The Role of Mast Cell Proteases in Allergic Disease and Apoptosis

Ida Waern
Faculty of Veterinary Medicine and Animal Science
Department of Anatomy, Physiology and Biochemistry
Uppsala

Acta Universitatis agriculturae Sueciae
2012:50

Cover: The two-faced art of a mast cell.

ISSN 1652-6880
ISBN 978-91-576-7697-9

Print: SLU Service/Repro, Uppsala 2012

The Role of Mast Cell Proteases in Allergic Disease and Apoptosis

Abstract

Mast cells (MCs) are key effector cells in allergic reactions, through the release of a wide variety of granule-stored and *de novo* synthesized inflammatory mediators. The MC secretory granules contain exceedingly high levels of serglycin proteoglycan and the heparin-binding proteases chymase, tryptase and carboxypeptidase A.

In this thesis the contribution of mouse mast cell protease (mMCP)-4, which is thought to be the functional homolog to the human chymase, was studied in the context of allergic airway inflammation. Using two models of allergic airway inflammation, wild-type (WT) and mMCP-4 deficient (mMCP-$4^{-/-}$) mice were treated with ovalbumin (OVA) or with house dust mite (HDM) extract. We found that the OVA challenged mMCP-$4^{-/-}$ mice displayed increased airway hyperreactivity and lung eosinophilia and in the HDM model they displayed increased serum IgE levels. Moreover, the level of IL-33, a pro-inflammatory cytokine, was enhanced in the lung tissue in mMCP-$4^{-/-}$ mice compared to WT mice after HDM-treatment.

The active proteases stored in MC granules have the ability to cleave a number of components upon degranulation. We could demonstrate that proteolytic degradation of IL-13 by MCs is mediated by a serine protease, dependent on serglycin proteoglycan for its storage.

Permeabilization of lysosomal membranes often leads to apoptosis and the released proteases take part in this process, activating pro-apoptotic compounds. We have found that serglycin$^{-/-}$ MCs are more resistant to apoptosis induced by secretory granule damage. We showed that serglycin$^{-/-}$ MCs exhibited reduced caspase-3 and protease activity in the cytosol compared to WT cells.

Taken together, the studies in this thesis suggest that MC chymase plays a protective role in the development of allergic airway inflammation and this could possibly be explained by chymases ability to degrade the pro-inflammatory cytokine, IL-33. In addition, we also suggest that serglycin proteoglycan and serglycin-dependent MC proteases participate in IL-13 degradation as well as in MC apoptosis induced by secretory granule damage.

Keywords: mast cells, serglycin proteoglycan, protease, chymase. allergic airway inflammation, asthma, apoptosis.

Author's address: Ida Waern, SLU, Department of Anatomy, Physiology and Biochemistry, P.O. Box 575, SE-751 23 Uppsala, Sweden.
E-mail: Ida.Waern@slu.se

Till min underbara familj

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Waern I., Jonasson S., Hjoberg J., Bucht A., Åbrink M., Pejler G. and Wernersson S. (2009). Mouse mast cell protease 4 is the major chymase in murine airways and has a protective role in allergic airway inflammation. *Journal of Immunology*. 183(10):6369-76.

II Waern I., Lundequist A., Pejler G. and Wernersson S. (2012). Mast cell chymase modulates IL-33 levels and controls allergic sensitization in dust-mite induced airway inflammation. *Manuscript*.

III Waern I., Karlsson I., Thorpe M., Schlenner S. M., Feyerabend T. B., Rodewald H-R., Åbrink M., Hellman L., Pejler G. and Wernersson S. (2012). Mast cells limit extracellular levels of IL-13 via a serglycin proteoglycan-serine protease axis. *Biological Chemistry* ISSN (online) 1437-4315. DOI: 10.1515/bc-2012-0189.

IV Melo F. R., Waern I., Rönnberg E., Åbrink M., Lee D. M., Schlenner S. M., Feyerabend T. B., Rodewald H-R., Turk B., Wernersson S. and Pejler G. (2011). A role for sergycin proteoglycan in mast cell apoptosis induced by a secretory granule-mediated pathway. *Journal of Biological Chemistry*. 286(7):5423-33.

Abbreviations

AHR	airway hyperresponsiveness
APC	antigen presenting cell
Arg	arginine
ASM	airway smooth muscle
BAL	bronchoalveolar lavage
BMMC	bone marrow derived mast cell
C_L	lung compliance
CPA	carboxypeptidase A
CR	complement receptor
CTMC	connective tissue mast cell
DPPI	dipeptidyl peptidase I
HDM	house dust mite
I.n.	intranasal
I.p.	intraperitoneal
Ig	immunoglobulin
IL	interleukin
kDa	kilodalton
Leu	leucine
LLME	H-Leu-Leu-OMe
Lys	lysine
MC	mast cell
MC_T	tryptase-positive human mast cell
MC_{TC}	tryptase- and chymase-positive human mast cell
Met	methionine
MHC	major histocompatibility complex
MMC	mucosal mast cell
mMCP	mouse mast cell protease
OVA	ovalbumin

PBS	phosphate-buffered saline
PCMC	peritoneal cell derived mast cell
PG	prostaglandin
Phe	phenylalanine
PI	propidium iodide
R_L	lung resistance
SCF	stem cell factor
Ser	serine
SMC	smooth muscle cell
T_H cell	T helper cell
Treg	regulatory T cell
Trp	tryptophan
Tyr	tyrosine
WT	wild type

1 Introduction

1.1 The immune system

The immune system protects us from potentially hazardous pathogens such as bacteria, fungi, viruses and parasites. The immune system is composed of two components i.e. the innate and adaptive (acquired) immunity. The innate immune system is always active and is often referred to as the first line of host defense that rapidly operates to eliminate foreign intruders. It includes anatomic barriers such as the skin, tears, saliva and mucosal surfaces as well as the complement system and various leukocytes. If innate immunity fails to eliminate a pathogen the adaptive immune system is activated. The adaptive immune response is specific towards one particular pathogen, and includes recognition of foreign molecules by antigen-specific receptors on B- and T-lymphocytes. After elimination of the foreign intruder some of the antigen-specific cells persist creating the unique property of immunological memory. Even though innate and adaptive immunity often are regarded as two separate components of the immune system, they are linked through cytokine secretion and cell-to-cell signaling (Crozat *et al.*, 2009).

1.2 The allergic immune response

The allergic immune response is a type I hypersensitivity reaction that involves the production of immunoglobulin (Ig) E antibodies toward an antigen mediated by the adaptive immune system. The reaction is initiated by antigen capture and processed by antigen-presenting cells (APCs) such as dendritic cells or macrophages. These cells mature and migrate to the lymph nodes to present peptides of the allergen on major histocompatibility complex (MHC) class II molecules to antigen-specific naïve CD4+ T lymphocytes, which in turn differentiate and become activated T helper type 2 cells (T_H2 cells). In

addition to the antigen presentation, other factors such as the cytokine milieu and antigen type and dose also influence the differentiation of naïve T cells. T_H2 cells produce the cytokines interleukin (IL)-4 and IL-13 and when interacting with antigen-specific B cells via MHC class II and co-stimulatory molecules they induce proliferation and class switching to IgE production. The primary response to an allergen, i.e. the sensitization phase, is summarized in figure 1. The antigens triggering allergic reactions are mostly innocuous environmental substances referred to as allergens.

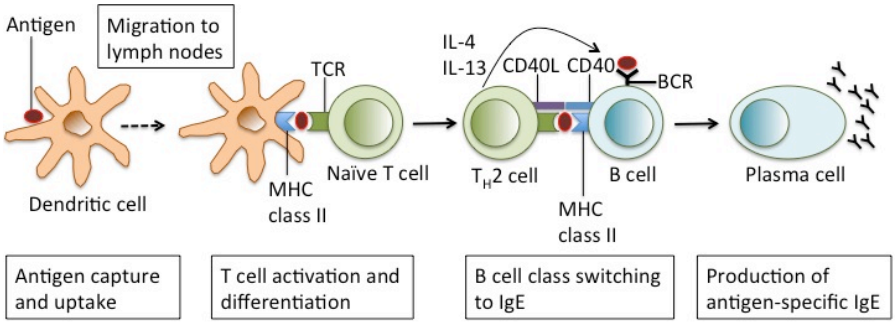

Figure 1. Mechanism for allergic sensitization and allergen-specific IgE production.

IgE production mainly takes place in the draining lymph nodes at the site of allergen entry. However, allergic sensitization may also occur in the mucosa of the airways where the produced IgE antibodies diffuse locally, enter the lymphatic vessels and can later be found in the blood stream. In the blood, IgE can bind to basophils, a type of circulatory granulocyte. The systemically distributed IgE subsequently binds to membrane receptors expressed on a tissue resident effector cells, i.e. the mast cell (MC). IgE binding to its receptors on the MCs is referred to as MC sensitization and does not produce any symptoms of allergy. Instead, once a MC is sensitized to an allergen, subsequent exposure to the same allergen mediates activation of the MCs via the IgE-receptor and an allergic reaction is initiated (figure 2). The immediate allergic reaction occurs within minutes after the second encounter with the allergen and is a result of MC and/or basophil activation and the release of inflammatory mediators.

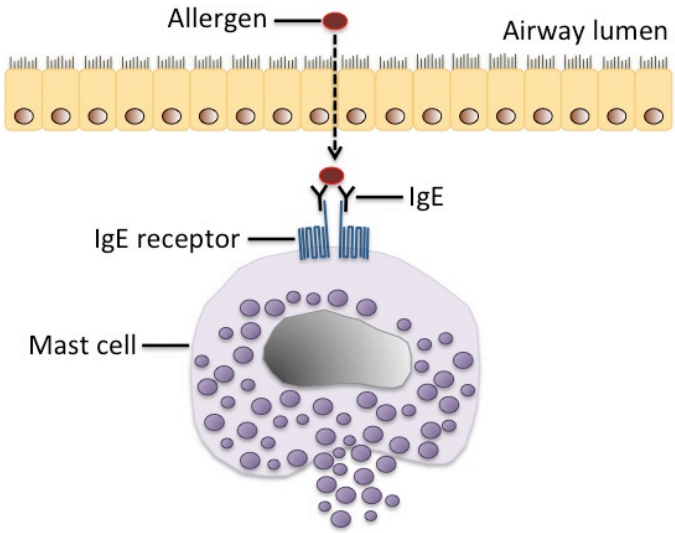

Figure 2. Immediate allergic reaction in response to subsequent exposure to the allergen via the airways.

1.3 Allergic asthma

The prevalence of allergic diseases has increased during the past decades. Today, more than one fifth of the population in industrial countries suffers from allergies, and allergic asthma is one of the most common diseases mediated by IgE antibodies. Allergic asthma is a chronic disorder characterized by reversible airway obstruction, airway hyperresponsiveness, airway inflammation and alterations in the structural cells and tissue of the airway, i.e., remodeling (Maddox & Schwartz, 2002). Airway hyperresponsiveness (AHR) is a key feature of asthma and is thought to be associated with airway inflammation and remodeling (Cockcroft & Davis, 2006). The allergic inflammation can be divided into three phases: early-phase reactions, late-phase reactions and chronic allergic inflammation. Secretion of inflammatory mediators by activated MCs are involved in the early inflammatory response that occurs within minutes after allergen exposure. This reaction mediates bronchoconstriction, vasodilation, mucous secretion, and an increased vascular permeability. The early reaction is often followed by a late response that develops after 2 hours, with a peak after 6-9 hours after allergen exposure. This phase is characterized by recruitment of other immune cells, in particular eosinophilic granulocytes, to the site of allergen exposure (Galli *et al.*, 2008).

Mediators involved in cell recruitment in the late-phase reaction originate from MCs and activated T_H2 cells. Clinical features of these events include narrowing of the airways and increased mucous secretion. Repeated exposure to the allergen may develop into chronic airway inflammation associated with remodeling events of the airways. Airway smooth muscle (ASM) hyperplasia and hypertrophy are alterations known to contribute to the pathophysiology of asthma. It is likely that thickening of the smooth muscle layer is an important component of airway AHR. In addition, remodeling events such as thickening of the airway wall and other mechanisms may also contribute to AHR and asthma (Cockcroft & Davis, 2006).

1.3.1 Cytokines involved in allergy and asthma

Cytokines are a group of soluble proteins that function as key regulatory signaling molecules in the immune system. Cytokines act by binding to specific cell surface receptors, thereby regulating gene expression in the target cells. Some cytokines are pro-inflammatory (inducing inflammation) whereas others are anti-inflammatory (suppressing inflammation). Dysregulation of cytokine expression may result in immune disorders. Therefore, the balance of effector cytokines associated with T_H1/T_H2 cells, regulatory T cells (Tregs) and other anti-inflammatory cytokines influences the onset of different diseases (Dinarello, 2000). As previously described, allergic disorders are dominated by T_H2 cytokines including IL-4, IL-5, and IL-13 to name but a few. IL-4 is a multifunctional cytokine produced by T_H2 cells, MCs, basophils and eosinophils. IL-4 induce B-cell switching to IgE synthesis, MC development, eosinophil and basophil activation as well as mucous secretion (Vercelli, 2001). It has been suggested that recruitment of eosinophils plays a role in the pathological process in asthmatic airways, and IL-5 is necessary for their differentiation, maturation and activation (O'Byrne *et al.*, 2001). A central player in allergic reactions and asthma is IL-13. Like IL-4, IL-13 is involved in B-cell switching to IgE production. Additionally, it has been demonstrated that IL-13 can induce smooth muscle cell (SMC) contractility and mucous secretion (Wills-Karp, 2004). IL-13 has been shown to be produced by a number of cells including T_H2 cells, MCs, basophils, dendritic cells and natural killer cells (Wills-Karp, 2004). IL-33 is a relatively newly characterized T_H2-associated cytokine, which has been shown to be involved in the recruitment of eosinophils, basophils and T_H2 cells (Suzukawa *et al.*, 2008; Komai-Koma *et al.*, 2007). It can also induce IL-5 and IL-13 production by T_H2 cells (Hsu *et al.*, 2010) and administration in mice leads to AHR and mucous production (Kondo *et al.*, 2008). IL-33 is a pro-inflammatory cytokine produced by a

variety of cells including epithelial cells, endothelial cells, MCs, SMCs, macrophages and dendritic cells (Moussion *et al.*, 2008). MCs can be activated by IL-33, which subsequently causes production of pro-inflammatory cytokines including IL-13 (Ho *et al.*, 2007). Basophils can also be activated by IL-33 to produce cytokines (Suzukawa *et al.*, 2008).

1.4 Mast cells

MCs are highly granulated cells found in large numbers throughout all vascularized tissue in the body, especially in tissues close to the exterior environment such as the skin, airways and gastro-intestinal tract. MCs are derived from multipotent hematopoietic stem cells that circulate as progenitors in the blood and mature when entering the tissue (Kirshenbaum *et al.*, 1991). Mature MCs are considered to be long-lived cells and have the potential to regenerate after a degranulation event (Xiang *et al.*, 2001). Due to their strategic distribution and their ability to sense pathogens and danger, MCs can rapidly respond to harmful intruders. They are therefore known as one of the immune cells participating in innate immunity. However, MCs are mainly known for their role in allergies. In allergic responses, MC activation is mediated by allergen-specific IgE antibodies through FcεRI receptor cross-linking causing a rapid release of the granule-stored mediators. In addition, MCs can also be activated by other mechanisms to secrete granule stored as well as *de novo* synthesized mediators. The mechanisms of MC activation as well as the granule-stored mediators are described in further detail later in this thesis.

1.5 Mast cell activation

A key MC feature is their ability to rapidly respond to various stimuli (figure 3). The different types of stimuli include binding to specific surface-bound receptors on the MC as well as compounds that have the ability to directly activate MCs. The classical and most well studied form of MC activation is through the high affinity receptor for IgE, FcεRI. This and other forms of MC activation will be described below.

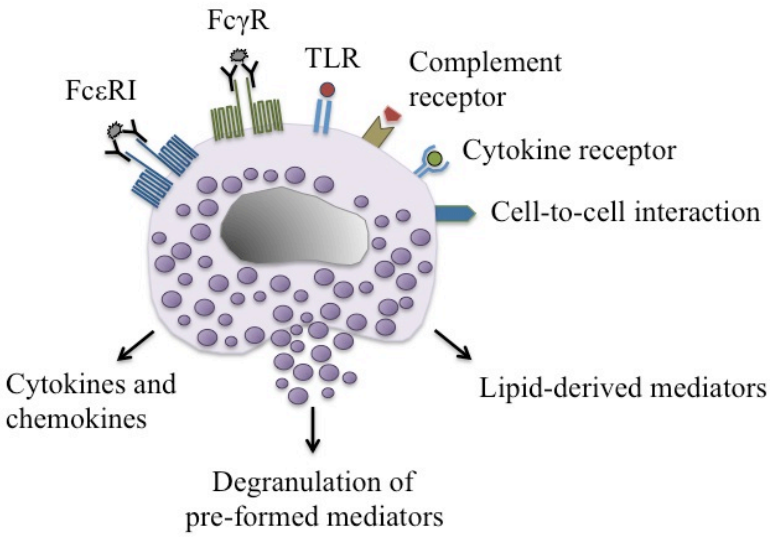

Figure 3. Different mechanisms of mast cell activation.

1.5.1 IgE-dependent activation

MCs express high numbers of the high affinity receptor for IgE (FcεRI) on their surface. Cross-linking of FcεRI-bound IgE by an antigen leads to MC activation. FcεRI is a tetrameric protein that consists of one α-, one β- and two γ-chains. The α-chain is responsible for the high affinity binding of the Fc part of IgE and the β- and γ-chains initiate intracellular signaling (Jouvin *et al.*, 1994; Hakimi *et al.*, 1990). The β- and γ-subunits of the receptor contain conserved cytoplasmic motifs, named immunoreceptor tyrosine based activation motifs (ITAMs). Upon FcεRI cross-linking, Lyn, a protein tyrosine kinase adds phosphate groups to the tyrosine residues in the ITAMs of the intracellular β- and γ-subunits. Phosphorylation of the ITAMs allows binding and activation of the protein tyrosine kinase Syk, which results in phosphorylation of a number of signaling proteins finally leading to degranulation, secretion and *de novo* synthesis of various mediators (Turner & Kinet, 1999).

1.5.2 IgG-mediated activation

Antigen-mediated activation of MCs can also be achieved via receptors for IgG (FcγRs). Human MCs express the high affinity receptor FcγRI and the low affinity receptor FcγRII, whereas murine MCs express the low affinity receptors FcγRII and FcγRIII (Okayama *et al.*, 2000; Katz & Lobell, 1995). It has been demonstrated that MC degranulation can be triggered by stimulation

of FcγRI or FcγRIII by polyvalent antigen (Daeron *et al.*, 1995). In contrast to the activating receptors, FcγRII contains an immunoreceptor tyrosine based inhibitory motif (ITIM). Crosslinking of FcγRII results in decreased signaling of the activating IgE- and IgG-receptors, thereby inhibiting MC degranulation and mediator release (Kepley *et al.*, 2000; Daeron & Vivier, 1999).

1.5.3 Toll-like receptors

Toll-like receptors (TLRs) are a group of pattern recognitions receptors detecting different pathogen-associated ligands. The TLR ligands can be divided into three categories: proteins, DNA/RNA and lipid based cell-wall components. TLRs may facilitate cooperative binding of ligands, thus exhibit a broad recognition spectrum. For example, TLR 1/2 recognizes tricylated lipoproteins whereas TLR 2/6 recognizes peptidoglycan from gram-positive bacteria. TLR 2, 3, 4, 6, 8, and 9 are expressed by MCs (Matsushima *et al.*, 2004; Takeda *et al.*, 2003). These may directly recognize a number of pathogens, leading to different MC responses. Stimulation of TLR 2 with peptidoglycan from gram-positive bacteria mediates cytokine production and degranulation, whereas stimulation with lipopolysaccharide via TLR 4 induces cytokine secretion but no degranulation (Supajatura *et al.*, 2002). Activation of MCs via TLR signaling is usually associated with cytokine, leukotriene and prostaglandin production without mediating degranulation (Marshall *et al.*, 2003; Okumura *et al.*, 2003; Varadaradjalou *et al.*, 2003). This demonstrates the importance of MCs in recruitment of inflammatory cells, for example in recruiting neutrophils to the local site of activation leading to antimicrobial responses and clearance of the invading pathogens.

1.5.4 Complement mediated activation

The complement system is composed of serum proteins and cell surface receptors that interact in a number of complex pathways in order to eliminate pathogens. The complement system operates in both innate and adaptive immunity and can be activated in three different ways; the classical pathway, the alternative pathway and the lectin pathway (Sarma & Ward, 2011). Tissue damage and different types of infections often lead to activation of these pathways. MCs can interact with the complement system by expressing complement receptor (CR) 3, CR4, C3aR and C5aR (Marshall, 2004). It is well established that C3a and C5a are MC-activating agents (Johnson *et al.*, 1975). In mice, connective tissue MCs (CTMCs), but not mucosal MCs (MMCs), express CRs and are able to respond to C3a and C5a (Mousli *et al.*, 1994). Complement-deficient mice are more sensitive and display reduced MC activation after caecal ligation and puncture (Prodeus *et al.*, 1997). Human

MCs also display different expression of receptors depending on surrounding environment. C5aR expression has been observed in skin and cardiac MCs, but not in MCs from the lungs, uterus and tonsils (Fureder *et al.*, 1995).

1.5.5 Other mechanisms of activation

In addition to the mechanisms mentioned above, MCs can be activated by other agents such as cytokines, chemokines, neuropeptides, calcium ionophores and drugs.

Several cytokines and chemokines including IL-1, IL-3, IL-8, granulocyte-macrophage colony stimulating factor, macrophage inflammatory protein (MIP)-1α, monocyte chemoattractant protein (MCP)-1 and stem cell factor (SCF) are able to induce mediator release (Mekori & Metcalfe, 2000; Taylor *et al.*, 1995; Alam *et al.*, 1994; Subramanian & Bray, 1987). The small peptide endothelin (ET)-1, a 21 amino acid peptide produced by endothelial cells, and neuropeptides such as substance P, calcitonin gene related peptide, vasoactive intestinal peptide (VIP) and neurotensin have been shown to activate MCs (Bauer & Razin, 2000; Metcalfe *et al.*, 1997). Other MC activators include the nucleoside adenosine and the opiates morphine and codeine (Mekori & Metcalfe, 2000). Many basic compounds (e.g. compound 48/80, mastoparan and polymers of basic amino acids) activate MCs directly, which leads to degranulation and histamine release (Metcalfe *et al.*, 1997). MC degranulation can also be mediated by elevating intracellular calcium levels. Calcium-mobilizing agents such as calcium ionophore (A23187) and ionomycin both have the ability to mediate MC degranulation (Metcalfe *et al.*, 1997). Furthermore, cell-to-cell contact has also been shown to have an impact on MC activation. Adhesion of activated T lymphocytes to MCs, via interaction of leukocyte function associated antigen (LFA)-1 and intracellular adhesion molecule (ICAM)-1, induces mediator release and cytokine production (Bhattacharyya *et al.*, 1998; Inamura *et al.*, 1998).

1.6 Apoptosis

MCs are long-lived cells that can regenerate after a degranulation event (Xiang *et al.*, 2001; Dvorak *et al.*, 1987; Kobayasi & Asboe-Hansen, 1969). It has been shown that the presence of SCF, the ligand for the receptor c-kit, is essential for MC survival both *in vitro* and *in vivo* (Iemura et al., 1994). Under normal conditions, the number of tissue MCs is constant. Dysregulation of apoptosis in MCs can cause accumulation of MCs, which may lead to diseases

such as mastocytosis. Therefore, induction of MC apoptosis may be an approach to treat MC-associated disorders, including asthma.

1.6.1 Apoptosis activation pathways

In order to maintain a constant number of cells in the body cell proliferation and programmed cell death occur in a controlled way. Apoptosis, the physiological process of programmed cell death, has been preserved during evolution and exists in all multicellular organisms. The process of apoptosis can be divided into two main signaling pathways, the mitochondrial/intrinsic- and the death receptor/extrinsic pathways (figure 4) (Ekoff & Nilsson, 2011). The intrinsic pathway is initiated by DNA damage, cytotoxic drugs and cytokine deprivation. When deprived of SCF, MCs have been shown to undergo apoptosis via the intrinsic pathway. Downstream signaling of the intrinsic pathway involves the Bcl-2 family of pro-apoptotic proteins (e.g. Bax, Bcl-Xs, Bik and Bad) and anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Mcl-1 and A1). Bcl-2 family members control the release of mediators of apoptosis triggered by permeabilization of the mitochondrial membrane. The extrinsic pathway is triggered by external signals from the environment that mediates signaling by surface-bound death receptors, e.g. the tumor necrosis factor (TNF) receptor family. Upon activation, these receptors interact with downstream molecules that activate a cascade of cysteine proteases called caspases. Both the intrinsic and extrinsic pathways involve intracellular activation of a family of cysteine proteases named caspases (Riedl & Shi, 2004). Activation of caspases directly or indirectly induces the morphological changes in the process of apoptosis including cell shrinking, chromatin condensation, DNA fragmentation and formation of apoptotic bodies (Kerr *et al.*, 1972). The apoptotic bodies are efficiently removed by phagocytic cells in the local tissue.

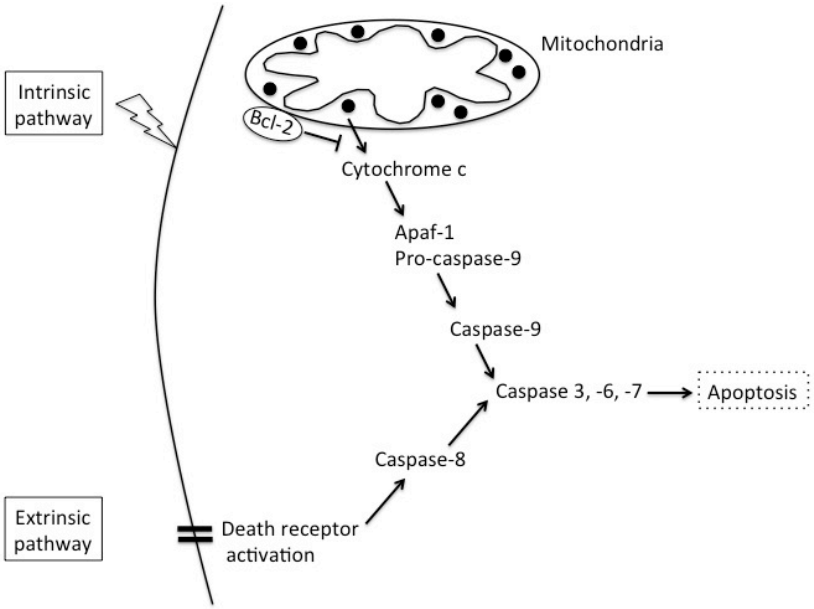

Figure 4. Intrinsic and extrinsic pathways of apoptosis.

1.7 Heterogeneity of mast cells

MCs can have different phenotypes and these are dependent on different factors including the species, environment and even age of the animal. Murine MCs are broadly divided into CTMCs and MMCs, summarized in table 1. This commonly used nomenclature for murine MCs originates from observations in rats, but the same phenotypes were also found in mice (Enerback, 1966). The CTMCs are located in the connective tissue of the skin and peritoneum and contain heparin proteoglycan, high amounts of histamine as well as the proteases: chymase, tryptase and carboxypeptidase A (CPA). MMCs are found at mucosal surfaces such as the lamina propria of the intestine and in the respiratory tract. In contrast to CTMCs, MMCs contain chondroitin sulphate proteoglycan and chymase, but do not express tryptase and CPA. Human MCs are classified according to their neutral protease content (table 1). The MC_T phenotype expresses only tryptase, whereas MC_{TC} contain tryptase, chymase, CPA and cathepsin G (Irani & Schwartz, 1994). A third, more rare, type of MC population, containing only chymase (MC_C) has also been reported (Weidner & Austen, 1993). However, it should be mentioned that combinations of MCs with different phenotypes could be found in murine as well as human tissues.

Table 1. Summary of the MC-specific protease and proteoglycan expression in humans and mice.

Species	Human		Mouse	
MC-subclass	MC_T	MC_{TC}	CTMC	MMC
Chymase	-	Chymase (human)	mMCP-4 mMCP-5	mMCP-1 mMCP-2
Tryptase	α-tryptase β-tryptase (I-III)	α-tryptase β-tryptase (I-III)	mMCP-6 mMCP-7	-
CPA	-	CPA (human)	CPA (mouse)	-
Proteoglycan	Heparin/ chondroitin sulfate	Heparin/ chondroitin sulfate	Heparin	Chondroitin sulfate

Abbreviations: MC, mast cell; mMCP, mouse mast cell protease; CPA, caboxypeptidase A. Protease name indicates expression of respective protease. (–) indicates no expression.

1.8 Pre-stored mediators in mast cell granules

Fully differentiated MCs can contain 500-1000 granules filled with pre-stored mediators such as proteoglycans, proteases, histamine and cytokines that can be released upon activation.

1.8.1 Proteases

The MC granules are composed of remarkably high amounts (up to 35%) that make up the total protein content of the cell, i.e., proteases that are active at a neutral pH (Schwartz *et al.*, 1987a; Schwartz *et al.*, 1987b). The granule-stored MCs proteases are divided into chymases, tryptases and CPA (Pejler *et al.*, 2010). The common feature for all proteases is that they cleave peptide bonds. One third of all proteases belong to a large family of proteolytic enzymes with a reactive serine (Ser) side chain. These are called serine proteases. Chymase and tryptase are serine proteases that hydrolyze bonds within the peptide chain of the substrate and are therefore known as endopeptidases. Chymases have chymotrypsin-like substrate specificity, i.e., they hydrolyze bonds after large hydrophobic amino acids such as tyrosine (Tyr), tryptophan (Trp), phenylalanine (Phe) and methionine (Met). Tryptases have trypsin-like cleavage specificity and have a preference for cleaving after the basic amino acids arginine (Arg) or lysine (Lys). CPA on the other hand is a zinc-dependent metalloprotease that cleaves bonds at the ends of peptides and is therefore an exopeptidase. Apart from the MC-specific proteases, MCs also express other proteases including lysosomal cathepsins, granzymes and cathepsin G (Pejler *et al.*, 2007).

1.8.2 Chymase

Chymases are stored in the MC granules as active monomeric enzymes in complex with heparin serglycin proteoglycan (Braga *et al.*, 2007; Henningsson *et al.*, 2006; Abrink *et al.*, 2004; Henningsson *et al.*, 2002). Chymases are activated by the proteolytic removal of an N-terminal acidic dipeptide by dipeptidyl peptidase I (DPPI) (Wolters *et al.*, 2001). Within the acidic granules, chymase is tightly bound to heparin and this is thought to prevent autolysis by the enzymes. Post degranulation, chymase remains in complex with heparin proteoglycan, which increases the enzymatic activity and offers protection from inhibitors outside the cell (Pejler & Sadler, 1999; Pejler & Berg, 1995).

Humans only express one chymase, which belongs to the group of α-chymases based on phylogenetic analyses (Chandrasekharan *et al.*, 1996; Caughey *et al.*, 1991). In mice, MMCs express two β-chymases: mouse mast cell protease (mMCP)-1 and mMCP-2 (Lunderius *et al.*, 2000; Lutzelschwab *et al.*, 1998; Huang *et al.*, 1991), although mMCP-2 lacks proteolytic activity (Pemberton *et al.*, 2003). CTMCs predominantly express the β-chymase mMCP-4 (Newlands *et al.*, 1993) and the α-chymase mMCP-5 (Huang *et al.*, 1991; Reynolds *et al.*, 1990) (table 1). mMCP-5 is the only α-chymase expressed by murine MCs and is the closest homolog to the human chymase based on sequence similarity. However, mMCP-5 has elastase-like cleavage specificity and is therefore not functionally a chymase (Karlson *et al.*, 2003; Kunori *et al.*, 2002). In contrast, mMCP-4 has a similar cleavage specificity and tissue distribution as the human chymase (Andersson *et al.*, 2008). This suggests that the functional homolog to the human chymase is mMCP-4.

In vivo and *in vitro* studies have revealed that chymase is involved in the processing of a wide array of proteins and peptides. Most attention been focused on chymase ability to cleave angiotensin I yielding angiotensin II, a peptide involved in vasoconstriction (Urata *et al.*, 1990; Reilly *et al.*, 1982). Considering that MCs are widely distributed in the connective tissue it is likely that the stored proteases may have a profound impact on the extracellular matrix (ECM). Indeed, fibronectin, an ECM component, has been shown to be a substrate for chymase (Tchougounova & Pejler, 2001; Vartio *et al.*, 1981). Further, it has been demonstrated that chymase induces apoptosis in ECM surrounded vascular smooth muscle cells (SMCs) (Leskinen *et al.*, 2001). In addition, chymase has the ability to inhibit mitogen-induced SMC proliferation (Lazaar *et al.*, 2002). Chymase can also modulate the EMC composition through the release of latent transforming growth factor β-1 (TGF-β1) from the matrix, which subsequently enhances the production of connective tissue

(Taipale *et al.*, 1995). In contrast, chymase has also been shown to be involved in ECM degradation through the activation of matrix metalloproteases and pro-collagenases. *In vivo* studies in mMCP-4-deficient mice provided support for this notion as these mice have an increased collagen-deposition in the skin (Tchougounova *et al.*, 2005). Chymase has been suggested to be involved in regulating the levels of other biological factors including ET-1 (Kido *et al.*, 1998), IL-1β (Mizutani *et al.*, 1991) and to cleave membrane-bound SCF mediating its release from the cell surface (de Paulis *et al.*, 1999). Taken together, chymase may have pro-inflammatory properties through the activation of various substances as well as anti-inflammatory by degrading others.

Chymase knockout mice and in vivo roles

To date, three chymase knockout mice have been generated: mMCP-1, mMCP-4 and mMCP-5. mMCP-1 is primarily expressed by intestinal MMCs and mice lacking this chymase have a delayed parasite expulsion in the intestine compared to WT mice (Knight *et al.*, 2000). The mMCP-4 knockout mice have a deletion in exon 1 of the chymase gene, which results in a complete loss of the protein (Tchougounova *et al.*, 2003). These mice have been used in several disease models to evaluate the *in vivo* functions of mMCP-4. For instance, a pathological role for mMCP-4 in experimental arthritis has been suggested since mMCP-$4^{-/-}$ mice had decreased passive and active collagen-induced arthritis compared to WT mice (Magnusson *et al.*, 2009). In addition, mMCP-4 contributes to the development of abdominal aortic aneurism (Sun *et al.*, 2009). mMCP-$4^{-/-}$ mice show increased skin blistering compared to WT mice in a model of bullous pemphigoid, suggesting a role for mMCP-4 in ECM and hemidesmosome degradation (Lin *et al.*, 2011). It is well known that MCs are important cells in the innate defense, and a role for mMCP-4 in reducing toxicity of Gila monster and scorpion venom was recently published (Akahoshi *et al.*, 2011). mMCP-4 is involved in maintaining homeostasis in the intestinal epithelium by regulating barrier properties and migration across the epithelium (Groschwitz *et al.*, 2009). mMCP-5 has been shown to contribute to ischemia reperfusion-induced injury of skeletal muscle (Abonia *et al.*, 2005). However, mMCP-5 knockout mice also lack CPA, which makes it difficult to interpret the data. A strategy for overcoming this issue would be to generate genetically targeted mice with a mutation in the active site of mMCP-5.

Several reports suggest that chymase has pro-inflammatory properties. For example, injection of chymase mediates accumulation of eosinophils and neutrophils *in vivo* (Terakawa *et al.*, 2005; He & Walls, 1998). A

polymorphism in the promoter region of the chymase gene has been associated with allergic asthma, possibly through regulation of IgE levels (Sharma *et al.*, 2005). There is also evidence suggesting a protective role for chymase in severe asthmatics, where the presence of chymase in the small airways correlates with preserved airway function (Balzar *et al.*, 2005).

1.8.3 Tryptase

Tryptases are serine proteases with trypsin-like substate specificity active in a tetrameric form. Tryptases, like chymases, are stored in complex with serglycin proteoglycan within the MC granules as active enzymes (Hallgren *et al.*, 2001; Schwartz & Bradford, 1986). Human MCs mainly express two types of tryptases: α- and β-type. β-tryptases are the main form of tryptases found in MCs and they are the most catalytically active (Marquardt *et al.*, 2002; Huang *et al.*, 1999). So far, three different forms of β-tryptases have been identified: βI, βII and βIII (Pallaoro *et al.*, 1999). The β-tryptases are very similar to each other and βI and βII differ in only one amino acid. The α-tryptases are further classified into αI and αII. In contrast to the β-tryptases, αI-tryptases can be detected in the circulation in absence of MC degranulation, which suggests that they are constitutively secreted (Schwartz *et al.*, 1995). Human tryptases also include δ-tryptase and the membrane anchored γ-tryptase form (Hallgren & Pejler, 2006).

To date, mice have been found to express four tryptases: mMCP-6, mMCP-7, mMCP-11 and mouse transmembrane tryptase (mTMT). mMCP-6, expressed by CTMCs, is the most similar to the human β-tryptases (Hallgren *et al.*, 2000). mMCP-6 and mMCP-7 are not constitutively secreted, but stored in MC granules and become exocytosed locally upon degranulation (Ghildyal *et al.*, 1996). mMCP-11 and mTMT have both been found to be mainly expressed during the early stages of MC development (Wong *et al.*, 2004; Wong *et al.*, 1999).

Like chymase, tryptase has been suggested to degrade a number of ECM components including fibrinogen (Schwartz *et al.*, 1985), fibronectin (Lohi *et al.*, 1992), type VI collagen (Kielty *et al.*, 1993) and to activate pro-MMP-3 (Gruber *et al.*, 1989). In addition, tryptase has been shown to activate protease-activated receptor (PAR)-2, which may lead to inflammatory events (Berger *et al.*, 2001). There are a number of studies suggesting a role for MC tryptase in allergic asthma. Tryptase stimulates the proliferation of SMCs, epithelial cells and fibroblasts, which may lead to features associated with asthma including AHR and remodeling events of the airways (Gruber *et al.*, 1997; Cairns &

Walls, 1996). Additionally, tryptase can degrade VIP, which, in the lungs, acts to relax bronchial smooth muscle (Caughey *et al.*, 1988). Further support for a pathological role of tryptase in asthma came from studies where tryptase inhibitors blocked airway inflammation and AHR in allergic sheep (Clark *et al.*, 1995). Incubation of isolated human bronchi with tryptase also mediates histamine release and promotes a subsequent *in vitro* bronchial reactivity to histamine (Berger *et al.*, 1999).

1.8.4 Carboxypeptidase A

CPA is an exopeptidase expressed by MCs and similarly to the other proteases, is stored as an active enzyme in the granules. CPA has a high content of positively charged amino acids that enable binding to negatively charged serglycin proteoglycan within the granules. It has been suggested that both CPA and chymase remain in complex with serglycin proteoglycan after degranulation. To date, only one CPA gene has been identified and its expression varies among the MC subtypes. In humans, CPA is only expressed in the MC_{TC} subtype and in mice CPA is restricted to CTMCs (Reynolds *et al.*, 1989). CPA has a preference for cleaving C-terminal aromatic or aliphatic residues (Vendrell *et al.*, 2000; Goldstein *et al.*, 1989). Interestingly, substrate processing by chymase generates products with a C-terminal cleavage preference for CPA.

CPA has been shown to be involved in the degradation of ET-1, a 21 amino acid peptide involved in septic shock and the development of high blood pressure (Metz *et al.*, 2006; Metsarinne *et al.*, 2002). In addition, CPA has an important role in protecting against the snake venom toxin, sarafotoxin, by preventing its toxicity through C-terminal degradation (Metz *et al.*, 2006).

1.8.5 Cathepsin G

Cathepsin G is a serine protease mainly expressed in neutrophils but has also been found in MCs. Humans and rodents express one cathepsin G protein, originating from the cathepsin G gene (*CTSG*) (Schechter *et al.*, 1990). The cleavage properties for cathepsin G are broader compared to the other serine proteases as it possesses both chymotryptic as well as tryptic activities, i.e., cleavage after aromatic and basic amino acids (Polanowska *et al.*, 1998).

1.8.6 Proteoglycans

Proteoglycans are ubiquitously expressed and highly abundant proteins with a variety of functions. For example, proteoglycans are involved in embryological development and functions in most organ systems of the body, including the

immune system (Handel *et al.*, 2005; Iozzo, 2005). Proteoglycans are composed of a core protein with covalently linked long, unbranched glycosaminoglycan (GAG) chains. Proteoglycans can be broadly divided into three subgroups i.e. the cell-surface spanning (syndecans and glypicans), the ECM associated (decorin, agrin and perlecan) and the intracellular proteoglycan, serglycin. Serglycin is synthesized by a number of hematopoietic cells including MCs, macrophages, lymphocytes, platelets and natural killer cells (Pejler *et al.*, 2009).

Serglycin proteoglycan consists of a 17.6 kDa core protein with an extended amino acid repeat of serine/glycine (Ser/Gly), where the Ser residues function as GAG attachment sites (Ronnberg *et al.*, 2012). The main GAGs found in connection with serglycin PGs are heparin/heparan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate and chondroitin sulfate B and E (Table 2) (Kolset & Tveit, 2008). The different combinations of GAG chains enable a multifaceted biological activity of serglycin proteoglycan.

Table 2. *Serglycin GAG chain expression in granulated cells (adapted from Kolset 2008).*

GAG chain	Cell type
Heparin/Heparan sulfate	MCs, macrophages
Chondroitin 4-sulfate	Platelets, monocytes, lymphocytes, natural killer cells
Chondroitin 6-sulfate	Guinea pig platelets
Chondroitin sulfate E	MCs, macrophages
Chondroitin sulfate B	Rat basophils

Heparin, the most well known GAG and one of the most negatively charged molecules in the body, is bound to serglycin in CTMCs (Kolset & Gallagher, 1990). Because of its highly negatively charged nature heparin can interact with a number of proteins, including the pre-stored proteases in the MC granules. In contrast to CTMCs, MMCs serglycin carries the less negatively charged chondroitin sulfate type GAGs (Enerback *et al.*, 1985). Human MCs can have both heparin as well as chondroitin sulfate GAG chains bound to the serglycin core protein.

Studies in knockout mice have demonstrated that serglycin proteoglycan serve as storage matrices for several of the proteases in the MC secretory granules (Abrink *et al.*, 2004). CTMCs deficient in serglycin proteoglycan have defective staining with cationic dyes and altered storage of a number of granule

compounds including mMCP-4, mMCP-5, mMCP-6, CPA as well as the biogenic amines histamine and serotonin (Ringvall *et al.*, 2008; Braga *et al.*, 2007; Abrink *et al.*, 2004). MCs lacking glucosaminyl N-deactylase/N-sulfotransferase 2 (NDST2), an enzyme involved in the initial step of heparin sulphation, have altered secretory granule protease storage due to the lack of highly negatively charged heparin (Forsberg *et al.*, 1999; Humphries *et al.*, 1999). Thus, serglycin proteoglycan and negatively charged heparin play a major role in the storage of a number of positively charged MC granule proteins.

Upon MC degranulation, serglycin is exocytosed in complex with compounds dependent on serglycin for storage, as well as with mediators that are independent on serglycin for their storage (figure 5) (Schwartz *et al.*, 1981). Histamine is dependent on serglycin for storage, but detaches from serglycin upon exocytosis because of the increase in pH outside the acidic granules. However, some proteases remain in complex with serglycin proteoglycan after their release, which may promote protease activity by enabling the close proximity of the enzymes with their heparin-binding substrates (Kolset *et al.*, 2004; Pejler & Sadler, 1999). For example, chymase and CPA remain bound to serglycin proteoglycan after degranulation and together may exert their biological functions at the site of MC activation. In addition to the biological functions mediated by serglycin-depedent proteases, serglycin is a ligand for CD44, a transmembrane glycoprotein involved in a number of cellular processes including regulation of growth, differentiation, motility and survival (Toyama-Sorimachi *et al.*, 1995). Other biological roles of serglycin may include protection of the serglycin-interacting proteins against proteolytic degradation, binding of inflammatory compounds as regulators of immune responses and delivering compounds to targets cells. The last notion was suggested in cytotoxic T lymphocytes, where granzyme B is released in complex with serglycin proteoglycan and delivered to the target cells to enable subsequent apoptosis (Froelich *et al.*, 1996).

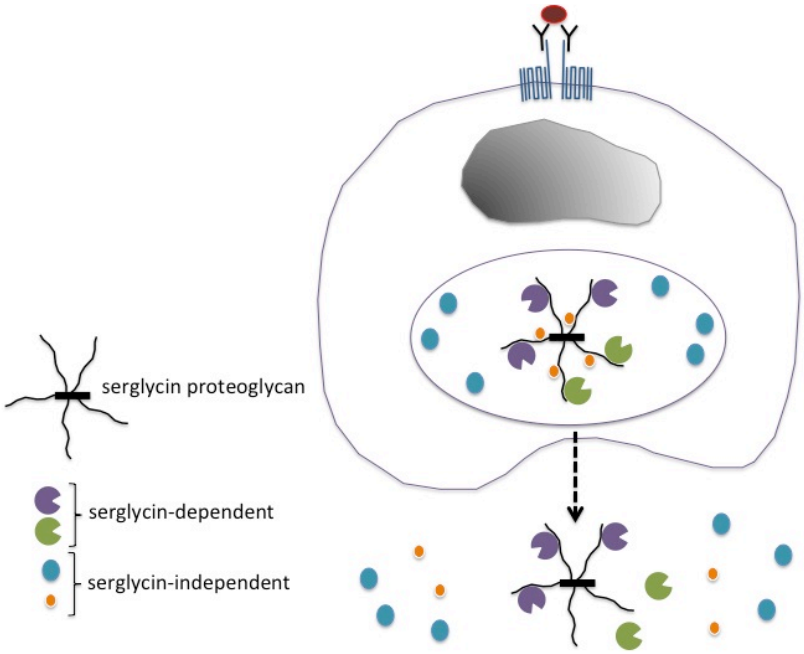

Figure 5. Mast cell granule storage and release of serglycin-dependent and serglycin-independent mediators.

In vivo studies using serglycin-deficient mice reported that *Klebisella pnemoniae* infection was less effectively cleared in the absence of serglycin proteoglycan (Niemann *et al.*, 2007). This could possibly be explained by the role of serglycin in neutrophil-elastase storage. It has also been shown that serglycin$^{-/-}$ mice show age-related enlargement of lymphoid organs including the spleen, Payer's patches and bronchus-associated lymphoid tissue (Wernersson *et al.*, 2009). This suggests a role for serglycin in maintaining homeostasis of the leukocyte populations, possibly through differentiation and/or apoptosis.

1.8.7 Biogenic amines

The biogenic amine histamine was discovered in the early 1900's and has been associated with many pathological and physiological conditions. For example, histamine mediates inflammation, increases vascular permeability, acts on SMCs, stimulates gastric acid secretion in the gastrointestinal tract and is a neurotransmitter in the CNS (Bachert, 2002). Histamine exerts its effects through the histamine receptors H1, H2, H3 and H4, which belong to the family of G-protein coupled receptors (Haaksma *et al.*, 1990; Hill, 1990).

Drugs targeting histamine receptors have successfully been in use since the 1940s. A number of cells including SMCs and endothelial cells express H1 receptors. The main function of H2 receptors is to stimulate the release of gastric acid. The H3 receptors are primarily expressed in the nervous system. Many hematopoietic cells, including MCs, express the H4 receptor. MCs also express receptors H1 and H2 receptors, and activation of these has an impact on MC mediator release (Lippert *et al.*, 2004).

1.9 *De novo* synthesized mediators

1.9.1 Eicosanoids

In addition to the pre-stored mediators, MCs also *de novo* synthesize a number of inflammatory mediators from the eicosanoid family. MC activation has been shown to generate production of prostaglandin D_2 (PGD_2) leukotriene B_4 (LTB_4) and LTC_4 (Boyce, 2007).

PGD_2, LTB_4 and LTC_4 are all derived from arachidonic acid released from phospholipid cell membranes by phospholipase A (Funk, 2001). The subsequent step in generating PGD_2 is conversion of arachidonic acid to an intermediate, PGH_2, by cyclooxygenase enzymes (COXs). PGH_2 is then converted to PGD_2 by specific synthases. A number of leukocytes act chemotactically to PGD_2 and mouse models of allergic airway inflammation suggest a pathological role for PGD_2 (Honda *et al.*, 2003; Fujitani *et al.*, 2002). In support of this idea, it has been shown that PGD_2 mediates bronchoconstriction via a Gq-coupled thromboxan receptor (Johnston *et al.*, 1992). In human asthmatics, increased levels of PGD_2 have been observed in BAL-fluids (Wenzel *et al.*, 1989). LTB_4 and LTC_4 are synthesized through a pathway in which the first step, arachidonic acid to LTA_4 conversion, is catalyzed by the enzyme 5-lioxygenase (5-LO) (Malaviya & Jakschik, 1993). LTA_4 can be then be converted to either LTB_4 (by LTA4-hydrolase) or LTC_4 (by LTC_4 synthase) (Lam *et al.*, 1994; Evans *et al.*, 1985). Both LTB_4 and LTC_4 are released and actively transported from the cell. Extracellularly, LTC_4 is converted to LTD_4 and subsequently LTE_4. Collectively, LTC_4, LTD_4 and LTE_4 are referred to as cysteinyl leukotrienes, where LTE_4 is the least reactive but most stable of the three (Boyce, 2007). Like PGD_2, leukotrienes mediate their biological effects via G-protein coupled receptors. Leukotrienes exert pro-inflammatory properties acting as a chemoattractants on a number of inflammatory cells, including MC progenitors (Weller *et al.*, 2005). Similarly to PGD_2, allergen-challenged asthmatics have increased levels of cysteinyl leukotrinenes in their BAL-fluid and cysteinyl leukotrienes mediate contraction

of the bronchi as well as pulmonary vascular smooth muscle (Wenzel *et al.*, 1990; Hanna *et al.*, 1981; Dahlen *et al.*, 1980).

1.9.2 Cytokines and chemokines

MCs are known to produce a number of pro-inflammatory as well as anti-inflammatory cytokines. Different routes of MC activation releases cytokines including IL-1β, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-33, basic fibroblast growth factor (bFGF)-2, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TGF-β1 (Ishizuka *et al.*, 1999; Kanbe *et al.*, 1999a; Kanbe *et al.*, 1999b; Qu *et al.*, 1998; Okayama *et al.*, 1995; Razin *et al.*, 1984). The majority of the secreted cytokines are newly synthesized, however, MCs are also capable of pre-storing cytokines in their granules, which was initially shown with tumor necrosis factor α (TNFα) (Walsh *et al.*, 1991; Young *et al.*, 1987). There is also evidence that MCs can store IL-4 (Gibbs *et al.*, 1997), SCF (Zhang *et al.*, 1998), TGFβ (Lindstedt *et al.*, 2001) and nerve growth factor (Leon *et al.*, 1994) in their secretory granules. Chemokines belong to a cytokine family of chemotactic proteins that are involved in trafficking and recirculation of various immune cells. They can also stimulate many immune cells including T cells, eosinophils and monocytes to produce cytokines. MCs secrete the chemokine IL-8 that acts to recruit neutrophils (Kasahara *et al.*, 1998). In addition, MCs also express MCP-1 and RANTES that are monocyte/macrophages chemoattractants (Ono *et al.*, 2003).

2 Present investigations

2.1 Aims of the present studies

The general aim has been to evaluate the role of serglycin proteoglycan and MC proteases in allergic disease and apoptosis. Specific attention was paid to the MC chymase, mMCP-4, and its role in allergic airway inflammation. More specifically, this thesis aims to:

- Investigate the role of mMCP-4 in ovalbumin (OVA)-induced allergic airway inflammation (**paper I**).

- Investigate the role of mMCP-4 and evaluate mechanisms by which mMCP-4 regulates airway inflammation induced by house dust mite (HDM)-extract (**paper II**).

- Determine how MC proteases regulate levels of the asthma-related cytokine IL-13 *in vitro* (**paper III**).

- Study the impact of serglycin proteoglycan-associated proteases on apoptosis induced by granule permeabilization (**paper IV**).

2.2 Results and Discussion

In this section, the main results from papers I – IV are summarized.

2.2.1 Paper I: Mouse mast cell protease 4 is the major chymase in murine airways and has a protective role in allergic airway inflammation.

MCs are known to be key effector cells in IgE-associated immune responses e.g. allergy and asthma. Activation of MCs by cross-linking of IgE bound to FcεRI receptors leads to the release of large amounts of newly synthesized and granule-stored pro-inflammatory mediators. These include histamine, serglycin proteoglycan and proteases such as chymases, tryptases and CPA.

The contribution of chymase in the context of allergic asthma is not completely understood. Therefore, defining the role of chymase is important for a deeper understanding of the molecular mechanisms of how MCs contribute to the disease. In paper I, the main aim was to define the role of MC chymase (mMCP-4) in a murine model of allergic airway inflammation.

The selection of a model for studying MCs in allergic airway inflammation is not trivial. Strong immunization protocols, including adjuvants, may diminish the role of MCs. Conversely, weak immunization protocols, using only the allergen for sensitization and provocation, have been shown to reveal a significant role for MCs in the development of allergic airway inflammation (Taube *et al.*, 2004; Kobayashi *et al.*, 2000; Williams & Galli, 2000). Therefore, we decided to use an acute model of allergic airway inflammation with immunizations/provocations with the antigen alone. This model involved seven intra peritoneal (i.p.) immunizations (sensitization) with OVA on day 1, 3, 6, 8, 10, 13 and 15 followed by three intra nasal (i.n.) challenges with OVA on day 31, 34 and 36.

Airway inflammation is a feature of asthma, and this is characterized by infiltration of eosinophils and other inflammatory cells to the airways. In both WT and mMCP-4$^{-/-}$ mice, OVA sensitization and challenge induced a markedly larger number of inflammatory cells in the BAL fluid. Differential count of BAL cells showed an increase mainly in the number of eosinophils, but also the number of lymphocytes and neutrophils. However, there were no significant differences when comparing BAL cells of WT and mMCP-4$^{-/-}$ mice. OVA-induced lung tissue inflammation was seen in both WT and mMCP-4$^{-/-}$ mice. Interestingly, lung tissue inflammation was more pronounced in the

absence of mMCP-4. These data suggests a role for mMCP-4 in regulating tissue inflammation.

Lung function analyses revealed that OVA sensitized/challenged mMCP-4$^{-/-}$ mice exhibited significantly higher average lung resistance (R_L) than corresponding WT mice in response to i.v. methacholine. Neither OVA sensitized/challenged WT mice, nor OVA sensitized control groups showed any AHR. The absence of AHR in OVA sensitized/challenged WT mice could possibly be explained by the use of the known low-responder C57BL/6J stain and weak immunization protocol. These results show that the presence of mMCP-4 protects from development of AHR in this model of allergic airway inflammation.

Genetic inactivation of mMCP-4 leads to a complete loss of chymotryptic activity in the peritoneum and ear tissue (Tchougounova *et al.*, 2003). However, there are several chymases expressed in mice. To further investigate the effect of mMCP-4 on chymotryptic activity in lung tissue we stained sections with the chloroacetate esterase assay. As shown by intense red staining of the MCs, chymotrypsin-like activity was detected in the lungs from WT mice compared to weak staining found in mMCP-4$^{-/-}$ mice. This finding shows that mMCP-4 is the major enzyme with chymotrypsin-like activity in murine lungs.

Airway inflammation in asthmatics may be accompanied by hyperplasia or hypertrophy of the smooth muscle layer in the lungs (Cockcroft & Davis, 2006). Chymase has previously been shown to regulate apoptosis in ASM by a secondary effect of fibronectin degradation (Leskinen *et al.*, 2003). In support of this notion, we detected fibronectin fragments in the lungs from WT, but not in mMCP-4$^{-/-}$ mice. Additionally, chymase has been shown to degrade the pericelullar matrix of ASM and inhibit mitogen-induced ASM proliferation (Lazaar *et al.*, 2002). In our model, OVA sensitization/challenge induced an increased ASM thickening in mMCP-4$^{-/-}$ mice but not in the corresponding WT mice, suggesting that mMCP-4 is involved in the regulation of ASM hyperplasia/hypertrophy. We therefore investigated whether mMCP-4 could cleave SMC mitogens. *In vitro* studies showed that mMCP-4 could cleave both platelet derived growth factor (PDGF)-BB and FGF. A possible explanation for the protective role of mMCP-4 in allergic asthma could be via the degradation of SMC mitogens or ECM components. However, the broad substrate specificity for mMCP-4 suggests that other protective mechanisms may also be involved.

To summarize, this study shows that the presence of mMCP-4 has an effect on airway reactivity to methacholine, tissue inflammation and ASM thickening in this model of allergic airway inflammation.

Summary (Paper I)

- Presence of mMCP-4 protected from the development of AHR and tissue inflammation in this OVA-induced model of allergic airway inflammation.
- As shown by chloroacetate esterase staining, mMCP-4 was the major chymotryptic enzyme in the murine airway MCs.
- ASM layer thickening was observed in OVA-sensitized and -challenged mMCP-4$^{-/-}$ mice.

2.2.2 Paper II: Mast cell chymase modulates IL-33 levels and controls allergic sensitization in dust-mite induced airway inflammation.

In paper I, we showed a protective role of the MC chymase, mMCP-4, in an acute model of allergic airway inflammation. However, the mechanism for this finding was not completely clear. Together with the search for the protective properties of mMCP-4 demonstrated in paper I, the objective of this study was also to investigate the role of mMCP-4 in a more chronic and physiologically relevant model of airway inflammation. HDMs are one of the most prominent airborne allergens causing asthma in humans. In murine models, repeated i.n. exposure of HDM generates features of airway inflammation similar to its human counterpart (Fattouh *et al.*, 2005). It has been shown that after continuous exposure to OVA, mice develop tolerance to the allergen rather than show features of chronic airway inflammation. On the other hand, repeated exposure of HDM induces a robust eosinophilic pulmonary inflammation, production of IgE-antibodies as well as airway reactivity to methacholine. Based on this information, we decided to use a HDM-induced model of pulmonary inflammation.

I.n. exposure of HDM-extract twice weekly for three weeks induced BAL and lung tissue eosinophilia, and this was significantly higher in mMCP-4$^{-/-}$ mice. The inflammatory response was accompanied by a significantly higher R_L to inhaled methacholine in these mice. Increased AHR may correlate with ASM hypertrophy and an increase in ASM thickness was found in HDM-treated mMCP-4$^{-/-}$ mice. In agreement with our pervious data (paper I), the presence of mMCP-4 limited airway inflammation, AHR and ASM thickening. In this

model mMCP-4 also contributed to the sensitization process, shown by the significantly higher IgE-levels in HDM-treated mMCP-4$^{-/-}$ compared to the corresponding WT mice. *In vitro* re-stimulation of splenocytes with HDM-extract demonstrated an increase in the IL-13 and IL-17A cytokine production, and this increase was more pronounced in mMCP-4$^{-/-}$ mice.

HDM allergens can induce MC activation and degranulation in the absence of allergen-specific antibodies (Machado *et al.*, 1996). In agreement with previous reports, we showed that peritoneal MCs degranulate and release both histamine and β-hexosaminidase in response to HDM-extract. We also found that chymase activity was detected in cultures of HDM-stimulated WT peritoneal cell-derived mast cells (PCMCs), yet almost absent in mMCP-4$^{-/-}$ PCMCs. This shows that mMCP-4 accounts for almost all chymase activity in peritoneal MCs. In murine lungs, the presence of mMCP-4 is essential for detection of chymotrypsin-like activity using the chloroacetate esterase assay (paper I). Together, these findings suggest that mMCP-4 is secreted in murine lungs post HDM-challenge.

Production of T_H2 cytokines is a characteristic of allergic airway responses. As described above, i.n. exposure of HDM-extract induced recruitment of inflammatory cells to the airways of the treated groups, which may be accompanied by increased levels of inflammatory mediators in the lung tissue. Therefore, we measured the levels of different T_H2 cytokines in lung homogenates. We did not detect any significant increases in the levels of T_H2 cytokines IL-5, IL-13 and thymic stromal lymphopoietin (TSLP). In contrast, HDM-treated mMCP-4$^{-/-}$ mice exhibited increased levels of IL-33 in the lungs, compared with corresponding WT mice as well as mMCP-4$^{-/-}$ controls. Chymase has relatively broad cleavage specificity and previous studies have shown that chymase can cleave a number of inflammatory mediators, ECM components, lipoproteins and angiotensin I. Our *in vitro* studies revealed that WT PCMCs degrade IL-33 more effectively than mMCP-4$^{-/-}$ PCMCs. Additionally, inhibitory studies showed that IL-33 degradation by PCMCs is blocked by Pefabloc SC, a serine protease inhibitor. These data demonstrate that mMCP-4, together with other serine proteases, contribute to IL-33 degradation *in vitro*.

In conclusion, we propose that the local secretion of mMCP-4 by MCs in response to HDM allergens dampens allergic airway inflammation, possibly through the effects on IL-33. Our results indicate that different MC mediators may have inflammatory or regulatory functions at sites of allergic

inflammation. This may be of clinical interest, in particular for approaches to target specific MC mediators in allergic asthma.

Summary (Paper II)

- Lack of mMCP-4 resulted in a higher R_L to inhaled methacholine in a HDM-model of asthma.
- Recruitment of inflammatory cells to BAL and lung tissue was enhanced in HDM-treated mMCP-$4^{-/-}$ mice.
- Lung tissue levels of IL-33 were enhanced in mMCP-$4^{-/-}$ mice but not in WT mice exposed to HDM-extract.
- *In vitro*, WT PCMCs degraded IL-33 more efficiently than mMCP-$4^{-/-}$ PCMCs and this degradation was blocked by a serine protease inhibitor.

2.2.3 Paper III: Mast cells limit extracellular levels of IL-13 via a serglycin proteoglycan-serine protease axis.

Some cells release pre-stored mediators in response to various stimuli thereby exerting their biological functions. MCs store large amounts of active proteases in their secretory granules. Studies in knockout mice have demonstrated that serglycin proteoglycan is implicated in the storage of several of the MC proteases, e.g. mMCP-4, mMCP-5, mMCP-6 and CPA. Chymase, tryptase and CPA belong to the abundant proteases stored in the MC granules, and may therefore have a large impact on many physiological and pathological processes upon degranulation.

The objective of this study was to investigate whether MCs deficient in serglycin-proteoglycan or in various serglycin-dependent proteases could regulate local levels of IL-13. Peritoneal cells from WT and different knockout strains were cultured *in vitro* in order to generate homogenous populations of MCs (Malbec *et al.*, 2007). Exogenous IL-13 was added to the cultures.

After activation with calcium ionophore, WT MCs reduced the levels of IL-13 in the cell supernatant whereas serglycin$^{-/-}$ MCs totally lacked this ability. Inhibitory studies demonstrated that proteolytic degradation of IL-13 was completely blocked by a serine protease inhibitor. Further inhibitory studies showed that degradation of IL-13 was dependent on the interaction of the serine proteases with heparin, since the heparin antagonist protamine blocked the proteolytic activities of the activated MCs. Additional studies with PCMCs deficient in various proteases revealed that CPA$^{-/-}$ PCMCs were unable to

degrade IL-13. However, cells expressing an inactive form of CPA exhibited IL-13 degradation similar to WT cells. In addition, studies using a metalloprotease inhibitor had no effect on the proteolytic degradation of IL-13. The exact mechanism behind these findings remains intriguing.

The results in this study show that proteolytic degradation of IL-13 by PCMCs is mediated by a serine protease, dependent on serglycin proteoglycan for its storage. Although MCs are mainly considered as effector cells during allergic responses, the present study provides an indication that serglycin-dependent serine proteases in MCs may also have immunomodulatory functions *in vivo*, down-regulating inflammatory responses through degradation of inflammatory cytokines.

Summary (paper III)

- WT PCMCs effectively reduced levels of exogenously added IL-13 *in vitro* after degranulation.
- Degradation of IL-13 by MCs was dependent on the presence of serglycin proteoglycan and CPA.
- Blocking of serine protease activity inhibited proteolytic degradation of IL-13.

2.2.4 Paper IV: A role for sergycin proteoglycan in mast cell apoptosis induced by a secretory granule-mediated pathway.

In this study we investigated the role of serglycin proteoglycan in cell death induced by H-Leu-Leu-OMe (LLME), a lysosomotropic agent shown to mediate apoptosis in a number of hematopoietic cells. Lysosomal membrane permeabilization often leads to apoptosis through the release of lysosomal proteases. This is followed by proteolytic activation of pro-apoptotic compounds as well as degradation of anti-apoptotic molecules. MC granules are similar to lysosomes in terms of membrane composition but also in their storeage of large amounts of proteases. Therefore we hypothesized that LLME-treatment of MCs may have an impact on cell death mediated via a lysosome/granule components-pathway.

In order to evaluate how serglycin proteoglycan affects cell death in MCs we cultured bone marrow-derived mast cells (BMMCs) *in vitro*. The fractions of apoptotic and necrotic/late-stage apoptotic cells were determined using staining for annexin V and propidium iodide (PI) respectively. After a 4h incubation

with LLME, at different concentrations, we showed that serglycin-deficient BMMCs were more resistant to apoptosis compared to WT BMMCs. This suggests that serglycin proteoglycan is involved in apoptosis, mediated via a secretory-granule pathway.

Acridine orange, a weak basic dye, is taken up by living cells and accumulates in acidic compartments such as lysosomes and secretory granules. High concentrations of acridine orange give rise to an orange fluorescence, whereas low concentrations yield a green fluorescent signal. To evaluate granule damage by LLME, WT and serglycin$^{-/-}$ BMMCs were stained with acridine orange and their fluorescence was analyzed. WT BMMCs exhibited a reduction in acridine orange staining intensity and cellular decomposition after LLME treatment, whereas serglycin$^{-/-}$ BMMCs showed a markedly reduced susceptibility to LLME treatment. Using a substrate for cysteine cathepsins, we detected protease activity in cytosolic extracts from WT BMMCs after LLME-treatment, but the cleaving activity was minimal in LLME-treated serglycin$^{-/-}$ BMMCs. Inhibitory studies revealed that cysteine cathepsins and serine proteases were released into the cytosol. These data demonstrate that apoptosis induced by LLME mediates the release of proteases into the cytosol and that presence of serglycin is essential for the proteolytic activity. Activation of caspase-3 plays a central role in apoptosis mediated by a number of pathways. Therefore, caspase-3 activity was analyzed in WT and serglycin$^{-/-}$ BMMCs in response to LLME. The WT BMMCs showed a substantial increase in caspase-3 activity, whereas activity in serglycin$^{-/-}$ BMMCs was at base-line levels except for a slight increase in activity at the highest concentrations of LLME.

MC proteases mMCP-4, mMCP-5, mMCP-6 and CPA are dependent on serglycin proteoglycan for storage in the secretory granules. To evaluate if the separate proteases could be involved in apoptosis mediated by secretory granule permeabilization we used knockout mice deficient in either of these proteases to generate BMMCs. These studies revealed that BMMCs deficient in mMCP-4, mMCP-6 or CPA were slightly less susceptible to apoptosis. Therefore, these proteases may account for some of the serglycin-dependent resistance to LLME-induced apoptosis.

This study uncovers the importance of serglycin proteoglycan in a lysosome/secretory granule pathway of apoptosis.

Summary (paper IV)

- Compared to WT BMMCs, serglycin$^{-/-}$ BMMCs were more resistant to apoptosis mediated by LLME and exhibited reduced protease activity in the cytosol as well as caspase-3 activity.
- As shown by inhibitory studies, apoptosis induced by secretory granule damage was dependent on cysteine cathepsins.
- LLME-mediated apoptosis was slightly reduced in BMMCs deficient for the serglycin-dependent proteases mMCP-4, mMCP-6 or CPA, indicating that MC proteases could contribute to the process.

3 Concluding remarks and future perspectives

MCs are key effector cells in allergic reactions but also important players in the innate defense against various pathogens. The striking feature of MCs are their secretory granules densely packed with various pre-formed mediators including proteases and proteoglycans. Large amounts of fully active proteases are released upon degranulation and these may therefore have a profound influence in different settings. The contribution of specific MC proteases remains to be clarified further. Therefore, the use of different knockout strains can contribute to a deeper understanding of the *in vivo* roles of specific proteases.

The first two studies of this thesis have focused on the biological role of MC chymase in allergic asthma. Animal models for human asthma can provide a useful understanding of the molecular mechanisms behind the disease. In the different murine models selected we used OVA or HDM-extract as allergens. Our models mimic several of the features in human allergic asthma, such as AHR, recruitment of eosinophils to the lungs and production of IgE antibodies. We have presented evidence that mMCP-4, the functional homolog to the human chymase, has a protective role in the disease, preventing eosinophilia and AHR, and this could possibly be explained by the defective ability to degrade the pro-inflammatory cytokine IL-33. Taken together, our findings suggest that MCs can possess both detrimental as well as protective properties in the development of allergic airway inflammation. We will continue to investigate the mechanism behind the increased IgE levels in mMCP-4$^{-/-}$ mice after HDM-treatment. It will be of particular interest to study whether chymase affects dendritic cell activation. Since our data provides information that the chymase has a protective role in the development of airway inflammation, it would also be of great interest to study the effects of chymase inhibitors in

these models. Today, guidelines for treatment of asthma mainly involve inhaled glucocorticoids and β_2-adrenoreceptor agonists. Selective protease inhibitors have been discussed as therapeutic applications. Chymase inhibitors are effective in reducing abdominal aortic aneurism and myocardial infarction (Tsunemi *et al.*, 2004; Hoshino *et al.*, 2003; Jin *et al.*, 2003). However, it is important to keep in mind that the MC proteases may have different roles depending on the physiological context and the aim is not to profoundly interfere with their beneficial functions. From the *in vitro* studies presented in this thesis MCs degrade IL-13, and this is dependent on serglycin proteoglycan. These data indicate a role for serglycin-dependent proteases in controlling extracellular levels of pro-inflammatory cytokines. We will look further into whether this is a specific/selective degradation using other cytokines and chemokines with pro- and anti-inflammatory properties. In summary, unraveling the underlying mechanisms for the protective role of mMCP-4 in allergic airway inflammation will hopefully provide new insights into the roles of MCs and chymase in asthma.

The intracellular proteoglycan serglycin is expressed by many hematopoietic immune cells. In MCs, serglycin proteoglycan is essential for the proper storage of a number of granule-stored mediators including chymase, tryptase and CPA. The *in vitro* studies presented in this thesis suggest a role for serglycin proteoglycan in MC apoptosis mediated by secretory granule damage. Since serglycin proteoglycan is expressed by a number of other hematopoietc cell types it is of interest to study whether serglycin affects apoptosis in for example macrophages, neutrophils or cytotoxic T cells.

The studies included in this thesis will hopefully provide a deeper understanding in some of the roles MCs and MC mediators play in allergic disease and apoptosis.

4 Populärvetenskaplig sammanfattning

Mastcellen är ofta en av de första immuncellerna som kommer i kontakt med de bakterier, virus och allergener vår kropp utsätts för dagligen. De finns rikligt representerade i vår hud och slemhinnor, såsom lungor och mag-tarmkanalen. Mastcellen innehåller en stor mängd sekretoriska vesiklar s.k. granula som vid aktivering kan frisättas och innehållet, bl.a. histamin, proteaser och proteoglykaner utsöndras i den närliggande miljön. Effekterna vid mastcells-aktivering kan vara goda då dess skyddande egenskaper kan eliminera angripande parasiter eller neutralisera toxiner från ormbett, men även skadliga i samband med symptomen som uppstår vid allergiska reaktioner.

Proteaser är en typ av proteiner vars funktion är att klyva olika typer av målmolekyler. Stora mängder, upp emot en tredjedel, av innehållet i granula utgörs av proteaserna kymas, tryptas och karboxypeptidas A. För att proteaserna ska kunna lagras i granula behövs en speciell proteoglykan, serglycin som består av en proteinkärna med flera negativt laddade kolhydratkedjor inbundna. Vid avsaknad av serglycin proteoglykan kan mastcellen i princip inte lagra några proteaser i sina granula.

För att undersöka vilken roll mastcellens kymas har vid allergisk astma har vi använt oss av experimentella musmodeller och genetiskt modifierade möss som med hjälp av knockoutteknologi saknar ett av mössens kymas, mMCP-4. Genom att exponera mössen för allergener från ägg (artikel I) eller kvalster (artikel II) efterliknas de symptom som man ser hos människor med allergisk astma.

Genom att använda allergener från ägg i en experimentell modell för akut luftvägsinflammation (artikel I) kunde vi visa att närvaro av kymas hade en skyddande roll då luftvägsreaktiviteten och inflammationsgraden i lungorna

var högre i mössen som saknade kymas. Vidare undersökning av den underliggande mekanismen visade att kymas kan ha en hämmande effekt på den glatta muskulaturen kring bronkerna.

Sökandet efter mekanismen bakom fyndet med kymas skyddande egenskaper studerades vidare i en mer kronisk experimentell modell för allergisk astma där mössen exponerades för kvalsterextrakt intranasalt (artikel II). Kvalster är luftburna allergener som är välkända för att ge upphov till astma hos människor och är därmed ett mer relevant allergen ur ett fysiologiskt perspektiv. Även i denna modell hade närvaro av kymas en skyddande funktion på luftvägreaktiviteten, inflammationen i lungan samt den glatta muskulaturen kring bronkerna. För att förstå mekanismen bakom dessa fynd analyserade vi proteiner i lungvävnaden. Vi fann att nivåerna av ett immunologiskt signalprotein, interleukin 33, var kraftigt förhöjda i mössen som saknade kymas. Genom vidare studier kunde vi visa att kymas är ett av proteaserna inuti mastceller som bidrar till att klyva sönder interleukin 33. Andra experimentella studier har visat att närvaron av interleukin 33 bidrar till ökad luftvägsreaktivitet och inflammation i luftvägarna hos möss. Nedbrytningen av interleukin 33 av kymas skulle kunna vara en förklaring till de skyddande egenskaper vi fann i våra experimentella modeller av allergisk astma. Det vi visat med våra studier är att trots att det är känt att mastcellen bidrar till patogenesen vid allergisk astma kan den även frisätta specifika ämnen med skyddande egenskaper.

Mastcellens lagrade proteaser har förmågan att klyva olika typer av inflammatoriska proteiner, t.ex. interleukiner. Vi ville i artikel III undersöka förmågan hos mastceller, som saknar proteoglykanen serglycin, att klyva ett interleukin som är viktigt vid utvecklandet av allergier och astma. Dessa studier gjordes på odlade mastceller som ursprungligen togs från genetiskt modifierade möss. Mastcellerna aktiverades i närvaro av interleukin 13 och nivåerna efter aktivering jämfördes med nivåerna från vild-typs (intakta) mastceller. Frånvaron av proteaser i granula gör att mastcellerna inte kan bryta ner interleukin 13. Genom att tillsätta enzymhämmare, som kan blockera olika typer av proteaser, kunde vi visa att klyvningen av interleukin 13 är beroende av s.k. serinproteaser.

I avhandlingens sista studie (artikel IV) undersökte vi hur odlade mastceller som saknar proteoglykanen serglycin påverkas vid inducerad celldöd s.k. apoptos. Ett ämne som tränger in i cellerna och väl inuti permeabiliserar vesiklar, såsom sekretoriska granula och lysosomer, användes för att inducera

apoptos. I denna studie kunde vi visa att serglycin proteoglykan är involverad i programmerad celldöd som inducerats genom intracellulär frisättning av reaktiva ämnen från granula och lysosomer.

Sammanfattning:

- Mastcellen är känd för att bidra till astma, men vi har visat att ett av proteaserna i dess granula, kymas, har skyddande egenskaper vid uppkomsten av allergisk luftvägsinflammation.
- Speciella proteaser som frisätts vid aktivering av mastcellen kan klyva sönder inflammatoriska signalproteiner.
- Mastceller som saknar proteoglykanen serglycin är mindre benägna att genomgå apoptos om den induceras via permeabilisering av intracellulära vesiklar.

Våra upptäckter kan förhoppningsvis bidra till bättre förståelse för mastcellens roll vid allergisk astma. Att förstå mekanismer som reglerar sjukdomsförloppen kan vara viktigt för att utveckla nya behandlingsmetoder.

References

Abonia, J.P., Friend, D.S., Austen, W.G., Jr., Moore, F.D., Jr., Carroll, M.C., Chan, R., Afnan, J., Humbles, A., Gerard, C., Knight, P., Kanaoka, Y., Yasuda, S., Morokawa, N., Austen, K.F., Stevens, R.L. & Gurish, M.F. (2005). Mast cell protease 5 mediates ischemia-reperfusion injury of mouse skeletal muscle. *Journal of immunology* 174(11), 7285-91.

Abrink, M., Grujic, M. & Pejler, G. (2004). Serglycin is essential for maturation of mast cell secretory granule. *The Journal of biological chemistry* 279(39), 40897-905.

Akahoshi, M., Song, C.H., Piliponsky, A.M., Metz, M., Guzzetta, A., Abrink, M., Schlenner, S.M., Feyerabend, T.B., Rodewald, H.R., Pejler, G., Tsai, M. & Galli, S.J. (2011). Mast cell chymase reduces the toxicity of Gila monster venom, scorpion venom, and vasoactive intestinal polypeptide in mice. *The Journal of clinical investigation* 121(10), 4180-91.

Alam, R., Kumar, D., Anderson-Walters, D. & Forsythe, P.A. (1994). Macrophage inflammatory protein-1 alpha and monocyte chemoattractant peptide-1 elicit immediate and late cutaneous reactions and activate murine mast cells in vivo. *Journal of immunology* 152(3), 1298-303.

Andersson, M.K., Karlson, U. & Hellman, L. (2008). The extended cleavage specificity of the rodent beta-chymases rMCP-1 and mMCP-4 reveal major functional similarities to the human mast cell chymase. *Molecular immunology* 45(3), 766-75.

Bachert, C. (2002). The role of histamine in allergic disease: re-appraisal of its inflammatory potential. *Allergy* 57(4), 287-96.

Balzar, S., Chu, H.W., Strand, M. & Wenzel, S. (2005). Relationship of small airway chymase-positive mast cells and lung function in severe asthma. *American journal of respiratory and critical care medicine* 171(5), 431-9.

Bauer, O. & Razin, E. (2000). Mast Cell-Nerve Interactions. *News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society* 15, 213-218.

Berger, P., Compton, S.J., Molimard, M., Walls, A.F., N'Guyen, C., Marthan, R. & Tunon-De-Lara, J.M. (1999). Mast cell tryptase as a mediator of hyperresponsiveness in human isolated bronchi. *Clinical and*

experimental allergy : journal of the British Society for Allergy and Clinical Immunology 29(6), 804-12.
Berger, P., Tunon-De-Lara, J.M., Savineau, J.P. & Marthan, R. (2001). Selected contribution: tryptase-induced PAR-2-mediated Ca(2+) signaling in human airway smooth muscle cells. *Journal of applied physiology* 91(2), 995-1003.
Bhattacharyya, S.P., Drucker, I., Reshef, T., Kirshenbaum, A.S., Metcalfe, D.D. & Mekori, Y.A. (1998). Activated T lymphocytes induce degranulation and cytokine production by human mast cells following cell-to-cell contact. *Journal of leukocyte biology* 63(3), 337-41.
Boyce, J.A. (2007). Mast cells and eicosanoid mediators: a system of reciprocal paracrine and autocrine regulation. *Immunological reviews* 217, 168-85.
Braga, T., Grujic, M., Lukinius, A., Hellman, L., Abrink, M. & Pejler, G. (2007). Serglycin proteoglycan is required for secretory granule integrity in mucosal mast cells. *The Biochemical journal* 403(1), 49-57.
Cairns, J.A. & Walls, A.F. (1996). Mast cell tryptase is a mitogen for epithelial cells. Stimulation of IL-8 production and intercellular adhesion molecule-1 expression. *Journal of immunology* 156(1), 275-83.
Caughey, G.H., Leidig, F., Viro, N.F. & Nadel, J.A. (1988). Substance P and vasoactive intestinal peptide degradation by mast cell tryptase and chymase. *The Journal of pharmacology and experimental therapeutics* 244(1), 133-7.
Caughey, G.H., Zerweck, E.H. & Vanderslice, P. (1991). Structure, chromosomal assignment, and deduced amino acid sequence of a human gene for mast cell chymase. *The Journal of biological chemistry* 266(20), 12956-63.
Chandrasekharan, U.M., Sanker, S., Glynias, M.J., Karnik, S.S. & Husain, A. (1996). Angiotensin II-forming activity in a reconstructed ancestral chymase. *Science* 271(5248), 502-5.
Clark, J.M., Abraham, W.M., Fishman, C.E., Forteza, R., Ahmed, A., Cortes, A., Warne, R.L., Moore, W.R. & Tanaka, R.D. (1995). Tryptase inhibitors block allergen-induced airway and inflammatory responses in allergic sheep. *American journal of respiratory and critical care medicine* 152(6 Pt 1), 2076-83.
Cockcroft, D.W. & Davis, B.E. (2006). Mechanisms of airway hyperresponsiveness. *The Journal of allergy and clinical immunology* 118(3), 551-9; quiz 560-1.
Crozat, K., Vivier, E. & Dalod, M. (2009). Crosstalk between components of the innate immune system: promoting anti-microbial defenses and avoiding immunopathologies. *Immunological reviews* 227(1), 129-49.
Daeron, M., Malbec, O., Latour, S., Arock, M. & Fridman, W.H. (1995). Regulation of high-affinity IgE receptor-mediated mast cell activation by murine low-affinity IgG receptors. *The Journal of clinical investigation* 95(2), 577-85.
Daeron, M. & Vivier, E. (1999). Biology of immunoreceptor tyrosine-based inhibition motif-bearing molecules. *Current topics in microbiology and immunology* 244, 1-12.

Dahlen, S.E., Hedqvist, P., Hammarstrom, S. & Samuelsson, B. (1980). Leukotrienes are potent constrictors of human bronchi. *Nature* 288(5790), 484-6.
de Paulis, A., Minopoli, G., Dal Piaz, F., Pucci, P., Russo, T. & Marone, G. (1999). Novel autocrine and paracrine loops of the stem cell factor/chymase network. *International archives of allergy and immunology* 118(2-4), 422-5.
Dinarello, C.A. (2000). Proinflammatory cytokines. *Chest* 118(2), 503-8.
Dvorak, A.M., Schleimer, R.P. & Lichtenstein, L.M. (1987). Morphologic mast cell cycles. *Cellular immunology* 105(1), 199-204.
Ekoff, M. & Nilsson, G. (2011). Mast cell apoptosis and survival. *Advances in experimental medicine and biology* 716, 47-60.
Enerback, L. (1966). Mast cells in rat gastrointestinal mucosa. I. Effects of fixation. *Acta pathologica et microbiologica Scandinavica* 66(3), 289-302.
Enerback, L., Kolset, S.O., Kusche, M., Hjerpe, A. & Lindahl, U. (1985). Glycosaminoglycans in rat mucosal mast cells. *The Biochemical journal* 227(2), 661-8.
Evans, J.F., Dupuis, P. & Ford-Hutchinson, A.W. (1985). Purification and characterisation of leukotriene A4 hydrolase from rat neutrophils. *Biochimica et biophysica acta* 840(1), 43-50.
Fattouh, R., Pouladi, M.A., Alvarez, D., Johnson, J.R., Walker, T.D., Goncharova, S., Inman, M.D. & Jordana, M. (2005). House dust mite facilitates ovalbumin-specific allergic sensitization and airway inflammation. *American journal of respiratory and critical care medicine* 172(3), 314-21.
Forsberg, E., Pejler, G., Ringvall, M., Lunderius, C., Tomasini-Johansson, B., Kusche-Gullberg, M., Eriksson, I., Ledin, J., Hellman, L. & Kjellen, L. (1999). Abnormal mast cells in mice deficient in a heparin-synthesizing enzyme. *Nature* 400(6746), 773-6.
Froelich, C.J., Orth, K., Turbov, J., Seth, P., Gottlieb, R., Babior, B., Shah, G.M., Bleackley, R.C., Dixit, V.M. & Hanna, W. (1996). New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. *The Journal of biological chemistry* 271(46), 29073-9.
Fujitani, Y., Kanaoka, Y., Aritake, K., Uodome, N., Okazaki-Hatake, K. & Urade, Y. (2002). Pronounced eosinophilic lung inflammation and Th2 cytokine release in human lipocalin-type prostaglandin D synthase transgenic mice. *Journal of immunology* 168(1), 443-9.
Funk, C.D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 294(5548), 1871-5.
Fureder, W., Agis, H., Willheim, M., Bankl, H.C., Maier, U., Kishi, K., Muller, M.R., Czerwenka, K., Radaszkiewicz, T., Butterfield, J.H., Klappacher, G.W., Sperr, W.R., Oppermann, M., Lechner, K. & Valent, P. (1995). Differential expression of complement receptors on human basophils and

mast cells. Evidence for mast cell heterogeneity and CD88/C5aR expression on skin mast cells. *Journal of immunology* 155(6), 3152-60.
Galli, S.J., Tsai, M. & Piliponsky, A.M. (2008). The development of allergic inflammation. *Nature* 454(7203), 445-54.
Ghildyal, N., Friend, D.S., Stevens, R.L., Austen, K.F., Huang, C., Penrose, J.F., Sali, A. & Gurish, M.F. (1996). Fate of two mast cell tryptases in V3 mastocytosis and normal BALB/c mice undergoing passive systemic anaphylaxis: prolonged retention of exocytosed mMCP-6 in connective tissues, and rapid accumulation of enzymatically active mMCP-7 in the blood. *The Journal of experimental medicine* 184(3), 1061-73.
Gibbs, B.F., Arm, J.P., Gibson, K., Lee, T.H. & Pearce, F.L. (1997). Human lung mast cells release small amounts of interleukin-4 and tumour necrosis factor-alpha in response to stimulation by anti-IgE and stem cell factor. *European journal of pharmacology* 327(1), 73-8.
Goldstein, S.M., Kaempfer, C.E., Kealey, J.T. & Wintroub, B.U. (1989). Human mast cell carboxypeptidase. Purification and characterization. *The Journal of clinical investigation* 83(5), 1630-6.
Groschwitz, K.R., Ahrens, R., Osterfeld, H., Gurish, M.F., Han, X., Abrink, M., Finkelman, F.D., Pejler, G. & Hogan, S.P. (2009). Mast cells regulate homeostatic intestinal epithelial migration and barrier function by a chymase/Mcpt4-dependent mechanism. *Proceedings of the National Academy of Sciences of the United States of America* 106(52), 22381-6.
Gruber, B.L., Kew, R.R., Jelaska, A., Marchese, M.J., Garlick, J., Ren, S., Schwartz, L.B. & Korn, J.H. (1997). Human mast cells activate fibroblasts: tryptase is a fibrogenic factor stimulating collagen messenger ribonucleic acid synthesis and fibroblast chemotaxis. *Journal of immunology* 158(5), 2310-7.
Gruber, B.L., Marchese, M.J., Suzuki, K., Schwartz, L.B., Okada, Y., Nagase, H. & Ramamurthy, N.S. (1989). Synovial procollagenase activation by human mast cell tryptase dependence upon matrix metalloproteinase 3 activation. *The Journal of clinical investigation* 84(5), 1657-62.
Haaksma, E.E., Leurs, R. & Timmerman, H. (1990). Histamine receptors: subclasses and specific ligands. *Pharmacology & therapeutics* 47(1), 73-104.
Hakimi, J., Seals, C., Kondas, J.A., Pettine, L., Danho, W. & Kochan, J. (1990). The alpha subunit of the human IgE receptor (FcERI) is sufficient for high affinity IgE binding. *The Journal of biological chemistry* 265(36), 22079-81.
Hallgren, J., Karlson, U., Poorafshar, M., Hellman, L. & Pejler, G. (2000). Mechanism for activation of mouse mast cell tryptase: dependence on heparin and acidic pH for formation of active tetramers of mouse mast cell protease 6. *Biochemistry* 39(42), 13068-77.
Hallgren, J. & Pejler, G. (2006). Biology of mast cell tryptase. An inflammatory mediator. *The FEBS journal* 273(9), 1871-95.
Hallgren, J., Spillmann, D. & Pejler, G. (2001). Structural requirements and mechanism for heparin-induced activation of a recombinant mouse mast cell tryptase, mouse mast cell protease-6: formation of active tryptase

monomers in the presence of low molecular weight heparin. *The Journal of biological chemistry* 276(46), 42774-81.
Handel, T.M., Johnson, Z., Crown, S.E., Lau, E.K. & Proudfoot, A.E. (2005). Regulation of protein function by glycosaminoglycans--as exemplified by chemokines. *Annual review of biochemistry* 74, 385-410.
Hanna, C.J., Bach, M.K., Pare, P.D. & Schellenberg, R.R. (1981). Slow-reacting substances (leukotrienes) contract human airway and pulmonary vascular smooth muscle in vitro. *Nature* 290(5804), 343-4.
He, S. & Walls, A.F. (1998). Human mast cell chymase induces the accumulation of neutrophils, eosinophils and other inflammatory cells in vivo. *British journal of pharmacology* 125(7), 1491-500.
Henningsson, F., Hergeth, S., Cortelius, R., Abrink, M. & Pejler, G. (2006). A role for serglycin proteoglycan in granular retention and processing of mast cell secretory granule components. *The FEBS journal* 273(21), 4901-12.
Henningsson, F., Ledin, J., Lunderius, C., Wilen, M., Hellman, L. & Pejler, G. (2002). Altered storage of proteases in mast cells from mice lacking heparin: a possible role for heparin in carboxypeptidase A processing. *Biological chemistry* 383(5), 793-801.
Hill, S.J. (1990). Distribution, properties, and functional characteristics of three classes of histamine receptor. *Pharmacological reviews* 42(1), 45-83.
Ho, L.H., Ohno, T., Oboki, K., Kajiwara, N., Suto, H., Iikura, M., Okayama, Y., Akira, S., Saito, H., Galli, S.J. & Nakae, S. (2007). IL-33 induces IL-13 production by mouse mast cells independently of IgE-FcepsilonRI signals. *Journal of leukocyte biology* 82(6), 1481-90.
Honda, K., Arima, M., Cheng, G., Taki, S., Hirata, H., Eda, F., Fukushima, F., Yamaguchi, B., Hatano, M., Tokuhisa, T. & Fukuda, T. (2003). Prostaglandin D2 reinforces Th2 type inflammatory responses of airways to low-dose antigen through bronchial expression of macrophage-derived chemokine. *The Journal of experimental medicine* 198(4), 533-43.
Hoshino, F., Urata, H., Inoue, Y., Saito, Y., Yahiro, E., Ideishi, M., Arakawa, K. & Saku, K. (2003). Chymase inhibitor improves survival in hamsters with myocardial infarction. *Journal of cardiovascular pharmacology* 41 Suppl 1, S11-8.
Hsu, C.L., Neilsen, C.V. & Bryce, P.J. (2010). IL-33 is produced by mast cells and regulates IgE-dependent inflammation. *PloS one* 5(8), e11944.
Huang, C., Li, L., Krilis, S.A., Chanasyk, K., Tang, Y., Li, Z., Hunt, J.E. & Stevens, R.L. (1999). Human tryptases alpha and beta/II are functionally distinct due, in part, to a single amino acid difference in one of the surface loops that forms the substrate-binding cleft. *The Journal of biological chemistry* 274(28), 19670-6.
Huang, R.Y., Blom, T. & Hellman, L. (1991). Cloning and structural analysis of MMCP-1, MMCP-4 and MMCP-5, three mouse mast cell-specific serine proteases. *European journal of immunology* 21(7), 1611-21.
Humphries, D.E., Wong, G.W., Friend, D.S., Gurish, M.F., Qiu, W.T., Huang, C., Sharpe, A.H. & Stevens, R.L. (1999). Heparin is essential for the storage of specific granule proteases in mast cells. *Nature* 400(6746), 769-72.

Iemura, A., Tsai, M., Ando, A., Wershil, B.K. & Galli, S.J. (1994). The c-kit ligand, stem cell factor, promotes mast cell survival by suppressing apoptosis. *The American journal of pathology* 144(2), 321-8.

Inamura, N., Mekori, Y.A., Bhattacharyya, S.P., Bianchine, P.J. & Metcalfe, D.D. (1998). Induction and enhancement of Fc(epsilon)RI-dependent mast cell degranulation following coculture with activated T cells: dependency on ICAM-1- and leukocyte function-associated antigen (LFA)-1-mediated heterotypic aggregation. *Journal of immunology* 160(8), 4026-33.

Iozzo, R.V. (2005). Basement membrane proteoglycans: from cellar to ceiling. *Nature reviews. Molecular cell biology* 6(8), 646-56.

Irani, A.M. & Schwartz, L.B. (1994). Human mast cell heterogeneity. *Allergy proceedings : the official journal of regional and state allergy societies* 15(6), 303-8.

Ishizuka, T., Okayama, Y., Kobayashi, H. & Mori, M. (1999). Interleukin-10 is localized to and released by human lung mast cells. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 29(10), 1424-32.

Jin, D., Takai, S., Yamada, M., Sakaguchi, M., Kamoshita, K., Ishida, K., Sukenaga, Y. & Miyazaki, M. (2003). Impact of chymase inhibitor on cardiac function and survival after myocardial infarction. *Cardiovascular research* 60(2), 413-20.

Johnson, A.R., Hugli, T.E. & Muller-Eberhard, H.J. (1975). Release of histamine from rat mast cells by the complement peptides C3a and C5a. *Immunology* 28(6), 1067-80.

Johnston, S.L., Bardin, P.G., Harrison, J., Ritter, W., Joubert, J.R. & Holgate, S.T. (1992). The effects of an oral thromboxane TP receptor antagonist BAY u 3405, on prostaglandin D2- and histamine-induced bronchoconstriction in asthma, and relationship to plasma drug concentrations. *British journal of clinical pharmacology* 34(5), 402-8.

Jouvin, M.H., Adamczewski, M., Numerof, R., Letourneur, O., Valle, A. & Kinet, J.P. (1994). Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. *The Journal of biological chemistry* 269(8), 5918-25.

Kanbe, N., Kurosawa, M., Nagata, H., Saitoh, H. & Miyachi, Y. (1999a). Cord blood-derived human cultured mast cells produce transforming growth factor beta1. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 29(1), 105-13.

Kanbe, N., Kurosawa, M., Yamashita, T., Kurimoto, F., Yanagihara, Y. & Miyachi, Y. (1999b). Cord-blood-derived human cultured mast cells produce interleukin 13 in the presence of stem cell factor. *International archives of allergy and immunology* 119(2), 138-42.

Karlson, U., Pejler, G., Tomasini-Johansson, B. & Hellman, L. (2003). Extended substrate specificity of rat mast cell protease 5, a rodent alpha-chymase with elastase-like primary specificity. *The Journal of biological chemistry* 278(41), 39625-31.

Kasahara, T., Oda, T., Hatake, K., Akiyama, M., Mukaida, N. & Matsushima, K. (1998). Interleukin-8 and monocyte chemotactic protein-1 production by a

human glioblastoma cell line, T98G in coculture with monocytes: involvement of monocyte-derived interleukin-1alpha. *European cytokine network* 9(1), 47-55.
Katz, H.R. & Lobell, R.B. (1995). Expression and function of Fc gamma R in mouse mast cells. *International archives of allergy and immunology* 107(1-3), 76-8.
Kepley, C.L., Cambier, J.C., Morel, P.A., Lujan, D., Ortega, E., Wilson, B.S. & Oliver, J.M. (2000). Negative regulation of FcepsilonRI signaling by FcgammaRII costimulation in human blood basophils. *The Journal of allergy and clinical immunology* 106(2), 337-48.
Kerr, J.F., Wyllie, A.H. & Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer* 26(4), 239-57.
Kido, H., Nakano, A., Okishima, N., Wakabayashi, H., Kishi, F., Nakaya, Y., Yoshizumi, M. & Tamaki, T. (1998). Human chymase, an enzyme forming novel bioactive 31-amino acid length endothelins. *Biological chemistry* 379(7), 885-91.
Kielty, C.M., Lees, M., Shuttleworth, C.A. & Woolley, D. (1993). Catabolism of intact type VI collagen microfibrils: susceptibility to degradation by serine proteinases. *Biochemical and biophysical research communications* 191(3), 1230-6.
Kirshenbaum, A.S., Kessler, S.W., Goff, J.P. & Metcalfe, D.D. (1991). Demonstration of the origin of human mast cells from CD34+ bone marrow progenitor cells. *Journal of immunology* 146(5), 1410-5.
Knight, P.A., Wright, S.H., Lawrence, C.E., Paterson, Y.Y. & Miller, H.R. (2000). Delayed expulsion of the nematode Trichinella spiralis in mice lacking the mucosal mast cell-specific granule chymase, mouse mast cell protease-1. *The Journal of experimental medicine* 192(12), 1849-56.
Kobayashi, T., Miura, T., Haba, T., Sato, M., Serizawa, I., Nagai, H. & Ishizaka, K. (2000). An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model. *Journal of immunology* 164(7), 3855-61.
Kobayasi, T. & Asboe-Hansen, G. (1969). Degranulation and regranulation of human mast cells. An electron microscopic study of the whealing reaction in urticaria pigmentosa. *Acta dermato-venereologica* 49(4), 369-81.
Kolset, S.O. & Gallagher, J.T. (1990). Proteoglycans in haemopoietic cells. *Biochimica et biophysica acta* 1032(2-3), 191-211.
Kolset, S.O., Prydz, K. & Pejler, G. (2004). Intracellular proteoglycans. *The Biochemical journal* 379(Pt 2), 217-27.
Kolset, S.O. & Tveit, H. (2008). Serglycin--structure and biology. *Cellular and molecular life sciences : CMLS* 65(7-8), 1073-85.
Komai-Koma, M., Xu, D., Li, Y., McKenzie, A.N., McInnes, I.B. & Liew, F.Y. (2007). IL-33 is a chemoattractant for human Th2 cells. *European journal of immunology* 37(10), 2779-86.
Kondo, Y., Yoshimoto, T., Yasuda, K., Futatsugi-Yumikura, S., Morimoto, M., Hayashi, N., Hoshino, T., Fujimoto, J. & Nakanishi, K. (2008). Administration of IL-33 induces airway hyperresponsiveness and goblet

cell hyperplasia in the lungs in the absence of adaptive immune system. *International immunology* 20(6), 791-800.

Kunori, Y., Koizumi, M., Masegi, T., Kasai, H., Kawabata, H., Yamazaki, Y. & Fukamizu, A. (2002). Rodent alpha-chymases are elastase-like proteases. *European journal of biochemistry / FEBS* 269(23), 5921-30.

Lam, B.K., Penrose, J.F., Freeman, G.J. & Austen, K.F. (1994). Expression cloning of a cDNA for human leukotriene C4 synthase, an integral membrane protein conjugating reduced glutathione to leukotriene A4. *Proceedings of the National Academy of Sciences of the United States of America* 91(16), 7663-7.

Lazaar, A.L., Plotnick, M.I., Kucich, U., Crichton, I., Lotfi, S., Das, S.K., Kane, S., Rosenbloom, J., Panettieri, R.A., Jr., Schechter, N.M. & Pure, E. (2002). Mast cell chymase modifies cell-matrix interactions and inhibits mitogen-induced proliferation of human airway smooth muscle cells. *Journal of immunology* 169(2), 1014-20.

Leon, A., Buriani, A., Dal Toso, R., Fabris, M., Romanello, S., Aloe, L. & Levi-Montalcini, R. (1994). Mast cells synthesize, store, and release nerve growth factor. *Proceedings of the National Academy of Sciences of the United States of America* 91(9), 3739-43.

Leskinen, M., Wang, Y., Leszczynski, D., Lindstedt, K.A. & Kovanen, P.T. (2001). Mast cell chymase induces apoptosis of vascular smooth muscle cells. *Arteriosclerosis, thrombosis, and vascular biology* 21(4), 516-22.

Leskinen, M.J., Lindstedt, K.A., Wang, Y. & Kovanen, P.T. (2003). Mast cell chymase induces smooth muscle cell apoptosis by a mechanism involving fibronectin degradation and disruption of focal adhesions. *Arteriosclerosis, thrombosis, and vascular biology* 23(2), 238-43.

Lin, L., Bankaitis, E., Heimbach, L., Li, N., Abrink, M., Pejler, G., An, L., Diaz, L.A., Werb, Z. & Liu, Z. (2011). Dual targets for mouse mast cell protease-4 in mediating tissue damage in experimental bullous pemphigoid. *The Journal of biological chemistry* 286(43), 37358-67.

Lindstedt, K.A., Wang, Y., Shiota, N., Saarinen, J., Hyytiainen, M., Kokkonen, J.O., Keski-Oja, J. & Kovanen, P.T. (2001). Activation of paracrine TGF-beta1 signaling upon stimulation and degranulation of rat serosal mast cells: a novel function for chymase. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 15(8), 1377-88.

Lippert, U., Artuc, M., Grutzkau, A., Babina, M., Guhl, S., Haase, I., Blaschke, V., Zachmann, K., Knosalla, M., Middel, P., Kruger-Krasagakis, S. & Henz, B.M. (2004). Human skin mast cells express H2 and H4, but not H3 receptors. *The Journal of investigative dermatology* 123(1), 116-23.

Lohi, J., Harvima, I. & Keski-Oja, J. (1992). Pericellular substrates of human mast cell tryptase: 72,000 dalton gelatinase and fibronectin. *Journal of cellular biochemistry* 50(4), 337-49.

Lunderius, C., Xiang, Z., Nilsson, G. & Hellman, L. (2000). Murine mast cell lines as indicators of early events in mast cell and basophil development. *European journal of immunology* 30(12), 3396-402.

Lutzelschwab, C., Huang, M.R., Kullberg, M.C., Aveskogh, M. & Hellman, L. (1998). Characterization of mouse mast cell protease-8, the first member of a novel subfamily of mouse mast cell serine proteases, distinct from both the classical chymases and tryptases. *European journal of immunology* 28(3), 1022-33.

Machado, D.C., Horton, D., Harrop, R., Peachell, P.T. & Helm, B.A. (1996). Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with antigen-specific IgE. *European journal of immunology* 26(12), 2972-80.

Maddox, L. & Schwartz, D.A. (2002). The pathophysiology of asthma. *Annual review of medicine* 53, 477-98.

Magnusson, S.E., Pejler, G., Kleinau, S. & Abrink, M. (2009). Mast cell chymase contributes to the antibody response and the severity of autoimmune arthritis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 23(3), 875-82.

Malaviya, R. & Jakschik, B.A. (1993). Reversible translocation of 5-lipoxygenase in mast cells upon IgE/antigen stimulation. *The Journal of biological chemistry* 268(7), 4939-44.

Malbec, O., Roget, K., Schiffer, C., Iannascoli, B., Dumas, A.R., Arock, M. & Daeron, M. (2007). Peritoneal cell-derived mast cells: an in vitro model of mature serosal-type mouse mast cells. *Journal of immunology* 178(10), 6465-75.

Marquardt, U., Zettl, F., Huber, R., Bode, W. & Sommerhoff, C. (2002). The crystal structure of human alpha1-tryptase reveals a blocked substrate-binding region. *Journal of molecular biology* 321(3), 491-502.

Marshall, J.S. (2004). Mast-cell responses to pathogens. *Nature reviews. Immunology* 4(10), 787-99.

Marshall, J.S., King, C.A. & McCurdy, J.D. (2003). Mast cell cytokine and chemokine responses to bacterial and viral infection. *Current pharmaceutical design* 9(1), 11-24.

Matsushima, H., Yamada, N., Matsue, H. & Shimada, S. (2004). TLR3-, TLR7-, and TLR9-mediated production of proinflammatory cytokines and chemokines from murine connective tissue type skin-derived mast cells but not from bone marrow-derived mast cells. *Journal of immunology* 173(1), 531-41.

Mekori, Y.A. & Metcalfe, D.D. (2000). Mast cells in innate immunity. *Immunological reviews* 173, 131-40.

Metcalfe, D.D., Baram, D. & Mekori, Y.A. (1997). Mast cells. *Physiological reviews* 77(4), 1033-79.

Metsarinne, K.P., Vehmaan-Kreula, P., Kovanen, P.T., Saijonmaa, O., Baumann, M., Wang, Y., Nyman, T., Fyhrquist, F.Y. & Eklund, K.K. (2002). Activated mast cells increase the level of endothelin-1 mRNA in cocultured endothelial cells and degrade the secreted Peptide. *Arteriosclerosis, thrombosis, and vascular biology* 22(2), 268-73.

Metz, M., Piliponsky, A.M., Chen, C.C., Lammel, V., Abrink, M., Pejler, G., Tsai, M. & Galli, S.J. (2006). Mast cells can enhance resistance to snake and honeybee venoms. *Science* 313(5786), 526-30.

Mizutani, H., Schechter, N., Lazarus, G., Black, R.A. & Kupper, T.S. (1991). Rapid and specific conversion of precursor interleukin 1 beta (IL-1 beta) to an active IL-1 species by human mast cell chymase. *The Journal of experimental medicine* 174(4), 821-5.

Mousli, M., Hugli, T.E., Landry, Y. & Bronner, C. (1994). Peptidergic pathway in human skin and rat peritoneal mast cell activation. *Immunopharmacology* 27(1), 1-11.

Moussion, C., Ortega, N. & Girard, J.P. (2008). The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PloS one* 3(10), e3331.

Newlands, G.F., Knox, D.P., Pirie-Shepherd, S.R. & Miller, H.R. (1993). Biochemical and immunological characterization of multiple glycoforms of mouse mast cell protease 1: comparison with an isolated murine serosal mast cell protease (MMCP-4). *The Biochemical journal* 294 (Pt 1), 127-35.

Niemann, C.U., Abrink, M., Pejler, G., Fischer, R.L., Christensen, E.I., Knight, S.D. & Borregaard, N. (2007). Neutrophil elastase depends on serglycin proteoglycan for localization in granules. *Blood* 109(10), 4478-86.

O'Byrne, P.M., Inman, M.D. & Parameswaran, K. (2001). The trials and tribulations of IL-5, eosinophils, and allergic asthma. *The Journal of allergy and clinical immunology* 108(4), 503-8.

Okayama, Y., Bradding, P., Tunon-de-Lara, J.M., Holgate, S.T. & Church, M.K. (1995). Cytokine production by human mast cells. *Chemical immunology* 61, 114-34.

Okayama, Y., Kirshenbaum, A.S. & Metcalfe, D.D. (2000). Expression of a functional high-affinity IgG receptor, Fc gamma RI, on human mast cells: Up-regulation by IFN-gamma. *Journal of immunology* 164(8), 4332-9.

Okumura, S., Kashiwakura, J., Tomita, H., Matsumoto, K., Nakajima, T., Saito, H. & Okayama, Y. (2003). Identification of specific gene expression profiles in human mast cells mediated by Toll-like receptor 4 and FcepsilonRI. *Blood* 102(7), 2547-54.

Ono, S.J., Nakamura, T., Miyazaki, D., Ohbayashi, M., Dawson, M. & Toda, M. (2003). Chemokines: roles in leukocyte development, trafficking, and effector function. *The Journal of allergy and clinical immunology* 111(6), 1185-99; quiz 1200.

Pallaoro, M., Fejzo, M.S., Shayesteh, L., Blount, J.L. & Caughey, G.H. (1999). Characterization of genes encoding known and novel human mast cell tryptases on chromosome 16p13.3. *The Journal of biological chemistry* 274(6), 3355-62.

Pejler, G., Abrink, M., Ringvall, M. & Wernersson, S. (2007). Mast cell proteases. *Advances in immunology* 95, 167-255.

Pejler, G., Abrink, M. & Wernersson, S. (2009). Serglycin proteoglycan: regulating the storage and activities of hematopoietic proteases. *BioFactors* 35(1), 61-8.

Pejler, G. & Berg, L. (1995). Regulation of rat mast cell protease 1 activity. Protease inhibition is prevented by heparin proteoglycan. *European journal of biochemistry / FEBS* 233(1), 192-9.
Pejler, G., Ronnberg, E., Waern, I. & Wernersson, S. (2010). Mast cell proteases: multifaceted regulators of inflammatory disease. *Blood* 115(24), 4981-90.
Pejler, G. & Sadler, J.E. (1999). Mechanism by which heparin proteoglycan modulates mast cell chymase activity. *Biochemistry* 38(37), 12187-95.
Pemberton, A.D., Brown, J.K., Wright, S.H., Knight, P.A., McPhee, M.L., McEuen, A.R., Forse, P.A. & Miller, H.R. (2003). Purification and characterization of mouse mast cell proteinase-2 and the differential expression and release of mouse mast cell proteinase-1 and -2 in vivo. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 33(7), 1005-12.
Polanowska, J., Krokoszynska, I., Czapinska, H., Watorek, W., Dadlez, M. & Otlewski, J. (1998). Specificity of human cathepsin G. *Biochimica et biophysica acta* 1386(1), 189-98.
Prodeus, A.P., Zhou, X., Maurer, M., Galli, S.J. & Carroll, M.C. (1997). Impaired mast cell-dependent natural immunity in complement C3-deficient mice. *Nature* 390(6656), 172-5.
Qu, Z., Huang, X., Ahmadi, P., Stenberg, P., Liebler, J.M., Le, A.C., Planck, S.R. & Rosenbaum, J.T. (1998). Synthesis of basic fibroblast growth factor by murine mast cells. Regulation by transforming growth factor beta, tumor necrosis factor alpha, and stem cell factor. *International archives of allergy and immunology* 115(1), 47-54.
Razin, E., Ihle, J.N., Seldin, D., Mencia-Huerta, J.M., Katz, H.R., LeBlanc, P.A., Hein, A., Caulfield, J.P., Austen, K.F. & Stevens, R.L. (1984). Interleukin 3: A differentiation and growth factor for the mouse mast cell that contains chondroitin sulfate E proteoglycan. *Journal of immunology* 132(3), 1479-86.
Reilly, C.F., Tewksbury, D.A., Schechter, N.M. & Travis, J. (1982). Rapid conversion of angiotensin I to angiotensin II by neutrophil and mast cell proteinases. *The Journal of biological chemistry* 257(15), 8619-22.
Reynolds, D.S., Gurley, D.S., Stevens, R.L., Sugarbaker, D.J., Austen, K.F. & Serafin, W.E. (1989). Cloning of cDNAs that encode human mast cell carboxypeptidase A, and comparison of the protein with mouse mast cell carboxypeptidase A and rat pancreatic carboxypeptidases. *Proceedings of the National Academy of Sciences of the United States of America* 86(23), 9480-4.
Reynolds, D.S., Stevens, R.L., Lane, W.S., Carr, M.H., Austen, K.F. & Serafin, W.E. (1990). Different mouse mast cell populations express various combinations of at least six distinct mast cell serine proteases. *Proceedings of the National Academy of Sciences of the United States of America* 87(8), 3230-4.
Riedl, S.J. & Shi, Y. (2004). Molecular mechanisms of caspase regulation during apoptosis. *Nature reviews. Molecular cell biology* 5(11), 897-907.
Ringvall, M., Ronnberg, E., Wernersson, S., Duelli, A., Henningsson, F., Abrink, M., Garcia-Faroldi, G., Fajardo, I. & Pejler, G. (2008). Serotonin and

histamine storage in mast cell secretory granules is dependent on serglycin proteoglycan. *The Journal of allergy and clinical immunology* 121(4), 1020-6.
Ronnberg, E., Melo, F.R. & Pejler, G. (2012). Mast Cell Proteoglycans. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*.
Sarma, J.V. & Ward, P.A. (2011). The complement system. *Cell and tissue research* 343(1), 227-35.
Schechter, N.M., Irani, A.M., Sprows, J.L., Abernethy, J., Wintroub, B. & Schwartz, L.B. (1990). Identification of a cathepsin G-like proteinase in the MCTC type of human mast cell. *Journal of immunology* 145(8), 2652-61.
Schwartz, L.B. & Bradford, T.R. (1986). Regulation of tryptase from human lung mast cells by heparin. Stabilization of the active tetramer. *The Journal of biological chemistry* 261(16), 7372-9.
Schwartz, L.B., Bradford, T.R., Irani, A.M., Deblois, G. & Craig, S.S. (1987a). The major enzymes of human mast cell secretory granules. *The American review of respiratory disease* 135(5), 1186-9.
Schwartz, L.B., Bradford, T.R., Littman, B.H. & Wintroub, B.U. (1985). The fibrinogenolytic activity of purified tryptase from human lung mast cells. *Journal of immunology* 135(4), 2762-7.
Schwartz, L.B., Irani, A.M., Roller, K., Castells, M.C. & Schechter, N.M. (1987b). Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells. *Journal of immunology* 138(8), 2611-5.
Schwartz, L.B., Riedel, C., Caulfield, J.P., Wasserman, S.I. & Austen, K.F. (1981). Cell association of complexes of chymase, heparin proteoglycan, and protein after degranulation by rat mast cells. *Journal of immunology* 126(6), 2071-8.
Schwartz, L.B., Sakai, K., Bradford, T.R., Ren, S., Zweiman, B., Worobec, A.S. & Metcalfe, D.D. (1995). The alpha form of human tryptase is the predominant type present in blood at baseline in normal subjects and is elevated in those with systemic mastocytosis. *The Journal of clinical investigation* 96(6), 2702-10.
Sharma, S., Rajan, U.M., Kumar, A., Soni, A. & Ghosh, B. (2005). A novel (TG)n(GA)m repeat polymorphism 254 bp downstream of the mast cell chymase (CMA1) gene is associated with atopic asthma and total serum IgE levels. *Journal of human genetics* 50(6), 276-82.
Subramanian, N. & Bray, M.A. (1987). Interleukin 1 releases histamine from human basophils and mast cells in vitro. *Journal of immunology* 138(1), 271-5.
Sun, J., Zhang, J., Lindholt, J.S., Sukhova, G.K., Liu, J., He, A., Abrink, M., Pejler, G., Stevens, R.L., Thompson, R.W., Ennis, T.L., Gurish, M.F., Libby, P. & Shi, G.P. (2009). Critical role of mast cell chymase in mouse abdominal aortic aneurysm formation. *Circulation* 120(11), 973-82.
Supajatura, V., Ushio, H., Nakao, A., Akira, S., Okumura, K., Ra, C. & Ogawa, H. (2002). Differential responses of mast cell Toll-like receptors 2 and 4 in

allergy and innate immunity. *The Journal of clinical investigation* 109(10), 1351-9.

Suzukawa, M., Iikura, M., Koketsu, R., Nagase, H., Tamura, C., Komiya, A., Nakae, S., Matsushima, K., Ohta, K., Yamamoto, K. & Yamaguchi, M. (2008). An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. *Journal of immunology* 181(9), 5981-9.

Taipale, J., Lohi, J., Saarinen, J., Kovanen, P.T. & Keski-Oja, J. (1995). Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells. *The Journal of biological chemistry* 270(9), 4689-96.

Takeda, K., Kaisho, T. & Akira, S. (2003). Toll-like receptors. *Annual review of immunology* 21, 335-76.

Taube, C., Wei, X., Swasey, C.H., Joetham, A., Zarini, S., Lively, T., Takeda, K., Loader, J., Miyahara, N., Kodama, T., Shultz, L.D., Donaldson, D.D., Hamelmann, E.H., Dakhama, A. & Gelfand, E.W. (2004). Mast cells, Fc epsilon RI, and IL-13 are required for development of airway hyperresponsiveness after aerosolized allergen exposure in the absence of adjuvant. *Journal of immunology* 172(10), 6398-406.

Taylor, A.M., Galli, S.J. & Coleman, J.W. (1995). Stem-cell factor, the kit ligand, induces direct degranulation of rat peritoneal mast cells in vitro and in vivo: dependence of the in vitro effect on period of culture and comparisons of stem-cell factor with other mast cell-activating agents. *Immunology* 86(3), 427-33.

Tchougounova, E., Lundequist, A., Fajardo, I., Winberg, J.O., Abrink, M. & Pejler, G. (2005). A key role for mast cell chymase in the activation of pro-matrix metalloprotease-9 and pro-matrix metalloprotease-2. *The Journal of biological chemistry* 280(10), 9291-6.

Tchougounova, E. & Pejler, G. (2001). Regulation of extravascular coagulation and fibrinolysis by heparin-dependent mast cell chymase. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 15(14), 2763-5.

Tchougounova, E., Pejler, G. & Abrink, M. (2003). The chymase, mouse mast cell protease 4, constitutes the major chymotrypsin-like activity in peritoneum and ear tissue. A role for mouse mast cell protease 4 in thrombin regulation and fibronectin turnover. *The Journal of experimental medicine* 198(3), 423-31.

Terakawa, M., Tomimori, Y., Goto, M., Hayashi, Y., Oikawa, S. & Fukuda, Y. (2005). Eosinophil migration induced by mast cell chymase is mediated by extracellular signal-regulated kinase pathway. *Biochemical and biophysical research communications* 332(4), 969-75.

Toyama-Sorimachi, N., Sorimachi, H., Tobita, Y., Kitamura, F., Yagita, H., Suzuki, K. & Miyasaka, M. (1995). A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. Possible involvement in lymphoid cell adherence and activation. *The Journal of biological chemistry* 270(13), 7437-44.

Tsunemi, K., Takai, S., Nishimoto, M., Jin, D., Sakaguchi, M., Muramatsu, M., Yuda, A., Sasaki, S. & Miyazaki, M. (2004). A specific chymase inhibitor, 2-(5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-[[3,4-dioxo-1-pheny l-7-(2-pyridyloxy)]-2-heptyl]acetamide (NK3201), suppresses development of abdominal aortic aneurysm in hamsters. *The Journal of pharmacology and experimental therapeutics* 309(3), 879-83.

Turner, H. & Kinet, J.P. (1999). Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature* 402(6760 Suppl), B24-30.

Urata, H., Kinoshita, A., Misono, K.S., Bumpus, F.M. & Husain, A. (1990). Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. *The Journal of biological chemistry* 265(36), 22348-57.

Varadaradjalou, S., Feger, F., Thieblemont, N., Hamouda, N.B., Pleau, J.M., Dy, M. & Arock, M. (2003). Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. *European journal of immunology* 33(4), 899-906.

Vartio, T., Seppa, H. & Vaheri, A. (1981). Susceptibility of soluble and matrix fibronectins to degradation by tissue proteinases, mast cell chymase and cathepsin G. *The Journal of biological chemistry* 256(1), 471-7.

Vendrell, J., Querol, E. & Aviles, F.X. (2000). Metallocarboxypeptidases and their protein inhibitors. Structure, function and biomedical properties. *Biochimica et biophysica acta* 1477(1-2), 284-98.

Vercelli, D. (2001). Immunoglobulin E and its regulators. *Current opinion in allergy and clinical immunology* 1(1), 61-5.

Walsh, L.J., Trinchieri, G., Waldorf, H.A., Whitaker, D. & Murphy, G.F. (1991). Human dermal mast cells contain and release tumor necrosis factor alpha, which induces endothelial leukocyte adhesion molecule 1. *Proceedings of the National Academy of Sciences of the United States of America* 88(10), 4220-4.

Weidner, N. & Austen, K.F. (1993). Heterogeneity of mast cells at multiple body sites. Fluorescent determination of avidin binding and immunofluorescent determination of chymase, tryptase, and carboxypeptidase content. *Pathology, research and practice* 189(2), 156-62.

Weller, C.L., Collington, S.J., Brown, J.K., Miller, H.R., Al-Kashi, A., Clark, P., Jose, P.J., Hartnell, A. & Williams, T.J. (2005). Leukotriene B4, an activation product of mast cells, is a chemoattractant for their progenitors. *The Journal of experimental medicine* 201(12), 1961-71.

Wenzel, S.E., Larsen, G.L., Johnston, K., Voelkel, N.F. & Westcott, J.Y. (1990). Elevated levels of leukotriene C4 in bronchoalveolar lavage fluid from atopic asthmatics after endobronchial allergen challenge. *The American review of respiratory disease* 142(1), 112-9.

Wenzel, S.E., Westcott, J.Y., Smith, H.R. & Larsen, G.L. (1989). Spectrum of prostanoid release after bronchoalveolar allergen challenge in atopic asthmatics and in control groups. An alteration in the ratio of bronchoconstrictive to bronchoprotective mediators. *The American review of respiratory disease* 139(2), 450-7.

Wernersson, S., Braga, T., Sawesi, O., Waern, I., Nilsson, K.E., Pejler, G. & Abrink, M. (2009). Age-related enlargement of lymphoid tissue and altered leukocyte composition in serglycin-deficient mice. *Journal of leukocyte biology* 85(3), 401-8.

Williams, C.M. & Galli, S.J. (2000). Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *The Journal of experimental medicine* 192(3), 455-62.

Wills-Karp, M. (2004). Interleukin-13 in asthma pathogenesis. *Immunological reviews* 202, 175-90.

Wolters, P.J., Pham, C.T., Muilenburg, D.J., Ley, T.J. & Caughey, G.H. (2001). Dipeptidyl peptidase I is essential for activation of mast cell chymases, but not tryptases, in mice. *The Journal of biological chemistry* 276(21), 18551-6.

Wong, G.W., Tang, Y., Feyfant, E., Sali, A., Li, L., Li, Y., Huang, C., Friend, D.S., Krilis, S.A. & Stevens, R.L. (1999). Identification of a new member of the tryptase family of mouse and human mast cell proteases which possesses a novel COOH-terminal hydrophobic extension. *The Journal of biological chemistry* 274(43), 30784-93.

Wong, G.W., Yasuda, S., Morokawa, N., Li, L. & Stevens, R.L. (2004). Mouse chromosome 17A3.3 contains 13 genes that encode functional tryptic-like serine proteases with distinct tissue and cell expression patterns. *The Journal of biological chemistry* 279(4), 2438-52.

Xiang, Z., Ahmed, A.A., Moller, C., Nakayama, K., Hatakeyama, S. & Nilsson, G. (2001). Essential role of the prosurvival bcl-2 homologue A1 in mast cell survival after allergic activation. *The Journal of experimental medicine* 194(11), 1561-69.

Young, J.D., Liu, C.C., Butler, G., Cohn, Z.A. & Galli, S.J. (1987). Identification, purification, and characterization of a mast cell-associated cytolytic factor related to tumor necrosis factor. *Proceedings of the National Academy of Sciences of the United States of America* 84(24), 9175-9.

Zhang, S., Anderson, D.F., Bradding, P., Coward, W.R., Baddeley, S.M., MacLeod, J.D., McGill, J.I., Church, M.K., Holgate, S.T. & Roche, W.R. (1998). Human mast cells express stem cell factor. *The Journal of pathology* 186(1), 59-66.

Acknowledgements

The work presented in this thesis was performed at the Department of Anatomy, Physiology and Biochemistry.

This thesis would not have been possible without encouragement from all wonderful colleagues, family and friends.

I would further like to express my sincere gratitude to the following people:

Sara Wernersson, my supervisor, for guiding me through the field of mast cell research. You are an excellent supervisor, inspiring person and a true role model. Thanks for sharing your scientific knowledge, helping me and teaching me how important the controls are. Many thanks for all your support during these years. It is a pleasure working with you!

Gunnar Pejler, my co-supervisor, for sending that e-mail about doing a project in you lab seven years ago! Thanks for sharing your scientific knowledge and for always having time for questions and coming up with new ideas when things did not go as planned.

Present and past members of the group:
Magnus, thanks for always having time for a chat or questions about anything, and helping me in the lab when I was just a rookie.
Elin, my roomie and no.1 Partypingla! I'm so happy we met in UGSBR and that you decided to join us in the GP-lab! I count myself lucky having a friend like you!
Anders, for your friendship, discussions about anything and everything and for always being helpful. I miss you in the lab, fingers crossed that you will receive the grants you applied for!

Tiago, for your support, all funny moments and being a good friend. Thanks for letting me teach you basic Swedish. You were really an excellent Swedish student…pause…NOOOT!
Fabio, you are a true gentleman! It has been a pleasure working with you. I hope the future will bring some more fruitful data.
Iulia, it has been fun working with you. Many thanks for the help with the IL-13 story.
Gianni, for nice discussions and for helping out whenever it's needed.
Mirjana, for valuable chats (mostly non-scientific) about life and kids.
Gabriela, you are so sweet and kind. I miss you in the lab, but I know that you are having a wonderful time at home with Klara.
Jane, the cool dude of the group. I was impressed in London when you managed to visit basically all the tourist attractions in one day. Good luck in Norway!
Helena, for nice conversations and suggestions regarding IHC stainings.
Annette, for bringing some hard rock culture to the lab. I hope you enjoy your beloved Göteborg.
Osama, for interesting discussions about life and for being a nice guy.
Maria Ringvall, thank you for always being so nice and positive. I really enjoyed going to Keystone with you.
Beata, for being an excellent project student. Good luck with everything!
Josefin, the newest member in the SW-group, You are such a sweet person. I'm looking forward working with you as well as having fika or just a chat at any time!

Collaborators:
Mike Thorpe, for the collaboration on paper III, and for the help with the English language in this thesis. Thanks for being a really nice guy and for always letting me bug you with questions.
Lars Hellman, for never ending enthusiasm and scientific discussions as well as the collaboration on paper III.
Sofia Jonasson and **Josephine Hjoberg** for the collaboration on paper I.
Anders Bucht, for the collaboration and sharing valuable comments on paper I.

Present and past members of the department:
Lena Holm, head of the department, thanks for the help with the microscope, valuable comments on histology and for help with grant-related issues.
Kerstin Nordling, thanks for always having a helping hand and bringing joy to the corridor. Too bad you moved to KI…

Piotr Wlad, I'm never worried when machines break down in the lab or when my computer goes nuts, because I know you can fix it! It's a gift to have you around.
Janne and **Staffan** for always sharing your scientific knowledge.
Liya, My, Anna R, Marlene, Sara N, Mona, Ronnie, Hanna, Louise, Elena, Charlotte, Jenny P, Jay, Hanan, Ingrid, Ulrika, Ekaterina, Kristina, Erik, Kiran, Ren and all the other people at B9:4 who makes going to work fun. I would also like to thank **Anna Wistedt** and **Gunilla Ericson Forslund** at Ultuna for the friendly attitude and help with the microscope and histological stainings.
I would like to thank **Carolina Wallström-Pan** and **Leena Grönberg** for always being helpful with administrational issues as well as other things.

The "Mast cell journal club" members for having nice discussions once a month! Especially I would like to thank **Jenny Hallgren Martinsson** and **Joakim Dahlin**.

Vänner:
Min kära vän **Jenny** och sambo **Henrik** för att ni alltid finns där, oavsett vad det kan vara. Att ha vänner som er betyder verkligen allt!
Greta, min vapendragare under tiden som student vid Uppsala Universitet. Så otroligt många roliga minnen vi delar! Vad sägs om labbarna i organisk kemi, breakfast for champions, övernattningar på "gårn", Egypten, Kreta, Klubban (med vinprovning på berget) eller bara "titta på bilderna" på stan…
Anna, du är så omtänksam, snäll och underbar! Tack för alla trevliga stunder tillsammans. Det är så roligt att du ska ha en liten bebis snart!
Tjejgänget från gymnasiet för alla trevliga middagar! **Lisa**, för alla trevliga pratstunder, promenader och pass på Ekeby. **Maria**, du är en fantastisk organisatör, kock och framförallt person. **Åsa**, det var jätteskönt att dela mammaledigheten tillsammans med dig. Du är så klok och får mig att inse att saker inte måste vara på ett visst sätt hela tiden, speciellt när det gäller småbarn. **Susanne,** för att du alltid finns där, bara ett telefonsamtal bort. **Elisabeth,** alltid så trevlig och smart. Säg bara till när du vill ha besök i Västerås – jag kommer. Jag vill också tacka **Lenita** och **Sandra** som förgyllde mammaledigheten med promenader, luncher, fika och sångstunder!

Sist med inte minst, min familj:
Min underbara syster **Johanna** och sambo **Linus**. Tack för att ni alltid finns där och är så omtänksamma och snälla! Tack för alla trevliga middagar,

pratstunder och att jag och Melvin får komma och klappa på Possible och alla andra roliga djur! Finns det plats för en ponny bland travhästarna?
Världens bästa **Mamma** och **Pappa**, för att ni stöttar mig i vått och torrt. Jag hade inte klarat det här utan er!
Melvin, Mammas lilla prins. Det är underbart att få leka, busa och gosa med dig. Du är mitt allt och jag älskar dig så mycket!