Mast Cells as Sentinels

Role of serglycin and mast cell proteases in infection and inflammation

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Dissertation presented at Uppsala University to be publicly examined in C8:305, Uppsala Biomedicine Centrum, Husargatan 3, Uppsala, Friday, August 17, 2012 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Mast cells (MCs), normally classified into connective tissue MCs and mucosal MCs, are highly granulated cells found in the interface between the interior and the exterior environment of our body, e.g. skin, airways and gastro-intestinal tract. They react to bacteria, parasites, viruses, and allergens by degranulation and release of preformed and newly synthesized inflammatory mediators. The MC-proteases (tryptases, chymases and carboxypeptidase A), histamine and serglycin (SG) proteoglycans are preformed mediators. Among these, SG is also expressed in a variety of other immune and non-immune cells. Heparin and chondroitin sulphate glycosaminoglycan chains confer highly negative charge to SG, by which MC-proteases are retained in secretory granules. Deletion of SG cause impaired packing and storage of most MC-proteases. During challenge with Toxoplasma gondii the SG-deficient mice showed significant lower inflammatory cytokine levels in comparison to wild-type mice. Results were consistently similar in vitro, bringing forward the importance of SG in inflammatory cytokine and innate immune responses towards T. gondii. Infection with Trichinella spiralis in SG/- mice caused increased intestinal enteropathy, a tendency of delayed worm expulsion and increased larval burden in the muscle tissue as compared to wild-type animals. An altered TH2 cytokine response was also observed, and all these effects were not repaired by wild-type MC reconstitution of the SG/- mice. Altogether, our results suggest that SG is important for tissue homeostasis, and that SG expressing cells seem capable of switching from a SG-dependent storage mode to a SG-independent secretory mode upon infection.

The chymase (MCPT4) expressed by connective tissue MC has been implicated to have a protective role during infection and in limiting inflammation. We explored a protective role by inducing T. gondii infection in the Mcpt4-null mice, and found MCPT4-mediated recruitment of neutrophils and eosinophils via control of cytokine signaling. Endogenous proteins “alarmins” released by dead cells can trigger tissue and cell damage. We conclusively show that chymase efficiently degrades Hsp70 both in vitro and in vivo and that the degradation of other alarmins, e.g. HMGB1, biglycan and IL-33 may also depend on chymase.

Keywords: mast cells, serglycin, chymase, infection, parasite

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Believe in yourself
List of Papers

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


*These authors contributed equally to the study.
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Abbreviations

• MC Mast cell
• SG Serglycin
• PG Proteoglycan
• GAG Glycosoaminoglycan
• DC Dendritic cell
• NK Natural killer cell
• APC Antigen-presenting cell
• TNF Tumor necrosis factor
• IL Interleukin
• IFN Interferon
• TGF Transforming growth factor
• GM-CSF Granulocyte macrophage colony stimulating factor
• MMP Matrix metalloprotease
• PAF Platelet activating factor
• MMC Mucosal
• CTMC Connective tissue mast cell
• PRR Pathogen recognition receptor
• DAMP Damage associated molecular pattern
• CTL Cytotoxic T lymphocyte
• HMGB1 High mobility group box 1
• BGN Biglycan
• HSP Heat shock protein
• NDST-2 N-deacetylase/N-sulfotransferase 2
• MCP Mast cell protease
• mMCP Mouse mast cell protease
• SCF Stem cell factor
• TLR Toll like receptor
• CPA Carboxypeptidase A
• FGF Fibroblast growth factor
• MCP-1 Monocyte chemotacting protein 1
• RANTES Regulated upon Activation, Normal T-cell Expressed and Secreted
Introduction

The immune system

Our body has a unique system that works as the defense frontier protecting us from external and internal threats. Of the various threats, pathogens like parasites, bacteria and viruses are the common invaders.

This defence system of the body, termed immune system has two components: i) the innate and ii) the adaptive immune system. The innate immune system is armed with leukocytes, which includes MCs (MCs), eosinophils, basophils, phagocytes (macrophages, neutrophils and dendritic cells) and natural killer cells. They provide immediate defence against an infection. The adaptive component provides the immune system to “recognise and remember” pathogens specifically and consequently mount a strong response. The leukocytes of the adaptive immune system are sub divided into B cell and T cell (killer T cell or CTL and T helper cell) lymphocytes. The B cells participate in the humoral immune system and the T cells in cell-mediated immune responses. CTLs possess receptors to bind to antigens or target cells and release mediators like granzyme, perforin, granulysin etc. thereby removing them. The innate cells act as mediators to activate the adaptive immune system. A cross-talk between the cells of the innate and adaptive immune system is essential for a proper immune response [1, 2].

Mast cells: their role in physiology

Over a century ago (1899), Paul Ehrlich received his noble prize in “Physiology or Medicine”. One of his major accomplishments was the description and characterization of tissue MCs, which he christened as “Mastzellen” [3]. He described them as cells filled with special type of granules that reacted metachromatically with aniline dyes (Ehrlich et al. 1877). This led him to postulate the novel idea that, MCs should be identified on the basis of their metachromatic reactivity and not only by their morphological appearance. During the years following his first observation, subsequent studies in his group and by other researchers have confirmed his findings. Even though Ehrlich observed an increase in MC numbers following chronic inflammation and other disorders the exact functionality of MCs have eluded him and scientists after him for a great length of time.
Now MCs are defined as highly granulated, sentinel cells. They originate from the bone marrow and circulate in the blood stream as precursors. Fully differentiated and mature MCs are usually not found in the circulation. From the vessels the precursors migrate to peripheral tissues where the environmental cues enable them to differentiate and mature to their destined subtype while becoming tissue residents. MCs are present in almost all vascularized tissues; in close proximity to blood and lymphatic vessels, nerve bundles and mucus producing glands. They reside in the interface between the interior and the exterior environment, e.g. the skin, airways and gastro-intestinal tract where a barrier function is essential for defense against external challenges.

The importance of MCs in immunity has heavily lied on their role as critical mediators of allergy, asthma and other hypersensitivity reactions. MCs participation in passive cutaneous anaphylaxis (PCA), a type I hypersensitivity reaction shows their importance in the early phase of allergic reaction. MC deficient mice are fully protected from PCA but reconstitution of these mice with MCs restores anaphylaxis [4]. During the last two decades research on MCs, focusing on the role in the immune system has brought forward their importance as a frontline participator in various physiological as well as other pathological conditions.

Infection and immunity towards various microbes (bacterial, parasitic and viral) is now one of the most studied branches of MC biology. MCs can function as antigen presenting cells (APCs) or release mediators upon recognizing bacteria or viruses [5-9].

The protective function of MCs in bacterial infections is demonstrated in MC deficient mice that show a reduced capacity to fight infections. However there are instances where these protective mediators are utilized by the pathogen against the host making MCs the “bad guys” in infection. *Shigella dysenteriae* and *Helicobacter pylori* are two classic examples. *S. dysenteriae* mediates pathogenesis via the shiga toxin that causes diarrhea by triggering excessive leukotriene C4 (LTC4) secretion [10]. In the case of *H. pylori* there is a sustained inflammatory process in the mucosa that is maintained by rapidly degranulating MCs in response to bacterial secretion [11-13].

Similarly in parasitic infection MCs have been shown to be important (during *Stronglyoides venezuelensis* infection) and in some cases very essential (*Trichinella spiralis* infection). Further studies have provided evidence that different MC mediators contribute to the pathogenesis and infection clearance of different nematodes.

In the past few years, MCs have even been implicated in a wide range of inflammatory diseases [14] that include migraines [15], arthritis [16-17], cardiovascular disease [18], interstitial cystitis of the urinary bladder [19], and irritable bowel syndrome [20].

Of the most studied influences of MCs on inflammatory disease, is the role in arthritis. MC deficient mice do not develop antibody-induced arthritis
at all [21], whereas in the collagen-induced model of arthritis there is a large accumulation and degranulation of MCs in the infected joints [22]. Similar degranulation patterns are also observed in the antibody-induced model. MC chymase was shown to be the aggressive mediator towards arthritis by contributing to the antibody response and severity of the disease [23].

Research in the field of MCs and cancer has brought forward the important role of MCs in a wide variety of cancers as well. MCs are most often located in the vicinity of the tumors. They are a rich source of growth factors and angiogenic cytokines and probably play both a positive and a negative role in tumor progression. The question that scientists are in search of answering now is what are the conditions that make a MC aid in tumor growth? Also conversely, what makes it decide to suppress and limit tumor growth.

One of the most interesting topics currently in MC research is the alleged protective role in limiting inflammation. Galli and coworkers successfully showed that MCs can significantly reduce snake and honeybee venom induced pathology in mice and reduces related morbidity and mortality as well [24]. This brought into light a previously unrecognized role of MCs.

Mast cells: Lineage and Heterogeneity

Lineage
MCs originate from hematopoietic stem cells in the bone marrow [25]; they migrate through the blood and enter their target tissue where they differentiate to attain their mature characteristic morphology. Until recently MCs were usually misunderstood as tissue invading basophils. However, in spite of the fact that both MCs and basophils have hematopoietic stem cell origins, they have distinct and different developmental pathways. While basophil development and maturation is concluded within the bone marrow, immature MCs leave the bone marrow as precursors and mature at the designated tissue sites (Fig. 1). Hence environmental cues from the tissue are vital for MC maturation [26].

What is the nature of the MC committed precursor? Earlier studies using colony formation assay indicated presence of low frequency MC precursor activity in the bone marrow, peripheral blood and lymph nodes of mice [27, 28]. However, these precursors were not purified from blood or bone marrow until Rodewald et al. isolated a cell population from murine fetal blood and identified them as MC-progenitors [29]. They characterized them as high expression c-kit (c-kit\textsuperscript{hi}) and low expression Thy-1 (Thy-1\textsuperscript{lo}) cells, containing cytoplasmic granules and transcripts for MC-specific proteases. They lack the high-affinity Fc-IgE receptor (FceRI) expression on their surface and, in vitro develop into MCs only and not other
hematopoietic lineages. Viability, tissue distribution and numbers of MCs are totally dependent on stem cell factor (SCF). This stromal cell derived cytokine triggers mast/stem cell growth factor receptor (c-kit) signaling which leads to dermal and connective tissue MC expression. The MC progenitors in humans are c-kit+/CD34+ agranular cells [30]. Additionally they express CD13 and are distinguishable from monocytes due to the lack of CD14 expression.

![Diagram](https://example.com/diagram)

**Figure 1.** Mast cell origin and maturation.

### Heterogeneity

MCs originating from the bone marrow and finally ending up in the different tissues in the body do not constitute a homogenous population. Histochemical and functional heterogeneity of MCs [31] are of immense importance for the proper understanding of their exact role in health and disease.

MCs cultured *in vitro* from mice and humans have provided a better platform for studying their heterogeneity. Most of the MC classifications in the past have been based on phenotypical differences. The rodent MC population was divided into connective tissue MCs (CTMC) and mucosal MCs (MMC). CTMCs were those located in the skin and peritoneal cavity, whereas MMCs were MCs of the intestinal lamina propria. Multiple studies have subsequently shown that CTMCs and MMCs (from rats and mice) apart from phenotypical differences vary in aspects of biochemistry and functionality as well. They differ in size, morphological and histochemical properties, histamine content, proteoglycan and neutral protease content as well as in their responsiveness on activation or inhibition. T cell-dependent immune
response to parasitic infection can trigger expansion of the MMC subpopu-
lation [32] [33], in contrast CTMCs are unresponsive to T cell-dependent sig-
nals and are present in athymic nude mice or rats [34]. Even though certain
parameters draw a line between CTMC and MMC distinction, at times cer-
tain MC population bears features common to both the subclasses [35]. Such
varieties in MC features indicate that there are different subpopulations of
MCs at different regions of the same anatomical site in an adult rodent.

Human MCs also exhibit a diverse heterogeneity similar to rodents, most
of which were originally identified by histological techniques [36]. Two
distinct human MC subtypes were found based on their distinctive neutral
protease patterns in different tissues [37-38]. Immunohistochemical staining
can distinguish these two subclasses accurately in the same tissue section,
which ensures an easy and efficient MC quantification in several anatomical
sites. This diverse heterogeneity further supports the effect of microenvi-
ronmental cues on MC development, maturation and functionality [31].

Mast cell subtypes

Currently MCs in rodents as well in humans are broadly classified based on
their heterogeneity that is distinct and comparatively easy to recognise. Mouse MCs are classified into CTMCs present in the mucosa and lamina pro-
pria of the respiratory and GI tract; and MMCs of the dermis, peritone-
um, synovia, and sub mucosal layers. In humans, they are classified on the
basis of their neutral protease contents into MCs that express “tryptase only”
(MC_T) and those that express “both tryptase and chymase” (MC_TC). The
MC_TC’s produce interleukin 4 (IL-4), interleukin 13 (IL-13) and prostaglan-
din D2 (PGD2), whereas MC_T’s synthesize interleukin 5(IL-5), interleukin 6
(IL-6) and LTC4 [39-43].

The MC_TC’s are mostly located in the alveolar lung partitions and in the
mucosa of the small intestine while the MC_TC resides in the skin and small
intestinal sub mucosa. Comparing human MCs to rodent MCs in terms of
tissue localisation, one can conclude that MC_TC are similar to rodent CTMCs
and MC_Ts are comparable to rodent MMCs. This might help to translate
certain effects of the MCs, as seen in the various rodent knockout models for
predictions for their human counterparts. However, it is also important to
remember that not all properties of human MC_T and MC_TC can be predicted
on the basis of rodent CTMC and MMC responses.
Mast cell classification in mouse and human.

Mast cell activation

MCs are versatile in responding to different stimuli (Fig. 3). This naturally suggests that they are able to recognise the different stimuli by means of different receptors located on their surface. MCs use different modes of receptor activation in various pathological processes. Activation in turn leads to the release of pre-formed inflammatory mediators, synthesis and release of cytokines and chemokines, thereby causing total degranulation. Conversely, the process can be independent of the degranulatory mechanism. The degree of this response is, again, governed by the specific stimulus that is modulated by a balance of positive and negative intracellular molecular events [44].
**FcεRI-IgE mediated activation**

The most broadly studied pathway of MC activation is the aggregation of the IgE-occupied high-affinity receptors for IgE (FCεRI) and the antigen. Three subunits of FCεRI including FcεRIα, FcεRIβ and two FcεRIγ are expressed and presented by MCs. The subunits are arranged in a tetramer (αβγ2). The α-chain is responsible for binding to the Fc part of IgE [45-47], resulting in the attachment of IgE to the cell surface. The β-chain interacts with either Lyn kinase via activation of Syk [48, 49] or through a Fyn dependent pathway [50] leading to signal amplification and thereby resulting in phosphorylation of the β- and the γ-chains. The FcεRI tetrameric form is a must for cell surface presentation in rodents, but in humans an isoform that lacks the β chain exists. In humans apart from MCs and basophils, other cells like monocytes, eosinophils and DCs also express α and γ-chains of FcεRI in its trimeric form [51-53].

*Figure 3. Various modes of mast cell activation.*
**FcγR- IgG mediated activation**

MCs can also mediate their activation via IgG receptors expressed on their surface. Human MCs express both the activatory (FcγRI and FcγRIII) and the inhibitory (FcγRII) IgG receptors [54, 55], whereas mouse CTMCs only express FcγRIII [56]. In humans, FcγRI can interact with IgG1 resulting in degranulation and cytokine production [98]. The FcεRI and FcγRI receptors share the common γ-subunits [99], but their activation pathways are not the same. The inhibitory activity of the FcγRII on MCs results in down-regulation of secretory responses [57,58]. The fact that MCs expresses FcγRI on their surface and degranulate upon IgG cross-linking, also makes them an ideal participant in T_{H1} type immune responses.

The Fc receptors (FccR & FcγR) bind to pathogen-specific antibodies thereby aiding MCs in pathogen recognition and elicitation of a proper T_{H1} or T_{H2} immune response.

**Complement-anaphylatoxin mediated activation**

MC degranulation can also be caused by the complement components C3a, C4a and C5a *in vivo* through specific receptors leading to anaphylactic reactions [59, 60]. There is no evidence of any crosstalk/desensitization between IgE-directed agents and anaphylatoxins [61]. In the human complement system C3a and C5a, expressed on skin have been shown to be capable of MC activation [62] however they have no effect on lung MCs [63]. C5a receptors are usually not expressed in normal physiological condition [64] and expression of C3a receptors are observed only during systemic mastocytosis [65]. In mice, only CTMCs express complement receptors [66].

**Direct recognition by pattern recognition receptors mediated activation**

MCs can very efficiently alert the host immune system to the presence of pathogens. This is achieved by directly recognizing pathogens through set of immune response expressed proteins receptors called pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), which are activated by specific molecules from the pathogens that have conserved molecular patterns, termed pathogen-associated molecular patterns (PAMPs) [67].

Currently there are eleven members of the TLR family [68-72] identified so far. MCs express TLR-2, 3, 4, 6, 7, 8 and 9 [73,74]. Different PAMPs subsequently stimulate different TLRs. For example, LPS (lipopolysaccharide) a gram-negative bacteria cell wall component stimulates rodent MCs via TLR4 and elicits cytokine secretion independent of degranulation. Peptidoglycans (a constituent of gram-positive bacteria) on the other hand, induce
cytokine production via TLR2 in a degranulation dependent manner [75] [76, 77]. Similarly in human cord blood cells both LPS and peptidoglycan induces a TH2 type cytokine response, but only peptidoglycan can elicit histamine release. Virus particles are identified via TLR3 on MCs that recognise the viral dsRNA[78]. Unlike IgE mediated activation, TLR based MC induction is usually degranulation independent.

Other modes of activation
Apart from the aforementioned receptor mediated pathways, certain cytokines, basic compounds, peptides and physical changes can also activate MCs. Cytokines like IL-3, IL-1, IL-8 and GM-CSF can release histamine at various concentrations [79-81]. SCF can degranulate MCs both in vivo and in vitro [82]. MIP-1α can degranulate MCs both in vivo [83].

A number of peptides have been shown to be capable of either degranulating MCs, releasing histamine or both. Neuropeptides such as substance P, neurtensin and vasoactive intestinal peptide (VIP), as well as Rab3A (a small monomeric GTP-binding peptide) are able to perturb adjacent MCs [84] leading to degranulation.

There are also substances that directly cause MC’s to degranulate. These include opiates, calcium ionophores, compound 48/80, endothelin and adenosine [85]. Exogenous stimuli following certain pathogen attacks that breach the skin barrier such as components of wasp venom or mosquito saliva [86] can lead to efficient MC degranulation. It is a small peptide called “mastoparan” in the wasp venom that causes potent degranulation [87].

Physical changes, such as stress due to changes in osmolarity and pressure, as well as cell-to-cell interactions can activate MCs. There is even evidence that activated T cells via ICAM-1 and LFA-1 are capable of activating MCs to release mediators and cytokines [88, 89].

In addition to the conventional Fc-receptors and PRR’s, MCs express, other receptors on their surface including histamine (H) receptors, CD40, CD48 and CD91. CD48 for example can detect presence of Escherichia coli, Mycobacterium tuberculosis and Staphylococcus aureus [90, 92].

Mast cell mediators
MCs store different groups of mediators that they secrete upon activation. The mediators vary in their functionality (Fig. 4); some possess unique biological activity at times, whereas at other times their activities can overlap. MC mediators can be broadly divided into 3 main classes: i) Preformed granule associated mediators, ii) newly generated lipid mediators, and iii) a wide array of cytokines and chemokines. These mediators can be constitutively released or released only upon activation.
Preformed granule associated mediators

The granule-associated mediators in MCs are packed inside the cells and contain biogenic amines (histamine and serotonin), neutral proteases, non MC-specific proteases, and proteoglycans (PGs).

Biogenic amines: MCs [93] and basophils [94] produce histamine, a biological amine whose role is diverse, varying from triggering the inflammatory response in tissues, to regulation of gut physiology and acting as neurotransmitter. In addition, small amounts of histamine are also synthesized by platelets and histaminergic neurons, for example. Inside cells, histamine is stored in the secretory granules (at acidic pH) by an ionic linkage with the carboxyl groups of peptides and PGs. However MCs are the major source of histamine, which is to-date the most vividly studied MC mediator in terms of its biological activity and mode of action. Synthesis of histamine occurs from the action of the enzyme L-histidine decarboxylase (HDC) on the amino acid histidine. HDC null mice have decreased MC numbers and decreased protease and PG levels [95].

Serotonin mainly functions as a neurotransmitter of the central nervous system, but is also present in many peripheral tissues including most immune cells (platelets, lymphocytes, monocytes, macrophages, MCs and pulmonary neuroendocrine cells) and enterochromaffine cells of the gut [96]. Studies show that brain cells and the enterochromaffine cells are the main producers of serotonin, which is then taken up and stored by other cells. Platelets have been denoted the “mobile storehouse" and MCs the stationary storehouse for serotonin [97]. Unlike rodents, human MCs do not contain serotonin [98, 99]. Some well-studied major functions of serotonin in immunity include T-cell and NK cell activation, delayed type hypersensitivity responses and production of chemotactic factors (e.g. from macrophages) [100].

Proteases: MC proteases comprise both neutral MC specific proteases and non-MC specific proteases. Non-MC specific proteases include lysosomal cathepsins, granzymes and neurolysin [101]. The MC-specific proteases are divided into three groups, the serine proteases, such as tryptases and chymases, and the zinc-dependent metalloprotease carboxypeptidase A (CPA).

The distribution of MC-protease amounts varies among species and also among MC subsets. In addition the MC-protease levels are also directly related to the stage of cellular differentiation and maturation and is grossly regulated by many other factors, disease being one of them. MC chymases and tryptases are sub-grouped based on their phylogenetic relationships [102] into alpha (α) or beta (β) classes.

The MC \(_T^\) subclass of human MCs express only tryptase (α and β) and the MC \(_TC^\) subclass expresses all types of MC proteases (tryptases, α-chymase
Mouse CTMCs express two different chymases (the \( \beta \)-chymase mouse MC protease 4 (Mcpt-4) and the \( \alpha \)-chymase (Mcpt-5) and tryptases (mMCP-6 and mMCP-7) as well as MC-CPA, whereas MMCs express two types of \( \beta \)-chymases (mMCP-1 and mMCP-2) only [103].

**Mast cell tryptases:** Tryptases are tetrameric serine proteases with trypsin-like substrate specificity (i.e. they cleave after Lys/Arg residues). Human MCs predominantly secrete two classes (\( \alpha \) and \( \beta \)) of tryptase, of which \( \beta \)-tryptases are of three subtypes: \( \beta I \), \( \beta II \) and \( \beta III \) [104]. Structural studies revealed a form of tryptase that was exposed to the cell surface upon MC degranulation. This form was distinctly different from the \( \alpha \) or the \( \beta \) forms and was named gamma (\( \gamma \)) tryptase or hTMT [105, 106]. Human MCs have been shown to also express another tryptase form, the \( \delta \)-tryptase, although its biological significance needs to be confirmed [105]. Interestingly, almost 29% of the human population is genetically deficient in \( \alpha \)-tryptase, with a wide variation existing between ethnic groups [107]. Mouse MCs express two tryptases, MCPT6 and MCPT7 and a transmembrane \( \gamma \) tryptase. The \( \gamma \) form is designated as mTMT [105]. In addition, murine MCs during their early stages of differentiation have also been shown to also express a tryptase MCPT11. Expression patterns of rat MC tryptases are similar to that of the murine counterparts and they express two tryptases, rMCPT-6 and rMCPT-7. In other species, two tryptases, 1 and 2 have been identified from sheep and bovine sources [108, 109], and one tryptase has been isolated from both canine and porcine origin [110, 111].

**Mast cell chymases:** Chymases are monomeric serine proteases with chymotrypsin-like specificity (i.e. they cleave after aromatic amino acid residues). Unlike tryptases, chymases are synthesized as inactive precursors and the removal of the acidic dipeptide by dipeptidylpeptidase [112] facilitates activation due to a conformational change [113]. The active form is then stored in the granules. Chymase remain attached to the glycosaminoglycan (GAG) chains even after degranulation. Human MCs express only one \( \alpha \)-chymase [114-116]. There are four mouse \( \beta \)-chymases designated MCPT-1, -2, -4 and -9 whereas only one \( \alpha \)-chymase, MCPT5 is known, although it seems to have more elastase-like cleavage specificity [117-123]. MCPT-9 is specifically expressed by MCs in the mouse uterus [124]. Rat MCs expresses one \( \alpha \)-chymase (rMCPT5) and four \( \beta \)-chymases (rMCPT1, rMCPT2, rMCPT3, and rMCPT4) [125-127]. Canine MCs express one \( \alpha \)-chymase [128]. rMCP-1 and mMCP-4 are the corresponding rodent \( \beta \)-chymases comparable to the human \( \alpha \)-chymase.

**Mast cell carboxypeptidase A:** CPA has a monomeric structure and shows exopeptidase-like specificity (i.e. cleaves amino acids from the C-terminal
end) [129]. Among all the species that have been investigated so far, only one MC-CPA gene has been identified [130-133]. The tissue localisation and other properties of MC-CPA are similar in both murines and humans [134]. The exact mechanism that converts proMC-CPA into its active form is not fully understood, but there is evidence that cathepsins E might play a role in this process [135]. Further evidence also highlights the importance of ser-glycin (SG) and its heparin linked side-chains in proMC-CPA processing [136,137]. Once processed, MC-CPA is stored inside the granules in its active form. Low pH levels (around pH 5.5) inside the granules keeps the protease activity to bare minimum.

Proteoglycans: PGs are protein cores with glycosaminoglycan (GAG) side chains, which are defined by both their core protein as well as their side chains. The GAG chains are repeating disaccharide subunits of uronic acid and hexosamine structures. Each disaccharide has between 0 to 3 sulphate groups that bestow the GAG’s with highly negative charges.

Most cell types express PGs and the same cell type often expresses more than one type of PG. They are present on the cell surface (syndecan and glypican) of most cells, or are present in various extracellular locations (decorin, aggrecan, perlecan), e.g. cartilage and basement membranes. Cell surface PGs have been proven to play an essential role as co-receptors for various growth factors. SG, an intracellular PG, is an essential component of MC granules. It is in fact the only “committed” intracellular PG identified so far. SG has a core protein of 153 amino acids attached to GAG chains. Twenty-five serine-glycine repeats in the core protein between amino acids 89 and 137 give this PG its name (ser-glycin). The repeats are the linkage sites for the negatively charged GAG’s that can be heparin or chondroitin sulphate, the former capable of carrying upto three sulphate groups per disaccharide unit. These highly negatively charged side chains offer ideal packaging for the storage of mediators in granules. The size of SG inside cells varies depending upon the number of GAG side chains and their chain length [138]. There have been studies that show an increase in size of this PG on stimulation of the cell as well [139, 140], suggesting the possibility of SG undergoing alterations during immunological challenges [141].

SG is highly expressed by several hematopoietic cell types, notably MCs, platelets, NK cells, CTLs, macrophages and monocytes [138, 142, 143-145]. Furthermore, it is also expressed by endothelial cells [146,147], smooth muscle cells [148], chondrocytes [149] as well as in pancreatic acinar cells [150], uterine decidua and early embryonic cells [151]. Various transformed cancerous cell types, e.g. metastatic carcinomas [152] and multiple myeloma cells [153] have also been shown to express considerable amounts of SG which in certain cases might have a negative implication [154]. In comparison to other cell types, MCs seem to express high levels of SG [155] which is supported by the fact that SG expression is highly up-regulated during MC
differentiation and maturation from bone marrow stem cells [156]. The variability in expression patterns of SG in different hematopoietic cells is not yet fully understood. There is some evidence that indicates differential expression of regulatory factors [157] as the reason.

Owing to the high anionic charges of the heparin chains of SG, it has long been implicated to play a major role in secretory granule storage. Generation of the SG knockout (SG\(^{-/-}\)) mouse proved this notion [158]. The knockout mouse showed a severe impairment in storage of granule proteases predominantly in MCs and other immune cells. However, the mRNA levels of these proteases were unaltered. This proves the importance of SG in storage of mediators inside cells. Interestingly, the MCs of SG\(^{-/-}\) mice showed similar characteristics and morphology to MCs that lack N-deacetylase/N-sulfotransferase 2 (NDST2\(^{-/-}\)). NDST2 is an enzyme that regulates sulphation of heparin, which makes heparin highly negatively charged. Given that most heparin in MCs is highly sulphated, it is evident that granule storage is heavily dependent on the strong anionic charge given to SG by highly sulphated heparin side-chains. Thus SG through interactions between its negatively charged sulphated GAG side-chains and basically charged granules, fulfills its storage capacity roles. Although MCs are the most affected cell type in the SG\(^{-/-}\) mice, subsequent studies have shown a whole range of other hematopoietic cells that show defective granule storage.

**Release of serglycin and regulation of protease activity**

Release of SG from the cells is accompanied in complex with the various intracellular compounds that interact with it. Some of these compounds are only loosely associated with SG and under this process may become detached from SG and thereby readily diffuse away from the degranulated cell. This detachment from SG can be dependent on pH difference. Other compounds attach to SG with high binding affinity, and are less dependent on pH changes when it comes to dissociation. Such compounds therefore remain in complex with SG even after cellular release. Some examples include MC chymase, MC-CPA, and granzyme B [159].

Studies indicate that SG-chymase and SG-CPA complexes tend to remain at the cellular surface even after exocytosis [160-162]. It can be postulated that SG’s interactions with the proteases probably anchor the compounds at the site of exocytosis and prevents them from diffusing away from the release site. This helps in restricting a given response to a specific anatomical site [159]. Anchoring of certain compounds/proteases with SG after exocytosis is also advantageous to prevent a proteolytic attack, or for better presentation of cytokines/chemokines to their target cells. There are studies suggesting that the formation of tight associations between granzyme B and SG aid the delivery of granzymes B into target cells following exocytosis [163, 161]. Secreted SG can also interact with CD44 and thus bind to other cell surfaces causing cell stimulation. The GAG side-chains makes SG a
very good candidate for binding to inflammatory compounds present in the extracellular environment by sequestering them for an enhanced immune response.

One of the most important functions of SG is probably maintaining homeostasis of the body’s immune cells, which is evident when one looks into aging SG−/− mice, which spontaneously develops enlargement of multiple lymphoid tissues including the spleen, BALT, and Peyer’s patches [164]. How SG mediates this function is still not clear, it might be via a balance between apoptosis and proliferation.

Non-mast cell specific mediators: In addition to the already mentioned mediators, MCs also releases certain matrix metalloproteases (MMPs). They are a family of Zn2+ and Ca2+ dependent metalloproteases with 25 members been described so far [165, 166]. Human MCs express MMP-9 [167-169] as well as MMP-1 [170, 171]. Mouse MCs express MMP-9 [172, 173]. SCF promotes up-regulation of MMP-9 production in human MCs [174] in contrast to murine MCs where MMP-9 production is down regulated [172] Apart from the above; MCs also express granzymes, neurolysin and lysosomal cathepsins.

**Newly generated lipid mediators**

Activation of MCs can initiate the de novo synthesis of certain lipid mediators. Lipid mediators act to stimulate the immune response, e.g. inducing vascular changes and attracting other immune cells. The important ones are the members of the cyclooxygenase (COX) and lipoxygenase family. MC’s produce prostaglandins and thromboxanes of the cyclooxygenase family and leukotrienes (LTs) and hydroperoxyeicosatetraenoic acids (HPETEs) of the lipoxygenase family.

The sequential actions of cytosolic phospholipase A2, 5-lipoxygenase, and leukotriene A4 (LTA4) hydrolase respectively on membrane phospholipids leads to the generation of all leukotrienes. The first step is the formation of LTB4 from 5-HPETE. MCs predominantly form LTC4 through glutathione and LTA4 conjugation mediated by LTC4 synthase [175]. Extracellular processing of LTC4 leads to the formation of LTD4 [176], which is then further metabolized into LTE4 [177]. LTB4 has been reported as a potent chemoattractant for various leukocytes including neutrophils, macrophages, eosinophils and monocytes [178-182], via the G protein coupled high affinity surface receptor BLT1 [183, 184]. BLT1 is also expressed on CD8+ effector memory T cells and helps in effector T cell accumulation at sites of inflammation [185, 186]. LTB4 secreted by activated MCs in turn acts as a chemoattractant for MC progenitors but not mature MCs. This was established by the fact that immature MCs express BLT1a receptor for LTB4, which is not present on mature cells. Thus LTB4 might be an auto-
crine regulator of tissue MC numbers [187]. LTB4 can also enhance lysosomal enzyme release, and augment superoxide anion production.

Cyclooxygenase enzymes (COXs) act on arachidonic acid to produce prostaglandins. The first step in this process is the production of prostaglandin H2 that then serves as the substrate for two enzymatic pathways leading to the formation of other prostaglandins or thromboxanes. Activated MCs predominantly produce PGD2 by up-regulation of PGD2 synthase (PGD2S) expression [188]. Although platelets, macrophages and certain T lymphocytes are known to secrete PGD2, MCs are exclusive cellular source of PGD2, producing levels almost 1000 times higher than any other cells [189, 190]. PGD2 secretion after MC degranulation [191, 192, 187] causes chemotaxis of leukocytes (eosinophils, basophils, T_{H2} cells), contributing in development of allergy [193-195]. PGD2 also plays a dual role of either inhibiting or mediating inflammation depending on the physiological or pathological condition. Furthermore, PGD2 can affect the orientation of an immune response towards a T_{H2} type response [196] and also affect the maturation of human monocyte derived DCs.

**Figure 4.** Physiological consequences of mast cell activation.
MCs can also secrete platelet-activating factor (PAF) upon activation by IgE crosslinking. PAF can aggregate and degranulated platelets, increase lung resistance and systemic hypotension: all symptoms of systemic anaphylaxis [197]. PAF can also act as a chemoattractant for certain leukocytes [198-202] as well as have the capability to activate them [203-206]. Cytokine production in mononuclear cells can also be modulated by PAF.

Cytokines and chemokines
Cytokines are immunomodulatory substances secreted by specific cells of the immune system to carry signals locally between cells, and exert the necessary effect on other cells. Chemokines are a small family of cytokines whose name originates from their chemotactic properties towards nearby responsive cells. Cytokines and chemokines show a broad spectrum of bioactivities, playing major roles in inflammation, the immune response, infection, cell repair and growth. MCs produce a wide variety of both pro and anti-inflammatory cytokines. MCs are involved in both storage [207, 208] and additional secretion [209-211] [74] of the classical pro-inflammatory cytokine TNF-α. The long list of cytokines that MCs are capable of producing includes IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, GMCSF, bFGF-2 and TGF-β1 [212-219]. Among the above mentioned cytokines IL-3 is vital for MC development and growth [220] and can differentiate bone marrow cells into functional MCs in vivo. MCs also express certain chemoattractant cytokines also known as chemokines. Secretion of RANTES and MCP-1 by MCs [221] results in the attraction/migration of macrophages or monocytes to the tissue site in question whereas secreted IL-8 [222] attracts neutrophils. There is also evidence that human MCs produce IL-16 and lymphotactin, both of which can contribute to lymphocyte recruitment at sites of MC degranulation [223, 224]. Cytokine release mostly occurs via receptor aggregation followed by degranulation, however cytokine/chemokine release can also be elicited without any degranulation (e.g. via LPS, SCF, thrombin and NGF).

Mast cell proteases: physiological role
Of the preformed mediators, the MC- specific proteases make up as much as 35% of the total protein content in the secretory granules [225, 38]. These proteases, tryptase, chymase and CPA that are released upon degranulation are active at neutral pH.

Among the three proteases the biological role of CPA is still elusive. However since they are localized in the same PG complex with chymase, suggestions have been made that CPA probably act in consortium with chymase [226]. Co-operation in activity was described in the processing of endothelin-1 (ET-1) [227] and in the formation and degradation of angiotensin II [228].
The biological role of chymase is potentially more carefully studied. Chymase have been implicated in the processing of a wide array of proteins in vitro. Human chymase can activate IL-1β [229], degrade albumin [230] and release cell-associated SCF [231], degrade fibronectin [232] to mention a few. In vivo chymase has been suggested to play a part in tissue remodeling, an example of which is a fibrosis model where chymase inhibitors were able to reduce the fibrotic response towards bleomycin [233, 234]. In another study, two independent groups in a model of scleroderma reported increased chymase levels on fibrosis development that could be counteracted upon administration of chymase inhibitors [235, 236]. Chymase can degrade thrombin and plasmin [237, 238] making it a possible candidate influencing the coagulation pathway. Chymase may also contribute to the pathology of atopic and chronic dermatitis [239, 240]. Into the transgenic era, mice lacking the gene encoding the MMC β-chymase Mcpt1 demonstrated for the first time chymase’s protective role in parasitic infections [241]. The β-chymase Mcpt4 lacking mice exhibited defective fibronectin and thrombin degradation [242]. Tryptase has a wide array of effect on coagulation and has been suggested to possess anticoagulant activity. It can limit blood clot formation by degrading fibrinogen [243], activating prekallikrin to produce bradykinin thereby increasing vascular permeability [244, 245]. Tryptase can also increase bronchial responsiveness by degrading bronchodilating neuropeptides [246] [247, 248]. This degradation effect is associated with asthma where tryptase has been proposed to induce fibroblast, smooth muscle cell and epithelial proliferation [249, 250] in association with airway inflammation.

Knock out models for mast cell and mast cell proteases

Mast cell knockout mice:
The very first MC knockout mice appeared in 1978 when Kitamura et al. reported their finding of an inbred mouse strain (WBB6F1-W/Wv or WBB6F1- Kit w/wv) that was MC deficient [251]. These mice have a double mutant allele at the W locus (i.e. c-kit), which results in a distinctive reduction in c-kit tyrosine kinase-dependent signaling, thereby causing disruption in MC development and survival. However, this mutation gives rise to other phenotypic alterations in the mice for example, loss of hair pigmentation, macrocytic anemia, total lack of interstitial cells of Cajal (ICC) and sterility [252]. Additionally they have a high incidence of spontaneously developing dermatitis [253], squamous papilloma in the stomach [254] and gastric ulcers [255]. Despite being a very interesting model, the studies using it had limitations.
Four years later, Lyon et al. reported the W-sash (W<sup>sh</sup>) mutation [256]. This is an inversion mutation in the 5’ regulatory sequences of Kit on mouse chromosome 5 [257]. The mutation leaves the gene otherwise structurally intact. The C57BL/6-Kit<sup>W-sh/W-sh</sup> mice were described in 1992 by Kitamura et al. but it was not used as a model for functional studies of MC in vivo until 2004, when MC dipeptidyl peptidase I was shown to provide protection from sepsis [258]. The W<sup>sh</sup> mutation had an advantage over the Kit<sup>W/Wv</sup> mutation in terms of fewer developmental abnormalities in the mice; to begin with, the adult Kit<sup>W-sh/W-sh</sup> mice were fertile, totally MC deficient [259], showed no signs of anemia, and the levels of hematopoietic and lymphoid cells, other than the MCs were normal [259]. Even though they lacked ICC, skin pigmentation and had a high bile reflux into the stomach, the Kit<sup>W-sh/W-sh</sup> mice did not exhibit a tendency for spontaneous pathology affecting the stomach, intestine or the skin [259].

Mast cell reconstitution:
The use of the MC deficient mouse model was just the beginning of an era to demonstrate the role of MC and their mediators in various biological responses. Researchers tried to combine them with adoptive transfer of cultured MCs. This concept of reconstituting a knockout mouse with the cells they lack boosted the studies for MC’s role as now one could use different populations of MCs deficient in a particular mediator for adoptive transfer. This would thereby help to pinpoint the contribution of that particular mediator.

The approach of reconstitution can be systemic or local depending upon the requirement of the researcher. Bone marrow cells from wild-type mice are grown in culture until they mature to form MCs under the influence of SCF and IL-3. For systemic reconstitution, the cells are then injected into the peritoneum (i.p.) (2.5 to 5 X 10<sup>6</sup> cells) or intravenously into the tail vein (i.v.) (2.5 to 5 X 10<sup>7</sup> cells). Both these methods can also be used in conjunction to attain maximum efficiency. Injected cells are allowed to circulate in the body for a minimum of 6-8 weeks before any experiment is commenced. For local reconstitution, the cells are injected into the dermis (i.d.) (1 to 4 X 10<sup>6</sup> cells).

There has been a lot of discussion regarding the efficiency and validation of MC reconstitution. A study done by Galli and coworkers [259] aimed to look at the distribution pattern of reconstituted MCs in the Kit<sup>W-sh/W-sh</sup> mice. According to their findings, 6-8 weeks after i.d. injection, a significant local repair of MCs was observed in the back skin and ear pinnae and the anatomical dermal distribution of MCs was comparable to the wild-type dermis. I.p. injection succeeded in repopulating the peritoneal cavity, mesentery, jejunum, ileum, colon and glandular part of the stomach, whereas the fore stom-
ach had less MC counts. MCs detected after reconstitution in the intestinal tissue were mostly in the outer layers of the muscularis propria. Ileum and colon of the reconstituted Kit\textsuperscript{W-sh/W-sh} mice had significantly higher MC counts compared to the wild-types. This was striking since it is quite impossible to find any MCs in the ileum and colon of wild-type mice. I.v. injection mediated reconstitution took twelve weeks, after which MC population from the donor could be detected in the back skin, mesentery, lung parenchyma, spleen and both fore and glandular stomach. I.v. transfer yielded significantly lower numbers of MCs in the dermis of back skin in comparison to i.d. reconstitution. I.v. transfer also failed to efficiently repopulate the peritoneal cavity. Adoptive transfer of MCs (i.v.) did not cause MCs to end up in the trachea, tongue, brain kidney, urinary bladder, or tail. Moreover the MC deficiency could not be rescued with adoptive transfer (i.v.) of wild-type bone marrow cells. This was however in strong contrast with previous findings by Galli and co-workers [260] and Kitamura et al. [251] where they showed successful MC reconstitution of Kit\textsuperscript{W/W-v} mice. Hematopoietic stem cells of Kit\textsuperscript{W/W-v} mice have absolutely no c-kit protein expression in contrast to the Kit\textsuperscript{W-sh/W-sh} mice that have sublime expression levels [261] and c-kit signalling is essential for stem cell survival and lymphopoiesis [262, 263].

Although the method of reconstitution is very well established, the distribution and functionality of the injected cells can still be debated.

MC protease knockouts:
The MC knockout models established a strong position for MCs in a myriad of pathological conditions. But whether these effects were a joint venture of all the MC mediators or a specific effect of a certain one was still to be answered. Development of a number of MC mediator and MC-protease specific knockout mice in the subsequent years, helped to find some answers.

N-deacetylase/N-sulfotransferase-2 (NDST-2) knockout mouse: Heparin and heparan sulphate are complex polysaccharide GAGs that are found both in extracellular matrixes as well on cell surfaces. Ongoing studies keep continuously highlighting their roles in a myriad of fundamental biological processes. Heparin is synthesized and stored exclusively by MCs (CTMCs specifically) in their secretory granules in complex with MC-proteases. Its biosynthesis and modification inside the cell involves a list of enzymatic reactions and sulphation processes [264]. An enzyme called NDST-2 mediates the first stage in the modification step, which paves way for further subsequent sulphations [265-267]. In the late 90’s two groups independently published [268, 269] the construction of a transgenic mice carrying targeted disruption of the NSDT-2 coding gene. This intended gene disruption led to non-sulphated heparin production.
The NDST-2\(^{-}\) mice are viable, healthy and fertile. They carry significantly fewer CTMCs, and those they have are defective with morphological and granular alterations [268, 269]. However, it should be kept in mind that the MC quantification was done mainly by relying upon metachromatic staining signals, and since they lacked a number of MC-proteases the cells might just stain less intensely making them harder to identify. They lack storage of most MC-specific proteases (MCPT-4, 5 and CPA), only MCPT6 and 7 levels were unaltered. The transcripts for these proteases, although were unaltered in the knockouts. Humphries et al. argued that mouse bone marrow MCs have higher NDST-2 transcript level than NDST-1, hence it is essentially NDST-2 that is involved in synthesizing heparin chains in MCs [268]. Data from the two knockout NDST2 mice supports this. MCs or more specifically connective tissue MCs seem to be the only affected immune cell in these knockouts.

Serglcrin knockout mouse: To date, SG is the only known “committed” intracellular proteoglycan. SG is expressed in most hematopoietic cells and in a number of non-hematopoietic cells. In addition, high level of SG expression has been observed in multiple myeloma cells [153] and metastatic carcinomas [152]. Given so many physiological importances, the construction of the SG knockout mouse [158] was a big step towards a better understanding of SG’s role in homeostasis and physiological disorders. The SG gene, approximately 15kb long, includes exon 1, 2 and 3 as well as regulatory sequences [270]. Exon 1 and the regulatory sequences were replaced with a neomycin cassette and this construct was eventually used for homologous recombination in embryonic cells to generate the SG\(^{-}\) mouse [158].

Following the generation of SG knockout mice, subsequent studies have been done to evaluate the effect of SG-deletion in different SG expressing cells. Consequences of SG-deletion have been studied in MCs [158, 137, 271, 272], cytotoxic T-lymphocytes [144], neutrophils [273], macrophages [145] and platelets [142]. Most of the effects were observed in naïve SG\(^{-}\) mice, which interestingly did not always give the same effects when challenged (Fig. 5). The most striking feature as a result of SG knockout was the inability of the cells to store inflammatory mediators in their secretory granules. This indicated that SG provides a scaffold or backbone for the proteases to be retained inside the vacuoles for release on demand.
Mcpt1 knockout mouse: Mcpt1 is a β-chymase homologue and is mostly expressed by intestinal mucosal MCs, IMMCs [274]. β-chymases play an important role during parasitic infection by increasing gut permeability allowing the transfer of proteins and antibodies to the niche occupied by the parasites. Mcpt1 is constitutively expressed with increased blood and intestine levels with rise in infection severity, reaching its maximum level during parasite expulsion [275]. Wastling et al. constructed the Mcpt1 null mouse in order to study the role of the MMC specific β-chymase [276]. They replaced all the five exons of the Mcpt1 gene with a target vector that had a neo cassette driven by a human β-actin promoter and a SV40 polyadenylation signal. The transgenic mice had normal transcription of Mcpt2, Mcpt4 and Mcpt5 whereas as expected it lacked transcription of the Mcpt1 gene. The mature MCPT1 protein was not detectable in the jejunal mucosa even after a parasitic infection. Intra-granular structural integrity of the IMMCs of the transgenic mice was changed profoundly compared to their wild-type counterpart. So the disruption of the Mcpt1 gene resulted in distinctive histochemical and ultra-structural changes in the IMMC granules [276].

Mcpt4 knockout mouse: The human chymase gene belongs to the alpha chymase family and Mcpt5; the only α-chymase in mice had long been attributed as it’s homologue. However, when both of them were structurally analysed, it revealed that Mcpt5 had the amino acid val instead of gly in position 216, meaning it lacked chymotrypsin-like activity [122]. Hence, the mouse counterpart to the human chymase was likely to be one from the mouse β-chymase family. Mcpt4, with its similar tissue distribution [277], heparin binding [278] and angiotensin I converting properties [279] as hu-
man chymase, was the likely candidate. Hence, mMCP-4 was suggested as the functional homologue to human chymase.

In 2003, Åbrink and co-workers published the generation of the Mcpt4 knockout mice [242]. They succeeded in deleting exon 1 of the Mcpt4 gene thereby rendering the Mcpt4 gene inactive in the mice. The Mcpt4<sup>−/−</sup> mice totally lacks any traces of MCPT4 protein and this absence did not cause any visible effect on the concentration of any other connective tissue MC (CTMC) proteases in the mutant. Neither the number of MCs in the peritoneum nor their size and morphology was affected. Also CPA or tryptase activity levels in the knockout mice were not affected at all [242]. Hence the Mcpt4<sup>−/−</sup> mouse is a good model for assessing the specific function of Mcpt4, which in turn can possibly be translated to functions of the human chymase. These mice are congenically inbred to two different genetic backgrounds, C57Bl/6J and DBA/1, which have been used for a number of different studies helping to gain insight into the role of MCPT4 during normal and pathological conditions.

**Mcpt6 knockout mouse:** The human β-tryptase homologues in mice are MCPT6 [118, 280] and MCPT7 [281]. Mouse MC protease 6 (MCPT6) is strictly expressed only in the MCs and is highly expressed in MCs of lung, peritoneal cavity and skin tissue [118, 282, 280]. Thakurdas et al. reported the generation of a Mcpt6 null mouse in 2007. They used a knock-in approach and inserted Cre cDNA with a SV40 polyA signal site into the mMCP-6 gene, downstream of the translation initiation site, thereby replacing three coding exons. They then used this construct for homologous recombination in 129svJxC57Bl/6J F1 ES cells. The construct lacked the essential catalytic triad residue His<sup>75</sup> encoding part of the Mcpt6 gene. This gene disruption led to production of truncated transcripts and was sufficient enough not to express any functional MCPT6 protein in the C57BL/6 knockout mice [283]. Since the C57BL/6 mouse strain do not express Mcpt7 [284], the Mcpt6<sup>−/−</sup> mice lack both Mcpt6 and 7.

The very next year Shin et al. developed another Mcpt6 null mouse by disruption of exon 1 and 2 and homologous recombination in 129/Sv ES cells [285]. They backcrossed the 129/Sv strain Mcpt6 knockout mice for five generations onto the C57BL/6 strain. The 129/Sv strain mice carry an intact Mcpt7 gene. Hence the Mcpt6 knockouts in the C57BL/6 strain expressed MCPT7 indicating the fact that MCPT6 and MCPT7 expression are not dependent on each other.

Both the knockouts had normal expression of other MC-specific and non MC-specific proteases, normal distribution of MCs in the skin and peritoneum, were fertile and had a normal life span. The MCs isolated from them showed no alterations in size, shape, granule morphology and density. This indicated that the absence of MCPT6 is not essential for the recruitment, migration, retention and maturation of MC progenitors.
CPA knockout and CPA inactive (inact) mice: To address the function of the MC specific CPA, Feyerabend and co-workers generated, the CPA null mouse. They constructed the target vector by disruption of the MC-CPA gene. This was achieved via insertion of the enhanced green fluorescent protein gene (EGFP) at the ATG start codon of the MC-CPA locus [286].

The knockout mice were healthy, fertile and phenotypically normal. Isolated MCs showed a total lack of CPA expression and had an immature histochemical phenotype and the CPA deficient MCs were never fully matured. However, unlike the other MC-protease knockouts, the CPA null mice showed defective expression of secretory granule proteases other than CPA. It lacked any functional MCPT5 expression in spite of an intact Mcpt5 mRNA expression and had an unregulated level of MCPT4. Other proteases were unaltered. Another interesting observation that still has not been addressed is the fact that the CPA knockout MCs possess a differential detergent sensibility (less tolerant) when compared to their wild-type counterparts. In spite of the above differences in the CPA knockout mice, the CPA+/− MCs were capable of normal degranulation and PCA responses.

Since MCPT5 was absent in the CPA−/− knockout, it was not clear if the phenotypes observed in the knockout, was a result of that absence. Also the inter dependability of MCPT5 for CPA [286] and vice versa was not answered yet. With this aim, they constructed the CPAinact mice, where the expression of CPA was not disrupted but the activity of the protease was hindered [287]. The CPAinact mice are fertile, normal and healthy and have unaffected expression of all other MC proteases. Experiments on this model showed that absence of CPA activity makes the mice susceptible to endothelin-1 intoxication.
Parasitic infections

“A parasite is an organism that lives on or in the host and harms the host or alters the host immune response.”

The definition of parasite in itself depicts a unique living relation between two organisms. A parasite needs to harmonize its coexistence within its host in order to propagate and survive. It should achieve this goal without raising too much attention from the immune system. A special evolutionary mechanism seems to have developed between the two. Parasitic infections are front-runners of disease, not only in the tropical and subtropical countries but worldwide. The infections are mostly caused by protozoa or helminths, parasitic infections can be either extracellular, for example, *Giardia lamblia* and *Trichuris trichiura* or intracellular, for example, *Toxoplasma gondii* and *Trichinella spiralis*. The capability of infecting a wide range of hosts with high efficiency and benign co-existence within the host are characteristics that make a successful and subtly harmful parasite. Both *T. spiralis* and *T. gondii* fits this profile perfectly.

Gastrointestinal helminth infection: *Trichinella spiralis*, an example.

Nematodes (roundworms) are simple structured, un-segmented species that may be free-living, predaceous or parasitic. The parasitic species are connected to many of the important diseases in plants, animals and humans. Around 2.9 billions of people worldwide are estimated by WHO to be affected by different worm infections. Nematode infections have a major long-term impact on our lives, directly or indirectly. The intensity of morbidity or disease in humans resulting from helminthiasis is a complex task to estimate. Harboring a parasitic infection does not always necessarily give a symptom and in some cases the symptoms do not comply with guidelines constituting a clinical disease scenario.

The mere fact that certain parasites can modulate immune responses towards other pathogens [288] makes helminthiasis, a disease of major public health significance. An infection can be totally harmless and symptomless by itself, or life threatening when combined with an intestinal obstruction [288], malaria-induced liver pathology [289] or might covertly cause impairment in nutrition, development and growth. In chronically infected individuals, nem-
atode infections can lead to the development of immune hypo-
responsiveness most probably via regulatory T-cells [290].

The hunt for a helminth equivalent of bacterial LPS or viral dsRNA that
function via TLR type mechanism akin to multicellular parasites is on. But
so far, no generic motifs have been isolated that specifically identifies eu-
karyotic pathogens [290]. Glycobiologists studying parasite carbohydrate are
hypothesizing a mechanism that recognises structural difference between the
sugar-chains, e.g. absence or presence of sialic acid from a glycan chain in
lower invertebrates. This approach is fairly new and generating a lot of inter-
est.

**Trichinella spiralis**

**The parasite:** Trichinella is the genus name for a group of parasitic
roundworms in the nematode phylum. Infection with this roundworm causes
“Trichinellosis”. The genus was first identified in a larval form in 1835. The
genus is subdivided into two sub-groups with a total of eight species. The
two sub-groups include: group 1 (*T. britovi, T. murrelli, T. nativa, T. nel-
soni, T. spiralis*) that encapsulates in the host muscle tissue and group 2 (*T.
papuae, T. pseudospiralis, T. zimbabwensis*) that does not. The non-
encapsulated group infects saurians, crocodilians and other nonavian archo-
saurs (*T. papuae, T. zimbabwensis*) and birds (*T. pseudospiralis*). The encap-
sulated group infects mammalian hosts. Of these above-mentioned species
the three Trichinella species that are known to cause trichinosis or trichinel-
losis are *T. spiralis, T. nativa,* and *T. britovi.*

**The prevalence:** Trichinellosis is prevalent worldwide. The number of
affected patients varies from year to year. Though a tendency of decline in
the disease prevalence in many European countries and the U.S. has been
observed it is still a substantial cause for morbidity. The U.S. has a trichinel-
losis prevalence rate of 4-20%, and it is still a major public health problem in
many Asian countries, including China, Japan, Korea and Thailand [291]. In
Europe, domestic pigs are apparently clear of *Trichinella sp.* but are present
in wild animals in many parts; Romania has had an increased disease inci-
dence in the past 25 years and trichinellosis still remains a public health is-
sue in Germany since the parasites are found in German wild animals [292].
Modus operandi: *Trichinella spiralis* has a direct life cycle completing all its developmental stages in one host. Life starts in the intestine of the host when an adult female worm sheds batches of live larvae in the intestinal lumen. The larvae then bore through the intestinal wall and enter the bloodstream and the lymphatic system, to draw nutrition. They are subsequently carried to the striated muscle tissue. On reaching the muscle they penetrate muscle cells enclose themselves in a capsule and mature to become infective for another host. Humans get trichinellosis on consumption of raw/undercooked meat of pork or wild game infected with infective encysted larvae [291]. Humans are ‘dead-end hosts’ for the parasite.

The entire life cycle of *T. spiralis* can be divided into an early and a late phase. The early phase includes the maturation of the larvae into the intestinal lumen to adult worms, mating and shredding of newborn larvae into the lumen. Around the time after the larvae matures into adult worms subsequently copulates; the host immune response creates a strong Th2 response accompanied by extensive MC and goblet cell hyperplasia, which in turn helps the host to remove the adult worms. In spite of this, adult worms continue to produce larvae for considerable length of time before they are totally expelled. The later phase is when the newly shredded larvae flows through
the circulation and encysts itself in the muscle tissue. This phase can give physiological symptoms (body pain, swelling, and rashes in humans) in the beginning but can also be asymptomatic. It is usually when challenged by another infection that an overall suppressed immune response of the body is noticed.

**The response:** Helminthic infection and the host immune response is a dynamic process that has co-evolved between the parasite and the host [293, 294]. The parasite needs to mislead the immune system into not developing a proper response, which thereby helps it to establish a niche for maturation and propagation. The host in turn tries to generate an effective immune response to expel the parasite and minimize its harmful effects. In this process the host should not lose its ability to mount an effective response to other pathogens either [295]. The fact that helminths and their antigens induce strong T helper 2 (T\text{H}2) responses, gave rise to the notion that parasitic infections have strongly influenced the evolved T\text{H}2 immunity that we see presently [296] [290].

T\text{H}2 cell mediated immune responses are an interplay of cytokine production in the lymph nodes and the periphery and infiltration of inflammatory cells to the regions of infection. Several cytokines including IL-9, IL-4, IL-13 and IL-5 are subsequently released. Cytokine signaling and other mediators lead to crosslinking of Fc\varepsilon RIIs resulting in enhanced MC and basophil activation and further release of mediators. IL-5 is involved in eosinophilia. An increase in IL-4 levels leads to class switching of B cells resulting in IgE production. IL-4 and IL-13 production also leads to i) increased smooth-muscle-cell contractility, ii) an increase in intestinal permeability and increased mucous secretion from goblet cells and iii) enhanced responsiveness of different secretory cells in the intestine towards mast-cell-derived mediators [297]. T regulatory cells produce TGF-\beta and IL-10, which have anti-inflammatory effects. Finally, IL-4 and IL-13 along with IL-21 induce alternatively activated macrophages with strong anti-inflammatory properties to enhance T\text{h}2 cell differentiation and development of fibrosis and repair at the site of injury [298]. All these processes ultimately result in the expulsion of the parasite.

MC-deficient W/W\textsuperscript{v} mice or mice where SCF-specific antibodies deletes MC function, have been used to evaluate the role of MCs in *T. spiralis* infection. These studies showed that, SCF is required for mucosal mastocytosis during helminth infection and also required for expulsion of the parasitic worm [299] and that MC deficient mice cannot effectively expel the worm from the gut [300]. Alizadeh *et al.* looked into the intestinal MC response and lymphoblast activity in w/w\textsuperscript{v} mice after *T. spiralis* infection. They concluded that the prolongation of infection and delay in worm expulsion in
w/w" mice was due to delayed MC appearance in the intestinal mucosa resulting in the slow generation of the intestinal inflammation [301].

Miller and co-workers showed in their experiments that Mcpt1 is constitutively secreted and essential for the enteropathy induced in the intestine [275]. Mast-cell protease 1 (Mcpt-1) deficient mice failed to expel T. spiralis [302]. This highlights the anthelminthic properties of the MCs cytoplasmic granules. However, the rat mucosal MC chymase RMCP II (the rat homologue of mouse mMCP-1) showed no major role in worm expulsion following a secondary infection [303].

In the case of chronic T. spiralis infection, both IgE-deficient mice and mouse MC tryptase Mcpt6 knockout mice demonstrated an identical defect in eosinophil recruitment to the site of larval infection. This emphasized an important function of Mcpt6 in linking the innate and the adaptive immunity during the chronic phase of infection in muscle tissue [285].

Of the MC specific proteases only Mcpt1 and Mcpt7 were found to be totally SG independent whereas the other proteases showed a clear SG dependency. Mcpt2 showed a partial dependence on SG PGs [271]. MCs have also been implicated to be directly responsible for an increased epithelial permeability during T. spiralis infection [304] and a deficiency or decrease in MC protease level fails to increase the permeability. This effect is probably mediated via mouse MC chymase Mcpt4 [305]. All these studies indicate MCs as an important participant and effector in immune response to T. spiralis infection.

Intracellular protozoan infection: Toxoplasma gondii, an example.

Protozoans are single celled organisms that are the causative agents for a number of parasitic infections. Clinicians usually classify these unicellular microorganisms into 4 broad groups based on their mode of transmission: enteric, sexual, arthropod and other alternative mode (fecal-oral, food-water). Almost all of the pathogenic protozoans have been associated with GI diseases. Toxoplasma is the only fecal-oral transmitted pathogenic protozoan that has not been linked to gastroenteritis.

Toxoplasma gondii

T. gondii is a facultative obligate intracellular parasite whose definitive host is the Felidae family, within which is propagates by sexual reproduction. It can also propagate asexually within its intermediate hosts, all of which are warm-blooded mammals including humans. Infection with T. gondii leads to a pathological condition called toxoplasmosis. The parasite gets its name partially from a rodent in North Africa called the common gundii (Ctenodac-
*tylus gundii* where it was first isolated from in 1908: *Toxoplasma* (Greek meaning “arc-like” form) and *gondii* (after the rodent).

**Infection**: There are a number of ways that humans can get infected with *T. gondii*. These include: eating tissue cyst contaminated meat, through contact with soil, other materials contaminated with cat faeces, through consumption of food or water contaminated by oocysts shed by cats or via blood transfusion. *Toxoplasma* infection is very common, speculations are that about one third of the world population carry this parasite asymptomatically, although the rate of infection and severity of symptoms vary from country to country. Primary infection is mostly subclinical. However, pregnant women, new-borns and immunologically impaired patients are at high risk from toxoplasmosis. When the host is immunosuppressed recurring infection can be fatal.

It is not the infection severity that is the only concern, but *Toxoplasma* can have more subtle effects on its host, which in turn can be fatal. It is now established that it causes behavioral changes in its host. A good example being the fearlessness of mice and rats to cats, which increases the likelihood of the parasite ending up inside the cat. This induced change is quite fascinating if one thinks that the cat is the only host where the parasite can mature and propagate. Although disputed several studies in the past few years have associated *T. gondii* infection with mental illness like schizophrenia [306, 307], anxiety and bipolar disorder [308-311], global differences in human culture [312], personality changes [313, 309, 310], increased risk for traffic accident [314] and as a putative cause for certain brain cancers [315].

**Life cycle**: *T. gondii* exists in three infectious forms, namely sporozoites (in oocysts), tachyzoites (in groups) and bradyzoites (in tissue cysts). These stages all contribute to a complex life cycle [316]. In its definitive host, where it propagates sexually, oocysts are produced. These oocysts are excreted in the faeces and can infect the intermediate host. Once inside the intermediate host, the oocysts develop into tachyzoites (a rapidly multiplying form of *T. gondii*). They spread the infection by entering the cells and dividing at a fast rate, thereby causing tissue destruction. In pregnant women they are capable of infecting the fetus leading to abortion. The tachyzoites final destination is muscle tissue and the central nervous system, where they convert to bradyzoites (tissue cysts). They form cysts inside the cells, which protects them from the host immune system. Bradyzoites, if ingested (e.g., from contaminated meat) can transform back to tachyzoites in the new host.

**Host immune response**: Mice are natural intermediate hosts for *T. gondii* indicating that the mouse immune system has evolved to cope well with the parasite. Moreover, the lifecycle of *Toxoplasma* in mice and hu-
mans are almost similar. This makes it easier to study the immune responses in inbred mice and also deduce the basis of protective immunity against intracellular pathogens in general. Studies demonstrating the regulation of immunopathology elicited by *T. gondii* [317-319] has brought to light an important feature of the immune response to this infection. *Toxoplasma* infection elicits a strong and persistent cell mediated immunity (CMI) [320, 321].

Induction of acquired immunity requires efficient antigen presentation of parasite peptides. *T. gondii* peptides are strong activators of antigen specific CD4+ and CD8+ T lymphocytes. The intracellular infection results in a Th1 cytokine environment that displays strong CTL activity and ability to produce large quantities of IFNγ [322]. IFNγ in turn prevents parasite coloniza-
tion in the organs [323] and mediates long-term immunity [324]. IFN-γ is of extremely important in controlling *T. gondii* growth [325-327].

Other proinflammatory cytokines such as TNF-α, IL-1 [328] and IL-6 [329] have also been shown to play a significant role in the host immune defense modulation against *T. gondii*. Early protection from *Toxoplasma* infection is mediated via production of IFN-γ by NK cells and IL-12 by splenic dendritic cells in the early phase and later macrophages. Given that macrophages and NK cells are the critical initiator of cytokine production resulting in further immune modulation, their down-regulation by TGF-β and IL-10 [330-332] aggravates the infection. However regulatory cytokines [333-335] limit the potency of infection and inflammation. Apart from cytokine secretion, macrophages regulate the immune response through generation of nitric oxide (NO) [336]. IFN-γ primed macrophages when in contact with antigen peptides or TNF-α secrete high levels of reactive nitrogen intermediates (RNI). *In vitro* and *in vivo* studies have revealed an immunosuppressive activity of RNI during early toxoplasma infection [337]. IFN-γ and TNF-α in turn plays a big role in the induction of iNOS and RNI levels [336]. This information is now well validated with experiments involving iNOS inhibitors and iNOS knockout mice [338]. Put together the various studies indicate that during acute infection NO plays a detrimental role by mediating pathologic changes whereas during a chronic infection NO down-regulates the pathological changes that are induced by the parasite[339, 327, 322].

So now the question would be “What in *Toxoplasma* turns the immune system on?” Activations of TLRs have been shown to be important for an immune response against *Toxoplasma* infection [340-342]. In search for a TLR agonist, TGPRF was indentified [343]. TGPRF the only *T. gondii* protein known so far as a TLR ligand act on TLR11 and was found to be the key mechanism for the infection of host cells [344] and also responsible for TGPRF induced IL-12 production by splenic DCs. Moreover the tachyzoite surfaces are rich in glycosylphosphatidylinositol (GPI) proteins which when attached to distinctive free lipids are recognizable by both TLR2 and TLR4 in mouse [345]. These GPI proteins, which are needed for parasite viability, in turn elicit cytokine production and start an immune response.

**Mast cells in parasitic infection**

MCs are considered as the first line of defence against infections. They exert their effects either via release of MC-specific proteases, non-MC specific proteases or cytokines/chemokines secretion, or through a combination of the three. The presence of MCs is essential for a proper immune response and expulsion of adult worms in case of *T. spiralis* infection [300].
This effect may as well in part be mediated by the chymase MCPT1, since Mcpt1-/- mice demonstrated delayed worm expulsion, reduced enteropathy and insufficient cytokine (TNF-α and NO) responses comparable to the MC deficient mice [302]. However the T12 responses in the Mcpt1+/+ mice were similar to the wild-type whereas they were much lower in the W/Wv mice [302]. In contrast, when infected with N. brasiliensis, the Mcpt1−/− mice were capable of combating the infection and clearing the parasite efficiently, both during primary and secondary challenge [241]. There was also no significant difference either in the faecal egg count or in the worm burden when compared to the wild-type. Interestingly, the MMC recruitment and distribution was altered in the T. spiralis infected Mcpt1−/− mice; MCs accumulated in the intestinal submucosa during the worm expulsion phase [241], which did not however help in the worm expulsion and subsequently led to a large muscle larvae burden. Infection with N. brasiliensis resulted in similar effects [276]. The exact mechanism by which this β-chymase exerts its effect is not yet fully understood, but a few studies have suggested a role of MCPT1 in cleavage of cell-cell interacting proteins, e.g., Occludin, ZO-1 and fibronectin. This in turn might lead to a disruption in the barrier function and proper response development [304, 346, 347].

Shin et al. showed that even though MCPT6 contributes in eliciting a proper innate immune response to T. spiralis infection, absence of the protease does not worsen the parasite clearance; neither does it hamper the IgE response [285]. However the chronic phase of the infection in the muscle tissue was affected due to poor recruitment of eosinophils, making the role of MCs in the innate immune response even more evident.
PAMPs and DAMPs

“Of all the mysteries in modern science, the mechanisms of self versus nonself recognition in the immune system ranks at or near the top”.

- D.E. Koshland Jr. [348]

The basic concept of immunology is based on the idea of our body’s (immune systems) capability to distinguish between “self” and “nonself” molecules in the system. In order for the immune system to defend our body from invaders, it must first detect the presence of microorganisms; this is where our innate immunity comes in to play. Innate immunity is designed to recognise molecules or molecular patterns that are common and essential to a group or groups of pathogens but are unrelated to mammalian cells. These unique molecules are called Pathogen Associated Molecular Patterns or PAMPs. They share some common biochemical features; either the entire molecule or partial molecule or certain polymeric assemblies [349]. Recognition of exogenous PAMPs by innate immune cells triggers numerous signaling pathways directed towards the pathogen or pathogen infected cells. This in turn triggers inflammation eventually resulting in an immune response in the tissue. At times the PAMPs are virulence factors released by the pathogens to cause tissue damage or suppression of a proper immune response.

But are pathogens the only threat for tissue and cell damage in our body? The answer is no; stress, mechanical injuries, wounds, extreme temperatures, chemical insults, toxins, hemodynamic changes and many other causes can rip and damage our tissue. Following such an injury, a state of inflammation is inevitable. So it is right to say that both pathogens and trauma leads to tissue and cell damage [350]. What triggers a response similar to that of pathogen infection? It has to be an endogenous signal that is generated to defend the host. This signal also has a structurally distinct pattern, posses the capacity to recruit and activate APCs and consequently is capable of enhancing the innate and adaptive immune responses [351, 352]. Owing to their unique activity, Oppenhiem et al proposed that this subset of mediators be named ‘alarmins’.

The nature of the signal both in case of pathogen infection and trauma is similar to “warning bells”. These signals: exogenous-pathogenic (PAMPs), endogenous-trauma mediated (alarmins) or intercellular inflammatory medi-
ators all generated with the aim of defending the host are coined “danger signal”. They in turn interact with receptors and eventually activate APCs [353, 354]. PAMPs can also activate effector cells of the immune system to secrete alarmins, thereby eliciting similar responses. Hence all of the signals can be considered as members of a larger family; the damage associated molecular patterns (DAMPs). However the term ‘DAMP’ is more often used in studies to describe an endogenous, non-microbial origin molecule that can elicit an immune response. Both PAMPs and DAMPs function via pattern recognition receptors (PRRs) [355]. Pattern recognition receptors include membrane associated PRRs, cytoplasmic PRRs and secreted PRRs.

![Figure 6. Schematic overview of PAMPs and DAMPs mechanism of action.](image_url)
In a recent review article Kono and Rock characterizes the DAMPs as intracellular molecules of non-microbial origin that are hidden from recognition by the immune system under normal physiological conditions. However, when cells get necrotic (not apoptotic) these molecules are released resulting in inflammation and eventual effector cell recruitment from the adaptive immune system [356]. In addition, there are extracellular DAMPs, which are released from extracellular matrix degradation following tissue injury [357].

When Polly Matzinger proposed the “danger hypothesis” in 1994, it was an entirely theoretical hypothesis without much experimental evidence experimental evidence to prove that cellular immunogens could also act as adjuvants. Since then there has been a big leap in scientific knowledge; it has been found that necrotic cells are capable of stimulating DCs, increasing the antigen-specific CD4+ and CD8+ T cell response and eliciting an immune response [358, 359]. Several intracellular molecules have been attributed with DAMP function, including HSPs, HMGB1, uric acid, certain classical cytokines, cytosolic calcium binding proteins of the S-100 family and some antibacterial peptides (defensin and cathelicidin) [360]; and some extracellular molecules like biglycan and hyaluronan.

PAMPs and DAMPs as physiological target for mast cells?

The general framework for MC activation is either degranulation followed by de novo synthesis of cytokines and lipid mediators or secretion of the de novo synthesised mediators only. Cytokines, preformed mediators and lipid mediators released by MCs play diverse roles in immune response towards various immune challenges, although not all mechanisms of action are totally understood yet. Researchers in the recent decade have attributed some of the MC functions to the proteases stored in their secretory granules. Many of the virulence factors or external toxins are capable of MC degranulation, releasing the proteases in the region. Three of the most important MC-specific proteases, tryptase, chymase and CPA are quite well studied and their release upon degranulation has mostly been linked to the pathology and tissue outburst that follows an infection. Although all three of them have strong and specific enzymatic activities, the functional consequence of their activities probably varies based on the nature of the substrate and the environmental cue.

Sets of biological peptides have already been identified as putative in vitro substrates for the proteases [361, 230, 242, 228]. However, many of these do not seem to have any effect or physiological significance in vivo, or that a biological relevance to the enzymatic activities has not been uncovered to date. The breakthrough in this aspect has been the identification of in vivo
targets for some of these proteases that puts MCs in a new perspective towards host defence. Metz et al. showed MCs capability to prevent mortality and morbidity induced by snake and bee venoms [24]. This was attributed to the enzymatic activity of MC-CPA. In 2010, Akahoshi et al. showed that MC chymase could limit the toxicity of venoms from Gila monsters and scorpions as well as toxicity induced by mammalian VIPs [362]. MCs have even been shown to cleave physiological peptides like the vasoconstrictor angiotensin and the vasodilator neurotensin, thereby reducing their adverse effects [363, 364]. These results establish MC-proteases as frontrunners for many other external or internal cell damaging peptides.

Most pathogenic parasites and bacteria release virulence factors to enhance their potential to cause disease. Virulence factors can be, for example, toxins (gene products) released by the parasites or bacteria, cell surface proteins or carbohydrates and hydrolytic enzymes. Given that many of these factors are able to cause MC degranulation, they serve as good targets for MC-specific proteases. Internal peptides released under stress, mechanical injury and hypoxia, can also act as chemotactic activators or alarms for the immune system. In tissues these alarms can lead to MC degranulation, which exposes these proteins to proteolytic cleavage by the MC-specific proteases. Hence the possibility that PAMPs and DAMPs can be targets for MC proteases is quite logical and promising.
Present investigation

Aim

Studies included in this thesis were carried out to better understand the role of serglycin and MC specific proteases in the immune response to parasitic infections.

Paper I and III addresses the contribution of SG in *T. gondii* and *T. spiralis* infection respectively. The role of SG was investigated not only as an important MC-PG but also as a major player in maintaining tissue homeostasis. In Paper II the focus was to determine the specific role of SG dependent chymase (mMcpt4) when challenged with *T. gondii*. Given the important roles of MCS in immune reactions to parasitic infection, we set forth to identify physiological targets for MC proteases during an infection. Paper IV focuses on this aspect of research where we identify a number of virulence factors from parasites and other danger signals from cells as potential target for MC chymase (MCPT4).

Results and Discussion

Manuscript I

**Serglycin contributes to cytokine secretion in Toxoplasma gondii infected mice**

Serglycin proteoglycans (SG-PG) are expressed by both immune and non-immune cells. Depending on the cell type, it is either secreted or stored. However, it is the only committed intracellular proteoglycan in MCs. In the peritoneum, the MCs are likely to be one of the first innate immune cells that respond to an infection, which activates them and thereby sends signals to other inflammatory cell types.

In this study we aim to investigate SG-PGs contribution in inflammatory cytokine responses during infection with the parasite *T. gondii*. Both *in vitro* and *in vivo* effects were investigated. Infection with *T. gondii* led to decreased levels of IL-12 and MCP-1/CCL2 both in the sera and peritoneal exudates of SG−/− mice. The peritoneal exudates of the infected SG−/− mice
also showed decreased IL-6 levels, delayed neutrophil recruitment and an increased parasite burden. *In vitro* stimulation of SG−/− peritoneal MCs with soluble *T. gondii* antigen induced significantly lower levels of IL-6, IL-12 and TNF-α secretion compared to their wild-type counterparts. In contrast, the MCP-1/CCL2 secretion was significantly higher in SG−/− MCs than wild-type cells.

Our results highlight an important role for SG-PGs in inflammatory cytokine secretion and innate immune response to *T. gondii* infection.

**Manuscript II**

**Mouse MC protease 4 regulates pro-inflammatory cytokine levels during Toxoplasma gondii infection**

MCs are important sentinel cells that play their role both as good and bad effector cells of the immune system. As a good effector cell, one of the prominent roles is their protective function during infections. Activation of MCs results in the release of mediators that leads to extracellular remodeling, leukocyte recruitment, cytokine signaling, degradation of peptides and many other physiological processes.

In this study, we looked in to the protective role of one of the pre-formed MC mediators, MC chymase (MCPT4) during *T. gondii* infection. When challenged with soluble *T. gondii* antigen, the Mcpt4−/− MCs secreted significantly lower levels of the inflammatory cytokines IL-6, TNF-α, IFN-γ, IL-12 and MCP-1/CCL2 compared to their wild-type counterparts. The infected Mcpt4−/− mice also had impaired neutrophil and eosinophil recruitment and sub-optimal secretion of the inflammatory cytokines both locally and systemically. Only the differences of *in vitro* levels of IL-12 and MCP1/CCL2 were dependent on their transcriptional levels.

Altogether, these results suggest a role for MC chymase in neutrophil and eosinophil recruitment by means of controlling the cytokine signaling.

**Manuscript III**

**Serglycin limits enteropathy in Trichinella spiralis-infected mice**

SG-PG, expressed in hematopoietic and non-hematopoietic cells, is required for maturation and granular storage of cationic proteases. This study investigates the potential role of SG in the host immune response after infection with *T. spiralis*, a nematode parasite that creates a strong Th2 response.

We infected mice of both genotypes (SG−/− and wild-type) with 500 muscle larvae via gavage and looked at the cellular and tissue response at 12 days and 5 weeks post infection. The absence of SG was associated with
significant intestinal enteropathy and a tendency of delayed expulsion of worms at 12 days p.i. These differences were reflected on the increased larval burden in the muscle tissue after 5 weeks of infection. Among other effects, the enteropathy was associated with increased levels of myeloperoxidase and elastase and less recruitment of MCs to the intestinal crypts.

We wanted to determine what role MCs played in this enteropathy. Wild-type bone marrow MCs were injected i.p. to SG⁻/⁻ mice and allowed to reconstitute for 8 weeks. The infection study was then repeated again using wild-type, SG⁺/⁺, reconstituted SG⁻/⁻ and NDST2⁻/⁻ mice. Reconstitution of the SG⁻/⁻ mice did not rescue the levels of enteropathy 12 d.p.i. but did increase pro-inflammatory cytokine levels in comparison to infected SG⁻/⁻ animals, depicting an important role of MCs in cytokine balance. However, the NDST2⁻/⁻ infected mice behaved similarly to the infected wild-type mice, indicating that connective tissue type MCs probably are not the front runners in *T. spiralis* infection.

These results show that SG⁻/⁻ mice are less protected from the infection and points to a regulatory role of serglycin in both innate and adaptive immunity. Moreover, the observation that neutrophil elastase was found in high levels in the infected SG⁻/⁺ mice indicates that when cells are challenged, they can switch from a SG dependent storage mode to SG independent secretory mode.

**Manuscript IV**

**MC chymase can limit danger-induced inflammation by degradation of heat shock protein 70, biglycan, HMGB1, and IL-33**

Our body detects pathogens through a receptor subpopulation, pathogen recognition receptors (PRR), which in turn can recognize pathogen associated molecular patterns (PAMPs). Pathogens are not the only causative agents for cell and tissue damage; stress, hypoxia, shock, and mechanical forces are also responsible. In such cases, it is endogenous proteins, “alarmins” released by dead cells that trigger cell and tissue damage. MC chymases have been shown to be capable of providing protection against snake and bee venom. Human chymase can degrade pro-inflammatory cytokines suggesting that chymase may function to limit inflammation. This project investigates if MC chymase can degrade “alarmins”, thereby modulating the level of infection and injury induced inflammation.

A fractionation protocol was standardized, whereby we were able to fractionate out different proteases from ear tissue and cultured MCs with various salt concentrations. Crude extracts and protein eluates at high salt concentrations of wild-type mice efficiently degraded heat shock protein 70 (Hsp70) from *T. spiralis*. However extracts from *W*<sup>sh</sup>, SG⁻/⁺ and NDST2⁻/⁻ mice lacked...
this activity, suggesting involvement of a MC-PG/heparin dependent protease. The same degradation pattern was observed using recombinant human Hsp70 as well. We used different protease inhibitors to elucidate the role of the participating protease and found that the addition of chymostatin, a chymase inhibitor, blocked this degradation activity. The entire degradation study was repeated using pure recombinant human chymase and we did indeed see the same activity pattern, which was efficiently blocked by chymostatin. We went further and investigated this activity of chymase in vitro and in vivo, using the Mcpt4−/− and wild-type MCs and animals. In both cases Mcpt4−/− MCs and animals failed to show any degradation of Hsp70, whereas wild-type MCs and animals could efficiently degrade Hsp70.

Our results show that chymase/MCPT4 efficiently degrades Hsp70 both in vitro and in vivo. Moreover, the degradation of HMGB1, biglycan and IL-33 may be chymase dependent.

Concluding remarks and continuation of studies

The use of MC deficient mouse strains established the contribution of MCs to inflammation and to the expulsion of parasites and bacteria, through a cooperation between innate and adaptive immune responses. The use of specific MC protease knockout mouse strains in similar studies have started to address the specific contribution of the individual and uniquely expressed MC mediators. The proteases rely to a large extent on the SG-PG for correct storage and function.

In our studies, we have identified several interesting functions for SG-PGs, effects that likely depend on SG-dependent mediators expressed in several cell types. Further, by using two different infection models we now see that the immune responses depend also on the infecting agents. This was most evident when comparing results between T. gondii and T. spiralis infection. In case of T. gondii infected SG−/− mouse strain had an initial delay in the cytokine responses that were restored to normal levels already at 48 hours and the morphological effects in spleen and liver was only modest. In contrast, the T. spiralis infected SG−/− mouse strain showed a reduced cytokine response both at 12 days and 5 weeks after infection and increased intestinal and muscle histopathology.

We would like to venture further with the infection studies and look into effect of serglycin deficiency in the muscle infection phase; 5 weeks post T. spiralis infection. We plan to reconstitute the serglycin knockout mice with wild-type MCs to elucidate their impact on the disease establishment and cyst formation. In another ongoing T. spiralis infection study with the Mcpt4 knockout mice we have observed significantly more MC infiltration into the muscle tissue at 5 weeks p.i. in the knockout animals compared to their wild-
type counter parts. This we need to study further to come to conclusion regarding chymase’s role in infection.

One of the projects included in this thesis has been the identification of possible in vivo target molecules for the MC proteases. Preliminary results identified mMCP-4 specific degradation of three to four T. spiralis antigens and a chymase/CPA cooperative degradation of 2 other T. spiralis antigens. When using soluble antigens from Listeria monocytogenes a combinatorial degradation of tryptase/chymase, tryptase/CPA was evident. One of the targets, HSP70 from T. spiralis was picked for further in vitro and in vivo studies. The data from HSP70 studies has been compiled in manuscript IV of this thesis. We reason in this paper the possible role of MCs in targeting DAMPs. We will continue to look into other soluble exogenous as well as endogenous proteins as prospective candidates.
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References


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Mastceller (MC) är viktiga i vårt försvar mot infektioner. MC, vilka normalt indelas i bindvävs-MC och mukosa-MC, är strategiskt placerade i kroppen t.ex. i hud, i luftvägar och i mag-tarmkanalen. MC reagerar vid bakterie-, parasit- och virus-infektion samt mot allergener genom frisättning, degranulering, av inflammatoriska substanser, framförallt MC-proteaser (tryptaser och kymaser och karboxipeptidas A), histamin och serglycin (SG) proteoglykan.


Kymaset, mast cell protease-4 (Mcpt4), har en försvarsroll och verkar begränsande vid infektion och inflammation i bindväven. Vi undersökte kymas försvarsroll vid infektion med *Toxoplasma gondii* i Mcpt4-knockout möss. Vi såg att MCPT4 förmedlar rekrytering av neutrofiler och eosinofiler genom aktivering/kontroll av cytokinsvaret. Det är inte bara patogener utan även endogena proteiner (alarminer) från döda eller skadade celler som kan aktivera ett immunsvar. Våra resultat visar att kymas är viktig för att reglera inflammation genom sin förmåga att degradera Hsp70 och andra alarminer t.ex. HMGB1, biglycan och IL-33 som frisätts vid vävnadsskada.
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