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Effects of mast cell proteases on lung structural cells

Implications for asthma

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Abstract

Zhao, X. 2024. Effects of mast cell proteases on lung structural cells. Implications for asthma. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 2048. 70 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-2122-6.

Asthma is a prevalent chronic inflammatory condition that impacts the airways of the lungs. Its hallmark symptoms include wheezing, difficulty of breathing, chest tightness and coughing. Allergic asthma represents the most common form of this condition. While previous investigations have established a pivotal role of mast cells in asthma, the precise underlying mechanisms remain unclear. Mast cells are immune cells with a high content of secretory granules, containing a diverse array of bioactive substances such as histamine, cytokines, proteoglycans, and mast cell-restricted proteases such as tryptase, chymase, and carboxypeptidase A3. This thesis aims to investigate the regulatory role of mast cell-restricted proteases in modulating airway responses across the three layers of the airway wall, including human lung fibroblasts (HLF), human small airway epithelial cells (HSAEC), and human bronchial/tracheal smooth muscle cells (SMC).

In Paper I, primary HLFs were subjected to *in vitro* challenges with two distinct mast cell proteases: human β-tryptase and recombinant human chymase. The chymase-treated group exhibited significant morphological alterations compared to minimal effects observed in the tryptase-treated group. Additionally, chymase demonstrated the ability to modulate the extracellular matrix (ECM), cytokine, and chemokine output from primary HLFs. Transcriptome analysis revealed that chymase induced a proinflammatory gene transcription profile in HLFs, whereas tryptase had minimal effects.

In Paper II, the effect of mast cell proteases on primary HSAEC monolayers was evaluated. The HSAECs were relatively refractory to tryptase. In contrast, chymase was found to suppress the expression of ECM-related genes, degrade fibronectin, and reduce the migratory capacity of HSAECs. Chymase also degraded the cell-cell contact protein E-cadherin on the epithelial cell surface.

In Paper III, the impact of mast cell proteases on primary human SMCs was investigated. Chymase induced a profound reduction in SMC metabolic activity and cell proliferation, while tryptase had minimal effects. Our findings also revealed that chymase partially suppressed SMC contractility and enhanced the migratory capacity of airway SMCs. Chymase also caused reorganization of major cytoskeletal components and degraded tight junction proteins expressed by SMCs.

Our investigations increased the current knowledge of the effects of mast cell proteases on lung structural cells. These findings have the potential to unravel novel mechanisms of relevance in the pathology of asthma.

Keywords: Mast cells, Asthma, Chymase, Tryptase

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To the untamed spirit of childhood dreams.

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List of Papers

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

I. **Zhao, X.O.**, Lampinen, M., Rollman, O., Sommerhoff, C.P., Paivandy, A., Pejler, G. (2022) Mast cell chymase affects the functional properties of primary human airway fibroblasts: Implications for asthma. *Journal of Allergy and Clinical Immunology*, 149(2):718-727.

II. **Zhao, X.O.**, Sommerhoff, C.P., Paivandy, A., Pejler, G. (2022) Mast cell chymase regulates extracellular matrix remodeling-related events in primary human small airway epithelial cells. *Journal of Allergy and Clinical Immunology*, 150(6):1534-1544.

III. **Zhao, X.O.**[#], Melo, F.R., Sommerhoff, C.P., Paivandy, A., Pejler, G[#]. (2023) Mast cell chymase suppresses functional parameters in primary human airway smooth muscle cells. *Allergy*, 26 Feb. 2024

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List of additional papers not included in this thesis

I. Lind, T., Melo, F. R., Gustafson, A. M., Sundqvist, A., **Zhao, X. O.**, Moustakas, A., Melhus, H., & Pejler, G. (2022). Mast cell chymase has a negative impact on human osteoblasts. *Matrix Biology: Journal of the International Society for Matrix Biology*, 112, 1–19.

II. **Pejler, G.***, **Zhao, X. O.***, Fagerström, E., Paivandy, A. (2024). Blockade of endolysosomal acidification suppresses toll-like receptor 3-mediated pro-inflammatory signaling in primary human airway epithelial cells: implications for respiratory virus infection. *Journal of Allergy and Clinical Immunology.* Submitted.

* Equal contributions

Contents

Abbreviations

α-SMA	α-smooth muscle actin
β-NGF	Nerve growth factor β
Ang	Angiotensin
ApoB-100	Apolipoprotein B100
AR	Amphiregulin
ATCC	American Type Culture Collection
BDNF	Brain-derived neurotrophic factor
CPA3	Carboxypeptidase A3
CTGF	Connective tissue growth factor
CXCL	C-X-C motif chemokine ligand
DEG	Differentially expressed gene
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
EdU	5-ethynyl-2'-deoxyuridine
ET-1	Endothelin 1
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF2	Fibroblast growth factor 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HGF	Hepatocyte growth factor
HLF	Human lung fibroblast
HSA	Human serum albumin
HSAEC	Human small airway epithelial cell
IgE	Immunoglobulin E
IGFBP	Insulin-like growth factor-binding protein
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's medium
MAPK	Mitogen-activated protein kinases
MCP	Monocyte chemoattractant protein
MIF	Migration inhibitory factor
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
MRGPRX2	Mas-Related G-Protein Coupled Receptor X2
NT-3	Neurotrophin-3
PAR	Protease-activated receptor

PDGF	Platelet-derived growth factor
PHM	Peptide histidine methionine
PI3K	Phosphoinositide 3-kinases
PRR	Pattern recognition receptor
rh EGF	Recombinant human epidermal growth factor
rh FGF b	Recombinant human fibroblast growth factor basic
rh insulin	Recombinant human insulin
SMC	Smooth muscle cells
SMMHC	Smooth muscle myosin heavy chain
TGF-β	Transforming growth factor
Th2	T helper type 2
TIMP	Tissue inhibitors of matrix metalloproteinase
TNF	Tumor Necrosis Factor
TLR	Toll-like receptor
uPAR	Urokinase-type plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
ZO	Zona occludens

Introduction

Picture this: As spring unfolds its vibrant canvas, sunshine bathes the landscape. Birch trees, adorned in delicate greenery, awaken from their winter slumber, and the air is saturated with the sweet fragrance of blossoms. Yet, in the middle of this scenic revival, nature delicately swirls with pollen. While many find this scene enjoyable, for allergy sufferers, it signals the onset of seasonal challenges, allergic asthma.

The intricate link between allergic asthma and mast cells has been historically recognized. Previous investigations have unveiled the pivotal role these sentinel cells play in the pathophysiology of allergic respiratory conditions. Mast cells are immune cells with a high content of secretory granules, containing a diverse array of bioactive substances such as histamine, cytokines, proteoglycans, and mast cell-restricted proteases such as tryptase, chymase, and carboxypeptidase A3. Despite previous research establishing the link between mast cells and asthma, the precise mechanisms underlying this association remain unclear.

The airway wall constitutes a diverse three-layered structure extending from the airway lumen to the connective tissue, comprising epithelial, submucosal, and smooth muscle layers. This thesis aims to elucidate the impact of mast cell proteases, particularly tryptase and chymase, across all three layers of the airway. Human lung fibroblasts predominantly reside in the submucosal layer, facilitating direct interactions between mast cells and fibroblasts, as explored in Paper I. Paper II uncovered the effects of mast cell proteases on small airway epithelial cells, while Paper III investigated the interaction between mast cell proteases and airway smooth muscle cells. The latter aspect is particularly intriguing as smooth muscle cells play a pivotal role in airway constriction during asthma due to their contractile nature.

Background

Asthma

Asthma is a chronic inflammatory disease of the respiratory system that primarily affects the airways within the lungs. It has been viewed as a non-communicable disease that can be a lifelong condition[1,2]. Individuals with asthma experience recurrent episodes characterized by wheezing, coughing, chest tightness, and shortness of breath[3,4]. These episodes are mainly caused by the narrowing of the airways, making it difficult to breathe[1].

Extensive investigations have identified three crucial features that contribute to the typical symptoms of asthma: airway inflammation, airway remodeling and airway hyperresponsiveness[1,4,5]. In asthmatic patients, inflammation has been recognized as a significant aspect of asthma pathology through both macroscopic morphological observations and histological studies of autopsy specimens spanning over a century[6,7]. Inflammatory changes are distributed throughout the entire airways during asthma, leading to airflow limitation and the infiltration of inflammatory cells within the airways[6,8]. This irritation exacerbates airway narrowing and excess mucus production, both of which hinder airflow and trigger asthma symptoms.

Over time, persistent irritation in asthma can result in chronic and irreversible structural changes and bronchial wall remodeling, a process known as airway remodeling. These structural alterations include thickening of the airway basement membrane, subepithelial fibrosis, increased vascularity, and abnormal deposition of Extracellular Matrix (ECM) components. Additionally, the epithelial lining of the airways may become damaged and scarred. Previous investigations have demonstrated a correlation between the degree of airway wall thickening and disease severity[9].

Furthermore, individuals with asthma exhibit heightened sensitivity to various triggers, including allergens such as pollen or dust mites, irritants like smoke or air pollution, and even physical exercise. This hypersensitivity causes the airways to react excessively to these triggers by constricting more readily and severely compared to healthy individuals. This increased constriction further narrows the airways, leading to airflow limitation and the characteristic symptoms of asthma.

Mast cells

Mast cells are multifaceted immune cells with a key role during inflammatory responses. They are first sourced from yolk sac erythromyeloid progenitors. In the later hematopoiesis waves, mast cells originate from pluripotent hematopoietic cells in the bone marrow and circulate in the blood as mast cell progenitors[10–12]. Once recruited, they mature and differentiate in the tissues where they reside. Mast cells are found in most tissues throughout the body, but are particularly abundant in the boundaries between the host tissue and the external environment, such as the skin, gastrointestinal tract, respiratory system, and intestinal epithelium[13].

Mast cells exhibit both pro-inflammatory and anti-inflammatory functions during the complex process of immune responses in both health and disease. They are strategically located in connective tissues beneath the epithelium and surrounding blood vessels, nerves, smooth muscle, and mucus[13], allowing them to fulfill distinct roles in almost all organs due to their sensitivity to local conditions and capacity to discharge a diverse array of pre-existing and newly synthesized substances. Moreover, mast cells express a broad range of receptors, allowing them to detect and react to various cytokines, chemicals, and pathogens. Upon activation, mast cells can undergo degranulation, releasing numerous granule contents, including histamine, heparin, cytokines, proteoglycans, and numerous proteases, contributing to the immunoregulatory processes[14].

Mast cells are an essential part in host defense against parasites and bacterial infections. Further, recent investigations have revealed their protective function during innate resistance to reptile or arthropod venoms[15]. However, mast cells are also notorious for their involvement in allergic disorders, such as asthma and fatal anaphylaxis. This “bad side” of mast cells is supported by a large amount of evidence from both clinical and experimental studies[15–18].

Mast cells in health and diseases

Mast cells play a pivotal role in T helper type 2 (Th2) immune responses, as their activation through FcεRI crosslinking initiates hypersensitivity reactions. The immediate release of mast cell mediators like histamine, prostaglandin D2, and leukotriene C4 contributes to asthma symptoms such as bronchoconstriction, mucus secretion, and mucosal swelling[19]. Mast cells also play a role in the later phases of allergic responses by promoting leukocyte migration and activating various immune cells including T-cells, dendritic cells, neutrophils, eosinophils, and monocytes[20]. The increased presence of mast cells in affected tissues underscores their significance in allergic reactions. Notably, patients with asthma exhibit elevated mast cell counts in specific areas such as the airway smooth muscle, mucus glands[21], and epithelium[22]. It is therefore understandable that this “bad side” of mast cells tends to overshadow their non-

redundant protective function in detoxifying honeybee or arthropod venom and defending against parasites.

While it may seem counterintuitive that a significant number of mast cells are present in normal, uninflamed and non-infected skin or mucosal tissue, idling until triggered by a random allergen[16], mast cells actually play a much broader role in tissue physiology beyond initiating inflammatory and vasodilatory responses to immunoglobulin E (IgE)-dependent environmental antigens. The cytoplasm of mature mast cells contains 50-200 lysosome-like secretory granules that contribute to various aspects of tissue health, including promoting homeostasis, aiding in wound healing, and interacting with neurons[23]. For example, histamine and Tumor Necrosis Factor (TNF) that are released by mast cells regulate the growth and regression of hair follicles during different phases of hair growth[16]. Additionally, mast cells secrete growth factors such as Platelet-Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), and Fibroblast Growth Factor 2 (FGF2), which facilitate tissue repair, collagen deposition, and matrix remodeling during wound healing[24]. However, these aforementioned growth factors, particularly VEGF and FGF2, can also stimulate early angiogenesis in tumor development. Clinical observations[25] of mast cell infiltration in various tumor settings, including breast, lung, pancreatic, and prostate cancer, Hodgkin's lymphoma, and malignant melanoma, further underline the potential role of mast cells in cancer progression[26].

Mast cell activation

IgE-dependent pathway is the most characterized mechanism of mast cell activation. In this pathway, dendritic cells residing in the airway epithelium act as antigen-presenting cells, capturing and presenting specific allergens to naïve $CD4^+$ T cells. This leads to the differentiation of T helper 2 cells, with the subsequent release of interleukin (IL)-4 and IL-13. These cytokines promote B-cell isotype switching, resulting in the production of allergen-specific IgE.

Most circulating IgE binds to high-affinity FcεRI receptors on mast cells. Upon re-exposure, the allergen binds to the IgE bound to FcεRI receptors, triggering the crosslinking of two or more FcεRI molecules. This initiates intracellular signaling cascades within seconds, leading to mast cell degranulation and the release of preformed inflammatory mediators stored in the granules.

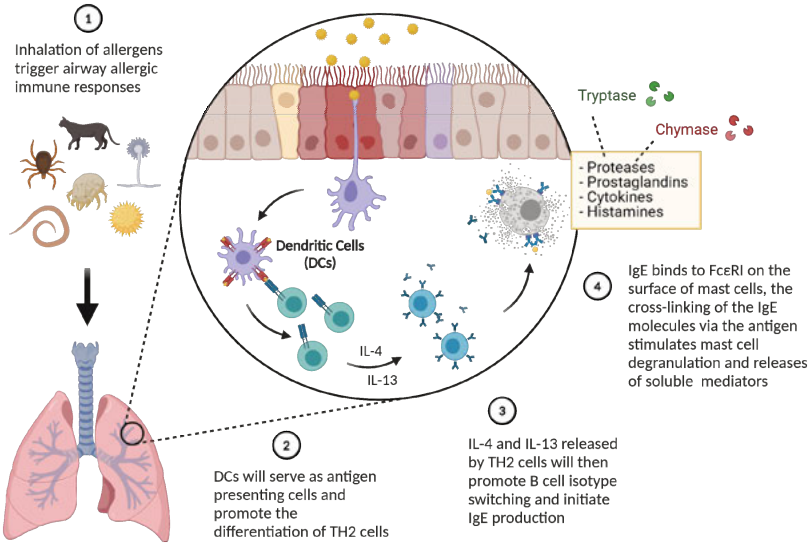

Figure 1. Classical scheme of IgE-dependent mast cell activation during allergen challenge. Dendritic cells within the human airway epithelium capture various allergens, such as house dust mites, pollens, or fungal spores, serving as antigen presenting cells. This process promotes Th2 cell differentiation and facilitates the release of IL-4 and IL-13, leading to isotype switching and IgE production. Mast cell activation occurs when IgE molecules bound to FcεRI receptors are cross-linked by antigens. Figure created via BioRender.

Beyond FcεRI receptors, mast cells express a diverse array of receptors for other molecules, including Fc receptors for IgG (FcγRs), complement receptor for C3a and C5a, chemokines receptors, Toll-Like Receptors (TLRs), and Pattern Recognition Receptors (PRRs). These receptors, along with Mas-Related G-Protein Coupled Receptor X2 (MRGPRX2), can activate mast cells through distinct mechanisms, influencing the immune response and contributing to mast cell activation.

Mast cell phenotypes

Not all mast cells are the same. Despite originating from a shared lineage and displaying a granulated morphology, mast cells exhibit remarkable diversity[19]. This heterogeneity is evident across species, including ultrastructure, receptor expression, mediator content, and responses to various stimuli[27]. In humans, mast cell diversity is notably shaped by their protease composition, leading to the classical categorization into two main subtypes: MC_T and MC_{TC}. MC_T mast cells exclusively express tryptase, whereas MC_{CT} mast cells possess the ability to store both tryptase and chymase, along with CPA3, within their granules[28]. The distribution of these mast cell subtypes varies across different tis-

sues. MC_T mast cells primarily reside in the bronchial lamina propria and intestinal mucosa, while MC_{CT} mast cells are predominantly located in the skin and intestinal submucosa. Together, these mast cell populations mediate crucial roles in maintaining tissue homeostasis and orchestrating immune responses within these compartments.

Interestingly, the landscape of mast cell phenotypes can shift under various physiological and pathological conditions. In healthy individuals, MC_T mast cells are the predominant subtype observed in lung tissues, whereas the percentage of MC_{CT} mast cells typically ranges from 0 to 13% [28,29]. However, in conditions such as asthma, there is a notable increase in the MC_{CT} subtype[21,29–32], attributed to mast cell protease phenotype switching, suggesting a potential role for MC_{CT} mast cells in the pathogenesis of asthma.

Mast cell proteases

Mast cells possess an exceptional capacity for storing an abundant supply of proteases within their granules, alongside other inflammatory mediators. These proteases are maintained at remarkably high levels, comprising 25% or more of the total protein content within these immune cells[33]. Stored as fully active enzymes, mast cell proteases are rapidly released into the extracellular environment during degranulation, potentially exerting a profound influence on conditions associated with mast cell degranulation.

The term "mast cell proteases" typically refers to chymases, tryptases, and mast cell carboxypeptidase A3 (CPA3), which are specifically expressed and stored in mast cell secretory granules[33]. However, it is important to note that mast cells also express additional proteases, including lysosomal cathepsins, granzymes and neurolysin[34]. While our focus will primarily delve into mast cell-restricted proteases, such as chymases and tryptases, it is essential to acknowledge the presence of non-mast cell-restricted proteases. These enzymes significantly impact cellular function upon release, yet determining their primary source *in vivo* poses challenges since they are also released by other cell types[33].

These potent mast cell-restricted proteases target a diverse range of substrates, including inflammatory mediators (which can be activated or inactivated by their hydrolytic activity), ECM constituents, and cell surface proteins, thereby disrupting epithelial layer functions[33]. Mast cell-restricted proteases also serve as potent chemoattractants, attracting various immune cells like eosinophils, neutrophils, and T lymphocytes to the site of inflammation. Moreover, their actions are characterized by remarkable specificity. Mast cell-restricted proteases can cleave and modify signaling molecules, thereby influencing the activation or inactivation of various pathways and shaping the overall inflammatory response.

Chymase

Mast cell chymase belongs to the serine protease family, and is selectively expressed by mast cells[35]. Unlike many proteases requiring activation, human mast cell chymase resides in a fully functional form within secretory granules. While prochymase undergoes intracellular activation and is stored in granules, its activity is restricted under low pH conditions inside the granules and reaches full activation when released extracellularly[36].

Chymases, as monomeric proteases[36], are recognized for their precise ability to cleave peptide bonds, demonstrating versatile substrate specificity. Furthermore, the cleavage of various substrates by chymase can result in diverse outcomes, ranging from the degradation of harmful or proinflammatory substances to the activation of molecules that worsen pathology. One target of chymase is ECM components like fibronectin, leading to the disruption of focal adhesions[37,38]. Thus, chymase may contribute to tissue remodeling and heightened epithelial permeability observed in numerous disease states.

Beyond the effects of chymase on the physical landscape, chymase profoundly influences cellular communication. It degrades tight junction proteins, which may compromise epithelial barrier function and disrupt communication within tissues[39,40]. The degradation of tight junction proteins by chymase could play a pivotal role in facilitating the migration of inflammatory cells from the circulation to sites of tissue injury[34].

Furthermore, chymase acts as a regulator of signaling molecules, with the capability to cleave and alter several essential components involved in the inflammatory cascade. These include cytokines like IL-6[41,42], IL-13[41], IL-15[42], and IL-33[42,43], as well as Transforming Growth Factor (TGF-β)[44,45]. Extensive evidence underscores the capacity of chymase to promote the recruitment of inflammatory cells across various contexts, including eosinophils, neutrophils, lymphocytes, and macrophages[46].

Tryptase

Tryptase is the most predominant secretory granule-derived serine protease in mast cells, although it is also present in smaller amounts in mature basophils[17]. It typically constitutes approximately 20% of the total cellular protein in mast cells[47]. Elevated levels of tryptase in serum can be observed during intense inflammatory conditions, like systemic anaphylaxis[17,18,48].

There are several types of mast cell tryptase in humans, including α-tryptase, β-tryptase, δ-tryptase, and γ-tryptase[49]. Among these, β-tryptase is the most extensively studied, often being referred to simply as "tryptase." Within the β-tryptase category, three similar forms have been recognized: βI, βII, and βIII[49]. It is noteworthy that βII and βIII may represent allelic variations at a single locus, while βI and α-tryptase are allelic variants at a neighboring locus[50].

In contrast to chymase, which functions in monomeric form, tryptase is arranged as a tetramer[47,51]. The mature tryptase tetramer has a three-dimensional structure resembling a flat rectangular frame, with one tryptase monomer situated at each corner. The active site of each tryptase monomer orients inward, forming a narrow central pore, making it difficult for macromolecular protease inhibitors and substrates to access[17].

While it is evident that active tryptase is primarily present in a tetrameric form, there have been suggestions that tryptase may also exhibit activity when in a monomeric state[52,53]. However, disrupting the tryptase tetramer into monomers normally results in the inactivation of tryptase[52]. The function of α-tryptase remains puzzling, as its tetramers lack protease activity, unlike β-tryptase tetramers which exhibit enzymatic activity. There is no indication that δ-tryptase is present in serum or serves a biological function. γ-tryptases feature an elongated hydrophobic C-terminal domain, followed by a small cytoplasmic tail that anchors them to plasma membranes on mast cell surfaces. Nonetheless, there is no evidence supporting the release or shedding of γ-tryptase in a soluble form[18].

Numerous studies have demonstrated that tryptase can induce proliferation of various cell types, including fibroblasts, epithelial cells and smooth muscle cells, thereby contributing to the airway remodeling during diseased conditions [54]. Tryptase can inactivate a large range of peptides by cleaving specific substrates, such as Vasoactive Intestinal Peptide (VIP)[55,56] and Peptide Histidine Methionine (PHM)[56], fibrinogen[35], gelatin[57], pro-matrix metalloproteinases (MMP)1[58] and complement factors[59].

CPA3

Mast cell CPA3, a pivotal component stored within mast cell secretory granules, is a member of the family of zinc-containing carboxypeptidases. However, no crystal structure of human CPA3 has been achieved to date[52]. Our understanding of the three-dimensional structure of CPA3 relies on predictive models extrapolated from known structures of other carboxypeptidases. Notably, human CPA3 is highly similar to pancreatic carboxypeptidases[60].

In contrast to chymase and tryptase, our knowledge of CPA3 remains relatively limited, particularly concerning its potential physiological substrates. Although previous research has pinpointed neurotensin[61], apolipoprotein B100 (apoB-100)[62], and Angiotensin (Ang) I [63] as potential targets of CPA3 cleavage, and highlighted the ability of CPA3 to inactivate endothelin 1 (ET-1)[64] and sarafotoxin[64,65], there remains a gap in comprehensive characterization. Furthermore, the absence of commercially available recombinant human CPA3 products with enzymatic activity restricts our ability to study its effects. Therefore, this thesis focuses only on the impact of mast cell chymase and tryptase, leaving the exploration of CPA3 for future investigations.

Respiratory system and asthma

While many associate the respiratory system primarily with breathing, its role extends far beyond mere air exchange. The respiratory system works in close collaboration with the circulatory system to ensure a continuous delivery of oxygen, essential for fuelling metabolic processes, and the removal of waste products generated by cellular activity. Moreover, the respiratory system plays a critical role in regulating blood pH levels, contributing significantly to overall physiological balance.[66]

Respiration is the process of gas exchange, which involves a coordinated motion of inhaling oxygen and exhaling carbon dioxide between the lungs and the circulatory system. Every few seconds, nerve impulses trigger ventilation, or the breathing process, which moves air through airways into and out of the lungs. Following ventilation, there occurs an exchange of gases between the lungs and the bloodstream, known as external respiration. The blood then transports these gases to and from the tissue cells. This exchange between blood and tissues is termed internal respiration. Finally, at the cellular level, oxygen is utilized for energy production through cellular respiration[67]. Collectively, these interconnected activities constitute the process of respiration.

The respiratory system comprises two main components: the conducting zone and the respiratory zone. The conducting zone includes the majority of the respiratory tract, extending from the airways of the mouth and nose to the smallest terminal bronchioles. Its primary function is to facilitate the passage of gases. In contrast, the respiratory zone primarily comprises the lung parenchyma, which includes the respiratory bronchioles and alveoli situated at the terminal ends of the bronchi. This region is where gas exchange takes place, enabling the absorption of oxygen and the release of carbon dioxide[68].

Asthma as a respiratory tract disease, predominantly affects the airways of the lung. The human airway wall is a heterogeneous, three-layered structure extending from the airway lumen to the connective tissue. It is composed of epithelium, submucosa and smooth muscle. During asthmatic conditions, regulatory mechanisms governing airway remodeling are disrupted across all layers of the airway wall[69]. This thesis centres on examining three key cell types that represent of each of these layers.

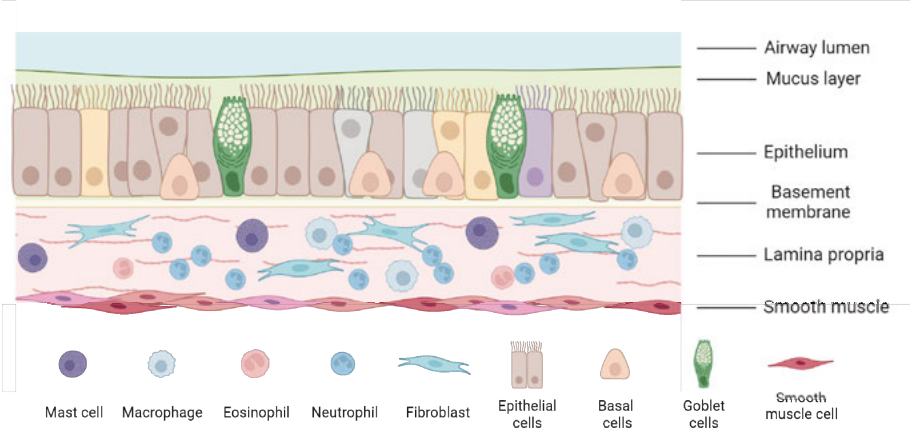

Figure 2. Magnification of a section of the airway wall, from the airway lumen to the smooth muscle. The human airway consists of three layers: epithelium, submucosa, and smooth muscle. Figure created via BioRender.

Fibroblasts

Fibroblasts are the most abundant cell type found in the lung interstitium and serve as the primary source of various components of the ECM[70,71]. These versatile cells have the ability to differentiate into other important airway mesenchymal cells, including myofibroblasts and smooth muscle cells[72].

Studies have shown noticeable differences[73] in cell morphology, particularly in cell shape, between Human Lung Fibroblasts (HLFs) from asthmatic and non-asthmatic donors when cultured under identical conditions. Asthmatic HLFs exhibit more pronounced stress fibers and matured focal adhesions compared to their non-asthmatic counterparts. This heightened tension in asthmatic HLFs influences the expression of ECM components and increases the ECM secretion into the surrounding microenvironment. Significant variations in the expression of various ECM components including type I collagen[74,75], proteoglycans[76], fibronectin[77,78], decorin[79], and tenascin C[80,81] have been noted. Additionally, while the synthesis of procollagens I and III is similar in asthmatic and non-asthmatic groups[82], the balance between collagen synthesis and degradation in asthmatic HLFs remains uncertain[83,84]. This imbalance is also linked to the unbalanced TIMP/MMP ratio in asthmatic HLFs[84], potentially leading to increased rearrangement and deposition of ECM components in asthmatic pathology.

Furthermore, HLFs derived from asthmatic donors secrete notably higher levels of Connective Tissue Growth Factor (CTGF)[85], IL-6[86], IL-8, IL-11[87], IL-17[87,88] and α-chemokines[89] compared to the non-asthmatic counterparts, indicating a potential immunomodulatory role of HLFs during asthmatic conditions.

HLFs are situated in the submucosa of the airway[90], alongside blood vessels, immune cells, and mesenchymal cells, positioning themselves strategically for direct interaction with mast cells[69], which are crucial in asthma pathology. There is growing evidence suggesting an elevated presence of mast cells in the submucosa of asthmatic individuals[12,91], highlighting the significance of studying the dynamic interplay between HLFs and mast cells. By elucidating the intricate interplay between these cell types, we may uncover new therapeutic targets and strategies aimed at managing asthma more effectively.

Small airway epithelial cells

From the trachea to the alveoli, human airways consist of approximately 23 generations of dichotomous branching tubes[92]. Small airways, typically emerging around generation 8, have a diameter of less than 2 mm, lack the supportive cartilage skeletons found in larger airways[93]. Additionally, they no longer contain mucous-producing cells. Excessive deposition of small, inhaled particles and pathogens are more prone to cause airflow obstruction in the site of small airways during asthma, and small airways are also susceptible to occlusion because of their size and structure[93]. However, our understanding of airway responsiveness and inflammation in asthma is derived predominantly from spirometric and plethysmographic measurements of central airways[94,95], as the traditional lung function tests have been largely limited to the central airways, leaving the small peripheral airways as "the quiet zone" of the lung during studies of asthma[93].

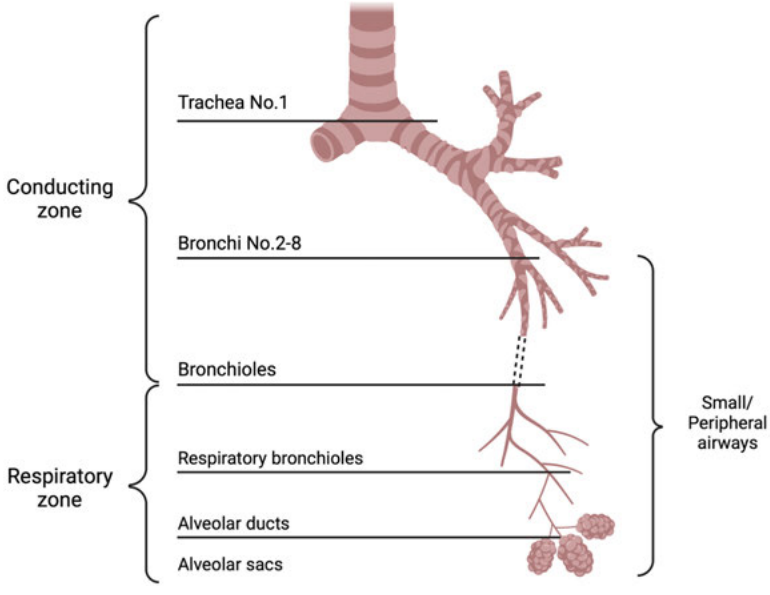

Figure 3. Structure of the airways in the respiratory system. Figure created via BioRender.

Recent studies have confirmed that small airways are the primary site of inflammation in asthma[96–98]. Patients suffering from fatal asthma display modifications in the ECM within their lungs, with notable alterations occurring in the outer lining of small airways[99]. These changes are characterized by increased levels of fibronectin, collagen I, and collagen III, as well as heightened activity of metalloproteinases, including MMP1, MMP2 and MMP9[98].

Mast cells are found more commonly in the small peripheral airways during asthmatic conditions[28,97]. It has been proven that there is a profound increase of a subset of MC_{TC} in the small airways of severe asthmatics[30,100]. This elevation in chymase expression correlates with improved lung function in severe asthma[100], suggesting the importance of mast cell chymase in asthma pathogenesis within small airways.

It is conceivable that mast cell chymase can have a positive effect on airway remodeling and epithelial repair considering their ability of activating pro-MMPs, TGF-β1[101], and Ang II[30,102], and degrading ECM components such as fibronectin and collagen IV[103–105]. Nevertheless, a comprehensive investigation of the direct impact of mast cell proteases on small airway epithelium is essential for a deeper understanding of asthma pathogenesis.

Smooth muscle cells

Within the intricate network of the respiratory system, airway smooth muscle cells (SMCs) play a crucial role. These dynamic and complex cells encircle the airway lumen throughout the bronchial tree[106,107], particularly in the upper airways where they constitute a significant portion (around 25%) of the cellular mass. While their presence diminishes in the lower airways (around 4-5% of cellular mass), their function remains vital[106].

SMCs are fundamental to regulating airflow through the airways. Their primary function is contraction[108], mediated by the expression of contractile proteins like α-Smooth Muscle Actin (α-SMA)[109], desmin[110], smoothelin[111], and Smooth Muscle Myosin Heavy Chain (SMMHC)[109]. This contractile response can be influenced by various factors, including cytokines such as TNFα[112], IL-1β[113,114], IL-5[112], and IL-13[115].

The impact of SMC contraction extends beyond regulating airflow. When excessive contraction occurs, the luminal diameter of the airway decreases, leading to a heightened bronchoconstrictive response. This phenomenon, known as airway hyperresponsiveness, is a hallmark feature of asthma[5,107]. Airway hyperresponsiveness can be attributed to two key changes in SMCs: modifications in their contractile properties and an increase in smooth muscle mass. These alterations make the airways more susceptible to stimuli, potentially leading to a prolonged state of constriction and airflow obstruction[108].

The severity of airway hyperresponsiveness often correlates with the extent of airway remodeling[116], a pathological process characterized by structural

changes in the airway wall. Airway remodeling refers to pathologic changes that occur to structural components of the airway wall, including changes in the epithelium, thickening of the basement membrane, increased ECM deposition, and an increase in subepithelial myofibroblasts[116,117]. The most prominent pathological change observed in the remodeling process is an increase in smooth muscle mass[107]. This is often observed with hypertrophy (enlargement of SMCs) and hyperplasia (increase in the number of SMCs)[107,118]. These changes are commonly associated with airway remodeling, resulting in thicker airway walls, further hindering airflow[107].

In addition to hypertrophy and hyperplasia, SMC motility has also been implicated in the development of airway remodeling[119–122]. The organization of the SMC cytoskeleton undergoes dynamic changes, SMCs can adapt their structure in response to variations in the external environment through this mechanism. Three key components of the cytoskeleton – the actin cytoskeleton, intermediate filaments, and microtubules – play a crucial role in regulating SMC motility[120]. Actin filaments are involved in processes such as actin elongation, depolymerization, and focal adhesion dynamics, while intermediate filaments like vimentin coordinate focal adhesion assembly/disassembly, contraction and nuclear rigidity. Microtubules, on the other hand, contribute to movement, communication within the cell, and the formation of structures like lamellipodia, which are essential for cell migration[120,121].

However, besides plastic and proliferative properties, recent evidence is emerging that airway SMCs also exhibit an immunomodulatory function. They can produce various cytokines, chemokines, growth factors and ECM components[106]. By releasing such compounds, airway SMCs can orchestrate and regulate chronic inflammation of the airways. These compounds can also modulate SMC function through autocrine mechanisms. For example, airway SMCs can modulate cell adhesion and leukocyte activation by inducing the expression of adhesion molecules on the cell surface. This will eventually lead to smooth muscle contractile or proliferative responses[123–125].

The interactions between mast cells and airway SMCs have been widely recognized in asthma pathophysiology[21,104,126]. However, although numerous studies have demonstrated the crosstalk between mast cells and airway SMCs, little is known regarding the effect of mast cell proteases on SMCs in cellular systems.

Present investigations

Aims

This thesis aims to investigate how mast cell-restricted proteases regulate airway responses across all layers of the airway wall, focusing on human lung fibroblasts, small airway epithelial cells, and bronchial/tracheal SMCs. Our research begins by analyzing the morphological changes in these cells after treatment with mast cell proteases, and then expands to a comprehensive exploration of various parameters.

The specific aims were:

Paper I

To assess how mast cell proteases impact primary human lung fibroblasts, from cellular morphology alterations to cytokine and growth factor regulation, and transcriptomic changes.

Paper II

To examine the influence of mast cell proteases, particularly chymase, on primary human small airway epithelial cells. Specifically, we focus on the effects of chymase on secreted ECM components, as well as the migratory capacity and transcriptome of these epithelial cells.

Paper III

To explore the interactions between mast cell proteases and primary human bronchial/tracheal SMCs. The initial focus was on assessing how chymase impacts the contraction ability of the cells, followed by an exploration of its effects on airway SMC cytoskeletal components and tight junction proteins.

Cell culture models

We employed mainly primary lung structural cells as research subjects in this thesis work. By utilizing primary cells sourced directly from human donors, we aimed to ensure the reliability and relevance of our findings. This approach better reflects the *in vivo* biology of cells, thereby enhancing the accuracy of our results. Additionally, for the cell proliferation assay, we incorporated established cell lines like HMC-1, LUVA cells and Hs578T cells as positive controls for the proliferation assays due to their higher proliferation rate.

Human lung fibroblast: Normal Human Primary Lung Fibroblast (HLF)

We utilized primary HLFs obtained from the American Type Culture Collection (ATCC; catalog PCS-201-013). These cells were isolated and cryopreserved by ATCC from various individual donors. For our study described in paper I, we utilized three vials of cells, each with distinct LOT numbers, indicating that the results were derived from three separate donors.

The primary HLFs were cultured at 37°C with 5% CO_2 in Fibroblast Basal Medium (ATCC, PCS-201-030) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and Fibroblast Growth Kit-Low Serum (ATCC, PCS-201-041). This growth kit contains 5 ng/mL recombinant human fibroblast growth factor basic (rh FGF b), 7.5 mM of L-glutamine, 50 µg/mL ascorbic acid, 1 µg/mL hydrocortisone hemisuccinate, 5 µg/mL recombinant human insulin (rh insulin), and 2% fetal bovine serum (FBS). The cells exhibit adhesion and display an elongated, spindle-shaped form within the *in vitro* cell culture setting.

Subculture of the cells was performed once the cells reached 80% confluency, and detachment was achieved using a trypsin/EDTA solution. Cells were then seeded at a density of 1×10^5 cells/mL into uncoated plastic cell culture flasks of suitable dimensions, given their adherent nature. Numbers of the cells were determined using trypan blue staining and an automated cell counter. Cells from passage 2-10 were used in the experiments. Mast cell protease treatments were conducted in serum-free conditions to prevent the inhibitory effect of serum on chymase.

Human small airway epithelial cells: Normal Human Primary Small Airway Epithelial Cells (HSAEC)

For paper II, we employed primary Human Small Airway Epithelial Cells (HSAECs) obtained from ATCC (catalog PCS-301-010). These cells were purified, prepared, and cryopreserved by ATCC until required for cell culture. Our experimental data were derived from samples collected from four independent donors.

Primary HSAECs were cultivated at 37°C with 5% CO_2 in Airway Epithelial Cell Basal Medium (ATCC, PCS-300-030), supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and the Bronchial Epithelial Cell Growth Kit (ATCC, PCS-300-040). This growth kit includes 500 µg/mL human serum albumin (HSA), 0.6 µM linoleic acid, 0.6 µg/mL lecithin, 6 mM L-glutamine, 0.4% extract P, 1.0 µM epinephrine, 5 µg/ml transferrin, 10 nM T3, 5 µg/mL hydrocortisone, 5 ng/mL recombinant human epidermal growth factor (rh EGF), and 5 µg/mL rh insulin. The cultured cells displayed an epithelial morphology, forming tightly packed cuboidal structures.

Cells were subcultured after reaching 80% confluency, detached with a trypsin/EDTA solution, and seeded at a concentration of 1×10^5 cells/mL in flat, uncoated, plastic cell culture flasks of appropriate size. Cell numbers were determined by trypan blue staining and an automated cell counter. Cells from passage 2-7 were used in the experiments.

Human airway smooth muscle cells: Normal Human Primary Bronchial/Tracheal Smooth Muscle Cells

In paper III, our study focused on investigating airway SMCs. To address this, we purchased primary Bronchial/Tracheal Smooth Muscle Cells (SMCs) from ATCC (catalog number PCS-130-011). Data were collected from cells sourced from three distinct donors, each with different LOT numbers.

Primary SMCs were grown at 37°C with 5% CO_2 in Vascular Cell Basal Medium (ATCC, PCS-100-030), supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and the Vascular Smooth Muscle Cell Growth Kit (ATCC, PCS-100-042). This growth kit comprised 5 ng/mL rh FGF b, 5 µg/mL rh Insulin, 5 ng/mL rh EGF, 10 mM L-glutamine, 50 µg/mL ascorbic acid and 5% FBS. These primary SMCs are attached cells, exhibiting an elongated, spindle-shaped morphology in culture.

The cells were subcultured after reaching 80% confluency, detached with a trypsin/EDTA solution, and seeded at a concentration of 1.2×10^5 cells/mL in uncoated plastic cell culture flasks of appropriate size. Cell counts were determined through trypan blue staining and automated cell counting. For this project, data collection was limited to passages 2 through 8. To avoid the inhibitory effect of serum on chymase, mast cell protease treatments were carried out under serum-free conditions.

Human mast cell line-1: HMC-1

The human mast cell line, HMC-1, derived from a mast cell leukemia patient, represents an established model closely resembling human mast cells[127]. In paper I, we employed HMC-1 cells as a positive control in the 5-ethynyl-2'-

deoxyuridine (EdU) staining method for assessing cell proliferation, demonstrating its efficacy as a proof of concept.

We obtained the HMC-1 cells originally as a kind gift from Mayo Foundation for Medical Education and Research. The HMC-1 cells were cultured under standard conditions. Specifically, they were maintained at 37°C with 5% CO_2 in Iscove's Modified Dulbecco's medium (IMDM), supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1.2 mM 1-thioglycerol.

Immortalized human mast cell line: LUVA

LUVA cells represent a human mast cell line sourced from an individual lacking a clonal mast cell disorder. These immortalized cells can sustain growth independent of stem cell factor, while also exhibiting robust expression of c-kit and FcεRI[128]. Just like the HMC-1 cells previously mentioned, LUVA cells were utilized as a positive control in the cell proliferation assay conducted via EdU staining, as described in paper I.

We obtained the LUVA cells from Kerafast (catalog number EG1701-FP). The cells were cultured at 37°C with 5% CO_2 in StemPro-34 SFM Complete Medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

Human breast cancer cell line: Hs578T cells

Hs578T cells are epithelial cells obtained from breast tissue sourced from a 74-year-old, Caucasian female diagnosed with breast cancer[129]. Alongside HMC-1 cells and LUVA, Hs 578T functioned as a positive control in the cell proliferation assay conducted via EdU staining in paper II.

We sourced Hs578T cells from ATCC (catalog number HTB-126). These cells were cultured under optimal conditions, maintained at 37°C with 5% CO_2. The culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Methods, results and discussions

Paper I

Mast cell chymase affects the functional properties of primary human airway fibroblasts: Implications for asthma

Fibroblasts coexist with various mesenchymal cells and immune cells, including mast cells, in the submucosa of the human airway[90]. The interaction between human lung fibroblasts and the surrounding inflammatory microenvironment may result in a switch from acute resolving inflammation to chronic persistent inflammation[130]. Here, we employed an *in vitro* model to evaluate the effect of mast cell proteases on various parameters of fibroblasts, from protein output to transcriptome analysis.

Methods

The effect of mast cell proteases - tryptase and chymase - was evaluated *in vitro* on primary HLFs. Cells were treated with 5 nM recombinant human β-tryptase and/or 5 nM recombinant human chymase in a serum-free environment for 24 hours. Morphological alterations of the primary HLFs after protease treatment were observed with a phase-contrast microscope and a live imaging system at different time points (0h, 2h, 4h, 24h). Cell viability was assessed by flow cytometry using Annexin V/DRAQ7 staining after 24 h of protease treatment. Proliferation was assessed by 5-Ethynyl-2'-deoxyuridine (EdU) labeling and flow cytometry. To evaluate the effect of mast cell proteases on the ECM produced by the primary HLFs, conditioned medium from untreated or protease-treated HLFs was assessed by western blot analysis and gelatin zymography. Effects on the output of inflammatory compounds from the primary HLF after mast cell protease treatment were assessed by a cytokine array approach, using conditioned medium from untreated or protease-treated HLFs. Selected findings were further verified by independent ELISA assays and western blot analysis. Next, the chemotactic activity of the conditioned medium from untreated or chymase-treated HLFs was assessed with a Boyden chamber technique using human neutrophils obtained from healthy donors. Finally, Ion AmpliSeq Transcriptome Human Gene Expression Kit analysis was applied to assess the effect of mast cell proteases on the transcriptome of primary HLFs.

Results and discussion

Mast cell chymase treatment resulted in major morphological alterations in primary HLFs compared to untreated or tryptase-treated HLFs. This was supported by both phase-contrast microscope and live imaging observations. Chymase-induced morphological alterations became observable 2 hours after the treatment was started and lasted up to at least 24 hours; no cell detachment was noted. These morphological alterations were not accompanied by changes

in cell viability. In fact, the cell viability assessment showed that neither chymase nor tryptase treatment induced apoptosis or necrosis in primary HLFs. Neither of the protease treatments affected the cell proliferation of primary HLFs.

Next, we sought to determine the effect of mast cell proteases on ECM-related compounds secreted from the primary HLFs. As a major component of ECM, fibronectin is primarily secreted by fibroblasts. Considering that fibronectin is a known substrate for chymase, we analyzed fibronectin in the conditioned medium from untreated or protease-treated primary HLFs using western blot. Indeed, we detected fibronectin in the samples from untreated cells, confirming that primary HLFs produce fibronectin. We also noted complete degradation of secreted fibronectin in the chymase-treated samples, whereas tryptase treatment resulted in only partial degradation of secreted fibronectin. Additionally, we detected the presence of another major ECM component, COL1A1, in the conditioned medium from primary HLFs by western blot. Samples from untreated cells showed a clear presence of COL1A1, confirming that COL1A1 is produced by primary HLFs. Again, we detected extensive COL1A1 degradation in the chymase-treated sample. Tryptase treatment resulted in a reduction of the band intensity on the membrane, but no degradation product was detected. Considering this extensive ECM degradation in the conditioned medium from chymase-treated primary HLFs, and also considering that chymase has been previously reported to activate pro-MMPs[131], we adopted gelatin zymography analysis. This analysis revealed that chymase can activate pro-MMP2 secreted by primary HLFs.

The effect of mast cell proteases on the functional properties of primary HLFs was further explored by a cytokine array approach, which detected 120 cytokines, chemokines, growth factors, and other soluble proteins in the conditioned medium from untreated samples, tryptase-treated samples, and/or chymase-treated samples. Chymase treatment resulted in an upregulated output of multiple proinflammatory chemokines and growth factors, including C-X-C motif chemokine ligand (CXCL) 1, CXCL5, Monocyte Chemoattractant Protein (MCP)-1, Insulin-like Growth Factor-Binding Protein (IGFBP)-2, CXCL6, urokinase-type Plasminogen Activator Receptor (uPAR), and Hepatocyte Growth Factor (HGF). Tryptase treatment had minimal effects on the output of cytokines and growth factors, whereas the co-treatment of tryptase and chymase had similar effects as seen after chymase treatment. However, we noticed that angiogenin, IL-1RA, and Brain-Derived Neurotrophic Factor (BDNF) were profoundly induced by the co-treatment of tryptase and chymase. We confirmed the upregulated output of CXCL6, IGFBP2, CXCL5, and uPAR in response to chymase treatment by ELISA.

The profound increase in the output of soluble uPAR is in line with previous findings of increased uPAR levels during asthma[132,133]. We performed western blot analysis of the cell pellets to provide more insight into the uPAR-releasing mechanism. We observed a significant reduction in the 43 kDa

uPAR in the chymase-treated primary HLFs, suggesting that chymase induced the release of the 43 kDa uPAR from the cells. It is also notable that chymase treatment resulted in the release of neutrophil-attracting CXCL-type chemokines, as shown by the cytokine array and subsequent ELISA results. Based on previous reports suggesting that soluble uPAR has chemotactic activity[134], we reasoned that the releasate from chymase-treated primary HLF might have chemotactic activity. Indeed, we observed a significantly higher chemoattractant effect on human neutrophils in conditioned medium from the chymase-treated primary HLFs compared to conditioned medium from non-treated HLFs, using the Boyden chamber technique.

Having identified an effect of mast cell proteases on fibroblast-mediated events related to tissue remodeling and proinflammatory cytokine release, we next sought to evaluate the impact of mast cell proteases on the transcriptome of primary HLFs. The results were first analyzed by multidimensional scaling plot analysis to present the similarities between each group. The samples from each group clustered closely together, indicating that the similarity within each group was high. Tryptase-treated samples clustered closer to the untreated samples, whereas the chymase-treated samples were clustered together with the samples from the co-treatment group, suggesting that chymase treatment and co-treatment had a more profound effect on the primary HLF transcriptome. The transcriptome analysis was also evaluated via volcano plots, where the distribution of mast cell protease-affected genes was visualized. Only 35 genes were differentially expressed (≥2-fold difference) after tryptase treatment compared to untreated cells. Chymase treatment resulted in a more drastic change, with 218 genes (133 genes upregulated and 85 genes downregulated) being differentially expressed compared to untreated cells. Similarly, 253 genes (162 genes upregulated and 91 genes downregulated) were differentially expressed compared to untreated cells after co-treatment. Pathway analysis of the impact of mast cell proteases on primary HLFs revealed a significant association between chymase treatment and the differential expression of genes involved in TGF-β signaling, cell adhesion, and ECM organization. Chymase treatment and co-treatment had a significant impact on pathways related to inflammatory responses, phosphorylation processes, cell-cell signaling, and smooth muscle cell growth.

Paper II

Mast cell chymase regulates extracellular matrix remodeling-related events in primary human small airway epithelial cells

The small airway epithelium acts as the major site for interactions with small inhaled particles and pathogens during asthma, due to its physical barrier function. In this study, we hypothesized that mast cell proteases play an important role in small airway obstruction and remodeling during asthma by affecting the airway epithelium. To test this hypothesis, we examined the effect of mast cell proteases on primary human small airway epithelial cell monolayers.

Methods

Two types of mast cell proteases were tested in this study - recombinant human β-tryptase and recombinant human chymase. Primary HSAECs were first treated with tryptase or chymase at various doses (1.25 nM, 5 nM, 10 nM, 50 nM, 100 nM). Cell morphological alterations were observed by phase-contrast microscopy at different time points (0h, 2h, 4h, 24h). Using Annexin V/DRAQ 7 staining and flow cytometry after 24 h of protease treatment, we established non-cytotoxic protease concentrations for the following experiments: 5 nM tryptase and 5 nM chymase. Cell proliferation was assessed by EdU labeling and flow cytometry. The impact of mast cell proteases on the output of cytokines, growth factors, and immunoregulatory molecules of primary HSAEC was evaluated by an unbiased cytokine array approach, with conditioned medium collected from protease-treated or untreated primary HSAECs. Selected findings were verified with corresponding ELISA measurements. The effect of mast cell proteases on cell migration was evaluated by a wound closure assay: cells were plated in a serum-deprived environment to eliminate the effect of cell proliferation, and observation photos were taken at different time points (0h, 0.5h, 2h, 4h, 24h) for data collection. Next, we assessed the effect of mast cell chymase on several epithelial cell surface and extracellular molecules (fibronectin, E-cadherin, and F-actin) by immunohistochemistry. We performed transcriptome analysis of the effect of mast cell tryptase and/or chymase on primary HSAECs using the Ion AmpliSeq Transcriptome Human Gene Expression Kit. Finally, to evaluate the effect of mast cell chymase on the MMPs secreted by primary HSAECs, we assessed the MMP content and processed the conditioned medium from mast cell protease-treated HSAECs via gelatin zymography analysis. We also examined the level of Tissue Inhibitors of Matrix Metalloproteinases (TIMP)-2 in the conditioned medium by ELISA.

Results and discussion

The morphology of primary HSAECs remained unchanged during 24 hours of treatment with tryptase across a range of concentrations (1.25 nM, 5 nM, 10 nM, 50 nM, 100 nM). A slight yet significant increase in the percentage of viable cells was observed under low concentrations (1.25 nM - 10 nM) of tryptase treatment, and no cell apoptosis was induced by tryptase. Next, we evaluated the effect of chymase on the morphology of primary HSAECs. Treatment with high concentrations (10 nM, 50 nM, 100 nM) of chymase resulted in major morphological changes of primary HSAECs. This was observed 4 hours after the treatment started and lasted for at least 24 hours, with partial cell detachment noted. Chymase treatment at 50 nM and 100 nM caused a significant increase in the population of apoptotic and necrotic/late apoptotic cells. We then investigated whether mast cell proteases can affect the proliferation rate of primary HSAECs at non-cytotoxic concentrations (5 nM). Judging by the EdU labeling and flow cytometry, tryptase treatment did not affect HSAEC proliferation, whereas chymase and co-treatment (tryptase + chymase) increased HSAEC proliferation at a similar rate.

We continued by evaluating the impact of mast cell proteases on the cytokine and growth factor output of primary HSAECs, by adopting a cytokine array approach. This analysis revealed that chymase treatment increased the output of several proinflammatory cytokines, including Macrophage migration Inhibitory Factor (MIF), IL-6R, CCL27, IFN-γ, Neurotrophin-3 (NT-3), uPAR, CXCL1, CXCL7, TIMP-2, IGFBP2, and IGFBP6. Interestingly, one major anti-inflammatory cytokine, IL-1RA, displayed a notable increase after chymase treatment. The production of several proinflammatory cytokines by the primary HSAECs decreased after chymase treatment, including Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and IL-12p70. Both tryptase and chymase treatment resulted in a lower output of the anti-inflammatory receptor IL-2Ra. Similarly, tryptase treatment also resulted in decreased production of IL-12p70. The output of proinflammatory cytokines, including Nerve Growth Factor β (β-NGF), VEGF-A and CXCL1, decreased in the tryptase-treated group. Tryptase treatment also induced increased production of MIF, IL-1R, CCL27, amphiregulin (AR), CXCL11, and IGFBP-6. Selected findings were verified by ELISA measurements, whereby the upregulated output of uPAR, CXCL7, IGFBP2, IL-1RA, and GM-CSF in response to chymase treatment was confirmed. The downregulated output of CXCL7 in response to tryptase treatment was also confirmed by ELISA.

Migration in response to airway damage during asthma is essential for airway epithelial cells. To address whether airway epithelial cell migration can be affected by mast cell proteases, we adopted a wound closure assay. This showed that chymase-treated primary HSAECs exhibited decreased migratory features and a lower wound closure percentage after 24 hours of treatment in comparison with untreated cells. To potentially provide more insight into this

delayed wound healing, we examined the effect of chymase on the deposition of fibronectin and E-cadherin from primary HSAECs. We first performed immunohistochemistry on either untreated or chymase-treated primary HSAECs. Untreated cells showed robust deposition of extracellular fibronectin, whereas the extracellular fibronectin signal was virtually absent after chymase treatment. This observation was confirmed by western blot analysis, where we detected degraded fibronectin in conditioned medium from chymase-treated cells. Degradation of E-cadherin secreted from primary HSAECs by chymase was confirmed by immunohistochemistry and western blot. Chymase treatment did not affect the F-actin network, as determined by phalloidin staining.

The regulatory effect of mast cell proteases on primary HSAECs was further examined by transcriptome analysis. Quadruplicate samples from 4 different treatment groups were subjected to transcriptome analysis: the untreated group, tryptase-treated group, chymase-treated group, and co-treatment group. Multidimensional scaling plot analysis revealed that the tryptase-treated group, chymase-treated group, and co-treatment group clustered separately from each other and from the untreated group. Moreover, the chymase-treated group and co-treatment group were clustered with a greater distance from the untreated group and tryptase-treated group, suggesting a more profound impact of chymase on the transcriptome of primary HSAECs. Heatmap and volcano plot analysis showed that chymase treatment and co-treatment had predominantly suppressing effects on gene expression in HSAECs. In comparison to the untreated cells, a total of 10, 60, and 79 genes were differentially expressed in the tryptase-treated group, chymase-treated group, and co-treatment group, respectively. Pathway analysis revealed that chymase predominantly affected processes related to metalloendopeptidase activity, protease binding, integrin binding, cell adhesion, collagen metabolism, and endodermal cell differentiation. Co-treatment affected similar pathways as those affected by chymase, with the addition of the angiogenesis category.

Having identified a role of mast cell chymase in altering the expression of genes related to metalloendopeptidase activities in primary HSAECs, we next assessed the impact of mast cell proteases on the output of MMPs using gelatin zymography. This revealed that primary HSAECs secreted both MMP2 and MMP9 as proenzymes. Chymase-treated cells secreted smaller amounts of pro-MMP9 and pro-MMP2 compared to untreated or tryptase-treated cells. The presence of active MMP2 was only observed after chymase treatment, suggesting that chymase activates pro-MMP2. As for MMP9, we only detected minimum presence of active MMP9 after chymase treatment. We suspected that an increased secretion of TIMPs from primary HSAECs after chymase treatment might have a counteractive effect on the presence of active MMP9 in the conditioned medium. As judged by the cytokine array approach, chymase treatment indeed caused an increased secretion of TIMP-2, pointing to the possibility of a counteracting effect of endogenously produced TIMP-2

on active MMP9. This was further confirmed by an independent ELISA analysis, which revealed enhanced secretion of TIMP-2 after chymase treatment. Moreover, a downregulatory effect of chymase on MMP9 was also confirmed on mRNA level.

Paper III

Mast cell chymase suppresses functional parameters in primary human airway smooth muscle cells

Increased airway smooth muscle mass is a common feature in asthma development, contributing to airway hyperresponsiveness and airway remodeling. Mast cell responses play a significant role in asthmatic conditions, releasing mediators and exceptionally large quantities of mast cell-restricted proteases upon activation. Here we investigated whether mast cell proteases may affect human bronchial/tracheal SMCs.

Methods

We initially evaluated the cytotoxic/cytostatic potential of mast cell proteases to determine their optimal working concentration. Primary SMCs underwent treatment with mast cell chymase or tryptase at various concentrations (0, 1.25, 5, 10, 50 and 100 nM). Cell viability was assessed using annexin V/DRAQ7 staining after a 24-hour treatment. The impact on cell metabolic activity/viability was assessed using the cell-permeable resazurin-based solution, Presto Blue Cell Viability Reagent. Considering the crucial role of metabolic activities in proliferation, we then investigated the effect of tryptase and chymase on cell proliferation through EdU labeling and flow cytometry. Non-cytotoxic protease concentrations for subsequent experiments were determined as 5 nM for both tryptase and chymase. As the primary function of SMC *in vivo* is contraction, we assessed the impact of mast cell proteases using a collagen gel contraction assay. Since smooth muscle contraction correlates with intracellular calcium fluctuations, we utilized Calbryte 520 AM to measure the effects of mast cell proteases on SMC intracellular calcium levels. Cell morphology was observed at various time points (0h, 1h, 6h, 24h) using a phase contrast microscope. We proceeded to investigate the effect of mast cell proteases on cell migration. To gain further insight into the mechanism by which chymase influences SMC morphology, we investigated the conformation of key cytoskeleton components, such as α-SMA, F-actin, vimentin and β-tubulin. Shifting focus, we examined the effect of chymase on primary SMC-ECM interactions, including fibronectin and Focal Adhesion Kinase (FAK), as well as intracellular adhesion complex proteins such as paxillin, vinculin, and phospho Myosin Light Chain Kinase (MLCK). Finally, we evaluated the effect of chymase on tight junction proteins (ZO-1, ZO-2, Claudin 1, and CD2AP) between primary SMCs.

Results and discussion

Neither tryptase nor chymase exhibited significant cytotoxic effects on primary airway SMCs, as indicated by Annexin V/DRAQ7 staining. Tryptase showed no noticeable impact on metabolic activity, as measured by the Presto

Blue assay. Conversely, chymase caused a marked reduction in SMC metabolic activity across all tested concentrations. However, considering that decreased cell proliferation could influence Presto Blue assay results, we conducted a cell proliferation assay using EdU staining. This analysis revealed that tryptase at concentrations ≥ 10 nM exerted a mild inhibitory effect on airway SMC proliferation, whereas chymase exhibited a more pronounced inhibitory effect, starting from concentrations ≥ 1.25 nM. Based on these findings, we determined that a concentration of 5 nM is optimal for both tryptase and chymase.

A hallmark feature of asthma is the excessive contractile activity of the airway SMCs[107]. Hence, we performed a collagen contraction assay to measure the contraction of SMCs. This assay measures the change in collagen gel size over a specific time frame following the initiation of contraction; a stronger contractility is indicated by a smaller gel size. In our study, profound collagen contraction was seen in the untreated wells over a 96h time period. A similar extent of collagen contraction was seen after addition of tryptase to the wells, suggesting that tryptase has minor effects on SMC contractility in this setting. In contrast, less prominent collagen contraction was seen in the presence of chymase, suggesting that chymase has the ability to suppress airway SMC contractility. SMC contraction is closely tied to fluctuations in intracellular calcium levels, with calcium ions pivotal in initiating and regulating the contractile process. Hence, we investigated the impact of mast cell proteases on SMC intracellular calcium levels. Both tryptase and chymase caused reductions in intracellular Ca^{2+} levels in SMCs.

Regarding the effects of mast cell proteases on cell morphology, untreated SMCs formed a monolayer in culture, apparently establishing cell-to-cell connections and attaching to the substratum. A similar morphology was seen after addition of tryptase over a 24h time period, suggesting that tryptase has minimal effects on airway SMC morphology. In contrast, the introduction of chymase into the cell cultures significantly altered SMC morphology over time, noticeable from approximately 6 hours onwards. Specifically, cells exhibited increased separation both from neighboring SMCs and the underlying surface, indicating a potential disruption of intercellular interactions and/or attachment to ECM components. We next proceeded to investigate whether the effects of mast cell proteases on SMC morphology can be translated into effects on cellular migration. Results from a scratch assay revealed a notable enhancement in SMC migration induced by chymase, whereas tryptase showed no significant impact on migration.

Cell morphology, migration and interaction with neighboring cells and ECM is intimately linked to cytoskeletal organization. Since our findings reveal that chymase can have the capacity to affect all of these parameters, we next investigated for effects of chymase on various key cytoskeleton components. We employed phalloidin staining to visualize F-actin. In untreated cells, F-actin exhibited longitudinal organization along the cell length, accompanied

by distinct stress fibers. Remarkably, upon chymase addition to SMCs, F-actin underwent dynamic remodeling, aligning more tightly and compactly. However, chymase did not alter the total amount of F-actin, as revealed by western blot analysis. SMCs exhibited high levels of α-SMA, evident through immunostaining and western blot analysis, where α-SMA predominantly localized in the cytoplasm as filamentous structures. Contrary to its effect on F-actin, chymase treatment did not induce noticeable changes in α-SMA organization or quantity. Next, we examined the impact of chymase on vimentin. Untreated cells showed robust vimentin expression, forming a fibrous network throughout the cytoplasm. However, 24 hours of chymase treatment led to a profound reorganization of the vimentin fibers, without affecting total vimentin levels. We also investigated the influence of chymase on β-tubulin organization. In untreated cells, β-tubulin was enriched circumferentially around the nuclear surface, radiating towards the periphery. Interestingly, after 24 h of chymase treatment, substantial reorganization of the β-tubulin network was observed, with β-tubulin showing a more concentrated and central location, being predominantly found in the close proximity to the nucleus. Additionally, chymase treatment resulted in the formation of β-tubulin degradation products, suggesting that chymase has the capacity to promote β-tubulin cleavage.

Our morphological examinations indicated a potential impact of chymase on the interaction of SMCs and ECM components. Therefore, we directed our focus towards this possibility. Fibronectin, a pivotal adhesive ECM component, is a primary substrate for chymase. Western blot analysis revealed abundant production of fibronectin by the SMCs. Additionally, chymase induced a significant, time-dependent proteolytic degradation of SMC-produced fibronectin. qPCR analysis confirmed high levels of fibronectin expression by the SMCs, and notably, chymase induced a substantial increase in fibronectin mRNA expression. These findings suggest that chymase has the potential to enhance fibronectin turnover by both stimulating fibronectin expression and promoting its degradation.

Next, we explored the effect of chymase on downstream signaling molecules associated with fibronectin, particularly focusing on FAK. Our findings indicate that chymase treatment reduced the levels of intact FAK while promoting the generation of FAK degradation products, suggesting its involvement in FAK proteolytic cleavage. Moreover, chymase treatment caused a reduction in the extent of FAK phosphorylation. We also investigated the impact of chymase on various intracellular adhesion proteins that may act downstream of FAK: paxillin, vinculin and phospho-MLCK. However, chymase had no apparent effect on either of these compounds.

We examined the effects of chymase on proteins implicated in cell-cell connections. Previous studies have demonstrated the potent ability of chymase to degrade proteins associated with tight junctions[135], suggesting their potential relevance in this context. Although tight junctions are typically associated

with epithelial cells rather than SMCs[136,137], we explored their presence in primary airway SMCs. Western blot analysis indeed revealed robust expression of several tight junction proteins, including zona occludens 1 (ZO-1), ZO-2, claudin-1 and CD2AP by the SMCs. Immunostaining and qPCR analysis further confirmed strong ZO-1 expression in airway SMCs. Chymase treatment significantly reduced the levels of intact ZO-1, ZO-2, claudin-1, and CD2AP, with distinct proteolytic fragments observed for ZO-2 and CD2AP. A reduction of ZO-1 levels in response to chymase treatment was also verified by immunostaining. Hence, these findings reveal that airway SMCs can express a panel of tight junction proteins, and that chymase has a strong capacity to promote the degradation of such compounds.

Concluding remarks and future perspectives

The investigations presented in this thesis explored the complex interrelationships between mast cell proteases and various cellular components in the context of asthma. We have studied the impact of mast cell-restricted proteases on primary HLFs, HSAECs, and SMCs. Through these examinations, we elucidate the multifaceted functions of mast cell proteases in shaping cellular responses and signaling pathways that are crucial to the development of asthma.

It is worth noting that the majority of our findings were derived from the interaction between chymase and various lung structural cells. In contrast, tryptase exhibited minimal effects on primary HLFs and HSAECs at a physiological relevant concentration, with only a minor impact on intracellular calcium levels and cell migration in primary SMCs. We have conducted a tryptase proteolytic activity assay using substrate S-2288, revealing that the recombinant human β-tryptase utilized in our study exhibits enzymatic activity. Further, the presence of tryptase activity is evident through its direct gelatin-degrading activity, as demonstrated in the gelatin zymography analysis detailed in paper I. These findings therefore appear to be in disagreement with prior research, where both tryptase and chymase were shown to stimulate proliferation and collagen production across diverse cell types within the human lung[138,139]. Currently, we are unable to provide a definitive explanation for these discrepancies. However, one plausible explanation might be that previous investigations primarily utilized transformed cell lines or non-airway cell types, whereas our study focused on primary lung structural cells. This highlights the critical role of primary cell cultures in research on asthma pathology. Another conceivable scenario is that some earlier studies employed purified tryptase of human origin, which includes naturally occurring human α/β-tryptase heterotetramers. A recent study has shown that the level of tryptase activity attributed to α/β-tryptase increases with the dosage of the α-tryptase gene. Furthermore, it was found that heparin-stabilized α/β-tryptase activates PAR2 in a dose-dependent manner, a property not observed with heparin-stabilized β-tryptase alone[17]. This finding could help elucidate the conflicting reports in the literature regarding the impact of tryptase on different cell types.

In the current investigation, all subsequent studies on the functional properties altered by mast cell chymase essentially originated from the same initial observation: alterations in cell morphology following chymase treatment. Particularly in paper I and paper III, there were pronounced transformations in

the HLF and SMC morphology during *in vitro* culture as monolayers. These alterations in morphology manifest in three key aspects. Firstly, there is a reduction in cell-substratum contact. Secondly, there is an observable loss of intercellular adhesion, indicated by increased empty space between cells. Thirdly, the cell area decreases, characterized by a more convex and contracted cell shape following chymase treatment. Each of these aspects will be addressed individually.

The reduced cell-substratum contact can be largely attributed to the proteolytic activity of chymase on various components of ECM, including fibronectin, MMPs and collagen. In our studies, we have proved that chymase can degrade the fibronectin that was secreted from primary HLF, HSAEC and SMCs. However, chymase manifests different effects on fibronectin gene expression on different types of cells. As transcriptome sequencing analysis revealed, chymase did not induce a significant change in the fibronectin gene expression in primary HLFs, whereas both transcriptome analysis and qPCR analysis revealed that chymase can decrease the expression of fibronectin in primary HSAEC. In contrast, chymase can increase fibronectin gene expression in the primary SMCs.

We utilized gelatin zymography analysis to examine the impact of chymase on MMPs derived from various cell types. Chymase has the capability to activate pro-MMP2, which is secreted by primary HLFs. This activation is illustrated by a faint yet distinguishable band corresponding to active MMP2 in the chymase-treated samples — a band that was virtually undetectable in both untreated and tryptase-treated samples. In the HSAEC study, we found that chymase treatment resulted in the secretion of reduced amounts of both proenzymatic and active forms of MMP9. This downregulatory effect of chymase on MMP9 was further confirmed at the mRNA level. Regarding MMP2, chymase treatment led to decreased levels of pro-MMP2; however, active MMP2 was only detected following chymase treatment, indicating a strong activating effect of chymase on pro-MMP2 across various cell types. Additionally, we observed an increase in the secretion of TIMP-2 following chymase treatment. TIMP-2 is known to regulate MMP activity in the extracellular environment following the activation of pro-MMP2[140]. Given that transcriptome analysis showed a decrease in MMP2 gene expression, the observed elevation in TIMP-2 levels may contribute to the overall reduction in metalloprotease activity within the tissue. This implies that mast cell chymase plays a complex role in regulating the ECM components in primary HSAECs, potentially explaining why the impact of chymase on the morphology of primary HSAECs was minimal.

Collagen represents a major component of the ECM. We observed that collagen 1, secreted by primary HLFs, undergoes degradation when exposed to chymase. This might suggest that chymase can mediate a direct cleavage of collagen. Yet, an alternate scenario is that chymase could indirectly contribute to collagen breakdown by proteolytically activating MMPs known for their

collagenolytic capabilities. This aligns with findings in the previous paragraph, which highlight the role of chymase in activating pro-MMPs in primary HLFs. Furthermore, our study involving primary HSAECs revealed that chymase suppresses the expression of multiple genes responsible for collagen production, including COL1A1, COL4A1, COL7A1, and COL22A1. The interplay between chymase and various ECM components, as detailed above, may lead to diminished cell-substratum contact, as manifested by the morphological changes induced by chymase.

The observed increase in intercellular space following chymase treatment can be credited to its potent proteolytic action on various cell junction molecules, such as tight junction proteins and adhesion junctions. These proteins are pivotal for cellular integrity, enabling the formation of a cohesive monolayer through intercellular adhesion and sealing intercellular gaps to control paracellular transport[141]. In our study on airway SMCs, we observed that chymase degrades tight junction proteins secreted by primary SMCs, including ZO-1, ZO-2, Claudin 1, and CD2AP. Moreover, although a significant morphological alteration in response to chymase was not noted in primary HSAEC, our findings from the HSAEC study reveal that chymase leads to the breakdown of E-cadherin, a crucial component of adheren junctions vital for cell-cell adhesion, as evident by both immunofluorescence staining and immunoblot analysis.

By degrading the junction molecules between cells, mast cells may exert their detrimental effects in asthma, disrupting the epithelial barrier function through chymase activity. Yet, this aforementioned degradation of tight junction molecules in primary SMCs by chymase may have a protective effect in asthma pathology. Our research demonstrates that chymase reduces primary SMC contraction, as shown in the collagen contraction assays presented in Paper III. In this experiment, primary SMCs were embedded in a collagen matrix, which was then allowed to polymerize, creating a stressed matrix. During this process, the cells formed intercellular connections and synthesized ECM components within the collagen gel lattice. Following the detachment of the gel lattice from its peripheral surface, the contraction of SMCs was initiated. This contraction pulled the collagen gel into a smaller volume. The outcome of the assay is based on the principle that a smaller gel size signifies a greater contraction force of SMCs. The ability of chymase to degrade the connections and matrix binding the cells within the collagen gel may explain why chymase is capable of partially reducing SMC contraction in our experimental setup.

Finally, a reduction in cell area following chymase treatment was observed in our studies, particularly in primary HLFs and SMCs. We put a large focus on this aspect in the primary SMC study, hypothesizing that the decrease in cell area might be attributable to the effects of chymase on cytoskeletal components. Indeed, our findings indicate that chymase profoundly affects the organization of key cytoskeletal elements, including F-actin, vimentin, and β-

tubulin. Therefore, the reduction in cell area observed in primary SMCs is likely due to chymase-induced modifications in cytoskeletal organization.

Cytoskeletal reorganization is very often correlated with increase cell mobility[142]. The facilitation of cell migration might be supported by the interaction of chymase with FAK present in primary SMCs. In our research, we have demonstrated that chymase is capable of degrading both FAK and its phosphorylated form in primary SMCs. Such degradation potentially leads to a quicker turnover of these proteins, enhancing cell motility by enabling faster detachment at the cell rear, thus promoting more efficient forward movement. Additionally, the ability of chymase to disrupt cell junctions, as previously mentioned, may further enhance this migratory capability. However, this hypothesis appears to conflict with findings from our second paper, where chymase was shown to degrade E-cadherin in primary HSAECs and to inhibit the wound healing process. Future studies should examine the impact of mast cell chymase on cytoskeletal elements in primary HSAECs and investigate additional cell migration markers to clarify the mechanisms involved.

Beyond the direct impact of mast cell chymase on the cellular component, we have also demonstrated profound immunoregulatory effects of chymase on primary HLFs and HSAECs. Utilizing a cytokine array approach, we found that chymase treatment led to a pronounced increase in the production of various proinflammatory chemokines and growth factors in primary HLFs. The influence of chymase on primary HSAECs, however, proved to be more intricate. Our analysis indicated that while chymase treatment increased the release of several proinflammatory cytokines and a major anti-inflammatory cytokine, IL-1RA. It also resulted in a decreased production of specific proinflammatory cytokines, including GM-CSF and IL-12p70, in primary HSAECs. Additionally, we observed a reduction in the output of the anti-inflammatory receptor IL-2Ra following chymase treatment in primary HSAECs. This immunoregulatory effect of chymase was further supported by an advanced analysis of transcriptome sequencing results, where the interactions among differentially expressed genes (DEGs) were analyzed through pathway-based assessments. Specifically, chymase treatment altered pathways related to TGF-β signaling, the inflammatory response, and cytokine-cytokine receptor interaction in primary HLFs, and impacted pathways associated with endodermal cell differentiation and angiogenesis in primary HSAECs.

Through this comprehensive evaluation of the immunoregulatory functions of chymase at both the protein and mRNA levels, we identified a novel role for chymase in primary HLFs: a chemoattractant effect on neutrophils. Treatment of primary HLFs with chymase resulted in the secretion of neutrophil-attracting CXCL-type chemokines, such as CXCL1, CXCL5, and CXCL6, suggesting that chymase-treated HLFs secrete factors with chemotactic activity towards leukocytes. To confirm this, we conducted a neutrophil chemoattractant assay using conditioned medium from chymase-treated primary

HLFs, which demonstrated a significantly enhanced chemoattractant effect on human neutrophils compared to medium from untreated HLFs. This finding indicates that factors released upon chymase treatment can recruit neutrophils, potentially contributing to a neutrophilic/monocytic infiltration in asthmatic conditions. Furthermore, HLF transcriptome gene pathway analysis also revealed that chymase treatment might also positively affect SMC proliferation. Future investigations should explore the interaction between lung fibroblasts and airway SMCs by treating primary SMCs with conditioned medium from chymase-treated primary HLFs to assess its impact on primary SMC proliferation.

Overall, our studies highlight the complex role of mast cell chymase in modulating cellular responses and immune pathways relevant to the pathophysiology of asthma. This has led to numerous intriguing questions. In paper III, which explored the interactions between airway SMCs and mast cell proteases, we observed the effects of chymase on several intracellular targets, including β-tubulin[143], FAK[144], ZO-1[145,146], and ZO-2[146]. Given that chymase was exogenously added to the SMC cultures, it suggests that chymase may induce the degradation of these proteins through indirect mechanisms. The process by which chymase facilitates this degradation is fascinating and highlights a new direction for future research. We have proposed three potential mechanisms to explain this phenomenon and have explored the interaction between chymase and intracellular targets using various methods, yielding some preliminary results.

One plausible scenario would be that chymase exerts its functions on the cells by cleaving Protease-Activated Receptors (PARs), which belong to a group of G protein-coupled receptors. Activation of PARs occurs through the specific cleavage of their N-terminal domain by specific proteases, exposing a tethered ligand that binds to and activates the receptor[147,148]. Notably, chymase can cleave PAR 1[147,149], a member of the PAR family. The activation of PARs by chymase and other proteases can trigger various cellular responses, including inflammation, vasodilation and tissue remodeling. To investigate this hypothesis, we have employed different types of PAR agonists (PAR 1-4). We started by adding the agonists into the SMCs and tried to reproduce the similar morphological alterations that were induced by chymase. However, no significant morphological changes were observed across all different types of PAR agonist treatment.

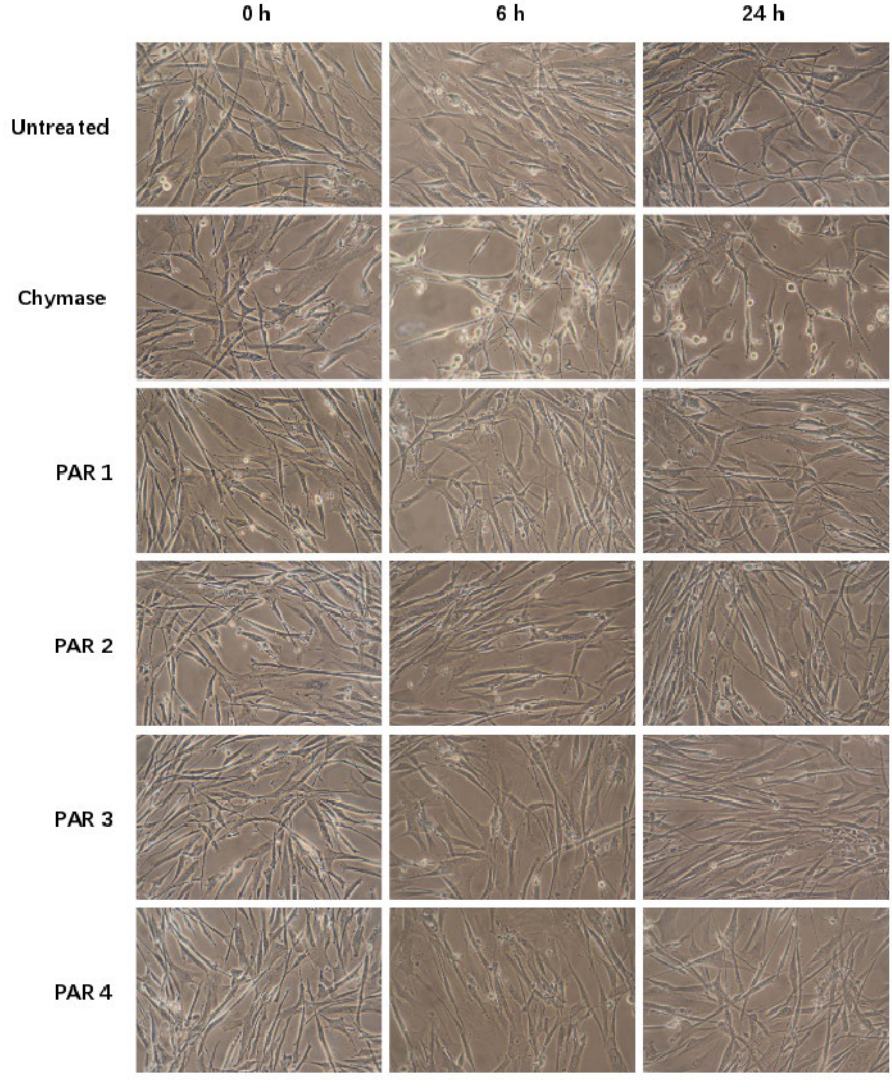

Figure 4. PAR agonist treatments do not induce the same level of morphological changes as induced by chymase. Phase contrast microscope observations of primary SMCs that were either untreated or treated with 5 nM chymase or with 50 μM of various PAR agonists (PAR1: TFLLRN-NH2, Anaspec, AS-62937. PAR2: SLIGKV-NH2, Tocris Bioscience, 3010. PAR3: SFNGGP-NH2, Anaspec, AS-62938. PAR4: AYPGKF-NH2, Anaspec, AS-60218-1).

An alternative hypothesis, building upon our earlier discoveries[148], involves the uptake of chymase by the lung structural cells. To address this possibility, we have used Alexa Fluor-488-labeled chymase and performed fluorescence microscopy analysis. As of now, we have confirmed preliminarily that chymase can be taken up by the primary HLFs. Subsequent experiments can be

conducted to pinpoint the corresponding cell surface receptor, with cell surface proteoglycans emerging as a primary candidate due to the affinity of chymase for this type of compounds.

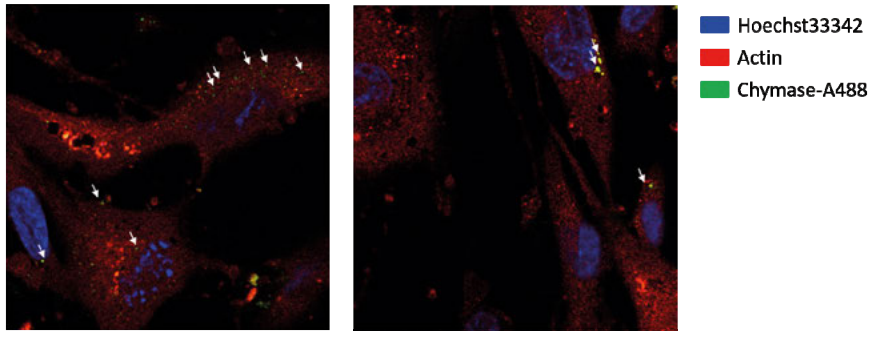

Figure 5. Alexa Fluor-488-labeled chymase is detected within primary HLF via confocal microscope observation.

The third potential scenario would be that chymase affects cell function by cleaving extracellular proteins, thereby disrupting intracellular signaling and initiating downstream events. The focus will be on the impact of chymase on major signaling pathways related to integrin, such as Mitogen-Activated Protein Kinases (MAPKs), FAK, and Phosphoinositide 3-Kinases (PI3K)[150,151], given the role of integrin in binding ECM components and forming focal adhesions that concentrate cytoskeletal components and signaling molecules. Our current findings on the impact of chymase on primary SMCs are aligned with targets within the integrin signaling pathway.

In conclusion, this thesis highlights the multifaceted role of mast cell chymase in modulating cellular and immune responses in various lung structural cells related to asthma, offering insights to the development of novel therapeutic/diagnostic strategies in the management of asthma.

Popular science summary

Asthma is a common disorder that affects millions of people worldwide. It is a chronic disease characterized by inflammation and narrowing of the airways, leading to symptoms like wheezing, chest tightness, coughing and breathlessness, which vary from mild to potentially life-threatening asthma attacks. Though there is currently no cure, asthma can be managed through treatment and prevention.

Allergic asthma is the most common type of asthma. It is triggered by allergens such as dust mites, animal dander, pollen and mold. My PhD project explores how mast cells, a type of white blood cell located at the interface of our body and the external environment, interact with various lung cell types.

For decades, researchers have studied the role of mast cell mediators in asthma and allergies. Histamine, one of the most famous mediators, serves as the target for widely-used antihistamine medications. However, mast cells also secrete other mediators, which may play a crucial role in the progression of asthma. For example, mast cells are known to produce a unique set of proteases, including tryptase, chymase, and carboxypeptidase A3, which are exclusive to mast cells. My research primarily focuses on the effects of tryptase and chymase.

Using *in vitro* cell culture systems, I examined the effects of treating cells with these proteases at physiologically relevant concentrations, starting with microscopic observations of changes in cell morphology. This led to investigations into how the proteases impact cell functions.

In paper I, looked into how mast cell proteases affect lung fibroblasts, spindle-shaped cells that are crucial for constructing connective tissue and are involved in repair processes post-injury, which could result from asthmatic coughing or other mechanical forces. Paper II focuses on small airway epithelial cells, which line the narrow passages in our lungs, serving as a protective barrier and facilitating gas exchange. Since small airways are significantly affected in asthma —with an increase in mast cell presence — understanding their interaction is vital. Paper III addresses airway smooth muscle cells, which, distinct from skeletal muscle, contract without conscious control. While this involuntary contraction plays a crucial role in regulating the rhythm of breathing, it also contributes to the pathophysiology of asthma by constricting the airways. This constriction triggers symptoms like breathlessness and

chest tightness. Gaining insight into the impact of mast cells on these cells may pave the way for improved management of asthma symptoms.

Overall, my thesis provides a thorough analysis of the impact of mast cell proteases on critical cell types within the human airway, uncovering distinct effects on each cell type.

Populärvetenskapligt Sammanfattning

Astma är en vanlig sjukdom som påverkar miljontals människor över hela världen. Det är en kronisk sjukdom som kännetecknas av inflammation och förträngning av luftvägarna, vilket leder till symtom som väsande andning, tryck över bröstet, hosta och andnöd, som varierar från milda till potentiellt livshotande astmaattacker. Även om det för närvarande inte finns något botemedel, kan astma hanteras genom behandling och förebyggande åtgärder.

Allergisk astma är den vanligaste typen av astma. Den utlöses av allergener såsom kvalster, djurpäls, pollen och mögel. Mitt doktorandprojekt utforskar hur mastceller, en typ av vit blodkropp som finns vid gränsen mellan vår kropp och den yttre miljön, interagerar med olika celltyper i lungorna.

I decennier har forskare studerat rollen av mastcellens utsöndrade mediatorer i astma och allergier. Histamin, en av de mest kända mediatorerna, fungerar som mål för vanligen använda antihistaminmediciner. Mastceller utsöndrar dock också andra mediatorer, som också kan spela en avgörande roll i utvecklingen av astma. Till exempel är mastceller kända för att producera en unik uppsättning proteaser, inklusive tryptas, kymas och karboxypeptidas A3, som är exklusiva för mastceller. Min forskning fokuserar främst på effekterna av tryptas och kymas.

Genom att använda *in vitro*-cellsystem undersökte jag effekterna av att behandla celler med dessa proteaser vid fysiologiskt relevanta koncentrationer, utgående från mikroskopiska observationer av förändringar i cellmorfologi. Detta ledde till undersökningar om hur proteaserna påverkar cellfunktionerna.

I artikel I undersökte jag hur mastcellsproteaser påverkar lungfibroblaster, spindelformade celler som är viktiga för att bygga upp bindväv och som är involverade i reparationsprocesser efter skador, vilket kan uppstå från astmatisk hosta eller andra mekaniska krafter. Artikel II fokuserar på små luftvägsepitelceller, som skyddar de smala passagerna i våra lungor, och som fungerar som ett skyddande lager och underlättar gasutbyte. Eftersom små luftvägar är starkt påverkade i astma – med en ökning av mastcellsnärvaro – är förståelsen för deras interaktion viktig. Artikel III tar upp glatta muskelceller i luftvägarna, som, till skillnad från skelettmuskel, drar ihop sig utan medveten kontroll. Medan denna ofrivilliga sammandragning spelar en avgörande roll för att reglera andningens rytm, bidrar den också till patofysiologin is astma genom att förtränga luftvägarna. Denna sammandragning utlöser symtom som

andnöd och tryck över bröstet. Att få insikt i mastcellers påverkan på dessa celler kan bana väg för förbättrad hantering av astmasymtom.

Sammanfattningsvis ger min avhandling en grundlig analys av mastcells-proteasers påverkan på kritiska celltyper inom luftvägarna hos människa, och påvisar distinkta effekter på varje celltyp.

科普总结

哮喘是一种影响全球数百万人的常见疾病。它是一种慢性疾病，典型特征为气道炎症和狭窄，导致喘息、胸闷、咳嗽和呼吸困难等等。这些症状被称为哮喘发作，它可能非常轻微，也可能危及生命。尽管目前没有有效的哮喘治愈方法，但我们可以通过治疗和预防哮喘发作来管理哮喘病情。

过敏性哮喘是最常见的哮喘类型，它是由一些常见的过敏原触发的，比如尘螨、动物皮屑、花粉和霉菌等等。我的博士论文主要研究肥大细胞如何影响人类肺部的各种细胞。

肥大细胞是一种白细胞，主要位于我们身体与外部环境的交界处，比如皮肤、肺部气道、肠道、血管周围和淋巴结附近。几十年来，研究人员一直在研究肥大细胞介质在哮喘和过敏中的作用。组织胺是最著名的肥大细胞介质之一，我们在过敏时常用的抗组胺类药物的靶点就是组织胺。但是，肥大细胞也分泌其他重要的介质，如蛋白酶，这些蛋白酶可能在哮喘的进展中发挥关键作用。

肥大细胞有一些特有的蛋白酶，比如胰蛋白酶、糜酶和羧肽酶 A3。我的研究主要关注胰蛋白酶和糜酶对各种人类肺部细胞的影响。我的研究主要使用体外细胞培养系统，为了更好的模仿体内系统，我使用了生理相关的蛋白酶浓度，从观察细胞形态的显微镜变化开始探讨蛋白酶如何影响细胞功能。

在第一篇论文中，我研究了肥大细胞蛋白酶对肺成纤维细胞的影响，这些纺锤形细胞对构建肺部的结缔组织至关重要。哮喘过程中的咳嗽或其他机械力会对肺部造成损失，这些肺成纤维细胞可以参与损伤后修复过程。

第二篇论文的研究对象是小气道上皮细胞，这些细胞守卫着我们肺部非常狭窄的气道，它们是防御污染物和病原体的屏障，并且帮助气体交换。过往的研究发现在哮喘病人中，肥大细胞数量在小气道中显著增加。因此，研究肥大细胞蛋白酶与小气道上皮细胞的相互作用非常重要。

第三篇论文研究的是气道平滑肌细胞。这是一类非常独特的肌肉细胞，与骨骼肌或心肌不同，这些细胞只有一个细胞核，且无法自主控

制收缩。这种非自愿收缩在调节呼吸节奏中起着关键作用，但在哮喘的病理生理成因中也会导致问题。常见的哮喘症状，比如呼吸困难和胸闷等症状都是因为气道平滑肌细胞不受控制地收缩引起的。深入了解肥大细胞蛋白酶对这些细胞的影响可能能为改善哮喘症状的药物研究提供新的思路。

总的来说，我的论文对肥大细胞蛋白酶对人类气道中主要细胞类型的影响进行了彻底分析。我的研究显示每种细胞类型都受到不同程度、不同类型的影响。

Populärwissenschaftliche Zusammenfassung

Asthma ist eine chronische Krankheit, die Millionen Menschen weltweit betrifft. Sie zeichnet sich durch Entzündung und Verengung der Atemwege aus was zu Symptomen wie Brustenge, Husten und Atemnot führt. Asthmaanfälle können dabei von mild bis hin zu lebensbedrohlich variieren. Obwohl eine vollständige Heilung derzeit nicht möglich ist kann Asthma durch Behandlung und Vorbeugung kontrolliert werden.

Allergisches Asthma ist die häufigste Form von Asthma. Es wird durch Allergene wie Hausstaubmilben, Tierhaare, Pollen und Schimmel ausgelöst. Mein PhD-Projekt erforscht, wie Mastzellen, eine Art von weißen Blutzellen, die sich an der Schnittstelle unseres Körpers zur Außenwelt befinden, mit verschiedenen Zelltypen in der Lunge interagieren.

Seit Jahrzehnten haben Forscher die Rolle von Mastzellmediatoren bei Asthma und Allergien untersucht. Histamin, einer der bekanntesten Stoffe, dient als Ansatzpunkt für weit verbreitete Antihistaminika. Mastzellen produzieren jedoch auch andere Mediatoren, die eine entscheidende Rolle im Fortschritt von Asthma spielen könnten. Beispielsweise ist bekannt, dass Mastzellen eine einzigartige Reihe von Proteasen produzieren, einschließlich Tryptase, Chymase und Carboxypeptidase A3. Meine Forschung konzentriert sich in erster Linie auf die Effekte von Tryptase und Chymase.

Unter Verwendung von *in-vitro*-Zellkultursystemen untersuchte ich die Auswirkungen der dieser Proteasen auf Zellen in physiologisch relevanten Konzentrationen, beginnend mit mikroskopischen Beobachtungen von Veränderungen in der Zellmorphologie. Dies führte zu Untersuchungen darüber, wie die Proteasen verschiedene Zellfunktionen beeinflussen.

In Publikation I untersuchte ich, wie Mastzellproteasen Lungenfibroblasten beeinflussen. Bei Lungenfibroblasten handelt es sich um spindelförmige Zellen, die für den Aufbau von Bindegewebe entscheidend sind und an Reparaturprozessen nach Verletzungen beteiligt sind Solche Verletzungen können zum Beispiel durch asthmatisches Husten oder andere mechanische Kräfte entstehen. Publikation II konzentriert sich auf Epithelzellen der kleinen Atemwege, die die engen Passagen in unseren Lungen auskleiden, als Schutzbarriere dienen und den Gasaustausch erleichtern. Da kleine Atemwege bei Asthma besonders starkbetroffen sind — mit einer Zunahme der Mastzellpräsenz — ist das Verständnis ihrer Interaktion entscheidend. Publikation III befasst sich mit den glatten Muskelzellen der Atemwege, die sich wie die

Herzmuskeln von “gewöhnlichen” Skelettmuskeln unterscheiden, da sie ohne bewusste Kontrolle kontrahieren. Während diese unfreiwillige Kontraktion eine entscheidende Rolle bei der Regulierung des Atemrhythmus spielt, trägt sie auch zur Pathophysiologie von Asthma bei, indem sie die Atemwege verengt. Diese Verengung löst Symptome wie Atemnot und Brustenge aus. Ein Einblick in die Auswirkungen von Mastzellen auf diese Lungenzellen-könnenden Weg für eine verbesserte Behandlung und Kontrolle von Asthma-symptomen ebnen.

Insgesamt bietet meine Dissertation eine gründliche Analyse der Auswirkungen von Mastzellproteasen auf für Asthma kritische Zelltypen innerhalb der menschlichen Atemwege und deckt unterschiedliche Effekte für diese Zelltyp auf.

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“Work and play can bleed into each other, become one and the same, until the very idea of their distinction feels meaningless.”

— Katalin Karikó,
Breaking Through, 2023

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