The Response of Mast Cells and Serglycin-dependent Proteases to Parasitic Infection

Studies on mast cells during *Toxoplasma gondii* (murine) and *Dictyocaulus viviparus* (bovine) infection

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2010

Acta Universitatis agriculturae Sueciae
2010:100

ISSN 1652-6880
ISBN 978-91-576-7545-3
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Print: SLU Service/Repro, Uppsala 2010

The response of mast cells and serglycin-dependent proteases to parasitic infection - Studies on mast cells during *Toxoplasma gondii* (murine) and *Dictyocaulus viviparus* (bovine) infection

Abstract

The proteoglycan (PG), serglycin (SG), is expressed in several hematopoietic cells and studies of the SG knockout mice ($SG^{-/-}$) revealed prominent effects on the storage of certain mouse mast cell proteases (mMCPs) such as mMCP-4, -5 and -6. In this thesis, the role of SGPG during parasitic infection was addressed. Both $SG^{+/+}$ and $SG^{-/-}$ animals infected with *Toxoplasma gondii* had significantly elevated levels of hyaluronan and chondroitin sulfate A PG in the peritoneum. In contrast, whereas heparin/heparan sulfate was confined to the peritoneal cells in $SG^{+/+}$ animals, it was almost undetectable in $SG^{-/-}$ animals. Surprisingly, both $SG^{+/+}$ and $SG^{-/-}$ animals were shown to secrete active MC proteases to almost the same levels in the peritoneal cavity, despite defective storage of proteases in $SG^{-/-}$ MCs. Furthermore, $SG^{-/-}$ animals showed a delayed neutrophil recruitment and decreased levels of proinflammatory cytokines such as IL-6, IL-12, TNF-α and MCP-1. *In vitro* stimulation of peritoneal derived MCs (PCMCs) with soluble *Toxoplasma* antigen induced significantly lower secretion of IL-6, IL-12 and TNF-α in $SG^{-/-}$ PCMCs than in $SG^{+/+}$ PCMCs.

In addition, when studying aging $SG^{-/-}$ animals, the SG-deficiency manifested as enlargements of lymphoid tissues, particularly spleens, Peyer's patches and bronchus associated lymphoid tissue, and was not shown to be associated with infection. Analysis of this phenomenon revealed an expansion of naïve lymphocytes through an increase in the $CD4^+$ and $CD45RC^+$ leukocyte population. Moreover, peritoneal macrophages were markedly increased in number in aging $SG^{-/-}$ animals.

In response to *Dictyocaulus viviparus* infection in calves, tryptase positive MCs in the bovine lung and BALF were shown to correlate with disease progression. The activity and expression levels of tryptase were increased in the lungs of infected calves compared to non-infected control animals.

In conclusion, MCs are actively involved in the host response to *Toxoplasma gondii* infection in mice and *Dictyocaulus viviparus* infection in calves.

Keywords: serglycin, mast cell, proteases, immune cells, proteoglycans, glycosaminoglycans, *Toxoplasma gondii, Dictyocaulus viviparus*.

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To my family

Contents

List of Publications

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

I Sawesi O., Spillman D., Lunden A., Wernersson S., Abrink M. (2010). Serglycin-independent Release of Active Mast Cell Proteases in Response to *Toxoplasma Gondii* Infection. *J Biol Chem 285(49), 38005-13.*

II Sawesi O., Lunden A., Feinstein R., Abrink M. (2010). Serglycin proteoglycans contribute to mast cell cytokine secretion *in vitro*, and to early neutrophil recruitment and cytokine secretion *in vivo*, in response to *Toxoplasma gondii*. *Manuscript*.

III Wernersson S., Braga T*., Sawesi O*., Waern I*., Nilsson K.E., Pejler G., Abrink M. (2009). Age-related enlargement of lymphoid tissue and altered leukocytes composition in serglycin-deficient mice. *Journal of Leukocyte Biology* 85(3), 401-408.

IV Sawesi O., Holmgren S., Andersson C., Wattrang E., Erjefält J., Lunden A., Abrink M. (2010). Tryptase-positive mast cells accumulate in bovine lung tissues during *Dictyocaulus viviparus* infection. *Manuscript*.

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⋆ Indicates equal contribution to the work.

Abbreviations

Ab	Antibody
BALF	Bronchoalveolar lavage fluid
BALT	Bronchus-associated lymphoid tissue
BMMC	Bone marrow mast cell
CD	Cluster of differentiation
CPA	Carboxypeptidase A
CS	Chondroitin sulphate
CTMC	Connective tissue mast cell
DPPI	Dipeptidyl peptidase I
DS	Dermatan sulphate
GAG	Glycosaminoglycans
Gal	Galactosamine
GalNac	N-acetylgalactosamine
GlcA	Glucoronic acid
GlcNac	N-acetylglucosamine
GPI	Glycosylphoshatidylinositol
HexA	Hexuronic acid
HS	Heparan sulphate
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
MC(s)	Mast cell(s)
MCP-1	Monocyte chemotactic protein-1
MMC	Mucosal mast cell
mMCP	Mouse mast cell protease
NK	Natural killer
NSDT	N-deacetylase/N-sulfotransferase

PAMP	Pathogen associated molecular pattern
PCMC	Peritoneal cell derived mast cell
PG	Proteoglycan
PRR	Pattern recognition receptors
SCF	Stem cell factor
SG	Serglycin
SGPG	Serglycin proteoglyucan
SMCP	Sheep mast cell proteinase
STag	Soluble Toxoplasma antigen
TLR	Toll-like receptor
TNF	Tumor necrosis factor

1 Introduction

In our daily life, we are constantly exposed to infectious agents and in most cases we are able to defeat them. To resist infections, we depend on our immune system, which consists of immune cells and soluble factors. Since the 1960s, there have been impressive advances in our understanding of the functional aspects of the immune system. The discovery and application of advanced techniques such as monoclonal antibody production, immunohisto/cytochemistry, recombinant DNA methodology and x-ray crystallography, in addition to the generation of genetically modified animals (transgenic and knockout mice) have aided the current understanding of immunology. The immune system is composed of two major subdivisions: the non-specific innate (natural or native) immune response and the specific, adaptive (acquired) immune response. The innate immune response is our first line of defense against invaders while the adaptive immune response acts as a second line of defense. Importantly, the adaptive system contributes protection through memory when re-exposure to pathogens occurs. A hallmark of the adaptive immune system is that it performs its specific actions in defined areas such as the lymph nodes that drain infected tissue. All blood cells originate from a common pluripotent, hematopoietic stem cell in the bone marrow that becomes committed to differentiate along one of two major hematopoietic branches to develop into lymphoid cells including NK, B and T cells or myeloid cells such as erythroid, megakaryocytic, granulocytic, and monocytic cells. Leukocytes can be distinguished by their expression of cluster of differentiation (CD) molecules. In addition, secreted cytokines and chemokines active in cellular crosstalk can also be used to define different leukocytic cell types.

The leukocyte cytokine family includes interleukins (IL), interferons (IFN), and colony- stimulating factors (CSF)(Abul K. Abbas, 2007) (Tizard, 2009).

1.1.1 Innate immunity

The first arm of innate immunity includes:

1) Physical barriers such as intact skin, epithelial cell layers, as well as the physical processes of vomiting and diarrhea in the gastrointestinal tract.
2) Chemical barriers, such as antimicrobial substances (saliva, tears, nasal secretions, mucus etc.) produced at the epithelial surface.
3) Biological factors such as the commensal flora of the skin and gastrointestinal tract, which can prevent colonization by pathogenic bacteria. The persistence and virulence of invading microorganisms, however, can enable them to eventually overcome and breach some of these physical obstacles. Once inside the host organism, they will encounter the second arm of innate immunity.

The second arm of innate immunity comprises:

1) The humoral system, consisting of antibodies (which are also part of adaptive immunity, see below) and the complement system. Antibodies are targeted to recognize foreign molecules (including those expressed by invading organisms) and contain activating domains for immune cells and complement in their constant regions. The complement cascade, which includes over 20 proteins, is designed to attack and lyse foreign organisms. In addition, cleaved products such as C3b/C4b can opsonize organisms and prepare them for phagocytosis by macrophages and granulocytes.
2) Sentinel cells, such as MCs, macrophages and dendritic cells (DCs), located in areas close to exterior surfaces.
3) The recruitment of granulocytic cell types such as NK cells, eosinophils, and neutrophils to the site of infection. Their main tasks are to eliminate pathogens either by direct contact or by phagocytosis, to serve as antigen-presenting cells (APCs) to T cells, and to produce a vast number of mediators for activation of the adaptive immune cells.

The MC, armed with multiple types of receptors capable of recognizing an intruder and releasing a multitude of essential signals, is believed to work as an alarm clock that wakes the immune system upon an assault. Macrophages are the main scavengers in tissues and are able to kill invaders, present antigen, control inflammation, repair damaged tissues and cells, and assist in the healing process. Immature, circulating monocytes are found mostly in the blood stream. When they extra-vasate into tissues, they undergo a maturation process to become tissue macrophages. DCs are the only sentinel cells that can activate naïve T cells and thus are essential for

initiating immune responses. NK cells, which originate from the lymphoid branch of the hematopoietic tree, are dedicated to killing tumor cells and virus infected cells via a non-specific mechanism. Eosinophils are mainly involved in the battle against helminthic parasites. Neutrophils engage by phagocytosis of infectious agents into phagosomes, which subsequently fuse with the lysosomes to form phagolysosomes, where the pathogen is destroyed by digestive enzymes. Despite the variety of the defensive measures that comprise innate cellular immunity, microbes can still occasionally evade these barriers. The most important function of innate immunity, then, may be to stall the speed of infection and thereby give the adaptive (acquired) immune system ample time to respond (Tizard, 2009; Abul K. Abbas, 2007).

1.1.2 Adaptive immunity

Adaptive or acquired immunity consists of B and T lymphocytes that generate highly antigen-specific responses, as well as memory cells against the invaders. The memory function enables rapid and specific responses on a second exposure to a pathogen. Once activated, B lymphocytes produce soluble antibodies (as a part of humoral immunity) that recognize, neutralize, and eliminate mainly extracellular microbes and microbial antigens (toxins). T lymphocytes, which lie at the core of cell-mediated immunity, target mainly intracellular organisms, such as viruses, as well as certain bacteria and parasites that survive and proliferate within host cells, making these pathogens inaccessible to circulating antibodies. Two distinct populations of T lymphocytes, helper T cells and cytotoxic T lymphocytes (CTLs), have been described. There are three subpopulations of helper T cells; Th1 cells secrete IL-2 and IFN-γ to promote cell-mediated responses such as macrophage activation, Th2 cells secrete IL-4 and IL-10 to enhance antibody production by B cells and Th17 cells secrete IL-17 and promote some inflammatory reactions. CTLs kill infected cells that present foreign antigens on their surface, such as cells infected with viruses and intracellular microbes. Regulatory T lymphocytes suppress immune responses and maintain self-tolerance (unresponsiveness to self antigen). These cells recently gained more attention as potential clinical targets for the suppression of certain types of cancers and autoimmune diseases (Tizard, 2009; Abul K. Abbas, 2007).

1.1.3 Host-pathogen interactions

Animals can be infected by a number of different microorganisms, such as viruses, bacteria, and protozoa. These microorganisms have fundamental differences from each other in their physiology, structures, and mechanisms

of propagation, all of which contribute to various effects on the immune response. Pattern recognition receptors (PRRs) on immune cells can recognize conserved pathogen-associated molecular patterns (PAMPs). The PRRs are activation units for the innate response during an infection. PAMPs can have quite distinct chemical structures, but all have common features recognized by PRRs, which are expressed only by microbial pathogens, and not by host cells. The best-known examples of PAMPs are bacterial lipopolysaccharides, peptidoglycans, lipoteichoic acids, mannans, bacterial DNA and glucans (Rasmussen *et al.*, 2009; Medzhitov & Janeway, 1997; Janeway, 1992). The PAMP-activated PRRs induce intracellular signaling, gene expression and on the initiation of antimicrobial as well as pro- and anti-inflammatory activities. The innate response is a rapid line of defense against infection, and also prompts the processes that ultimately lead to the expansion of the adaptive immune response and the establishment of immunological memory (Rasmussen *et al.*, 2009; Janeway, 1989) (Ishii *et al.*, 2008).

The expression of glycophoshatidylinostitol (GPIs) lipids in cell membranes varies widely among different organisms, species and even cell types. GPIs are expressed ubiquitously by eukaryotes as well as pathogenic parasitic protozoa such as *Trypansoma*, *Leishmania*, and *Plasmodium* species, which express GPIs abundantly, thereby constituting the major PAMPs in these parasites. The main function of GPIs is to anchor functionally important proteins to cell surfaces. *Plasmodium*-derived GPIs, referred to as malaria toxins, induce pro-inflammatory responses by the host through TLR2/MyD88 signaling. This recognition is essential not only for combating parasite growth before the development of adaptive immunity, but also for initiating and regulating adaptive immunity for effective parasite clearance. Additionally, the proteins anchored via GPIs in *P. falciparum* are involved in the invasion of erythrocytes and are thus essential for parasite surviva (Mayor & Riezman, 2004) (Gowda, 2007). Generally, PRRs are expressed on many effector cells of the innate immune system, most importantly on macrophages, dendritic cells, and B lymphocytes, i.e. the specialized antigen presenting cells. PRRs can be further divided into secreted, endocytic, and signaling types. The secreted types function as opsonins, by binding to microbial cell walls and flagging them for recognition by the complement system and phagocytes. The best-characterized example of secreted receptors is the mannan-binding lectin, which binds to carbohydrates on gram-positive and gram-negative bacteria and yeast as well as parasites and activates the complement cascade (Epstein *et al.*, 1996) (Medzhitov & Janeway, 1997). Endocytic PRRs are expressed

on the surface of phagocytes, mediating the uptake and delivery of pathogens into lysosomes, which after lysosomal processing are presented as pathogen-derived proteins by major histocompatibility-complex (MHC) molecules on the surface of the APCs (Fraser *et al.*, 1998). In addition, signaling receptors recognize PAMPs, activate signal-transduction pathways and induce the expression of various immune response genes, including inflammatory cytokines. The best example of signaling receptors is the Toll-like receptor (TLR) family, which appear to play a major role in the induction of immune and inflammatory responses (Ishii *et al.*, 2008). The Toll receptors were first identified in Drosophila spp. as a component of a signaling pathway. Orthologues of Drosophila toll have been identified in mammals and are referred to as TLRs (Rock *et al.*, 1998).

TLRs are cell-surface or endosome membrane-bound PRRs able to recognize PAMPs in the extracellular space and intracellularly. The first human TLR identified was designated as TLR4 (Rock *et al.*, 1998). Today, 10 TLRs have been identified in human (TLRs 1-10) and 12 in mouse (TLRs 1-9 and 11-13) (Rasmussen *et al.*, 2009). TLRs recognize a wide range of microbial molecules, including bacterial lipopolysaccharide (LPS) (TLR4), lipids and carbohydrates expressed by gram-positive bacteria (TLR1, 2, 6), bacterial flagellin (TLR5), and microbial nucleic acids (TLR3, 7, 8, and 9). TLRs 3, 7, 8, and 9, commonly engaged in viral infections (Diebold *et al.*, 2004) (Hemmi *et al.*, 2000), are usually expressed intracellularly within cytoplasmic membrane-bound compartments (Barton *et al.*, 2006) (Akira & Sato, 2003). TLR-activation induces recruitment of adaptor molecule MyD88 and IL-1 receptor-associated kinases, which in turn activate and trigger mitogen-activated protein kinases (MAPK) and nuclear factor (NF)-κB signaling pathways and eventually induce the production of pro-inflammatory cytokines (Yamamoto *et al.*, 2003).

2 Parasitic infection

2.1 *Toxoplasma gondii* infection and host response

T. gondii, an obligate intracellular protozoan parasite, was discovered 1908 by a French group (Nicolle and Manceaux) and a Brazilian group (Spendore)(Innes *et al.*, 2009). The parasite was isolated from a rodent in North Africa called *Ctenodactylus gundi*, which gave the parasite its name. The genus name, toxo, comes from the Greek word toxo, meaning arc, and which indicates the shape of the parasite (cresent). A unique feature of *T. gondii* is its ability to replicate in most mammalian nucleated cells and today, infections caused by *T. gondii* are considered the most prevalent and well-studied parasitic zoonotic diseases in humans (Black & Boothroyd, 2000). It has been estimated that approximately 25% of the human population is chronically infected worldwide. In general, only immunocompromised individuals, for example HIV patients, suffer from life threatening complications such as toxoplasmic encephalitis. In addition, the parasite could be transmitted to the fetus during pregnancy, causing congenital disorders accompanied by a prominent encephalomyelitis, or stillbirth with hydrocephalus and retinochoroitis. Ingestion of undercooked meat or direct contact with cat feces are the main routes of human infection (Innes *et al.*, 2009) (Sibley, 2003) (Black & Boothroyd, 2000). Interestingly, the parasite has been shown to be capable of manipulating the behavior of rats, the intermediate host, in order to increase the chance of being killed and ingested by cats, the definite host, thereby ensuring the completion of its life cycle (M. Berdoy, 2000).

The parasite is also of great importance in the sheep industry. It is considered the main cause of infectious ovine abortion in Australia, New Zealand, the UK, Uruguay, USA, and many other countries. The neonatal losses in sheep flocks caused by *T. gondii* has been estimated to be about 1-2% annually, which corresponds to approximately 0.5 to 1.5 million lambs each year in the UK and Europe, respectively, and represents a significant economical loss for the farmer. The infection incidence in sheep has been associated with contaminations of grazing land with sporulated oocysts and the presence of cats in the farm area. However, congenital transmission of the parasite from the pregnant ewe to the fetus is the most common route of infection. The disease outcome depends on the stage of pregnancy when transplacental transmission occurs. An early infection frequently leads to fetal death, mid-stage infection leads to stillbirth, while lambs exposed to a

late stage infection may be asymptomatic (Freyre *et al.*, 1997) (Innes *et al.*, 2009) (Dubey, 2009).

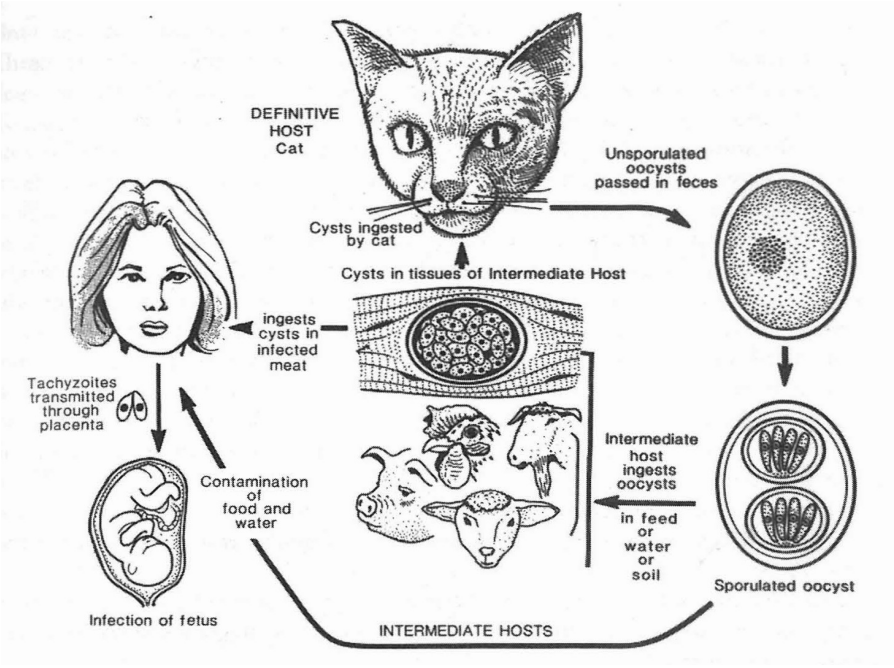

Figure 1. Toxoplasma gondii life cycle (adapted from J.P. Dubey, 1998)

The life cycle of *T. gondii* (Fig. 1) involves two hosts; the definite host (cat) and intermediate hosts, which can be human, rodent, ruminant, or avian. *T. gondii* has two stages of asexual type in the intermediate host: a) tachyzoites, which rapidly multiply within the host cells via a specialized form of reproduction called endodyogeny (the formation of two daughter tachyzoites within the mother). Infected cells eventually rupture and tachyzoites spread to neighboring cells which then become infected (acute toxoplasmosis); b) the bradyzoites, which represent the chronic or cyst forming stage. In the definite host, a third sexual stage, the sporozoites (oocysts), forms (Dubey *et al.*, 1998). Ingestion of cysts by hosts leads to the rupture of the cysts and the release of bradyzoites into the intestinal epithelia where they differentiate back to the rapidly multiplying tachyzoite form, thereby defining the asexual part of the cycle. Ingestion of tissues containing cysts is the main route of infection in the cat, while the fecal-oral (oocyst) route is more prevalent in other hosts (Dubey *et al.*, 1998) (Sibley, 2004) (Dubey, 2008) (Black & Boothroyd, 2000). The

infection activates the host immune system, which limits the invasiveness of the tachyzoites. *T. gondii* responds to the host immune activation by formation of bradyzoites, and 7-10 days later completes the formation of tissue cysts, which are thoroughly distributed in muscle tissues and in the central nervous system (Fig.1).

2.1.1 Mechanisms of cellular entry

Some subgroups of bacteria, such as *Listeria monocytogenes* (facultative intracellular bacteria) and *Chlamydia trachomatis* (obligate intracellular bacteria), are known to propagate inside host cells. The entry of these bacteria is through the endosomal system (uptake mechanism of the cells). The bacteria modify the identity of the endosomes to avoid fusion with lysosomes, thereby preventing proteolytic processing and subsequent presentation together with MHC molecules, and thus blocking the establishment of an adaptive response to the infection (Gruenberg, 2006) (Vazquez-Boland *et al.*, 2001). In contrast, *T. gondii* does not use the endocytic machinery to penetrate, but instead uses its gliding motility to find its way into the cell. Once inside the cell, *T. gondii* forms a non-fusogenic compartment termed a parasitophorous vacuole (PV), surrounded by a parasitophorous vacuole membrane (PVM), in which parasite replication takes place. In addition, PVM forms a high affinity association with the mitochondria and endoplasmic reticulum of the host cell, and by this means is believed to hinder host cell metabolism (Martin *et al.*, 2007) (Sinai *et al.*, 1997).

2.1.2 Host immune responses to *Toxoplasma gondii*

It has been shown that $CD4^+$ and $CD8^+$ T cells as well as MCs engage in the immune response directed towards *T. gondii* infection. The infection induces a strong innate response and expression of many different genes. This ultimately leads to the production of inflammatory mediators and an adaptive immune reaction, with production of specific antibodies, which kills extracellular but not intracellular parasites. The acquired immune response against *T. gondii* is characterized by strong $CD4^+$ and $CD8^+$ T cell activities (Denkers & Gazzinelli, 1998). The acute stage of *T. gondii* infection is characterized by markedly elevated levels of IFN-γ and IL-12, as well as other pro-inflammatory cytokines such as TNF-α, IL-6, IL-1, and the anti-inflammatory cytokine, IL-10. These cytokines are produced by many innate cells such as macrophages, dendritic cells and NK cells. Of these, IFN-γ was found to predominate during infection and it has been shown to prevent parasite colonization in many organs (Suzuki *et al.*, 1988)

(Gazzinelli *et al.*, 1991) (Ferreira *et al.*, 2004) (Dubey, 2008). In addition, activated macrophages liberate high levels of nitric oxide (NO), which limits replication of tachyzoites and thus reduces the immunopathological alterations induced by the parasite (Suzuki, 2002).

Mice lacking MyD88 are highly susceptible to infection with *T. gondii,* suggesting that activation of TLRs is important in the host immune response (Scanga *et al.*, 2002). One protein, the *T. gondii* profiling-like protein (TGPRF), was identified as a potent agonist for TLR11 in mice and was shown to be responsible for the potent IL-12 production by splenic DCs when stimulated with STag (Yarovinsky *et al.*, 2005) (Yarovinsky, 2008). TGPRF remains the only *T. gondii* protein identified as a ligand of TLRs. Moreover, the use of parasites deficient in TGPRF revealed that TGPRF recognition by TLR11 is required for the infection of host cells and TLR11-dependent activation (Plattner *et al.*, 2008). In addition, tachyzoite surfaces are dominated by GPI anchored proteins, which are essential for parasite viability (Wichroski & Ward, 2003), and biochemical studies have shown that *T. gondii* GPI molecules attached to distinctive free lipids are recognized by both TLR2 and TLR4. In mouse macrophages, both TLR2 and TLR4 were found to mediate recognition of *T. gondii* GPIs, as measured by the induction of TNF-α synthesis (Debierre-Grockiego *et al.*, 2007).

2.2 Dictyocaulus viviparus infection

The nematode, *D. viviparus* belongs to the parasitic super family *Trichostrongyloidea*. *D. viviparus* infections cause great economic losses to producers and to the livestock economy worldwide, as it affects young cattle during the first grazing season on pasture (Taylor, 2007) (Corwin, 1997). The infection is also known as parasitic bronchitis, or verminous pneumonia because the main predilection sites are trachea and bronchi. The disease is distributed worldwide although it is more significant in high rainfall areas where the infection might persist for long time(Taylor, 2007).

The *D. viviparus* life cycle (Fig. 2) starts with the first stage L_1 larvae, which, within 5 days and under optimal weather conditions differentiate into the second stage L_2 larvae, and further into the third stage L_3 larvae. Following grazing of the third stage L_3 larvae (infective stage), the larvae penetrate the mucosa of the small intestine and continue migration into the mesenteric lymph node. Here, the larvae moult into the fourth stage L_4 larvae and thereafter, the L_4 larvae reaches the lung via lymph. Within a week of L_3 larvae ingestion, L_4 larvae can be detected in lung alveoli. However, a faster migration rate has been noted when the infection dose increases (Poynter & Silverman, 1960).

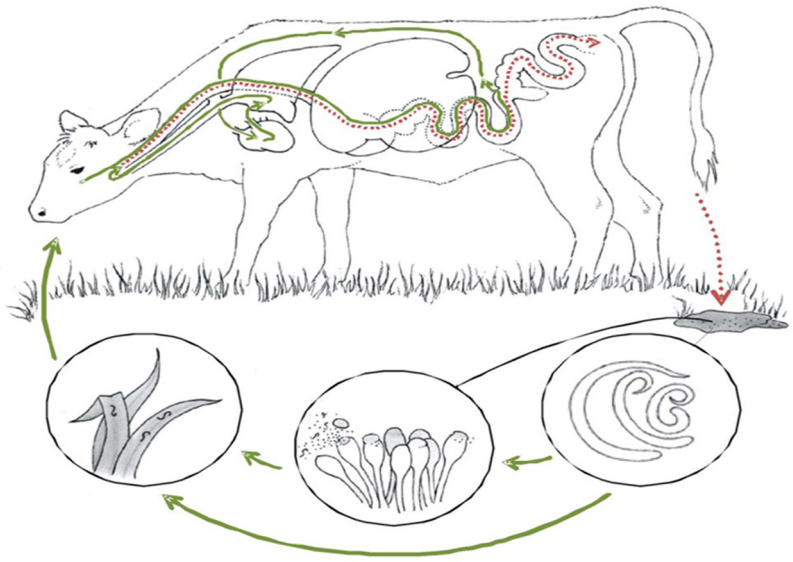

Figure 2. Dictyocaulus viviparus life cycle. The migration route of the life stages from third stage larvae to adult worms is drawn as a green line, and the route of first stage larvae is drawn as a red dotted line (adapted from Hagberg M, 2008, drawn by Katarina Naslund).

The fourth stage L_4 larvae develop into adult worms in lung alveoli, and then migrate to inhabit the bronchial tree. The eggs produced by adult females hatch directly into first stage L_1 larvae, which are then partly exhaled, re-swallowed to the gut and spread with the feces into the grazing field (Fig. 2) (Taylor, 2007).

The clinical signs of disease range from coughing, nasal and oral discharge, respiratory distress, weakness and malnutrition in mild infections to anorexia, dyspnoea and sudden death in massively infected calves

(Choquette, 1954) (Akpavie & Pirie, 1993) (Taylor, 2007). *D. viviparus* induces a strong host immune response characterized by local and circulating eosinophilia and high levels of antigen-specific IgE (Schnieder & Daugschies, 1993). In addition, a positive correlation between the infection and total IgE serum levels (Kooyman *et al.*, 2002), and an increase in specific IgG1 and IgA toward *D.viviparus* has been described (Scott *et al.*, 1996). Moreover, a transient increase in the proportion of γ/δ TCR-expressing T cells in bronchoalveolar lavage fluid (BALF) 2 weeks post-infection has been reported (Hagberg *et al.*, 2005).

3 Mast cell development, physiology and response to pathogens

In 1878, Paul Ehrlich described a cell type located in human connective tissue that stained reddish-purple (referred to as metachromasia) with aniline dyes. He called them "mästzellen", a term that referred to an apparent quality of being well-fed, and was based on the appearance of numerous intracellular granules (Bloom, 1984). The origin of these cells remained obscure for many years, but Kitamura and co-workers observed that the WBB6F1-W/Wv mice (devoid of MCs) developed MCs after receiving bone marrow cells from a normal littermate. This was the first evidence that mouse MCs develop from bone marrow precursor cells. It is now accepted that MCs arise from $CD34^{+}$ pluripotential hematopoietic cells in the bone marrow (Liu *et al.*, 2009) (Sonoda *et al.*, 1983) (Kitamura *et al.*, 1978). Progenitor cells were further characterized and were found to express high levels of c-kit and low levels of Thy-1. They were also shown to contain a few cytoplasmic granules, and to express RNAs encoding the MC-associated proteases, while lacking the expression of the high-affinity IgE receptor (FcεRI). In humans, MCs are also known to arise from $CD34^{+}$/c-kit^{+} progenitor cells. Isolated Thy-1^{lo}c-kit^{hi} cells generated pure, functionally competent MC colonies at high frequencies *in vitro* when cultured in the presence of recombinant stem cell factor (SCF) and interleukin-3 (IL-3), but lacked differentiation potential for other hematopoietic lineages. An intra-peritoneal injection of this population reconstituted the peritoneum of genetically MC-deficient W/Wv mice with functional MCs back to the same levels as that of wild type mice (Rodewald *et al.*, 1996). MCs progenitors leave the bone marrow and circulate in blood before entering the tissue where they complete their differentiation (Hallgren & Gurish, 2007). Growth factors, integrins, and chemokines, e.g. MCP-1/CCL2, MIP/CCL3, RANTES/CCL5 all contribute to MC migration and

attraction. MCs express several chemokine receptors such as CXCR2, CCR3, CXCR4, and CCR5, which are involved in directing the MCs from the circulation into the tissue. Furthermore, IgE alone or in combination with antigens can also promote the migration of MCs (Kitaura *et al.*, 2005; Gurish *et al.*, 2001; Meininger *et al.*, 1992). Mature MCs resident in tissues display different functions and properties depending on the location, and these differences will be described below.

3.1 *In vitro* studies of mast cells

MCs, which are usually few in number and scattered in the tissue of interest, are difficult to purify in sufficient amounts for reliable analysis. The development of *in vitro* protocols to isolate and culture primary MCs, usually derived from bone marrow, thus proved to be very useful. The cells were stimulated with conditioned media derived from concanavalin A-activated T cells or with WEHI-3B tumor cell-conditioned media, to obtain virtually unlimited numbers of mouse bone marrow-derived MCs (BMMCs), which resemble mucosal MCs (MMCs) (Sredni *et al.*, 1983) (Nabel *et al.*, 1981) (Nagao *et al.*, 1981) (Razin *et al.*, 1981) (Tertian *et al.*, 1981). BMMCs cultured in WEHI-3-conditioned medium as a source of IL-3 give rise to a population of cells, more than 85% of which express IgE-receptors and synthesize chondroitin sulfate E and histamine after 3-5 weeks (Razin *et al.*, 1984) (Rottem *et al.*, 1993) (Metcalf, 1997). Addition of IL-4, IL-9 and SCF promotes proliferation and maturation of mouse BMMCs and peritoneal MCs (Rottem *et al.*, 1994) (Schmitt *et al.*, 1987) (Hultner *et al.*, 1990). In contrast, culturing of human cord blood or bone marrow derived mononuclear cells, in IL-3 induced the differentiation of basophils over MCs after 3 weeks of culture (Ishizaka *et al.*, 1985). However, human peripheral blood cells cultured on medium supplemented with SCF and IL-6 form MC colonies within 6 weeks (Saito *et al.*, 2006). Murine BMMCs supplemented with IL-4 and IL-10 up-regulate mMCP-2 mRNA, which is characteristic of MMCs, whereas addition of SCF induces BMMCs into a serosal or connective tissue MC (CTMC) phenotype (Ghildyal *et al.*, 1992) (Metcalf, 1997). These studies implicated that the maturation of MCs is highly dependent on the local microenvironment.

One drawback of the widely used BMMCs, however, is that they are fairly immature and have no known physiological equivalent in tissues. Fortunately, a new method for culturing mature-differentiated serosal-type MCs peritoneal MC (PCMCs) was recently presented by Marc Daëron and co-workers (Malbec *et al.*, 2007). These cells not only respond to IgE, but

also to IgG antibodies through FcγRIIIA receptors. In addition, PCMCs contain and release massive amounts of preformed granular vasoactive mediators and proteases. Since murine peritoneal MCs represent <4% of cells recovered in peritoneal lavage from normal mice (Malbec *et al.*, 2007), this protocol to enable the collection of unlimited numbers of mature CTMCs is highly useful.

In other species, such as ovine and caprine, only a few studies have led to models for culturing MCs from bone marrow. By the use of recombinant ovine interleukin-3 (rOvIL-3) and recombinant ovine stem cell factor (rOvSCF), more than 65% of the cells arising after 3 weeks in culture were MCs, which could be maintained for at least 6 weeks. Cultured ovine MCs contained significantly more serine protease but similar amounts of β-hexosaminidase when compared with caprine cells during the second week of culture (Macaldowie *et al.*, 1997) (Haig *et al.*, 1988). Stimulation of BMMCs with the calcium ionophore, A23187, led to dose-dependent secretion of granule constituents and the generation of leukotriene C4 (Huntley *et al.*, 1992).

3.2 Murine, human and bovine mast cell heterogeneity

Knowledge of MC heterogeneity, first described by Enerbäck (Enerback, 1966), arose from studies comparing MC populations in rodent cell lines. Based on their tissue location, granule and PG content, murine MCs can be divided into two major subclasses: connective tissue MCs (CTMCs) and mucosal MCs (MMCs). The CTMCs can be found mainly in the connective tissues of the skin and peritoneal cavity, and express tryptase, chymase, carboxypeptidase A (CPA), high levels of heparin PGs and histamine. In contrast, MMCs, which are found mainly in the lamina propria of intestine and airways, express chondroitin sulfate (CS) PGs, and chymase (Table 1) (Miller & Pemberton, 2002). Human MCs are also divided into two major subclasses, the mucosal type MC (MC_T) and the connective tissue type MC (MC_{TC}), according to their protease content (Table 1). MC_T cells predominantly express tryptase and are usually found in the lamina propria of airways and in the gastrointestinal tract. MC_{TC} cells express tryptase, chymase, carboxypeptidase A, and dominate the connective tissue (Krishnaswamy *et al.*, 2006). In a simplified comparison, human MC_T cells correspond to rodent MMCs, whereas MC_{TC} cells correspond to rodent CTMCs (Metcalf, 1997). MC numbers in the mucosa of human, rat, canine, and ruminant intestines are relatively high compared to other species, such as mouse.

	Mouse		Human	
	CTMC	MMC	MC_T	MC_{TC}
Tryptase	mMCP-6 mMCP-7		+	+
Chymase	mMCP-4 mMCP-5	mMCP-1 mMCP-2	–	+
CPA	+	–	–	+
PGs	Heparin	CS	Heparin/CS	Heparin/CS

Table 1. *Mast cell heterogeneity in human and mouse*

Ovine MC heterogeneity reflects the distribution of sheep MC proteinases (SMCP) in distinct organs such as trachea, bronchus, bronchial lymph node, lung, thymus, spleen, liver, flank skin, abomasums, small intestine, and mesenteric lymph node. Based on reactivity to the anti-SMCP antibody, two populations of MCs were defined, one in gastrointestinal tissues, analogous to the mucosal MC subset, and the other present in skin, the putative ovine connective tissue MC subset (Sture *et al.*, 1995).

In *Bos taurus* (bovine), three MC subtypes have been described: T-MC, expressing tryptase; TC-MC, expressing tryptase and chymase; and C-MC, expressing chymase. The heterogeneity and density were defined according to tissue location, methods of fixation (Carnoy's fluid) and chemical staining in addition to double-enzyme-immunohistochemical staining techniques. T-MC was the prevailing subtype in all investigated organs and tissue locations. Only tryptase-positive MCs could be demonstrated in bovine skin and uterus, regardless of the fixation type (Kuther *et al.*, 1998). In large vertebrates, the density of MCs in the peripheral bronchioles is substantially greater than around the conducting airways (Miller & Pemberton, 2002).

3.3 Preformed mast cell mediators; expression and function

Once activated, MCs release a massive amount of preformed or *de novo* synthesized mediators (Fig. 3), which are involved in many physiological

and pathophysiological processes. The function of most of these mediators has only recently been understood.

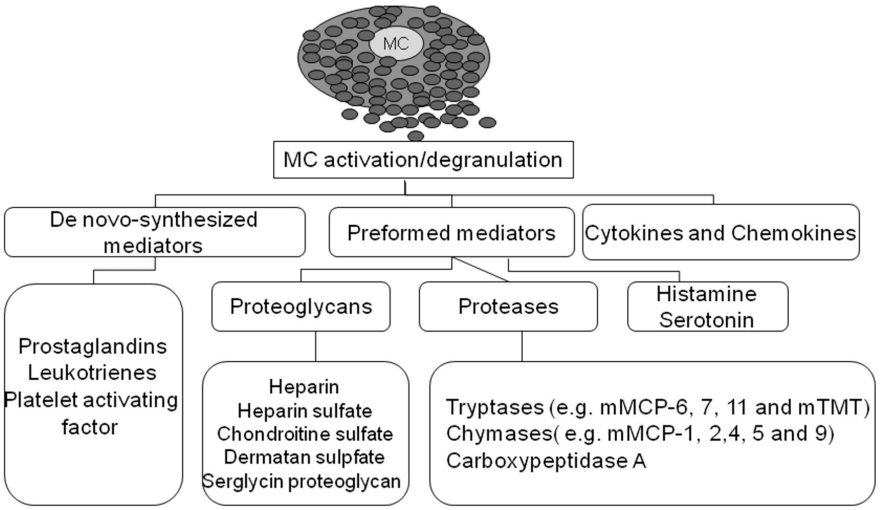

Figure 3. Scheme of different mast cell mediators.

3.3.1 Histamine

Histamine (2-[4-imidazolyl]ethylamine) was discovered in 1910 (Dale & Laidlaw, 1910) and in 1932 it was identified as a mediator of anaphylactic reactions (Maintz & Novak, 2007). Histamine belongs to the biogenic amines and is synthesized from the amino acid histidine by L-histidine decarboxylase (HDC). In addition to MCs and basophils, which are the main source of histamine in the body, synthesis of histamine occurs in platelets, histaminergic neurons, and enterochromaffine cells (Riley & West, 1952) (Ishizaka *et al.*, 1972). In MCs and basophils, histamine is stored in secretory granules, associated by ionic linkage at acidic pH with the carboxyl groups of proteins and PGs. Binding of histamine to the histamine receptors, H1R, H2R, H3R, and H4R exerts variable effects on target cells in various tissues, such as smooth muscle cell contraction, vasodilatation, increased vascular permeability and mucus secretion, tachycardia, and gastric secretion. MCs also express histamine receptors H1R, H2R, and H4R indicating an autocrine/paracrine activation mechanism (Lippert *et al.*, 2004). Mice deficient in HDC showed a decrease in MC numbers and

decreased storage of PGs and proteases in MC granules compared to normal mice (Ohtsu *et al.*, 2001).

3.3.2 Tryptases

Tryptase, with trypsin-like activity, is one of the major constituents of MC granules, and belongs to the serine protease family. Human MCs express two classes of tryptases, known as α (with variants αI and αII) and β (with variants βI, βII and βIII) (Pallaoro *et al.*, 1999) (Miller *et al.*, 1989). In addition, human MCs express a transmembrane tryptase, γ-tryptase or hTMT, which seems to be exposed on the cell surface after MC degranulation, and another tryptase, the δ-tryptase, with as yet unknown biological function (Caughey *et al.*, 2000) (Wong *et al.*, 2002).

In contrast, murine MC tryptase expression seems to be restricted to CTMCs. CTMC tryptases so far described in mouse are mMCP-6 and -7, where the expression of the latter is strain dependant, e.g. C57BL/6 strain does not express mMCP-7 because of a mutation affecting a splice site (McNeil *et al.*, 1992; Reynolds *et al.*, 1991). Mouse MCs also express a transmembrane form of tryptase, mTMT (Wong *et al.*, 1999), and a tryptase-like enzyme known as mMCP-11, which was mainly expressed at early stages of MC differentiation, in a similar to mMCP-7 (Wong *et al.*, 2004). Rat MMCs, like their murine counterparts, do not express tryptases, but rather express several members of the rMCP-8 family such as (rMCP-8, -9, and -10), a protease family of unknown substrate specificity and biological function. Further, rat CTMCs were found to express two tryptase genes, rMCP-6 and -7 (Lutzelschwab *et al.*, 1997).

In sheep, two tryptases have been described, tryptase-1 and -2, as well as two other MC proteases, sMCP-1 and -3 (Pemberton *et al.*, 2000) (McAleese *et al.*, 1998). Two similar bovine MC tryptases, bovine lung tryptase (BLT) and bovine liver capsule tryptase (BLCT) have been isolated from lung and liver, respectively (Gambacurta *et al.*, 2003). Dogs also express a tryptase, known as mastin (Raymond *et al.*, 2005) (Vanderslice *et al.*, 1989).

Activation

MC proteases, including tryptases, are synthesized in preproform in the endoplasmic reticulum. A subsequent cleavage process leads to generation of the proforms, propeptide, and the mature enzymes. Dipeptidyl peptidase I DPPI (cathepsin C) may be involved in the processing of the N-terminal dipeptide of prochymases (Pejler *et al.*, 2007), which was supported by studies in the $DPPI^{-/-}$ mouse, which fail to generate active chymases *in vivo*.

The N-terminal processing mechanism was less characterized for tryptases. However, the level of mature tryptases was lower in DPPI$^{-/-}$ MCs than in wild type counterparts. All MC proteases, including tryptase, are stored as fully processed enzymes, in contrast to some other enzymes. MCs, therefore, most likely need a protective system to overcome any protease leakage from granules to the cytoplasm. The serpin class of protease inhibitors have been proposed as one potential protective mechanism (Pejler *et al.*, 2007; Wolters *et al.*, 2001). Tryptase in MC granules is stored as an active tetramer in complex with SGPG, with an active site facing a central pore. Dissociation of this tetrameric structure might result in inactive monomers. Additionally, previous studies have also shown that tryptase can be active in the monomeric form (Schwartz & Austen, 1980) (Schwartz & Bradford, 1986) (Schwartz *et al.*, 1981) (Hallgren *et al.*, 2001) (Addington & Johnson, 1996).

Function

Immunologically, MC tryptases are implicated in a variety of inflammatory disorders. The prominent proinflammatory role in allergies has been extensively studied in humans as well as in animal models of allergic inflammation (Caughey, 2007). Injection of human tryptase into guinea pig or recombinant mMCP-6 into mice induced the influx of neutrophils and eosinophils into skin and peritoneum, mediating peritonitis (Huang *et al.*, 1998), and increased microvascular leakage in the skin (He & Walls, 1997). Tracheal administration of human tryptase βI to MC-deficient mice was shown to have a protective role against *Klebsiella pneumoniae* infection in the lung (Huang *et al.*, 2001), and conversely, inhibition of MC tryptases in inflammatory arthritis was found to have a therapeutic effect (McNeil *et al.*, 2008). MC tryptases can activate protease-activated receptors (PAR2), which leads to inflammation in the airways, joints and kidney, degrading fibrinogen and thereby limiting blood clot formation (Ossovskaya & Bunnett, 2004). Additionally, tryptases have been shown to play a role in many other physiological functions and diseases such as fibrosis, angiogenesis, itching, sepsis, multiple sclerosis, tissue remodeling, arthritis and sudden infant death syndrome (Fiorucci & Ascoli, 2004) (Pejler *et al.*, 2007) (Shin *et al.*, 2009).

3.3.3 Chymases

MC chymases with chymotrypsin-like activity also belong to the serine protease family (Benditt & Arase, 1959). Expression profiles vary among MC subtypes and species. Humans express only one chymase gene

belonging to the α-family expressed by MC_{TC} cells (Caughey *et al.*, 1991) (Urata *et al.*, 1991). Murine MCs express several chymases: MMCs express two chymases belonging to the β-family designated as mMCP-1 and -2 (Chu *et al.*, 1992) (Serafin *et al.*, 1990) (Chu *et al.*, 1992). In addition, CTMCs express mMCP-4 (β-chymase), mMCP-5 (α-chymase, but with elastase like activity) and mMCP-9 (particularly uterine MCs). It is noteworthy that mMCP-4 constitutes the major chymotrypsin-like activity in peritoneum and ear connective (Pejler *et al.*, 2007)

Activation

Like the tryptases, the MC chymases are synthesized as inactive proproteins and are activated intracellularly by the enzyme, DPPI (Wolters *et al.*, 2001). Active MC chymases are then stored in low pH secretory granules in a tight complex with serglycin PGs. In a neutral environment, chymases are known to cleave peptides at specific locations in polypeptide chains following aromatic amino acids (Powers *et al.*, 1985).

Function

Previous work from our lab showed that mMCP-4 (which together with CPA is involved in the formation and degradation of angiotensin II) plays a key role in the activation of pro-matrix metalloprotease-9, as well as in thrombin regulation and fibronectin turnover (Tchougounova *et al.*, 2005) (Lundequist *et al.*, 2004) (Tchougounova *et al.*, 2003). We further showed that mMCP-4 contributes to the antibody response and severity in autoimmune arthritis (Magnusson *et al.*, 2009), and that it has a protective role in inflammation of the airways induced by allergens (Waern *et al.*, 2009). Prominent roles in the regulation of homeostatic intestinal epithelial migration and barrier function during inflammatory conditions, and in aneurysm formation has also been described (Groschwitz *et al.*, 2009) (Sun *et al.*, 2009).

3.3.4 Carboxypeptidase A

In 1979, MC carboxypeptidase A (MC-CPA) was partially purified from rat liver mitochondria, and was thought to reside in the inner surface of mitochondrial membranes, although an MC origin was also suggested (Haas & Heinrich, 1979). Normally, CTMCs express MC-CPA with a molecular weight of 30-35 kDa in human, mouse and rat (Goldstein *et al.*, 1987) (Serafin *et al.*, 1987) (Everitt & Neurath, 1980). Murine MC-CPA is a zinc-dependent metalloprotease with exoprotease activity that hydrolyzes C-terminal residues with aliphatic or aromatic amino acids. MC-CPA was

found to form a macromolecular complex with PGs in the secretory granules. In human, MC_{TC} cells appear to be the source of MC-CPA, and it thus has similar tissue distribution in both rodents and humans (Irani *et al.*, 1991). Positively charged amino acids in MC-CPA are thought to mediate CPA storage of MC-CPA in MC granules through high affinity interactions with the negatively charged serglycin PGs containing heparin, or chondroitin sulfate. Storage is highly dependent on heparin and MC-CPA storage and processing was shown to be altered in mice deficient in N-deacetylase/N-sulfotransferases 2 (NDST-2), the downstream enzyme in heparin synthesis (Humphries *et al.*, 1999) (Henningsson *et al.*, 2002) (Forsberg *et al.*, 1999). Storage of MC-CPA was also found to be defective in SG-deficient mice (Abrink *et al.*, 2004). Like tryptase and chymase, MC-CPA is stored as an active form with lowered enzymatic activity (Henningsson *et al.*, 2002).

Activation

The preproenzyme of MC-CPA in both mouse and human consists of about 417 amino acids, with a 15 amino acid N-terminal signal peptide and a 94 amino acid activation peptide. Activation produces a mature MC-CPA of 308 amino acids (Reynolds *et al.*, 1989b) (Reynolds *et al.*, 1989a). Work performed in mice deficient in cathepsin E showed that cathepsin E is important for MC-CPA processing (Henningsson *et al.*, 2005). Storage of MC-CPA and mMCP-5 was strongly correlated in MC-CPA knockout mice. The MC-CPA deficient strain was also deficient in mMCP-5, although other proteases, such as mMCP-4 and mMCP-6 were not affected (Feyerabend *et al.*, 2005).

Function

MC-CPA is implicated in endothelin-1 (ET-1) degradation. ET-1 is a major vasoconstrictor and plays an important role in sepsis. However, addition of CPA inhibitors did not inhibit the degradation of ET-1 (Maurer *et al.*, 2004), suggesting that other MC-derived proteases possibly contribute to this process. MC-CPA has also been reported to reduce snake and honeybee venom-induced pathology, by degrading certain snake venom components and reducing honeybee venom morbidity and mortality (Metz *et al.*, 2006).

3.3.5 Proteoglycans (PGs)

PGs comprise a wide range of ubiquitously distributed macromolecules and structural diversity suggests involvement in a multitude of functions. PGs

are also found in more simple organisms, such as *Drosophila spp.* and *Caenorhabditis elegans* (Kolset & Tveit, 2008). The influence of PGs on biological processes is mediated through interaction with a huge variety of proteins and components of the extracellular matrix (ECM) (Handel *et al.*, 2005) (Iozzo *et al.*, 1994).

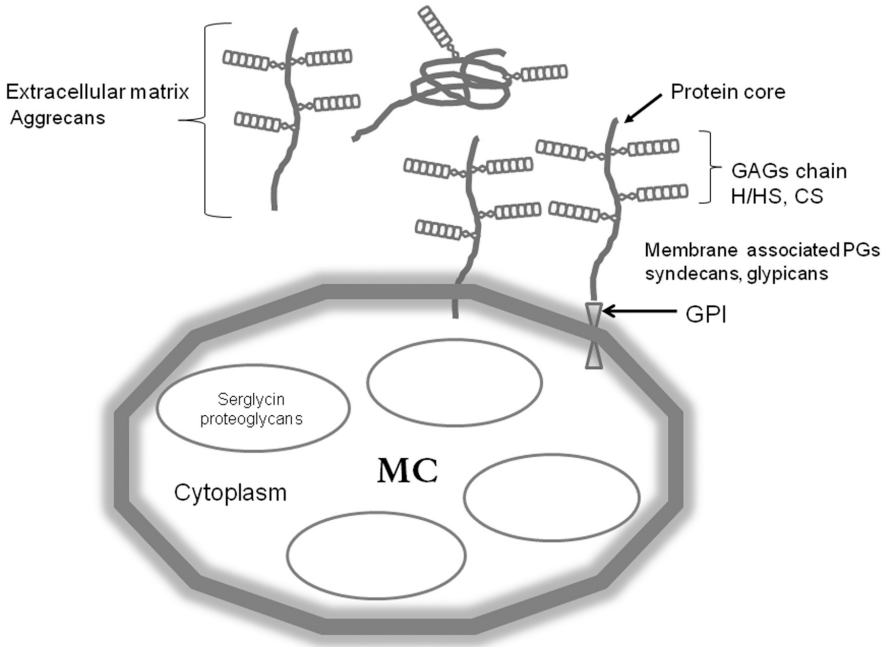

Figure 4. Classes of proteoglycans

Glycosaminoglycan (GAG) chain biosynthesis takes place in the Golgi apparatus and comprises a multi-step enzymatic process. Depending on cell type and location in the tissue, GAG chains attached to PGs can vary. GAGs can be grouped into three main classes known as heparan sulfate/heparin, keratan sulfate, and chondroitin sulfate/dermatan sulfate. PGs can be located at the cell surface, e.g. syndecans and glypicans, the extracellular matrix, e.g. perlecan and agrin, and intracellularly, e.g. serglycin proteoglycans (SGPG) (Esko & Lindahl, 2001) (Lindahl *et al.*, 1998) (Fig. 4). Interestingly, hyaluronic acid (hyaluronan), the non-sulfated form of GAGs, is not attached to any core protein. Hyaluronan was shown to be expressed in rodent and human skin MCs (Eggli & Graber, 1993). In addition, it was demonstrated earlier that MC granules are a rich source of

hyaluronan, suggesting that hyaluronan is possibly involved in pathological processes in asthma and allergy (Soderberg *et al.*, 1989).

Synthesis of glycosaminoglycans (GAGs)

PGs consist of a protein core with long covalently attached, un-branched and negatively charged polysaccharide chains, or GAGs. The GAG chains are composed of repeated disaccharide units made up of one alternating amino sugar, N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc), bound to either hexuronic acid (HexA) or galactosamine (Gal) (Fig. 5). Attachment of the sugar chains to the core protein occurs through an O-glycosidic bond, usually to serine or threonine residues (Bernfield *et al.*, 1999) (Kjellen & Lindahl, 1991). The first polymerization step in CS/DS and heparin/HS chain PG synthesis is the formation of the linker tetrasaccharide region (xylose-galactose-galactose-GlcA), followed by chain elongation by alternating addition of GalNAc and glucuronic acid (GlcUA), driven by chondroitin sulfate polymerase in the case of CS and EXT1/EXT2 in the case of heparin/HS (Fig.5) (Lind *et al.*, 1998).

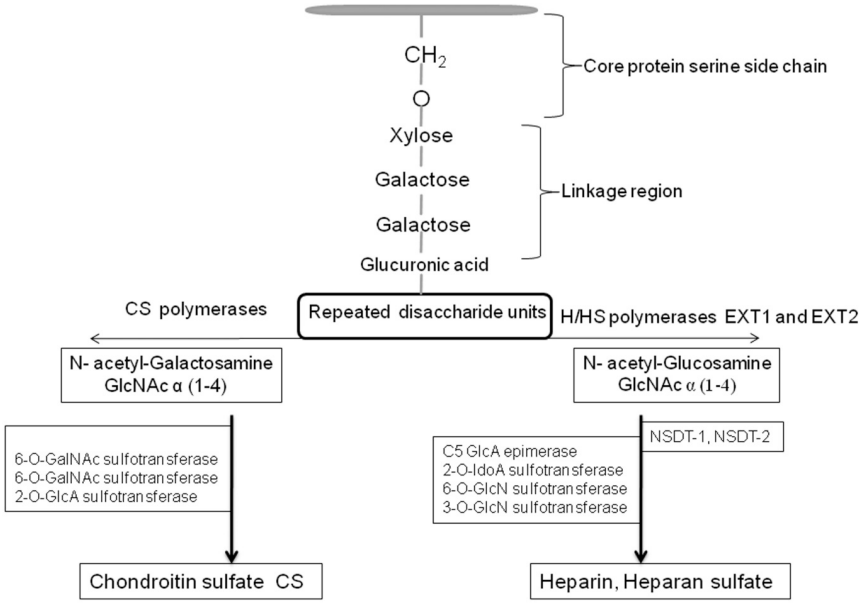

Figure 5. Heparin/heparin sulfate and chondroitin sulfate biosynthesis (modified from Prydz *et al.*, 2000).

Other modifications occur by the action of N-deacetylase/N-sulfotransferases (NDSTs), C5-epimerase and 2, 3, and 6 O-sulfotransferase enzymes that introduce sulfate groups at various positions, and which finally result in the production of a highly sulfated heparin or the less sulfated HS (Prydz & Dalen, 2000)

Until recently, four isoforms of the NDST enzyme family have been identified in mice; NDST-1 and NDST-2 are expressed in embryonic and adult tissue, whereas NDST-3 and NDST-4 are principally expressed during embryogenesis (Aikawa *et al.*, 2001) (Eriksson *et al.*, 1994). Heparin is mainly expressed by CTMCs. Genetic targeting of the genes encoding the enzymes, NDST-2 and NDST-1, demonstrated that NDST-2 is essential for heparin biosynthesis whereas NDST-1 is essential for heparan sulfate synthesis (Forsberg *et al.*, 1999) (Ringvall *et al.*, 2000).

Function

The syndecans, which are transmembrane HSPGs, possess an extracellular domain with GAG-attachment sites on that side. Mammalian cells express four types of syndecans, syndecans 1 to 4 (Saunders *et al.*, 1989), and nearly all cells express at least one type (Kim *et al.*, 1994). Syndecans are involved in cell-cell, cell-matrix adhesion and signaling, and syndecan-1 has been shown to bind a broad range of extracellular proteins, via its HS chains, thus serving as a matrix receptor. Glypicans constitute another family of cell surface PGs that carry HS GAG chains, which are linked to the cytoplasmic membrane through a GPI anchor. In mammals, this family consists of six members, glypicans 1 to 6 (Saunders *et al.*, 1997) (David, 1993).

Perlecan, located in all basement membranes and many other extracellular matrices, is one of the most well studied extracellular PGs. The perlecan core protein can interact with fibronectin, laminin, growth factors and cytokines (Isemura *et al.*, 1987). Agrin is a molecule that plays a key role in the aggregation of acetylcholine receptors during the development of the neuromuscular junction during embryogenesis (Nitkin *et al.*, 1987).

3.3.6 Serglycin proteoglycan

Serglycin proteoglycan (SGPG), the first gene for the core protein of a PG to be cloned (Bourdon *et al.*, 1985), was initially disclosed as a secretory and membrane associated product isolated from a rat L2 yolk sac carcinoma cell line (Oldberg *et al.*, 1981), and was found to be expressed in a broad range of cells of hematopoietic origin (Tantravahi *et al.*, 1986). The SG gene from mouse and human consists of three exons (Avraham *et al.*, 1989), which seems to be the conserved genomic structure in most species (Genbank,

2010). Exon 1 encodes for the first 25 amino acid residues, which encompass the signal peptide of the core protein, exon 2 encodes for the N-terminal part of the protein and exon 3 encodes a 79 amino acid sequence that contains the GAG attachment site, i.e. the serine/glycine repeats. SGPG is the dominating PG identified in secretory granules, but is constitutively secreted in some cell types. The GAGs attached to SGPGs, with different sulfation patterns, are chondroitin 4-sulfate, chondroitin 6-sulfate, chondroitin 4, 6-sulfate, HS or heparins, depending on the cell type in which SG is expressed. Heparin is considered to be the most negatively charged GAG *in vivo* (Kolset & Gallagher, 1990). In c-kit ligand/SCF-induced growth of MCs, heparin links to the SG protein core, whilst IL-3-induced growth leads to chondroitin 4,6-sulfate linkage to the SG core protein (Levi-Schaffer *et al.*, 1986) (Razin *et al.*, 1982). The sulfation pattern of the GAG chains influences their biological functions, e.g. MCs and basophils contain highly sulfated GAG chains, whereas NK cells and CTLs posses less sulfated GAG chains. Interestingly, the MC subtypes, CTMCs and MMCs, express different types of GAGs. Mouse CTMCs contain SG-heparin, whereas mouse MMCs contain SG-CS (Enerback *et al.*, 1985). On the other hand, human MCs seem to express both GAG-types in the same MC-subclass (Stevens *et al.*, 1988).

3.3.7 Functional consequences of serglycin-deficiency

Previous work on NDST-2-deficient mice showed an impairment in the storage of the MC-specific proteases, MC-CPA, mMCP-4, mMCP-5 and mMCP-6(Forsberg *et al.*, 1999; Humphries *et al.*, 1999). Furthermore, mMCP-6 was dependent on heparin for the formation of functional tetramers, and the mMCP-4-heparin complex was shown to play an important role in extravascular coagulation (Hallgren *et al.*, 2000) (Tchougounova & Pejler, 2001) (Tchougounova & Pejler, 2001). In line with these results, the deletion of the SG gene, which also produced knockout mice with normal development and fertility, showed that SGPGs had a profound impact on MC granule hemostasis (Abrink *et al.*, 2004). Briefly, this work showed that many of the MC-proteases, such as mMCP-4, -5, -6, and MC-CPA were highly dependent on SGPGs for their storage in CTMCs, although SG was not essential for the expression of protease mRNA. The lack of dependence of other proteases on SG for storage was also discussed, e.g mMCP-1 and mMCP-7 were not dependent on SG for storage, whereas mMCP-2 was partially SG-dependent. The SG-dependency for storage was mostly attributed to the electrostatic nature of the protein surfaces, where positively charged proteases interact with

negatively charged PGs (Braga *et al.*, 2007). CTLs failed to store granzyme B and were impaired in their ability to produce secretory granules of high density (Grujic *et al.*, 2005). In addition, neutrophil elastase, a protease present in the azurophil granules of neutrophils, was lacking in SG-deficient neutrophils (Niemann *et al.*, 2007).

The correct packaging of granule components such as hormones, peptides, and other enzymes into secretory granules is crucial for the proper assembly of a functional secretory vesicle. Correctly packed, the granule will appear dark and dense in electron micrographs. Immunocytochemical studies of MC chymase and tryptase in association with SG suggested that chymase may be located in the electron-dense parts while tryptase is located in the translucent parts of secretory vesicles (Whitaker-Menezes *et al.*, 1995). However, in IL-3-derived BMMCs, the lack of SG did not interfere with the formation of functional secretory vesicles. Formation of the dense core was defective, and it was suggested that SG was involved in the retention of proteases after entry into the secretory vesicles (Henningsson *et al.*, 2006). Together, these studies clearly demonstrated that SG is important in the process of granulopoiesis, i.e. in the generation of storage granules.

3.4 *De novo*-synthesized mast cell mediators

3.4.1 Lipid mediators, leukotrienes and prostaglandins

Leukotrienes were identified in the late 1970s (Murphy *et al.*, 1979). They are a family of inflammatory lipid mediators synthesized from arachidonic acid by various cell types, including MCs, eosinophils, neutrophils, basophils, and macrophages. The cleavage of arachidonic acid from the nuclear membrane by phospholipase A2 (PLA2) initiates the synthesis of the leukotrienes (Uozumi *et al.*, 1997). Interaction of arachidonic acid with the biosynthetic proteins, 5-lipoxygenase (5-LO) and 5-lipoxygenase activating protein (FLAP), results in the formation of the intermediate, 5-HPETE (5-hydroxyperoxy-6, 8, 11, 14-eicosatetraenoic acid), which is quickly converted to LTA4. LTA4 hydrolase or LTC4 synthase then converts LTA4 into LTB4 or LTC4, respectively. LTC4 is converted extracellularly to LTD4 and LTE4 by sequential amino acid removal from the glutathione tripeptide moiety. Thus, LTC4, LTD4, and LTE4 are together referred to as cysteinyl leukotrienes (CysLTs). CysLTs exert their actions through activation of two receptors, CysLT subtype 1 receptor (CysLT1) and CysLT subtype 2 receptor (CysLT2). LTE4 is the most stable form of the

CysLTs, and accordingly, can be measured in the urine. LTE4 is often used as a marker of 'whole body' leukotriene synthesis. A complex inter-regulation between CysLTs and a variety of other inflammatory mediators exists, where CysLTs are believed to play roles in the maturation and tissue recruitment of inflammatory cells. CysLT1 has been suggested as a potential drug target (Peters-Golden *et al.*, 2006).

Prostaglandins are produced through the activity of cyclooxygenase (COX) on arachidonic acid. The major COX product in MCs after allergen-induced activation is prostaglandin D2 (PGD2) (Lewis *et al.*, 1982). Besides being a potent inhibitor of platelet aggregation, PGD2 is chemotactic for human neutrophils, and together with LTD4, may mediate the accumulation of neutrophils in human skin (Metcalf, 1997).

3.4.2 Platelet activating factor (PAF)

The name refers to its ability to aggregate and degranulate platelets, besides many other biological activities. PAF has been shown to induce bronchoconstriction, most likely through contraction of airway smooth muscles, to constrict blood vessels, and to increase endothelial cell permeability. Furthermore, PAF is proinflammatory, i.e. chemoattractant for eosinophils, neutrophils, and lymphocytes, and activates eosinophils and neutrophils, inducing the release of granule- associated and newly formed mediators (Hines, 2002).

3.4.3 Mast cell-derived cytokines and chemokines

Plaut *et al.* were the first to demonstrate that murine MCs are capable of expressing many cytokines (Plaut *et al.*, 1989). Since then, others have shown that MCs express a spectrum of cytokines and chemokines, such as interleukin (IL)-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-12, IL-13, IL-16, tumor necrosis factor (TNF-α), interferon (IFN-γ), granulocyte/macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein 1 (MCP- 1), and macrophage inflammatory proteins (MIP) 1 and 2 (Hines, 2002) (Nakano *et al.*, 2007), in response to IgE, TLR, or substance P activation (Bradding & Holgate, 1996) (Bradding, 1996). The release of cytokines and chemokines can also be induced by factors that do not cause degranulation, including lipopolysaccharides, stem cell factor, thrombin, and nerve growth factor (NGF). In human, IL-4 is preferentially expressed by MC_{TC} cells, whereas IL-5 and IL-6 were generally restricted to the MC_T subset. Furthermore, MCs have been shown to produce IL-16 and the chemokine, lymphotactin, both contributing to lymphocyte recruitment at sites of MC degranulation (Metcalf, 1997).

The proinflammatory cytokine, TNF-α, has been shown to be stored in the secretory granules and released upon activation, and is thus classified as one of the preformed mediators (Walsh *et al.*, 1991). TNF-α is a multi-functional cytokine involved in leukocyte extravasation through regulation of endothelial leukocyte adhesion molecule-1 (Messadi *et al.*, 1987).

3.5 Mast cell activation and function

In order to release their mediators and become functional, MCs need to be activated by one of many different pathways. Activation of MCs through FcεRI and its ligand, IgE, is still among the most extensively studied. In addition, TLRs and FcεRI were found to induce rapid and selective MC activation. However, other pathways for MC activation have been recognized and need to be discussed (Fig. 6).

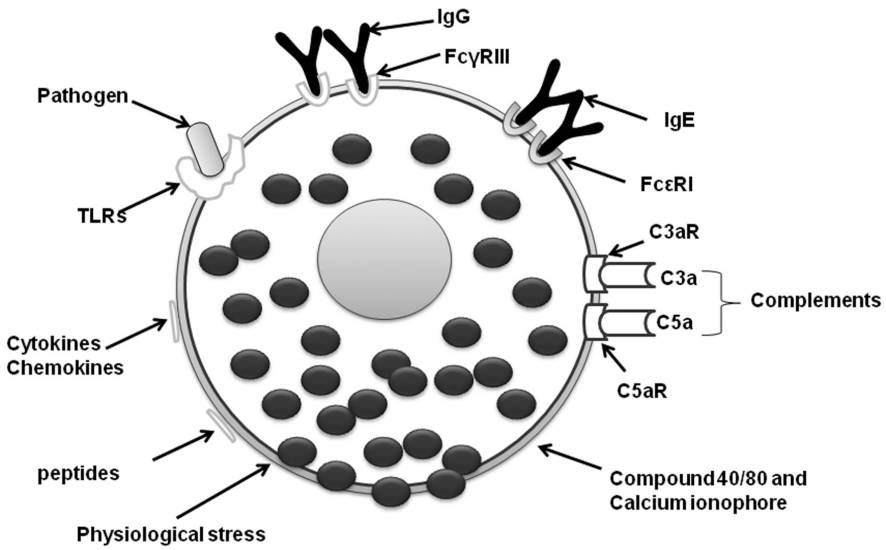

Figure 6. Different mechanisms of mast cell activation

3.5.1 IgE-dependant mast cell activation

The most well studied event of MC activation is via antigen-crosslinked IgE/FcεRI receptors, which is a crucial event in type I hypersensitivity reactions (Gilfillan & Tkaczyk, 2006). The FcεRI on MCs includes three

subunits: FcεRIα, FcεRIβ, and FcεRIγ (Gilfillan & Tkaczyk, 2006) (Rivera & Gilfillan, 2006). IgE is bound to the α-chain through its Fc domain (Lin et al., 1996), thereby securing attachment to the MC surface. Crosslinking triggers activation of the kinase, Lyn, which in turn phosphorylates FcεRI ITAMs (immunoreceptor tyrosine-based activation motifs) followed by downstream activation processes for many enzymes and adaptor-molecules and leading to MC degranulation (Kalesnikoff & Galli, 2008). Interestingly, murine MCs exposed to a high level of IgE in the absence of specific antigen increased surface expression of their FcεRIs and, subsequently increased mediator release after activation by cross-linking of surface-bound IgE by allergen. Thus, IgE not only mediates MC activation, but also regulates MC functions by itself (Kawakami & Galli, 2002).

3.5.2 IgG-regulated activation

Surprisingly, IgE-deficient mice were found to exhibit anaphylaxis, suggesting another mechanism, presumably mediated via IgG/Fcgamma receptors, to compensate for IgE deficiency (Oettgen *et al.*, 1994). In support of this, murine MCs do express low affinity receptors for IgG, including FcγRIIb1, FcγRIIb2 and FcγRIII (Benhamou *et al.*, 1990) (Katz & Lobell, 1995). Of those, FcγRIII has been revealed to induce MC-degranulation (Katz & Lobell, 1995) and promote adhesion after IgG-mediated aggregation (Dastych *et al.*, 1997). Peritoneal mouse MCs were found to express receptors capable of binding mouse monoclonal IgG1, IgG2a, and IgG2b, but not IgG3 antibodies (Tigelaar *et al.*, 1971). Other FcγRs such as FcγRIIB and FcγRIIIA were also found to bind IgE aggregates or complexes. Human MCs were demonstrated to express FcγRI (CD64) following IFN-γ treatment (Okayama *et al.*, 2000). In addition, aggregation of FcγRI on the surface of human MCs induced degranulation, generation of arachidonic acid-derived mediators, and up-regulatation of cytokine and chemokine mRNAs (Malbec & Daeron, 2007).

3.5.3 Complement receptor-mediated activation

MCs have long been recognized to interact with the complement system: C3a and C5a, for example, activate MCs through complement receptors CR3 (CD11b) and CR4 (CD11c). Constitutive expression in MC populations is low and expression is up-regulated only after cellular activation. Furthermore, the expression levels of complement receptors also varies depending on the differentiation status of the MCs and the effects of local cytokines. In humans, the C5aR was observed to be expressed by some skin and cardiac MCs but not by MCs from the uterus, lungs or

tonsils, which indicates that MCs from different anatomical locations might vary in their capacity to respond to complement components (Fureder *et al.*, 1995). In addition to their function as MC activating agents, C3a and C5a have been shown to be chemotactic for MCs (Nilsson *et al.*, 1996). In a mouse model of bacterial infection by caecal ligation and puncture, both $C3^{-/-}$ and $C4^{-/-}$ mice were more susceptible to infection, and these mice also showed reduced MC activation during peritoneal infection (Prodeus *et al.*, 1997).

3.5.4 Toll-like receptor activation

Numerous studies report the expression of TLR1 to TLR9 in murine MC populations (Mrabet-Dahbi *et al.*, 2009). Through the expression of TLRs, MCs are able to recognize different PAMPs, For example, TLR4 recognizes and responds to lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria (Zhang *et al.*, 2004) (Supajatura *et al.*, 2001), and TLR2 recognizes peptidoglycan, a constituent of the cell wall of gram-positive bacteria (Takeda *et al.*, 2003). In addition, TLR3 mediated activation of fetal skin-derived mouse MCs and cultured human MCs, inducing pro-inflammatory cytokine production and type I IFNs, respectively (Matsushima *et al.*, 2004) (Kulka *et al.*, 2004). TLR1 and TLR6, which form functional heterodimers with TLR2, are also expressed by rodent and human MCs, and TLR7 and TLR8 are crucial for responses to single-stranded RNA sequences associated with viral infection (Marshall, 2004). The interaction between PAMPs and TLRs on MCs cause activation, induction of expression and release of cytokines, leukotrienes and prostaglandins (Marshall, 2004; Okumura *et al.*, 2003).

3.5.5 Other mast cell activators

MCs are also activated by other means, such as substance P, compound 48/80, mastoparan, polymyxin B, peptides such as melitin (bee venom peptides) and adrenocorticotropic hormone (ACTH), which can cause histamine release. Certain cytokines and chemokines, such as IL-1, IL-3, SCF, GM-CSF, macrophage inflammatory protein-1 (MIP-1) and monocyte chemoattractant peptide-1 (MCP-1), are also able to elicit a response (Mekori & Metcalfe, 2000). In addition, cell-to-cell interactions, opiates, calcium ionophores, endothelin, adenosine, as well as physiological stress such as changes in osmolarity, pressure and UV light can also provoke MC activation (Metcalf, 1997).

3.6 Mast cell function

Tissue-resident MCs are large, round cells scattered throughout the body, close to external and internal surfaces such as skin, airways, mucosa, and blood vessels (Bischoff *et al.*, 2000). MCs exert physiological and pathopysiological functions at these strategically important sites (Maurer *et al.*, 2003). MCs are mostly associated with allergy and anaphylaxis, but have also been shown to play a key role in innate immunity through the regulation of the inflammatory response and to possibly link innate and adaptive immunity. MCs contribute to allergic inflammation through a variety of complex events, including the synthesis and release of mediators such as histamine and serotonin. Cross-linking of FcεRI receptors by specific allergens, as well as neuropeptides, complement factors, cytokines, hyperosmolarity, lipoproteins, adenosine, superoxidases, hypoxia, chemical and physical factors (extreme temperatures, traumas), or alcohol and certain food and drugs, have been shown to activate MCs and induce the release of histamine (Maintz & Novak, 2007) (Vlieg-Boerstra *et al.*, 2005).

The physiological sequels for releasing these mediators are increased vasodilatation, local secretion by mucosal tissues, and an increase in smooth muscle reactivity In addition, MCs are also involved in bidirectional communication with nerves through the release of MC mediators and the capacity to respond to factors released by the nerves (Maurer *et al.*, 2003). Further, MCs have been suggested to play a role in various contexts, such as peripheral tolerance, phagocytosis of pathogens, endocrine function, metabolism of lipid/vitamins/calcium, tissue growth, response to stress, aging, protection from cancer, and in autoimmune diseases such as multiple sclerosis, bullous pemphigoid, and rheumatoid arthritis (Fig. 7). MCs are intimately involved in maintaining blood vessel tone, blood pressure regulation, vascular permeability, and angiogenesis (Gregory & Brown, 2006; Maurer *et al.*, 2003) (Galli *et al.*, 2005). Furthermore, MCs in the gut have been shown to play important roles in multiple organ function such as fluid and electrolyte secretion by epithelial cells and motor functions of smooth muscle cells (Crowe *et al.*, 1997)(Fig.7). Additionally, as a source of heparin, MCs could possibly regulate blood coagulation (Tchougounova & Pejler, 2001) (Sillaber *et al.*, 1999). Finally, the recruitment of MCs to sites of tissue repair and fibrosis also suggest involvement in these processes (Metcalf, 1997).

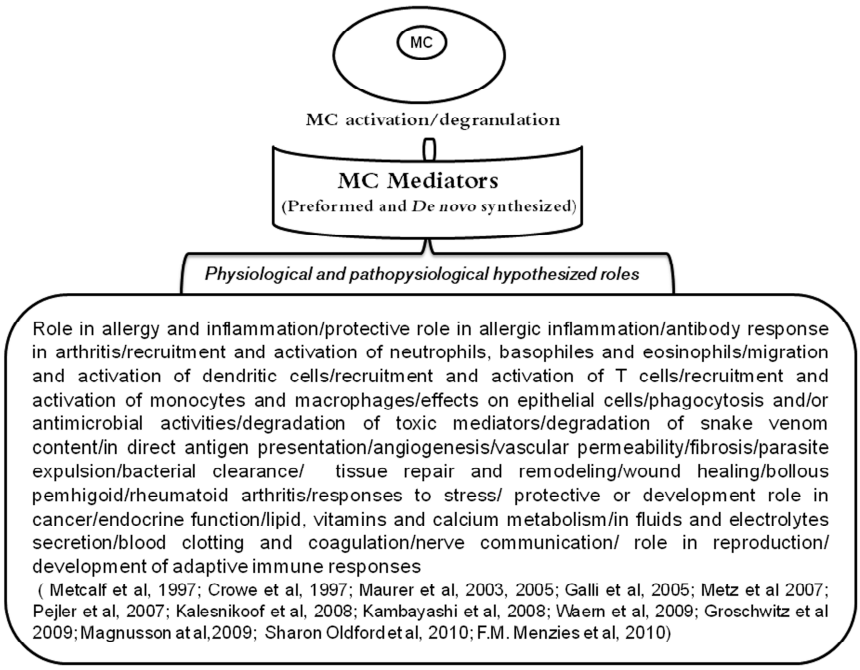

Figure 7. The hypothesized physiological and pathophysiological roles of mast cells.

3.7 Mast cells and infection

Both human and mouse MCs have been shown to be capable of presenting antigens through MHC class I- and MHC class II-dependent pathways *in vitro* (Poncet *et al.*, 1999) (Malaviya *et al.*, 1996b). Moreover, MCs may indirectly present antigen by internalization of IgE-bound antigen for presentation to specific T cells (Kambayashi *et al.*, 2008). The release of MC-specific mediators after activation seems to be crucial for initiating an efficient early response at sites of infection. The release of cytokines, such as TNF-α and the protease mMCP-6, induces recruitment of neutrophils, resulting in pathogen clearance and can improve survival in mice during septic peritonitis (Malaviya *et al.*, 1996a) (Henz *et al.*, 2001) (Marshall, 2004; Echtenacher *et al.*, 1996). In addition, MC proteases have been suggested to degrade endogenous and exogenous toxic peptides and to enhance resistance to parasites (Knight *et al.*, 2000) (Metz *et al.*, 2006). Moreover, MCs produce lipid mediators, which modulate further MC mediator release and improve the innate host response by recruitment of proinflammatory cells (Metz & Maurer, 2007).

In response to ectoparasites, gastrointestinal helminths and nematodes, MCs could be influenced and up-regulated by Th-2 responses (McDermott *et al.*, 2003; Lantz *et al.*, 1998) (Woodbury *et al.*, 1984). Interestingly, mMCP-1 has been shown to be important for a full intestinal host response during *Trichinella spiralis* infection (Knight *et al.*, 2000). In addition, a possible involvement of MCs in effective responses toward many parasites such as *Schistosomes, Filaria, Giardiasis* and *Toxoplasma* have been demonstrated (Cutts & Wilson, 1997) (Li *et al.*, 2004) (Ferreira *et al.*, 2004). The use of MC-deficient WBB6F1-W/W^{v} mice established that MCs may have a protective role in *Plasmodium spp.* infection and that they control skin T cell-dependent host defense in *Leishmania major* infections (Furuta *et al.*, 2006) (Maurer *et al.*, 2006).

Overall, the mechanisms by which MCs elicit these protective functions are diverse and dependent on the site, kinetics and pathogen involved. For example, in acute septic peritonitis, endothelin (ET)-1 is known to induce degranulation of MCs. Conversely, signals through TLRs do not induce MC degranulation but rather induce the secretion of newly synthesized mediators such as cytokines and chemokines, for example, IL-12, which may improve host survival during polymicrobial infections (Marshall, 2004) (Nakano *et al.*, 2007). The possible contribution of MCs in the response to the protozoan parasite, *Toxoplama gondii*, has not been extensively studied. In one study, the leukotriene, LTB4, released from MCs was found to damage the tachyzoites, suggesting an important role of eicosanoids in host defense (Henderson & Chi, 1998). Further, a significant influx of MCs into the site 1 h after i.p injection of the parasite was observed, suggesting that MCs may be involved in the acute phase of the infection. In addition, MC-deficient mice displayed a defect in polymorphonuclear (PMN) leukocyte migration after i.p injection with *T. gondii*, which indicated MCs as a source of chemokines driving the recruitment of PMN cells (Del Rio *et al.*, 2001) (Ferreira *et al.*, 2004).

In ovine, bovine and equine species, the protective role of MCs against parasites has not been so extensively studied. However, the few studies performed support the experimental evidence in the mouse model and suggest a role for MCs in livestock in their responses to parasitic infection. Lambs and kids challenged with the gastrointestinal parasite, *Teladorsagia circumcincta*, displayed an increased number of MMCs in the tissues (Macaldowie *et al.*, 2003). Additionally, alveolar mastocytosis developed in the lungs of lambs with the naturally acquired nematode, *Protostrongylus rufescens* infections (Mansfield & Gamble, 1995). Further, extensive MC degranulation in cases of bovine respiratory syncytial virus-

associated paroxystic respiratory distress syndrome has been observed (Jolly *et al.*, 2004). Calves infected with *Pasteurella heamolytica* displayed a reduction in numbers of pulmonary MCs in the areas of acute inflammation by pneumonia (Ramirez-Romero *et al.*, 2000). Lastly, equine MC and MC proteinases have been implicated in the response to infection by the intestinal nematode, *Cyathostomin* (du Toit *et al.*, 2007).

4 Present investigation

4.1 Aims

Since the development of the SG-deficient mouse strain (Abrink *et al.*, 2004), a number of studies have addressed the potential role and possible biological function of SGPGs in different hematopoietic cell types. In particular, the functional role of SGPGs for the storage and activity of MC-proteases has been studied intensively. However the possible roles during infection remain less well studied. In this thesis, the possible roles of MC, MC proteases and SGPGs in immune responses towards parasitic infection by *T. gondii* have been addressed, using the mouse as a model system. As the role of MCs during infections in large farm animals is rarely studied, this issue has been addressed in an experimental lung worm infection by analyzing the recruitment of tryptase-positive MCs, and tryptase expression in lung tissue.

4.2 Serglycin-independent release of active mast cell proteases in response to *Toxoplasma gondii* infection

SG-deficient mice display defects in the storage of neutrophil elastase, granzyme B, platelet factor 4, β-thrombotubulin, and platelet derived growth factor (Grujic *et al.*, 2005) (Niemann *et al.*, 2007) (Woulfe *et al.*, 2008). In addition, SG is the main secreted PG in peritoneal macrophages and TNF-α secretion by macrophages in response to LPS was found to be regulated by SG (Zernichow *et al.*, 2006). However, the most dramatic effects caused by the targeted deletion of SG were noted in MCs. SG-deficient MCs displayed defects in the morphology of their secretory

granules and failed to retain MC-specific proteases, mMCP-4, -5, -6, and MC-CPA (Abrink *et al.*, 2004) (Braga *et al.*, 2007) (Ringvall *et al.*, 2008).

In this study, we sought to reveal the possible contribution of SGPGs during parasitic infections, using *T. gondii* as the infectious agent. We injected the virulent RH strain into the peritoneum and followed the mice up to 8 days. Early effects of SG-deficiency were found, such as delayed neutrophil recruitment and significant reduction in the recruitment of CD117-positive cells (probably MCs) into the peritoneum. Moreover, the number of degranulated MCs was found to be significantly higher in infected WT compared to $SG^{-/-}$ animals at 12 h and 24 h, which indicates the involvement and activation of MCs in response to parasitic infection. However, as these differences vanished by 48 h, possibly suggesting that other SG-independent mechanisms could take over by this time point, we focused our analysis on the early time points.

The surprising restoration of the SG-dependent MC-specific proteases in the peritoneum of infected $SG^{-/-}$ mice prompted us to investigate the possible mechanism, compensatory or not, behind this result. A sequential assessment of the enzymatic activities related to the MC-specific proteases showed that both $SG^{-/-}$ and WT animals have increased activities, which in consistent with the reappearance of MC-proteases in Western blot analyses.

A possible explanation for the restored activation of MC-proteases in the SG-deficient mice *in vivo*, is that other PGs may compensate. Another possibility is that MC-proteases could act independently of SGPGs once released from the MC. To this end, we isolated and analyzed GAGs from peritoneum of infected WT and $SG^{-/-}$ mice at 48 h post-infection. Interestingly, a massive SG-independent release of hyaluronan and CS was noted, but only minimal levels of heparin/HS were found in the lavage fluid. A high salt extraction of heparin/HS did not increase the recovered amounts, suggesting that most of the heparin/HS is cell-associated. As others have reported that heparin is the main GAG involved in MC-protease activation and possibly acts in a complex with proteases (Hallgren *et al.*, 2001) (Henningsson *et al.*, 2002), we next analyzed whether the heparin/HS pool in the peritoneum was confined to the cellular pool or reabsorbed by surrounding tissues after secretion. In support of a cellular association of heparin, analysis of the GAG-content in the peritoneal cell pool revealed heparin/HS in both challenged and naïve WT mice, although the amount of HS in the $SG^{-/-}$ mice was less by a factor of 10 and was less sulfated.

Our results suggest that MC-specific proteases can be functionally active during infection *in vivo* independently of SGPGs. GAG analysis indicates

that SG is the major heparin/HS-containing PG in the body and that heparin/HS is dispensable for secretion and activation of MC-specific proteases. In conclusion, our results indicate that other sulfated CS/HS GAG chains induced during infection may provide a compensatory mechanism or that SGPGs are dispensable for the function of MC specific proteases *in vivo* when infected with *Toxoplasma gondii*.

4.3 Serglycin proteoglycans contribute to mast cell cytokine secretion *in vitro*, and to early neutrophil recruitment and cytokine secretion *in vivo,* in response to *Toxoplasma gondii*

We next conducted a study to evaluate the role of SG *in vivo* and *in vitro* after *Toxoplasma gondii* infection by measuring the cytokine profiles, parasite burden and other changes in the animals and in MC cultures. In line with study I, $SG^{-/-}$ mice were found to recruit similar total leukocyte numbers and to have similar weight losses to WT mice, due to the infection. However, $SG^{-/-}$ mice had significantly delayed neutrophil recruitment, which may be attributed to a lack of certain MC proteases, such as mMCP-6, previously shown to play a role in the recruitment of neutrophils (Huang *et al.*, 1998). In addition, the delay in production or secretion of the cytokines IL-6, IL-12, TNF-α and IFN-γ, possibly contributes to the observed delay in neutrophil recruitment. *In vitro* analysis of cytokine secretion from STag-stimulated PCMCs indicated a significant role for SG in the production of these cytokines. Interestingly, MCP-1 levels in PCMC cultures was significantly up-regulated in the $SG^{-/-}$ PCMCs compared to WT PCMCs. However, as judged by qPCR, the expression levels of these cytokines were similar in $SG^{-/-}$ and $SG^{+/+}$ both *in vivo* and *in vitro*.

The *T. gondii* infection caused a systematic decrease of these cytokines in the serum of $SG^{-/-}$ mice. Histopathological examination of the spleens from infected animals revealed striking differences at 48 h and 5 days post-infection. The periarteriol lymphoid sheath (PALS) in infected mice exhibited clear areas populated with cells having poorly demarcated borders, and with features corresponding to dendritic cells. In $SG^{-/-}$, these areas were more diffuse and infiltrated with polymorphonuclear cells. The parasite load inside the peritoneal cells at 48 h and numbers of free parasites in peritoneal exudates at 24 h were significantly higher in $SG^{-/-}$ compared to WT animals.

Our results indicate that SGPG may play a regulatory role in the secretion of proinflammatory cytokines and host immune responses toward infection in general, processes which may depend on SG-dependent MC-mediators.

4.4 Age-related enlargement of lymphoid tissue and altered leukocyte composition in serglycin-deficient mice

Serglycin is the dominant PG in MCs, macrophages, neutrophils, CTLs and platelets, where it regulates the storage, secretion and activity of secretory granule constituents. The SG-deficient mouse strain appears normal, with no gross abnormality, normal lifespan and reproduction is not affected (Abrink *et al.*, 2004). However, and surprisingly, aging SG-deficient mice were found to develop spontaneous enlargements in lymphoid organs. In particular the spleen, Peyer's patches (PP), and bronchus-associated lymphoid tissue (BALT) were enlarged. We speculated that SG-deficient mice may be more susceptible to spontaneous infections and so attempted to clarify the observed phenomena. Careful systematic analysis showed that the incidence of spleen enlargement was ~37% versus ~7%, and PP enlargement was ~95% versus ~20% when SG-deficient animals were compared with WT animals. Further analysis of $SG^{-/-}$ spleens by histomorphology showed increased cellularity in the white pulp areas with less defined demarcation zones. Analyses using flow cytometry showed a significant increase in the percentage of CD45RC expressing cells, a surface molecule normally expressed mainly by mature, naïve lymphocytes. Morphological examination of $SG^{-/-}$ PP also showed increased leukocyte infiltration, whereas no major difference in the cellular profile could be seen when cells from SG and WT animals were analyzed by flow cytometry. Together, this suggests that the enlargements develop from an influx of multiple leukocytes rather than one specific cell type. Next, we investigated the profiles of the peritoneal cell population in young and old mice, and found that the macrophage proportion in aging $SG^{-/-}$ mice decreased significantly when compared with WT mice. Furthermore, although similar numbers of MCs were present in $SG^{-/-}$ and WT animals, the level of c-kit (CD117) surface expression was significantly lower in aging $SG^{-/-}$ mice. Interestingly, we did not find any proof of ongoing infections, as the proinflammatory cytokine levels in BALF and serum were similar in $SG^{-/-}$ and WT animals. This suggested that SG-deficiency spontaneously leads to the observed phenotype in aging mice.

Finally, we investigated whether SG-deficiency influenced the general immune responsiveness on challenge with LPS, a bacterial cell wall component. In aging $SG^{-/-}$ mice, intranasal provocation with LPS showed a significant increase in the total cell number and a trend toward higher counts of neutrophils and lymphocytes in BALF, when compared with WT mice. Lung tissues displayed more lesions in peribronchial and perivascular

tissue, an effect possibly due to increased leukocyte infiltration, and suggesting a regulatory role for SG in the inflammatory response.

These results confirm that SG is an important component of the immune system and for the homeostasis of granule components. Moreover, the diverse changes associated with a lack of SG may be attributed to a role for SG in the regulation of cytokines necessary for some differentiation processes.

4.5 Tryptase-positive mast cells accumulate in bovine lung tissues during *Dictyocaulus viviparus* infection

D. viviparus induces a host immune response with a potential role for γ/δ TCR-expressing cells in the immune response of the airways, as previously shown during experimental infection (Hagberg *et al.*, 2005). In addition, the levels of IgE correlated positively with the infection, which implied that *D. viviparus* induces a Th2 profile (Kooyman *et al.*, 2002). However, as few studies have been performed to address MC responses during *D. viviparus* infection, we investigated the airway MC responses during experimental infection. To this end, we analyzed lung tissues, BAL-fluid and blood samples from infected calves, collected at different time points during disease progression, and noted a significant increase in plasma histamine levels on days 3 and 5, and a significant increase of tryptase-positive MCs in the airways on day 14.

The sequential movement of *D. viviparus* from the intestine through lymph nodes to lung tissue in experimentally infected calves causes different host immune responses. The increased histamine levels probably reflect the early response by the MCs in the intestinal mucosa and lymph nodes, rather than those located in the lung. Toluidine blue and immunohistochemistry staining of the lung tissue, with significantly increased MC numbers on day 14, suggests that a major MC recruitment to the infection site only occurs when the parasite has reach its preferred tissue site. The increased MC numbers were then further evaluated. Expression of tryptase was confirmed by qPCR, and was shown to consistently increase during the course of infection. As expected, counting of BAL cells from infected animals on day 14 indicated a massive infiltration of leukocytes, but interestingly, MCs were also found in significant numbers among the BAL cells, as confirmed by qPCR for the tryptase gene. Previous studies (Welle *et al.*, 1995) (Kuther *et al.*, 1998) indicated that the use of an anti-human tryptase antibody enabled the detection of bovine tryptase. We used both a

monoclonal and a polyclonal anti-human tryptase antibody to detect bovine tryptase in Western blots, but were unable to obtain reliable data.

Together, our results show that tryptase-positive MCs are recruited to infection sites in response to *D. viviparus*, where MCs are likely to play a role in the pathogenesis of the disease.

5 Concluding remarks and future perspectives

MCs have been regarded as the "bad guy" in many pathological conditions, particularly due to their devastating role in allergies. On the other hand, recent studies using naturally occurring MC- deficient and/or genetically targeted mice have shown that MCs have beneficial and protective functions both in normal physiology and during severe pathological conditions. With the development of SG-deficient mice, we and others were able for the first time to address the potential role of SGPGs during *in vivo* infections. However, many questions about exact mechanistic roles still remain to be answered.

In this thesis, we addressed the issue of how SGPG-deficiency may affect the immune response towards the protozoan parasite, *T. gondii*. We observed unexpected consequences in the absence of SG; the most interesting was the restoration of SG-dependent MC proteases in infected animals. We have proposed some possible solutions to this mystery, but further investigations are required to reveal more information about the mechanisms at work. Our future work will be aimed at resolving the mechanism that compensates for the absence of SG after MC-specific proteases are released. Intracellular immunostaining may be used to further study different cytokines, particularly MCP-1, which showed a striking difference in secretion when $SG^{-/-}$ and WT PCMCs were compared in tissue culture. Furthermore, it will be interesting to continue work that reveals more about the mechanism on how a lack of SG results in an early transit defect in recruitment of neutrophils and then try to identify the precise role of each of the SG dependent/independent MC-mediators. A first approach will include the use of gene/cDNA microarrays, which may indicate the genes that are most up- and down-regulated in our in vivo and in vitro infection models. Another way to address this apparent delay in neutrophil recruitment is with the use of mouse strains selectively and

genetically targeted for MC-proteases. We have access to mMCP-4 (chymase), mMCP-6 (tryptase), and MC-CPA knockout strains as well as the MC deficient Wsash mouse strain, which can be functionally restored with MCs, including those with specific deletions, through intraperitoneal adoptive transfer.

As peritoneal injection is far from the natural route of infection, we also plan to study the chronic form of the disease by oral administration of infection, where we can conduct a study on the involvement of MMCs in addition to CTMCs through tissue cyst formation.

Peritoneal-derived MCs (PCMCs) produce more mature MCs than cultures of BMMCs. This is why they are likely to be useful for detailed *in vitro* studies of the interaction between MCs and pathogens in studies on parasite recognition receptors expressed on MCs.

An important goal will be to study the diffuse functions of MCs in domestic animals, with the emphasis on parasitic diseases, by characterization of the cells and the development of new methods to enable the optimal performance of these studies.

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Acknowledgments

During these years of tough experience, I realized that there are unseen people who deserve acknowledgement.

First and foremost, my supervisor, **Magnus Åbrink**, I would like to express my heartfelt thank and gratitude for your guidance through my PhD journey. It was really a great experience for me to be under your supervision and inspired by your immense, vast knowledge in biomedical science. Thanks with all help with writing, your tremendous patience, endless encouragement, and the constructive and aimed discussions. Thank you so much for understanding my circumstances and not giving up on me!

My co-supervisors, Gunnar Pejler; you have introduced me to Magnus and the mast cell world. I have really appreciated all your valuable comments, scientific consultations regarding my studies. Thank you! Sara Wernersson; thank you for being helpful and for all your efforts and the knowledge you have added to our manuscript

Co- authors, Anna Lundén; for your continuous energy and interest in discussing most of the topics from mice to cows. Thank you for inviting me to be a member of the bovine projects. Dorothe Spillmann; for being always talented in the field of proteoglycans and for taking care of the GAG analysis. Your optimistic view on referee's comments made it easy. Ricardo Feinstein; with you I really liked looking down the microscopes for hours, you really made it perfect with your expert and fantastic camera. Ananya Roy, thank you for all the help and your time for discussions, Sofia Holmgren, Eva Watterang and Sofia Magnusson; thank you all for the assistance and the nice moments we had during our experimental work. Special thanks to Eva Britt, Cecilia Andersson and Jonas Erjefält for the lab assistance and the very positive response regarding the collaboration.

The ex and present members of the department

Tiago, Jay, Ida, Elin, Fabio, Annette, Anders, Maria R, Gabriella, Helena, Julia, Gianni, Kerstin N, Piotr Wald, Lena H, Janne J, Staffan E, Liya W, Stefan G, Mona, Hanna J, Anna R, Anna S, Charlotte, Rahama, Cecilia, Jenny P, Urmi, My, Louise, Ren, Erik, Ronnie, Kiran, Diana, Carolina Wallström, Lena Holm; thank you all for being ready to help smoothly, whenever I asked for your help in the lab. Thanks for the nice company and for making B9:4 a really lovely work environment. Alexis; thanks for your company and all the help. To all the Libyan students in Uppsala, Jamal Ali, Nizar, Hanan, Khaled, Shiama, and Juma's family; for the best friendship, all help and support from you were really great! To all the other Libyan students here in Sweden, thank you for the best friendship and company.

Special thanks go to Dr Khaled and Dr Hesham; my best friends and colleagues; thank you for all the support and recommendations to go through with it. Dr Abdalla Albergli, for introducing me to MENA members and education. Professor Mikael Kubista, MENA organization founder, thank you for all the help from the beginning.

Thanks to the General People's Committee of Higher Education and to all staff at the Libyan People's Bureau in Stockholm.

إلى والدتي ووالدي، لا أجد الكلمات الكافية لشكركم على تضحيتكم وحبّكم، آمل أن أستطيع رد ولو جزءًا بسيطاً من فضلكم بإذنه تعالى.

إلى زوجتي الغالية، الشكر الجزيل لك، لحُبك، ووضوحك، ومشاركتك لي جميع لحظات مشواري الطويل؛ بالفعل لقد كنت زوجة رائعة، وأمًّا لأحسن زهرتين، رؤى وريان، الجوهر والمعنى الحقيقي لحياتنا.

الشكر الجزيل لإخوتي وأخواتي وعائلاتهم المحترمة، وإلى عائلة جمعة وفتحي الرطيب للمساندة وللمشاعر الطيبة.

أخيراً، أنا ممتن وشاكر لجميع أصدقائي وزملائي في ليبيا، وإلى كل الزملاء في كلية الطب البيطري بجامعة الفاتح- طرابلس.