasia.

Defining the role of mast cells in guinea pig models of asthma

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Jielu Liu

The thesis will be defended in public at Biomedicum, room A0315 (Peter Reichard), Stockholm, Friday the 3rd of June at 13 PM

Principal Supervisor:
Associate Professor Mikael Adner
Karolinska Institutet
Institute of Environmental Medicine
Division of Experimental Asthma and Allergy Research

Co-supervisor(s):
Professor Gunnar Nilsson
Karolinska Institutet
Department of Medicine, Solna
Division of Immunology and Allergy

Assistant Professor Jesper Säfholm
Karolinska Institutet
Institute of Environmental Medicine
Division of Experimental Asthma and Allergy Research

Professor Sven-Erik Dahlén
Karolinska Institutet
Institute of Environmental Medicine
Division of Experimental Asthma and Allergy Research

Opponent:
Professor Hans Jürgen Hoffmann
Aarhus University
Department of Clinical Medicine
Division of Respiratory Diseases and Allergy

Examination Board:
Associate Professor Mattias Carlström
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Reactive Oxygen Radicals and Nitric Oxide in Renal and Cardiovascular Research

Professor Maria Jenmalm
Linköping University
Department of Biomedical and Clinical Sciences
Division of Inflammation and Infection (II)

Associate Professor Nanna Fyhrquist
Karolinska Institutet
Institute of Environmental Medicine
Division of System Toxicology

Focus is more important than intelligence, focus on what matters and let go what doesn't!

To my dearest family

POPULAR SCIENCE SUMMARY OF THE THESIS

All over the world there are more than 300 million people suffering from a respiratory disease called asthma. Individuals who have this disease usually have several symptoms such as cough, feeling shortness of breath and hearing the high-pitched sound when breathing (also called wheezing). These symptoms are due to the narrowing of the airways. Even though the main symptoms are similar, asthmatic subjects have different clinical characteristics allowing them to be clustered into different groups (asthma phenotype). Among all the asthma subjects, there is a group of people whose symptoms appear very early in their lives, and usually they are allergic to allergens. This group is defined as allergic asthma which is the most common type of asthma and takes up around a half of the asthma population.

Several features of allergic asthma have been defined. For example, the airways of these subjects will constrict if exposed to specific allergens (**allergen induced bronchoconstriction**). They have exaggerated responses to stimuli that healthy individuals usually do not respond or respond very weakly to (**airway hyperresponsiveness, AHR**). In addition, there are increased eosinophils in their airways (**eosinophilic inflammation**). Several airway structural changes can be found, such as the increased numbers of mucus secreting goblet cells, deposition of collagen in the airways and the thickening of smooth muscle bundle (**airway remodeling**). There are many evidence showing that asthma people have increased mast cells in their airways, indicating that mast cells are important in asthma. However, the role of mast cells in the development of the pathophysiological features in allergic asthma is not clear. Mice are commonly used to modeling human diseases for mechanistic research. However, the airways of mice differ from humans. In addition, the murine mast cells are scarce and respond differently to human mast cells. Thus, mouse is not an ideal specie for mast cell research. In contrast, guinea pigs have more similarities with humans anatomically, physiologically, pharmacologically, and also when it comes to mast cell distributions and responses. Thus, the aim of this thesis was to establish guinea pig asthma models using human relevant allergens: house dust mite (HDM) and cat dander extract (CDE). An additional aim was to investigate the role of mast cells in the asthma-like features in guinea pigs.

In **paper I**, we exposed guinea pigs to HDM solutions through the nose for seven weeks. Animals which inhaled these solutions demonstrated asthma-like features (*e.g.*, 'allergen' induced bronchoconstriction, AHR, eosinophilic inflammation, airway remodeling and an increase of mast cells). This model can be used further to define the role of mast cells in asthma.

In **paper II**, we investigated the time effect of HDM and CDE on the development of airway inflammation, airway remodeling and the increase of mast cells. Both 'allergens' induced airway inflammation and airway remodeling of guinea pigs after 4 weeks' exposures and both features still could be found after 8- and 12-week 'allergen' exposures. The increase of

mast cells appeared after 8- and 12-week exposures. This increase was predominated by mucosal mast cells.

In **paper III**, we compared the responses of guinea pig trachea to 'allergen' (HDM) and another mast cell agonist Compound 48/80 (C48/80). We found that mast cell mediators (histamine, prostaglandins and leukotrienes) are important in both HDM and C48/80 induced contractions of guinea pig tracheal segments. Exposures to HDM and C48/80 induced the release of several mediators, including histamine, prostaglandin D_2 and leukotriene B_4. Compound 48/80 induced a broader release of lipid mediators than HDM, however, the leukotriene E_4 increased only after HDM challenge.

In **paper IV** and **V**, we identified that an antibiotic (monensin) can induce mast cell death. By exposing the guinea pig tracheal segments or human small airways to monensin, we found that the bronchoconstrictions induced by mast cell agonists (HDM or anti-human monoclonal IgE antibody) totally disappeared. Low dose of monensin did not affect tissue viability. In addition, the animals treated with monensin demonstrated reduced AHR, airway inflammation and mast cell numbers.

To conclude, HDM and CDE are suitable for allergic asthma model establishments in guinea pigs which can be used for studying the mechanisms behind asthma, especially the role of mast cells in the development of asthma. The mast cells in guinea pig airways can respond differently to 'allergen' and 'non-allergen' agonists. Monensin can be a useful tool to induce mast cell death. The 'allergen' induced airway constriction is mediated by mast cells both in guinea pig and human airways. An expansion of mast cells is important in the development of AHR and airway inflammation in the used guinea pig model. This PhD study facilitates further investigations of the role of mast cells in both allergic and non-allergic asthma.

ABSTRACT

Asthma is a common respiratory disease characterized by several pathophysiological features, such as allergen induced bronchoconstriction (in allergic asthma), airway hyperresponsiveness (AHR), airway inflammation, airway remodeling and mast cell hyperplasia. An increase of mast cells has been found in asthma patients. However, how these cells are involved in the development of asthma are not well defined. To investigate the role of mast cells in the pathophysiological characteristics of asthma, we established asthma models in guinea pigs, which have many similarities with humans, by exposing the animals to human relevant allergens: house dust mite (HDM) and cat dander extract (CDE). The involvement of mast cells in asthma-like features was investigated either by the addition of mast cell mediator antagonists or inhibitors, or inducing mast cell death.

In **paper I**, we repeatedly exposed guinea pigs to HDM via intranasal instillation for seven weeks and successfully recaptured the antigen induced bronchoconstriction, the production of HDM specific immunoglobulins, AHR, eosinophilic inflammation with an increase of IL-13, airway remodeling (*e.g.*, subepithelial collagen deposition and goblet cell hyperplasia) and mast cell hyperplasia. This model can be further used to study the role of mast cells in asthma.

In **paper II**, we exposed guinea pigs to HDM or CDE intranasally for different time. Both HDM and CDE induce airway inflammation and airway remodeling after 4 weeks' antigen exposures. These increases maintained after 8- and 12-week exposures. Exposing to both antigens for 8 weeks and 12 weeks induced a clear expansion of mast cells which is predominated by mast cells expressing tryptase. An increase of mast cells expressing both tryptase and chymase were also observed.

In **paper III**, we isolated guinea pig trachea for comparing the effect of different mast cell agonists (HDM and Compound 48/80 (C48/80)) on airway smooth muscle responses and mediator release. We found that histamine, prostaglandins and 5- lipoxygenase products mediated the bronchoconstriction induced by HDM and C48/80. Both agonists induced a release of histamine, prostaglandin D_2 and leukotriene B_4. However, distinct of lipid mediator profiles were observed. The leukotriene E_4 was only elevated by HDM, whereas C48/80 induced a broader release of lipid mediators.

In **paper IV** and **V**, we identified an antibiotic monensin that can induce mast cell death. To examine if monensin can be a tool for investigating the role of mast cells in asthma, we cultured guinea pig tracheal segments from HDM sensitized guinea pigs and human bronchi with different concentrations of monensin for different time. We found that monensin has robust effects on causing mast cell death and totally blocked the HDM (in guinea pig trachea) and anti-IgE (in human bronchi) induced bronchoconstriction after 2 to 72h exposure without affecting the general tissue viability at low concentration. In the *in vivo* investigations, we exposed the guinea pigs to HDM repeatedly with or without monensin interventions.

Monensin reduced the AHR, airway inflammation and mast cell hyperplasia in the HDM induced guinea pig model.

In conclusion, exposing to human relevant allergens (HDM and CDE) are suitable for modeling of allergic asthma in guinea pigs. The increase of mast cells by HDM and CDE helps to investigate the role of mast cells in asthma models. Mast cells in guinea pig airways can respond differently to antigen and non-antigen agonists. Monensin can be a robust tool to induce mast cell death. The antigen induced bronchoconstriction by HDM in guinea pig trachea and anti-IgE in human bronchi are purely mast cell mediated. Our findings emphasize that mast cells have important roles in the development of AHR and airway inflammation in the guinea pig model used in this PhD study. The findings in this thesis highlight the importance of mast cells in asthma and the models we developed can be used as important tools for defining the mechanisms behind asthma.

LIST OF SCIENTIFIC PAPERS

I. Ramos-Ramírez P, Noreby M, Liu J, Ji J, Abdillahi SM, Olsson H, Dahlén SE, Nilsson G, Adner M.
A new house dust mite–driven and mast cell–activated model of asthma in the guinea pig
Clinical and experimental allergy. 2020; 50: 1184-1195.

II. Ramos-Ramírez P, Liu J, Mogren S, Gregory J, Noreby M, Petrén A, Lei Y, Olsson H, Hage MH, Kervinen J, Hellman L, Andersson C, Nilsson G, Adner M.
House dust mite and cat dander extract induce asthma-like histopathology with an increase of mucosal mast cells in a guinea pig model
Manuscript submitted.

III. Liu J, Kolmert J, Säfholm J, Johnsson AK, Zurita J, Wheelock CE, Dahlén SE, Nilsson G, Adner M.
Distinct effects of antigen and compound 48/80 in the guinea pig trachea
Allergy. 2021;76(7):2270-2273.

IV. Maccarana M, Liu J, Lampinen M, Rollman O, Adner M, Pejler G, Paivandy, A.
Monensin induces selective mast cell apoptosis through a secretory granule mediated pathway
Allergy.2022;77(3):1025-1028.

V. Liu J, Nie M, Dong C, Säfholm J, Pejler G, Nilsson G, Adner M.
Monensin inhibits mast cell mediated airway contractions in human and guinea pig asthma models
Manuscript submitted.

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Säfholm J, Abma W, Liu J, Balgoma D, Fauland A, Kolmert J, Wheelock CE, Adner M, Dahlén SE
Prostaglandin D_2 inhibits mediator release and antigen induced bronchoconstriction in the Guinea pig trachea by activation of DP1 receptors
European journal of pharmacology. 2021; 907:174282.

II. Stark JM, Liu J, Tibbitt CA, Christian M, Ma J, Wintersand A, Dunst J, Kreslavsky T, Murrell B, Adner M, Grönlund H, Gafvelin G, Coquet J
A recombinant multimeric dog allergen prevents airway hyperresponsiveness in a model of asthma marked by vigorous TH2 and TH17 cell responses
Allergy (accepted).2022.

CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AHR	Airway hyperresponsiveness
AIB	Allergen/Antigen induced bronchoconstriction
APC	Antigen presenting cell
Al (OH)3	Aluminum hydroxide
ASM	Airway smooth muscle
AUC	Area under curve
BALF	Bronchoalveolar lavage fluid
BPT	Bronchial provocation test
C48/80	Compound 48/80
CCh	Carbachol
CDE	Cat dander extract
COX	Cyclooxygenase
CRC	Concentration response curve
CYP450	Cytochrome p450
CysLTs	Cysteinyl leukotrienes
DHA	Docosahexaenoic acid
DHGLA	Dihomo-γ-linolenic acid
EAR	Early allergic reaction
EPA	Eicosapentaenoic acid
FcεRI	Fc epsilon receptor 1
FEV_1	The forced expiratory volume in the first second
5-HpETE	5-hydroperoxyeicosatetraenoic acid
5-LOX	5-lipoxygenase
FLAP	5-lipoxygenase-activating protein
FOT	Forced oscillation technique
FWBP	Flow whole-body plethysmography
GAG	Glycosaminoglycan
HETE	Hydroxyeocosatetraenoic acid
HDM	House dust mite

Ig	Immunoglobulin
IL	Interleukin
ILC2	Type 2 innate lymphoid cell
LA	Linoleic acid
LAR	Late allergic reaction
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LPS	Lipopolysaccharides
LT	Leukotriene
MC	Mast cell
MCh	Methacholine
MC_T	Mast cell expressing only tryptase
MC_{TC}	Mast cell expressing both tryptase and chymase
MCp	Mast cell progenitor
MRGPRX2	Mas-related G-protein coupled receptor X2
NGF	Nerve growth factor
OVA	Ovalbumin
PAS	Periodic Acid-Schiff
PEF	Peak expiratory flow
Penh	Enhanced pause
PG	Prostaglandin
PGDS	Prostaglandin D synthase
PGES	Prostaglandin E synthase
PGFS	Prostaglandin F synthase
PGIS	Prostaglandin I synthase
PIF	Peak inspiratory flow
PLA2	Phospholipase A2
PUFA	Polyunsaturated fatty acid
PWBP	Pressure whole-body plethysmography
SCF	Stem cell factor
SCG	Sodium cromoglycate
SER	Subepithelial area

Syk	Spleen tyrosine kinase
T2	Type 2
TGF-β_1	Transforming growth factor-β_1
Th	T helper
TSLP	Thymic stromal lymphopoietin
TX	Thromboxane
TXAS	Thromboxane synthase
WBP	Whole-body plethysmography

1 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The word '*asthma*' is a noun derived from the Greek verb *aazein,* meaning shortness of breath. The earliest written record of asthma can be sourced back around 2800 years to the 8th century BC, where Homer depicted a warrior in the *Iliad* who died from asthma-like manners. Later, it was found that '*asthma*' was first used as a medical term in the school of Hippocrates of Kos (c. 460-360 BC.)[1]. In the year 1860, Henry Hyde Salter defined asthma as '*Paroxysmal dyspnea of a peculiar character, generally periodic with intervals of healthy respiration between the attacks*'. Spastic contraction of bronchial tubes was thought to be the mechanism behind asthma based on the dyspnea and the sounds of asthmatic patients[2-4]. Therefore, the treatment development for asthma was focused on bronchodilators in the beginning, such as theophylline, ephedrine, adrenaline, isoprenaline and selective β_2-adrenoceptor agonists (*e.g.,* salbutamol, terbutaline, remiterol and fenoterol *etc.*) and anticholinergics (*e.g.,* ipratropium)[2,5]. However, the use of bronchodilators did not change the outcome of asthma, overreliance and overuse of β_2- agonists, on the contrary, increased the mortality of asthma patients[6]. It was emphasized later that airway inflammation is crucial in asthma. Eosinophils, mast cells (MCs), lymphocytes and macrophages were found increased in the bronchoalveolar lavage fluid or bronchial biopsies of asthmatic patients[7-9]. Several treatments targeting mast cells or their mediators (*e.g.,* mast cell stabilizers and anti-histamines) demonstrated limited treatment effectiveness[10,11]. As anti-leukotrienes have been shown in some studies to reduce asthma exacerbations in children and adults[12,13], they are now suggested as add-on therapies of asthma. Systemic corticosteroids, administered either intravenously or orally, have been shown to improve asthma outcomes[14,15]. However, the long-term adverse consequences of systemic corticosteroids are apparent[16]. In contrast, inhaled corticosteroids, with less side effects, have been shown to reduce asthma symptoms and pathophysiological features by reducing inflammatory cells in the lung[17]. The use of inhaled corticosteroids revolutionized the treatment of asthma, and they are now regarded as the standard treatments for this disease. With the developed understanding of asthma immunology and pathophysiology, more and more biologics have been approved as treatments for specific asthma phenotypes (will be discussed later)[18].

1.2 AIRWAY ANATOMY

The respiratory system, where gas exchange occurs, includes the airway tract and the lung. During respiration, ambient air passes through the upper (including nasal cavity, pharynx, and larynx) to the lower respiratory tract, also known as the conducting airways, which starts from the windpipe (trachea). The trachea is a ventilatory tube consisting of 18 to 20 membrane connected D-shaped tracheal rings with the C-shaped cartilages cover the anterior and lateral wall and the smooth muscle bundles in the posterior[19]. At the carina, trachea divides 20 to 23 times to bronchi (two main bronchus to the lobar, segmental and subsegmental bronchus) and bronchioles (conducting, terminal and respiratory bronchioles).

After passing the airway tract, air reaches the lungs (alveolar duct, sac, alveolus) for the exchange of oxygen with carbon dioxide in the blood stream[20].

Starting from the airway lumen, the conducting airways consist of epithelial layer, basement membrane, lamina propria (loose connective tissue), gland, smooth muscle bundle, cartilage, and fibrous membrane surrounded with vascular. The epithelium of airways changes from pseudostratified columnar epithelium in the trachea and bronchi to simple columnar epithelium in the bronchioles with a gradual reduce of mucus producing goblet cells, ciliated cells, basal cells but increase of club cells[21,22]. In the bronchioles and alveolar, there are no mucus producing cells. Changes of glands and cartilages can also be found. For instance, the mucus glands are numerous in trachea, whereas the number of glands is reduced in the bronchi. The cartilages in bronchi become more complete circles. Furthermore, both glands and cartilages disappear in the bronchioles. In contrast, the airway smooth muscle bundle can be found from the trachea to the bronchioles.

1.3 ASTHMA DEFINITION

Asthma is now defined as a heterogenous respiratory disease with 1-18% people suffering worldwide. A proper diagnosis of asthma is based on recurrent respiratory symptoms, such as wheezing, chest tightness, dyspnea and cough, together with variable expiratory airflow limitation[23]. The heterogeneity of asthma includes several aspects. First, asthmatic symptoms can be worsened by various stimuli, for instance, respiratory viruses[24] and allergens[25], exercise[26], and environmental and indoor pollutants[27,28]. In addition, the asthmatic symptoms are viable, changing with weather or resolve with or without treatments. Moreover, the expiratory airflow limitation of asthma patients changes overtime and with treatments. However, not all asthmatic patients respond well to treatments. Depending on the demography, up to 4-10% of asthma population[29-31] respond poorly even with high dose of standard treatments. Therefore, these individuals are regarded as severe asthma who experience recurrent exacerbations, which affects their daily life quality and causes huge economic burden to society. Moreover, asthma is not a single disease, but an ‘umbrella’ of diseases with different clinical grouping features (phenotypes) and distinct underlying pathophysiological mechanisms (endotypes). In the beginning, asthma was simply classified into extrinsic and intrinsic asthma[32] based on age of disease onset and atopy. Extrinsic patients usually start with an early disease onset and are allergic, while the intrinsic asthma develops later without clear allergies[33]. However, these two phenotypes are not enough to group all asthma patients. By combining different clinical manifestations (disease onset, triggers, atopy, smoking history and BMI) with inflammation, more and more phenotypes have been defined, such as early-onset allergic, late-onset eosinophilic, exercise-induced, obesity-related and neutrophilic asthma[34]. Among them, allergic asthma is the most common form (approximately 50%), with increasing prevalence in the younger population[35]. Despite the differences, there are some pathophysiological features shared by asthma phenotypes including airway lumen narrowing, airway inflammation, mucus hypersecretion, airway hyperresponsiveness (AHR) and thickening of the airway wall[36].

1.4 ASTHMA PATHOPHYSIOLOGY

1.4.1 Allergen induced bronchoconstriction

When exposed to specific allergens, atopic asthmatics suffer from bronchoconstriction due to the early allergic reaction (EAR), which, in some patients (around 50%), are followed by a second phase of airway contraction, the late allergic reaction (LAR). The EAR, also known as immediate allergic reaction, usually starts within 10 minutes after allergen exposures, reaches maximal bronchoconstriction after 30 minutes and resolves within 1-3 hours, whereas the LAR develops slowly, generally appearing at least 3-4 hours, post allergen challenges[37]. Mast cells are key players both in EAR and LAR. The EAR is initiated by the allergen-induced crosslinking of high-affinity IgE receptors (FcεRI) on the mast cells of sensitized individuals that causes the release of spasmogenic mediators, such as histamine, cysteinyl leukotrienes (CysLTs; LTC_4, LTD_4 and LTE_4), prostaglandin (PG) D_2 and thromboxane (TX) A_2 which cause bronchoconstriction[38]. In addition to the above-mentioned mediators, mast cells release proteases, numerous cytokines, chemokines and growth factors which can recruit and activate inflammatory cells (*e.g.*, T cells, eosinophils, basophils and neutrophils). All these factors have properties to influence smooth muscle contraction, mucus hypersecretion, vasodilation and vascular leakage causing edema of airways inducing the LAR[37,39].

In specific clinics, bronchial allergen provocation test can be applied in the lower respiratory tract of allergic asthmatics to induce EAR and LAR[40]. Spirometry is often used to identify the responses. A positive allergic response is defined as a drop of the forced expiratory volume in the first second (FEV_1) $\geqslant$ 20% (EAR) or $\geqslant$ 15% (LAR) of the baseline condition[41].

In experimental settings, several techniques can be used to monitor antigen induced responses of animals, reflected as changes of lung functions. The ventilator-based technologies (*e.g.*, flexiVent) measure the lung mechanics of anesthetized and ventilated animals and are invasive and non-repetitive. Therefore, they can only be used at the terminations of animals. In contrast, the plethysmography (*e.g.*, whole-body, head-out or double chamber plethysmography) can be non-invasive and harmless to animals and provides repeatable measurements without anesthesia[42]. Thus, the non-invasive plethysmography can be used during the whole period of model establishments.

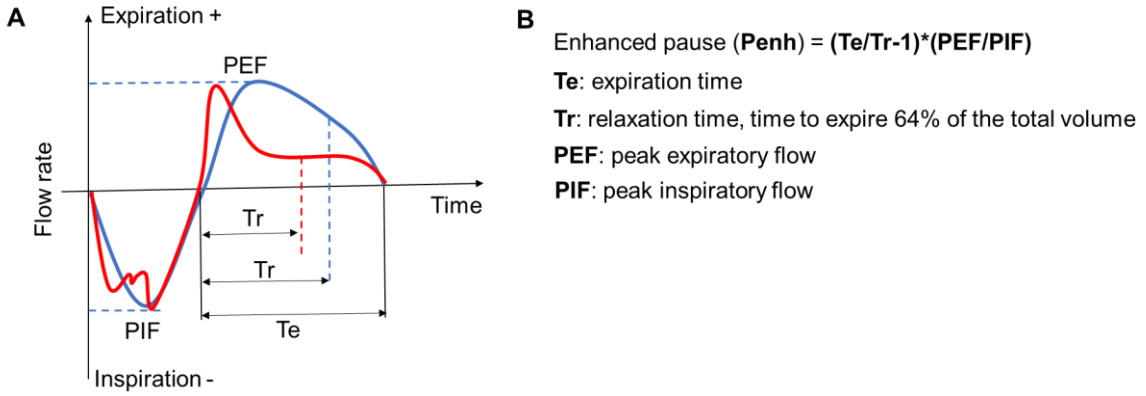
1.4.1.1 flexiVent

The flexiVent applies a method, namely forced oscillation technique (FOT), to give reliable measurements of the lung mechanics of animals. The FOT, which applies a single or broad band frequency waveform to the airway openings, measures the pressure, flow, and volume signals of the animals' responses to the applied waveforms. The measured input (impedance) is afterwards fitted into different mathematical models, such as single compartment model (for single frequency maneuver) or constant phase model (for broadband maneuver), to generate parameters that reflect lung mechanics[43].

From the single compartment model, three important parameters are obtained: Resistance (*R*), Elastance (*E*) and Compliance (*C*). The *R* reflects the overall constriction of the lung, whereas *E* resembles the general elastic stiffness of the airways, lung tissue and chest wall. The *C* is the reciprocal of *E* and stands for the ease to extend the respiratory system. With the constant phase model, the mechanics between airways and tissues can be separated reflected in three parameters, that are Newtonian resistance (*Rn*, reflecting the resistance of the central or conducting airways), tissue damping (*G*, reflecting the energy dissipation in the alveoli, represents the tissue resistance) and tissue elastance (*H*, as energy conservation in the alveoli)[43,44].

1.4.1.2 Whole-body plethysmography

Whole-body plethysmography (WBP) is a non-invasive technique for breathing measurements where enhanced pause (Penh), a parameter reflecting the shape of breathing curve[45], is computed (**Figure 1**). Two types of the WBP are broadly used in research: sealed plethysmography or pressure plethysmography (PWBP) and flow plethysmography (FWBP) with a pneumotachograph[46]. With the PWBP, the Penh can be used to accurately predict airway resistance only under very strict conditions[47]. In contrast, increased Penh was observed after exposures to spasmodic agonists in FWBP, and it is correlated with airway resistance[45,46,48]. Though correlated, Penh is not a measurement of airway resistance. The bronchoconstriction can result in the increase of Penh, but the increase in Penh is not evidence for bronchoconstriction. Conditions that can affect animals' breathing patterns can also cause the change of Penh. For instance, a slight increase of Penh can be detected while guinea pigs fall asleep.



Blue curve: baseline breathing; **Red curve**: breathing of HDM sensitized guinea pigs after *i.n.* HDM challenge

Figure 1: Schematic drawing of guinea pig breathing waveforms and computation of enhanced pause in flow whole-body plethysmography used in this PhD study. (A) Breathing curve before (blue) and after (red) *i.n.* HDM challenge in HDM sensitized guinea pigs. (B) Computation of enhanced pause.

1.4.2 Airway hyperresponsiveness

Airway hyperresponsiveness is a crucial pathophysiological feature of asthma, which is defined as the excessive bronchoconstriction to stimuli that normally have no or very limited effects in healthy subjects[49]. Clinically, bronchial provocation tests (BPTs) are used to

measure AHR. These include direct and indirect BPTs. The direct BPTs refer to the provocation by airway smooth muscle (ASM) cell receptor agonists, such as histamine, methacholine or CysLTs which bind to the H_1, M_3 and $CysLT_1$ receptors, respectively, that induce ASM contraction directly. The indirect BPTs use stimuli, such as allergen, mannitol or adenosine monophosphate that contract ASM indirectly by acting on inflammatory cells (*e.g.*, mast cells), causing the release of contractile mediators[50]. Clinically, there are two components in the AHR, *i.e.*, the variable and fixed component. The former changes with environmental exposures and can be affected by treatments (*e.g.*, inhaled corticosteroids), the latter remains constant which mainly relates to the structural changes of the airways[51]. Direct BPTs usually reflect the persistent component whereas indirect BPTs reflect variable component of AHR[52]. The bronchoconstriction can either be accessed by spirometry measuring a 15-20% drop in FEV_1 or by respiratory mechanics by forced oscillation technique (FOT)[49]. In animal experiments, FOT technique is broadly used to assess AHR[53].

1.4.3 Airway inflammation

Airway inflammation in asthma patients can generally be divided into two major types: type (T) 2 and non-type 2 (non-T2) inflammation. Furthermore, based on the increased proportion of eosinophils and neutrophils in induced sputum, four inflammatory subtypes are identified, that are: eosinophilic, mixed granulocytic (increase of both eosinophils and neutrophils), neutrophilic and paucigranulocytic inflammation (no increase of eosinophils and neutrophils)[54]. The development of either T2 or non-T2 inflammation depends on the conditions of the asthmatic individuals and the exposures to different stimuli. However, both inflammatory types are influenced by responses of the airway epithelial layer which is the first line defense of airborne agents.

Airway epithelium works as both a physical barrier and airway modulator by releasing different cytokines, chemokines, growth factors, lipid mediators, extracellular matrix, protein mediators and gases to respond to external stimuli[22]. Asthma patients demonstrate impaired epithelial barrier with malfunction of tight junctions by losing of E-cadherin[55] and claudin-18[56]. Meanwhile, some protease containing allergens, such as house dust mite (HDM)[57] and cockroaches[58], directly cleavage tight junctions, which increase the permeability of epithelial barrier. The inflammatory processes are initiated by exposing to allergens and other environmental triggers (such as diesel exhaust particles) that stimulate epithelial cells to produce alarmins, such as interleukin (IL) -25, IL-33 and thymic stromal lymphopoietin (TSLP)[59,60]. These alarmins have potential to affect various immune cells (*e.g.*, dendritic cells, lymphocytes, type 2 innate lymphoid cells (ILC2s), mast cells, basophils and eosinophils).

1.4.3.1 T2 inflammation

The T2 inflammation, characterized as the presence of high numbers of eosinophils and T2 cytokines (*e.g.*, IL-4, IL-5, IL-9, and IL-13), accounts for around 50% of asthma[61]. Both adaptive and innate immune system are involved in the development of T2 inflammation, the

former relates to the typical allergic responses and the latter causes non-allergic eosinophilic inflammation.

The adaptive immune responses for T2 inflammation start with the access of allergens to subepithelial antigen presenting cells (APCs), primarily dendritic cells. The APCs recognize, process and present allergens to $CD4^+$ T helper (Th) 0 cells in the nearby lymph nodes and polarize the cells to Th2 cells. Th2 cells together with ILC2s can produce type 2 cytokines. IL-4 causes the transformation of B cells into plasma cells where antibody class switching, and antigen specific immunoglobulin (Ig) E production occur[62]. The produced IgEs circulate in the blood stream, to reach, and bind to the surface of mast cells and basophils via FcεRI receptors. When allergen encounter the mast cells again and bind to allergen specific IgE bound to FcεRI, allergic reaction occurs. Mast cells release cytokines, chemokines and growth factors which facilitate the development of inflammation. Both IL-4 and IL-13 cause smooth muscle hyperreactivity[63,64] and induce mucus secretion from goblet cells[65-67]. Interlukin-5 and IL-9 contribute to eosinophil recruitment and survival[68,69]. Interlukin-5 promote the proliferation and survival of mast cell progenitors[70]. Interlukin-9 induces mast cell hyperplasia[71-73]. All these cytokines together contribute to the development of the T2 asthma (**Figure 2**).

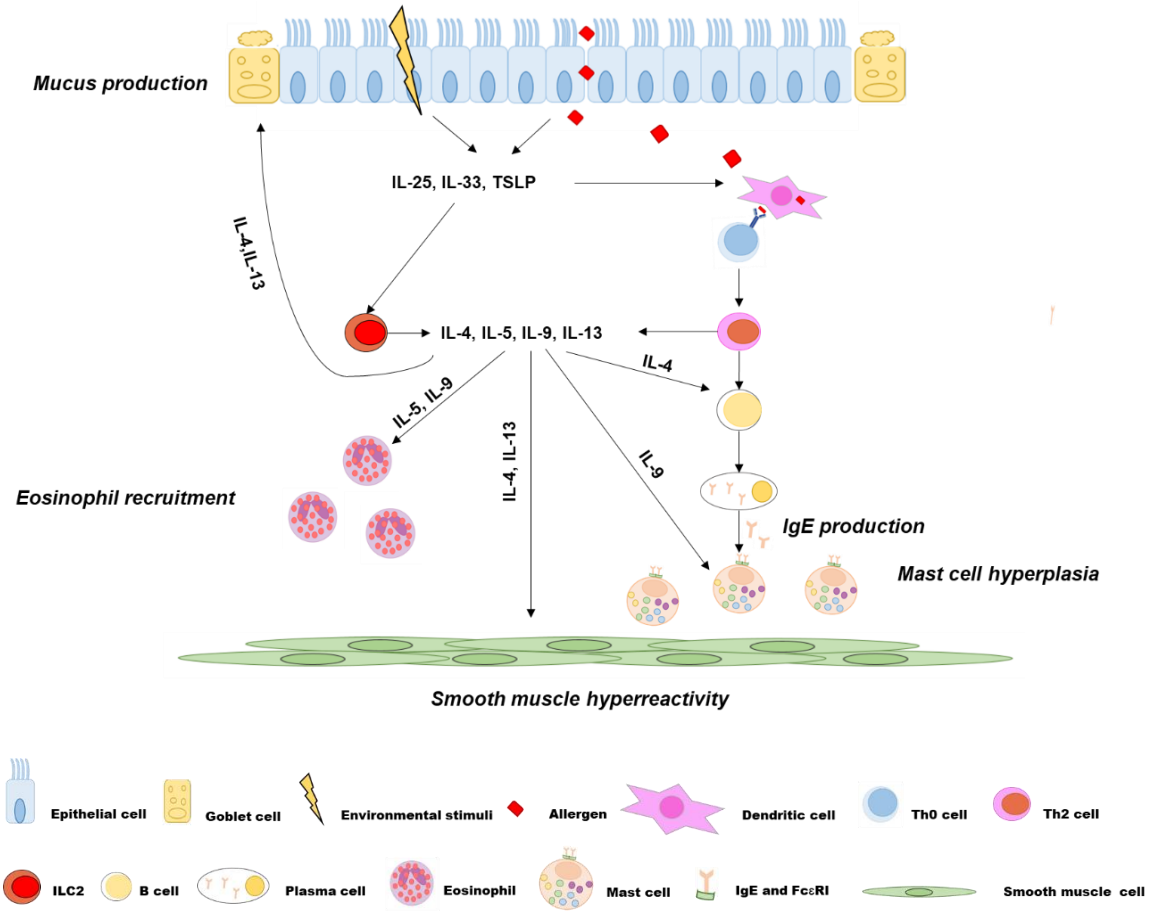


Figure 2: Schematic drawing of T2 inflammation

Biomarkers of T2 inflammation include sputum and blood eosinophils, fractional exhaled nitric oxide, periostin, allergen specific IgE, urine LTE_4 and PGD_2[74-76]. These biomarkers

often correlate with type 2 cytokines and can be used to guide treatments with anti-T2 biologics which are denoted as antibodies against specific T2 cytokines or their receptors[74-76]. In recent years, several biologics have been approved for severe asthma treatments. These including anti-IgE (Omalizumab), anti-IL-5 (Mepolizumab and Reslizumab) or IL-5Rα (Benralizumab), anti-IL-4Rα (Dupilumab, blocking signaling of both IL-4 and IL-13)[18] and anti-TSLP (Tezepelumab)[77].

1.4.3.2 Non-T2 inflammation

Compared to T2 inflammation, non-T2 inflammation, including neutrophilic and paucigranulocytic inflammation (lack of inflammatory cells) of asthma, is less characterized. Several cells (Th1, Th17 and ILC3 cells) are suggested to be involved in non-T2 inflammation by releasing cytokines or chemokines, such as interferon-γ, TNF-α, IL-1β, IL-6, IL-8 (CXCL8), IL-17A, IL-17F, IL-21, IL-22 and IL-23[78-83].

Some stimuli can facilitate the formation of neutrophilic inflammation, such as viral infections[84,85], exposures to environmental pollutants (*e.g.*, ozone[86] and diesel exhaust particulates[87]), smoking[88] and occupational chemicals (*e.g.*, toluene diisocyanate[89] and grain dust[90]). By sensing those irritants (*e.g.*, through the pattern recognition receptors (PRRs)), the epithelial cells release IL-8 (CXCL8)[86,88-90] and LTB_4[91], which chemoattract neutrophils. Th17 cells are also able to enhance the recruitment of neutrophils by releasing IL-8[92] and IL-17[93]. Several non-T2 cytokines, *e.g.*, interferon-γ[94], TNF-α[95] and IL-17[96], have been shown to induce hyperreactivity of airway smooth muscle.

The mechanism for paucigranulocytic asthma is poorly understood. It is suggested that the altered neuronal control of smooth muscle might be involved in this type of asthma as the nerve growth factor (NGF) has been shown to induce AHR in murine models[97]. Mast cells have been shown to be a source of NGF[98], therefore, the interactions between mast cells and nerves might also have roles in paucigranulocytic asthma.

1.4.4 Airway remodeling

Airway remodeling, another cardinal feature of asthma, refers to the structural changes of airways in asthmatics, including epithelium changes (*e.g.*, epithelium denudation, goblet cell hyperplasia and mucin hypersecretion)[99], submucosal glands volume increase[100,101], reticular basement membrane thickening, subepithelial fibrosis (the deposition of collagen I, collagen III and fibronectin[102]), smooth muscle hyperplasia and hypertrophy[101], extracellular matrix glycoprotein deposition[103] and increased bronchial wall vascularity[104]. The onset of airway remodeling can be very early in childhood asthma[105] and in adult asthma patients[106]. However, due to the difficulties of investigations in humans, the mechanisms behind airway remodeling and its role in asthma are far from clear. Whether it is reversible or not after treatments needs to be further investigated.

1.5 MAST CELLS

Mast cells (MCs) are mononuclear innate immune cells filled with secretory granules which were first described and named by Paul Ehrlich in 1878[107]. They have widespread distribution in the body, commonly at surfaces facing the external environment, such as skin, airways, and intestine[108]. Mast cells recognize and respond to various internal and external agents, participating in both innate and adaptive immunity. They have important roles in physiological processes such as hemostasis, tissue repair and host defense against pathogens. Furthermore, MCs are also involved in many diseases including allergy, inflammatory disorders such as allergy and autoimmune diseases, heart disease and cancer[109-111].

1.5.1 Origin

Human MCs are tissue resident cells originating from the yolk sac and $CD34^+$ bone marrow hematopoietic MC progenitors (MCps)[112]. Unlike other myeloid cells that circulate in mature forms, MCps enter the circulation as immature cells that migrate to barrier tissues such as skin, lung and gastrointestinal system, where they undergo differentiation, maturation and survival with the help of stem cell factor (SCF) acting on the KIT receptor (CD117), and other microenvironmental factors[113-116].

1.5.2 Phenotypes

Human lung MCs are heterogenic with various phenotypes. Oversimplified, human MCs can be divided into two subpopulations according to the expression of neutral proteases, that are: MC_T ($tryptase^+/chymase^-$) and MC_{TC} ($tryptase^+/chymase^+$)[117]. The MC_T and MC_{TC} coexist in all lung compartments, where MC_T is the most abundant in the airways and parenchyma[117,118], MC_{TC} prevails in the pleura, and the numbers of the two phenotypes are about the same around pulmonary vessels[118]. Phenotypic changes can occur between MC subpopulations according to tissue environment[113]. There are site-specific MCs in the lung, which show distinct expression of receptors and enzymes[118]. Functionally, MC_T and MC_{TC} respond differently when encounter a stimulus. For instance, MC_T respond to platelet activating factor, whereas MC_{TC} react to opiates[115]. Both MC_T and MC_{TC} can be activated by FcεRI cross-linking, however, only MC_{TC} by substance P[115,119].

1.5.3 Mast cell receptors

In addition to FcεRI and KIT, human MCs express a variety of surface receptors, such as chemokine receptors (*e.g.*, CXCR1-4, CX3CR1, CCR1, CCR3-5), interleukin receptors (*e.g.*, IL-15R, IL-18R, IL-33R/ST2), TSLPR, pattern recognition receptors (*e.g.*, Toll-, NOD- and RIG- like receptors), amine receptors (*e.g.*, H1-4R and 5-HTRs), purinergic receptors (*e.g.*, P1, P2X and P2Y receptors), corticotricotropin releasing hormone receptors, cannabinoid receptors (CB1 and CB2), Mas-related G-protein coupled receptor X2 (MRGPRX2; which will be highlighted below) and inhibitory receptors (*e.g.*, FcϒRIIB, CD300, CD72, and sialic acid binding Ig-like lectins (Siglec-6, -7 and -8)[120,121]. Thus, MCs have the potential to

recognize and respond to plenty of agents that might results in degranulation and/or release of cytokine/chemokine and lipid mediators[111].

1.5.4 Mast cell activation

1.5.4.1 IgE/FcεRI

The most well-defined MC activation signaling pathway is through the allergen-IgE complex crosslinking its high affinity cell surface receptor, FcεRI. This receptor is composed of an IgE-binding α subunit and a signaling module $\beta\gamma_2$[122]. When crosslinked by allergen, FcεRI activation signaling is initiated by the receptor related Lyn (a Src family tyrosine kinase) resulting phosphorylation of downstream tyrosine kinases (*e.g.*, Syk, SLP-76, Vav, Shc, Grb2 and Sos), enzymes (*e.g.*, $PLC\gamma_1$ and $PLC\gamma_2$) and GTPases (*e.g.*, Rac and Ras). Another transducing pathway is through PI3K via phosphorylation of Fyn and Gab2. The aforementioned proteins further transfer signals into MCs resulting degranulation, cytokine/chemokine production and arachidonic acid metabolism[110,123].

1.5.4.2 MRGPRX2

Mas-related G-protein coupled receptor X2 is a seven transmembrane receptor that is mainly expressed on MCs[124], especially on MC_{TC}[125], albeit some expressions in blood basophils and eosinophils have been reported[126]. The ligands for MRGPRX2 are various including natural peptides (*e.g.*, substance P, cortistatin 14, β-defensin, LL37, PAMP 9-20 and mastoparan), bacterial products (quorum-sensing molecules), and synthetic drugs with tetrahydroisoquinoline motif[127-129]. Compound 48/80 (C48/80) is a condensation product of N-methyl-p-methoxyphenethylamine with formaldehyde[130]. It has been shown that C48/80 activate MCs[131,132] through human MRGPRX2 receptors[125,127].

Activation of MCs by MRGPRX2 is distinct from the FcεRI pathway, where the former induces a rapid and transient MC degranulation with small, individual and spherical granules, whereas the latter demonstrates slower but sustained Ca^{++} influx with larger and heterogeneous granule sizes[133]. In addition, activation of MRGPRX2 pathway has been shown to release less PGD_2, PGE_2 and cytokines compared to activation of FcεRI. The intracellular signaling pathway for MRGPRX2 remains to be defined. However, recently, it has been found that, unlike FcεRI, MRGPRX2-mediated degranulation does not require the involvement of IKK-β, SNARE proteins synaptosomal-associated protein-23 and syntaxin-4[133]. The store-operated Ca^{++} entry via the calcium sensor, stromal interaction molecule 1 regulate both FcεRI- and MRGPRX2-mediated responses[134]. The G proteins (Gαi and Gαq) together with Ca++ Channels, ERK1/2 and PI3K are required for MGRPRX2 induced degranulation of skin MCs[135].

1.5.5 Mediator release

After activation, MCs release a wide range of bioactive mediators including preformed and *de novo* synthesized mediators through different ways. Preformed mediators are released through exocytosis of secretory granules. Lipid mediators are synthesized in the lipid body of MCs and released through membrane transporters. Moreover, the newly synthesized cytokines, chemokines and growth factors are usually released via constitutive exocytosis[136].

1.5.5.1 Preformed mediators

Mast cells store various mediators in their secretory granules including bioactive amines (*e.g.*, histamine and serotonin), lysosomal enzymes (*e.g.*, β-hexosaminidase and cathepsins), proteases (*e.g.*, tryptase, chymase and carboxypeptidase A), proteoglycans (*e.g.*, heparin and chondroitin sulphate), cytokines (*e.g.*, TNF-α, IL-4 and IL-15), chemokines (*e.g.*, CCL5, CCL11, CXCL8, CCL2, CCL7 and MCP-4), growth factors (*e.g.*, TGF-β, bFGF-2, VEGF, NGF and SCF) and peptides (*e.g.*, endothelin-1, LL-37, substance P and vasoactive intestinal peptide)[137].

1.5.5.2 Newly synthesized mediators

In complement to the stored mediators, there is *de novo* synthesis of proinflammatory cytokines (T2 cytokines: IL-4, IL-5, IL-9, IL-13 and non-T2 cytokines: IL-1, IL-2, IL-3, IL-12, IL-17, IL-18, IL-33, TNF-α, and IFN-γ), and anti-inflammatory cytokines, such as IL-10 and TGF-β. The *de novo* synthetized chemokines include CCL5 and CXCL8[109].

1.5.5.3 Lipid mediators

Lipid mediator synthesis starts when phospholipase A2 (PLA2) is activated and liberate polyunsaturated fatty acids (PUFAs) from membrane bound phospholipids. The most studied PUFA is arachidonic acid (AA), which is metabolized into various downstream mediators called eicosanoids through different enzymatic pathways.

One major pathway is the cyclooxygenase (COX) pathway. Arachidonic acid is catalyzed into unstable PGG_2 by either of the two COX isoenzymes: COX-1 and COX-2. This is followed by the metabolism of PGG_2 to PGH_2 which is a common precursor of five major prostanoids: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 (**Figure 3**). These are derived through specific enzymes (hematopoietic PGD synthase or lipocalin-type PGDS for PGD_2, microsomal PGE synthase (mPGES)-1, mPGES-2, or cytosolic PGES for PGE_2, PGF synthase, PGI synthase and thromboxane synthase for $PGF_{2\alpha}$, PGI_2 and TXA_2 respectively).

In addition to the COX pathway, the 5-lipoxygenase/5-lipoxygenase-activating protein (5-LOX/FLAP) pathway is another important route of AA metabolism. This pathway generates leukotrienes: LTB_4 and CysLTs (LTC_4, LTD_4 and LTE_4). All the leukotrienes are metabolized from AA intermediate product 5-hydroperoxyeicosatetraenoic acid (5-HpETE) and LTA_4. Leukotriene B_4 is formed via LTA_4 hydrolase, whereas LTC_4 by LTC_4 synthase.

Leukotriene C_4 is metabolized by successive cleavage of the glutathionyl conjugated at C6 into LTD_4 and LTE_4 by the stepwise actions of the gamma-glutamyl transpeptidase and a specific dipeptidase iso-enzyme (**Figure 3**)[138,139].

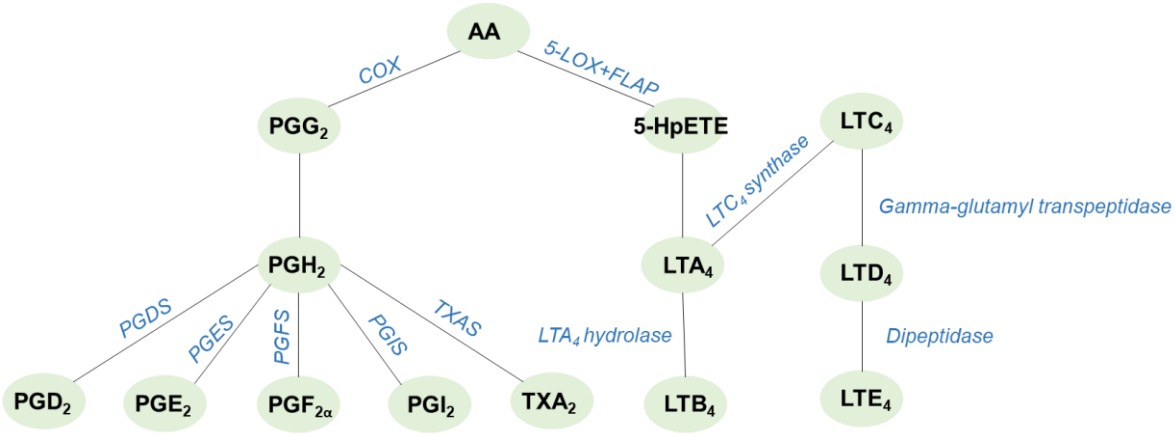


Figure 3: Arachidonic acid metabolism

There are also other enzymatic/non-enzymatic metabolites of AA and other PUFAs (*e.g.*, linoleic acid, eicosapentaenoic acid and docosahexaenoic acid), however, compared to prostanoids and leukotrienes, the importance of these mediators in asthma is less understood.

1.6 MAST CELLS IN ASTHMA

Mast cells may have crucial roles in the pathophysiology of asthma. Mast cells are present in the epithelium, submucosa and airway smooth muscle (ASM) layer of human asthmatic airways[140-142] which have increased MC numbers, especially MC_{TC}[143] in the ASM bundle. On the other hand, MC_T predominates in mild asthma[142]. Compared to mild asthma, there is a higher proportion of MC_{TC} in severe asthma with increased level of PGD_2 in bronchoalveolar lavage fluid[142], indicating the importance of MCs in asthma. The influence of MCs on inflammatory cells may contribute to the development of airway inflammation, whereas the crosstalk between MCs and airway structural cells offers the possible basis for the progress of AHR and airway remodeling.

1.6.1 Mast cells and epithelium

Human MCs have avid adhesion to epithelium via carbohydrate-mediated mechanism[144]. The settled MCs can respond directly to the exogeneous stimuli, such as airborne allergens, hyperosmolar or inhaled adenosine. In addition, upon activation, MCs releases mediators (*e.g.*, tryptase, IL-4 and IL-13) that cause epithelium malfunction, such as denudation and increased permeability, initiating the development of asthma pathology[145].

1.6.2 Mast cells and submucosa

Asthmatic patients have increased MCs in submucosal mucous glands and mucus plugging[146], which may be due to increased adhesion to glandular cells, induced by released

chemokines such as CXCL8, CXCL10, CXCL12, CCL5 and CCL11[145]. Mast cells, in return, release mediators, such as tryptase, chymase, histamine, PGD_2, LTC_4, IL-13, TNF-α and amphiregulin, inducing mucous gland and goblet cell hyperplasia and mucus hypersecretion[145]. In addition, amphiregulin induces the proliferation of lung fibroblasts, contributing to the subepithelial fibrosis[147].

1.6.3 Mast cells and airway smooth muscle

The released mediators from MCs can modulate airway smooth muscle (ASM) responses. The MC derived spasmogenic mediators (*e.g.*, histamine, PGD_2, TXA_2 and CysLTs) cause ASM contraction. In addition, tryptase, TGF-β and FGF participate in ASM hyperplasia and hypertrophy. Moreover, tryptase, IL-4 and IL-13 induce hyperreactivity of ASM[63,145]. On the other hand, ASM cells attract MCs by releasing CXCL10 or SCF which binds to the MC expressed receptors CXCR3[148] and KIT[145].

1.7 TARGETING MAST CELLS

Since MCs are suggested to have important roles in the pathogenesis of asthma. Interventions targeting MCs might be beneficial to asthmatic individuals. These strategies include the prevention of MCs from activation, inhibition or antagonism the effect of MC mediators, and interference with MC survival[149].

1.7.1 Prevention of mast cell activation

One type of compounds that stabilizes MCs is MC stabilizers, for instance, sodium cromoglycate (SCG). By agonizing the inhibitory G-protein-coupled receptor 35 on MCs[150], SCG demonstrates the ability to inhibit histamine release from MCs[151]. However, the efficacy of SCG is limited[152]. Though some improvements were observed in asthmatic symptoms, no significant changes of lung function were found in patients receiving SCG[153]. In asthmatic children, no beneficial effect was found after the treatment with SCG[154].

Another maneuver is to interfere with the classical IgE-mediated MC activation. Anti-IgE (Omalizumab), which is a monoclonal IgG_1 antibody, neutralize free serum IgE[155], preventing the binding to FcεRI and therefore prevent the activation of MCs by allergen[156]. In allergic asthma, both EAR and LAR could be reduced by anti-IgE treatment[157]. In addition, omalizumab reduced asthma exacerbations and lower the required inhaled corticosteroids for symptom control in patients with elevated IgE[158-160]. However, anti-IgE cannot prevent MCs from activation by IgE-independent MC activators.

The activation signaling pathway of MCs is another alternative to prevent MCs from activation. R112, an inhibitor against spleen tyrosine kinase (Syk), completely inhibited IgE induced MC degranulation, lipid mediator and cytokine synthesis[161]. However, in a clinical trial, R112 failed to reduce histamine and tryptase release post allergen challenge[162]. Furthermore, nemiralisib, an inhibitor against phosphoinositide 3-kinase δ (PI3Kδ), even

though reduced sputum IL-5, IL-13, IL-6, and IL-8 levels, did not show any clinical improvements of asthma patients[163].

1.7.2 Inhibition or antagonism of mast cell mediators

As aforementioned, MC releases both preformed and newly synthesized mediators upon activation. Targeting a single contractile mediator or its receptors seems far from enough. For instance, antagonism of histamine H_1, $CysLT_1$, or TP receptors alone did not have good effect on allergen or exercise-induced bronchoconstriction in asthma patients[164,165]. In contrast, only the combined blockade demonstrated predominant inhibition of both EAR and LAR[164,166,167]. Fevipiprant, which targets the DP_2 receptor and block the effect of PGD_2, showed promising effect in reducing sputum eosinophils and improvement of lung function in phase II trials[168,169]. However, it failed to show significant changes of asthma exacerbations in the phase III study[170].

1.7.3 Interference with mast cell survival

Stem cell factor is important for MC survival and maturation[171]. In human asthmatic airways, an increased expression of SCF and its receptor (KIT) were observed[172]. The KIT inhibitor (imatinib) reduced AHR and MCs in severe asthma patients[173]. However, in addition to inhibit KIT, imatinib also has effects on other tyrosine kinases, such as BCR-ABL and PDGFRA[174].

Induction of MC apoptosis is another novel strategy to reduce MC numbers. ABT-737 is an inhibitor of antiapoptotic proteins (*e.g.*, Bcl-2, Bcl-XL, and Bcl-w). Injection of ABT-737 resulted in the abolishment of peritoneal MCs in mice[175]. Mast cells are highly granulated cells. Since the contents of MC granules have many similarities with lysosomes, for instance, both have low pH, contain typical lysosomal enzymes (*e.g.*, cathepsins[176] and β-hexosaminidase[177]), MC granules are therefore named as secretory lysosomes[178]. By permeabilization of those MC granules, lysosomotropic agents, such as mefloquine[179] and siramesine[180], can selectively induce MC apoptosis. The mechanism behind the apoptosis is suggested to be due to the escaped granule proteases activate the pro-apoptotic compounds in the cytosolic compartment of MCs.

1.8 ASTHMA MODELS

In addition to experiments in cells and tissues from human origin, there is a need for animal models to define the mechanisms for asthma. Mice models are often used because of the availability of various immunological and gene editing investigating tools. However, the limited translation of murine research data to humans makes researchers reconsider the limitations of this specie[181]. Guinea pig is an alternative with more similarities with human than mouse regarding lung anatomy, physiological and pharmacological responses[182,183].

To establish animal models of allergic asthma, repeated antigen exposures are usually needed. The most commonly used antigen is ovalbumin (OVA). Depending on different protocols, repeated OVA exposures can induce bronchoconstriction, AHR, airway

inflammation or airway remodeling in guinea pigs which mimic some asthmatic features[183]. However, since OVA is not an airborne allergen to humans and normally OVA need to be given intraperitoneally or subcutaneously with adjuvant, which is not a natural way of exposure to allergens in humans, the use of OVA is therefore questioned.

House dust mite (HDM) is a common and relevant allergen with around 50% sensitization rate for allergic asthma[184]. There are many HDM species widely spread over the world, the most common two species are *Dermatophagoides pteronyssinus* (European house dust mite) and *Dermatophagoides farinae* (American house dust mite), with 85% and 47% occurrence respectively[184]. HDM contains at least 30 allergenic proteins and microbial compounds, such as lipopolysaccharides (LPS) and β-glucans, which can activate both the innate and adaptive immune system promoting allergic responses[185,186].

Cat dander is another human relevant allergen with the sensitization rate around 9 to 15% among adults worldwide[187-189]. In Japan, 70% of asthmatic children who kept cats at home were found sensitized to cat, the prevalence was still high (34%) even in children did not have cats[190]. Several cat dander antigens have been identified, 10 out of 15 have the possibility to bind to antigen specific IgE[191]. Among the antigens, *Fel d1*, also known as *Cat Ag 1* is the most abundant and potent[191].

Taken together, exposing guinea pigs to human relevant allergens (*e.g.*, HDM and Cat dander extract (CDE)) might be promising to establish models that mimic human allergic asthma.

2 RESEARCH AIMS

Except for the bronchoconstriction elicited in allergic reaction and after exercise in certain individuals, the role of mast cells in asthma is still not well defined. The general aim of this PhD project was to investigate the role of mast cells in allergic asthma using guinea pig models exposed to human relevant allergens.

Specific aims:

I. To establish a guinea pig asthma model using a human relevant allergen.
II. To define the histopathological changes of guinea pig lungs by different antigens.
III. To determine mast cell responses to different mast cell agonists in guinea pig airways.
IV. To find a tool to induce mast cell apoptosis.
V. To investigate the role of mast cells on asthma-like features in human and guinea pig models.

3 MATERIALS AND METHODS

3.1 ANIMALS

All the animal experiments were performed with the approvement from Stockholm ethics committee (Permit number: N143-14 and 10973-2019). Both male and female guinea pigs (Dunkin-Hartley), around 300 g, were purchased from Envigo. Animals were housed four to five per cage in the animal facility under 12h light/dark circles with food and water *ad libitum* and daily supply of fresh vegetables or fruits. Animals were acclimatized at least one week before experiments.

3.2 GUINEA PIG SENSITIZATION AND CHALLENGES

In **paper I** and **II**, male guinea pigs, start at approximate 500 g, were exposed to HDM extract (*Dermatophagoides pteronyssinus)* by intranasal instillations under anesthesia with 5% isoflurane for induction and 2% for maintenance. In paper **I**, animals were sensitized twice by 50 µg and 100 µg HDM protein at day 1 and day 4. From day 15, animals were challenged weekly with 25 µg HDM for five consecutive weeks **(Figure 4A)**. In paper **II**, guinea pigs received 200 µg HDM or CDE twice on the first week then challenged with 100 µg antigens, two times per week. In total, animals were exposed to HDM or CDE for 4 weeks, 8 weeks and 12 weeks **(Figure 4B)**.

Male guinea pigs in **paper III**, and female in **paper IV** and **paper V** were sensitized by a single intraperitoneal injection of 100 µg HDM with 100 mg aluminum hydroxide (Al $(OH)_3$) at day 1. Two weeks after sensitization, animals were euthanized by CO_2 and trachea was isolated for *ex vivo* experiments **(Figure 4C)**. For *in vivo* study in **paper V**. Awake animals were challenged with 25 µg HDM through the intranasal route once per week for three or five weeks **(Figure 4D)**. All the antigens were prepared in sterile PBS.

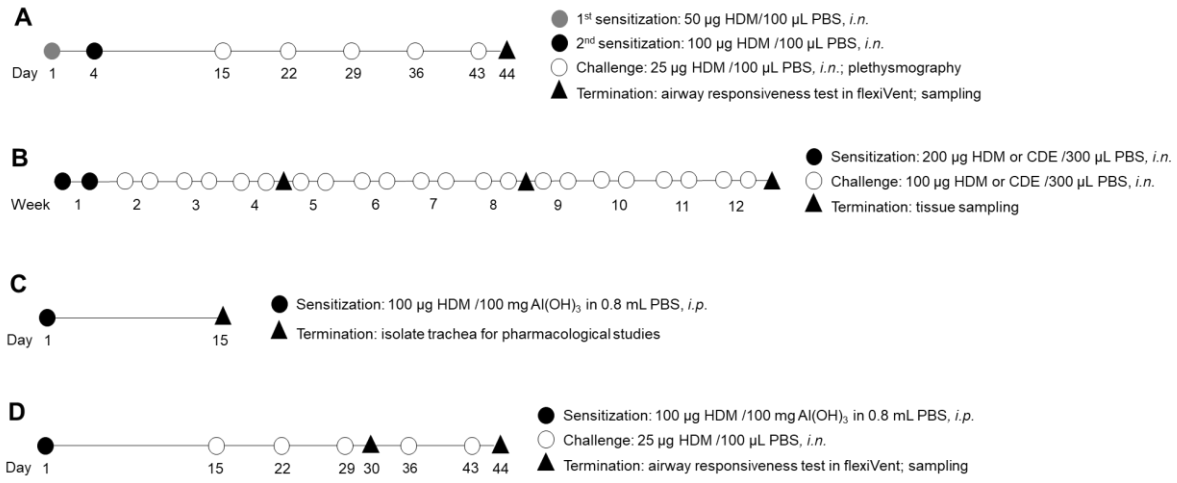


Figure 4: Antigen exposure protocols. (A) paper I; (B) paper II; (C) paper III, IV and V and (D) paper V

3.3 GUINEA PIG TRACHEA AND HUMAN BRONCHI DISSECTION

In **paper III**, **IV** and **V**, after euthanization of guinea pigs, trachea was quickly dissected out and further cut into eight segments after fine dissection in cold Krebs-Henseleit buffer.

In **paper V**, with the consent from patients that underwent lobectomy and approval from reginal ethical committee (Permit number: 2018/1819-31/1), healthy parts of the lung tissues were obtained and human bronchi (diameter between 0.5 and 2 mm), were dissected out in cold Krebs buffer.

Tracheal segments and human bronchi were either used freshly or incubated in 0.5-1 mL DMEM/F-12 medium supplemented with 1% penicillin (100 $IU \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$) with or without interventions in a humified incubator at 37 °C and 5% CO_2 before pharmacological studies in tissue organ bath or myography.

3.4 TISSUE ORGAN BATH AND MYOGRAPHY

Guinea pig tracheal segments in **paper III, IV and V** and human bronchi in **paper V** were mounted in 5 mL organ baths or myograph chambers which were constantly heated and bubbled by carbogen (5% CO_2 in 95% O_2) to keep buffer temperature at 37 °C and pH at 7.4. The force changes of airway smooth muscle were recorded, amplified and displayed in LabChart software. After 45 to 60 min equilibration, a mechanical tension of 30 mN (guinea pig trachea) or 1.5 mN (human bronchi) was applied before tissue viability test with histamine (10 nM - 1 mM), carbachol (1 nM - 0.1 mM), or 60 mM KCl. A combination of histamine (1 mM), acetylcholine (1 mM) and KCl (60 mM) were used to test the viability at the end of experiments.

3.5 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Cytokines in bronchoalveolar lavage fluid (**paper I**) and serum total IgE (**paper I and III)** were measured according to manufacturer's instructions (Nordic Biosite, Täby, Sweden). In **paper I and III**, HDM specific IgG_1, IgG_2 and IgE were measured by antigen capture ELISA where precoated HDM was bind to biotinylated anti-guinea pig antibodies (Nordic Biosite, Täby, Sweden), followed by development with streptavidin HRP and TMB substrate. Absorbance differences at 450 nm and 490 nm were calculated (Δ ABS 450/490 nm).

In **paper III**, histamine in organ bath fluid was measured using HR-Test RLA210 Plate Kit (RefLab, Copenhagen, Denmark). In brief, samples were added to plates which consists of pre-coated microtiter plates with the histamine standard (50 ng/mL). Histamine release was detected fluorometrically (o-phthalaldehyde (OPA)-method) with a HISTAREADER™ 501-1 and concentrations of histamine in samples were calculated based on the histamine standard.

3.6 MASS SPECTROMETRY

Organ bath fluid in **paper III** was stored in -80°C directly after collection for measuring lipid mediators which were enriched and quantified using liquid chromatography coupled to

tandem mass spectrometry (LC-MS/MS) using an Acquity UPLC™ coupled to Xevo TQ-XS™ instrument[192].

3.7 WHOLE-BODY PLETHYSMOGRAPHY

Sensitized guinea pigs (**paper I**) were placed in the calibrated flow plethysmography chambers for 5 to 20 min acclimatization followed by baseline recordings of breathing parameters. After that, animals were challenged intranasally with HDM under light anesthesia by isoflurane. The parameters were recorded again for 90 min after HDM instillation.

3.8 AIRWAY RESPONSIVENESS TEST IN FLEXIVENT

One day after the last challenge, guinea pigs were anesthetized by intraperitoneal injection of 40 mg/kg ketamine with 5 mg/kg xylazine (**paper I**) or with 0.5 mg/kg medetomidine (**paper V**). Animals were tracheostomized, incubated and ventilated in the flexiVent system with FX4 module where airway resistances were measured. Airway responsiveness was obtained by measuring airway resistances to nebulized methacholine with increasing concentrations.

3.9 HISTOLOGY

To capture the morphological changes of tissues (**paper I, II, IV** and **V**), different staining methods were used. Most samples were fixed in 4% buffered formaldehyde. Tissues for mast cell quantification were fixed in Carnoy solution (60% ethanol, 30% chloroform, 10% acetic acid). Samples were fixed for at least 5 to 24h before dehydration and embedding. Five µm sections were obtained and slides were stained with Hematoxylin Eosin, Picro-Sirius Red, Periodic Acid-Schiff (PAS), Astra blue to identify airway inflammation, subepithelial collagen deposition, goblet cell hyperplasia and mast cells respectively. To identify mast cell phenotypes (**paper II**), anti-human tryptase (clone: G3, Chemicon, Temecula, CA) and anti-guinea pig chymase antibodies were used. The polyclonal antibodies against guinea pig chymase were produced in rats which were immunized by the recombinant guinea pig chymase (Innovagen AB, Lund, Sweden). The recombinant guinea pig chymase was produced in baculovirus-infected insect cells which express the chymase coding region obtained from the Swiss Prot/Tr EMBL database (P85201) and purified as previously described[193]. All the quantifications were performed on blinded sections.

3.10 CALCULATIONS AND STATISTICAL ANALYSIS

Smooth muscle contractions were normalized to the maximal contraction induced by histamine (10 nM - 0.1 mM**; paper III**), carbacol (1 nM - 0.1 mM; guinea pig trachea in **paper IV** and **V**) and KCl (60 mM; human bronchi in **paper V**). All the data was analyzed in GraphPad Prism (version 8.0.1(244), San Diego, California, USA) and presented as mean ± standard error of the mean (SEM). One-way or two-way ANOVA or t-test were performed to identify group differences. Significances were set at $p < 0.05$.

4 RESULTS AND DISCUSSION

4.1 ESTABLISHMENT OF AN ALLERGIC ASTHMA MODEL IN GUINEA PIGS USING HOUSE DUST MITE (PAPER I)

To establish an animal model that mimic human allergic asthma, we initiated the sensitization of guinea pigs to HDM by two times of antigen exposures (50 µg and 100 µg) via intranasal instillation with a 3-day interval. Two weeks after the first sensitization, guinea pigs were canulated and exposed to nebulized HDM aerosol in the flexiVent system and airway resistances were measured using the FOT technique (**Figure 5A**) to investigate the antigen induced bronchoconstriction (AIB). After HDM challenge, an increase of *Rn* and *G* were observed in sensitized guinea pigs (**Figure 5B**), suggesting the contraction of the airways. This contraction was only found in sensitized but not in control animals indicates the sensitization is required for AIB. However, no increase of *H* was found after antigen exposures, this might be due to that mild homogeneous constriction of the airways only increases lung resistance but not the elastance[194]. The AIB was totally inhibited by pretreatment of antagonist of the histamine H_1 receptors and inhibitors for the formation of prostanoids and leukotrienes. This is in accordance with a previous study[195], indicating that the responses were mediated by mast cell mediators.

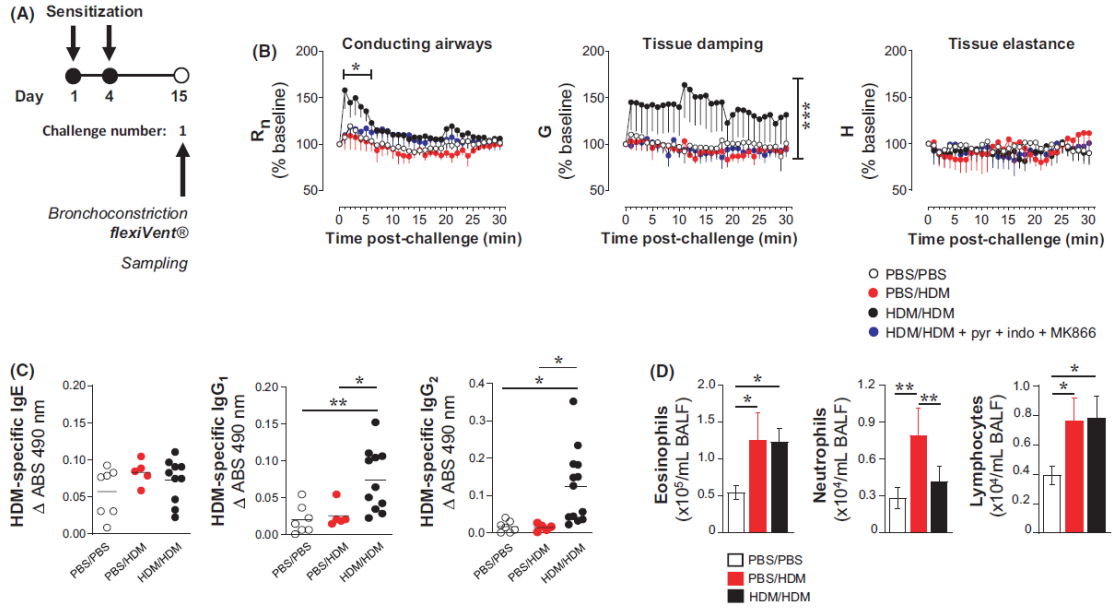


Figure 5: The effect of HDM sensitization and the first challenge on antigen induced bronchoconstriction, production of immunoglobulins and inflammation. (A) Protocol. Animals received two sensitizations intranasally at day 1 and 4 with 50 µg and 100 µg HDM or PBS. The first challenge was performed in flexiVent at day 15. (B) The challenge in flexiVent. The normalized changes of Newtonian resistance (*Rn*), tissue damping (*G*) and tissue elastance (*H*) after PBS or HDM aerosol exposures to guinea pigs that were sensitized with PBS or HDM. PBS/PBS: animals sensitized and challenged with PBS; PBS/HDM: sensitized with PBS, challenged with HDM; HDM/HDM: sensitized and challenged with HDM; HDM/HDM+pyr+indo+MK886: sensitized, challenged with HDM and pretreated intraperitoneally with pyrilamine (pyr; 10 mg/kg), indomethacin (indo; 2 mg/kg), and MK886 (3 mg/kg) for 30 min before the challenge. (C) Levels of serum HDM-specific IgE, IgG_1 and IgG_2. (D) Differential cell counts from bronchoalveolar lavage fluid (n=4-13 in each group). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Serum HDM-specific immunoglobulins were quantified by ELISA. The HDM- specific IgG_1 and IgG_2 were significantly increased two weeks after HDM sensitization (**Figure 5C**). In contrast, no change of HDM-specific IgE was observed. This finding indicates that antigen specific IgGs appear earlier than IgE, the latter appears only after repeated antigen exposures, which has also been shown in a guinea pig model induced by OVA[196]. The increase of immunoglobulins could only be found in animals that were sensitized to HDM indicating that the sensitization phase is crucial for the production of immunoglobulins. It has been shown that, in addition to IgE, both IgG_1 and IgG_2 have the possibility to induce smooth muscle contractions in guinea pigs[197,198], therefore the possible mechanism behind HDM induced bronchoconstriction might be as follows: The sensitizations led to the production of HDM-specific IgGs which bind to the surface of mast cells. The binding of HDM to IgGs during the challenge cross-linking the Fc receptors which induce mast cell activation releasing the contractile mediators. However, further experiments need to be performed for detailed analysis of this pathway.

To investigate the effect of HDM to the development of inflammation, we examined the inflammatory cells in the bronchoalveolar lavage fluid (BALF). The HDM exposures increased both eosinophils and lymphocytes (**Figure 5D**) which support that HDM could induce airway inflammation. However, neutrophils increased only in animals that encounter HDM for the first time which suggests the activation of the innate immune responses. No significant increase of neutrophils was found in sensitized animals which might be due to the sensitization modifies the immune system and therefore decrease the recruitment of neutrophils[199].

To induce other features of allergic asthma in guinea pigs, we prolonged the HDM challenges to once a week for five weeks. One day after the last challenge, airway responsiveness to methacholine was assessed in flexiVent and lung samples were collected to assess airway inflammation and remodeling (**Figure 6A**). The response of guinea pigs in each of the five challenges were monitored using the whole-body plethysmography. House dust mite exposures induced a gradual increase of Penh which reached maximum around 40 to 70 min and resolved in 90 min (**Figure 6B**). Even though Penh is not a true measurement of airway resistance, the increase of Penh has been shown to correlate with bronchoconstriction[46]. Thus, the increase of Penh after each challenge reflects the antigen induced bronchoconstriction. The difference of the response pattern measured in flexiVent (transient) and plethysmography (slow onset but longer duration) might be due to the particle size of HDM. The nebulized HDM aerosol in the flexiVent was small and could be quickly dispersed into the airways and lung, while the HDM solution given by intranasal instillation before plethysmography could be retained in the nasal cavity and slowly be breathed into the lung to induce a contractile response. After each HDM challenge, an increase of the maximal Penh value could be observed (**Figure 6C**), supporting that MCs can be degranulated repeatedly with enough time in between the challenges[200,201].

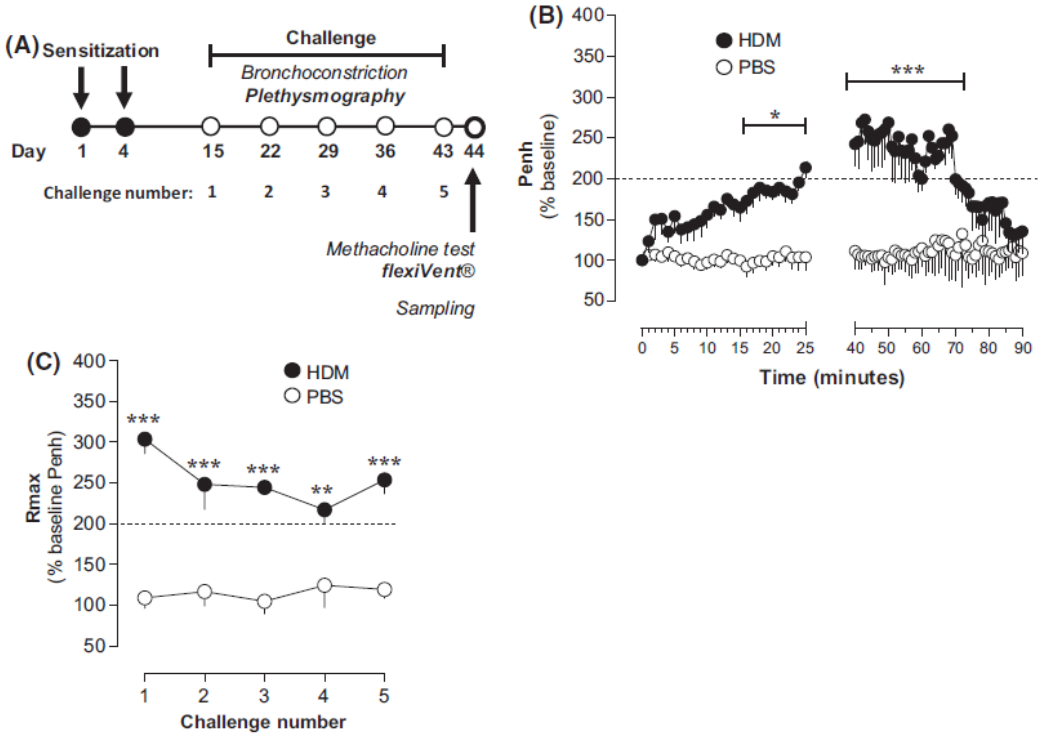


Figure 6: The responses induced by HDM recorded in plethysmography. (A) Protocol. Animals received two sensitizations intranasally at day 1 and 4 with 50 µg and 100 µg HDM or PBS followed by five weekly challenges with 25 µg HDM or PBS. (B) The normalized Penh values at the first intranasal challenge with HDM or PBS. (C) The maximal Penh (Rmax) of the five challenges (n=7-8 in each group). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

To measure the airway responsiveness, guinea pigs were anesthetized, canulated and ventilated in flexiVent. Increasing concentrations of methacholine (MCh) aerosol were given to test the responses of animals. Methacholine, also known as acetyl-β-methylcholine, activates the muscarinic receptors (M_3 receptors) in the airways, causing smooth muscle constrictions. Depending on the condition of the airways, the responses to MCh can be different. Significant increases of *Rn*, *G* and *H* were found in animals that were previously exposed to HDM (**Figure 7**). This finding indicates that the guinea pigs developed AHR to MCh both in the conducting airways and peripheral lung tissue which is similar to the AHR of asthma patients examined with impulse oscillometry technique[202].

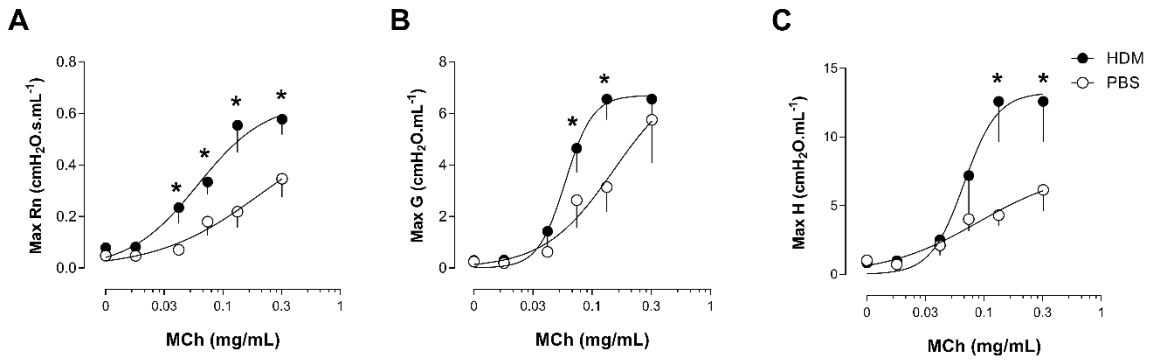


Figure 7: Responses of guinea pigs to methacholine after repeated HDM exposures. Airway responsiveness to methacholine (MCh) was assessed in flexiVent one day after the last challenge. Differences of resistance in

conducting airways (*Rn*; A), tissue damping (*G*; B) and tissue elastance (*H*; C) were compared between control (PBS) and HDM exposed animals (n=8 in each group). * $p<0.05$.

We next examined the effects of HDM on the development of airway inflammation. A significant increase of eosinophils in the BALF (**Figure 8A)** and inflammatory area in the airways (**Figure 8B)** were found in guinea pigs that underwent HDM exposures for seven weeks.

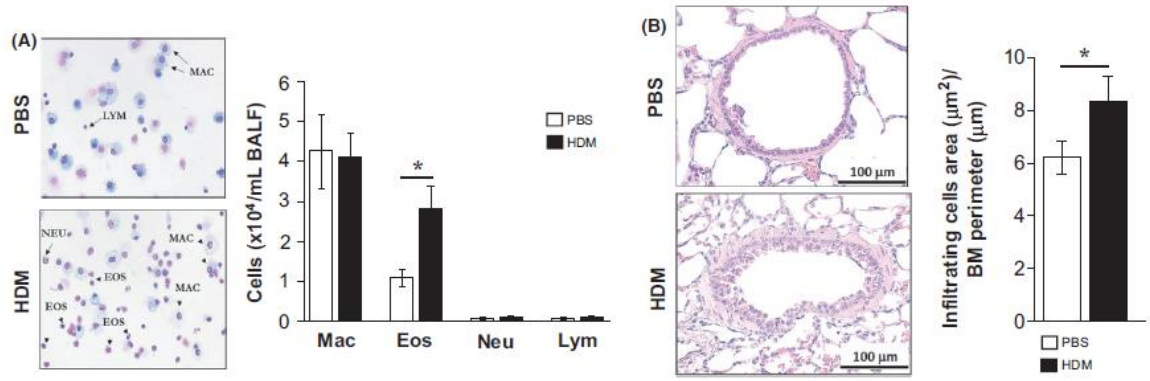


Figure 8: The change of inflammatory cells in guinea pigs after repeated HDM exposures. (A) May-Grünwald Giemsa staining of BALF cells and (B) Hematoxylin-Eosin staining of lung tissues from PBS and HDM exposed guinea pigs (n= 8 in each group). * $p<0.05$.

Moreover, we measured several typical T2 cytokines (IL-4, -5 and -13) together with CCL11 and IL-6 in the BALF. The levels of IL-13 were significantly higher in HDM treated animals than controls that received PBS (**Figure 9**), however, no significant increases of other cytokines and CCL11 were observed.

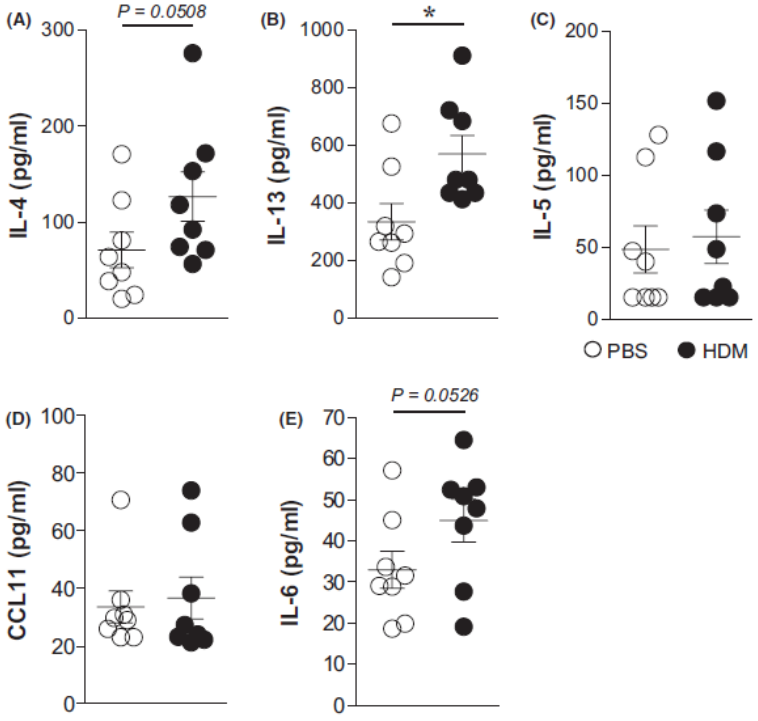


Figure 9: Cytokines, CCL11 and IL-6 in the BALF. (A) IL-4, (B) IL-13, (C) IL-5, (D) CCL11 and (E) IL-6 level in the BALF from PBS and HDM exposed guinea pigs (n=8 in each group). * $p<0.05$.

Taken together, our findings suggest that repeated HDM exposures can induce eosinophilic inflammation in guinea pig lungs with an increase of T2 cytokine IL-13, which resembles the T2 inflammation of allergic asthma. It has been shown that HDM could induce eosinophilia in guinea pigs[203] and IL-5 and CCL11 were suggested to have a close correlation with eosinophils[204]. The non-elevated IL-5 and CCL11 in the BALF might be due to that the sampling time is not appropriate since the eosinophils were already in the airways.

To investigate whether HDM exposures have effects on the structure of airways, guinea pig lungs were fixed, sectioned, and stained with different staining methods. In the sections stained with Hematoxylin-Eosin, an increase of subepithelial layer was found in HDM exposed animals (**Figure 10A**). However, the increase of airway smooth muscle area did not reach statistical significance (p=0.0671). In addition, the Picro-Sirius Red positive area of the airways (**Figure 10B**) and the PAS positive cells in the epithelium (**Figure 10C**) were significantly increased in the sections from HDM exposed animals. These histological findings suggest that HDM can induce airway remodeling characterized with the enlargement of airways, subepithelial collagen deposition and goblet cell hyperplasia. These successfully capture the airway remodeling features of human asthma[205].

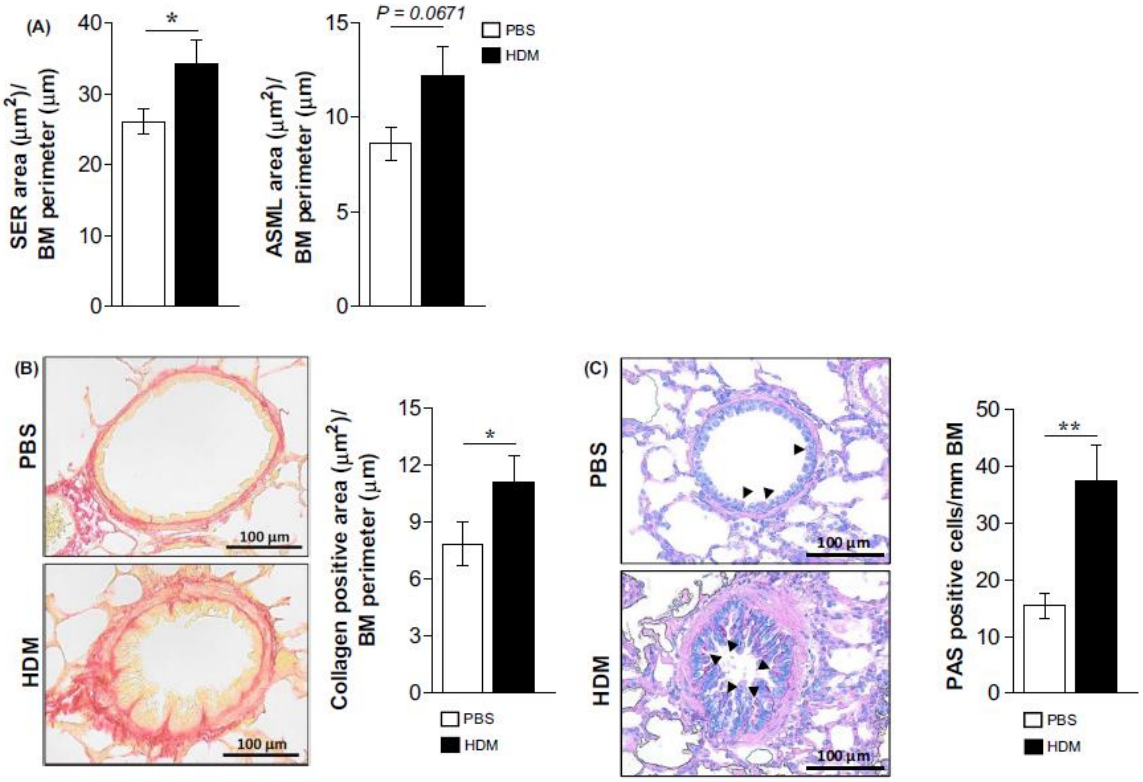


Figure 10: The changes of airway structure in guinea pigs after repeated HDM exposures. (A) Subepithelial area (SER) and airway smooth muscle layer (ASML) area calculated from Hematoxylin-Eosin-stained guinea pig lung tissue. (B) Picro-Sirius Red and (C) Periodic Acid-Schiff (PAS) staining of the of lung tissue from PBS and HDM exposed guinea pigs (n= 8 in each group). * p<0.05, ** p < 0.01.

To investigate the long-term effect of HDM exposures on immunoglobulins and mast cells. Serum HDM-specific IgE, IgG_1 and IgG_2 were examined by ELISA. Notably, in contrast to the single HDM challenge, animals received five challenges demonstrated elevation of both

HDM-specific IgE and IgGs (**Figure 11A**), which confirms that repeated antigen exposures increase both IgE and IgG in guinea pigs[196]. Furthermore, a marked increase of mast cells was observed in HDM treated animals (**Figure 11B**). The mast cell hyperplasia found in our guinea pig model also recaptures the finding in human asthmatics[143].

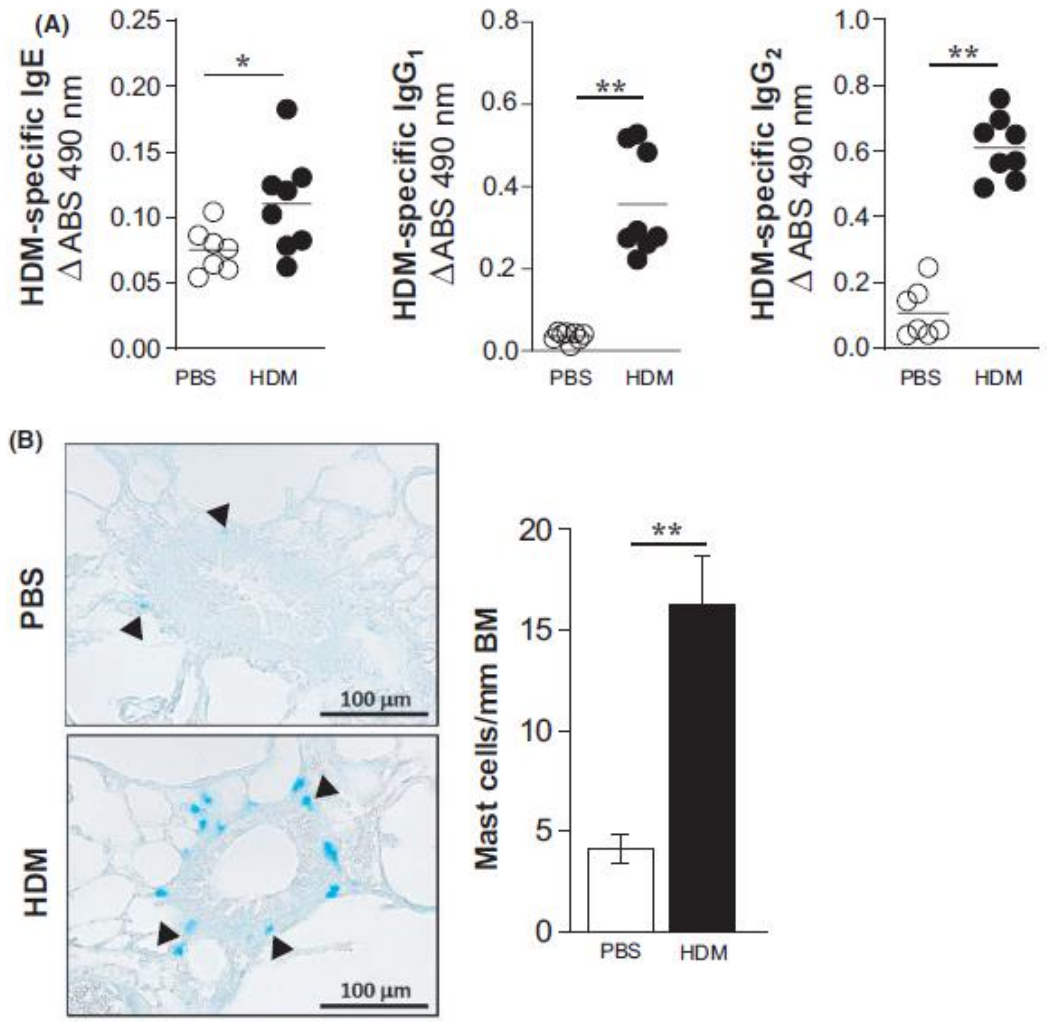


Figure 11: The changes of serum immunoglobulins and mast cells in guinea pigs after repeated HDM exposures. (A) Levels of serum HDM-specific IgE, IgG_1 and IgG_2. (B) Astra blue staining of lung tissues from PBS and HDM exposed guinea pigs (n= 8 in each group). * $p<0.05$, ** $p < 0.01$.

4.1.1 Summary

In this study, we exposed guinea pigs to HDM repeatedly for seven weeks by intranasal instillation and successfully established a model that generates several typical features of allergic asthma, they are antigen induced bronchoconstriction, AHR, T2 inflammation, airway remodeling and mast cell hyperplasia. The marked increase of mast cells found in this model suggests that these cells might have important roles in the development of other asthma features. Due to the similarities between guinea pigs and humans[181], this model provides possibilities for further investigations of the role of mast cells in asthma. To our knowledge, this is the first guinea pig model of asthma demonstrates AHR measured by flexiVent after airway exposures of HDM.

4.2 DEFINING THE HISTOPATHOLOGY INDUCED BY REPETATIVE EXPOSURES TO HOUSE DUST MITE AND CAT DANDER EXTRACT IN GUINEA PIGS (PAPER II)

To investigate the effect of different human relevant allergens on the development of airway inflammation, remodeling and mast cell hyperplasia overtime, guinea pigs were exposed to HDM or CDE intranasally for 4, 8 or 12 weeks before lungs were collected for histological examinations (**Figure 12**).

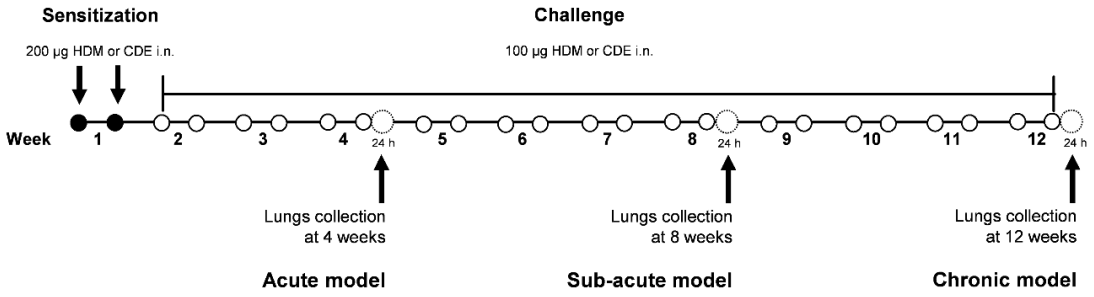


Figure 12: Protocol for HDM and CDE exposures. The protocol included the sensitization and challenge phases. During sensitization, guinea pigs received two times of 200 µg HDM or CDE intranasally at the first week. The challenges started at the second week where 100 µg HDM or CDE were given twice weekly. In total, animals were exposed to HDM and CDE for 4, 8 or 12 weeks. Lungs were collected the day after the last exposure. Control animals received PBS.

The airway inflammation was examined by Hematoxylin-Eosin staining of the lung sections. An increased of inflammatory area was observed after 4 weeks' exposures to either HDM or CDE and maintained after 8- and 12-week exposures compared to the controls (**Figure 13A** and **B**). The inflammation induced by HDM and CDE is in accordance with previous studies in guinea pigs showing that the infiltration of inflammatory cells into the airways occurred at early time points[195] and maintained after 8- and 12-week OVA exposures[206,207]. The airway inflammation was found exist in the large and small airways and perivascular, which is a typical feature observed in human asthma[208,209]. The ASML area of the airways were markedly increased after 4-, 8- and 12-week antigen exposures (**Figure 13A** and **C**). The increase of ASM mass has also been shown in guinea pigs exposed to OVA for 5 weeks[210]. However, in a 13-week protocol, no thickening of ASM were found in guinea pigs exposed to OVA aerosols[207]. Therefore, the exposure time is not the only factor that affects airway remodeling, but also other aspects, such as the dose and frequency of antigen exposures. Another reason might be due to those natural antigens (*e.g.*, HDM and CDE) which have complex compositions, are more potent than OVA.

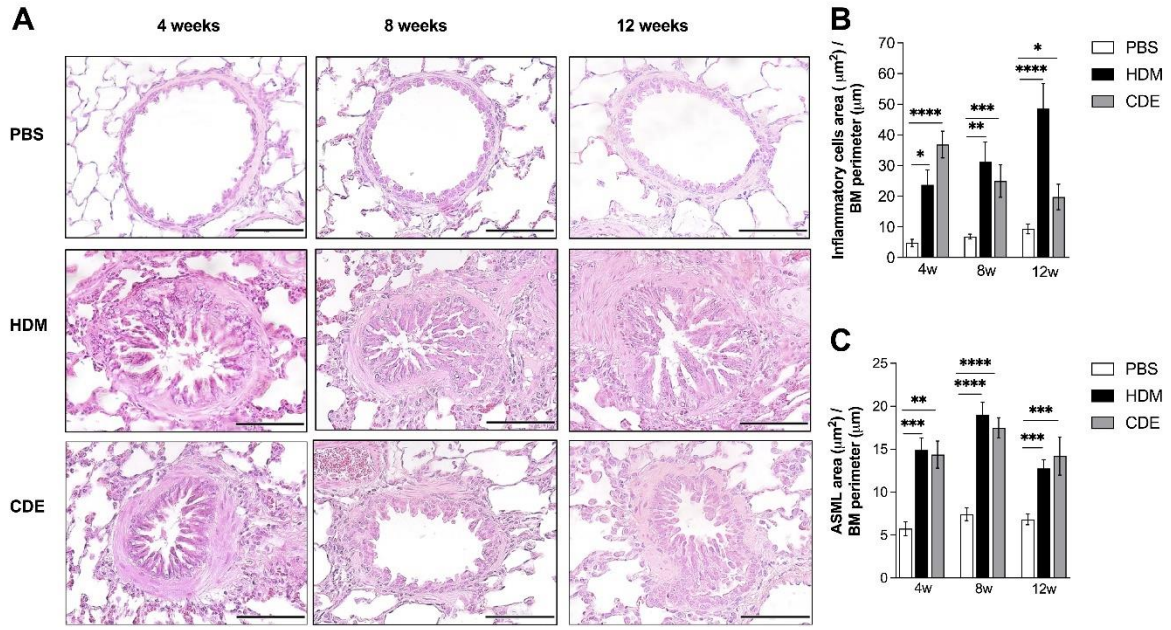


Figure 13: The airways of guinea pigs after repeated HDM or CDE exposures. (A) Hemotoxylin-Eosin staining of guinea pig lungs. (B) Inflammatory and (C) ASML areas of the airways (n= 4-5 in each group). * p<0.05, **p<0.01. *** p<0.001, **** p<0.0001.

The other features regarding airway remodeling were also investigated. The collagen deposition, reflected as Picro-Sirus Red positive areas, was increased after 4 weeks' HDM exposures and continued after 8 and 12 weeks (**Figure 14**). No such increase was found after 4-week exposures to CDE. However, marked augments were observed after 8 and 12 weeks' CDE exposures (**Figure 14**). The difference indicates that HDM and CDE affect the airway structures in different ways.

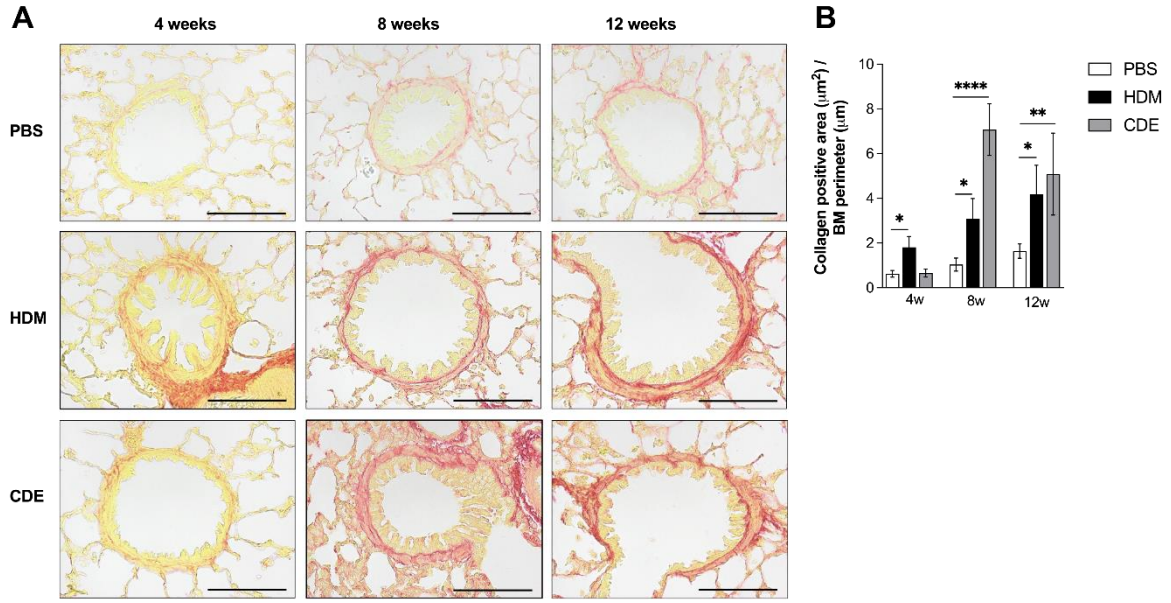


Figure 14: The airways of guinea pigs after repeated HDM or CDE exposures. (A) Picro-Sirius Red staining of guinea pig lungs. (B) Positive staining areas (n= 4-5 in each group). * p<0.05, ** p<0.01, **** p<0.0001.

The PAS positive cells were increased after HDM exposures at all three time points (**Figure 15**). Though failed to reach statistical significance after 4-week exposures, the PAS positive

cells were significantly increased after 8- and 12- week exposures to CDE. The subepithelial collagen deposition and goblet cell hyperplasia has been found in OVA model after 15 days[211] or 13 weeks of OVA exposures and in the previous 7-week HDM model in **paper I**. Indeed, the airway remodeling occurs very early in childhood asthma[105].

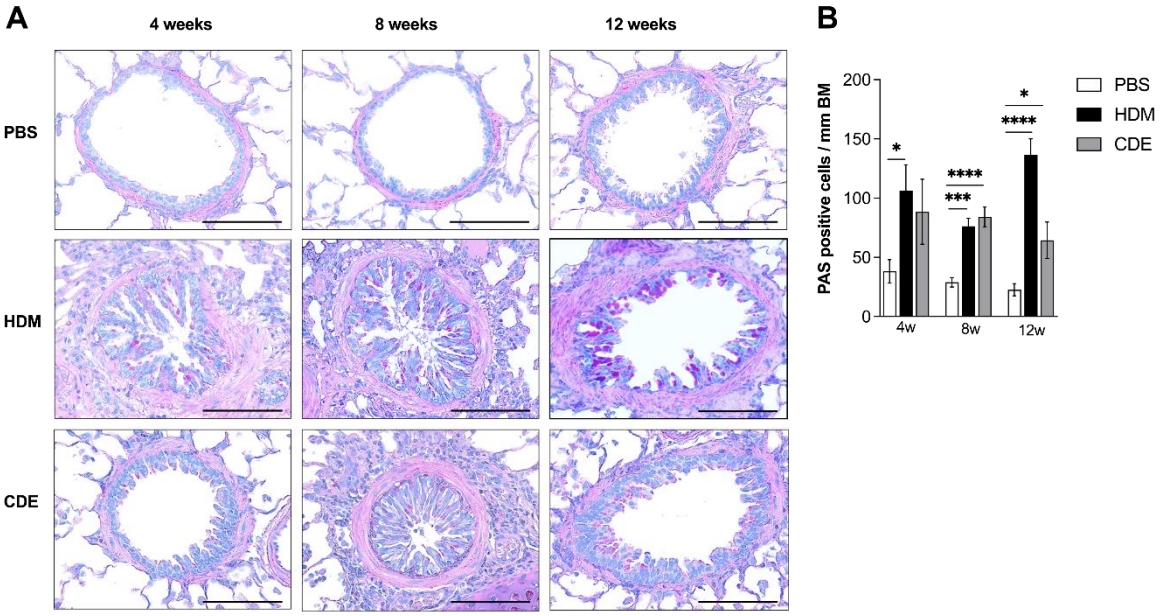


Figure 15: The airways of guinea pigs after repeated HDM or CDE exposures. (A) Periodic Acid-Schiff (PAS) staining of guinea pig lungs. (B) Positive cells (n= 4-5 in each group). * $p<0.05$, *** $p<0.001$, **** $p<0.0001$.

Mast cells were identified by Astra blue staining and mast cell numbers were quantified. No increases of mast cells were observed after 4 weeks' exposures to either HDM or CDE. However, marked increases were found after 8- and 12- week exposures to both antigens (**Figure 16**). This finding indicates that mast cell hyperplasia develops slower than airway inflammation and other remodeling parameters after repeated intranasal instillation of HDM and CDE.

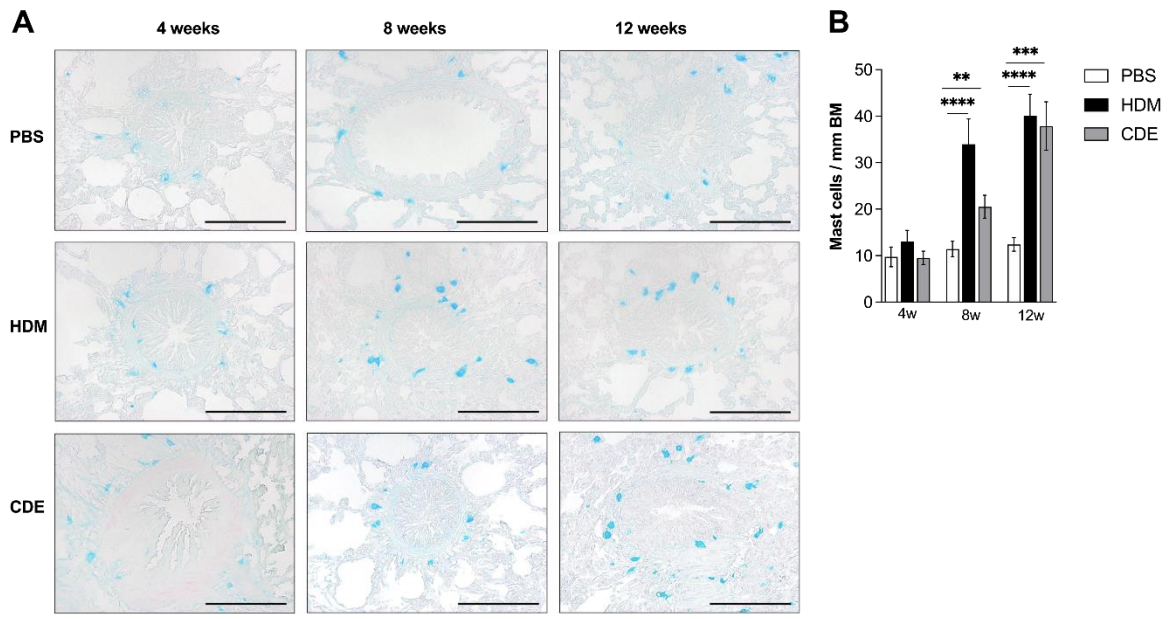


Figure 16: Mast cells in guinea pig lungs after repeated HDM or CDE exposures. (A) Astra blue staining of guinea pig lungs. (B) Positive cells (n= 4-5 in each group). ** p<0.01, *** p<0.001, **** p<0.0001.

Mast cell phenotypes were identified by immunohistochemistry staining using antibodies against tryptase and chymase in the lung sections of guinea pigs after 8- and 12-week exposures to HDM or CDE. In the control animals, both tryptase- and chymase- positive mast cells were found in the airways and parenchyma (**Figure 17C, D, J, K, L and M**). In addition, most chymase$^+$ cells were found to be tryptase$^+$ in consecutive slides, suggesting that the mast cells present are mainly tryptase and chymase double positive. The number of tryptase$^+$ mast cells (reflecting total MCs) was significantly increased after each antigen exposure for 8 weeks or 12 weeks and the mast cells dominate in the airways (**Figure 17A, F, H, J and K**). The quantity of increased chymase$^+$ cells was less than tryptase$^+$ mast cells in the airways after 8-week antigen exposures, however, the change of chymase$^+$ cells after 12-week exposures did not reach statistical significance and neither the cells in parenchyma (**Figure 17B, E, G, L and M**). It is noticeable that chymase$^+$ mast cells were found closely to the ASM layer after HDM and CDE exposures (**Figure 17B and E**). Thus, both HDM and CDE exposures primarily induced an increase of MC_T (mast cells containing only tryptase; reflected as total tryptase$^+$ cells- chymase$^+$ cells) in the airways and parenchyma, whereas MC_{TC} (mast cells containing tryptase and chymase) only increased in the airways but not the parenchyma. These findings in accordance with a study showing that MC_T is predominate in mild asthmatics without treatment[142] and MC_{TC} is in proximity with ASM bundles[143]. However, even though less in quantity, airway MC_{TC} was found to have a correlation with lung function of asthmatics[212], which might be due to the released mast cell mediators that modulate ASM reactions.

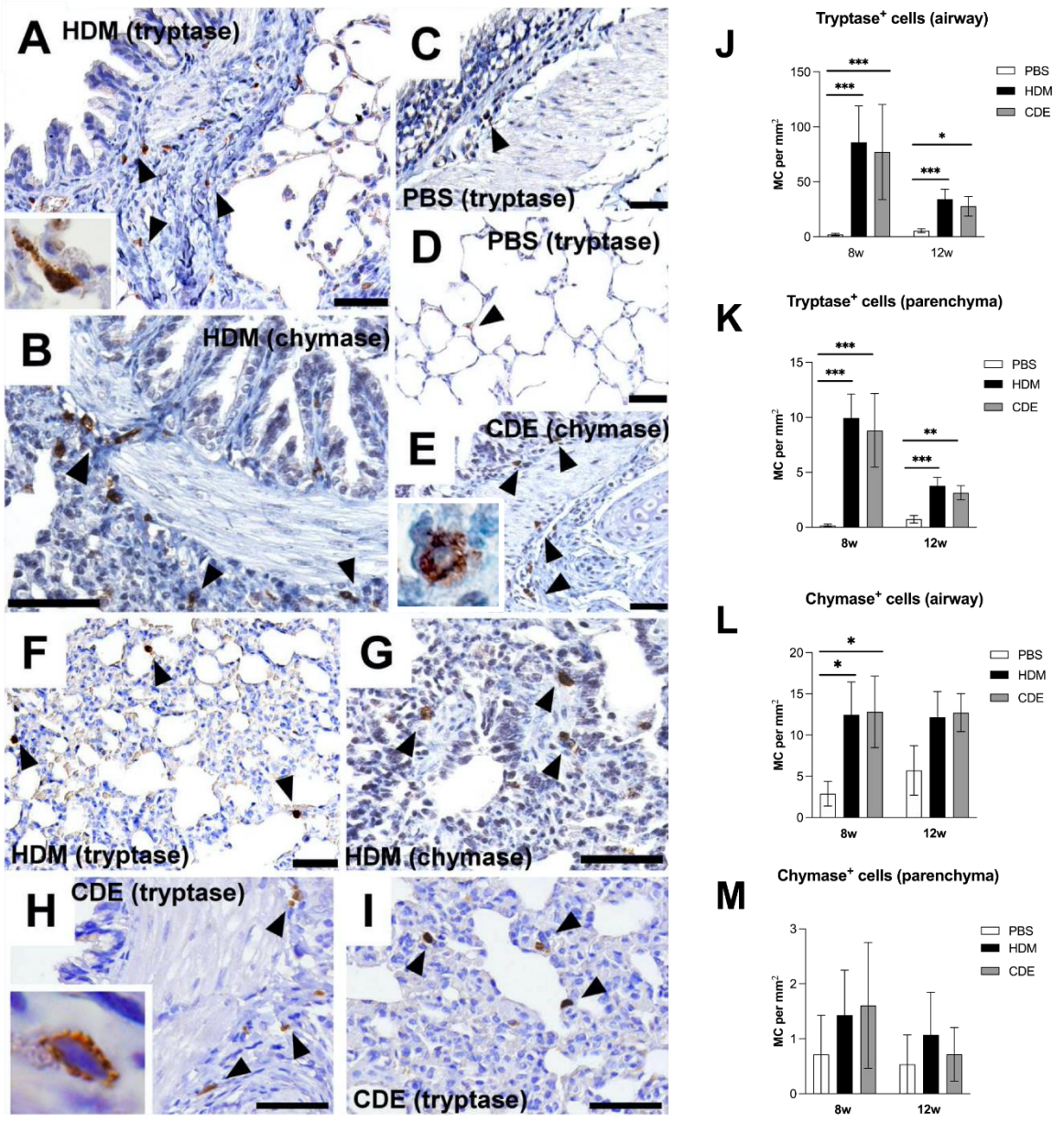


Figure 17: Immunohistochemistry staining of mast cells in guinea pig lungs after repeated HDM or CDE exposures. Micrographs of tryptase (A, C, D, F, H, I) and chymase (B, E, G) staining in airways and parenchyma after 8 weeks' antigen exposures. PBS treated animals: C-D, HDM: A-B, F-G, and CDE: E, H-I. Quantifications of tryptase positive mast cells in (J) airways and (K) alveolar parenchyma, chymase positive mast cells in (L) airways and (M) alveolar parenchyma. Scale bar: 50 µm. High magnification micrographs (60x) are shown as inserts in A, E and H (n= 4-5 in each group). * p<0.05, ** p<0.01, *** p<0.001.

4.2.1 Summary

In this paper, we exposed guinea pigs to HDM and CDE through the intranasal route for different time points (4, 8 and 12 weeks) and investigated the effects of both antigens on histological changes of guinea pig lungs. Despite some differences, HDM and CDE both could induce airway inflammation, increase of ASM mass, collagen deposition and goblet cell hyperplasia. The antigen induced mast cell hyperplasia appeared later than other features. The increase of mast cells was dominated by $MC_{T,}$ but MC_{TC} was found increased in the airways after 8-week antigen exposures with proximity to the ASM bundle. This study

support that both HDM and CDE might be possible antigens to be used in animal model establishments of asthma and provide possibilities to investigate the role of different mast cell phenotypes on asthma.

4.3 COMPARISON OF RESPONSES INDUCED BY DIFFERENT MAST CELL AGONSITS IN GUINEA PIG TRACHEA (PAPER III)

Mast cell activation is suggested to be important for the development of asthma. In addition to antigen, mast cells have shown to be activated by MRGPRX2 agonists[127]. With the evolving interest of MRGPRX2 in mast cell mediated responses, we hypothesize that there are differences and similarities compared to classical antigen induced responses. In this paper, we therefore compared the ASM response and release of both preformed and *de novo* synthesized mediators in guinea pig tracheal segments exposed to C48/80, a human MRGPRX2 agonist, or HDM. Airway smooth muscle contractions were assessed in organ baths where buffer samples were collected for subsequent measurement of histamine release using ELISA, and profiling of lipid mediator metabolites applying the previously described LC−MS/MS platform[192].

Guinea pigs were sensitized via a single intraperitoneal injection of HDM with aluminum hydroxide (100 µg/100 mg). Serum and trachea were collected two weeks after sensitization. Sensitization of animals was evaluated by measuring serum immunoglobulins from sensitized and naïve animals. HDM sensitized animals demonstrated significantly higher absorbance value than naïve, both in HDM-specific IgG_1 and IgG_2, but not in total IgE (**Figure 18**). Levels of HDM specific IgE were below detection limit.

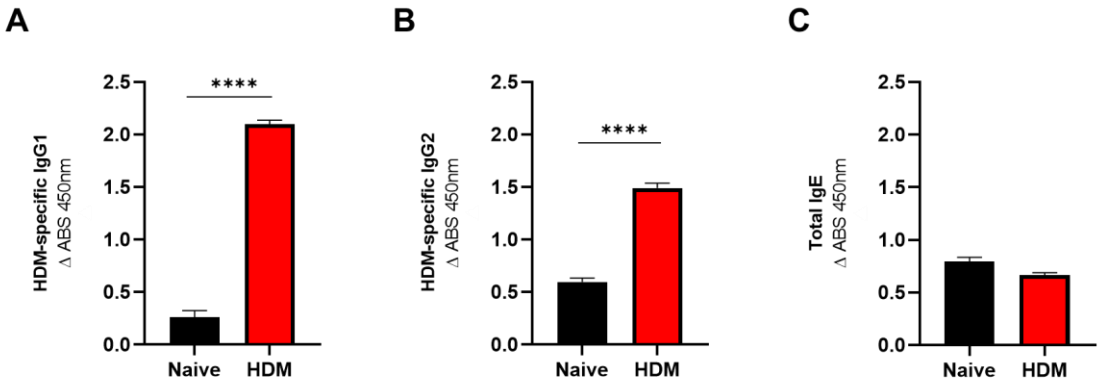


Figure 18: Guinea pig serum immunoglobulins. (A) HDM-specific IgG_1, (B) IgG_2 and (C) total IgE from sensitized (HDM/ aluminum hydroxide (100 µg/100 mg, *i.p.*) or naïve guinea pigs (n=5-14 in each group). **** $p<0.0001$.

The contractions in guinea pig trachea induced by C48/80 and HDM were first investigated using a bolus dose of the stimuli where smooth muscle contractility was monitored over time. The nonselective COX inhibitor indomethacin (3 µM) was used to ablate the constitutive release of PGE_2, which mediates an intrinsic smooth muscle tone of about 20% of referral maximal contraction as previously described[213]. Challenge with C48/80 (500 µg/mL) or HDM (10 µg/mL) induced a sustained smooth muscle contraction (**Figure 19A and B**). As neither total nor specific IgE were increased, the effects of HDM were most likely due to

specific IgGs which, in addition to IgE, has been shown to induce smooth muscle contractions in guinea pig trachea[197].

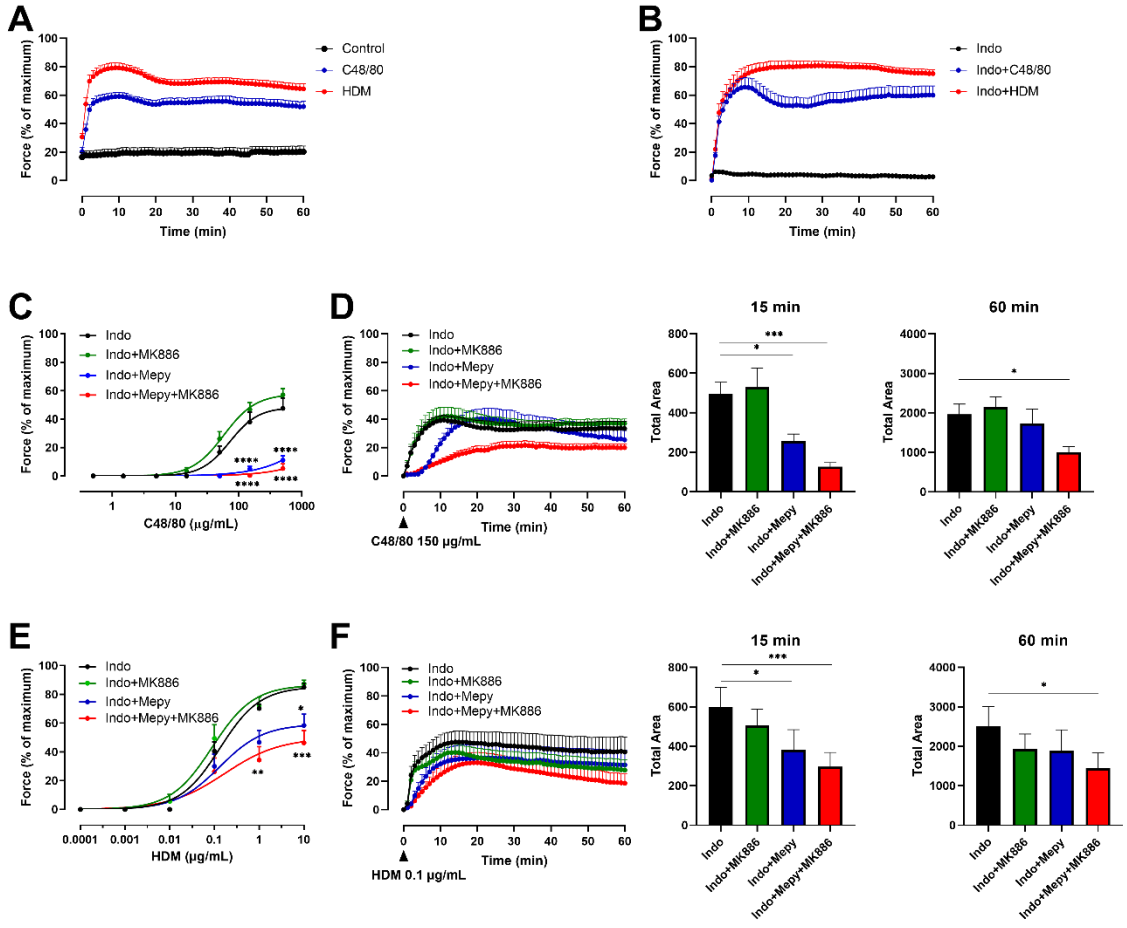


Figure 19: Responses of guinea pig trachea to C48/80 or HDM. Responses of guinea pig tracheal segments to a single dose of C48/80 (500 µg/mL) and HDM (10 µg/mL) in the absence (A) or (B) with presence of indomethacin (Indo (3 µM); n=5-6 in each group). (C) Concentration response curves to C48/80 (n=11-12 in each group) and (E) HDM (n=7 in each group), (D) The bolus dose challenge of C48/80 (150 µg/mL; n=6-8 in each group) and (F) HDM (0.1 µg/mL; n=7 in each group) with area under curve (AUC) area of the first 15 minutes and entire 60 minutes' responses. (C) to (F) were performed with the presence of indomethacin (Indo; 3 µM). C48/80 challenges in (C) and (D) were performed in naïve and HDM challenges in sensitized guinea pigs. Mepyramine (Mepy; 1 µM), MK886 (10 µM) or the combination of both pharmacological treatments were added 45 minutes before the challenges. Group differences were compared with control segments (Indo). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

To compare the mediators that cause the ASM responses to C48/80 and HDM, concentration response curves (CRC) or bolus dose challenges were performed in presence or absence of receptor antagonists or enzyme inhibitors. To remove the production of COX products which participate in the ASM contractions, indomethacin (3 µM) was present during the experiments. Both C48/80 and HDM caused concentration dependent contractions with the maximal response (E_{max}) higher in HDM response (E_{max}: 52±6% and 85±3%, respectively; $p<0.05$). The FLAP inhibitor MK886 (10 µM) alone did not affect either C48/80 or HDM responses. In contrast, the histamine H_1 receptor antagonist mepyramine (1 µM) significantly reduced E_{max} for both C48/80 and HDM (11±3% and 58±8%, respectively). Combining both

histamine H_1 receptor antagonism and FLAP inhibition had no additional effect compared to histamine H_1 receptor antagonism alone (**Figure 19C** and **E**).

To investigate the responses over time, a submaximal bolus dose (approximately 40% of E_{max}) of either C48/80 (150 µg/mL) or HDM (0.1 µg/mL) was added, and smooth muscle contractions were monitored for 60 minutes. Similar to the CRC, pretreatment with MK886 (10 µM) alone did not modify the responses, whereas mepyramine (1 µM), either alone or in combination with MK886 (10 µM), postponed both C48/80 and HDM responses and reduced the total contraction, expressed as area under curve (AUC) during the first 15 minutes. For the entire 60 minutes, only the combination of both mepyramine and MK886 significantly reduced responses of C48/80 and HDM (**Figure 19D** and **F**). Antagonizing the histamine H_1 receptors significantly inhibited the CRCs and the initial 15 minutes of responses induced by submaximal bolus dose challenges indicating that the early response of both treatments was mediated by histamine. On the other hand, the whole phase responses could only be dampened by inhibition of both histamine and the leukotriene pathways, which is in agreement with earlier studies[131,132,214,215], suggesting that C48/80 and HDM caused smooth muscle contractions mediated through mast cell activation releasing both histamine and leukotrienes.

However, despite a combined inhibition of COX-/5-LOX products and the histamine H_1 receptors, a residual contraction remained for both C48/80 and HDM treated segments. This residual contraction was greater after HDM exposure compared to C48/80 when performing the CRC. However, after the bolus dose, the same level of the residual component appeared for both stimuli, albeit this effect was more slowly developed for C48/80. This residual response has previously been found[216,217]. Of interest, several pharmacological interventions have been evaluated to elucidate the cause of this residual component, but none of the muscarinic receptors, serotonin 5-HT_{2A} or bradykinin BK-B_2 receptors seems to be involved[216]. Thus, although our observed responses indicate that both stimuli activate mast cells, the difference in response pattern suggest that there are dissimilarities in the resulting mediator profile released.

As the contractile responses to both C48/80 and HDM were inhibited by mepyramine, the release of histamine into the organ bath was measured. Histamine was found to be elevated after 15- and 60- min post addition of C48/80 (500 µg/mL) or HDM (10 µg/mL). Both challenged segments demonstrated similar increases of histamine. Neither time nor indomethacin had an impact on the total amount of histamine release (**Figure 20**). This finding might explain why inhibiting synthesis of leukotrienes alone could not affect the contractions at all, because histamine presented both in the 15- and 60- min of the responses. It is not likely that the cause of this release is due to activation of basophils, which is another source of histamine[218], as these cells are mainly circulating cells and do not appear in lungs during healthy conditions[65,219]. Moreover, the basophil number has shown to be much lower than mast cells in atopic conditions[220].

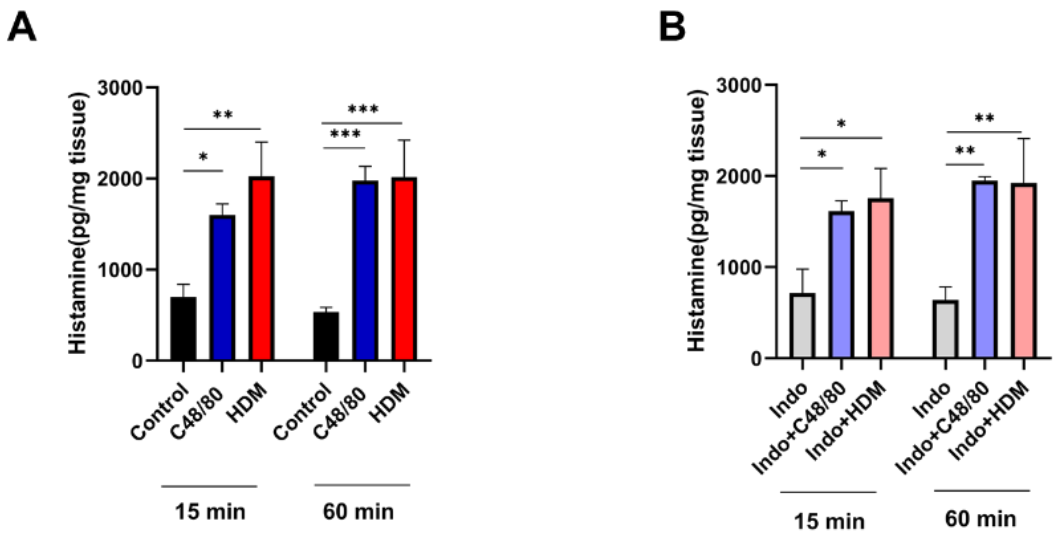


Figure 20: Histamine release after challenge with C48/80 (500 µg/mL) or HDM (10 µg/mL). Histamine release in (A) absence and (B) presence of indomethacin (Indo (3 µM) (n=5-6 in each group). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

To further assess mediators potentially involved in the contractions, a mass spectrometry-based analysis was conducted to investigate the appearance and alteration of a broad range of lipid mediators[192] beside the known mast cell related contractile mediators, such as CysLTs, PGD_2 and TXA_2[216,221]. Sixty minutes after challenge with C48/80 (500 µg/mL) or HDM (10 µg/mL), a total of 67 of 112 measured lipid mediators were detected, among which 39 were significantly increased by challenges compared to basal release (unstimulated control segments). Of note, several metabolites from linoleic acid (LA) pathway were found in the HDM extract itself (LA, 9-HODE, 9-KODE, 13-HODE, 13-KODE, 12(13)-EpOME, 12,13-DiHOME, 9(10)-EpOME, 9,10-DiHOME) and 20-HETE from arachidonic acid (AA) pathway, and consequently these 10 metabolites were removed from further analysis as C48/80 challenge did not increase these mediators. Among the 29 confirmed changes, 15 were elevated by both C48/80 and HDM challenge, and an additional 13 only by C48/80, while LTE_4 was only elevated by HDM (**Figure 21**).

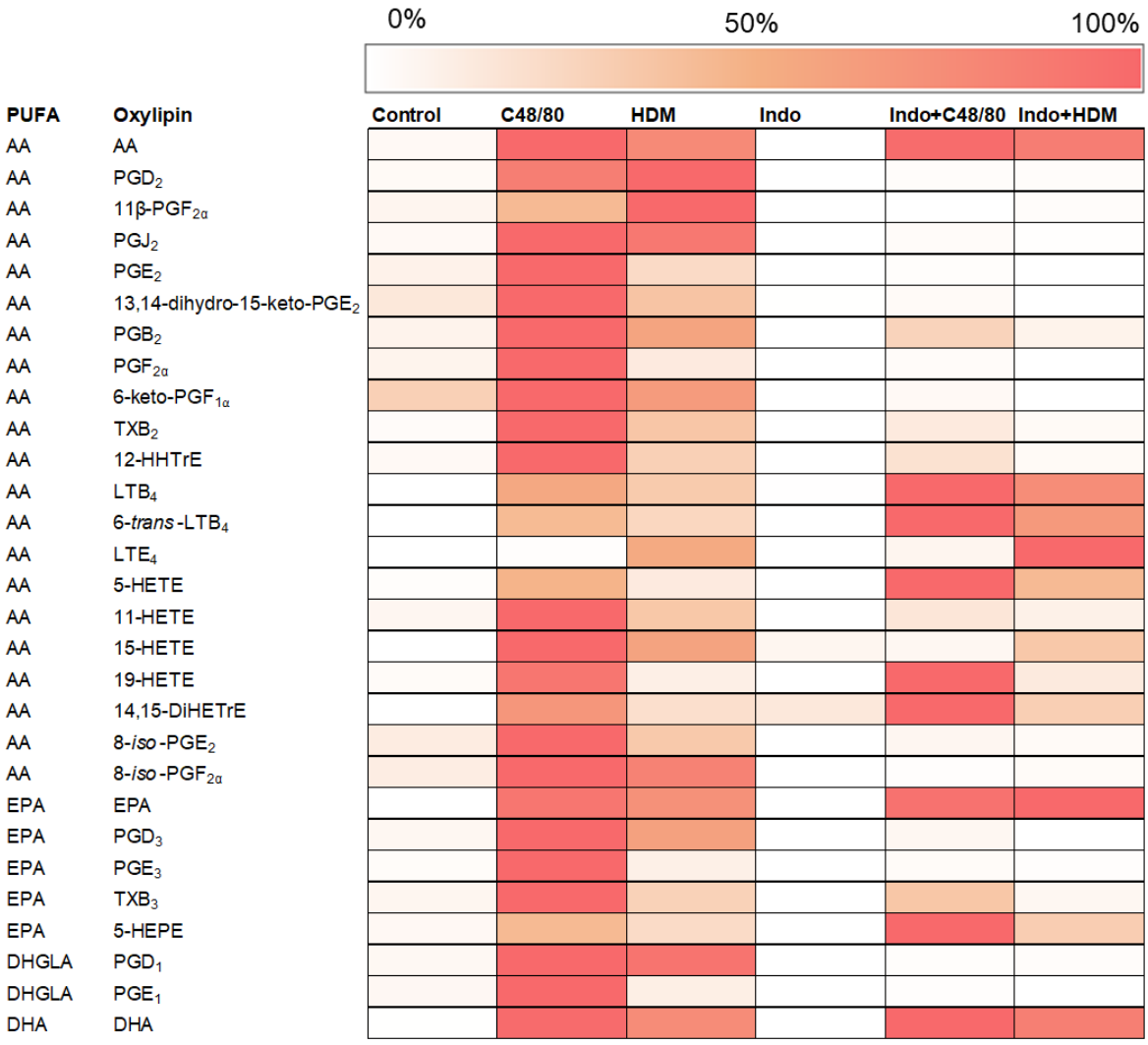


Figure 21: Overview of the elevated lipid mediators after stimulation with C48/80 or HDM. Segments were challenged with C48/80 (500 µg/mL) or HDM (10 µg/mL) with or without the presence of indomethacin (Indo; 3 µM). Organ bath fluid was collected 60 minutes post challenge. For each lipid mediator, red is 100% (highest value), white is 0% (lowest value) (n=5-6 in each group). Abbreviations: arachidonic acid (AA), eicosapentaenoic acid (EPA), dihomo-γ-linolenic acid (DHGLA), docosapentaenoic acid (DHA).

The three polyunsaturated fatty acid (PUFA) precursors AA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), were significantly increased by stimulation with either C48/80 or HDM. Arachidonic acid was elevated 3-fold whereas EPA and DHA increased more than 2-fold (**Table 1**). It is known that mast cells release *de novo* synthesized lipid mediators derived from PUFAs that are liberated by phospholipase A_2 from phospholipids[222]. Activation of mast cells in guinea pig trachea by C48/80 and HDM not only caused liberation of AA, the most abundant n-6 PUFA, but also n-3 PUFAs (EPA and DHA) which, however, resulted in markedly few n-3 derived metabolites.

Table 1. Elevated PUFAs after challenges (concentration unit: pg/mg tissue)

PUFA	Control	C48/80	HDM	Indo	Indo+C48/80	Indo+HDM
AA	6.5 ± 1.5	19.5 ± 1.6***	16.4 ± 1.7**	5.9 ± 1.8	19.2 ± 1.8***	17.6 ± 1.8**
EPA	3.6 ± 0.7	7.7 ± 0.8**	6.8 ± 0.9*	3.6 ± 1.2	7.8 ± 1.0*	8.1 ± 0.7*
DHA	2.8 ± 0.4	7.4 ± 0.4****	6.3 ± 0.7**	2.8 ± 0.7	7.4 ± 0.7**	6.7 ± 0.7**

Segments were challenged with C48/80 (500 µg/mL) or HDM (10 µg/mL) with or without the presence of indomethacin (Indo; 3 µM). Organ bath fluid was collected 60 minutes after challenge. Multiple comparisons were made between challenged segments with control segments (Control or Indo) (n=5-6 in each group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Several of the COX products that were released have previously been described to be involved in mast cell-induced contraction[216,223]. Downstream of AA, both C48/80 and HDM induced a similar increase of PGD_2 and two of its metabolites (11β-$PGF_{2\alpha}$ and PGJ_2). Moreover, TXA-synthase activity was observed by the increase in TXB_2 and 12-HHTrE after both challenges, but with 3 times greater amounts following stimulation with C48/80. Supplemental to the above-mentioned mediators, C48/80 elevated a broad range of other COX-related products, such as PGE_2 and its metabolites (13,14-dihydro-15-keto-PGE_2 and PGB_2), $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ (**Figure 22**). Similar to a previous study[224], treatment with non-selective COX inhibitor (indomethacin) during the experiments significantly reduced all COX dependent metabolites, except the non-enzymatic dehydration product PGB_2.

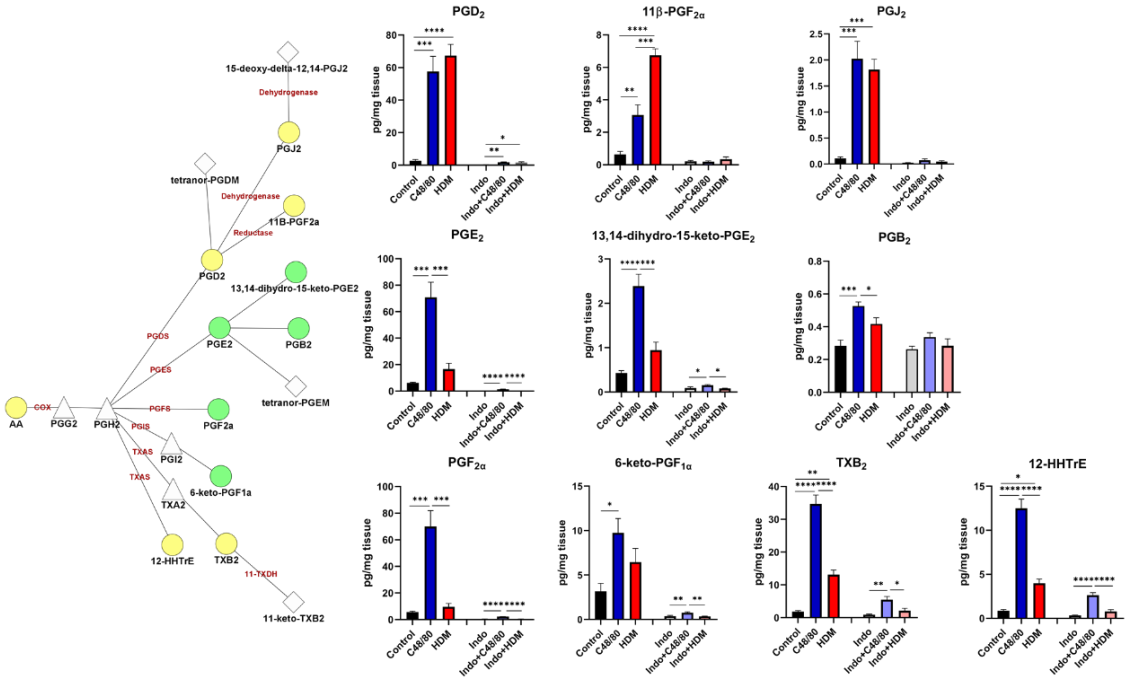


Figure 22: Prostaglandins and thromboxane after challenge with C48/80 (500 µg/mL) or HDM (10 µg/mL). Lipid mediators not monitored are denoted (Δ), below limit of detection (◊), detected but not changed

(○), significantly changed by both C48/80 and HDM (○) (yellow), changed only by C48/80 (green) and changed only by HDM (pink) (n=5-6 in each group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

Since, PGD_2 is a biomarker of mast cell activation[225], the increase of PGD_2 metabolites providing further evidence that both stimuli activate mast cells. On the other hand, TXB_2, the breakdown metabolites of TXA_2, which may also be derived from mast cells[226], was to a greater extent released following C48/80 than HDM, suggesting that C48/80 and HDM induces activation of the intracellular pathways in different ways. The other prostanoids, PGE_1, PGE_2 and its metabolites, PGE_3, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_3 were solely increased by C48/80. These mediators can be derived from many cell types, such as tracheal epithelial cells, smooth muscle cells, activated platelets, granulocytes[138,227,228]. As MRGPRX2 has mainly shown to be expressed on mast cells and nerves[125,229], the C48/80 induced increase can be due to direct activation of exclusively mast cells and embedded nerve endings in guinea pig trachea. The activation could also be due to indirectly through a release from other cells triggered by specific signals from C48/80-activated mast cells and nerves. Collectively, the broader and stronger release of prostanoids obtained by C48/80 indicates that there is a distinct signal routes compared to the one induced by HDM. Of further interest, not only contractile mediators such as PGF_{2a}, and TXB_2 were elevated after C48/80 challenge, but also, the main PGI_2 metabolite, which relaxes smooth muscle[230]. Moreover, the increase of PGE_2, having contractile and relaxant abilities to both human and guinea pig smooth muscles[213,230], further contributes to the complexity of C48/80 induced bronchoconstriction. A limitation with the study was that we used indomethacin in many of the functional studies. This removes all COX products, both contractile and relaxant, as well as those with regulatory functions on mast cells. Future studies using selective inhibition of COX isoenzymes and terminal prostaglandin synthases might resolve some of the questions from this study.

As the main contractions by the bolus dose of both C48/80 and HDM were only reduced by mepyramine and MK886 together, one component of the contraction was expected to be mediated through the 5-LOX products. In agreement with this, a strong increase in LTE_4 was observed following HDM stimulation (**Figure 23**). By an additional analysis method covering all individual CysLTs, it was confirmed that most of the LTC_4 and LTD_4 were metabolized to LTE_4 by the end of the 60 minutes challenge (LTC_4 took up 0%, LTD_4 1.7% and LTE_4 98.3% of measured responses). However, no increase in LTE_4 appeared after C48/80 stimulation. Elevation of LTA_4-hydrolase products, such as LTB_4 and the non-enzymatic product 6-*trans*-LTB_4 were increased following stimulus with C48/80 or HDM (in absence of indomethacin, HDM failed to reach statistical significance). 5-HETE was only increased after C48/80 stimulation.

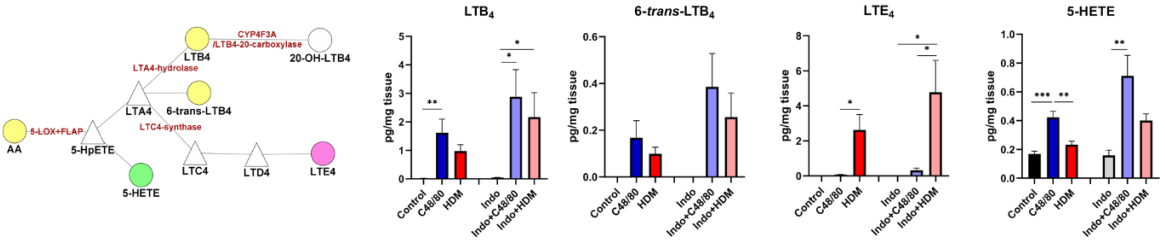


Figure 23: 5-LOX/FLAP metabolites after challenge with C48/80 (500 µg/mL) or HDM (10 µg/mL). Lipid mediators not monitored are denoted (Δ), below limit of detection (◊), detected but not changed (○), significantly changed by both C48/80 and HDM (○) (yellow), changed only by C48/80 (green) and changed only by HDM (pink) (n=5-6 in each group). * p<0.05, ** p<0.01 and *** p<0.001.

It is known that mast cells have the capacity to synthesize leukotrienes (LTB_4 and CysLTs) and 5-HETE[231] through the 5-LOX pathway. In our setting, both C48/80 and HDM increased the LTB_4 whereas only HDM triggered the CysLTs production, reflected in the elevation of LTE_4. This difference is probably due to different intracellular signaling pathways for G-protein coupled receptors and Fc-receptors and agrees with a previous study demonstrating that FcεRI and MRGPRX2 has different signaling pathways, where only the former released LTC_4 from activated human skin mast cells[232]. An additional possible explanation for the distinct LTE_4 release is that antigen and C48/80 activate different mast cell phenotypes. It has been shown that FcεRI cross-linking in humans[119] and antigen-induced activation in guinea pig[233] activate both MC_T and MC_{TC}, whereas C48/80 selectively activates MC_{TC}[119]. As human MC_T, but not MC_{TC}, has been shown to express high levels of LTC_4 synthase in central airways[118], a presence of both mast cell phenotypes in the guinea pig trachea may explain the HDM-induced release of LTE_4. However, the inhibitory effect caused by MK886, together with mepyramine, on the whole phase response for C48/80 indicate that products from the 5-LOX/FLAP pathway was involved in the contraction. This can either be explained by a small amount of released CysLTs being directly bound to their receptors and not reaching the buffer solution, due to LTB_4 contracting guinea pig trachea[234] or due to a release of 5-HETE, which has previously been shown to potentiate the histamine response[235].

Hydroxyeicosatetraenoic acids (HETEs) and dihydroxyeicosatrienoic acid (DiHETrE) metabolites derived from AA with less known bioactive functions, were found to be affected by C48/80 and HDM. Two monohydroxylated metabolites, 11- and 15-HETE, were found significantly increased by both C48/80 and HDM challenge, with highest levels detected after C48/80 exposure. However, the increased levels of 11-HETE by both stimuli and 15-HETE after C48/80 challenge were reduced by indomethacin which in accordance with a study showing that 15-HETE is a COX product in human mast cells[236]. From the cytochrome p450 (CYP450) pathway, 19-HETE and 14,15-DiHETrE were increased by C48/80, but not by HDM (**Figure 24**).

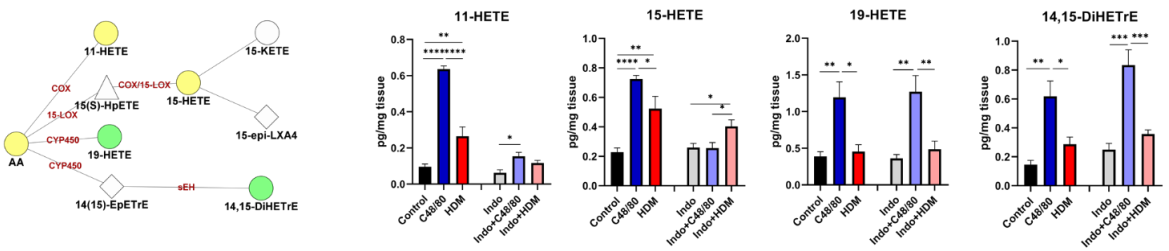


Figure 24: HETEs and DiHEtrE metabolites after challenge with C48/80 (500 µg/mL) or HDM (10 µg/mL). Lipid mediators not monitored are denoted (Δ), below limit of detection (◊), detected but not changed (○), significantly changed by both C48/80 and HDM (○) (yellow), changed only by C48/80 (green) and changed only by HDM (pink) (n=5-6 in each group). * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

This finding demonstrates again that both similarities and differences exist in response to C48/80 and HDM. To what extent some of these mediators participate in the observed contractions remained to be further investigated, especially as the amount of these was comparably low in relation to the major prostaglandin and leukotriene metabolites.

As for the isoprostanes, 8-*iso*-PGE_2 was significantly increased after C48/80 challenge and 8-*iso*-$PGF_{2\alpha}$ were elevated after both C48/80 and HDM addition. Treatment with indomethacin significantly abolished the formation of both (**Figure 25**). Although it has been shown that isoprostanes are formed both from COX pathway and non-enzymatically[237-239], our data indicate that 8-*iso*-PGE_2 and 8-*iso*-PGF_{2a} are products much dependent on COX enzyme activity in guinea pig trachea.

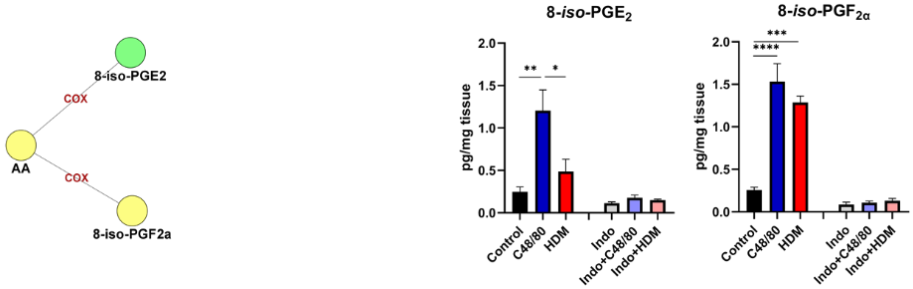


Figure 25: Isoprostanes after challenge with C48/80 (500 µg/mL) or HDM (10 µg/mL). Lipid mediators not monitored are denoted (Δ), below limit of detection (◊), detected but not changed (○), significantly changed by both C48/80 and HDM (○) (yellow), changed only by C48/80 (green) and changed only by HDM (pink) (n=5-6 in each group). * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

In addition to AA metabolites, products from different PUFAs were also observed after challenges. Metabolic products of EPA were released in relatively low levels (<2 pg/mg tissue) where PGD_3 was significantly elevated by both C48/80 and HDM, whereas PGE_3, TXB_3 and 5-HEPE only increased by C48/80 (**Figure 26**).

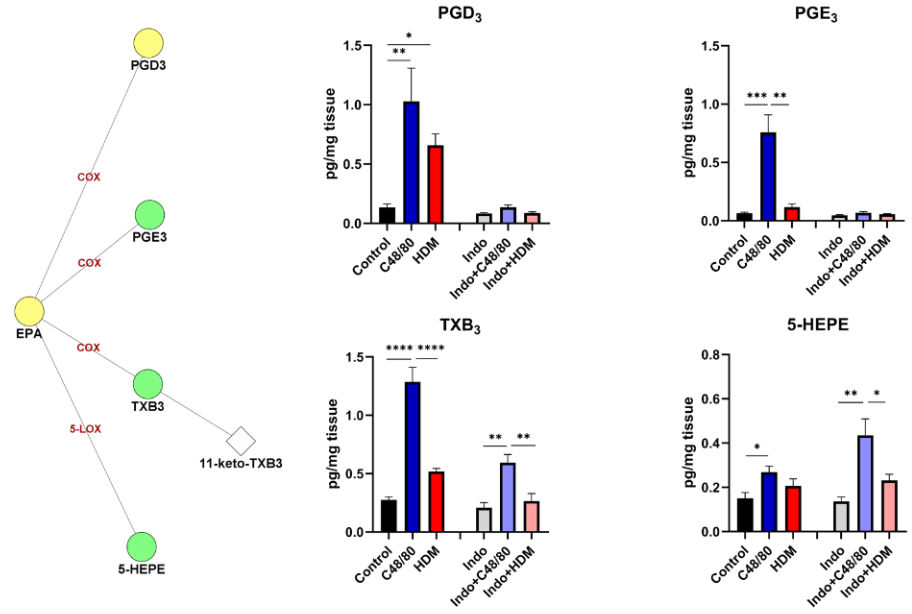


Figure 26: EPA metabolites after challenge with C48/80 (500 µg/mL) or HDM (10 µg/mL). Lipid mediators not monitored are denoted (Δ), below limit of detection (◊), detected but not changed (○), significantly changed by both C48/80 and HDM (○) (yellow), changed only by C48/80 (green) and changed only by HDM (pink) (n=5-6 in each group). * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

Prostaglandin D_1 and PGE_1 which are derived from DHGLA were significantly modified by challenges. Whereas the former increased by both C48/80 and HDM stimulations, the latter only by C48/80 (**Figure 27**).

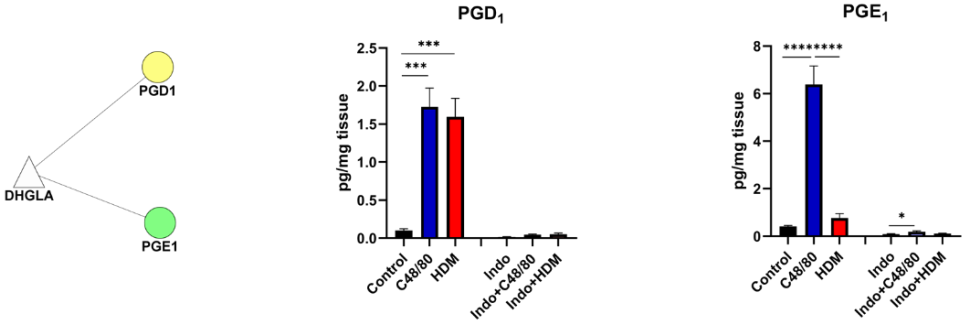


Figure 27: DHGLA metabolites after challenge with C48/80 (500 µg/mL) or HDM (10 µg/mL). Lipid mediators not monitored are denoted (Δ), below limit of detection (◊), detected but not changed (○), significantly changed by both C48/80 and HDM (○) (yellow), changed only by C48/80 (green) and changed only by HDM (pink) (n=5-6 in each group). *** p<0.001 and **** p<0.0001.

The simultaneous elevation of PGD_1 and PGD_3 further strengthen the activity of mast cell specific PGD synthases.

None of the metabolites downstream of the main substrates of docosapentaenoic acid, 11,14-eicosadienoic acid, α- and γ-linolenic acids, or mead acid were significantly changed during any of the conditions evaluated.

The responsible receptor for C48/80 induced response in guinea pig trachea is not completely clarified, but it is likely that the response is mediated through a MRGPRX2 orthologue in guinea pig trachea. There are at least two MRGPRX2 gene orthologues that exist in guinea pigs[240]. Due to absence of tools, we could not define which of these that were responsible for the response. However, we tested some other known MRGPRX2 agonists, including substance P, LL-37, cortistatin-14, PAMP (9–20) and mastoparan, all of them could induced smooth muscle contraction in guinea pig trachea (**Figure 28**). The contraction induced by PAMP (9–20) was totally inhibited by pretreatment of histamine H_1 receptor antagonist, indicating that this response is mast cell mediated.

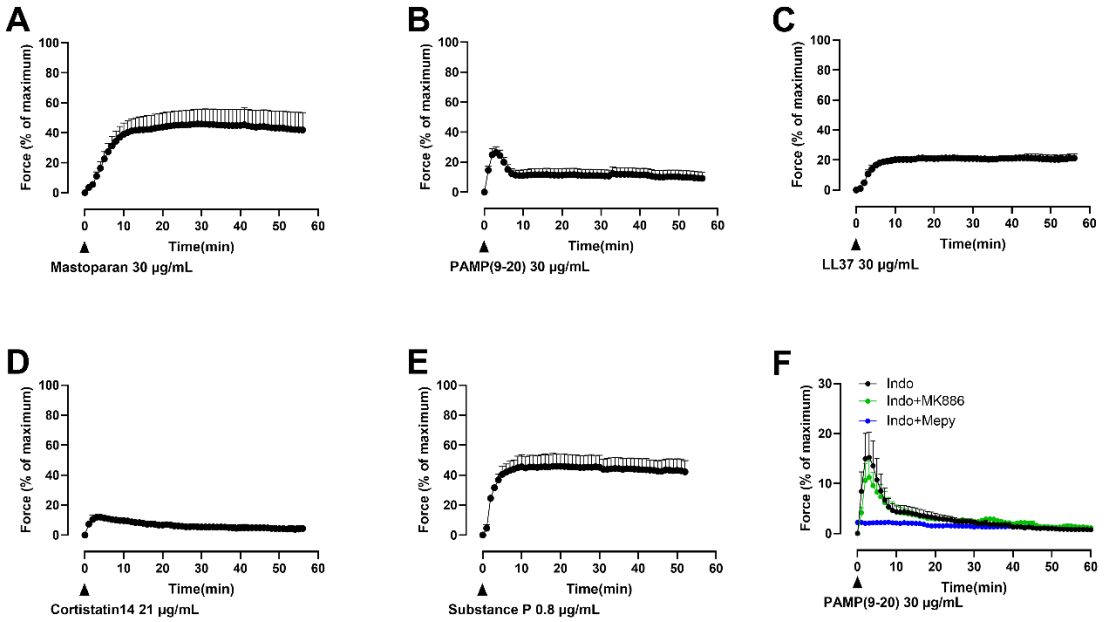


Figure 28: Responses of guinea pig trachea to MRGPRX2 agonists. All the tracheal segments were treated with indomethacin (Indo; 3 µM). Responses of guinea pig trachea to (A) Mastoparan (30 µg/mL) (n=5), (B) PAMP (9-20) (30 µg/mL) (n=13), (C) LL37 (30 µg/mL) (n=2), (D) Cortistatin14 (21 µg/mL) (n=2), (E) Substance P (0.8 µg/mL) (n=2) and (F) PAMP (9-20) (n=4-7) with or without antagonist/inhibitor, mepyramine (Mepy; 1 µM), MK886 (10 µM).

4.3.1 Summary

In this paper, we isolated guinea pig trachea for comparing the effect of different mast cell agonists on the ASM response and mediator release. Though some differences existed, the contractions induced by HDM and C48/80 were both mediated by mast cell mediators. Both agonists induced the release of histamine and PGD_2 to similar levels. However, distinct of lipid mediator profiles were observed. The CysLTs were only elevated by HDM, whereas C48/80 induced a broader release of lipid mediators. This project provides novel insights for future guinea pig studies to investigate the role of mast cell mediators on both allergic and non-allergic asthma.

4.4 INVESTIGATION OF THE ROLE OF MAST CELLS IN ASTHMA RELATED FEATURES IN HUMAN AND GUINEA PIG MODELS (PAPER IV AND V)

Due to the interest of investigating the role of mast cells on the pathophysiological features in asthma models, the study to find an efficient tool to induce mast cell death was performed. After screening the Prestwick compound library which contains 1,200 approved drugs, monensin, an ionophoric antibiotic, was found to be a promising compound to induce mast cell death. We exposed several types of mast cells to different doses of monensin for different time. Two markers, namely Annexin V and DRAQ7 were used to distinguish the apoptotic and necrotic cell death (**Figure 29A**), After exposing to monensin, the population of viable cells (Annexin V^- and $DRAQ7^-$) were gradually decreased in bone marrow-derived mast cells (BMMCs; **Figure 29B**), peritoneal cell-derived mast cells (PCMCs; **Figure 29C**), immortalized human MCs (LUVA cells; **Figure 29D**), human mast cell leukemia cells (HMC-1 cells; **Figure 29E**) and human skin MCs (**Figure 29F**). On the contrary, the increases of apoptotic cells (Annexin V^+ and $DRAQ7^-$) and necrotic cells (Annexin V^+ and $DRAQ7^+$) were observed in all the MCs. The proportion of apoptotic cells was larger than of necrotic cells (**Figure 29B to F**). All these findings suggest that monensin can be a tool for delineating the role of MCs in functional studies.

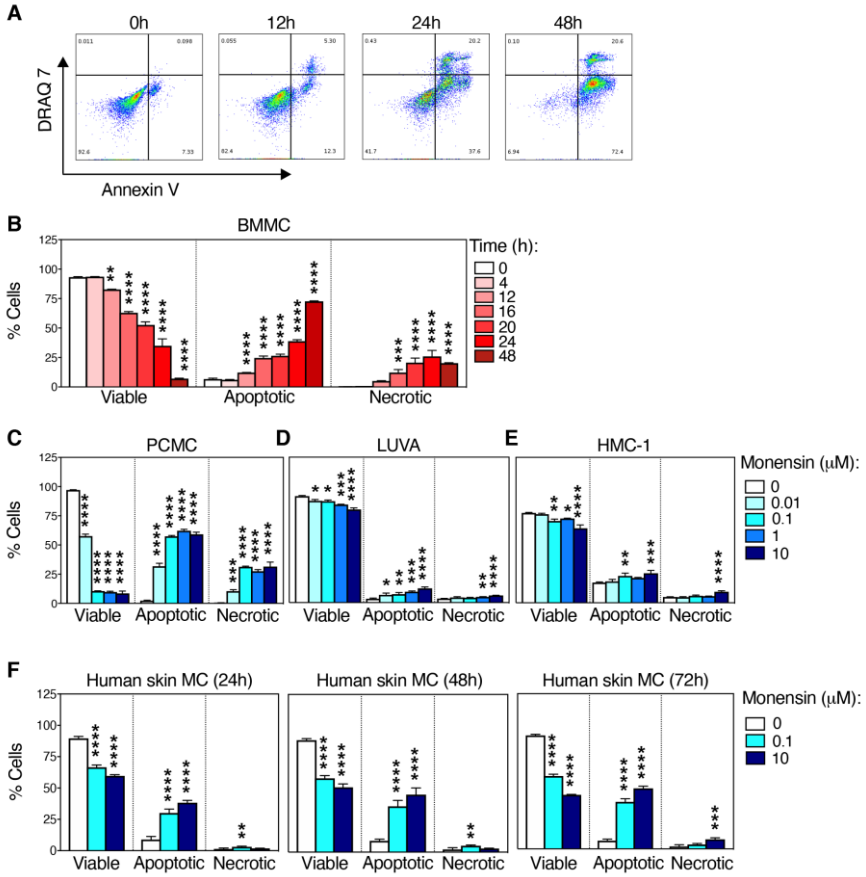


Figure 29. The effect of monensin on mast cells. (A) Representative dot plots used to distinguish between viable (Annexin $V^-/DRAQ7^-$), apoptotic (Annexin $V^+/DRAQ7^-$) and necrotic (Annexin $V^+/DRAQ7^+$) cells. (B) Time effect of monensin (10 μM) to BMMCs (0.5×10^6 cells) (n = 3 in each group). Effect of different concentrations and time course of monensin on (C) PCMCs, (D) LUVA cells, (E) HMC-1 and (F) human skin MCs (n = 3-4 in each group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

To investigate the effect of monensin on the MCs of guinea pig airways. The trachea was isolated from HDM sensitized (a single intraperitoneal injection of 100 µg HDM with 100 mg aluminum hydroxide) animals. Tracheal segments were cultured with or without monensin, at different concentrations, for 24 to 72h. The segments were fixed in Carnoy fixatives (60% Ethanol, 30% chloroform and 10% acetic acid) and stained with Astra blue, which has been shown to stain MCs[241]. Compared to the control segments, the staining intensity of MCs, assessed as intensity score, were found significantly reduced in the segments exposed to monensin for 24h (**Figure 30A**). However, no significant decrease of MC numbers was observed (**Figure 30B**). More clear reductions of granule staining intensity were found in the segments exposed to monensin for 48h (**Figure 30C**) and 72h (**Figure 30E**). The numbers of detectable MCs were significantly reduced after both 48- (**Figure 30D**) and 72h (**Figure 30F**) exposure to monensin (10 µM).

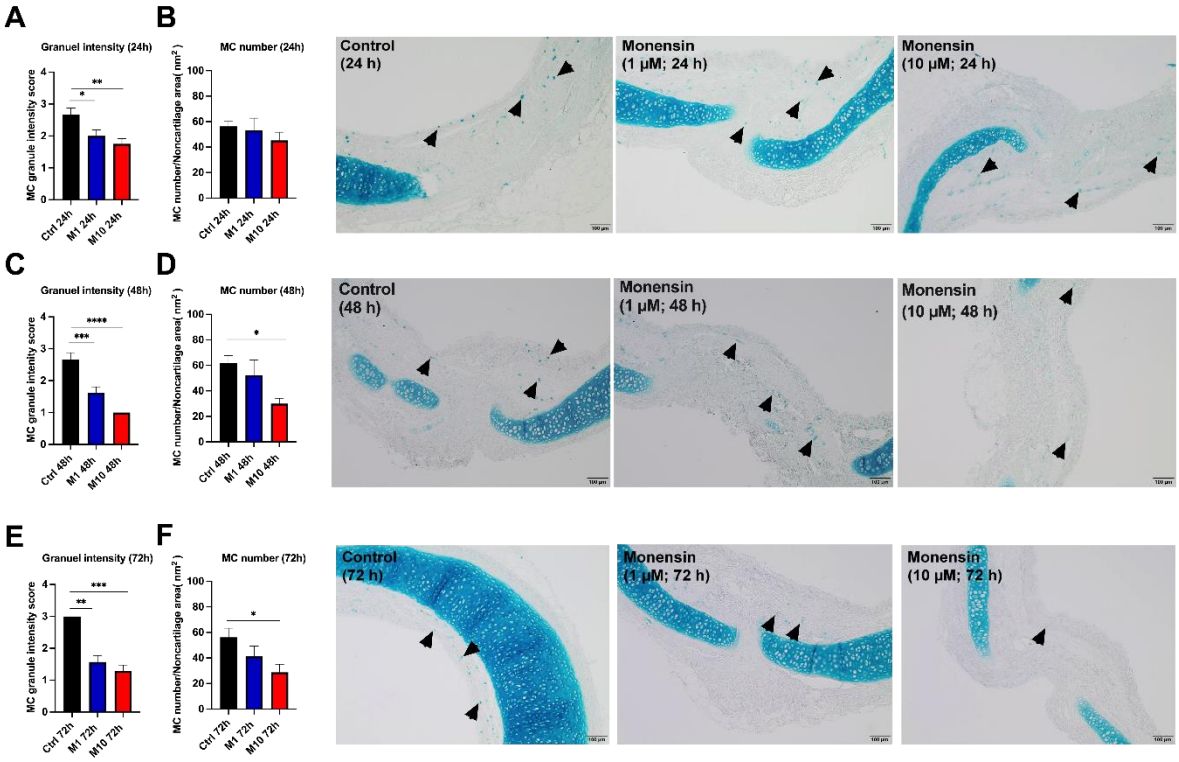


Figure 30: Astra blue staining of guinea pig trachea. The staining intensity of mast cells was scored (1: pale, 2: medium, 3: strong) after (A) 24h, (C) 48h and (E) 72h culture with 1 µM (M1) or 10 µM (M10) monensin, or vehicle (1% ethanol, Ctrl). The mast cell numbers after culturing with monensin or vehicle for (B) 24h, (D) 48h and (F) 72h. Representative pictures were listed on the right (n=6-8 in each group). Arrows denote mast cells. * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

It is known that Astra blue binds to the glycosaminoglycans (GAGs) which are negatively charged polysaccharides present in tissues including heparin/heparan sulfate, chondroitin sulfate/dermatan sulfate, hyaluronic acid, and keratan sulfate[242]. The basis for identify MCs by Astra blue is that MCs contain heparin[243] and chondroitin sulphate[244,245] in their granules. The rapid penetration properties of Carnoy fixatives causes the precipitation of GAGs, in addition, the acidity of the fixative facilitate the binding of Astra blue and the ionic linkages between GAG and the basic cationic dye[246]. Therefore, more MCs can be identified in sections fixed with Carnoy than formaldehyde. Our findings suggest MCs gradually lose their

granule contents by monensin exposures. However, whether the contents were released outside MCs or be degraded inside the cells were not clear.

To investigate whether monensin induce the death of MCs, the tracheal segments after 2 to 72h treatment with or without monensin were examined by Astra blue/Hematoxylin staining to stain the nuclei of MCs. The staining revealed that monensin treated tracheal segments demonstrated marked decrease of MCs that have intact nuclei after exposing to monensin (**Figure 31**). This decrease was discovered directly after 2h monensin exposure (**Figure 31A**), further decreased after prolonged exposure to 24h (**Figure 31B**) and maintained after 48h (**Figure 31C**) and 72h exposure to monensin (**Figure 31D**).

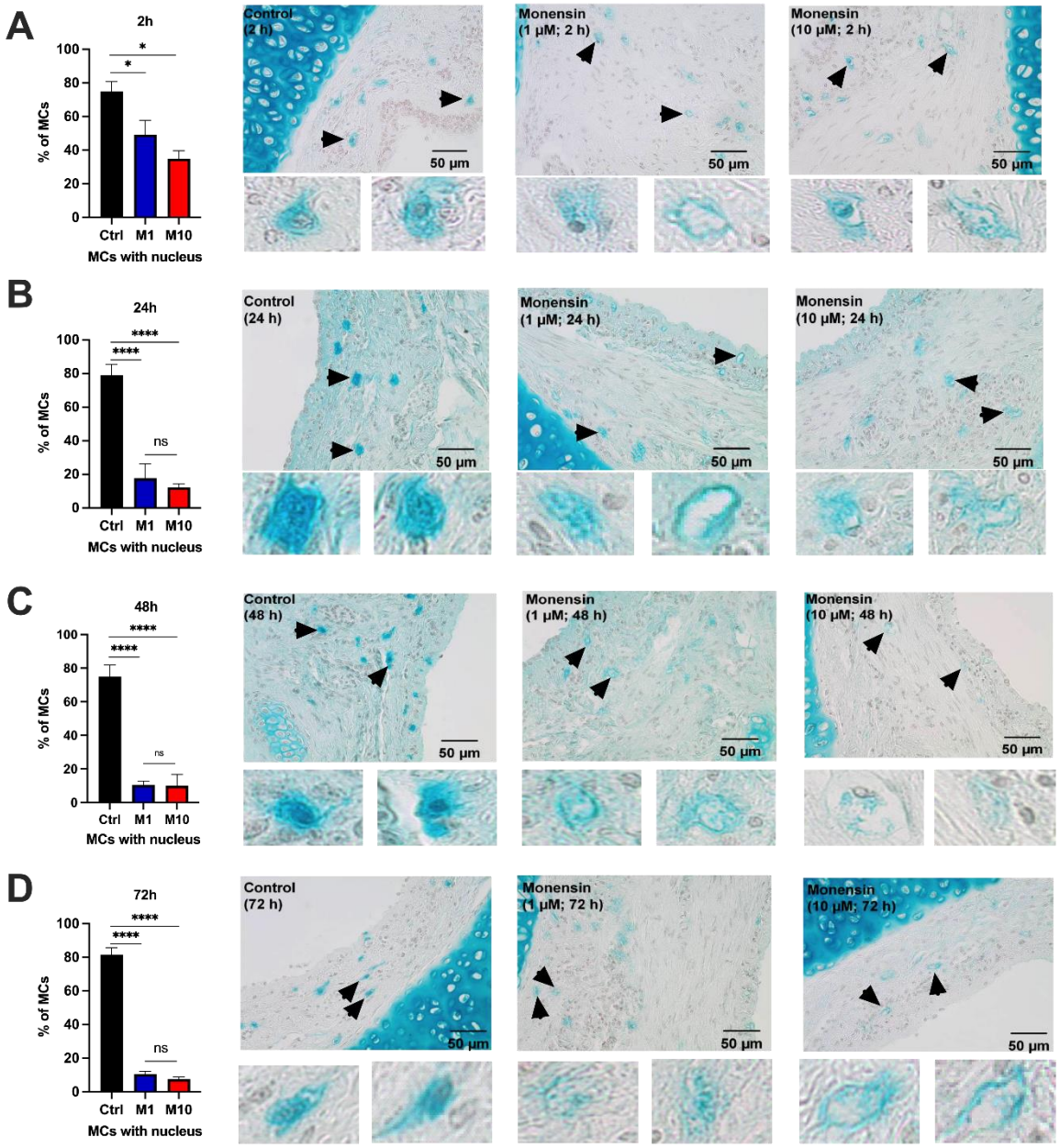


Figure 31: Astra blue/Hematoxylin staining of guinea pig trachea. Percentage of mast cells that have detectable nuclei after (A) 2h, (B) 24h, (C) 48h and (D) 72h culture with 1 µM (M1) or 10 µM (M10) monensin, or vehicle (1% ethanol; Ctrl) with representative pictures (n=3-8 in each group). Arrows denote the magnified mast cells. *p<0.05 and **** p<0.0001.

The decreased percentage of MCs that have intact nuclei indicate that monensin causes the death of MCs and the effect is robust and time dependent.

To study whether there are releases of MC contents by monensin releasing spasmodic mediators, guinea pig tracheal segments were mounted in the organ baths where smooth muscle contractions were recorded. As could be seen from **Figure 32A**, rapid contractions were found directly after monensin exposure which could be totally removed by histamine H_1 receptor antagonist (mepyramine). The smooth muscle constrictions were also observed in human bronchi (**Figure 32B**) and again, these contractions could be inhibited by mepyramine.

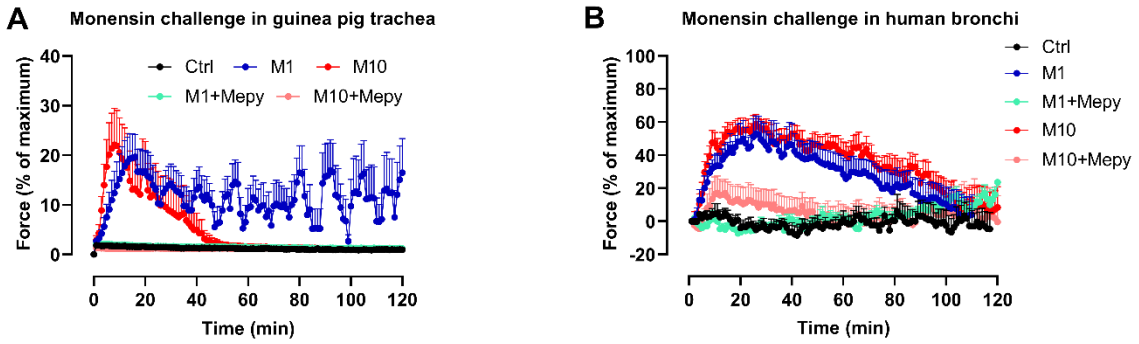


Figure 32: Airway responses to monensin. (A) The contractions of guinea pig tracheal segments were monitored in organ baths and (B) human bronchi response in the myographs after exposure to 1 µM (M1) or 10 µM (M10) monensin with or without the presence of mepyramine (1 µM; Mepy) (n=5-6 in each group).

These findings confirm that the monensin induced bronchoconstrictions in guinea pig and human airways were both mediated by histamine. This is in accordance with a previous study showing that monensin induces histamine release from MCs[247]. Unlike cell necrosis which causes karyolysis and loose of cell contents[248], the apoptotic cells usually have cell contents kept inside[249]. Hence, this release suggests that monensin can cause MC necrosis.

To study the effect of monensin on the general tissue viability, both guinea pig trachea and human bronchi were exposed to the muscarinic receptor agonist (carbachol; CCh) after exposing the segments to monensin for different time periods. The absolute contractile force (mN) was recorded and used for comparisons. No significant differences in CCh responses were found after exposing to low concentration of monensin (1 µM) for 24 to 72h (**Figure 33A to C**). Although, no significant effect of the high concentration of monensin (10 µM) on CCh response after 24h exposure (**Figure 33A**), significant dampened responses were observed after 48h (**Figure 33B**) and 72h exposure (**Figure 33C**). This indicates that monensin, at low concentration or short incubation time, has no significant effect on the contractility of guinea pig trachea. However, monensin can cause reduction of the smooth muscle cell responses at high doses and for prolonged exposure time.

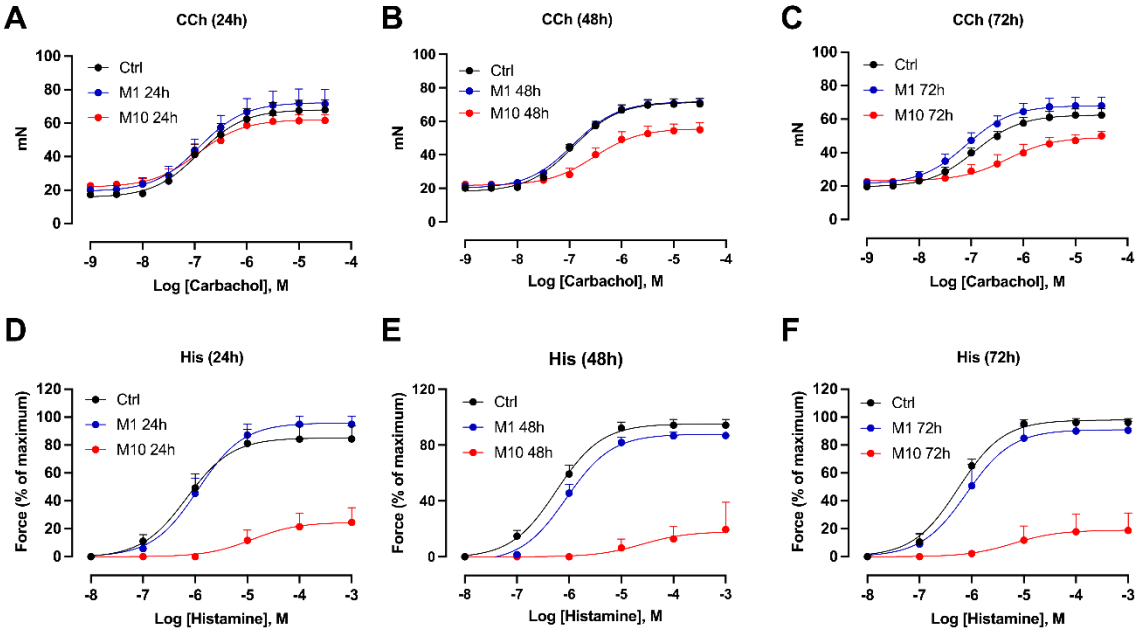


Figure 33: Responses of guinea pig trachea to carbachol and histamine after monensin exposure. Responses to carbachol (CCh) after (A) 24h, (B) 48h, and (C) 72h exposure to 1 µM (M1) or 10 µM (M10) monensin, or vehicle (1% ethanol; Ctrl). Responses to histamine (His) after (D) 24h, (E) 48h, and (F) 72h incubation (n=6 to 14 in each group).

Since histamine releases were found, this could affect the functions of the histamine receptors. Thus, the exogenous histamine with different concentrations were added to examine the effect of monensin on the histamine H_1 receptors. No significant changes of the histamine responses were found after culturing with low concentration of monensin at any time points (**Figure 33D to F**). Notably, these responses were markedly right shifted and maximal responses (E_{max}) dampened after 24 to 72h exposure to the high dose of monensin (**Figure 33D to F**).

Monensin, cultured for 24h at high concentration, did not affect the CCh but dampened histamine responses. This can be explained by that the monensin induced release of histamine could cause the desensitization of the histamine H_1 receptors. This is in accordance with a previous study demonstrating that homologous desensitization of the H_1 receptors could be induced by pre-exposure to histamine in guinea pig tissues[250].

In human bronchi, the 24h monensin treatment did not affect the carbachol responses (**Figure 34A**), indicating that monensin does not dampen the smooth muscle contractions. However, the histamine curve was significantly left shifted by 10 µM monensin with the pEC_{50} changed from 6.8 in the control to 7.2 in the treated group (**Figure 34B**). The potentiation effect of monensin seen in human bronchi might be explained by the release of MC mediators, for instance, IL-4[63,251], that makes the airway smooth muscle hyperreactive.

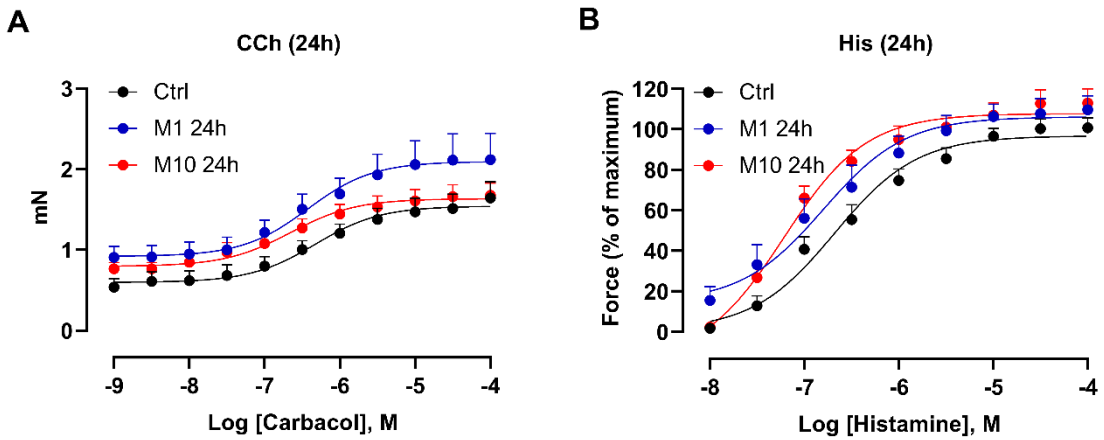


Figure 34: Responses of human bronchi to carbachol and histamine after monensin exposure. Responses to (A) carbachol (CCh) and (B) histamine (His) after 24h culture with 1 µM (M1) or 10 µM (M10) monensin, or vehicle (1% ethanol; Ctrl) (n=8-13 in each group).

To investigate the role of MCs on antigen induced bronchoconstriction, both guinea pig trachea and human bronchi were used. Guinea pigs were sensitized intraperitoneally to HDM, tracheas were isolated and incubated with monensin. The segments were exposed to HDM again in the organ baths after the incubations. As it is not possible to actively sensitize humans beforehand, we use anti-IgE antibodies (anti-IgE) to activate MCs in human bronchi. The anti-IgE binds to the bounded IgE on MCs that attached to the high affinity IgE receptor and thereby cause MC activation[221]. Two hours after monensin exposure, no HDM induced responses were observed in guinea pig trachea (**Figure 35A**). The inhibition effect continued after 24 to 72h monensin culture (**Figure 35 B to D**).

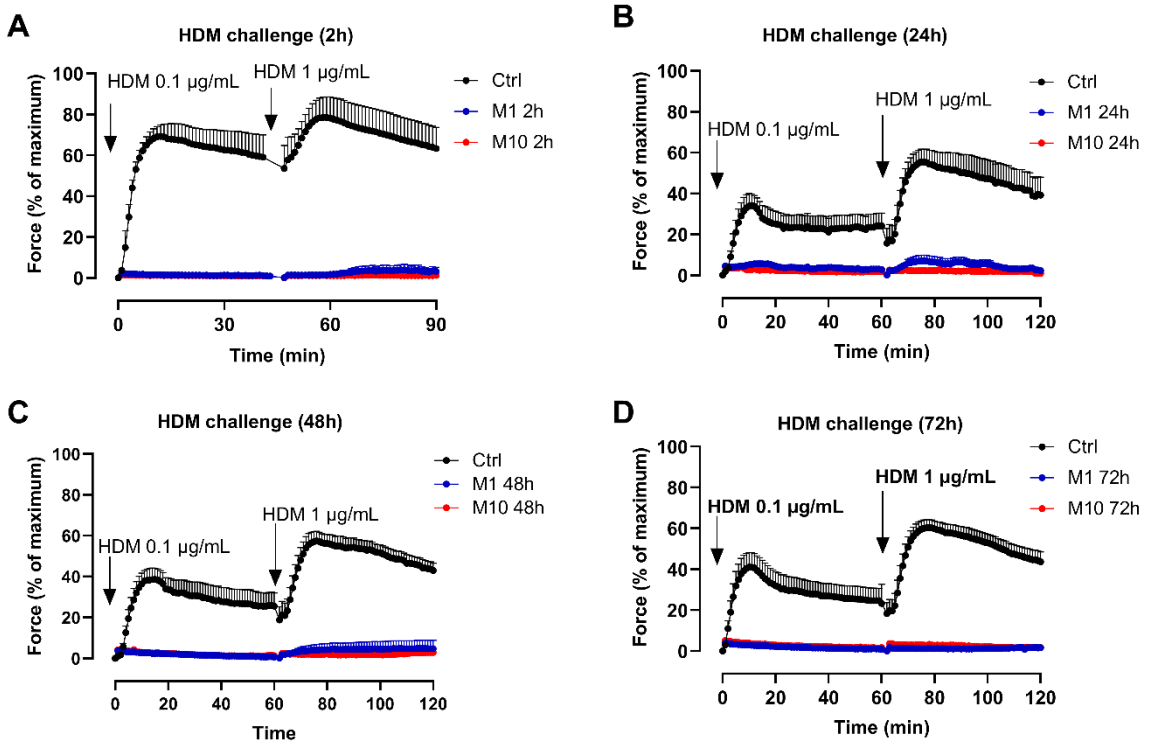


Figure 35: Responses of guinea pig tracheal segments to HDM after monensin exposure. Guinea pigs were

sensitized by a single intraperitoneal injection of 100 µg HDM with 100 mg aluminum hydroxide for two weeks. Tracheal segments were challenged with HDM after culturing with 1 µM (M1) or 10 µM (M10) monensin, or vehicle (1% ethanol; Ctrl) for (A)2h, (B) 24h, (C) 48h and (D) 72h (n=3-8 in each group).

In human bronchi, the anti-IgE induced contractions were also inhibited after 2h (**Figure 36A**) and 24h (**Figure 36B**) exposure to monensin.

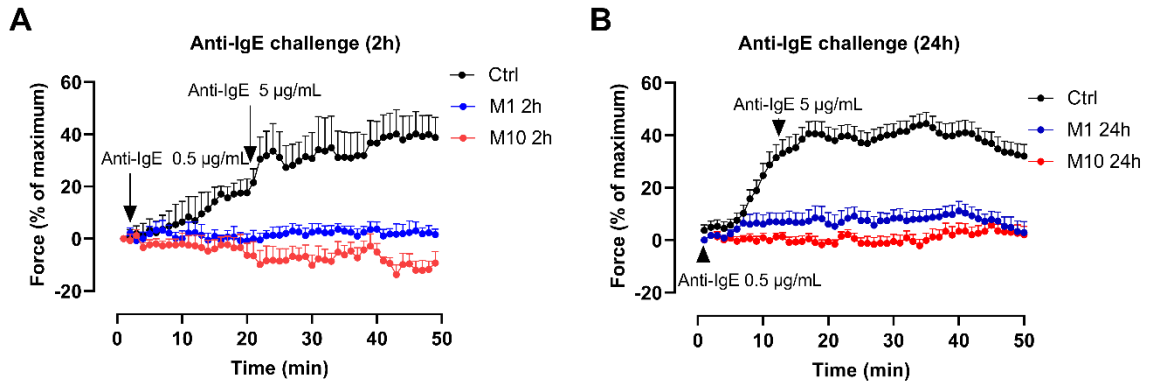


Figure 36: Responses of human bronchi to anti-IgE challenge after monensin exposure. Segments were challenged with anti-IgE after culturing with 1 µM (M1) or 10 µM (M10) monensin, or vehicle (1% ethanol; Ctrl) for (A)2h and (B) 24h (n=5-13 in each group).

Both HDM and anti-IgE represent antigen-induced activation of MCs, since the increased release of MC mediators was observed, such as histamine, CysLTs and PGD_2 from guinea pig trachea[252] and human MCs[253,254]. These spasmodic mediators induce the smooth muscle contraction. Our findings suggest that monensin has a fast and strong effect on reducing the antigen induced bronchoconstriction both in guinea pig and human airways. In addition, no remaining HDM responses were observed after monensin exposure, suggesting that the remaining antigen response found in **paper III**, were solely mediated by MCs.

To investigate the role of MCs on AHR, monensin was used as a tool *in vivo* in the *i.p.* sensitized guinea pigs that received weekly intranasal HDM challenges for three and five consecutive weeks (**Figure 37**). Monensin was given 24h before each challenge.

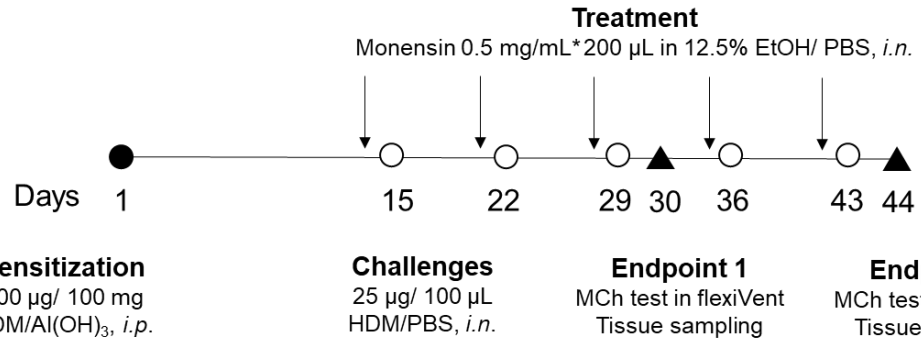


Figure 37: Protocol for HDM exposures. Guinea pigs were sensitized by a single intraperitoneal injection of 100 µg HDM with 100 mg aluminum hydroxide for two weeks. Followed by three or five intranasal HDM challenges (25 µg/ 100 µL HDM/PBS). Monensin (0.5 mg/mL* 200 µL) was given 24h before each challenge. 12.5% ethanol (EtOH) in PBS was used as the vehicle. One day after the last challenge, the airway responsiveness to methacholine (MCh) was performed and lungs were collected for histological examinations.

Compared to naïve animals, the HDM sensitized animals demonstrated enhanced responses to methacholine after three weeks challenges in the three parameters generated from the constant phase model, namely *Rn* (**Figure 38A**), *G* (**Figure 38B**) and *H* (**Figure 38C**). However, all the increases were significantly reduced by pretreatment with monensin (**Figure 38A to C**). The increased methacholine responses could also be found after five-week HDM challenge, and again, could be inhibited by monensin (**Figure 38D to F**).

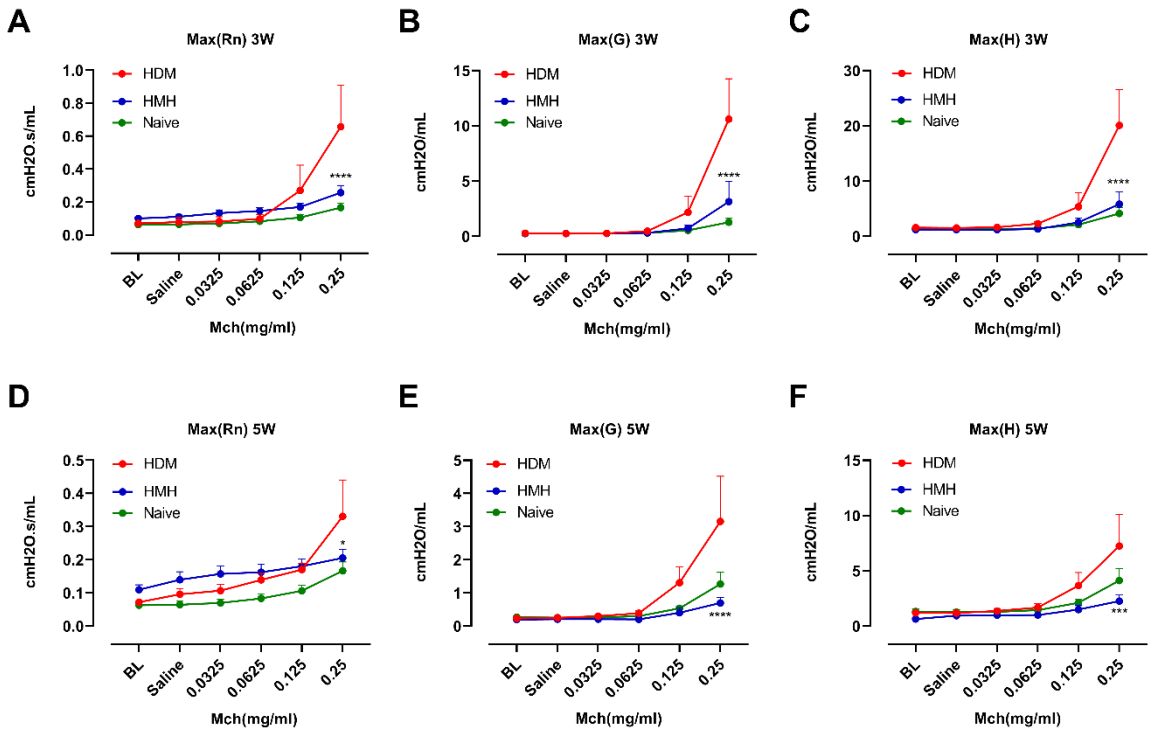


Figure 38: Effect of monensin on airway responsiveness to methacholine. Animals were sensitized with a single *i.p.* injection of of 100 µg HDM with 100 mg aluminum hydroxide at day 1 followed by intranasal HDM challenges (25 µg per challenge). Monensin (0.5mg/mL in 200 µL 12.5%EtOH/PBS) was given 24h before each challenge. Airway responsiveness to methacholine (Mch) was assessed in the flexiVent one day after 3- and 5-week challenge. Differences of resistance in conducting airways (*Rn*; A and D), tissue damping (*G*; B and E) and tissue elastance (*H*; C and F) were compared between the animals exposed to HDM with or without monensin treatment (n=4-7 in each group). HMH, animals sensitized and challenged with HDM that received monensin pretreatment. * $p<0.05$, *** $p<0.001$ and **** $p<0.0001$.

These findings indicate that the HDM exposed animals demonstrate AHR to mechacholine and MCs have important role in the development of AHR. In humans, the AHR can be found early in life in infants who have family histories of asthma[255]. The mechanisms behind AHR are not yet defined. Many factors are suggested to have roles in the development of AHR. For instance, the inflammatory cytokines and the changes of structure cells (*e.g.*, epithelial dysfunction and increase of smooth muscle mass)[256,257]. The reduced AHR by monensin might be due to the reduced MC mediators that known to induce hyperreactivity of the smooth muscle cells, such as tryptase, IL-4 and IL-13[63,145].

The effect of monensin on airway inflammation was studied in the Hematoxylin-Eosin-stained lung sections from animals received five weeks of challenges. Significant increases of

the areas of infiltration of inflammatory cells were found in HDM sensitized and challenged animals (**Figure 39**), which could be alleviated by monensin.

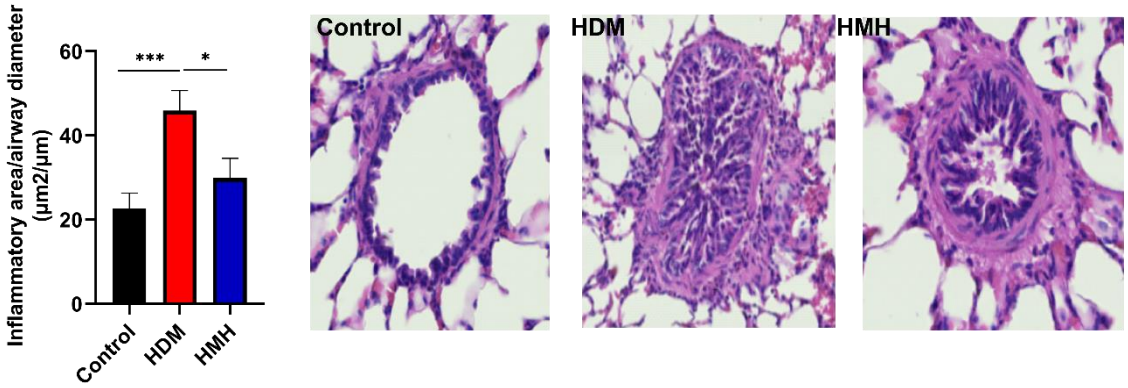


Figure 39: Airway inflammation of guinea pigs. Animals were sensitized by *i.p.* injection of HDM with adjuvant followed by five weeks' intranasal HDM challenges. Monensin or vehicle were given 24h before the last challenge. Inflammatory areas were compared in Hematoxylin-Eosin-stained slides between control animals, animals exposed to HDM without and with monensin treatment (HMH). Control: animals exposed to PBS, received vehicle (12.5% EtOH/PBS) or monensin as pretreatment. HDM: animals exposed to HDM and received vehicle as pretreatment. HMH: animals exposed to HDM and received monensin as pretreatment (n=6-7 in each group). * $p<0.05$ and *** $p<0.001$.

Lung sections were stained with Astra blue/Hematoxylin to quantify MCs. Increases of MCs after repeated HDM exposures were found both after 3-week (**Figure 40A**) and 5-week challenges (**Figure 40B**). The MC hyperplasia could be significantly dampened by pre-exposure to monensin (**Figure 40**).

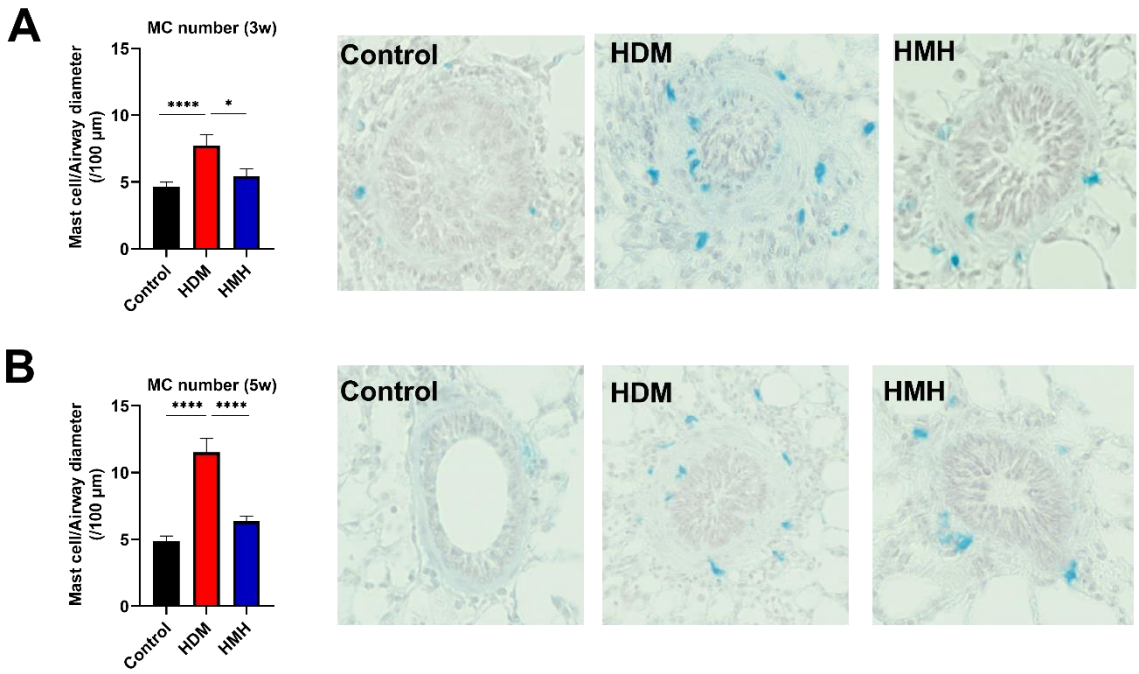


Figure 40: Mast cells in the airways of guinea pigs. Animals were sensitized by *i.p.* injection of HDM with adjuvant followed by weekly intranasal HDM challenge. Monensin or vehicle were given 24h before the last challenge. Mast cell number was counted in Astra blue stained sections after (A) 3-week and (B) 5-week HDM challenges. Control: animals exposed to PBS, received vehicle (12.5% EtOH/PBS) or monensin as pretreatment. HDM: animals exposed to HDM and received vehicle as pretreatment. HMH: animals exposed to HDM and received monensin as pretreatment (n=6-7 in each group). * $p<0.05$ and **** $p<0.0001$.

It is known that HDM exposures can cause MC hyperplasia both in humans[258] and in animal models[259,260]. This may be due to direct activation of MCs or due to indirect effects on other cells, which release MC chemoattractants that recruit MC progenitors from the circulation[116]. In the present study, repeated exposures to monensin caused a decline in MC numbers to the same level as in controls. However, we cannot at present be certain as to whether this is due to inhibited MC recruitment or due to cell death of resident MCs.

In the *in vivo* experiments, no abnormal behaviors and weight changes were observed in animals received monensin (**Figure 41**). As monensin is considered to be a toxic compound with narrow treatment window for several animal species (*e.g.*, horses[261], cattle[262] and poultry[263]), the dose of monensin therefore needs to be carefully considered. Previous studies have shown that the toxicity of monensin is dose-dependent, and only high doses of monensin reduced the growth of animals[263]. Thus, we did not find any signs of toxicity in this study at the monensin dose that efficiently reduced AHR, airway inflammation and MC hyperplasia, suggesting that monensin at this dose was well tolerated by animals.

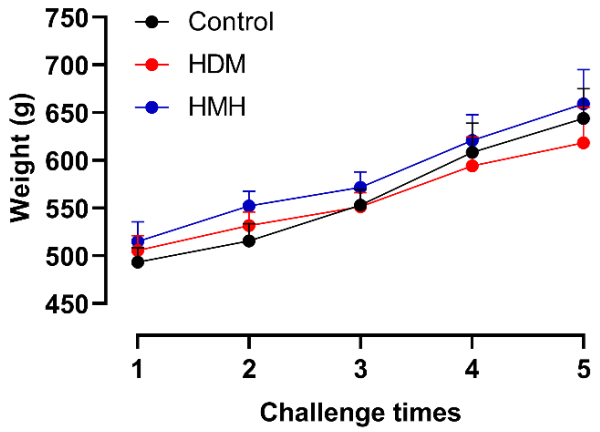


Figure 41: Weights of guinea pigs. The weights of animals from the first to the fifth HDM challenge. Control: animals exposed to PBS, received vehicle (12.5% EtOH/PBS) or monensin as pretreatment. HDM: animals exposed to HDM and received vehicle as pretreatment. HMH: animals exposed to HDM and received monensin as pretreatment (n=6-7 in each group).

4.4.1 Summary

In these two papers, we found monensin to be an efficient tool to reduce MC numbers and MC related antigen induced bronchoconstriction in guinea pig and human airways. With due consideration of dosing and timing, the study shows that monensin treatment provides a new strategy in experimental asthma and allergy research to elucidate the role of MCs in allergic and non-allergic diseases. Finally, the study highlights that an expansion of MC populations is fundamental for the development of AHR and airway inflammation in the used models which are highly relevant for reactions seen in allergic asthma.

5 GENERAL DISCUSSION

The general aim of this Ph.D. project was to investigate the role of mast cells on asthma-like features in guinea pig models. To fulfil the aim, we exposed guinea pigs to human relevant antigens (HDM or CDE) through different routes and successfully recaptured several asthma-like features in guinea pigs, such as antigen induced bronchoconstriction, AHR, T2 inflammation, airway remodeling and mast cell hyperplasia (both MC_T and MC_{TC}). By inducing MC death using monensin, we show that the antigen induced bronchoconstriction is merely mediated by MCs. In addition, MCs participate in the development of AHR and airway inflammation.

The specie of guinea pig used in this study is the outbred Dunkin-Hartley albino guinea pig. Several guinea pig strains have previously been used in 'allergic' model establishments, such as inbred strains[264] and euthymic hairless strain[265,266]. However, due to the paucity of inbred guinea pigs, the most used strain in model establishments is the Dunkin-Hartley guinea pigs[266]. Even though the outbred strain increases the variability of the experiments, it is an advantage in modeling asthma as the disease is highly heterogeneous. Both male and female guinea pigs were used in this study and the asthma like features (*e.g.*, antigen induced bronchoconstriction, AHR, airway inflammation and MC hyperplasia) could be replicated in both sexes.

The production of antigen specific immunoglobulins by HDM show that guinea pigs were sensitized to HDM after the exposures to antigen either through intranasal or intraperitoneal route with or without adjuvant. This finding indicates that the adaptive immune system is involved in the HDM induced responses. The complex contents of HDM extract (*Dermatophagoides pteronyssinus*) forms the basis for the production of immunoglobulins. However, the content of *Der p* varies among the batches of commercially available HDM[267]. In humans, *Der p 1* and *Der p 2* have the highest rates to elicit allergic reactions in mite sensitized asthmatic children[268]. *Der p 7* and *14* demonstrate strong IgE binding capacity[269,270]. The access of antigens to the antigen presenting cells is the initiation of the production of immunoglobulins. Several of the HDM contents have been shown to be proteases. *Der p 1* is a cysteine protease, *Der p 3*, *6* and *9* are serine proteases. Those proteases can affect the proteins in the epithelial tight junctions, such as occludin, claudin-1 and ZO-1[271,272], therefore impair the airway barrier. The disrupted barrier function of airways facilitates the HDM proteins to the APCs in the subepithelial layer. The APCs in the airways (the intranasal route) and in the abdominal cavity (the *i.p.* injection) present the accessed antigens to T cells and facilitate the production of antigen specific immunoglobulins by plasma cells. Though not mandatory, the addition of adjuvant enhances the HDM induced immune response. It is possibly due to that aluminum hydroxide absorbs antigens and form complexes which keep the exposure to antigen longer. In addition, it promotes the interactions between the complexes and APCs[273].

After sensitization, the antigen induced bronchoconstriction can be elicited through *in vivo* and *in vitro* exposures to HDM. The produced immunoglobulins bind to the surface of MCs, when challenged with HDM again, MCs release several mediators that cause the contraction, such as histamine, CysLTs and prostaglandins. Two weeks after sensitization, either by *i.p.* or *i.n.*, only HDM specific IgG_1 and IgG_2 were found elevated, but not HDM specific IgE. The HDM specific IgE appeared only after repeated HDM challenges. Therefore, the antigen induced bronchoconstrictions after two weeks sensitization, were possibly mediated by HDM specific IgGs instead of IgE. In addition to IgE, IgG_1 and IgG_2 have been shown to activate guinea pig MCs[274] and induce smooth muscle contraction of guinea pig trachea[275]. However, in humans, the allergic responses are mediated by allergen specific IgEs. The mechanisms behind the differences between guinea pigs and humans in the specific Igs induced allergic responses need to be further investigated. In the *ex vivo* setting, the HDM induced bronchoconstriction is totally blocked by monensin treatment which causes the death of MCs. This confirms that the antigen induced bronchoconstriction is pure MC mediated. However, the residual MC dependent component of the broncoconstriction after combined blockade of histamine, leukotrienes and prostanoids was only found in the *i.p.* (**paper III**), but not *i.n.* (**paper I**) sensitized guinea pigs nor in human bronchi[221]. The mechanisms behind need to be further explored.

Repeated exposures to HDM induce an expansion of eosinophils in the BALF and recruit inflammatory cells in the airways together with an increase of IL-13 in the BALF of guinea pigs. This indicates that HDM induce T2 inflammation. The production of HDM specific IgE and IgGs indicate that the cells in adaptive immune system (*e.g.*, APCs, T cells and B cells) might be involved in the development of eosinophilic inflammation. However, in addition to the adaptive immune response, the innate immune system can also induce eosinophilic inflammation by exposure to HDM, as the contents of HDM can interact with airway structure cells and inflammatory cells. *Der p 1*, *3* and *9* can induce the release of IL-6, IL-8, GM-CSF and eotaxin from airway epithelial cells through the activation of PAR2 receptors[276,277]. The activation of PAR2 contributes to the development of airway inflammation and the production of antigen specific immunoglobulins[278]. Endotoxin (lipopolysaccharide, LPS) from the wall of Gram negative bacteria[279] is apparent in HDM extract. Toll-like receptor 4 (TLR4) is responsible for the LPS triggered production of IL-25, IL-33 and TSLP from airway epithelium[280] and activate mucosal dendritic cells to drive the eosinophilic inflammation[280]. However, the high doses of LPS can switch the Th2 to Th1 response where the eosinophils and T2 cytokines in the BALF reduced and the neutrophils and non-T2 cytokines increased[281]. The content of endotoxin varies between batches of HDM extracts. Therefore, the batch differences need to be taken into consideration when comparing the results between experiments. Both the variations of LPS and the *Der p* have great impact on the responses and create a problem making the experiments highly batch dependent[267,282,283].

The decreased inflammation by monensin indicate that MCs take an important role in the development of the eosinophilic inflammation. Mast cells express receptors for direct

interactions with HDM components and release mediators that take part in the development of inflammation, in addition, MCs respond to the T2 cytokines released by other cells. *Der p 1* can activate cultured human MCs[284]. *Der p 3* induce MC migration through the activation of the PAR4 receptors[285]. It is known that MCs express TLR4 receptors that can bind to the LPS in HDM extracts, the activation of TLR4 causes the release of TNF-α, IL-1β, IL-6 and IL-13, but not IL-4 or IL-5[286]. In addition, MCs can respond to the alarmins induced by HDM. For instance, MCs express ST2 receptors that can respond to IL-33, in addition, MCs can also release IL-33[287,288]. IL-33 promote MC proliferation, survival, adhesion and activation through the ST2 receptors[289,290], increasing the release of IL-8 and IL-13[291]. Activated MCs also release both preformed and newly synthesized mediators that are involved in the development of T2 inflammation. For instance, tryptase and chymase can cleave IL-33 which lead to more potent activation of ILC2 cells[292]. Leukotriene E_4 activate MCs and cause the release of PGD_2 through $CysLT_1$ receptors[293]. Prostaglandin D_2 activate ILC2 through the DP_2/CRTH2 receptors, cause ILC2 migration, activation and release T2 cytokines IL-4, IL-5 and IL-13[294]. Moreover, MCs have the possibility to secrete IL-4, -5, -6, -13 and TNF-α[295,296]. The MC degranulated TNF-α induces the increased expression of E-selectin in the vessels which facilitate leukocyte trafficking[297].

The decreased AHR by monensin supports that MCs are important in AHR. Several MC mediators are suggested to have influences on the ASM. For example, tryptase[298,299], TNF-α[300,301], IL-4[63] and IL-13[63,287] and the activation of PAR2 receptors[302] increases the airway responsiveness. Airway hyperresponsiveness and airway inflammation are common features of human asthmatics. However, whether MCs are crucial for developing AHR and airway inflammation is quite debatable as there are conflicting results. Most data regarding the role of MCs in asthma features was obtained from murine models where genetically modified mice deficient in MCs were used. In a study, MCs were found to be important in enhancing airway inflammation and AHR in mice exposed to OVA without adjuvant, however, the importance of MCs disappeared in the models adding adjuvant[303]. In another study using different allergens, no AHR and inflammation differences were found between the wild type and the MC deficient mice[304,305], indicating MCs are not important at all in developing AHR or airway inflammation in mice. In contrast, other studies demonstrated that MC derived mediators are crucial in AHR development in murine asthma models[306-308]. The reasons for the broad using of mice to establish asthma models were due to the availability of various immunological and gene editing investigating tools. However, compare to mouse, guinea pig might be a more appropriate specie for MC and asthma research due to their similarities with humans regarding lung anatomy, physiological and pharmacological responses[182,183] and, in particular, MC location. Compared to inbred mouse MCs that mainly are located in central airways and only scarcely found in the lung[309,310], the guinea pig MCs are located both in airways and lung parenchyma[195,311], which is similar to humans[118]. Moreover, like humans, histamine, prostaglandins and CysLTs are MC mediators that responsible for allergic response in guinea pigs[195], whereas the antigen induced bronchoconstriction in mice is majorly mediated by serotonin[312] and neither histamine nor CysLTs contract airway smooth

muscle in mice[183]. Taken together, the guinea pig is a suitable animal for MC and asthma research.

Both HDM and CDE induced airway remodeling (*e.g.*, collagen deposition and goblet cell hyperplasia) of the guinea pig airways. Mast cells may also contribute to the development of airway remodeling. For instance, In the alveolar parenchyma of uncontrolled asthmatics, there is an increased population of the profibrotic TGF-β^+ MC_{TC}[313]. Tryptase from MCs promote the growth of lung fibroblast by binding to the PAR-2 receptors[314], which contribute to the airway fibrosis. IL-4 and IL-13 have important roles in the development of goblet cell hyperplasia[315]. However, further experiments need to be performed to investigate the role of MCs on airway remodeling.

In this study, we found that HDM and CDE can induce the expansion of MCs detected by both Astra blue and immunohistochemistry staining against tryptase and chymase. It has been shown that generally more MCs could be detected by immunohistochemistry staining against tryptase than conventional metachromatic staining[316] (*e.g.*, Toluidine blue and Astra blue). Both the MC_T and MC_{TC} were found increased in the airways of guinea pigs, with proximity to the ASM bundle. This is in accordance with the findings in humans and animal models. An increase of MCs were found both in allergic and non-allergic asthma patients, with a higher population of activated MCs in allergic asthma[317]. The MC_{TC} is increased and close to the ASM bundle[143]. Repeated low dose (1/5 of that induce an allergic response) of cat allergen challenge for two weeks induced an expansion of MCs in the bronchus of asthma patients[318]. In murine models, repeated exposures to HDM induce a marked expansion of MC numbers[260,319], both the MC_T and MC_{TC} in bronchi and alveolar[319].

It is not well defined whether the increase of MCs by antigen comes from the migration of MC progenitors or the proliferation of resident MCs. Therefore, the mechanisms need to be further investigated. Several cytokines, chemokines, growth factors, mediators and adhesion molecules are suggested to have impacts on the antigen induced MC hyperplasia[320]. For instance, several studies found that the antigen induced MC hyperplasia is mediated by IL-9[72,321,322], which increases the numbers of MCps in the lungs[322]. The IL-10 and TGF-β_1 released by T regulatory cells are found be important in recruiting MCps into the murine lungs[323]. However, IL-10 is not a chemoattractant for rat MCs, instead it inhibits the migration of MCs induced by RANTES, TNF-α, and NGF[324]. The expression of CXCR2 in the lung promote the recruitment of MCps[325]. The TGF-β_1 show chemotactic effect for human MCs[326,327]. The chemokine CCL2/CCR2 and CXCL10/CCR3 also facilitate the migration of MCs[148,328].Stem cell factor, in addition to promoting MC growth and differentiation, is a chemoattractant for both mouse[329] and human MCs[330]. Histamine enhances the CXCL12 induced MC recruitment via the H_4 receptors[331]. The PGE_2 is a chemoattractant for both mouse and human MCs through the EP_3 receptors[332,333], whereas the EP_2 receptors inhibit the MC migration[333]. The PGD_2/CRTH2 axis is important for the migration of murine MCs[334]. Leukotriene B_4 is also a potent chemoattractant for the immature MC progenitors[335]. The adhesion molecules might also have important roles in the

migration of MC progenitors. The cultured human MC progenitors express several integrins, such as $\alpha_4\beta_1$ and β_7, and selectin ligand (PSGL-1). The interaction between MC progenitors with the activated human umbilical vein endothelial cells is through the α_4 integrins, vascular cell adhesion molecule 1(VCAM-1), PSGL-1 and E-selectin[336]. The migration of mouse MCps by antigen is through the interactions with VCAM-1 and both $\alpha_4\beta_1$ and β_7[337].

In this study, we showed that the monensin has direct effect on MCs and causing cell death *in vitro*. However, we also showed that monensin with the dose used in the *in vivo* study does not reduce MC numbers. Instead, it reduced the MC hyperplasia induced by HDM. This might be due to that the newly recruited MCs, compared to the resident mature MCs, are more sensitive to monensin. Another possible reason is that monensin cause the malfunction of the resident MCs, which reduces the production of MC mediators that affect the chemotaxis and migration of MCps into the lungs. For instance, IL-9[338], PGD_2[334] and LTB_4[335]. In addition, tryptase can stimulate human ASM cells (which express PAR2 receptors) to release TGF-β_1 which works as a chemoattractant for MCs[326]. However, the effect of monensin on other cells besides MCs on guinea pig and human tissues need to be further investigated.

In summary, the general aim of this Ph.D. study was fulfilled. We successfully established an allergic asthma model in guinea pig using HDM. Further, we found both HDM and CDE are suitable for asthma model establishments and MCs indeed have important roles in antigen induced bronchoconstriction, AHR and airway inflammation. However, future studies are needed for investigating the role of MCs in the airway remodeling.

6 CONCLUSIONS

In the five papers included in this thesis, several conclusions can be drawn.

- Repeated exposures to house dust mite extract can be used to establish guinea pig models of allergic asthma.
- Both house dust mite and cat dander extract can induce asthma like histopathology with expansions of mucosal mast cells.
- Guinea pig trachea has distinct response to ‘allergen’ and ‘non-allergic’ mast cell agonists.
- Monensin is an effective tool to reduce mast cell numbers.
- An expansion of mast cells is fundamental for the development of AHR and airway inflammation in the used guinea pig models.

7 POINTS OF PERSPECTIVE

For continuing studying the role of mast cells in asthma, guinea pigs are suitable for asthma model establishments. In this thesis, we initiated the model establishment using human relevant allergens which opens more possibilities for further mechanistic studies both *in vitro*, *ex vivo* and *in vivo* to investigate the role of MCs in asthma.

In vitro studies: Instead of using the trachea, we now have the possibility to isolate the small intralobular airways from guinea pigs to do pharmacology studies. For instance, we can isolate small airways from naïve animals and culture them with mediators that are known to induce airway hyperreactivity (*e.g.*, IL-4, IL-13, IL-33, TNF-α and tryptase). To investigate if MCs have a role in the ASM hyperreactivity induced by those mediators, we can co-culture the segments with or without monensin which induces MC death. The responses of airway segments to different agonists (histamine, carbachol or KCl) can be measured in the myography.

Ex vivo studies: A special advantage of *ex vivo* study is that we can isolate the airways from animals that receive different treatments. This is almost impossible to achieve in humans. Moreover, the guinea pig modes are very relevant for pharmacological studies as both MC responses and smooth muscle responses are very similar to humans. For mechanistic studies of which mediators of MCs participated in the development of AHR, we can isolate small airways from the intraperitoneal HDM sensitized guinea pigs (without airway inflammation) followed by repeated activation of MCs by culturing the segments with HDM with or without the pretreatment of MC mediator antagonists. The airway reactivity of those airway segments can be measured in the myography. In addition, we can investigate how the inflammation and the increase of MCs in the asthma models alters the smooth muscle properties. This can be achieved by isolating the small airways from the guinea pig models with or without targeting MCs for RNA sequencing. The alterations of smooth muscle contraction can be studied by activating several G-protein coupled receptors (*e.g.*, histamine H_1, $CysLT_1$ and TP receptors) as well as receptor-operated channels (*e.g.*, purinergic P2X receptors and transient receptor potential (TRP) channels). In addition to investigate the changes of smooth muscle properties, the function of MCs can also be affected by inflammation and MC hyperplasia. The responses of isolated airways from the guinea pig models can be studied in myography under the stimulation with MC agonists, such as HDM, C48/80 and mannitol.

In vivo studies: We can investigate the role of MCs in asthma models by different exposure route of antigens. In addition to the exposure of antigens through the intraperitoneal injection or through intranasal instillation, we have the possibility to expose guinea pigs to antigen aerosols generated by the PreciseInhale system where the inhaled doses of antigens are monitored. Furthermore, it is very interesting to establish models using different MC agonists (*e.g.*, antigen and C48/80) to examine the similarities and differences of those agonists on asthma features. In addition, investigating the role of MCs in non-allergic asthma models. For instance, establishing Th17 model using dog allergens or Th1 model using environmental

pollutants (*e.g.*, ozone and diesel exhaust particulates) or occupational chemicals (*e.g.*, toluene diisocyanate) with or without interventions target MCs.

All in all, this PhD thesis presents several unique guinea pig models that can be used for providing new insights of the mechanism behind the development of asthma.

8 ACKNOWLEDGEMENTS

Time flies so fast! Four and a half year has passed and suddenly it comes to the end of my Ph.D. study. The journey towards a PhD is tough! However, during this period, I obtained so many precious memories ……

The first and most important person I want to express my appreciation is my main supervisor **Mikael Adner**. Thank you, **Mikael**, for choosing me as your Ph.D. student and guided me to the interesting scientific area of asthma. It is my great honor to have a true scientist as my main supervisor. Your broad and profound knowledge, your kindness and encouragements, your unconditional supports and always reachable supervisions made my PhD study easier and happier. You are the best supervisor I have ever seen!!! You are not only a scientist and supervisor to me, but also a very good friend. Thank you for giving me all the nice words and love to me, they will encourage me to bravely face challenges in my future.

I am so lucky to have such great co-supervisors. **Gunnar Nilsson**, for always participating my project meetings and sharing your knowledge, experiences and suggestions which greatly promoted the progress of my PhD study. I really like your smile and enjoy the discussions with you. **Jesper Säfholm**, for your guidance both in experiments and writing. You are such a trustworthy supervisor that I can always ask for help without hesitation. **Sven-Erik Dahlén**, for sharing your encyclopedic knowledge of asthma and your nice and critical comments on my projects and manuscripts. I also would like to thank my previous co-supervisor **Patricia Ramos-Ramírez**, for guiding me into the *in vivo* study, teaching me handling animals and all the experimental supports.

I feel so happy in our group because of all the nice current/previous group members at EAAF: **Willem Abma**, **Johan Kolmert**, **Ingrid Delin**, **Anne Petrén**, **Mu Nie**, **Caijuan Dong**, **Lingling Wu**, **Maria Belikova**, **Anna-Karin Johnsson**, **Anna James**, **Anita Sydbom**, **Maria Sparreman Mikus**, **Lars I. Andersson**, **Roelinde Middelveld**, **Alexandra Ek**, **Malin Noreby**. Thank you, **Willem**, for being a very nice friend of me. I appreciated the time you stood beside me when we could have free talks regardless of topics, from research, life to politics. You are like a big brother to me, give me encouragements and supports. **Johan**, for sharing your solid knowledge in lipids, mass spectrometry and your life experiences to me and the help with the mass spectrometry analysis. I really appreciate your passion towards work and life. **Ingrid**, for all your help with the orders and keep the lab works safe, it made me have the possibility to focus on research. Meanwhile, thank you for your patients on teaching me Swedish and your knowledge in ELISA. Thank you, the kind and gentle **Anne**, for all the help and supervisions on histology and ELISA. Also, the help with ordering stuffs. **Mu**, for being together with me supporting the *in vivo* experiments and opening your heart to me, I really enjoy the talks with you. **Caijuan**, for being such a great helper in experiments. You are such a fast learner of high efficacy. **Lingling**, for sharing your experiences in mice studies and the joyful time we spent together both in and outside the lab. **Maria B**, for teaching and helping me with my first guinea pig organ bath experiment and the

help in dissecting human bronchi. With you in the lab, it is always clean and in good order. **Anna-Karin**, for your help with the mass spectrometry study and your knowledge in mast cells. You are a very nice person to talk to. **Anna**, for sharing your knowledge in asthma biomarkers and being a nice teacher and instructor in the *Pulmonary Inflammation* course. Also, a special thanks for your suggestions of the title of my PhD thesis. **Anita**, for sharing your knowledge in histamine. I really like your dances, songs, and your self-made traditional Swedish dresses. **Maria M**, for the sharing knowledge of proteomics. I appreciated the times we sat together talking about cats and journeys. **Lars**, for the help with ELISA analysis. I really appreciate your serious attitude towards the quality controls of ELISA and other experiments. **Roelinde**, for your help in processing financial issues and told me the correct pronunciation of 'Mikael'. **Alexandra**, for being a very nice person in the lab. I like the smiles on your face and all the talks we had together. **Malin**, for being a cheerful colleague and sharing your knowledge in animal handling and anesthesia. I really enjoyed the time we spent together in the lab and in Germany where I attended my first international conference of my PhD study.

I would like to express my appreciation for persons outside our group. **Susanna Kumlien Georén**, for sharing your knowledge in guinea pig studies and your microscopes for histological analysis. **Olivia Larsson**, for your teaching of intra-tracheal instillation technique in mice and your experiences in guinea pigs. **Nailin Li**, for being my mentor and your efforts in the KI-CSC collaboration which gave me the possibility to come to KI. **Jonathan Coquet** and **Julian Stark**, for your knowledge in mice asthma model. It was an unforgettable and exciting memory when all of us agreed to work until 3 a.m. to finish the collaborating study. **Ewa Selg** and **Per Gerde** for sharing your knowledge in inhalations. **Javier Zurita** and **Craig Wheelock** for your help with the mass spectrometry studies. **Aida Paivandy** and **Gunnar Pejler** for sharing your knowledge in mast cells.

I am also grateful for all the staff at AFL for taking good care of the experimental animals and being very helpful in solving issues. Especially **Sabina De Villiers** and **Bengt Eriksson**. **Sabina**, for your always well-timed animal ordering help and being a good and trustworthy coordinator. You are always the first person at AFL I want to contact when I have practical issues. **Bengt**, for helping me order anesthetics and your valuable suggestions on animal anesthesia.

I spent most of my spare time with the families of my daughter's closest friends: **Simon**, **Chen Liu** and **Daniel Ingmar Rhodin**; **Yian**, **Yichuan**, **Li Song** and **Hui Wang**; **Yiyi**, **Lina Zhao** and **Xianning Zhan**; **Emilia**, **Alejandra Zarate Delgado** and **Erik Olausson**; **Taotao**, **Miao Wang** and **Hao Liu**. Thank you all for the joyful time we spent together.

For my friends at KI: **Feifei Yan** and **Long Jiang**, **Zhengbing Zhuge** as well as **Jinyan He**. **Feifei**, for all the joyful times we spent together in the last 14 years, thanks for introducing KI to me when I planned to apply a PhD position and also for the wonderful times we spent together in the forests investigating mushrooms! **Long**, for sharing your experiences and

suggestions on PhD studies. **Zhengbing**, for always being so nice to me and for the times we were out exploring forests to find our secret mushroom places. **Jinyan**, for the nice food and snacks you prepared for us. I really like your charming smile, it warms me as the sunshine.

For my friends in China, **Meifang Li**, **Yue Bi**, **Meiling Sun**, **Zihuan Luo**, **Guan Zhen**, **Hongwei Luo** and **Xiao Yang**. Thank you all for your accompany when I went back to China. Especially **Meifang (Xiao Wanzi)**, thank you for being my sincere friend, you are the one I can always trust and seek help from. Whenever I go back to China, you are the one I always eager to meet and you always have time for me regardless how busy you are.

Now it is time to express my sincere appreciation for my family members. For my parents and parents-in-law: 感谢我的妈妈欧嫦秀把我拉扯长大，没有你对教育的坚持，我没有办法从农村一步步走来瑞典留学。感谢你的言传身教，教会我坚毅，努力以及上进，赐予我终身受益的品格。感谢你无私的爱，时时温暖着我。感谢我的继父吴华将我当作亲生女儿一般对待，二十年来从未与我红脸，给予我温暖与帮助，也非常感谢你对妈妈的照顾。感谢我的公公婆婆对我们小家庭的无私照顾与支持。我的婆婆朱俊芳，不远万里独自一人多次往返瑞典和中国，不为别的，只为能帮上我们一点忙。我的公公李龙鹤，时时心系我们，为我们的事业规划提供宝贵的建议以及支持。感谢我的父母们让我真正体会到: 母爱若水，水之所处，无微不至；父爱如山，山之高昂，令人仰止。

To my husband, **Yuanzhe Li**. Without your love and supports, I could never survive my PhD study! Thank you for digesting my endless depressing emotions and giving me sunshine. You are the shiniest star in the darkest sky that gives me direction and guides me home. Thank you also for the delicious food you cooked and daily lunch boxes you prepared for me.

To my dearest daughter, **Yiyi Li**. I am so grateful that you chose me to be your mom. Thanks for giving me the opportunity to grow up together with you. Thank you for your trust and unconditional love to me. I am so proud of you! Your independence, courage to face challenges, enthusiasm towards learning and kindness to friends and family members will benefit you lifelong. Thanks for your drawing '*The story between mast cells and guinea pigs*' for my thesis.

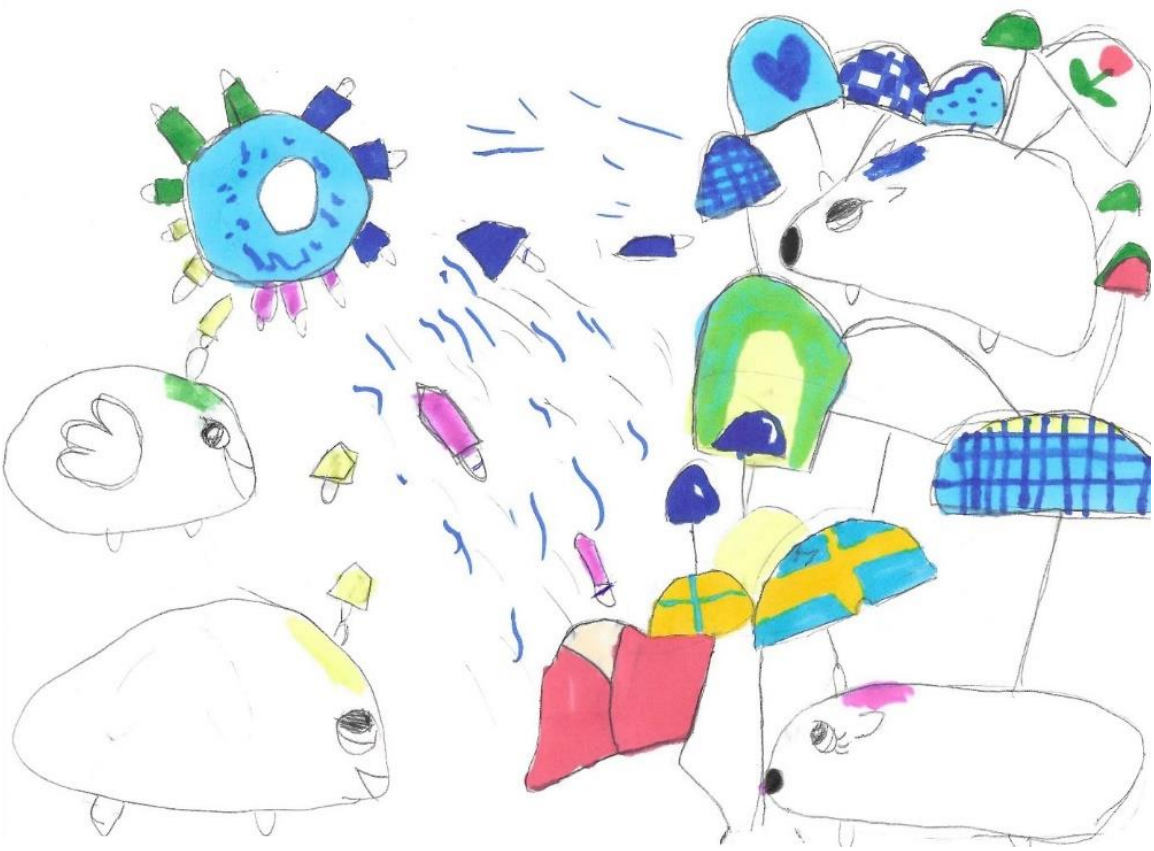


Figure Yiyi: **The story between mast cells and guinea pigs**. The activated mast cell releases harmful substances to the colored guinea pigs (When doing experiments, I usually mark the white guinea pigs with green, red, yellow, or blue), the guinea pigs protect themselves with different umbrellas and discharge weapons against the mast cell.

To my two cats: **Chocolate** and **Cookie**; four guinea pigs at home: **Feifei**, **Qiuqiu**, **Wanmei** and **Xiaofei**. Thank you for the accompany and comforts to me.

Finally, I would like to say 'thank you' to myself. Thank you, **Jielu**, for not giving up after endless self-doubting and emotional breakdown. You are not as bad as you thought, cheer up and face new life challenges with all the love you have!

9 REFERENCES

1. Marketos SG, Ballas CN. Bronchial asthma in the medical literature of Greek antiquity. *The Journal of asthma : official journal of the Association for the Care of Asthma.* 1982;19(4):263-269.

2. Holgate ST. A brief history of asthma and its mechanisms to modern concepts of disease pathogenesis. *Allergy Asthma Immunol Res.* 2010;2(3):165-171.

3. Sakula A. Henry Hyde Salter (1823-71): a biographical sketch. *Thorax.* 1985;40(12):887-888.

4. Salter HH. On asthma: its pathology and treatment. In:1860: https://wellcomecollection.org/works/xsk58bgh. Accessed 2022-01-28.

5. Diamant Z, Boot JD, Virchow JC. Summing up 100 years of asthma. *Respir Med.* 2007;101(3):378-388.

6. Spitzer WO, Suissa S, Ernst P, et al. The use of beta-agonists and the risk of death and near death from asthma. *The New England journal of medicine.* 1992;326(8):501-506.

7. Bousquet J, Chanez P, Lacoste JY, et al. Eosinophilic inflammation in asthma. *The New England journal of medicine.* 1990;323(15):1033-1039.

8. Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, Kay AB. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *The American review of respiratory disease.* 1988;137(1):62-69.

9. Laitinen LA, Laitinen A, Haahtela T. Airway mucosal inflammation even in patients with newly diagnosed asthma. *The American review of respiratory disease.* 1993;147(3):697-704.

10. Sridhar AV, McKean M. Nedocromil sodium for chronic asthma in children. *Cochrane Database Syst Rev.* 2006;2006(3):Cd004108.

11. Hoshino M, Nakamura Y, Sim JJ, Tomioka H. A comparative study of the effects of ketotifen, disodium cromoglycate, and beclomethasone dipropionate on bronchial mucosa and asthma symptoms in patients with atopic asthma. *Respir Med.* 1998;92(7):942-950.

12. Bisgaard H, Zielen S, Garcia-Garcia ML, et al. Montelukast reduces asthma exacerbations in 2- to 5-year-old children with intermittent asthma. *American journal of respiratory and critical care medicine.* 2005;171(4):315-322.

13. Virchow JC, Bachert C. Efficacy and safety of montelukast in adults with asthma and allergic rhinitis. *Respir Med.* 2006;100(11):1952-1959.

14. Djukanović R, Homeyard S, Gratziou C, et al. The effect of treatment with oral corticosteroids on asthma symptoms and airway inflammation. *American journal of respiratory and critical care medicine.* 1997;155(3):826-832.

15. Ellul-Micallef R, Fenech FF. Intravenous prednisolone in chronic bronchial asthma. *Thorax.* 1975;30(3):312-315.

16. Price DB, Trudo F, Voorham J, et al. Adverse outcomes from initiation of systemic corticosteroids for asthma: long-term observational study. *J Asthma Allergy.* 2018;11:193-204.

17. Djukanović R, Wilson JW, Britten KM, et al. Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *The American review of respiratory disease.* 1992;145(3):669-674.

18. McGregor MC, Krings JG, Nair P, Castro M. Role of Biologics in Asthma. *American journal of respiratory and critical care medicine.* 2019;199(4):433-445.

19. Furlow PW, Mathisen DJ. Surgical anatomy of the trachea. *Ann Cardiothorac Surg.* 2018;7(2):255-260.

20. tracheobronchial-tree. https://radiopaedia.org/articles/tracheobronchial-tree. Accessed 02-01, 2022.

21. Ball M HM, Padalia D. Anatomy, Airway. https://www.ncbi.nlm.nih.gov/books/NBK459258/. Updated [Updated 2021 Jul 31]. Accessed 02-01, 2022.

22. Knight DA, Holgate ST. The airway epithelium: structural and functional properties in health and disease. *Respirology.* 2003;8(4):432-446.

23. GINA. GINA Guidlines. https://ginasthma.org/gina-reports/. Published 2021. Updated 2022-01-20. Accessed 2022-01-20, 2022.

24. Minor TE, Dick EC, Baker JW, Ouellette JJ, Cohen M, Reed CE. Rhinovirus and influenza type A infections as precipitants of asthma. *The American review of respiratory disease.* 1976;113(2):149-153.

25. Langley SJ, Goldthorpe S, Craven M, Morris J, Woodcock A, Custovic A. Exposure and sensitization to indoor allergens: association with lung function, bronchial reactivity, and exhaled nitric oxide measures in asthma. *The Journal of allergy and clinical immunology.* 2003;112(2):362-368.

26. McFadden ER, Jr., Gilbert IA. Exercise-induced asthma. *The New England journal of medicine.* 1994;330(19):1362-1367.

27. Guarnieri M, Balmes JR. Outdoor air pollution and asthma. *Lancet (London, England).* 2014;383(9928):1581-1592.

28. Ostro BD, Lipsett MJ, Mann JK, Wiener MB, Selner J. Indoor air pollution and asthma. Results from a panel study. *American journal of respiratory and critical care medicine.* 1994;149(6):1400-1406.

29. von Bülow A, Kriegbaum M, Backer V, Porsbjerg C. The prevalence of severe asthma and low asthma control among Danish adults. *The journal of allergy and clinical immunology In practice.* 2014;2(6):759-767.

30. Larsson K, Ställberg B, Lisspers K, et al. Prevalence and management of severe asthma in primary care: an observational cohort study in Sweden (PACEHR). *Respiratory research.* 2018;19(1):12.

31. Backman H, Jansson S-A, Stridsman C, et al. Severe asthma among adults: Prevalence and clinical characteristics. *European Respiratory Journal.* 2018;52(suppl 62):PA3918.

32. Cohen MB. BRONCHIAL ASTHMA: CLASSIFICATION BASED ON ETIOLOGICAL AND PATHOLOGICAL FACTORS. *Annals of Internal Medicine.* 1944;20(4):590-596.

33. Rackemann FM. Intrinsic asthma. *Journal of Allergy.* 1940;11(2):147-162.

34. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nature medicine.* 2012;18(5):716-725.

35. Pakkasela J, Ilmarinen P, Honkamäki J, et al. Age-specific incidence of allergic and non-allergic asthma. *BMC Pulm Med.* 2020;20(1):9.

36. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *American journal of respiratory and critical care medicine.* 2000;161(5):1720-1745.

37. Weersink EJ, Postma DS, Aalbers R, de Monchy JG. Early and late asthmatic reaction after allergen challenge. *Respir Med.* 1994;88(2):103-114.

38. Wenzel SE, Larsen GL, Johnston K, Voelkel NF, Westcott JY. Elevated levels of leukotriene C4 in bronchoalveolar lavage fluid from atopic asthmatics after endobronchial allergen challenge. *The American review of respiratory disease.* 1990;142(1):112-119.

39. Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nature medicine.* 2012;18(5):693-704.

40. Gauvreau GM, Davis BE, Scadding G, et al. Allergen Provocation Tests in Respiratory Research: Building on 50 Years of Experience. *The European respiratory journal.* 2022.

41. Agache I, Bilò M, Braunstahl GJ, et al. In vivo diagnosis of allergic diseases--allergen provocation tests. *Allergy.* 2015;70(4):355-365.

42. Hoymann HG. Lung function measurements in rodents in safety pharmacology studies. *Front Pharmacol.* 2012;3:156-156.

43. SCIREQ. flexivent techniques and measurements. https://www.scireq.com/flexivent/techniques-measurements/. Published 2022. Accessed 0315, 2022.

44. Hantos Z, Daróczy B, Suki B, Nagy S, Fredberg JJ. Input impedance and peripheral inhomogeneity of dog lungs. *Journal of applied physiology (Bethesda, Md : 1985).* 1992;72(1):168-178.

45. Hamelmann E, Schwarze J, Takeda K, et al. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *American journal of respiratory and critical care medicine.* 1997;156(3 Pt 1):766-775.

46. Lomask M. Further exploration of the Penh parameter. *Exp Toxicol Pathol.* 2006;57 Suppl 2:13-20.

47. Lundblad LK, Irvin CG, Adler A, Bates JH. A reevaluation of the validity of unrestrained plethysmography in mice. *Journal of applied physiology (Bethesda, Md : 1985).* 2002;93(4):1198-1207.

48. Chong BTY, Agrawal DK, Romero FA, Townley RG. Measurement of bronchoconstriction using whole-body plethysmograph: comparison of freely moving versus restrained guinea pigs. *Journal of Pharmacological and Toxicological Methods.* 1998;39(3):163-168.

49. Chapman DG, Irvin CG. Mechanisms of airway hyper-responsiveness in asthma: the past, present and yet to come. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2015;45(4):706-719.

50. Brannan JD, Lougheed MD. Airway hyperresponsiveness in asthma: mechanisms, clinical significance, and treatment. *Front Physiol.* 2012;3:460.

51. Cockcroft DW. Direct challenge tests: Airway hyperresponsiveness in asthma: its measurement and clinical significance. *Chest.* 2010;138(2 Suppl):18s-24s.

52. Busse WW. The relationship of airway hyperresponsiveness and airway inflammation: Airway hyperresponsiveness in asthma: its measurement and clinical significance. *Chest.* 2010;138(2 Suppl):4s-10s.

53. Shalaby KH, Gold LG, Schuessler TF, Martin JG, Robichaud A. Combined forced oscillation and forced expiration measurements in mice for the assessment of airway hyperresponsiveness. *Respiratory research.* 2010;11(1):82.

54. Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. *Respirology.* 2006;11(1):54-61.

55. Trautmann A, Kruger K, Akdis M, et al. Apoptosis and loss of adhesion of bronchial epithelial cells in asthma. *International archives of allergy and immunology.* 2005;138(2):142-150.

56. Sweerus K, Lachowicz-Scroggins M, Gordon E, et al. Claudin-18 deficiency is associated with airway epithelial barrier dysfunction and asthma. *The Journal of allergy and clinical immunology.* 2017;139(1):72-81.e71.

57. Reithofer M, Jahn-Schmid B. Allergens with Protease Activity from House Dust Mites. *International journal of molecular sciences.* 2017;18(7).

58. Kale SL, Agrawal K, Gaur SN, Arora N. Cockroach protease allergen induces allergic airway inflammation via epithelial cell activation. *Sci Rep.* 2017;7:42341.

59. Wang W, Li Y, Lv Z, et al. Bronchial Allergen Challenge of Patients with Atopic Asthma Triggers an Alarmin (IL-33, TSLP, and IL-25) Response in the Airways Epithelium and Submucosa. *Journal of immunology (Baltimore, Md : 1950).* 2018;201(8):2221-2231.

60. Bleck B, Tse DB, Curotto de Lafaille MA, Zhang F, Reibman J. Diesel exhaust particle-exposed human bronchial epithelial cells induce dendritic cell maturation and polarization via thymic stromal lymphopoietin. *J Clin Immunol.* 2008;28(2):147-156.

61. Woodruff PG, Modrek B, Choy DF, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *American journal of respiratory and critical care medicine.* 2009;180(5):388-395.

62. Finkelman FD, Katona IM, Urban JF, Jr., et al. IL-4 is required to generate and sustain in vivo IgE responses. *Journal of immunology (Baltimore, Md : 1950).* 1988;141(7):2335-2341.

63. Manson ML, Säfholm J, James A, et al. IL-13 and IL-4, but not IL-5 nor IL-17A, induce hyperresponsiveness in isolated human small airways. *The Journal of allergy and clinical immunology.* 2020;145(3):808-817.e802.

64. Akiho H, Blennerhassett P, Deng Y, Collins SM. Role of IL-4, IL-13, and STAT6 in inflammation-induced hypercontractility of murine smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol.* 2002;282(2):G226-232.

65. Kubo M. Innate and adaptive type 2 immunity in lung allergic inflammation. *Immunological reviews.* 2017;278(1):162-172.

66. Dabbagh K, Takeyama K, Lee H-M, Ueki IF, Lausier JA, Nadel JA. IL-4 Induces Mucin Gene Expression and Goblet Cell Metaplasia In Vitro and In Vivo. *The Journal of Immunology.* 1999;162(10):6233-6237.

67. Kibe A, Inoue H, Fukuyama S, et al. Differential regulation by glucocorticoid of interleukin-13-induced eosinophilia, hyperresponsiveness, and goblet cell hyperplasia in mouse airways. *American journal of respiratory and critical care medicine.* 2003;167(1):50-56.

68. Louahed J, Zhou Y, Maloy WL, et al. Interleukin 9 promotes influx and local maturation of eosinophils. *Blood.* 2001;97(4):1035-1042.

69. Clutterbuck E, Hirst E, Sanderson C. Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: comparison and interaction with IL-1, IL-3, IL-6, and GMCSF. *Blood.* 1989;73(6):1504-1512.

70. Wu C, Boey D, Bril O, et al. Single-cell transcriptomics reveals the identity and regulators of human mast cell progenitors. *Blood Adv.* 2022.

71. Matsuzawa S, Sakashita K, Kinoshita T, Ito S, Yamashita T, Koike K. IL-9 Enhances the Growth of Human Mast Cell Progenitors Under Stimulation with Stem Cell Factor. *The Journal of Immunology.* 2003;170(7):3461-3467.

72. Kearley J, Erjefalt JS, Andersson C, et al. IL-9 governs allergen-induced mast cell numbers in the lung and chronic remodeling of the airways. *American journal of respiratory and critical care medicine.* 2011;183(7):865-875.

73. Ochi H, Hirani WM, Yuan Q, Friend DS, Austen KF, Boyce JA. T helper cell type 2 cytokine-mediated comitogenic responses and CCR3 expression during differentiation of human mast cells in vitro. *J Exp Med.* 1999;190(2):267-280.

74. Parulekar AD, Diamant Z, Hanania NA. Role of T2 inflammation biomarkers in severe asthma. *Current opinion in pulmonary medicine.* 2016;22(1):59-68.

75. Coverstone AM, Seibold MA, Peters MC. Diagnosis and Management of T2-High Asthma. *The journal of allergy and clinical immunology In practice.* 2020;8(2):442-450.

76. Kolmert J, Gomez C, Lefaudeux D, et al. Non-invasive urinary lipid mediator excretion profiles identify sub-phenotypes of asthma in the U-BIOPRED study. *European Respiratory Journal.* 2016;48(suppl 60):OA4954.

77. Brusselle GG, Koppelman GH. Biologic Therapies for Severe Asthma. *New England Journal of Medicine.* 2022;386(2):157-171.

78. Kaur R, Chupp G. Phenotypes and endotypes of adult asthma: Moving toward precision medicine. *The Journal of allergy and clinical immunology.* 2019;144(1):1-12.

79. Samitas K, Zervas E, Gaga M. T2-low asthma: current approach to diagnosis and therapy. *Current opinion in pulmonary medicine.* 2017;23(1):48-55.

80. Kuruvilla ME, Lee FE, Lee GB. Understanding Asthma Phenotypes, Endotypes, and Mechanisms of Disease. *Clinical reviews in allergy & immunology.* 2019;56(2):219-233.

81. Nakajima H, Hirose K. Role of IL-23 and Th17 Cells in Airway Inflammation in Asthma. *Immune Netw.* 2010;10(1):1-4.

82. Sze E, Bhalla A, Nair P. Mechanisms and therapeutic strategies for non-T2 asthma. *Allergy.* 2020;75(2):311-325.

83. Simpson JL, Phipps S, Baines KJ, Oreo KM, Gunawardhana L, Gibson PG. Elevated expression of the NLRP3 inflammasome in neutrophilic asthma. *The European respiratory journal.* 2014;43(4):1067-1076.

84. Zhu J, Message SD, Qiu Y, et al. Airway Inflammation and Illness Severity in Response to Experimental Rhinovirus Infection in Asthma. *Chest.* 2014;145(6):1219-1229.

85. Wark PAB, Johnston SL, Moric I, Simpson JL, Hensley MJ, Gibson PG. Neutrophil degranulation and cell lysis is associated with clinical severity in virus-induced asthma. *European Respiratory Journal.* 2002;19(1):68-75.

86. Scannell C, Chen L, Aris RM, et al. Greater ozone-induced inflammatory responses in subjects with asthma. *American journal of respiratory and critical care medicine.* 1996;154(1):24-29.

87. Nightingale JA, Maggs R, Cullinan P, et al. Airway inflammation after controlled exposure to diesel exhaust particulates. *American journal of respiratory and critical care medicine.* 2000;162(1):161-166.

88. Chalmers GW, MacLeod KJ, Thomson L, Little SA, McSharry C, Thomson NC. Smoking and airway inflammation in patients with mild asthma. *Chest.* 2001;120(6):1917-1922.

89. Park H, Jung K, Kim H, Nahm D, Kang K. Neutrophil activation following TDI bronchial challenges to the airway secretion from subjects with TDI-induced asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 1999;29(10):1395-1401.

90. Park HS, Jung KS, Hwang SC, Nahm DH, Yim HE. Neutrophil infiltration and release of IL-8 in airway mucosa from subjects with grain dust-induced occupational asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 1998;28(6):724-730.

91. McBride DE, Koenig JQ, Luchtel DL, Williams PV, Henderson WR, Jr. Inflammatory effects of ozone in the upper airways of subjects with asthma. *American journal of respiratory and critical care medicine.* 1994;149(5):1192-1197.

92. Pelletier M, Maggi L, Micheletti A, et al. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood.* 2010;115(2):335-343.

93. Roussel L, Houle F, Chan C, et al. IL-17 promotes p38 MAPK-dependent endothelial activation enhancing neutrophil recruitment to sites of inflammation. *Journal of immunology (Baltimore, Md : 1950).* 2010;184(8):4531-4537.

94. Raundhal M, Morse C, Khare A, et al. High IFN-γ and low SLPI mark severe asthma in mice and humans. *The Journal of clinical investigation.* 2015;125(8):3037-3050.

95. Abma W, Noreby M, Wheelock CE, Dahlén SE, Adner M, Säfholm J. Lipoxin A(4) reduces house dust mite and TNFα-induced hyperreactivity in the mouse trachea. *Prostaglandins & other lipid mediators.* 2020;149:106428.

96. McKinley L, Alcorn JF, Peterson A, et al. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *Journal of immunology (Baltimore, Md : 1950).* 2008;181(6):4089-4097.

97. Braun A, Quarcoo D, Schulte-Herbrüggen O, Lommatzsch M, Hoyle G, Renz H. Nerve growth factor induces airway hyperresponsiveness in mice. *International archives of allergy and immunology.* 2001;124(1-3):205-207.

98. Nilsson G, Forsberg-Nilsson K, Xiang Z, Hallböök F, Nilsson K, Metcalfe DD. Human mast cells express functional TrkA and are a source of nerve growth factor. *European journal of immunology.* 1997;27(9):2295-2301.

99. Fahy JV. Remodeling of the airway epithelium in asthma. *American journal of respiratory and critical care medicine.* 2001;164(10 Pt 2):S46-51.

100. Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. *Nat Rev Immunol.* 2015;15(1):57-65.

101. Cho SH, Seo JY, Choi DC, et al. Pathological changes according to the severity of asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 1996;26(10):1210-1219.

102. Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet (London, England).* 1989;1(8637):520-524.

103. Laitinen A, Altraja A, Kämpe M, Linden M, Virtanen I, Laitinen LA. Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *American journal of respiratory and critical care medicine.* 1997;156(3 Pt 1):951-958.

104. Hashimoto M, Tanaka H, Abe S. Quantitative analysis of bronchial wall vascularity in the medium and small airways of patients with asthma and COPD. *Chest.* 2005;127(3):965-972.

105. Barbato A, Turato G, Baraldo S, et al. Epithelial damage and angiogenesis in the airways of children with asthma. *American journal of respiratory and critical care medicine.* 2006;174(9):975-981.

106. Payne DN, Rogers AV, Adelroth E, et al. Early thickening of the reticular basement membrane in children with difficult asthma. *American journal of respiratory and critical care medicine.* 2003;167(1):78-82.

107. Ehrlich P. *Beiträge für Theorie und Praxis der histologischen Färbung*. [Leipzig]1878.

108. Yong LCJ. The mast cell: origin, morphology, distribution, and function. *Experimental and Toxicologic Pathology.* 1997;49(6):409-424.

109. da Silva EZ, Jamur MC, Oliver C. Mast cell function: a new vision of an old cell. *J Histochem Cytochem.* 2014;62(10):698-738.

110. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu Rev Immunol.* 2005;23:749-786.

111. Dahlin JS, Maurer M, Metcalfe DD, Pejler G, Sagi-Eisenberg R, Nilsson G. The ingenious mast cell: Contemporary insights into mast cell behavior and function. *Allergy.* 2022;77(1):83-99.

112. Gentek R, Ghigo C, Hoeffel G, et al. Hemogenic Endothelial Fate Mapping Reveals Dual Developmental Origin of Mast Cells. *Immunity.* 2018;48(6):1160-1171.e1165.

113. Kitamura Y. Heterogeneity of mast cells and phenotypic change between subpopulations. *Annu Rev Immunol.* 1989;7:59-76.

114. Kirshenbaum AS, Kessler SW, Goff JP, Metcalfe DD. Demonstration of the origin of human mast cells from CD34+ bone marrow progenitor cells. *Journal of immunology (Baltimore, Md : 1950).* 1991;146(5):1410-1415.

115. Ribatti D. The development of human mast cells. An historical reappraisal. *Exp Cell Res.* 2016;342(2):210-215.

116. Dahlin JS, Hallgren J. Mast cell progenitors: origin, development and migration to tissues. *Mol Immunol.* 2015;63(1):9-17.

117. Irani AA, Schechter NM, Craig SS, DeBlois G, Schwartz LB. Two types of human mast cells that have distinct neutral protease compositions. *Proceedings of the National Academy of Sciences of the United States of America.* 1986;83(12):4464-4468.

118. Andersson CK, Mori M, Bjermer L, Lofdahl CG, Erjefalt JS. Novel site-specific mast cell subpopulations in the human lung. *Thorax.* 2009;64(4):297-305.

119. Oskeritzian CA, Zhao W, Min HK, et al. Surface CD88 functionally distinguishes the MCTC from the MCT type of human lung mast cell. *The Journal of allergy and clinical immunology.* 2005;115(6):1162-1168.

120. Gangwar RS, Landolina N, Arpinati L, Levi-Schaffer F. Mast cell and eosinophil surface receptors as targets for anti-allergic therapy. *Pharmacology & therapeutics.* 2017;170:37-63.

121. Bradding P, Arthur G. Mast cells in asthma--state of the art. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2016;46(2):194-263.

122. Rivera J, Fierro NA, Olivera A, Suzuki R. New insights on mast cell activation via the high affinity receptor for IgE. *Advances in immunology.* 2008;98:85-120.

123. Siraganian RP. Mast cell signal transduction from the high-affinity IgE receptor. *Current opinion in immunology.* 2003;15(6):639-646.

124. Motakis E, Guhl S, Ishizu Y, et al. Redefinition of the human mast cell transcriptome by deep-CAGE sequencing. *Blood.* 2014;123(17):e58-67.

125. Tatemoto K, Nozaki Y, Tsuda R, et al. Immunoglobulin E-independent activation of mast cell is mediated by Mrg receptors. *Biochemical and biophysical research communications.* 2006;349(4):1322-1328.

126. Wedi B, Gehring M, Kapp A. The pseudoallergen receptor MRGPRX2 on peripheral blood basophils and eosinophils: Expression and function. *Allergy.* 2020;75(9):2229-2242.

127. McNeil BD, Pundir P, Meeker S, et al. Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. *Nature.* 2015;519(7542):237-241.

128. Subramanian H, Gupta K, Guo Q, Price R, Ali H. Mas-related gene X2 (MrgX2) is a novel G protein-coupled receptor for the antimicrobial peptide LL-37 in human mast cells: resistance to receptor phosphorylation, desensitization, and internalization. *J Biol Chem.* 2011;286(52):44739-44749.

129. Galli SJ, Gaudenzio N, Tsai M. Mast Cells in Inflammation and Disease: Recent Progress and Ongoing Concerns. *Annu Rev Immunol.* 2020;38:49-77.

130. Sigma-Aldrich. Compound 48/80. 2022.

131. Burka JF. EFFECTS OF COMPOUND-48/80 AND FORMYL-METHIONYL-LEUCYL-PHENYLALANINE ON ISOLATED GUINEA-PIG AIRWAYS. *International archives of allergy and applied immunology.* 1984;73(4):309-313.

132. Tudoric N, Coon RL, Bosnjak ZJ. INHIBITION OF NEUTRAL ENDOPEPTIDASE POTENTIATES COMPOUND 48/80-INDUCED CONSTRICTION OF GUINEA-PIG TRACHEAL SMOOTH-MUSCLE. *Pulmonary pharmacology & therapeutics.* 1994;7(5):343-347.

133. Gaudenzio N, Sibilano R, Marichal T, et al. Different activation signals induce distinct mast cell degranulation strategies. *The Journal of clinical investigation.* 2016;126(10):3981-3998.

134. Occhiuto CJ, Kammala AK, Yang C, et al. Store-Operated Calcium Entry via STIM1 Contributes to MRGPRX2 Induced Mast Cell Functions. *Frontiers in immunology.* 2019;10:3143.

135. Wang Z, Franke K, Bal G, Li Z, Zuberbier T, Babina M. MRGPRX2-Mediated Degranulation of Human Skin Mast Cells Requires the Operation of G(αi), G(αq), Ca++ Channels, ERK1/2 and PI3K-Interconnection between Early and Late Signaling. *Cells.* 2022;11(6).

136. Moon TC, Befus AD, Kulka M. Mast cell mediators: their differential release and the secretory pathways involved. *Frontiers in immunology.* 2014;5:569.

137. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. *Nat Rev Immunol.* 2014;14(7):478-494.

138. Fanning LB, Boyce JA. Lipid mediators and allergic diseases. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology.* 2013;111(3):155-162.

139. Bäck M, Kumlin M, Cotgreave IA, Dahlén SE. An alternative pathway for metabolism of leukotriene D(4): effects on contractions to cysteinyl-leukotrienes in the guinea-pig trachea. *British journal of pharmacology.* 2001;133(7):1134-1144.

140. Fajt ML, Wenzel SE. Mast cells, their subtypes, and relation to asthma phenotypes. *Annals of the American Thoracic Society.* 2013;10 Suppl:S158-164.

141. El-Shazly A, Berger P, Girodet PO, et al. Fraktalkine produced by airway smooth muscle cells contributes to mast cell recruitment in asthma. *Journal of immunology (Baltimore, Md : 1950).* 2006;176(3):1860-1868.

142. Balzar S, Fajt ML, Comhair SA, et al. Mast cell phenotype, location, and activation in severe asthma. Data from the Severe Asthma Research Program. *American journal of respiratory and critical care medicine.* 2011;183(3):299-309.

143. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *The New England journal of medicine.* 2002;346(22):1699-1705.

144. Sanmugalingam D, Wardlaw AJ, Bradding P. Adhesion of human lung mast cells to bronchial epithelium: evidence for a novel carbohydrate-mediated mechanism. *Journal of leukocyte biology.* 2000;68(1):38-46.

145. Bradding P, Walls AF, Holgate ST. The role of the mast cell in the pathophysiology of asthma. *The Journal of allergy and clinical immunology.* 2006;117(6):1277-1284.

146. Carroll NG, Mutavdzic S, James AL. Increased mast cells and neutrophils in submucosal mucous glands and mucus plugging in patients with asthma. *Thorax.* 2002;57(8):677-682.

147. Wang SW, Oh CK, Cho SH, et al. Amphiregulin expression in human mast cells and its effect on the primary human lung fibroblasts. *The Journal of allergy and clinical immunology.* 2005;115(2):287-294.

148. Brightling CE, Ammit AJ, Kaur D, et al. The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. *American journal of respiratory and critical care medicine.* 2005;171(10):1103-1108.

149. Paivandy A, Pejler G. Novel Strategies to Target Mast Cells in Disease. *J Innate Immun.* 2021;13(3):131-147.

150. Yang Y, Lu JY, Wu X, et al. G-protein-coupled receptor 35 is a target of the asthma drugs cromolyn disodium and nedocromil sodium. *Pharmacology.* 2010;86(1):1-5.

151. Leung KB, Flint KC, Brostoff J, et al. Effects of sodium cromoglycate and nedocromil sodium on histamine secretion from human lung mast cells. *Thorax.* 1988;43(10):756-761.

152. Church MK, Hiroi J. Inhibition of IgE-dependent histamine release from human dispersed lung mast cells by anti-allergic drugs and salbutamol. *British journal of pharmacology.* 1987;90(2):421-429.

153. Bernstein IL, Siegel SC, Brandon ML, et al. A controlled study of cromolyn sodium sponsored by the Drug Committee of the American Academy of Allergy. *J Allergy Clin Immunol.* 1972;50(4):235-245.

154. Tasche MJ, van der Wouden JC, Uijen JH, et al. Randomised placebo-controlled trial of inhaled sodium cromoglycate in 1-4-year-old children with moderate asthma. *Lancet (London, England).* 1997;350(9084):1060-1064.

155. Chang TW. The pharmacological basis of anti-IgE therapy. *Nature Biotechnology.* 2000;18(2):157-162.

156. Presta LG, Lahr SJ, Shields RL, et al. Humanization of an antibody directed against IgE. *The Journal of Immunology.* 1993;151(5):2623-2632.

157. Fahy JV, Fleming HE, Wong HH, et al. The effect of an anti-IgE monoclonal antibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects. *American journal of respiratory and critical care medicine.* 1997;155(6):1828-1834.

158. Busse W, Corren J, Lanier BQ, et al. Omalizumab, anti-IgE recombinant humanized monoclonal antibody, for the treatment of severe allergic asthma. *The Journal of allergy and clinical immunology.* 2001;108(2):184-190.

159. Solèr M, Matz J, Townley R, et al. The anti-IgE antibody omalizumab reduces exacerbations and steroid requirement in allergic asthmatics. *The European respiratory journal.* 2001;18(2):254-261.

160. Milgrom H, Berger W, Nayak A, et al. Treatment of childhood asthma with anti-immunoglobulin E antibody (omalizumab). *Pediatrics.* 2001;108(2):E36.

161. Rossi AB, Herlaar E, Braselmann S, et al. Identification of the Syk kinase inhibitor R112 by a human mast cell screen. *J Allergy Clin Immunol.* 2006;118(3):749-755.

162. Guyer BJ, Shimamoto SR, Bradhurst AL, Grossbard EB, Dreskin SC, Nelson HS. Mast cell inhibitor R112 is well tolerated and affects prostaglandin D2 but not other mediators, symptoms, or nasal volumes in a nasal challenge model of allergic rhinitis. *Allergy Asthma Proc.* 2006;27(3):208-213.

163. Khindri S, Cahn A, Begg M, et al. A Multicentre, Randomized, Double-Blind, Placebo-Controlled, Crossover Study To Investigate the Efficacy, Safety, Tolerability, and Pharmacokinetics of Repeat Doses of Inhaled Nemiralisib in Adults with Persistent, Uncontrolled Asthma. *The Journal of pharmacology and experimental therapeutics.* 2018;367(3):405-413.

164. Roquet A, Dahlén B, Kumlin M, et al. Combined antagonism of leukotrienes and histamine produces predominant inhibition of allergen-induced early and late phase airway obstruction in asthmatics. *American journal of respiratory and critical care medicine.* 1997;155(6):1856-1863.

165. Finnerty JP, Twentyman OP, Harris A, Palmer JB, Holgate ST. Effect of GR32191, a potent thromboxane receptor antagonist, on exercise induced bronchoconstriction in asthma. *Thorax.* 1991;46(3):190-192.

166. Richter K, Grönke L, Janicki S, Maus J, Jörres RA, Magnussen H. Effect of azelastine, montelukast, and their combination on allergen-induced bronchoconstriction in asthma. *Pulmonary pharmacology & therapeutics.* 2008;21(1):61-66.

167. Davis BE, Illamperuma C, Gauvreau GM, et al. Single-dose desloratadine and montelukast and allergen-induced late airway responses. *The European respiratory journal.* 2009;33(6):1302-1308.

168. Bateman ED, Guerreros AG, Brockhaus F, et al. Fevipiprant, an oral prostaglandin DP(2) receptor (CRTh2) antagonist, in allergic asthma uncontrolled on low-dose inhaled corticosteroids. *The European respiratory journal.* 2017;50(2).

169. Gonem S, Berair R, Singapuri A, et al. Fevipiprant, a prostaglandin D2 receptor 2 antagonist, in patients with persistent eosinophilic asthma: a single-centre, randomised, double-blind, parallel-group, placebo-controlled trial. *The Lancet Respiratory Medicine.* 2016;4(9):699-707.

170. Brightling CE, Gaga M, Inoue H, et al. Effectiveness of fevipiprant in reducing exacerbations in patients with severe asthma (LUSTER-1 and LUSTER-2): two phase 3 randomised controlled trials. *The Lancet Respiratory Medicine.* 2021;9(1):43-56.

171. Kirshenbaum AS, Goff JP, Kessler SW, Mican JM, Zsebo KM, Metcalfe DD. Effect of IL-3 and stem cell factor on the appearance of human basophils and mast cells from CD34+ pluripotent progenitor cells. *Journal of immunology (Baltimore, Md : 1950).* 1992;148(3):772-777.

172. Al-Muhsen SZ, Shablovsky G, Olivenstein R, Mazer B, Hamid Q. The expression of stem cell factor and c-kit receptor in human asthmatic airways. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2004;34(6):911-916.

173. Cahill KN, Katz HR, Cui J, et al. KIT Inhibition by Imatinib in Patients with Severe Refractory Asthma. *The New England journal of medicine.* 2017;376(20):1911-1920.

174. Iqbal N, Iqbal N. Imatinib: a breakthrough of targeted therapy in cancer. *Chemother Res Pract.* 2014;2014:357027-357027.

175. Karlberg M, Ekoff M, Huang DCS, Mustonen P, Harvima IT, Nilsson G. The BH3-Mimetic ABT-737 Induces Mast Cell Apoptosis In Vitro and In Vivo: Potential for Therapeutics. *The Journal of Immunology.* 2010;185(4):2555-2562.

176. Dragonetti A, Baldassarre M, Castino R, et al. The lysosomal protease cathepsin D is efficiently sorted to and secreted from regulated secretory compartments in the rat basophilic/mast cell line RBL. *J Cell Sci.* 2000;113 (Pt 18):3289-3298.

177. Schwartz LB, Austen KF, Wasserman SI. Immunologic Release of β-Hexosaminidase and β-Glucuronidase from Purified Rat Serosal Mast Cells. *The Journal of Immunology.* 1979;123(4):1445-1450.

178. Blott EJ, Griffiths GM. Secretory lysosomes. *Nature Reviews Molecular Cell Biology.* 2002;3(2):122-131.

179. Paivandy A, Calounova G, Zarnegar B, Ohrvik H, Melo FR, Pejler G. Mefloquine, an anti-malaria agent, causes reactive oxygen species-dependent cell death in mast cells via a secretory granule-mediated pathway. *Pharmacology research & perspectives.* 2014;2(6):e00066.

180. Spirkoski J, Melo FR, Grujic M, et al. Mast cell apoptosis induced by siramesine, a sigma-2 receptor agonist. *Biochemical pharmacology.* 2012;84(12):1671-1680.

181. Adner M, Canning Brendan J, Meurs H, et al. Back to the future: re-establishing guinea pig in vivo asthma models. *Clinical Science.* 2020;134(11):1219-1242.

182. Canning BJ, Chou Y. Using guinea pigs in studies relevant to asthma and COPD. *Pulmonary pharmacology & therapeutics.* 2008;21(5):702-720.

183. Adner M, Canning BJ, Meurs H, et al. Back to the future: re-establishing guinea pig in vivo asthma models. *Clinical science (London, England : 1979).* 2020;134(11):1219-1242.

184. Calderon MA, Linneberg A, Kleine-Tebbe J, et al. Respiratory allergy caused by house dust mites: What do we really know? *The Journal of allergy and clinical immunology.* 2015;136(1):38-48.

185. Jacquet A. The role of innate immunity activation in house dust mite allergy. *Trends Mol Med.* 2011;17(10):604-611.

186. Thomas WR, Hales BJ, Smith WA. House dust mite allergens in asthma and allergy. *Trends Mol Med.* 2010;16(7):321-328.

187. Plaschke P, Janson C, Norrman E, et al. Skin prick tests and specific IgE in adults from three different areas of Sweden. *Allergy.* 1996;51(7):461-472.

188. Freidhoff LR, Meyers DA, Marsh DG. A genetic-epidemiologic study of human immune responsiveness to allergens in an industrial population. II. The associations among skin sensitivity, total serum IgE, age, sex, and the reporting of allergies in a stratified random sample. *The Journal of allergy and clinical immunology.* 1984;73(4):490-499.

189. Roost HP, Künzli N, Schindler C, et al. Role of current and childhood exposure to cat and atopic sensitization. European Community Respiratory Health Survey. *The Journal of allergy and clinical immunology.* 1999;104(5):941-947.

190. Ichikawa K, Iwasaki E, Baba M, Chapman MD. High prevalence of sensitization to cat allergen among Japanese children with asthma, living without cats. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 1999;29(6):754-761.

191. Løwenstein H, Lind P, Weeke B. Identification and clinical significance of allergenic molecules of cat origin. Part of the DAS 76 Study. *Allergy.* 1985;40(6):430-441.

192. Kolmert J, Fauland A, Fuchs D, et al. Lipid Mediator Quantification in Isolated Human and Guinea Pig Airways: An Expanded Approach for Respiratory Research. *Analytical chemistry.* 2018;90(17):10239-10248.

193. Kervinen J, Crysler C, Bayoumy S, et al. Potency variation of small-molecule chymase inhibitors across species. *Biochem Pharmacol.* 2010;80(7):1033-1041.

194. Lutchen KR, Gillis H. Relationship between heterogeneous changes in airway morphometry and lung resistance and elastance. *Journal of applied physiology (Bethesda, Md : 1985).* 1997;83(4):1192-1201.

195. Riley JP, Fuchs B, Sjoberg L, et al. Mast cell mediators cause early allergic bronchoconstriction in guinea-pigs in vivo: a model of relevance to asthma. *Clinical science (London, England : 1979).* 2013;125(11):533-542.

196. Nabe T, Shinoda N, Yamada M, et al. Repeated antigen inhalation-induced reproducible early and late asthma in guinea pigs. *Jpn J Pharmacol.* 1997;75(1):65-75.

197. Graziano FM, Gundersen L, Larson LA, Harley P, Buckner CK. Receptor-specific mediation by immunoglobulin E of antigen-induced contraction of tracheal and lung parenchymal strips isolated from the guinea pig. *The Journal of clinical investigation.* 1984;73(4):1215-1222.

198. Fraser DG, Graziano FM, Larsen CP, Regal JF. The role of IgG1 and IgG2 in trimellitic anhydride-induced allergic response in the guinea pig lung. *Toxicol Appl Pharmacol.* 1998;150(2):218-227.

199. Habibzay M, Saldana JI, Goulding J, Lloyd CM, Hussell T. Altered regulation of Toll-like receptor responses impairs antibacterial immunity in the allergic lung. *Mucosal Immunol.* 2012;5(5):524-534.

200. Xiang Z, Block M, Löfman C, Nilsson G. IgE-mediated mast cell degranulation and recovery monitored by time-lapse photography. *The Journal of allergy and clinical immunology.* 2001;108(1):116-121.

201. Suurmond J, Habets KLL, Tatum Z, et al. Repeated FcεRI triggering reveals modified mast cell function related to chronic allergic responses in tissue. *J Allergy Clin Immunol.* 2016;138(3):869-880.

202. Short PM, Anderson WJ, Manoharan A, Lipworth BJ. Usefulness of impulse oscillometry for the assessment of airway hyperresponsiveness in mild-to-moderate adult asthma. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology.* 2015;115(1):17-20.

203. Hsiue TR, Lei HY, Hsieh AL, Wang TY, Chang HY, Chen CR. Mite-induced allergic airway inflammation in guinea pigs. *International archives of allergy and immunology.* 1997;112(3):295-302.

204. Wu D, Zhou J, Bi H, et al. CCL11 as a potential diagnostic marker for asthma? *The Journal of asthma : official journal of the Association for the Care of Asthma.* 2014;51(8):847-854.

205. Bergeron C, Tulic MK, Hamid Q. Airway remodelling in asthma: from benchside to clinical practice. *Can Respir J.* 2010;17(4):e85-e93.

206. Santing RE, Olymulder CG, Zaagsma J, Meurs H. Relationships among allergen-induced early and late phase airway obstructions, bronchial hyperreactivity, and inflammation in conscious, unrestrained guinea pigs. *J Allergy Clin Immunol.* 1994;93(6):1021-1030.

207. Bazan-Perkins B, Sanchez-Guerrero E, Vargas MH, et al. Beta1-integrins shedding in a guinea-pig model of chronic asthma with remodelled airways. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2009;39(5):740-751.

208. Djukanovic R, Roche WR, Wilson JW, et al. Mucosal inflammation in asthma. *Am Rev Respir Dis.* 1990;142(2):434-457.

209. Wenzel SE, Vitari CA, Shende M, Strollo DC, Larkin A, Yousem SA. Asthmatic granulomatosis: a novel disease with asthmatic and granulomatous features. *Am J Respir Crit Care Med.* 2012;186(6):501-507.

210. Pazhoohan S, Raoufy MR, Javan M, Hajizadeh S. Effect of Rho-kinase inhibition on complexity of breathing pattern in a guinea pig model of asthma. *PLoS One.* 2017;12(10):e0187249.

211. Smith N, Broadley KJ. Optimisation of the sensitisation conditions for an ovalbumin challenge model of asthma. *Int Immunopharmacol.* 2007;7(2):183-190.

212. Balzar S, Chu HW, Strand M, Wenzel S. Relationship of small airway chymase-positive mast cells and lung function in severe asthma. *American journal of respiratory and critical care medicine.* 2005;171(5):431-439.

213. Safholm J, Dahlen SE, Delin I, et al. PGE2 maintains the tone of the guinea pig trachea through a balance between activation of contractile EP1 receptors and relaxant EP2 receptors. *British journal of pharmacology.* 2013;168(4):794-806.

214. Regal JF. IgG vs IgE: mediators of antigen-induced guinea pig tracheal contraction. *Immunopharmacology.* 1984;8(3-4):111-119.

215. Sundström E, Låstbom L, Ryrfeldt A, Dahlén SE. Interactions among three classes of mediators explain antigen-induced bronchoconstriction in the isolated perfused and ventilated guinea pig lung. *The Journal of pharmacology and experimental therapeutics.* 2003;307(1):408-418.

216. Safholm J, Dahlen SE, Adner M. Antagonising EP1 and EP2 receptors reveal that the TP receptor mediates a component of antigen-induced contraction of the guinea pig trachea. *European journal of pharmacology.* 2013;718(1-3):277-282.

217. Jonsson EW, Dahlen SE. Interactions between leukotrienes and histamine in the anaphylactic contraction of guinea pig lung parenchyma. *The Journal of pharmacology and experimental therapeutics.* 1994;271(2):615-623.

218. Kabashima K, Nakashima C, Nonomura Y, et al. Biomarkers for evaluation of mast cell and basophil activation. *Immunological reviews.* 2018;282(1):114-120.

219. Koshino T, Teshima S, Fukushima N, et al. Identification of basophils by immunohistochemistry in the airways of post-mortem cases of fatal asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 1993;23(11):919-925.

220. Macfarlane AJ, Kon OM, Smith SJ, et al. Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. *The Journal of allergy and clinical immunology.* 2000;105(1 Pt 1):99-107.

221. Safholm J, Manson ML, Bood J, et al. Prostaglandin E2 inhibits mast cell-dependent bronchoconstriction in human small airways through the E prostanoid subtype 2 receptor. *The Journal of allergy and clinical immunology.* 2015;136(5):1232-1239.e1231.

222. Marone G, Casolaro V, Patella V, Florio G, Triggiani M. Molecular and cellular biology of mast cells and basophils. *International archives of allergy and immunology.* 1997;114(3):207-217.

223. Schulman ES, Newball HH, Demers LM, Fitzpatrick FA, Adkinson NF, Jr. Anaphylactic release of thromboxane A2, prostaglandin D2, and prostacyclin from human lung parenchyma. *The American review of respiratory disease.* 1981;124(4):402-406.

224. Selg E, Låstbom L, Ryrfeldt A, Kumlin M, Dahlén SE. Effects of selective and non-selective COX inhibitors on antigen-induced release of prostanoid mediators and bronchoconstriction in the isolated perfused and ventilated guinea pig lung. *Prostaglandins Leukot Essent Fatty Acids.* 2008;78(2):89-97.

225. Dahlen SE, Kumlin M. Monitoring mast cell activation by prostaglandin D2 in vivo. *Thorax.* 2004;59(6):453-455.

226. Wei YF, Heghinian K, Bell RL, Jakschik BA. Contribution of macrophages to immediate hypersensitivity reaction. *Journal of immunology (Baltimore, Md : 1950).* 1986;137(6):1993-2000.

227. Delamere F, Holland E, Patel S, Bennett J, Pavord I, Knox A. Production of PGE2 by bovine cultured airway smooth muscle cells and its inhibition by cyclo-oxygenase inhibitors. *British journal of pharmacology.* 1994;111(4):983-988.

228. Churchill L, Chilton FH, Resau JH, Bascom R, Hubbard WC, Proud D. Cyclooxygenase metabolism of endogenous arachidonic acid by cultured human tracheal epithelial cells. *The American review of respiratory disease.* 1989;140(2):449-459.

229. Dong X, Han S, Zylka MJ, Simon MI, Anderson DJ. A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. *Cell.* 2001;106(5):619-632.

230. Peebles RS, Jr. Prostaglandins in asthma and allergic diseases. *Pharmacology & therapeutics.* 2019;193:1-19.

231. Salari H, Chan-Yeung M. Mast cell mediators stimulate synthesis of arachidonic acid metabolites in macrophages. *Journal of immunology (Baltimore, Md : 1950).* 1989;142(8):2821-2827.

232. Benyon RC, Robinson C, Church MK. Differential release of histamine and eicosanoids from human skin mast cells activated by IgE-dependent and non-immunological stimuli. *British journal of pharmacology.* 1989;97(3):898-904.

233. Yu S, Li Q, Cavanaugh S, Undem BJ, Ouyang A. Characterization of mast cell subtypes, distribution, and antigen-induced activation in the guinea pig esophagus. *Diseases of the esophagus : official journal of the International Society for Diseases of the Esophagus.* 2009;22(7):600-605.

234. Lawson C, Bunting S, Holzgrefe H, Fitzpatrick F. Leukotriene B4 and 20-hydroxyleukotriene B4 contract guinea-pig trachea strips in vitro. *The Journal of pharmacology and experimental therapeutics.* 1986;237(3):888-892.

235. Copas JL, Borgeat P, Gardiner PJ. The actions of 5-, 12-, and 15-HETE on tracheobronchial smooth muscle. *Prostaglandins Leukot Med.* 1982;8(2):105-114.

236. Johnsson AK, Rönnberg E, Fuchs D, et al. COX-1 dependent biosynthesis of 15-hydroxyeicosatetraenoic acid in human mast cells. *Biochim Biophys Acta Mol Cell Biol Lipids.* 2021;1866(5):158886.

237. Morrow JD, Harris TM, Roberts LJ, 2nd. Noncyclooxygenase oxidative formation of a series of novel prostaglandins: analytical ramifications for measurement of eicosanoids. *Analytical biochemistry.* 1990;184(1):1-10.

238. Voynow JA, Kummarapurugu A. Isoprostanes and asthma. *Biochimica et biophysica acta.* 2011;1810(11):1091-1095.

239. Pratico D, Lawson JA, FitzGerald GA. Cyclooxygenase-dependent formation of the isoprostane, 8-epi prostaglandin F2 alpha. *J Biol Chem.* 1995;270(17):9800-9808.

240. Ensembl. MRGPRX2 orthologues http://www.ensembl.org/Homo_sapiens/Gene/Compara_Ortholog?db=core;g=ENSG00000183695;r=11:19054455-19060717;t=ENST00000329773. Published 2020. Accessed May 28, 2020.

241. Blaies DM, Williams JF. A simplified method for staining mast cells with astra blue. *Stain Technol.* 1981;56(2):91-94.

242. Casale J CJ. Biochemistry, Glycosaminoglycans. . 2021.

243. Metcalfe DD, Lewis RA, Silbert JE, Rosenberg RD, Wasserman SI, Austen KF. Isolation and characterization of heparin from human lung. *The Journal of clinical investigation.* 1979;64(6):1537-1543.

244. Mulloy B, Lever R, Page CP. Mast cell glycosaminoglycans. *Glycoconj J.* 2017;34(3):351-361.

245. Thompson HL, Schulman ES, Metcalfe DD. Identification of chondroitin sulfate E in human lung mast cells. *Journal of immunology (Baltimore, Md : 1950).* 1988;140(8):2708-2713.

246. Strobel S, Miller HR, Ferguson A. Human intestinal mucosal mast cells: evaluation of fixation and staining techniques. *J Clin Pathol.* 1981;34(8):851-858.

247. Decorti G, Bartoli Klugmann F, Crivellato E, Mallardi F, Candussio L, Baldini L. Effect of the carboxylic ionophore monensin on histamine release from rat peritoneal mast cells. *Agents Actions.* 1991;32(3-4):253-260.

248. Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect Immun.* 2005;73(4):1907-1916.

249. Hengartner MO. The biochemistry of apoptosis. *Nature.* 2000;407(6805):770-776.

250. Leurs R, Smit MJ, Bast A, Timmerman H. Different profiles of desensitization dynamics in guinea-pig jejunal longitudinal smooth muscle after stimulation with histamine and methacholine. *British journal of pharmacology.* 1990;101(4):881-888.

251. Venkayya R, Lam M, Willkom M, Grünig G, Corry DB, Erle DJ. The Th2 lymphocyte products IL-4 and IL-13 rapidly induce airway hyperresponsiveness through direct effects on resident airway cells. *Am J Respir Cell Mol Biol.* 2002;26(2):202-208.

252. Liu J, Kolmert J, Säfholm J, et al. Distinct effects of antigen and compound 48/80 in the guinea pig trachea. *Allergy.* 2021;76(7):2270-2273.

253. Lewis RA, Soter NA, Diamond PT, Austen KF, Oates JA, Roberts LJ, 2nd. Prostaglandin D2 generation after activation of rat and human mast cells with anti-IgE. *Journal of immunology (Baltimore, Md : 1950).* 1982;129(4):1627-1631.

254. Koziol-White CJ, Jia Y, Baltus GA, et al. Inhibition of spleen tyrosine kinase attenuates IgE-mediated airway contraction and mediator release in human precision cut lung slices. *British journal of pharmacology.* 2016;173(21):3080-3087.

255. Young S, Le Souëf PN, Geelhoed GC, Stick SM, Turner KJ, Landau LI. The influence of a family history of asthma and parental smoking on airway responsiveness in early infancy. *The New England journal of medicine.* 1991;324(17):1168-1173.

256. Cockcroft DW, Davis BE. Mechanisms of airway hyperresponsiveness. *J Allergy Clin Immunol.* 2006;118(3):551-559.

257. O'Byrne PM, Inman MD. Airway Hyperresponsiveness. *Chest.* 2003;123(3, Supplement):411S-416S.

258. Mitchell EB, Crow J, Williams G, Platts-Mills TA. Increase in skin mast cells following chronic house dust mite exposure. *Br J Dermatol.* 1986;114(1):65-73.

259. Ramos-Ramírez P, Noreby M, Liu J, et al. A new house dust mite-driven and mast cell-activated model of asthma in the guinea pig. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2020.

260. Li S, Aliyeva M, Daphtary N, et al. Antigen-induced mast cell expansion and bronchoconstriction in a mouse model of asthma. *American journal of physiology Lung cellular and molecular physiology.* 2014;306(2):L196-206.

261. Gy C, Leclere M, Bélanger MC, Allano M, Beauchamp G, Lavoie JP. Acute, subacute and chronic sequelae of horses accidentally exposed to monensin-contaminated feed. *Equine Vet J.* 2020;52(6):848-856.

262. Varga A, Schober KE, Holloman CH, Stromberg PC, Lakritz J, Rings DM. Correlation of serum cardiac troponin I and myocardial damage in cattle with monensin toxicosis. *J Vet Intern Med.* 2009;23(5):1108-1116.

263. Wagner DD, Furrow RD, Bradley BD. Subchronic toxicity of monensin in broiler chickens. *Vet Pathol.* 1983;20(3):353-359.

264. Bautsch W, Hoymann HG, Zhang Q, et al. Cutting edge: guinea pigs with a natural C3a-receptor defect exhibit decreased bronchoconstriction in allergic airway disease: evidence for an involvement of the C3a anaphylatoxin in the pathogenesis of asthma. *Journal of immunology (Baltimore, Md : 1950).* 2000;165(10):5401-5405.

265. Mishra NC, Rir-sima-ah J, March T, et al. Sulfur mustard induces immune sensitization in hairless guinea pigs. *International immunopharmacology.* 2010;10(2):193-199.

266. Taylor DK, Lee VK. Chapter 25 - Guinea Pigs as Experimental Models. In: Suckow MA, Stevens KA, Wilson RP, eds. *The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents.* Boston: Academic Press; 2012:705-744.

267. Casset A, Mari A, Purohit A, et al. Varying allergen composition and content affects the in vivo allergenic activity of commercial Dermatophagoides pteronyssinus extracts. *International archives of allergy and immunology.* 2012;159(3):253-262.

268. Kidon MI, Chiang WC, Liew WK, et al. Mite component-specific IgE repertoire and phenotypes of allergic disease in childhood: the tropical perspective. *Pediatr Allergy Immunol.* 2011;22(2):202-210.

269. Shen HD, Chua KY, Lin WL, Chen HL, Hsieh KH, Thomas WR. IgE and monoclonal antibody binding by the mite allergen Der p 7. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 1996;26(3):308-315.

270. Epton MJ, Dilworth RJ, Smith W, Thomas WR. Sensitisation to the lipid-binding apolipophorin allergen Der p 14 and the peptide Mag-1. *International archives of allergy and immunology.* 2001;124(1-3):57-60.

271. Wan H, Winton HL, Soeller C, et al. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *The Journal of clinical investigation.* 1999;104(1):123-133.

272. Wan H, Winton HL, Soeller C, et al. The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of Dermatophagoides pteronyssinus. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2001;31(2):279-294.

273. He P, Zou Y, Hu Z. Advances in aluminum hydroxide-based adjuvant research and its mechanism. *Hum Vaccin Immunother.* 2015;11(2):477-488.

274. al-Laith M, Weyer A, Havet N, Dumarey C, Vargaftig BB, Bachelet M. Immunoglobulin-G-dependent stimulation of guinea pig lung mast cells and macrophages. *Allergy.* 1993;48(8):608-614.

275. Regal JF. Immunoglobulin G- and immunoglobulin E-mediated airway smooth muscle contraction in the guinea pig. *The Journal of pharmacology and experimental therapeutics.* 1984;228(1):116-120.

276. Sun G, Stacey MA, Schmidt M, Mori L, Mattoli S. Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. *Journal of immunology (Baltimore, Md : 1950).* 2001;167(2):1014-1021.

277. Asokananthan N, Graham PT, Stewart DJ, et al. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *Journal of immunology (Baltimore, Md : 1950).* 2002;169(8):4572-4578.

278. Davidson CE, Asaduzzaman M, Arizmendi NG, et al. Proteinase-activated receptor-2 activation participates in allergic sensitization to house dust mite allergens in a murine model. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2013;43(11):1274-1285.

279. Valerio CR, Murray P, Arlian LG, Slater JE. Bacterial 16S ribosomal DNA in house dust mite cultures. *The Journal of allergy and clinical immunology.* 2005;116(6):1296-1300.

280. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nature medicine.* 2009;15(4):410-416.

281. Daan de Boer J, Roelofs JJ, de Vos AF, et al. Lipopolysaccharide inhibits Th2 lung inflammation induced by house dust mite allergens in mice. *Am J Respir Cell Mol Biol.* 2013;48(3):382-389.

282. Klimov P, Molva V, Nesvorna M, et al. Dynamics of the microbial community during growth of the house dust mite Dermatophagoides farinae in culture. *FEMS Microbiol Ecol.* 2019;95(11).

283. Jacquet A. Characterization of Innate Immune Responses to House Dust Mite Allergens: Pitfalls and Limitations. *Front Allergy.* 2021;2:662378.

284. Machado DC, Horton D, Harrop R, Peachell PT, Helm BA. Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with antigen-specific IgE. *European journal of immunology.* 1996;26(12):2972-2980.

285. Lin YP, Nelson C, Kramer H, Parekh AB. The Allergen Der p3 from House Dust Mite Stimulates Store-Operated Ca(2+) Channels and Mast Cell Migration through PAR4 Receptors. *Mol Cell.* 2018;70(2):228-241.e225.

286. Supajatura V, Ushio H, Nakao A, et al. Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *The Journal of clinical investigation.* 2002;109(10):1351-1359.

287. Kaur D, Gomez E, Doe C, et al. IL-33 drives airway hyper-responsiveness through IL-13-mediated mast cell: airway smooth muscle crosstalk. *Allergy.* 2015;70(5):556-567.

288. Tung HY, Plunkett B, Huang SK, Zhou Y. Murine mast cells secrete and respond to interleukin-33. *J Interferon Cytokine Res.* 2014;34(3):141-147.

289. Schmitz J, Owyang A, Oldham E, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity.* 2005;23(5):479-490.

290. Saluja R, Hawro T, Eberle J, Church MK, Maurer M. Interleukin-33 promotes the proliferation of mouse mast cells through ST2/MyD88 and p38 MAPK-dependent and Kit-independent pathways. *J Biol Regul Homeost Agents.* 2014;28(4):575-585.

291. Iikura M, Suto H, Kajiwara N, et al. IL-33 can promote survival, adhesion and cytokine production in human mast cells. *Laboratory investigation; a journal of technical methods and pathology.* 2007;87(10):971-978.

292. Lefrançais E, Duval A, Mirey E, et al. Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. *Proceedings of the National Academy of Sciences of the United States of America.* 2014;111(43):15502-15507.

293. Lazarinis N, Bood J, Gomez C, et al. Leukotriene E(4) induces airflow obstruction and mast cell activation through the cysteinyl leukotriene type 1 receptor. *The Journal of allergy and clinical immunology.* 2018;142(4):1080-1089.

294. Xue L, Salimi M, Panse I, et al. Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells. *The Journal of allergy and clinical immunology.* 2014;133(4):1184-1194.

295. Bradding P, Roberts JA, Britten KM, et al. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol.* 1994;10(5):471-480.

296. Toru H, Pawankar R, Ra C, Yata J, Nakahata T. Human mast cells produce IL-13 by high-affinity IgE receptor cross-linking: enhanced IL-13 production by IL-4-primed human mast cells. *The Journal of allergy and clinical immunology.* 1998;102(3):491-502.

297. Klein LM, Lavker RM, Matis WL, Murphy GF. Degranulation of human mast cells induces an endothelial antigen central to leukocyte adhesion. *Proceedings of the National Academy of Sciences of the United States of America.* 1989;86(22):8972-8976.

298. Sekizawa K, Caughey GH, Lazarus SC, Gold WM, Nadel JA. Mast cell tryptase causes airway smooth muscle hyperresponsiveness in dogs. *The Journal of clinical investigation.* 1989;83(1):175-179.

299. Berger P, Compton SJ, Molimard M, et al. Mast cell tryptase as a mediator of hyperresponsiveness in human isolated bronchi. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 1999;29(6):804-812.

300. Thomas PS, Heywood G. Effects of inhaled tumour necrosis factor alpha in subjects with mild asthma. *Thorax.* 2002;57(9):774-778.

301. Thomas PS, Yates DH, Barnes PJ. Tumor necrosis factor-alpha increases airway responsiveness and sputum neutrophilia in normal human subjects. *American journal of respiratory and critical care medicine.* 1995;152(1):76-80.

302. Ebeling C, Forsythe P, Ng J, Gordon JR, Hollenberg M, Vliagoftis H. Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathways. *The Journal of allergy and clinical immunology.* 2005;115(3):623-630.

303. Williams CM, Galli SJ. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J Exp Med.* 2000;192(3):455-462.

304. Pohlmeier L, Sonar SS, Rodewald HR, Kopf M, Tortola L. Comparative analysis of the role of mast cells in murine asthma models using Kit-sufficient mast cell-deficient animals. *Allergy.* 2021;76(7):2030-2043.

305. Takeda K, Hamelmann E, Joetham A, et al. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J Exp Med.* 1997;186(3):449-454.

306. Mendez-Enriquez E, Alvarado-Vazquez PA, Abma W, et al. Mast cell-derived serotonin enhances methacholine-induced airway hyperresponsiveness in house dust mite-induced experimental asthma. *Allergy.* 2021;76(7):2057-2069.

307. Fuchs B, Sjöberg L, Möller Westerberg C, et al. Mast cell engraftment of the peripheral lung enhances airway hyperresponsiveness in a mouse asthma model. *American journal of physiology Lung cellular and molecular physiology.* 2012;303(12):L1027-1036.

308. Kobayashi T, Miura T, Haba T, et al. An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model. *Journal of immunology (Baltimore, Md : 1950).* 2000;164(7):3855-3861.

309. Xing W, Austen KF, Gurish MF, Jones TG. Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue. *Proceedings of the National Academy of Sciences of the United States of America.* 2011;108(34):14210-14215.

310. Fuchs B, Sjoberg L, Moller Westerberg C, et al. Mast cell engraftment of the peripheral lung enhances airway hyperresponsiveness in a mouse asthma model. *American journal of physiology Lung cellular and molecular physiology.* 2012;303(12):L1027-1036.

311. Chen ZY, Zhou SH, Zhou QF, Tang HB. Inflammation and airway remodeling of the lung in guinea pigs with allergic rhinitis. *Experimental and therapeutic medicine.* 2017;14(4):3485-3490.

312. Cyphert JM, Kovarova M, Allen IC, et al. Cooperation between mast cells and neurons is essential for antigen-mediated bronchoconstriction. *Journal of immunology (Baltimore, Md : 1950).* 2009;182(12):7430-7439.

313. Andersson CK, Weitoft M, Rydell-Törmänen K, Bjermer L, Westergren-Thorsson G, Erjefält JS. Uncontrolled asthmatics have increased FceRI(+) and TGF-β-positive MC(TC) mast cells and collagen VI in the alveolar parenchyma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2018;48(3):266-277.

314. Akers IA, Parsons M, Hill MR, et al. Mast cell tryptase stimulates human lung fibroblast proliferation via protease-activated receptor-2. *American journal of physiology Lung cellular and molecular physiology.* 2000;278(1):L193-201.

315. Leigh R, Ellis R, Wattie JN, et al. Type 2 cytokines in the pathogenesis of sustained airway dysfunction and airway remodeling in mice. *American journal of respiratory and critical care medicine.* 2004;169(7):860-867.

316. Atiakshin D, Samoilova V, Buchwalow I, Boecker W, Tiemann M. Characterization of mast cell populations using different methods for their identification. *Histochem Cell Biol.* 2017;147(6):683-694.

317. Amin K, Janson C, Boman G, Venge P. The extracellular deposition of mast cell products is increased in hypertrophic airways smooth muscles in allergic asthma but not in nonallergic asthma. *Allergy.* 2005;60(10):1241-1247.

318. Kassel O, De Blay F, Duvernelle C, et al. Local increase in the number of mast cells and expression of nerve growth factor in the bronchus of asthmatic patients after repeated inhalation of allergen at low-dose. *Clinical & Experimental Allergy.* 2001;31(9):1432-1440.

319. Schmit D, Le DD, Heck S, et al. Allergic airway inflammation induces migration of mast cell populations into the mouse airway. *Cell Tissue Res.* 2017;369(2):331-340.

320. Collington SJ, Williams TJ, Weller CL. Mechanisms underlying the localisation of mast cells in tissues. *Trends in immunology.* 2011;32(10):478-485.

321. Jones TG, Hallgren J, Humbles A, et al. Antigen-induced increases in pulmonary mast cell progenitor numbers depend on IL-9 and CD1d-restricted NKT cells. *Journal of immunology (Baltimore, Md : 1950).* 2009;183(8):5251-5260.

322. Sehra S, Yao W, Nguyen ET, et al. TH9 cells are required for tissue mast cell accumulation during allergic inflammation. *The Journal of allergy and clinical immunology.* 2015;136(2):433-440.e431.

323. Jones TG, Finkelman FD, Austen KF, Gurish MF. T regulatory cells control antigen-induced recruitment of mast cell progenitors to the lungs of C57BL/6 mice. *Journal of immunology (Baltimore, Md : 1950).* 2010;185(3):1804-1811.

324. Pietrzak A, Misiak-Tłoczek A, Brzezińska-Błaszczyk E. Interleukin (IL)-10 inhibits RANTES-, tumour necrosis factor (TNF)- and nerve growth factor (NGF)-induced mast cell migratory response but is not a mast cell chemoattractant. *Immunology letters.* 2009;123(1):46-51.

325. Hallgren J, Jones TG, Abonia JP, et al. Pulmonary CXCR2 regulates VCAM-1 and antigen-induced recruitment of mast cell progenitors. *Proceedings of the National Academy of Sciences of the United States of America.* 2007;104(51):20478-20483.

326. Berger P, Girodet PO, Begueret H, et al. Tryptase-stimulated human airway smooth muscle cells induce cytokine synthesis and mast cell chemotaxis. *Faseb j.* 2003;17(14):2139-2141.

327. Olsson N, Piek E, ten Dijke P, Nilsson G. Human mast cell migration in response to members of the transforming growth factor-beta family. *Journal of leukocyte biology.* 2000;67(3):350-356.

328. Collington SJ, Hallgren J, Pease JE, et al. The role of the CCL2/CCR2 axis in mouse mast cell migration in vitro and in vivo. *Journal of immunology (Baltimore, Md : 1950).* 2010;184(11):6114-6123.

329. Meininger CJ, Yano H, Rottapel R, Bernstein A, Zsebo KM, Zetter BR. The c-kit receptor ligand functions as a mast cell chemoattractant. *Blood.* 1992;79(4):958-963.

330. Nilsson G, Butterfield JH, Nilsson K, Siegbahn A. Stem cell factor is a chemotactic factor for human mast cells. *Journal of immunology (Baltimore, Md : 1950).* 1994;153(8):3717-3723.

331. Godot V, Arock M, Garcia G, et al. H4 histamine receptor mediates optimal migration of mast cell precursors to CXCL12. *The Journal of allergy and clinical immunology.* 2007;120(4):827-834.

332. Weller CL, Collington SJ, Hartnell A, et al. Chemotactic action of prostaglandin E2 on mouse mast cells acting via the PGE2 receptor 3. *Proceedings of the National Academy of Sciences of the United States of America.* 2007;104(28):11712-11717.

333. Duffy SM, Cruse G, Cockerill SL, Brightling CE, Bradding P. Engagement of the EP2 prostanoid receptor closes the K+ channel KCa3.1 in human lung mast cells and attenuates their migration. *European journal of immunology.* 2008;38(9):2548-2556.

334. Boehme SA, Franz-Bacon K, Chen EP, Ly TW, Kawakami Y, Bacon KB. Murine bone marrow-derived mast cells express chemoattractant receptor-homologous

molecule expressed on T-helper class 2 cells (CRTh2). *Int Immunol.* 2009;21(6):621-632.

335. Weller CL, Collington SJ, Brown JK, et al. Leukotriene B4, an activation product of mast cells, is a chemoattractant for their progenitors. *The Journal of experimental medicine.* 2005;201(12):1961-1971.

336. Boyce JA, Mellor EA, Perkins B, Lim YC, Luscinskas FW. Human mast cell progenitors use alpha4-integrin, VCAM-1, and PSGL-1 E-selectin for adhesive interactions with human vascular endothelium under flow conditions. *Blood.* 2002;99(8):2890-2896.

337. Abonia JP, Hallgren J, Jones T, et al. Alpha-4 integrins and VCAM-1, but not MAdCAM-1, are essential for recruitment of mast cell progenitors to the inflamed lung. *Blood.* 2006;108(5):1588-1594.

338. Stassen M, Arnold M, Hültner L, et al. Murine bone marrow-derived mast cells as potent producers of IL-9: costimulatory function of IL-10 and kit ligand in the presence of IL-1. *Journal of immunology (Baltimore, Md : 1950).* 2000;164(11):5549-5555.