Mast Cells in Bacterial Infections

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Abstract

Mast cells are implicated in immunity towards bacterial infection, but the molecular mechanisms by which mast cells contribute to the host response are only partially understood. Previous studies have examined how mast cells react to purified bacterial cell wall components, such as peptidoglycan and lipopolysaccharide. To investigate how mast cells react to live bacteria we co-cultured mast cells and the gram-positive bacteria *Streptococcus equi* (*S. equi*) and *Staphylococcus aureus* (*S. aureus*). Gene array analysis showed that mast cells upregulate a number of genes in response to live bacteria. Many of these corresponded to pro-inflammatory cytokines, but also numerous additional genes, including transcription factors, signaling molecules and proteases were upregulated. The release of cytokines was confirmed on the protein level by antibody-based cytokine/chemokine arrays and/or ELISA.

Granzyme D, a protease mainly expressed in cytotoxic T cells, was one of the genes that were upregulated by *S. equi*. We showed that granzyme D is expressed by murine mast cells and that its level of expression correlated positively with the extent of mast cell maturation. Granzyme D expression was also induced by stem cell factor, IgE receptor cross-linking and calcium ionophore stimulation.

Previous studies investigating the role of mast cells in bacterial infection *in vivo* have used mice that are mast cell deficient due to mutations in Kit-signaling. However, these mutations also influence other cell types than mast cells. Thus, to study the role of mast cells during *in vivo* infection with *S. aureus* we used the newly developed Mcpt5-Cre^{+} x R-DTA mice whose expression of diphteria toxin under the Mcpt5 promoter selectively depletes mast cells. *S. aureus* was injected intraperitoneally into Mcpt5-Cre^{+} x R-DTA mice using littermate mast cell-sufficient mice as controls. We did not observe any difference between mast cell-deficient and control mice in regard to weight loss, bacterial clearance, inflammation or cytokine production. We conclude that, despite mast cells being activated by *S. aureus in vitro*, mast cells do not influence the *in vivo* manifestations of *S. aureus* intraperitoneal infection. However, to make more general conclusions about the role of mast cells in bacterial infections, more studies in the new mast cell-deficient mice are needed using other bacterial strains and other routes of administration.

Keywords: mast cells, bacterial infections, proteases, granzyme D, *Streptococcus equi*, *Staphylococcus aureus.*

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Dedication

Till min familj

Det är när du stöter på motgångar som du lär känna din verkliga styrka.
- Okänd

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List of Publications

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

I Rönnberg E., Guss B. and Pejler G. (2010). Infection of mast cells with live streptococci causes a toll-like receptor 2- and cell-cell contact-dependent cytokine and chemokine response. *Infection and Immunity.* 78(2):854-64

II Rönnberg E., Calounova G., Guss B., Lundequist A. and Pejler G. (2013) Granzyme D is a novel murine mast cell protease, highly induced by multiple pathways of mast cell activation. *Infection and Immunity*. 81(6):2085-94

III Rönnberg E., Johnzon CF., Calounova G., Garcia Faroldi G., Grujic M., Hartmann K., Roers A., Guss B., Lundequist A., Pejler G. (2014) Mast cells are activated by *Staphylococcus aureus in vitro* but do not influence the outcome of intraperioneal *Staphylococcus aureus* infection *in vivo*. *Immunology.*

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1 Abbreviations

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motif
bFGF	basic fibroblast growth factor
BMMC	Bone-Marrow derived Mast Cells
CCL	C-C motif ligand
CPA	Carboxypeptidase A
CLP	Caecal ligation and puncture
CR	Complement receptor
CRAMP	Cathelicidin- related antimicrobial peptide
CTMC	Connective tissue mast cell
CXCL	C-X-C motif ligand
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
GAG	Glycosaminoglycan
GM-CSF	granulocyte-macrophage colony-stimulating factor
i.d.	intradermally
i.p.	intraperitoneally
i.v.	intravenously
ICAM	Intracellular adhesion molecule
IE	Intronic enhancer
Ig	Immunoglobin
IL	Interleukin
LFA	Leukocyte function associated antigen
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccaride
LT	Leukotiene
Mcl-1	Myeloid cell leukemia sequence 1

MCP	Monocyte chemoattractant protein
MC_T	Human mast cells that contain tryptase
MC_{TC}	Human mast cells that contain tryptase and chymase
MIP	Macrophage inflammatory protein
MMC	Mucosal mast cell
mMCP	Mouse mast cell protease
MMP	Matrix metalloprotease
mTMT	Mouse transmembrane tryptase
NF-κβ	Nuclear factor-κβ
NFAT	Nuclear factor of activated T-cells
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PAR	Protease activated receptor
PCMC	Peritoneal cell derived mast cell
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PG	Proteoglycan
PGN	Peptidoglycan
PKC	Protein kinase C
SCF	Stem cell factor
TCR	T cell receptor
TGF	Transforming growth factor
T_H	T helper
TLR	Toll-Like Receptor
TNF	Tumor necrosis factor

2 Introduction

2.1 The immune system

In our everyday life we encounter many different pathogens (bacteria, viruses, fungi and parasites). It is the immune system's job to protect us from these pathogens. The immune system is divided into two components: the innate and the adaptive immune response. The innate immune system is often referred to as the first line of defense, which enables us to protect ourselves even on the first encounter with the pathogen. It includes anatomic barriers such as the skin, tears, saliva and mucosal surfaces as well as the complement system and various cells including mast cells, eosinophils, basophils and phagocytes (macrophages, neutrophils and dendritic cells). The adaptive immune system is specific toward one particular pathogen or antigen, which develops as a consequence of exposure to that particular pathogen. The leukocytes of the adaptive immune system are divided into B cells and T cells. The B cells participate in antibody-mediated responses and T cells in cell-mediated immune responses. Even though the innate and adaptive responses are often regarded as two separate components, cross-talk between them through cytokine secretion and cell to cell signaling is essential for a proper immune response (Crozat *et al.*, 2009).

2.2 Mast cells

Mast cells are highly granulated cells found throughout all vascularized tissues, often close to blood vessels. They are common at host-environment interfaces such as the skin and mucosal tissues. Because of their location, as well as being equipped with innate receptors that can react directly upon invading pathogens, they are known as a "first line of defense" cell. The mast cell granules are filled with mediators that can be released immediately upon activation. In

addition, mast cells can also produce new ("de novo") mediators upon activation (Metcalfe *et al.*, 1997).

Mast cells derive from pluripotent hematopoietic stem cells in the bone marrow. However, they do not go through their final stages of maturation until the precursors have entered the tissues (Kirshenbaum *et al.*, 1991). Mast cells are long-lived cells, which can regranulate after having degranulated (Xiang *et al.*, 2001).

2.3 Heterogeneity of mast cells

In rodents, mast cells are divided into connective tissue type mast cells (CTMCs) and mucosal mast cells (MMCs). As indicated by their names, they are located at different anatomical sites, but they also differ in their mast cell protease and proteoglycan (PG) content (Enerback, 1966) (Table 1). In humans, mast cells are classified depending on their mast cell protease content and are divided into MC_T, containing tryptase, and MC_{TC}, containing both tryptase and chymase (Irani & Schwartz, 1994) (Table 1).

Table 1. *Summary of the mast cell specific protease and proteoglycan content in humans and mice.*

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

2.4 Mast cell activation

Mast cells can rapidly respond to various stimuli by degranulating but they can also be activated to produce *de novo* mediators. Activation may occur by various mechanisms, including cross-linking of surface-associated immunoglobulin (Ig)E or IgG receptors, exposure to bacterial components and complement factors. This is described below.

2.4.1 IgE-dependent activation

The most studied way of mast cell activation, especially in the context of allergic reactions, is cross-linking of their high-affinity IgE receptors, FcεRI. When an antigen enters the body it may provoke production of IgE antibodies. The IgE antibodies bind tightly to the FcεRI on the mast cells and exposure to the antigen a second time will cross-link FcεRI on the mast cell surface. Cross-linking of the receptors leads to intracellular signaling events and consequent degranulation, as well as *de novo* synthesis of various mediators (Turner & Kinet, 1999). Mast cell FcεRI is a tetrameric protein complex (Blank *et al.*, 1989; Ra *et al.*, 1989), consisting of an IgE-binding α-subunit (Hakimi *et al.*, 1990), a signal-amplifying membrane-spanning β-subunit as well as a homodimeric disulphide-linked γ-subunit (Perez-Montfort *et al.*, 1983), which gives the receptor the ability to signal. (Jouvin *et al.*, 1994).

2.4.2 IgG-mediated activation

Mast cell activation can also be achieved via receptors for IgG (FcγRs). Human mast cells express the high-affinity receptor, FcγRI, and the low-affinity receptor, FcγRII. In contrast, murine mast cells express the low-affinity receptors, FcγRII and FcγRIII (Okayama *et al.*, 2000; Katz & Lobell, 1995). Mast cell degranulation can be triggered by stimulation of FcγRI and FcγRIII (Daeron *et al.*, 1995). However, cross-linking of FcγRII results in decreased signaling from activated IgE- and IgG-receptors, thereby inhibiting mast cell degranulation (Kepley *et al.*, 2000; Daeron & Vivier, 1999).

The Fc receptors bind to pathogen-specific antibodies that aid mast cells in pathogen recognition and elicitation of a proper immune response.

2.4.3 Toll-like receptors

Mast cells can recognize and be activated by pathogens through their toll-like receptors (TLRs). TLRs are activated by specific molecules from the pathogens, termed pathogen-associated molecular patterns (PAMPs). TLR-2, -3, -4, -6, -8 and -9 are expressed on mast cells (Matsushima *et al.*, 2004; Takeda *et al.*, 2003; Supajatura *et al.*, 2001). Different PAMPs stimulate different TLRs. For example, lipopolysaccaride (LPS), a gram-negative bacterial cell wall component, activates TLR-4 and viral dsRNA activates TLR-3. TLRs may also facilitate cooperative binding of ligands, and thereby exhibit a broad recognition spectrum. For example, TLR-2/6 recognizes peptidoglycan (PGN), a component of the gram-positive bacterial cell wall, whereas TLR-1/2, recognizes triacylated lipoproteins (Takeda *et al.*, 2003). Activation of mast cells via their TLRs is usually associated with cytokine, leukotriene and prostaglandin production without causing degranulation

(Marshall *et al.*, 2003). However, activation of TLR-2 has been shown to cause degranulation as well (Supajatura *et al.*, 2002). Mast cells may thus recruit other immune cells to the site of infection by responding to various bacterial, viral and fungal components through TLR signaling pathways.

2.4.4 Complement-mediated activation

The complement system is composed of serum proteins and cell surface receptors, which interact in a number of complex pathways and can be activated by invading pathogens or tissue damage. The complement system is associated with both the innate and adaptive immune responses (Sarma & Ward, 2011). Mast cells express complement receptor (CR)3, CR4, C3aR and C5aR (Marshall, 2004). In mice, only CTMCs, but not MMCs, express CRs, which are able to respond to C3a and C5a (Mousli *et al.*, 1994). Complement deficient mice are more sensitive to cecal ligation and puncture (CLP), a model for bacterial sepsis, and display reduced mast cell activation (Prodeus *et al.*, 1997). Human mast cells display varying expression levels of CRs depending on the surrounding milieu. Skin and cardiac mast cells express C5aR but mast cells from the lungs, uterus or tonsils do not (Fureder *et al.*, 1995). In addition to their function as mast cell activating agents, C3a and C5a have been shown to be chemotactic for mast cells (Nilsson *et al.*, 1996).

2.4.5 Other mechanisms of activation

In addition to the previously mentioned modes of mast cell activation, there are a number of other ways they can be activated, including activation by cytokines/chemokines, neuropeptides, calcium ionophores and drugs.

Many different cytokines can influence and activate mast cells, including interleukin (IL)-1, IL-3, IL-8, granulocyte-macrophage colony stimulating factor, macrophage inflammatory protein (MIP)-1, monocyte chemoattractant protein (MCP)-1 and stem cell factor (SCF) (Mekori & Metcalfe, 2000; Taylor *et al.*, 1995; Alam *et al.*, 1994; Subramanian & Bray, 1987). In addition, production of various cytokines can be induced by the lipid mediators prostaglandin E_2 (PGE_2) and leukotriene (LT)C_4 (Abdel-Majid & Marshall, 2004; Mellor *et al.*, 2002; Leal-Berumen *et al.*, 1995).

Mast cells are often situated close to nerve endings and can also be activated by neuropeptides such as substance P, calcitonin gene-related peptide, vasoactive intestinal peptide and neurotensin. Other small peptides, such as endothelin-1, expressed by endothelial cells, also activate mast cells (Bauer & Razin, 2000; Metcalfe *et al.*, 1997).

Mast cell degranulation can be mediated by elevating intracellular calcium levels. Therefore calcium mobilizing agents, such as calcium ionophores,

A23187 and ionomycin, have the ability to cause mast cell degranulation. Basic compounds, for example compound 48/80, can also directly activate mast cells and cause degranulation (Metcalfe *et al.*, 1997).

Furthermore, cell-cell contact can also cause mast cell activation. Interaction of mast cells with activated T cells via leukocyte function associated antigen (LFA)-1 and intracellular adhesion molecule (ICAM)-1, induces cytokine production and mediator release (Bhattacharyya *et al.*, 1998; Inamura *et al.*, 1998).

In addition to the TLRs, mast cells can also react to pathogens through CD48 that recognizes the FimH protein, which can be expressed, for example, by *Escherichia coli (E. coli*) (Malaviya *et al.*, 1999), as well as nucleotide-binding oligomerization domain (NOD) receptors, which are intracellular sensors of PAMPs (Enoksson *et al.*, 2011; Wu *et al.*, 2007). Other mast cell activators include the nucleoside adenosine and the opiates morphine and codine (Mekori & Metcalfe, 2000).

Individual stimuli of mast cells can elicit distinct, but sometimes overlapping patterns of mediator release. For example, IgE-mediated activation leads to degranulation, *de novo* synthesis and release of mediators, whereas LPS via stimulation of TLR-4, induces release of certain cytokines without degranulation.

2.5 Mast cell mediators

Mast cells have the potential to produce and release many mediators. Some of these are preformed and stored in granules; these include neutral proteases, PGs, biogenic amines and some cytokines. Other mediators, including lipid mediators and cytokines, are only synthesized and released after the mast cell has been activated.

2.5.1 Mast cell proteases

The granules of mast cells contain large amounts of proteases; up to 35% of the total protein content. The mast cell specific proteases include tryptases, chymases and carboxypeptidase A3 (CPA3), but mast cells also express other proteases including lysosomal cathepsins, granzymes, matrix metalloproteases (MMPs), and A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) (Garcia-Faroldi *et al.*, 2013; Pejler *et al.*, 2010; Pejler *et al.*, 2007).

Tryptase

Tryptases are serine proteases with trypsin-like cleavage specificity, i.e., they cleave after lysine/arginine residues, which are active in a tetrameric form with the active sites facing the central pore. The tetrameric structure of tryptase provides its selective substrate specificity, as it can only cleave relatively small substrates that can enter the central pore. The structure also protects tryptase against macromolecular inhibitors that cannot enter the pore. Tryptase is stored in an active form in complex with serglycin PG within the mast cell granules (Pejler *et al.*, 2007; Hallgren *et al.*, 2001; Schwartz & Bradford, 1986). Humans mainly express two types of tryptases: α-tryptases and β-tryptases. β-tryptases are further classified into βI-, βII- and βII-tryptases and the α-tryptases into αI- and αII-tryptases (Pallaoro *et al.*, 1999). β-tryptases are the main form found in mast cells and are the most catalytically active (Marquardt *et al.*, 2002; Huang *et al.*, 1999). α-tryptases can be found in the circulation in the absence of mast cell degranulation, which suggests that they are constitutively secreted (Schwartz *et al.*, 1995). In addition to the α- and β-tryptases, human tryptases also include δ-tryptase and the membrane anchored γ-tryptase (Hallgren & Pejler, 2006).

Mice have been found to express four tryptases: mouse mast cell protease (mMCP)-6, mMCP-7, mMCP-11 and mouse transmembrane tryptase (mTMT). mMCP-6 is the most similar to human β-tryptase, both in sequence homology and cleavage specificity (Pejler *et al.*, 2007). mMCP-11 and mTMT have both been found to be mainly expressed in the early stages of mast cell development (Wong *et al.*, 2004; Wong *et al.*, 1999).

Tryptase has been suggested to degrade a number of extracellular matrix (ECM) components, such as fibrinogen (Schwartz *et al.*, 1985), fibronectin (Lohi *et al.*, 1992), type VI collagen (Kielty *et al.*, 1993) and to activate pro-MMP-3 (Gruber *et al.*, 1989). Additionally, tryptase has been shown to cleave and thereby activate protease-activated receptor (PAR)-2, which may lead to inflammatory events, such as recruitment of eosinophils (Matos *et al.*, 2013; Berger *et al.*, 2001). Tryptase can also recruit neutrophils (Huang *et al.*, 1998) and a study in mMCP-6 knockout mice has also shown that mMCP-6 contributes to the defense against intraperitoneal *Klebsiella pneumoniae* (*K. pneumoniae*) infection by influencing early neutrophil recruitment (Thakurdas *et al.*, 2007). In addition to a role in bacterial infections, studies of mMCP-6 knockout mice have shown that mMCP-6 is involved in eosinophil recruitment to skeletal muscle infected by the parasite *Trichinella spiralis* (Shin *et al.*, 2008) and that mMCP-6 has a harmful role in arthritis (McNeil *et al.*, 2008).

Chymase

Chymases are serine proteases with chymotrypsin-like cleavage specificity, i.e., they preferentially cleave after aromatic amino acid residues. They are monomeric, and like tryptases, are stored fully active in the secretory granules in complex with serglycin PG (Braga *et al.*, 2007; Henningsson *et al.*, 2006; Abrink *et al.*, 2004; Henningsson *et al.*, 2002). Once released, they remain attached to the proteoglycan, which increases the activity of the enzyme and protects it from macromolecular inhibitors (Pejler & Sadler, 1999; Pejler & Berg, 1995). Chymases are divided into α- and β-chymases. Humans only express one α-chymase (Caughey *et al.*, 1991), whereas mice express several different chymases. In mice, MMCs express the β-chymases mMCP-1 and -2, and CTMCs express the β-chymase mMCP-4 and the α-chymase mMCP-5 (Huang *et al.*, 1991; Reynolds *et al.*, 1990). Phylogenetically, mMCP-5 is the closest homolog to the human chymase. However, mMCP-5 has elastase-like cleavage specificity and is therefore not functionally a chymase (Karlson *et al.*, 2003; Kunori *et al.*, 2002). mMCP-4 on the other hand, has a similar cleavage specificity and tissue distribution as the human chymase and is therefore considered to be the functional homolog to the human chymase in mice (Andersson *et al.*, 2008; Pejler *et al.*, 2007).

Studies have revealed that chymase is involved in processing of a wide array of proteins and peptides. Chymase can cleave angiotensin I yielding angiotensin II, a peptide that causes vasoconstriction (Urata *et al.*, 1990; Reilly *et al.*, 1982). Chymase has been shown to be involved in ECM remodeling via cleavage of the ECM component fibronectin (Tchougounova *et al.*, 2003; Tchougounova *et al.*, 2001; Vartio *et al.*, 1981), as well as activation of MMPs and pro-collagenases (Tchougounova *et al.*, 2005). A number of different cytokines have been shown to be substrates for chymase, including IL-1β (Mizutani *et al.*, 1991), membrane-bound SCF (de Paulis *et al.*, 1999), tumor necrosis factor (TNF) (Piliponsky *et al.*, 2012) and IL-33 (Waern *et al.*, 2013).

To date, three chymase knockout mice have been generated: mMCP-1, mMCP-4 and mMCP-5. mMCP-1 is expressed in MMCs, primarily in the intestine and the mMCP-1 knockout mice have been shown to have delayed parasite expulsion in the intestine (Knight *et al.*, 2000). The mMCP-5 knockouts are, in addition to lackingmMCP-5, also lacking CPA, which makes it difficult to interpret data from these mice. Since mMCP-4 is functionally the closest homolog to human chymase, this knockout is perhaps the most interesting to study (Pejler *et al.*, 2010). These mice have been used in many mouse models to evaluate the *in vivo* functions of mMCP-4. In a model of sepsis, CLP, mMCP-4 has been shown to enhance survival, in part by degrading and limiting the harmful effects of TNF (Piliponsky *et al.*, 2012).

mMCP-4 has also been shown to have a detrimental role in models of arthritis (Magnusson *et al.*, 2009), abdominal aortic aneurism (Sun *et al.*, 2009) and bullous pemphigoid (Lin *et al.*, 2011), yet a protective role in models of allergic airway inflammation (Waern *et al.*, 2013; Waern *et al.*, 2009). In addition, mMCP-4 has been shown to reduce the toxicity from a number of venoms, including from the gila monster and scorpion (Akahoshi *et al.*, 2011).

Carboxypeptidase A3

CPA3 is a Zn-dependent metalloprotease that, similarly to the other mast cell proteases, is stored in an active form in complex with serglycin PG in the secretory granules. It is an exopeptidase that has a preference for cleaving C-terminal aromatic and aliphatic residues (Pejler *et al.*, 2007; Goldstein *et al.*, 1989). Both CPA3 and chymase have been suggested to remain in complex with serglycin PG after degranulation and, interestingly that cleavage of substrates by chymase can generate C-terminal ends that are substrates for CPA3 (Pejler *et al.*, 2007). A CPA3 knockout mouse has been generated, but in analogy with the mMCP-5 knockout that also lacks CPA3, the CPA3 knockout lacks mMCP-5. To circumvent this problem, a $CPA3^{inact}$ mouse has been generated that retains the CPA3 protein but where the active site of CPA3 has been mutated, leading to enzymatically inactive CPA3. In these mice, the mMCP-5 protein is unaffected. CPA3 has been shown to be important for degrading certain toxic peptides, including endothelin-1 and certain snake venoms (Metz *et al.*, 2006; Metsarinne *et al.*, 2002).

Other proteases expressed by mast cells

In addition to the mast cell-specific proteases, mast cells also express many other proteases that are not exclusive to mast cells.

Both human and murine mast cells have been shown to express granzyme B, which is primarily expressed by cytotoxic T cells (Pardo *et al.*, 2007; Strik *et al.*, 2007). Granzyme B derived from mast cells can cause cell death in susceptible adherent cells *in vitro* (Pardo *et al.*, 2007).

Cathepsin G is a serine protease mainly expressed by neutrophils but also expressed by mast cells. It has broad cleavage properties with both chymotrypic and tryptic activities, i.e., cleaving after both aromatic and basic amino acids (Polanowska *et al.*, 1998; Schechter *et al.*, 1990).

MMPs are a family of Zn^{2+}- and Ca^{2+}-dependent metalloproteases, mainly involved in ECM remodeling. Human mast cells express MMP-1 (Di Girolamo & Wakefield, 2000) and MMP-9 (Kanbe *et al.*, 1999c), and mouse mast cells express MMP-9 (Tanaka *et al.*, 1999).

Mast cells also express ADAMTS-5, -6 and -9. The ADAMTSs are metalloproteases, which have been shown to possess aggrecanase activity (Garcia-Faroldi *et al.*, 2013).

2.5.2 Proteoglycans

PGs are essential components of the mast cell secretory granules. PGs are glycoproteins that consist of a protein core with glycosaminoglycan (GAG) side chains. GAGs are polysaccharides that consist of repeating disaccharide units, which are highly anionic due to the presence of sulfate and carboxyl groups. There are several types of PGs, based on the protein core and the GAG chains. However, the dominant protein core expressed by mast cells is of serglycin type, while the side chains differ in different mast cell subtypes. In murine CTMCs, the PGs have heparin side chains (Kolset & Gallagher, 1990) and in MMCs, the side chains are of chondroitin sulfate type (Enerback *et al.*, 1985). In contrast, human mast cells contain both heparin and chondroitin sulfate, regardless of subtype (Pejler *et al.*, 2009). Because of the highly negatively charged nature of serglycin PG it can interact with a number of positively charged molecules, including many of the mast cell proteases in the secretory granules. Indeed, serglycin knockout mast cells show defective storage of a number of different proteases including mMCP-4, mMCP-5, mMCP-6 and CPA3, as well as the biogenic amines histamine, serotonin and dopamine (Ronnberg *et al.*, 2012a; Ringvall *et al.*, 2008; Braga *et al.*, 2007; Abrink *et al.*, 2004).

Upon mast cell degranulation, serglycin PG in complex with different compounds is released. Because of the change in pH outside the granules, some of the compounds will be released from serglycin, such as histamine, whereas others will remain attached, such as chymase and CPA3. Outside the granules, serglycin functions to protect the attached proteases from macromolecular inhibitor, as well as increasing the activity of chymase, and potentially attracting substrates to the proteases (Ronnberg *et al.*, 2012b).

2.5.3 Biogenic amines

Mast cells are one of the main sources of histamine, although many other cell types produce it as well. Histamine has been widely studied and is associated with many physiological and pathological conditions. For example, histamine mediates inflammation, increases vascular permeability, acts on smooth muscle cells, stimulates gastric acid secretion and is a neurotransmitter in the central nervous system (Bachert, 2002). Histamine exerts its effects through histamine receptors H1, H2, H3 and H4 (Haaksma *et al.*, 1990; Hill, 1990). In addition to

producing histamine, mast cells also express the H1, H2 and H4 receptors (Lippert *et al.*, 2004).

Mast cells also produce the biogenic amines serotonin and dopamine, both of which are mostly known as neurotransmitters (Kushnir-Sukhov *et al.*, 2007; Freeman *et al.*, 2001).

2.5.4 Lipid mediators

Activation of mast cells can initiate *de novo* synthesis of certain lipid mediators, prostaglandins and LTs, both members of the eicosanoid family. Mast cells have been shown to produce prostaglandin D_2 (PGD_2), LTB_4 and LTC_4 (Boyce, 2007). They are all derived from arachidonic acid, then released and actively transported from the cell (Funk, 2001). Once outside the cell, LTC_4 is converted to LTD_4 and subsequently LTE_4 (Boyce, 2007). LTs mediate their biological effects via G-protein coupled receptors. They act as chemoattractants for neutrophils, macrophages, eosinophils, monocytes and mast cell progenitors. In addition, their physiological effects include bronchoconstriction, induction of cytokine production, vascular leakage, endothelial activation and tissue repair (Weller *et al.*, 2005; Beller *et al.*, 2004; Kanaoka & Boyce, 2004; Machida *et al.*, 2004; Mellor *et al.*, 2002; Peters-Golden *et al.*, 2002; Laitinen *et al.*, 1993; Dahlen *et al.*, 1980). Similarly to the LTs, PGD_2 exerts its actions though G-protein coupled receptors. PGD_2 is also a chemoattractant for a number of leukocytes (neutrophils, basophils and T helper 2 (T_H2) cells) and it also mediates bronchoconstriction (Honda *et al.*, 2003; Fujitani *et al.*, 2002; Johnston *et al.*, 1992).

2.5.5 Cytokines and chemokines

Cytokines are molecules that are involved in cell signaling, with both systemic and local immunomodulatory effects. Chemokines are a family of cytokines named after their chemotactic properties. Cytokines and chemokines have a broad spectrum of functions, playing a major role in inflammation, infection, cell repair and growth. The list of cytokines and chemokines that mast cells can express is very long, including IL-1β, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IL-33, basic fibroblast growth factor-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, transforming growth factor (TGF)-β1, MCP-1 and RANTES (Ono *et al.*, 2003; Lindstedt *et al.*, 2001; Ishizuka *et al.*, 1999; Kanbe *et al.*, 1999a; Kanbe *et al.*, 1999b; Kasahara *et al.*, 1998; Qu *et al.*, 1998; Zhang *et al.*, 1998; Gibbs *et al.*, 1997; Okayama *et al.*, 1995; Razin *et al.*, 1984). The list includes both classical pro-inflammatory cytokines, such as TNF-α and IL-1β, and cytokines that are associated with anti-inflammatory or immunomodulatory effects, such as IL-10 and TGF-β.

The majority of the secreted cytokines are newly synthesized upon mast cell activation. However, mast cells can also pre-store certain cytokines in their granules, which was initially shown for TNF-α (Walsh *et al.*, 1991; Young *et al.*, 1987).

2.6 Mast cell-deficient mice

2.6.1 Kit-dependent mast cell-deficient mice

Most of the *in vivo* mast cell data is based on studies of mast cell-deficient Kit mutant mice. Kit is the tyrosine receptor for SCF, which is the main growth factor for mast cells *in vivo*, but SCF also has many other functions. Kit is expressed both inside and outside of the hematopoietic lineage, in cell types of diverse developmental origin. In the immune system, Kit is an important hematopoietic stem and progenitor cell marker. In most lineages Kit expression is lost with differentiation, the exception to this being mast cells, which mainatin a high Kit expression throughout their development. Kit also has many functions outside the immune system: it is important in germ cells, melanocytes (Besmer *et al.*, 1993), intestinal pacemaker cells (Sergeant *et al.*, 2002), neuronal cells (Milenkovic *et al.*, 2007) and in liver metabolism (Magnol *et al.*, 2007).

The Kit mutant mice exist with variable phenotypes, depending on naturally occurring alleles, the most relevant for mast cell studies being Kit^{W}, $Kit^{W\text{-}v}$ and $Kit^{W\text{-}sh}$. The majority of the mast cell literature is based on the mutant $Kit^{W/W\text{-}v}$ mouse. The Kit^{w} protein cannot be expressed on the cell surface and the $Kit^{W\text{-}v}$ protein has impaired kinase activity (Nocka *et al.*, 1990). By breeding these two mice, mast cell-deficient $Kit^{W/W\text{-}v}$ mice are produced. The $Kit^{W/W\text{-}v}$ mice are severely affected by their Kit deficiency. They are sterile, have severe macrolytic anemia (Waskow *et al.*, 2004), impaired T-cell development in the thymus (Waskow *et al.*, 2002), have a shift in intraepithelial T cells in the gut in favor of T cell receptor (TCR) $\alpha\beta^{+}$, instead of $\gamma\delta^{+}$ cells (Puddington *et al.*, 1994), and they have reduced numbers of neutrophils (Zhou *et al.*, 2007) and basophils (Feyerabend *et al.*, 2011; Mancardi *et al.*, 2011). $Kit^{W\text{-}sh}$ mice have a large genomic inversion that affects the transcriptional regulatory elements upstream of the Kit gene (Berrozpe *et al.*, 1999). This genomic rearrangement does not only affect the expression of Kit itself but can potentially lead to the dysregulation of 27 other genes (Nigrovic *et al.*, 2008). The $Kit^{W\text{-}sh}$ mice have been used more recently since they have fewer abnormalities than the $Kit^{W/W\text{-}v}$ mice; they are fertile and have normal red blood cell numbers (Grimbaldeston *et al.*, 2005). However, they do have neutrophilia, megakaryocytosis,

thrombocytosis and are associated with splenomegaly and histological aberrations of the spleen (Nigrovic *et al.*, 2008).

A way to prove that the phenotype seen in these mice is due to their mast cell deficiency has been to reconstitute them with mast cells derived either from bone marrow, bone marrow derived mast cells (BMMCs), or embryonic stem cells. The *in vitro* derived mast cells can be administered intravenously (i.v.), intraperitoneally (i.p.) or intradermally (i.d.) to create a so-called mast cell knock-in mouse (Figure 1) (Kawakami, 2009; Nakano *et al.*, 1985). However, depending on the route of administration and the number of mast cells injected, the final numbers and anatomical distribution of the mast cells in reconstituted Kit mutant mice may not reflect the native mast cell population in wild type mice. For example, after i.v. injection of BMMCs, few or no mast cells are detectable in the trachea, whereas the number of mast cells in the periphery of the lungs is greater than in wild-type mice (Grimbaldeston *et al.*, 2005). However, depending on the administration, the mast cell population in certain anatomical sites is similar in number to wild-type, for example i.p. injection leads to similar numbers of mast cells in the peritoneum (Grimbaldeston *et al.*, 2005). Studies have shown that, with time, the reconstituted mast cells will closely resemble the native population (Otsu *et al.*, 1987; Nakano *et al.*, 1985). However, relatively few studies of this type have been done. Additionally, it is not possible to define every aspect of the mast cells *in vivo*, that is, it cannot be ruled out that adoptively transferred cells might have phenotypic differences to native mast cells.

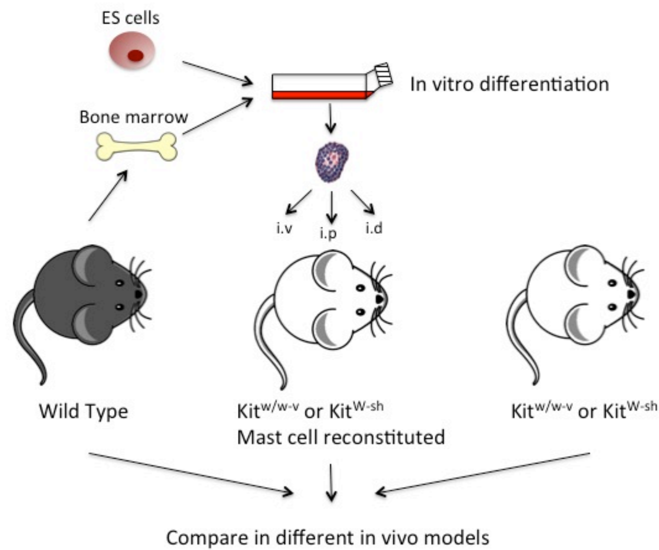

Figure 1. Mast cells derived from wild-type embryonic stem (ES) cells or bone marrow can be reconstituted into the mast cell-deficient mice (intravenously, i.v., intraperitoneally, i.p., or intradermally, i.d.). By comparing the 3 different groups of mice, wild-type, mast cell-deficient and reconstituted mast cell-deficient mice, conclusions can be made about the roles of mast cells in different *in vivo* models.

2.6.2 Kit-independent mast cell-deficient mice

Recently, several Kit-independent mast cell-deficient mice have been developed. A common approach has been to generate mice that express Cre-recombinase under a promoter thought to be mast cell specific or at least mast cell associated. Cre-recombinase is a site-specific recombinase that catalyses a recombination event between two specific DNA fragments, loxP sites. DNA fragments found between two loxP sites are said to be "floxed". The fate of this fragment, after Cre-mediated recombination, depends on the orientation of the loxP sites: if they are oriented in the same direction, the fragment will be excised, and if they are in opposite direction, it will be inverted (Brault *et al.*, 2007).

Mcpt5-Cre; R-DTA mice

These mice express Cre-recombinase under the control of the Mcpt5 (the gene for mMCP-5) promoter, a mast cell specific protease expressed in CTMCs.

They also have the gene for diphtheria toxin (DT) with a loxP floxed stop codon. Therefore, the DT is not expressed unless the floxed stop codon is removed by Cre-recombinase. When the stop codon is removed (i.e., in CTMCs), DT is expressed and the cell dies. These mice are basically devoid of mast cells in the peritoneum and only have 10% residual mast cells in the skin. Since mMCP-5 is a CTMC protease the MMCs are not affected. They have no other detected abnormalities (Dudeck *et al.*, 2011).

Cre-Master mice

Cre-Master stands for Cre-mediated mast cell eradication. In these mice Cre-recombinase is knocked-in under the control of the *Cpa3* (the gene for CPA3) promoter, while deleting 28 nucleotides of the first exon of *Cpa3*. Unexpectedly, the $Cpa3^{Cre/+}$ mice were virtually completely lacking mast cells. This depletion of mast cells appeared to be mediated by Cre-induced genotoxicity. When Cre-recombinase expression is very strong or long lasting, it can become promiscuous and toxic, independently of the presence of loxP sites. However, CPA3 is not only highly expressed in mast cells; it is also expressed in basophils and in some T-cell populations. Consistent with this, Cre activity can be seen in T cells in the Cre-Master mice and they have a 60 % reduction in spleen basophils (Feyerabend *et al.*, 2011).

Cpa3-Cre; Mcl-1$^{fl/fl}$- "Hello Kitty" mice

These mice express Cre-recombinase under the control of a promoter fragment of *Cpa3* and the floxed gene of the anti-apoptotic factor, myeloid cell leukemia sequence 1 (Mcl-1). They have a reduction in numbers of mast cells (92-100%) but also show a reduction of basophils (58-78 %). These mice also showed a 56% increase in splenic neutrophils and suffered macrolytic anemia (Lilla *et al.*, 2011).

2.6.3 Inducible depletion of mast cells

In certain studies it can be interesting to induce a selective depletion of mast cells. One way to achieve this is by injection of DT into transgenic mice bearing the DT receptor (DTR) only on the particular cell type you want to deplete. Two different groups recently used this approach to deplete mast cells in adult mice.

Mcpt5-Cre; iDTR mice

Mcpt5-Cre mice were mated with $iDTR^{fl/fl}$ mice expressing a floxed simian DTR transgene in order to achieve Cre-dependent expression of DTR in mast

cells. Repeated injections of DT, once a week for 4 weeks, led to a complete ablation of mast cells in the peritoneal cavity and the skin, when assessed 1 week after the last DT injection. The injections did not, however, affect MMCs, reflecting that Mcpt5 is not expressed in MMCs (Dudeck *et al.*, 2011).

Mas-TRECK mice

Mas-TRECK stands for Mast cell-specific enhancer-mediated Toxin Receptor-mediated Conditional cell Knock out. These mice express the human DTR gene under the control of an intronic enhancer (IE) element of the *il-4* gene. This IE element has been shown to be mast cell specific. Repeated injections with DT lead to a complete loss of mast cells in the skin, peritoneal cavity, stomach and mesenteric windows, 3 days after the last DT treatment. Skin mast cells were also absent 12 days after DT treatment. However, DT treatment also transiently depleted blood basophils, while other major types of leukocytes were unaffected (Sawaguchi *et al.*, 2012; Otsuka *et al.*, 2011).

A summary of the new Kit-independent mast cell-deficient mice can be seen in Table 2. Some of the mast cell related results from Kit-deficient mice have been confirmed in the new Kit-independent mice, including their roles in allergic airway hyperresponsiveness (Sawaguchi *et al.*, 2012). However, there are also some conflicting data between the Kit-dependent and -independent mast cell-deficient mice, including the role of mast cell derived IL-10 in contact hypersensitivity (Dudeck *et al.*, 2011) as well as the proposed role of mast cells in autoimmunity (Feyerabend *et al.*, 2011). This has lead to a need to also re-evaluate other proposed roles of mast cells derived from experiments using the Kit-deficient mice (Reber *et al.*, 2012; Rodewald & Feyerabend, 2012).

Table 2. *A summary of Kit-independent mast cell-deficient mice.*

	Mcpt5-Cre		Mas-TRECK	Cre-Master	Cpa3-Cre
Reference	(Dudeck *et al.*, 2011)		(Sawaguchi *et al.*, 2012; Otsuka *et al.*, 2011)	(Feyerabend *et al.*, 2011)	(Lilla *et al.*, 2011)
Construct	Cre under the control of the Mcpt5 promoter		Human DTR under the control of an intronic enhancer of the Il4 gene	Cre expression under the control of the Cpa3 promoter	Cre under the control of a Cpa3 promoter fragment
Additional loci	R-DTA floxed	Inducible DTR floxed			Mcl-1 floxed
MC numbers	Up to 97% of CTMCs; MMCs are not depleted		Deficient for CTMCs and	Deficient for CTMCs and	92-100% including

		MMCs	MMCs	CTMCs and MMCs
Basophils	Not affected	Depleted	Reduced to 38%	Reduced to 22-42%
Other alterations	Not detected	Not detected	Cre activity in T cells	Macrolytic anemia, neutrophilia

2.7 Mast cells in bacterial infections

Mast cells are common at sites exposed to the external environment, such as the skin, the airways and the intestine. In these locations, they are well placed to function in host defense against invading pathogens.

Many of the modes of mast cell activation discussed earlier can come into play during bacterial infections. Bacteria can activate mast cells both directly and indirectly. Direct detection of bacteria can be achieved through TLRs, NODs and CD48. Indirectly, mast cells can be activated by complement and through their FcRs, by the action of pathogen-specific antibodies. Bacterial superantigens can also activate mast cells through their FcRs, such as *S. aureus* derived protein A and *Peptostreptococcus magnus* derived protein L (Genovese *et al.*, 2000). Mast cells can respond with widely differing cytokine and mediator profiles depending on the type of pathogen-associated signal they encounter. For example, rodent mast cells produce TNF-α, IL-1β, IL-6, GM-CSF and several chemokines in response to LPS but they do not degranulate. *In vivo,* the mast cell response following LPS injection leads tot the recruitment of neutrophils (Supajatura *et al.*, 2002; Leal-Berumen *et al.*, 1994). On the other hand, PGN leads to production of a different range of cytokines, including IL-4, IL-5, IL-6 and GM-CSF, as well as induction of degranulation, which results in the development of local edema and increased vascular permeability *in vivo* (Supajatura *et al.*, 2002). However, in another study, PGN, which binds TLR-2/6 heterodimers, was shown to induce proinflammatory cytokines and cysteinyl LTs with little or no degranulation. In contrast, tri-palmitoylated lipopeptide, which binds to TLR-1/2 heterodimers, induced considerable degranulation and production of IL-1β and GM-CSF without the generation of LTs (Marshall *et al.*, 2003; McCurdy *et al.*, 2003).

Mast cells can also produce compounds that have direct bactericidal activity, including several antimicrobial peptides, such as the human cathelicidin, LL37 or the mouse cathelicidin-related antimicrobial peptide (CRAMP). Mast cells deficient in CRAMP have a reduced ability to kill group A streptococci (Di Nardo *et al.*, 2003). These compounds are both

constitutively produced by mast cells as well as induced in response to various stimuli such as LPS (Di Nardo *et al.*, 2003).

2.7.1 Enhancement of effector cell recruitment

Possibly the most important consequence of mast cell activation, in the context of bacterial infection, is the release of mediators that aid in the rapid recruitment of effector cells. Mast cells provide signals that can enhance each of the crucial stages of recruitment from the vasculature. IL-1, TNF, vascular endothelial growth factor, histamine and proteases can increase the permeability of the endothelial cell barrier as well as upregulate the expression and affinity of adhesion molecules. In addition, mast cells can produce chemoattractant compounds, such as C-X-C motif ligand (CXCL)8, CXCL11 and LTs. Finally, mast cells also produce factors that can aid in the long-term survival of recruited cells, such as IL-1 and GM-CSF (Marshall, 2004). *In vivo*, the recruitment of neutrophils depends on mast cell-derived TNF, which increases adhesion molecule expression on the endothelium, and on the potent neutrophil chemoattractant LTB_4 (Malaviya & Abraham, 2000). Mast cell-derived tryptases have also been shown to be important for mobilization of neutrophils. When human tryptase is administered intratracheally, it induces more than a 100-fold increase in local neutrophil numbers and substantially decreases the susceptibility to airway infection by *K. pneumoniae* (Huang et al., 2001). In addition, mMCP-6 injected i.p. induces a substantial CXCL8-independent recruitment of neutrophils that is dependent on the protease function (Huang *et al.*, 1998). mMCP-6 knockout mice show early impaired neutrophil recruitment and decreased survival in response to intraperitoneal *K. pneumoniae* infection (Thakurdas *et al.*, 2007). The mechanism by which tryptases induce neutrophil recruitment might involve the activation of PAR2, which is known to have an important role in leukocyte recruitment (Lindner *et al.*, 2000; Vergnolle, 1999). In addition, mast cell chymase has been shown to recruit monocytes and neutrophils *in vitro* (Tani *et al.*, 2000).

2.7.2 Influence on adaptive immunity

After stimulation, mast cells produce many factors that can influence the adaptive immune response. Mast cell-derived TNF induces upregulation of E-selectin expression on the endothelium, promoting the influx of dendritic cells, which are subsequently increased in the draining lymph nodes (Shelburne *et al.*, 2009). C-C motif ligand (CCL)20 from mast cells can also contributes to the recruitment of dendritic cells (Lin *et al.*, 2003). Additionally, mast cells can activate Langerhans cells, a skin resident dendritic cell, which leads to increased numbers of Langerhans cells in the draining lymph nodes, in

response to PGN and gram-negative bacteria (Shelburne *et al.*, 2009; Jawdat *et al.*, 2006). Histamine has been suggested to promote antigen uptake and cross-presentation (Amaral *et al.*, 2007) as well as upregulate co-stimulatory molecules on dendritic cells required for T-cell activation (Caron *et al.*, 2001).

In addition to influencing cell trafficking to the lymph nodes, mast cell TNF is also crucial for retention of lymphocytes in the lymph nodes 24 hours after *E. coli* infection (McLachlan *et al.*, 2003). This increases the probability of antigen-specific lymphocytes being present in the lymph nodes, and together with the ability of mast cells to mobilize dendritic cells, should increase the magnitude and specificity of the adaptive immune response. In a related study, wild-type mice were shown to have enhanced humoral immunity to *E. coli* compared to mast cell-deficient mice, including increased *E. coli*-specific antibodies and protection after passive immunization (Shelburne *et al.*, 2009).

There is also evidence that mast cells themselves can present antigen to T cells. Activated mast cells upregulate expression of MHC II and co-stimulatory molecules and they have been visualized physically interacting with T cells *in vivo* (Metcalfe *et al.*, 1997). However, the *in vivo* the role of mast cells in antigen presentation during bacterial infections has not been studied. Conversely, T cells can modulate mast cells through the production of chemokines such as CCL3 (also known as MIP1α) and CCL2, as well as through physical contact between each other (Mekori & Metcalfe, 1999). This suggests that feedback regulation exists, where the adaptive immune system can modulate mast cell function during an ongoing infection.

2.7.3 Mast cells in bacterial infections *in vivo*

The first *in vivo* evidence that mast cells were important during bacterial infections came from two studies in $Kit^{W/W-v}$ mice, where mast cells were shown to be protective in bacterial infection following either CLP (where the intestine of the mouse is punctured, releasing the cecal microflora into the peritoneum, and then ligated) or i.p. injection of live fimbriated *E. coli* and *K. pneumoniae*. Reconstitution of mast cells in the peritoneum restored the ability of the mice to survive the infections and efficiently clear the bacteria. Since then, mast cells have been shown to be protective in a number of different bacterial infections, including *Mycoplasma pneumonia* and *Citrobacter rodentium* (summarized in Table 3). However, mast cells and mast cell-derived TNF have also been reported to exacerbate mortality in severe CLP. This suggests that there is a delicate balance where the extent of the bacterial infection seems to determine the outcome of the mast cell response (Piliponsky *et al.*, 2010). mMCP-4 has also been shown to be protective in CLP, at least in

part, by degrading TNF and thereby limiting the detrimental effects of TNF (Piliponsky *et al.*, 2012).

Table 3. *A summary of in vivo studies looking at the role of mast cells in bacterial infections.*

Bacteria	Mice	Comments	Reference
Cecal microflora	$Kit^{W/W-v}$	Decreased survival in $Kit^{W/W-v}$ mice, a role for mast cell-derived TNF	(Echtenacher *et al.*, 1996)
	$Kit^{W/W-v}$	Reconstitution of wild-type but not IL-12-deficient mast cells corrects the defect	(Nakano *et al.*, 2007)
	Kit^{W-sh}	Mast cell-derived TNF can exacerbate mortality in severe CLP.	(Piliponsky *et al.*, 2010)
	$Kit^{W/W-v}$	The protective role of the mast cells is mediated by TLR-4.	(Supajatura *et al.*, 2001)
	$IL\text{-}15^{-/-}$	IL-15 inhibits chymase activity and thereby constrains mast cell-dependent antibacterial defenses	(Orinska *et al.*, 2007)
	Wild-type	Repeated injections of SCF increases the numbers of mast cells and these mice show enhanced survival	(Maurer *et al.*, 1998)
	$mMCP\text{-}4^{-/-}$	mMCP-4 promotes survival by degrading TNF and limiting the detrimental effects of TNF.	(Piliponsky *et al.*, 2012)
Escherichia coli	$Kit^{W/W-v}$	Decreased survival of mast cell-deficient mice, TNF important for the response. The FimH protein on the bacteria is important for the mast cell response.	(Malaviya *et al.*, 1996)
	$Kit^{W/W-v}$	Mast cell LTs have a role in neutrophil recruitment and bacterial clearance	(Malaviya & Abraham, 2000)
	$Kit^{W/W-v}$	A protective role of mast cells in urinary tract infection.	(Malaviya *et al.*, 2004)
	Kit^{W-sh}	Mast cell-derived TNF increases E-selectin expression and dendritic cell recruitment to the infected skin. The serum antibody response is diminished and less protective in a urinary tract infection.	(Shelburne *et al.*, 2009)
	$Kit^{W/W-v}$	Mast cell-derived TNF induces hypertrophy of the draining lymph nodes.	(McLachlan *et al.*, 2003)
Klebsiella pneumoniae	$Kit^{W/W-v}$	Decreased survival of mast cell-deficient mice.	(Malaviya *et al.*, 1996)
	$IL\text{-}6^{-/-}$ and Kit^{W-sh}	Mast cell IL-6 improves survival by enhancing neutrophil killing.	(Sutherland *et al.*, 2008)
	$mMCP\text{-}6^{-/-}$	Impaired clearance of the bacteria and impaired early recruitment of neutrophils.	(Thakurdas *et al.*, 2007)

Mycoplasma pneumonia	$Kit^{W\text{-}sh}$	Impaired clearance of the bacteria associated with increased lung pathology.	(Xu *et al.*, 2006)
Citrobacter rodentium	$Kit^{W/W\text{-}v}$	Decreased survival, increased histopathology in the gut and increased bacterial spread.	(Wei *et al.*, 2005)
Pseudomonas aeruginosa	$Kit^{W/W\text{-}v}$	Larger skin lesions in mast cell deficient-mice and impaired neutrophil recruitment. Wild-type mice show pronounced mast cell degranulation.	(Siebenhaar *et al.*, 2007)
Listeria monocytogenes	$Kit^{W/W\text{-}v}$ and α2-intergrin$^{-/-}$	Recruitment of neutrophils to the site of peritoneal infection dependent on mast cell $\alpha_2\beta_1$-intergrins.	(Edelson *et al.*, 2004)
Streptococcus pyogenes	$Kit^{W/W\text{-}v}$	Increased progressive tissue necrosis by the subcutaneous infection.	(Matsui *et al.*, 2011)
Helicobacter Pylori	$Kit^{W/W\text{-}v}$	Mast cells are critical mediators of vaccine-induced Helicobacter clearance.	(Velin *et al.*, 2005)
Francisella tularensis	$Kit^{W/W\text{-}v}$ and IL-4$^{-/-}$	Mast cells increase in the lung early (48 h) after infection and mast cell-deficient mice show decreased survival and bacterial clearance. *In vitro* studies showed mast cell inhibition of bacterial replication in macrophages by contact dependent events and secreted IL-4.	(Ketavarapu *et al.*, 2008)

3 Present investigations

3.1 Aims of the present studies

The general aim has been to investigate the role of mast cells during bacterial infections. Specific attention was paid to a novel mast cell protease, granzyme D.

- Investigate the global effect of live streptococci on mast cells *in vitro*. (**paper I**).

- Investigate the expression and regulation of the novel mast cell protease granzyme D (**paper II**)

- Investigate the global effect of live *S. aureus* on mast cells *in vitro* and study the role of mast cells in *S. aureus* infections *in vivo* (**paper III**)

3.2 Results and Discussion

3.2.1 Paper I: Infection of mast cells with live streptococci causes a toll-like receptor 2- and cell-cell contact-dependent cytokine and chemokine response.

Mast cells have been implicated in immunity toward bacterial infections but the mechanisms by which mast cells contribute to the host defense are not fully understood. Several studies on the effects of bacterial components, such as LPS and PGN on mast cells have been done. However, very few studies have looked at the effect of live bacteria on mast cells. Therefore, the aim of the study (paper I) was to investigate the global effect of live streptococci on mast cells by using a number of approaches including unbiased strategies.

We chose to use a gram-positive pathogen *S. equi* subspecies equi, a serological group C streptococcus that causes a severe upper respiratory tract infection in horses known as strangles (Timoney, 2004). *S. equi* can also infect mice. The bacteria were co-cultured with BMMCs, which were derived from mouse bone marrow by culturing them with WEHI-conditioned medium containing IL-3.

To examine if co-culture with *S. equi* caused mast cell degranulation we measured histamine release from the cells. As a control, calcium ionophore was used, which caused a rapid and robust secretion of histamine. *S. equi* also caused histamine release but the response was much slower and lower compared to calcium ionophore. The cells were also examined with transmission electron microscopy and also here it was evident that no major degranulation had taken place. However, the cells had a dilated rough endoplasmic reticulum, indicative of elevated transcriptional activity. Furthermore, there were no signs of phagocytosis of bacteria by the mast cells.

As an unbiased approach to look at cytokines produced by the mast cells in response to *S. equi* we used an antibody-based cytokine/chemokine array system. From this array it was evident that mast cells secrete multiple cytokine/chemokines when stimulated with *S. equi*, in particular IL-6, MCP-1, IL-13, TNF-α and IL-4. To examine whether this response was dependent on the bacteria being alive, BMMCs were stimulated with heat-inactivated *S. equi*. However, this only caused a minimal release of cytokines/chemokines as deduced by the array approach. To verify and quantify the cytokine/chemokine array results we used specific enzyme-linked immunosorbent assay (ELISA)s, where the secretion of high levels of IL-6, MCP-1, IL-13 and TNF-α in response to live *S. equi* were detected.

Next, we investigated whether the mast cells and the bacteria needed to be in cell-cell contact for the mast cells to be activated. We did this by using transwell polystyrene plates, where the mast cells and bacteria were separated with a membrane with 0.4 μm pores. In this case only small amounts of cytokines (IL-6, MCP-1, IL-13 and TNF-α) were produced in response to *S. equi* indicating that the mast cells and bacteria needed to be in cell-cell contact for optimal activation. To further investigate this, we looked into which pattern recognition receptors were responsible for this activation. We chose to focus on TLR-2 and TLR-4, which have previously been shown to be expressed by mast cells. Using BMMCs derived from TLR-2$^{-/-}$ and TLR-4$^{-/-}$ mice, we could observed that the secretion of cytokines was markedly reduced in TLR-2$^{-/-}$ BMMCs compared to wild-type controls. There was also a reduction in TLR-4$^{-/-}$ BMMCs. However, this reduction was not as pronounced as in the TLR-2$^{-/-}$ BMMCs. These results are consistent with the fact that *S. equi* is a gram-

positive bacterium that contains PGN, which is recognized by TLR-2. However, the secretion of cytokines from the TLR-$2^{-/-}$ BMMCs were higher than in the non-infected controls, indicating that other receptors are also involved in the activation.

To study the effects of *S. equi* co-culture on the BMMCs at the mRNA level, we used an Affymetrix gene chip microarray. Using this approach, we could confirm the upregulation of the cytokines that were shown to be induced at the protein level (using the cytokine array). In addition, several other cytokines/chemokines were also upregulated at the mRNA level, including IL-3, IL-2, CCL7 (MCP-3), CCL4 (MIP-1β), CCL1 (I-309), CXCL-2 (MIP-2) and CCL3 (MIP-1α). Using specific ELISAs, the upregulation of MCP-3 and MIP-2 at the protein level was confirmed. A large number of other genes were also profoundly upregulated in response to *S. equi*; among these were many transcription factors, growth factors and genes implicated in signaling processes.

Summary (paper I)

- Mast cells are activated by *S. equi* and produce and release a number of different cytokines and chemokines, including TNF-α, MCP-1, IL-6, IL-13, MIP-2 and MCP-3.
- The co-culture of mast cells with bacteria induces a slow release of histamine but no major degranulation takes place.
- The mast cells do not phagocytose *S. equi*.
- The bacteria need to be alive and in cell-cell contact to the mast cells for the activation to take place.
- The activation is dependent on TLR-2, but also on other unknown receptors.
- At the mRNA level, 155 genes were significantly (more then 4-fold) upregulated. Many of these genes were cytokines/chemokines, transcription factors, growth factors and genes implicated in signaling processes.

3.2.2 Paper II: Granzyme D is a novel murine mast cell protease, highly induced by multiple pathways of mast cell activation.

In paper I, we looked at which genes were upregulated in mast cells in response to *S. equi,* a gram-positive bacterium. One of the most highly upregulated genes was the one coding for granzyme D. Granzyme D is a protease that is mainly expressed in cytotoxic T cells, and there was no previous report indicating its expression by mast cells. Therefore, the aim of

this paper was to investigate the expression of granzyme D by mast cells more thoroughly.

The expression of granzyme D in BMMCs was confirmed by real-time PCR, where the expression was correlated to mast cell maturation. In addition, the expression was upregulated in response to SCF, the main growth factor for mast cells. The expression of granzyme D was relatively low in resting mast cells but was highly inducible, occurring when activating mast cells in a number of different ways. The upregulation by the gram-positive bacteria *S. equi* was confirmed and granzyme D was also shown to be upregulated by the gram-negative bacteria *E. coli,* although to a much lower extent than to *S. equi.* Granzyme D was also directly upregulated by the bacterial cell wall components LPS and PGN. However, slightly contradicting results showed that granzyme D was upregulated to a lower extent by PGN, despite being the main component in gram-positive (i.e., *S. equi*) bacteria cell walls, than to LPS, the main component in gram-negative bacteria cell walls (i.e., *E. coli*). However, as shown in paper I, *S. equi* do not only activate the mast cells through TLR-2 and PGN - other unknown receptors are also involved. Granzyme D was also shown to be upregulated through the classical way of activating mast cells, namely through IgE-receptor crosslinking and also directly through mobilizing calcium by using a calcium ionophore. The upregulation of granzyme D was shown to be dependent on a protein kinase C (PKC) (PKC inhibitor Gö6976, which inhibits PKC α/β1 and PKCD1), the transcription factor Nuclear factor of activated T-cells (NFAT) and to some extent the transcription factor nuclear factor-κβ (NF-κβ).

Due to the difficulty of finding a specific granzyme D antibody that did not cross-react with the closely related granzyme B, which is abundantly expressed in mast cells, we developed a new antibody. Our new antibody did not cross-react with granzyme B and we could see that granzyme D protein expression was linked to mast cell maturation, and it was released after calcium ionophore stimulation. Using immunohistology, we could also see that the granzyme D antibody stained mainly the perinuclear region in the mast cells.

Summary (paper II)

- Mast cells express granzyme D, which is associated with mast cell maturation.
- Mast cell granzyme D is upregulated by live bacteria, LPS, PGN, SCF, IgE-receptor crosslinking and calcium ionophore A23187.
- The upregulation of granzyme D is dependent on a PKC and the transcription factor NFAT and to some extent the transcription factor NFκβ.

3.2.3 Paper III: Mast cells are activated by *Staphylococcus aureus in vitro* but do not influence the outcome of intraperioneal *Staphylococcus aureus* infection *in vivo*.

In paper III we studied the mast cell response to *S. aureus in vitro* and also the contribution of the mast cell response to the course of the *in vivo* infection. Previous studies have shown that mast cells can degranulate in response to *S. aureus* and release TNF-α and IL-8. They can also exert anti-microbial activity against *S. aureus* by releasing extracellular traps and antimicrobial compounds (Abel *et al.*, 2011). *S. aureus* evades the extracellular antimicrobial activity of mast cells by promoting its own uptake (Rocha-de-Souza *et al.*, 2008). However, the global mast cell response to *S. aureus* has not been investigated. Therefore, we performed an Affymetrix microarray analysis of *S. aureus*-infected murine peritoneal-cell derived mast cells (PCMCs), to study how *S. aureus* affected the mast cell gene expression. The mast cells responded by significantly upregulating 52 genes, with a higher than 2 log2fold change. Of these genes, several were cytokines and chemokines, indicating that *S. aureus* induces a strong pro-inflammatory response in mast cells. The cytokine IL-3 was the gene induced to the highest extent of all genes. Other cytokines/chemokines that were upregulated included: IL-13, CCL3, TNF-α, CCL7, IL-6 and Leukemia inhibitory factor (LIF). Analysis by ELISA confirmed the upregulation of IL-3, IL-13 and TNF-α at the protein level.

Mast cells have been shown to be important in combating many different bacteria *in vivo,* as shown by assessment of the Kit-dependent mast cell-deficient mice, $Kit^{W/W-v}$ and Kit^{W-sh} (Abraham & St John, 2010). However, the importance of mast cells in *S. aureus* infection has not been investigated. Furthermore, the new Kit-independent mast cell-deficient mice have not been used in the context of bacterial infection. Therefore, we did i.p. infection with *S. aureus* in $Mcpt5\text{-}Cre^{+}$ x R-DTA mice, with littermate $Mcpt5\text{-}Cre^{-}$ x R-DTA mice as mast cell-sufficient controls. We euthanized the mice after 4 hours as well as 3 days, and studied changes in weight, bacterial clearance and inflammatory cell influx in the peritoneum. In this infection model, the mast cells did not affect any of these parameters. Since we had seen that mast cells responded to *S. aureus* by releasing various cytokines *in vitro* we decided to look at the cytokine profile in the peritoneum. However, also here, we could not see any effect of the mast cell deficiency. There could be many reasons for the discrepancy between our results and the earlier published results showing that mast cells are important in bacterial infections. Many of the earlier published infection models are severe and have death as an end point; in contrast our infection is a quite mild one that the mice recover from. It is thus possible that mast cells do not have a big impact on mild infections. There are

also published results from the CLP model, where the extent of the infection determines whether mast cells promote or decrease survival (Piliponsky *et al.*, 2010). Another major difference is the use of different mouse strains. The Kit-dependent mast cell-deficient mice have, in addition to lacking mast cells, many other abnormalities. A role of mast cells has therefore been proven by reconstituting the mast cell-deficient mice with mast cells. However, it is not certain that the reconstituted mast cells that are derived from BMMCs will have the same distribution and function as normal mast cells. Moreover, studies on the recently developed Kit-independent mast cell-deficient mice have provided some conflicting data in comparison with those based on the use of $Kit^{W/W-v}$ and Kit^{W-sh} mice (Reber *et al.*, 2012; Rodewald & Feyerabend, 2012). This has led to the need to re-evaluate results based on the $Kit^{W/W-v}$ and Kit^{W-sh} mice in the new Kit-independent mice, including the protective role in bacterial infections. Our data may thus indicate that mast cells might not play as big a role in bacterial infections as previously believed. However, to make more general conclusions about the roles of mast cells in bacterial infections, it will be imperative to perform more extensive studies in the novel Kit-independent mast cell-deficient mice, using different bacterial strains and different experimental setups.

Summary (paper III)

- Mast cells upregulate many genes in response to live *S. aureus in vitro*. Many of these genes are cytokines/chemokines including IL-3, IL-13, CCL3, TNF-α and LIF.
- Mast cells have no impact on *in vivo* i.p. *S. aureus* infection in respect to weight loss, bacterial clearance and inflammatory cell influx.
- Mast cells do not contribute to the total cytokine profile in the peritoneum of i.p. *S. aureus* infected mice.

4 Concluding remarks and future perspectives

Mast cells are mostly known for their detrimental effects in allergy and asthma but they also have important functions in the immune response towards pathogens. Their location at sites that are exposed to the external environment, such as the skin, gut and the lungs, plus the fact that they are equipped with many receptors, which can recognize pathogens, makes them a good candidate for a “first line of defense” cell.

Most of the evidence of how mast cells react to bacteria is based on *in vitro* studies involving responses to purified bacterial cell wall components, such as LPS and PGN. To investigate how mast cells react to live bacteria, we have done *in vitro* experiments where we co-cultured mast cells with *S. equi* or *S. aureus*. They reacted by producing many cytokines and chemokines. Despite both *S. equi* and *S. aureus* being gram-positive bacteria the response was quite different, with the mast cells reacting more strongly to *S. equi*. This difference could also be due to the use of different mast cells: BMMCs in the case of *S. equi* and PCMCs in the case of *S. aureus*. It would be interesting to investigate the response of the more mature PCMCs to *S. equi,* and deduce what is making the mast cells react stronger to *S. equi*. In addition, it would be interesting to see how cultured mast cells react to a spectrum of other bacterial strains in order to see if the mast cells have a specific response to certain types of bacteria.

One of the genes that were upregulated in response to live *S. equi* was granzyme D, a protease that had not been shown to be expressed by mast cells before. Naturally, it is important to understand function of granzyme D. Generation of a granzyme D knockout mouse would greatly increase the possibilities to study this. The optimal tool to study the function of granzyme D in mast cells would be a mouse that has the granzyme D gene floxed with loxP sites. This mouse could be crossed with one that expresses Cre-recombinase in

mast cells, such as the Mcpt5-Cre, to generate a mouse that is lacking granzyme D only in mast cells.

Mast cells have been shown to be important in combating many different bacteria in studies that used Kit-deficient mice. However, since Kit not only effects mast cells but also many other cell types within and outside the immune system, interpretation of data from these mice is not straight forward. Therefore, a new generation of Kit-independent mast cell-deficient mice has been generated. There are some reported discrepancies between results from the Kit-dependent and the Kit-independent mice. Therefore, it is important to re-evaluate the results obtained in Kit-dependent mast cell-deficient mice in the new generation of mast cell-deficient mice, including the roles of mast cells in bacterial infections. We have investigated the roles of mast cells in i.p. *S. aureus* infection in the Mcpt5-Cre$^+$ x R-DTA mice, and found no impact of the mast cell-deficiency on weight loss, bacterial clearance and inflammatory cell influx. However, to make more general conclusions about the role of mast cells in bacterial infections, it is imperative to investigate these mice in other models of bacterial infection, for example via other routes of infection and other bacterial strains. Since mast cells in culture reacted stronger to *S. equi* than to *S. aureus,* it would be interesting to use this bacterial strain in the Mcpt5-Cre$^+$ x R-DTA mice. It would also be interesting to investigate other aspects of the mast cells during a bacterial infection, such as their effects on the adaptive immune system. This could be done by looking at the recruitment of dendritic cells to the site of infection, the effects on the draining lymph nodes, the generation of bacterial-specific antibodies and the effect of mast cells in a secondary infection.

5 Populärvetenskaplig sammanfattning

Varje dag träffar vår kropp på många organismer, såsom bakterier, virus och parasiter, som potentiellt kan infektera oss. Det är vårt immunförsvars uppgift att ta hand om dessa organismer så att de inte orsakar sjukdom. Immunförsvaret består av en rad olika celler, en av dessa celler är mastcellen. Mastceller innehåller en stor mängd sekretoriska vesiklar (granula) och vid aktivering frisätts innehållet i dessa granula till den närliggande miljön. Den mest kända granulakomponenten är histamin men även en rad cytokiner (signalmolekyler mellan celler), proteoglykaner (består av en proteinkärna med kolhydrater kopplade till sig) och proteaser (klipper sönder andra proteiner i kroppen) finns lagrade. Förutom att frisätta innehållet i dess granula kan aktiverade mastceller också nyproducera och frisätta olika substanser. Förutom att hjälpa till att försvara oss mot sjukdomsalstrande organismer är mastcellen också involverad i olika typer av sjukdomstillstånd, främst astma och allergier.

Denna avhandling har fokuserat på hur mastcellerna reagerar och fungerar vid en bakteriell infektion. Tidigare studier har visat hur mastcellen reagerar på komponenter från bakteriens cellvägg men vi var intresserade av hur dem reagerar på levande bakterier. Vi har därför odlat mastceller tillsammans med de levande bakterierna *Streptococcus equi* (artikel I) och *Staphylococcus aureus* (artikel III). Vi kunde se att mastcellerna bland annat reagerade genom att producera och frisätta ett antal olika cytokiner. Cytokiner används i kroppen för att signalera mellan olika delar av immunförsvaret. Genom att frisätta cytokiner kan alltså mastcellen kommunicera till andra celler att vi är utsatta för ett bakterie angrepp.

En annan molekyl som vi såg att mastceller producerade när de odlades tillsammans med bakterier var proteaset granzyme D (artikel II). Detta proteas har inte visats att det produceras av mastceller tidigare. Vi kunde också se att mastcellen producerade granzyme D i respons till andra stimuleringar än bakterier, bland annat aktivering via antikroppar.

Mastceller är beroende av en tillväxtfaktor som heter stamcellfaktor och dess receptor Kit för att kunna växa. Möss som är defekta i Kit har därför inga mastceller. Studier på Kit-defekta möss har visat att mastceller är viktiga vid bakterieinfektioner. Problemet är att Kit-defekta möss inte bara saknar mastceller, utan också har många andra problem, eftersom stamcellfaktor är en viktig tillväxtfaktor för många celler. Därför har det nyligen utvecklats nya möss som saknar mastceller men inte har några andra defekter. En av dessa möss, de så kallade Mcpt5-Cre^{+} x R-DTA-mössen, har vi använt för att utreda om mastcellen är viktig vid *Staphylococcus aureus* infektion i bukhålan. Vi kunde dock inte se någon skillnad på dessa möss och möss som har mastceller när vi tittade på viktminskning, bakteriell ”clearance” (hur bra de dödade bakterierna), rekrytering av andra immunceller och produktion av cytokiner. Men för att kunna dra mer generella slutsatser om vilken roll mastceller har vid bakterieinfektion måste det dock utföras försök med dessa nya möss och andra infektionsmodeller (andra bakteriestammar och andra infektionsställen).

Slutsatser:

- Mastceller producerar en rad olika cytokiner när de odlas tillsammans med levande bakterier (*Streptococcus equi* (artikel I) och *Staphylococcus aureus* (artikel III)).
- Mastcellen innehåller proteaset granzyme D, och det produceras till högre grad när mastcellen aktiveras bland annat via bakterier och antikroppar (artikel II).
- Mastcellen har ingen betydelse vid *Staphylococcus aureus* infektion i bukhålan i möss, vad det gäller viktminskning, bakteriell clearance, rekrytering av immunceller och produktion av cytokiner (artikel III).

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