Inflammatory mechanisms in bacterial infections: focus on mast cells and mastitis

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Abstract

Inflammation is an unspecific response of the immune system to pathogens, for example, invasion by bacteria. This thesis focuses on two aspects of inflammation in the context of bacterial infections: (1) mast cells and (2) mastitis. Mast cells are potent pro-inflammatory leucocytes that have been implicated in the defence against bacterial infections. Mastitis is an inflammation of the mammary tissue and is one of the most economically destructive disease in the dairy industry worldwide.

Here, mast cell synthesis of the potent pro-angiogenic vascular endothelial growth factor (VEGF) in response to stimuli with *Staphylococcus aureus* (*S. aureus*) was studied using an *in vitro* model of primary mouse mast cells. VEGF synthesis was found to be dependent on the presence of live whole bacteria.

Previous *in vivo* investigations of the roles of mast cells in bacterial infections have been conducted using c-Kit-dependent mast cell-deficient mice. These mice suffer from numerous abnormalities in addition to the lack of mast cells. Instead, we used newer, c-Kit-independent mast cell-deficient mice (Mcpt5-Cre), which have fewer non-mast cell related abnormalities. We found no impact of the mast cell deficiency on the course of intraperitoneal *S. aureus* infection (e.g., bacterial clearance and cytokine production).

We differentiated the virulence of, and response to, a set of clinical bacterial strains of bovine mastitis origin. *Escherichia coli* (*E. coli*) and *S. aureus* strains were injected intraperitoneally into mice. One *E. coli* strain (strain 127) was found to consistently cause more severe infection (judged by a clinical score) and induce a distinct profile of cytokines (CXCL1, G-CSF, CCL2). The concentrations of these cytokines correlated with both the clinical score and bacterial burden. The kinetics of the clinical and molecular changes that occurred during acute bovine mastitis were studied using a bovine *in vivo* model in which mastitis was induced by an intramammary infusion of *E. coli* lipopolysaccharide. Changes in clinical parameters (clinical score, milk changes, rectal temperature) as well as in milk and plasma cytokine concentrations and changes in the metabolome were registered. The progression of these changes occurred in the following order: (1) signs of inflammation in the udder and an increase in milk cytokine concentrations (after/at two hours), (2) visible changes in the milk and an increase in milk somatic cell counts (SCCs) (four hours), (3) changes in the plasma metabolome (four hours) and (4) changes in the milk metabolome (24 hours).

Keywords: mast cell, mastitis, inflammation, vascular endothelial growth factor

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Inflammatoriska mekanismer i bakterieinfektion: fokus på mastceller och mastit

Sammafattning

Inflammation är ett ospecifikt immunsvar, exempelvis mot bakterier. Denna avhandling fokuserar på två aspekter av inflammation i kontexten av bakterieinfektion: (1) mastceller och (2) mastit. Mastceller är potenta pro-inflammatoriska leukocyter som tros vara inblandande i försvaret mot bakterier. Mastit är en inflammation av mjölkkörtelvävnaden.

Mastcellens syntes av den potenta pro-angiogenes faktorn vaskulär endotelcellstillväxtfaktor (VEGF) studerades i kontexten av *Staphylococcus aureus* (*S. aureus*) infektion. En *in vitro* modell baserad på primära musmastceller användes. Vi fann att mastcellens syntes av VEGF var beroende av närvaron av levande bakterier.

Tidigare *in vivo* studier av mastcellens roll in bakterieinfektion har varit begränsade av deras beroende av c-Kit mastcellsdefekta möss. Dessa möss lider av flera fysiska defekter utöver frånvaron av mastceller. Vi använde oss av en ny musmodell där mastcellsdefekten induceras oberoende av c-Kit. I infektionsstudier med *S. aureus* fann vi att frånvaron av mastceller inte påverkade sjukdomsförloppet.

En intraperitoneal musmodell användes för att studera och särskilja virulensen hos en selektion av bakteriestammar ursprungligen isolerade från kor med akut klinisk mastit. Möss infekterades med *Escherichia coli* (*E. coli*) och *S. aureus* genom intraperitoneal injektion. *E. coli* stammen 127 orsakade allvarligare infektioner (bedömdes med kliniskscore). Immunsvaret mot stammen genererade även en distinkt cytokinprofil (CXCL-1, G-CSF, CCL2). Koncentrationen av dessa cytokiner korrelerade mot både kliniskscore och antalet bakterier i bukhålan.

Förändringar i kliniska och molekylära parametrar som sker i akut klinisk bovin mastit studerades med en bovin *in vivo* modell där mastit induceras med en intramammär *E. coli* lipopolysackarid infusion. Kliniska parametrar (kliniskscore, mjölkförändringar, temperatur), cytokinkoncentration i mjölk och plasma, och förändringar i metabolitkoncentrationer registrerades över tid. Vi fann att dessa förändringar skedde i följande ordning: (1) inflammatoriska tecken i juvret och ökade cytokinkoncentrationer i mjölken (två timmar), (2) synliga förändringar i mjölken och förhöjda somatiska cell antal i mjölken (SCCs) (fyra timmar), (3) förändringar i metabolitkoncentrationer i plasman (fyra timmar) och (4) förändringar i metabolitkoncentrationer i mjölken (24 timmar).

Nyckelord: mastcell, mastit, inflammation, vaskulär endotelcellstillväxtfaktor

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Preface

I thought that I would begin this thesis with a whimsical little poem. It may have some bearing on the matter at hand. Thus:

The cheese-mites asked how the cheese got there,
And warmly debated the matter;
The Orthodox said that it came from the air,
And the Heretics said from the platter.
They argued it long and they argued it strong,
And I hear they are arguing now;
But of all the choice spirits who lived in the cheese,
Not one of them thought of a cow.

A Parable (1898)
Sir Arthur Conan Doyle

Dedication

To my parents, and also to many dear friends and colleagues, whose sentiments on the passage of time I decidedly *do not* share

Things that just keep passing by – A boat with its sail up.
People's age.
Spring. Summer. Autumn. Winter.
Sei Shōnagon

Cold-hearted waves are these; not the waves but the years pass over the waiting pine.
Michitsuna no Haha

Now like a traveller who has tried two ways in vain
I stand perplexed where these sad seasons meet.
Murasaki Shikibu

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 Johnzon, CF., Rönnberg, E., Guss, B., Pejler, G*. (2016). Live *Staphylococcus aureus* induces expression and release of vascular endothelial growth factor in terminally differentiated mouse mast cells. *Frontiers in Immunology*, DOI: 10.3389/fimmu.2016.00247.

II 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 intraperitoneal *S. aureus* infection *in* vivo. *Immunology*, 143 (2), 155 – 163.

III Johnzon, CF., Artursson, K., Söderlund, R., Guss, B., Rönnberg, E., Pejler, G*. (2016). Mastitis pathogens with high virulence in a mouse model produce a distinct cytokine profile *in vivo*. *Frontiers in Immunology*, DOI: 10.3389/fimmu.2016.00368.

IV Johnzon, CF*., Dahlberg, J., Gustafson, AM., Waern, I., Moazzami, AA., Östensson, K., Pejler, G*. The effect of lipopolysaccharide-induced experimental bovine mastitis on clinical parameters, inflammatory markers and the metabolome: a kinetic approach. (Manuscript)

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The contribution of Carl-Fredrik Johnzon to the papers included in this thesis was as follows:

I Planned and performed the majority of the experimental work, analysed and interpreted data, performed statistical analyses, prepared figures and wrote the manuscript

II Planned and performed experimental work, analysed and interpreted data, and revised the manuscript

III Planned and performed the greater part of the experimental work, analysed and interpreted data, performed the majority of the statistical analyses, prepared figures and wrote the manuscript

IV Planned and performed the experimental work, analysed and interpreted the majority of the data, performed the majority of the statistical analyses, prepared most figures and wrote the manuscript

Abbreviations

BMMC	Bone marrow-derived mast cell
BTSCC	Bulk tank somatic cell counts
CCL	Chemokine (C-C motif) ligand
CFU	Colony-forming unit
CTMC	Connective tissue mast cell
CXCL	Chemokine (C-X-C motif) ligand
DAMP	Damage-associated molecular pattern
DT	Diphtheria toxin
ELISA	Enzyme-linked immunosorbent assay
FcγR	Fc-gamma (γ) receptor
FcεR	Fc-epsilon (ε) receptor
GAG	Glycosaminoglycan
G-CSF	Granulocyte colony-stimulating factor
ICSCC	Individual cow somatic cell counts
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMI	Intramammary infection
iNOS	Nitric oxide synthase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MC_C	Mast cell containing chymase (human)
MCP	Monocyte chemoattractant protein
MC_T	Mast cell containing tryptase (human)
MC_{TC}	Mast cell containing both tryptase and chymase (human)
MEC	Mammary epithelial cell
MMC	Mucosal mast cell
mMCP	Mouse mast cell protease

mTMT	Mouse transmembrane tryptase
NETs	Neutrophil extracellular traps
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-κB
NLR	Nucleotide-binding oligomerisation domain (NOD)-like receptor
NMR	Nuclear magnetic resonance
PAM3	Pam3CSK4
PAMP	Pathogen-associated molecular pattern
PCMC	Peritoneal cell-derived mast cell
PGN	Peptidoglycan
PRR	Pattern-recognition receptor
ROS	Reactive oxygen species
SCC	Somatic cell count
SCF	Stem cell factor
SLB	Swedish Friesian (cattle breed)
SptP	*Salmonella typhimurium* tyrosine phosphatase
SRB	Swedish Red-and-White (cattle breed)
TLR	Toll-like receptor
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor

1 Introduction

1.1 Bacteria & the Immune System

Immunology is, to an extent, the study, on the one hand, of a system that is noticeable by our imperceptions of its effects on the body and, on the other hand, of the discomfort its activity causes during moments of stress. I find myself that it is easier to recall with precision instances of sickness than the much longer periods of healthy everyday activity. This is perhaps a natural consequence of the human condition. After all, the immune system is a complex network of tissues that are largely unseen by the unaided human eye, which react to exposure to organisms and particles so numerous and infinitesimal that it is difficult to comprehend their existence. With these ruminations on perspective in mind, I was reminded of a short article written by Sir Arthur Conan Doyle[1] on knowledge of the immune system and bacteria in the late 19th century, titled "Life and Death in the Blood" (Doyle, 1883). The opening passage of that article proceeds as follows:

> "Had a man the power of reducing himself to the size of less than the one-thousandth part of an inch, and should he, while of this microscopic stature, convey himself through the coats of a living artery, how strange the sight that would meet his eye!" (Doyle, 1883).

1.1.1 Bacterial Life

Bacteria are prokaryotes, unicellular organisms that are structurally distinguished by a lack of internal membranes separating the genetic material or enzymatic machinery into isolated compartments (Stanier & Van Niel, 1962).

1. Then simply Arthur Conan Doyle

They are morphologically and metabolically highly diverse organisms (Salton & Kim, 1996; Jurtshuk, 1996). Consequently, bacteria are present in a diverse set of environments, ranging from seawater to soil to the digestive systems of metazoans (Foster *et al.*, 2017).

1.1.2 The Immune System

Animals, specifically mammals within the context of this thesis, are constantly exposed to a multitude of bacterial organisms[2]. From birth until death, the totality of a mammalian body represents fertile territory for these organisms to colonise. Indeed, they are found in virtually every surface or space available (Foster *et al.*, 2017). It is the function of the immune system to protect the 'self' against these visitors. Interactions between the host and bacteria are not exclusively antagonistic. Indeed, the host is continuously exposed to and colonised by microbes – comprising the 'microflora' – without eliciting any negative effects. The mechanisms of these seemingly paradoxical interactions are the focus of a great deal of research (Chu & Mazmanian, 2013). The antagonistic interactions are the topic of this thesis.

The immune system is broadly divided into an innate component and an adaptive component. These components are distinguished by the speed and specificity of their response. The innate response is immediate, whereas the adaptive response is specific but slow (days to weeks). The innate and adaptive immune systems, though composed of different cells, are not two entirely separate entities, and a fully functional immune system requires extensive interactions between the two (Crozat, Vivier & Dalod, 2009).

Innate. The innate immune system is composed of anatomical barriers (e.g., the skin, mucosal surfaces), the complement system, a number of different leucocytes (neutrophils, monocytes, macrophages, natural killer cells, mast cells) and various molecular immune mediators (cytokines, acute-phase proteins). It is capable of separating "self" from "non-self" and is the first defence that a microorganism will encounter when it enters the body (Parkin & Cohen, 2001).

Adaptive. The basis of the adaptive immune response are the T cells and B cells. T cells participate in cell-mediated mechanisms. B cells participate in immunoglobulin/antibody mediated responses (IgM, IgG, IgA, IgE, IgD). This is a stronger and more selective immunological response (i.e., it can distinguish between one species of bacteria and another and selectively react to one but not the other). This response is also characterised by immunological memory, which

2. To the bacteria, one must add the yeasts, moulds, protozoans, parasites and viruses

enables a more rapid response against a foreign particle upon subsequent re-exposure (Parkin & Cohen, 2001).

1.1.3 Inflammation

Inflammation is an unspecific response of the immune system to pathogens or trauma, which is activated within minutes of exposure. It has two purposes: (1) defend the host against infection and (2) facilitate tissue-repair (Medzhitov, 2008). In the context of this thesis, it is the former role, in relation to bacterial infections, which is relevant.

Inflammation develops in vascularised tissues and is characterised by increased capillary permeability, vascular dilation, leucocyte infiltration and accumulation, and increased blood flow (Freire & Van Dyke, 2013; Ashley, Weil & Nelson, 2012). These effects culminate in the elimination or isolation of the invading bacteria. However, the unspecific nature of these effects does not distinguish between self and the bacteria, causing collateral damage to the affected tissue. Hence, the inflammatory response represents both a cost and an, albeit short-term, benefit to the host. The immediate noticeable effects of inflammation are concisely summarised by its five cardinal signs: *calor* (heat), *dolor* (pain), *rubor* (redness), *tumor* (swelling) and *functio laesa* (loss of function) (Ashley, Weil & Nelson, 2012). Unsurprisingly for such a distinct and visible activity of the body, references to inflammation are present in historical records dating as far back as ancient Egypt and ancient Mesopotamia (Ryan & Majno, 1977; Granger & Senchenkova, 2010).

Inflammation from Activation to Resolution in Bacterial Infection

The events of the inflammatory cascade are broadly divided into: Detection, Signalling, Response, Infiltration and Resolution.

Detection. Inflammation is induced by the introduction of bacterial components into a tissue. These components take the form of either pathogen-associated molecular patterns (PAMPs), which are produced by all bacteria, and virulence factors, which are restricted to pathogenic bacteria (Medzhitov, 2008). PAMPs are conserved molecular structures that are structurally distinct from self-molecules (Kumar, Kawai & Akira, 2011). They are detected by a corresponding set of host germline-encoded proteins called pattern-recognition receptors (PRRs) expressed primarily on immune cells (e.g., tissue resident macrophages). There are several different classes of PRRs. Two major receptor classes that detect bacterial PAMPs are the Toll-like receptors (TLRs), which are located on the plasma membrane and intracellular membranes, and the NOD-like receptors (NLRs), which are located intracellularly (Takeuchi & Akira,

2010). In contrast, virulence factors trigger an inflammatory response through their effects on the host cells (e.g., pore formation) or direct tissue damage rather than through a set of conserved dedicated receptors that bind to specific foreign ligands (Medzhitov, 2008). In the latter case, the inflammatory response is activated by the detection of damage-associated molecular patterns (DAMPs). These are endogenous molecular structures that are not normally present in intact tissues. DAMPs are, like PAMPs, detected by PRRs (Sharma & Naidu, 2016).

Signalling. The intracellular mechanism that will be initiated is determined by the identity of the activated receptor. TLRs commonly activate a signalling pathway that is dependent on the adaptor protein MyD88. The MyD88 pathway terminates with activation and translocation into the nucleus of the transcription factor nuclear factor-κB (NF-κB). Once in the nucleus, NF-κB upregulates the expression of pro-inflammatory genes. Signalling by NLRs leads to assembly of the inflammasome (an intracellular protein complex) that associates with and activates the enzyme caspase-1, which in turn activates inactive immune compounds by proteolytic cleavage (Ashley, Weil & Nelson, 2012; Sharma & Naidu, 2016). The inflammasome can also be activated by an efflux of K^+ resulting from pore formation, an example of activation in response to a pore-forming virulence factor (Mariathasan *et al.*, 2006).

Response. Inflammation is mediated by a large number of compounds divided into seven distinct categories: (1) cytokines, (2) chemokines, (3) eicosanoids, (4) proteolytic enzymes, (5) complement components, (6) vasoactive amines and (7) vasoactive peptides (Medzhitov, 2008).

Cytokines and *chemokines* are small soluble signalling proteins. Cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α enhance the activity of leucocytes, whereas chemokines, for example the neutrophil attractant IL-8, promote leucocyte chemotaxis (migration towards an increasing concentration of a chemoattractant) (Turner *et al.*, 2014). *Eicosanoids* are a class of lipid-derived mediators that are synthesised enzymatically from phospholipids present on the plasma membrane. They include prostaglandins, thromboxanes and leukotrienes. These substances are, for example, involved in vasodilation (Dennis & Norris, 2015). *Proteolytic enzymes* promote inflammation through degradation of the extracellular matrix and promotion of leucocyte migration (Sharony *et al*., 2010). The *complement system* promotes inflammation by enhancing leucocyte migration (Parkin & Cohen, 2001). The *vasoactive amines* histamine and serotonin, increase vascular permeability and vasodilation (Barnes, 2001). *Vasoactive peptides* promote vascular permeability and vasodilation either directly or by inducing the release of vasoactive amines from immune cells (Medzhitov, 2008). The cumulative effect of all these

different compounds is the facilitation of leucocyte recruitment into the disturbed tissue.

Infiltration. Neutrophils and monocytes migrate into the disturbed tissue from the blood through chemotaxis and extravasation (movement from the capillaries into the surrounding tissue) (Ashley, Weil & Nelson, 2012). Neutrophils kill bacteria through one of three mechanisms. First, they can engulf a bacterial cell by phagocytosis and kill it internally using reactive oxygen species (ROS). Second, they can degranulate and release antibacterial proteases as well as toxic chemicals including ROS. Third, they can trap bacteria using so-called neutrophil extracellular traps (NETs). NETs are composed of core DNA elements together with various antimicrobial compounds (Kolaczkowska & Kubes, 2013). It is the release of toxic chemicals into the extracellular environment by neutrophils that is a major contributor to the damage that inflammation causes to the host tissue (Ashley, Weil & Nelson, 2012). Monocytes differentiate into macrophages inside inflamed tissue (Geissmann *et al.*, 2010), where they proceed to phagocytose and destroy bacteria. They also promote inflammation through the synthesis and release of many pro-inflammatory compounds (Zhang & Wang, 2014).

Resolution. Resolution of inflammation is important to prevent extensive damage to the host tissue and to promote healing. It is dependent on a number of interlinked processes which aim to (1) deplete the supply of neutrophil chemoattractants in the tissue, (2) ensure neutrophil apoptosis, (3) clear away apoptotic neutrophils by macrophages, and (4) switch the phenotype of the macrophages from a pro-inflammatory to a pro-resolution phenotype. These pro-resolution processes are driven by proteases, ROS, cytokines and other factors. The pro-resolution phenotype macrophages synthesise and release the pro-resolution lipid-derived mediator lipoxin and the fatty acid-derived resolvins and protectins. These block further neutrophil recruitment and promote neutrophil apoptosis. The tissue returns to homeostasis and functionality by the combined actions of macrophages, stem cells and progenitor cells (Ashley, Weil & Nelson, 2012; Ortega-Gómez, Perretti & Soehnlein, 2013).

1.2 The Mast Cell

1.2.1 A Brief History of the Mast Cell

The mast cell has a long evolutionary history. Mast cells are found in all classes of vertebrates, and mast cell-like cells are present in some invertebrate classes (Crivellato & Ribatti, 2010). The mast cell was originally described by the

German biologist Paul Ehrlich in 1878. He named these cells "Mastzelle" (from the German "mast", meaning "fattening"[3]). The name reflects Paul Ehrlich's belief of a nutritional role for his newly described cell type (Crivellato *et al.*, 2003). Subsequent researchers with a wider array of tools at their disposal than the light microscope and dyes with which Ehrlich's conducted his investigations, have classified the mast cell as a potent pro-inflammatory leucocyte with a range of putative functions, implicating it in many different pathologies. To date, the only truly well-established activity attributed to the mast cell is its involvement in IgE-mediated hypersensitivity (allergy) (Crivellato *et al.*, 2003; Beaven, 2009; Rodewald & Feyerabend, 2012).

1.2.2 Introduction to the Mast Cell

Mast cells are tissue-resident granulocytes (i.e., leucocytes containing cytoplasmic granules). They are distributed throughout the body but are particularly numerous in tissues that are directly exposed to the external environment (Marshall, 2004). Mast cells originate from bone marrow-derived pluripotent stem cell. Unlike other haematopoietic cells, mast cells circulate as immature progenitors and will only undergo the final steps in the maturation process once they enter a tissue (Dahlin & Hallgren, 2015). Morphologically and biochemically, mast cells are characterised by: (1) cytoplasmic secretory granules, (2) biogenic amines, (3) proteases, (4) proteoglycans, and (5) receptors for IgE.

Granules. Mast cell secretory granules – also called secretory lysosomes – are membrane enclosed cytoplasmic particles and act as storage units for a wide range of compounds. Biogenic amines, proteases and proteoglycans are major granule compound classes. Additionally, some cytokines have been found in mast cell granules – e.g., TNF-α (Gordon & Galli, 1990: Wernersson & Pejler, 2014). Up to 50 – 55% of the cytoplasmic space inside a mast cell is occupied by these granules (Yong, 1997).

Biogenic Amines. Biogenic amines are synthesised from amino acids and have a wide range of effects when released into the extracellular milieu (Barnes, 2001). The biogenic amines synthesised by mast cells are histamine, serotonin and dopamine (Barnes, 2001; Freeman *et al.*, 2001). Histamine in particular is associated with mast cells, which constitute a major source of that amine. Histamine has different effects depending on the exposed cell type and the histamine receptors expressed by those cells (H_1 – H_4) (Barnes, 2001; Parsons & Ganellin, 2006).

3. With regards to the foci of this thesis, it is interesting to note that the word "mast" derives from the Greek μαστός, meaning breast

Proteases. Mast cells synthesise two categories of proteases: (1) mast cell-specific proteases and (2) proteases that are not exclusively found in mast cells (Wernersson & Pejler, 2014). Mast cell proteases are distinguished by their high level of expression, which may account for more than 25% of the total mast cell protein content, and that they are stored in their active form. Mast cells express three classes of cell-specific proteases: tryptase, chymase and carboxypeptidase A3 (Pejler *et al.*, 2010). Tryptases are homotetrameric serine proteases with trypsin-like specificity (cleave after lysine/arginine residues). Human mast cells contain αI-, αII-, βI-, βII- and βIII-tryptase (Pallaoro *et al.*, 1999). Mouse mast cells express four tryptases: transmembrane tryptase (mTMT), mouse mast cell protease (mMCP)-6, mMCP-7 and mMCP-11 (Pejler *et al.*, 2007). Chymases are serine proteases. They are monomeric and have a chymotrypsin-like specificity (they cleave after aromatic amino acids). Chymases are divided into α- and β-chymases. Human mast cells only express one α-chymase. Mice express several chymases: the α-chymase mMCP-5 and the β-chymases mMCP-1, mMCP-2 and mMCP-4 (Pejler *et al.*, 2007). Carboxypeptidases are monomeric zinc-dependent metalloproteases that cleave after aromatic amino acids (Pejler *et al.*, 2010). Unlike the other proteases, human and mouse mast cells only express one carboxypeptidase each (Pejler *et al.*, 2007). Mast cell proteases have been attributed a wide range of physiological activities, including the recruitment of leucocytes, arthritis, the maintenance of tissue homeostasis and the degradation of toxins (Tchougounova *et al.*, 2005; Metz *et al.*, 2006; Schneider *et al.*, 2007; Thakurdas *et al.*, 2007; Shin *et al.*, 2009). Non-mast cell-restricted proteases found in granules include cathepsins and granzyme B, amongst several others (Wernersson & Pejler, 2014).

Proteoglycans. Proteoglycans are glycoproteins composed of a protein core with numerous glycosaminoglycans (GAG) attached covalently as side chains. In mast cells, the dominant core protein is serglycin (Åbrink, Grujic & Pejler, 2004). Serglycin contains lengthy regions of repeated serine and glycine residues. The length of these regions varies from species to species, but in mice and humans they are 18 – 21 aa long. These regions act as attachment sites for GAGs (Rönnberg, Melo & Pejler, 2012), polysaccharides composed of repeating sulphated disaccharide units. Due to the high degree of sulphation, proteoglycans are highly anionic. Mast cells synthesise GAGs of the heparin and chondroitin sulphate types. Proteoglycans have an essential role in the regulation of storage inside the granules. Serglycin-deficient mast cells show defective storage of proteases and biogenic amines (Åbrink, Grujic & Pejler, 2004; Ringvall *et al.*, 2008). It seems paradoxical that mast cells store large amounts of proteases and proteoglycans in a small confined space without degradation of the latter. It has been hypothesised that the proteoglycan core protein is protected

from proteolytic degradation by clustering of GAGs (Rönnberg, Melo & Pejler, 2012).

FcεRI. FcεRI is a high-affinity receptor for the Fc portion of IgE[4]. Mast cell FcεRI is a tetrameric protein complex composed of an α-subunit (IgE binding), β-subunit (signalling) and γ-homodimer (signalling). The complete tetramer structure is referred to as $\alpha\beta\gamma_2$. IgE binding to FcεRI results in the release of granule content – so called degranulation (see *Activation* below) (Kraft & Kinet, 2007).

1.2.3 The Mast Cell in Mice & Humans

Mast cell research makes great use of mouse and human models. Hence, it is of interest to consider the species-dependent differences in mast cells in mice and humans. Within either species, mast cells are present as distinct subpopulations. At the species level, mast cells differ by the contents of protease, proteoglycan and biogenic amines. At the subpopulation level, they are distinguished by the contents of protease (human) or protease and proteoglycan, as well as the tissue localisation (mouse). Human mast cells are categorised as MC_T (containing tryptase), MC_C (containing chymase) or MC_{TC} (containing tryptase and chymase). Mouse mast cells are classified as either MMC (mucosal type) or CTMC (connective tissue type) (Buckley *et* al., 2006; Moon *et al.*, 2010) (Table 1).

Table 1. *Mast cell subpopulations in mice and humans.*

	Mouse		Human		
	MMC	CTMC	MC_T	MC_C	MC_{TC}
Tryptase	-	mMCP-6	+	-	+
		mMCP-7			
Chymase	mMCP-1	mMCP-4	-	+	+
	mMCP-2	mMCP-5			
Carboxypeptidase A3	-	+	-	+	+
Proteoglycan	Chondroitin sulphate	Heparin	Heparin	Heparin	Heparin
			Chondroitin sulphate	Chondroitin sulphate	Chondroitin sulphate
Biogenic Amines	Histamine[a]	Histamine[a]	Histamine	Histamine	Histamine
	Serotonin	Serotonin	Serotonin	Serotonin	Serotonin

[a]Less in MMC than in CTMC (<1 pg/cell compared with >15 pg/cell)

4. The low-affinity receptor FcεRII is expressed on B cells (Stone, Prussin & Metcalfe, 2010)

1.2.4 Mast Cell Mediators

Mast cells produce two distinct categories of mediators: preformed mediators and *de novo* synthesised mediators (Gri *et al.*, 2012). Preformed mediators are stored inside the cytoplasmic secretory granules and are released within seconds of activation. These mediators include biogenic amines, proteoglycans, proteases and lysosomal enzymes (Wernersson & Pejler, 2014). The *de novo* synthesised mediators are generated first upon mast cell activation and are released within minutes to hours of activation, depending on the mediator type. These mediators include eicosanoids, cytokines, chemokines, growth factors and antimicrobial species. Eicosanoids generated by mast cells include prostaglandins (PGD_2) and leukotrienes (LTB_4, LTC_4) (Boyce, 2005). Mast cells can synthesise a plethora of cytokines, chemokines and growth factors, with effects that range from pro-inflammatory (e.g., IL-1β, TNF-α) to anti-inflammatory (e.g., IL-10) to immunomodulatory (e.g., TGF-β) (Mukai *et al.*, 2018). Compounds with direct antimicrobial effects have also been found to be produced by mast cells, e.g., antimicrobial peptides (Di Nardo, Vitiello & Gallo, 2003; Di Nardo *et al.*, 2008). Taken together, mast cell mediators have the potential to exert a wide range of effects on the immune system and on microbes (Table 2).

Table 2. *Major mast cell mediator classes and examples of mediators belonging to those classes.*

Mediator Class	Effects (e.g.)	Reference
Preformed		
Biogenic Amines	Vasodilation, Leucocyte regulation, Vasoconstriction	Barnes, 2001
Restricted Proteases	Recruitment of neutrophils, Tissue homeostasis, Toxin degradation	Pejler *et al.*, 2010
Non-restricted Proteases	Tissue remodelling	Wernersson & Pejler, 2014
Proteoglycans	Affect protease activity	Rönnberg, Melo & Pejler, 2012
Lysosomal enzymes	Tissue remodelling	Gri *et al.*, 2012
De novo		
Cytokines	Pro-inflammatory, Anti-inflammatory	Mukai *et al.*, 2018
Chemokines	Leucocyte chemotaxis	Mukai *et al.*, 2018
Growth Factors	Promotion of cell growth (various cell types)	Mukai *et al.*, 2018
Eicosanoids	Leucocyte recruitment, Vascular permeability, Smooth muscle constriction	Boyce, 2005
Antimicrobial Compounds	Direct antimicrobial effects	Gri *et al.*, 2012

1.2.5 Mast Cell Receptors

Mast cells express a great variety of receptors belonging to several different receptor families, including: (1) Fc receptors, (2) TLRs, (3) mannosylated receptors, (4) complement receptors, (5) cytokine receptors and (6) chemokine receptors (Gri *et al.*, 2012) (Table 3).

Table 3. *Major receptor families expressed by mast cells, examples of receptors belonging to those families and examples of ligands.*

Family/Type	Receptor (e.g.)	Ligands (e.g.)	Mouse	Human	Reference
Fc	FcεRI	IgE	+	+	Kraft & Kinet, 2007
	FcγR[a]	IgG	+	+	Malbec & Daëron, 2007
TLRs[b]	TLR1	Lipopeptides	+	+	Applequist, Wallin & Ljunggren, 2002
					Kulka *et al.*, 2004
	TLR2	PGN, LTA	+	+	Applequist, Wallin & Ljunggren, 2002
					Kulka & Metcafle, 2006
	TLR3	dsRNA	+	+	Matsushima *et al.*, 2004
					Kulka *et al.*, 2004
	TLR4	LPS	+	+	Applequist, Wallin & Ljunggren, 2002
					Kubo *et al.*, 2007
	TLR5	Flagellin		+	Kulka *et al.*, 2004
	TLR6	LTA	+	+	Applequist, Wallin & Ljunggren, 2002
					Kulka *et al.*, 2004
	TLR7	ssRNA	+	+	Matsushima *et al.*, 2004
					Kulka *et al.*, 2004
	TLR8	ssRNA	+	+	Supajatura *et al.*, 2001
					Kulka *et al.*, 2004
	TLR9	DNA	+	+	Matsushima *et al.*, 2004
					Kulka *et al.*, 2004
Mannosylated	CD48	FimH	+		Malaviya *et al*., 1999
Complement	C3aR	C3a	+	+	Schäfer *et al.*, 2013
					el-Lati, Dahinden & Church, 1994
	C5aR	C5a	+	+	Schäfer *et al.*, 2013
					Füreder *et al.*, 1995
Cytokine	c-kit	SCF	+	+	Chen *et al.*, 1994
					Hauswirth *et al.*, 2006

Family/Type	Receptor (e.g.)	Ligands (e.g.)	Mouse	Human	Reference
	IL-3R	IL-3	+	+	Wright *et al*., 2006 Dahl *et al.*, 2004
Chemokine	CCR1	CCL5	+	+	Amin *et al.*, 2005 Oliveira & Lukacs, 2001
	CCR2	CCL2	+		Oliveira & Lukacs, 2001
	CCR4	CCL17		+	Amin *et al.*, 2005

[a]FcγRI in human, FcγRIII in mouse, FcγRII in both human and mouse

[b]Ligands: Akira & Takeda, 2004

1.2.6 Mast Cell Activation

Mast cells can be activated dependently or independently of IgE. IgE-independent activation can be mediated by, for example, IgG, TLR ligands and complement components. The response of the mast cell varies with the stimuli (Frossi, De Carli & Pucillo, 2004).

IgE-dependent. IgE binds with high affinity to FcεRI receptors on the mast cell surface. Upon exposure to IgE and antigen, FcεRI receptors will cross-link and initiate an intracellular signalling cascade (Kraft & Kinet, 2007). A key feature of this cascade is the mobilisation of intracellular Ca^{2+} (Wernersson & Pejler, 2014), which ends in the release of mast cell granule content, degranulation, as well as *de novo* synthesis of mediators (Kraft & Kinet, 2007). Degranulation occurs within seconds, release of eicosanoids within minutes and *de novo* protein mediators within hours of activation (Abraham & St John, 2010). After activation, mast cells replenish their granules. Granule replenishment occurs over a long period of time, up to 72 hours. Upon full regranulation, mast cells can participate in a new activation cycle (Blank, 2011).

IgE-independent. IgG-mediated activation of mast cells occurs through the FcγR receptor upon antibody binding to antigen. IgG-mediated activation results in mast cell degranulation (Malbec & Daëron, 2007). TLR-mediated activation of mast cells typically results in the release of mediators in the absence of degranulation, i.e., the synthesis and release of *de novo* mediators such as eicosanoids and cytokines (Sandig & Bulfone-Paus, 2012). However, some TLR ligands can induce degranulation, such as peptidoglycan (PGN) (Supajatura *et al.*, 2002). Activation of mast cells by complement compounds has been observed to enhance IgE-mediated degranulation (Schäfer *et al.*, 2013).

1.2.7 The Mast Cell in the Immune System

The mast cell constitutes something of a paradox. It is evolutionary preserved in all vertebrates, expresses a wide range of receptors and can synthesise a broad range of mediators (Crivellato & Ribatti, 2010; Gri *et al.*, 2012). These observations would lead one to suppose that the mast cell has an indispensable and multifunctional role in the immune system. However, the most well documented activity attributed to the mast cell to date is its essential role in mediating allergic disease, i.e., the inflammation resulting from IgE-mediated mast cell activation and degranulation in response to innocuous antigens (Rodewald & Feyerabend, 2012). Beyond this well-documented activity, mast cells have been reported to participate in a wide range of contexts, such as the degradation of animal toxins, in tumour development, angiogenesis, diabetes and obesity (Norrby, 2002; Metz *et al.*, 2006; Schneider *et al.*, 2007; Ribatti & Crivellato, 2012; Shi & Shi, 2012). These seemingly contradictory reports have posited the mast cell as both a negative and positive modulator of immunity (Galli, Grimbaldeston & Tsai, 2008).

The notion that mast cells are involved in host responses towards bacterial exposure is based on the observation that these cells: (1) express receptors for detecting bacterial compounds (Table 3), (2) synthesise compounds that can modulate other immune cells or have direct antimicrobial effects (Table 2) and (3) are localised at the ideal position to detect bacterial pathogens (i.e., tissues directly exposed to the external environment). Taken together, these observations are the basis of the idea of the mast cell as a 'sentinel cell', a first line of defence against invading pathogens (Galli, Maurer & Lantz, 1999). In agreement with the general notion of the mast cell as a mediator of both positive and negative effects, it has been reported to have both a protective and a detrimental impact on the course of infections (Johnzon, Rönnberg & Pejler, 2016).

Protective. The protective functions of mast cells in bacterial infection have been reported to be dependent on the (1) recruitment of immune cells to the site of infection, (2) modulation of inflammatory cell function, (3) interactions with cells of the adaptive immune system and (4) direct antimicrobial effects (Table 4). *Recruitment.* The first studies that suggested a role for mast cells in bacterial infection attributed an essential role for mast cell-derived TNF-α in the recruitment of neutrophils (Echtenacher, Männel & Hultner, 1996; Malaviya *et al.*, 1996). Other mast cell-derived neutrophil chemoattractants are leukotrienes, CXCL1 and CXCL2, and tryptase (Malaviya & Abraham, 2000; Thakurdas *et al.*, 2007; De Filippo *et al.*, 2013). *Modulation.* Mast cells have been shown to enhance the antimicrobial activity of neutrophils against the bacteria *Klebsiella* via an IL-6-dependent mechanism (Sutherland *et al.*, 2008). Mast cells have also

been observed to inhibit replication of the bacteria *Francisella tularensis* (*F. tularensis*) inside macrophages in an IL-4 and contact-dependent manner (Ketavarapu *et al*., 2008). *Adaptive Immune System.* Mast cells have been shown to impact the recruitment of $CD4^+$ cells to draining lymph nodes during *Escherichia coli* (*E. coli*) infection, the recruitment of dendritic cells into draining lymph nodes in response to *Staphylococcus aureus* (*S. aureus*) PGN and into infected tissues during *E. coli* infection (McLachlan *et al.*, 2003; Shelburne *et al.*, 2009; Dawicki *et al*., 2010). *Antimicrobial Effects.* Several studies have shown that mast cells possess the ability to directly kill bacteria via phagocytosis, the secretion of antimicrobial peptides and the release of mast cell extracellular traps (Malaviya *et al*., 1994; Di Nardo, Vitiello & Gallo, 2003; Di Nardo *et al*., 2008; von Köckritz-Blickwede *et al*., 2008).

Detrimental. The detrimental impact of mast cells in the context of bacterial infections have been reported in terms of (1) mast cell activation, (2) immune evasion and (3) suppression (Table 4). *Activation.* In models of severe polymicrobial sepsis and *Salmonella typhimurium* (*S. typhimurium*) infection, mast cells have been shown to have a negative impact on the host, an effect that is dependent on TNF-α and IL-4. IL-4 has been observed to aggravate the disease by inhibiting phagocytosis by macrophages (Piliponsky *et al.*, 2010; Dahdah *et al.*, 2014). *Evasion.* Both *Mycobacterium tuberculosis* (*M. tuberculosis*) and *S. aureus* have been shown to persist intracellularly inside mast cells (Muñoz, Rivas-Santiago & Enciso, 2009; Abel *et al*., 2011). Survival inside immune cells is a common mechanism exploited by pathogens to evade the immune system (Finlay & McFadden, 2006). Mast cells appear to be no exception to this phenomenon. *Suppression. S. typhimurium* have been shown to be able to suppress the neutrophil-recruiting activity of mast cells by using a tyrosine phosphatase (SptP) *in vivo*. The bacterium delivers this protein into mast cells, thereby inhibiting their activation (Choi *et al*., 2013). Similarly, commensal bacteria have been shown to inhibit mast cell activation, possibly as a mechanism of homeostasis. High densities of non-pathogenic *E. coli* have been found to inhibit the activation of mast cells both *in vitro* and *ex vivo* (Magerl *et al*., 2008). A combination of four different probiotic bacterial strains upregulate the expression of anti-inflammatory genes in mast cells (Oksaharju *et al*., 2011).

Table 4. *Protective and detrimental effects mediated by mast cells in response to bacterial infections or stimuli with bacterial components.*

Effect	Example	Reference
Protective		
Recruitment	Neutrophil recruitment	Echtenacher, Männel & Hultner, 1996
		Malaviya *et al.*, 1996
		Malaviya & Abraham, 2000
		Thakurdas *et al.*, 2007
		De Filippo *et al*., 2013
Modulation	Enhance neutrophil activity	Sutherland *et al*., 2008
	Inhibition of intramacrophage *F. tularensis* replication	Ketavarapu *et al*., 2008
Adaptive Immune System	Recruitment of $CD4^+$ cells and dendritic cells into draining lymph nodes during *E. coli* infection	McLachlan *et al.*, 2003
		Shelburne *et al.*, 2009
	Recruitment of dendritic cells into draining lymph nodes in response to *S. aureus* PGN	Dawicki *et al*., 2010
Antimicrobial Effects	Antimicrobial peptides	Di Nardo, Vitiello & Gallo, 2003
		Di Nardo *et al*., 2008
	Phagocytosis	Malaviya *et al*., 1994
	Extracellular traps	von Köckritz-Blickwede *et al*., 2008
Negative		
Activation	Increased mortality rate in enterobacterial infection models	Piliponsky *et al.*, 2010
		Dahdah *et al.*, 2014
Evasion	Intracellular survival of *S. aureus*	Abel *et al*., 2011
	Intracellular survival of *M. tuberculosis*	Muñoz, Rivas-Santiago & Enciso, 2009
Suppression	Suppression by *S. typhimurium* via SptP	Choi *et al*., 2013
	Upregulation of anti-inflammatory genes by probiotic bacteria	Oksaharju *et al*., 2011

1.2.8 The Mast Cell & Vascular Endothelial Growth Factor

Human and mouse mast cells have been shown to store vascular endothelial growth factor (VEGF; also called VEGF-A) in their granules and release it in response to a variety of stimuli, including IgE receptor cross-linking (Boesiger

et al., 1998; Grützkau *et al.*, 1998). The observation that mast cells express VEGF has led to the notion that mast cells are involved in angiogenesis, for example, in the context of tumour growth (Norrby, 2002; Ribatti & Crivellato, 2012). VEGF is a member of the VEGF gene family of growth factors (Byrne, Bouchier-Hayes & Harmey, 2005).[5] VEGF is a glycoprotein with an essential role in angiogenesis (the formation of blood vessels from pre-existing vessels) and vasculogenesis (*de novo* formation of blood vessels) (Robinson & Stringer, 2001). In humans, the VEGF gene produces at least eight different isoforms ($VEGF_{110}$, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$) (Hoeben *et al.*, 2004). In mice, three splice variants are known ($VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$) (Ng *et al.*, 2001). The isoforms vary in terms of their potency in inducing angiogenesis, ability to diffuse, extent of expression and tissue specificity (Berse *et al*., 1992; *Ng et al*., 2001; Hoeben *et al*., 2004). Interestingly, in the context of mast cells, VEGF diffusibility is determined by its ability to bind heparin/heparan sulphate. Isoforms lacking heparin binding domains are more diffusible than those expressed with these domains (Ng *et al*., 2001; Hoeben *et al*., 2004).

1.2.9 Mast Cell Models

Mast cells are traditionally studied using *in vitro* models (human, mouse) and *in vivo* models (mouse). Both primary cells and cell lines are routinely employed in mast cell *in vitro* studies. Primary mast cells can be derived from the bone marrow (bone marrow-derived mast cells; BMMCs) or peritoneum (peritoneal cell-derived mast cells; PCMCs) of mice, or from human tissue (Malbec *et al.*, 2007; Arock *et al.*, 2008; Passante, 2014). An important tool in *in vivo* studies of mast cells are mast cell-deficient mice. There are two classes of these models: (1) kit-dependent deficient mice and (2) kit-independent deficient mice. c-Kit is the receptor for stem cell factor (SCF), the principal growth factor for mast cells (Moon *et al.*, 2010).

c-Kit-dependent. Mutants with deficient Kit proteins have different phenotypes: Kit^{W}, Kit^{Wv}, $Kit^{W\text{-}sh}$ and $Kit^{W/Wv}$. In Kit^{Wv} mice, c-Kit has impaired kinase activity (Nocka *et al.*, 1990). $Kit^{W\text{-}sh}$ mice have impaired expression of Kit due to a genomic rearrangement (Berrozpe *et al.*, 1999). $Kit^{W/Wv}$ mice are produced by crossing Kit^{W} and Kit^{Wv} mice and have been extensively used in mast cell research. A key aspect of these phenotypes is that the c-Kit receptor is also expressed by many other cells during development; hence, mutations affecting c-Kit have consequences for tissues and cells beyond the lack of mast cells. These effects may influence the outcome of experiments and lead to

5. Also includes the members VEGF-B, VEGF-C and VEGF-D

erroneous conclusions regarding the roles of mast cells (Rodewald & Feyerabend, 2012).

c-Kit-independent. Mice in which the mast cell deficiency results from an alteration independent of c-Kit were developed in response to the concerns raised regarding the validity of the older c-Kit-dependent models. These deficiencies are either constitutive or inducible. Constitutively mast cell-deficient mice are established from birth. In inducible models, the mast cell deficiency only develops in response to infusion of a toxin into a genetically modified animal (Rodewald & Feyerabend, 2012). The mast cell deficiency in these models depends either on the Cre recombinase system or on the addition of constitutively expressed diphtheria toxin receptors. By ensuring that the expression of the added genetic elements is under the control of mast cell-specific genes (proteases, enhancers), the alteration is restricted to mast cells (i.e., Cre recombinase is only active in mast cells) (Dudeck *et al.*, 2011; Otsuka *et al.*, 2011; Feyerabend *et al.*, 2011; Lilla *et al*., 2011; Sawaguchi *et al.*, 2012). For example, in *Mcpt5-Cre R-DTA* mice, the Cre-recombinase is controlled by the promoter for *Mcpt-5* (gene encoding mMCP5). Cre-recombinase catalyses the recombination of specific DNA fragments (loxP) located at either ends of a larger DNA sequence, in this case a sequence containing the stop codon for the genes encoding diphtheria toxin (DT). Expression of *Mcpt-5* leads to expression of the Cre-recombinase and removal of the DT stop codon, enabling the expression of DT. DT expression leads to mast cell death (Brault *et* al., 2007; Dudeck *et al.*, 2011). Regardless of how the c-Kit-independent mast cell deficiency is induced, these mice have fewer abnormalities compared with the older c-Kit-dependent models. Hence, they are believed to be a more powerful research tools (Feyerabend & Rodewald, 2012).

1.3 Mastitis

1.3.1 Modern Domestic Cattle

Cattle are large domesticated ruminant ungulates (Adelsköld *et al.*, 1923). Modern domestic cattle are split into two species, taurine cattle (*Bos taurus*) and zebus (*Bos indicus*) descended from aurochs (*Bos primigenius*). A third species, sanga cattle, is an African species of mixed taurine:zebu ancestry (Ajmone-Marsan *et al.*, 2010). In 2016, the global population of cattle was estimated to be in excess of one billion individuals (FAOSTAT, 2016). In Sweden, the cattle population is estimated to include approximately 1400000 individuals (Grönvall,

2018). The Swedish Red-and-White (SRB) and Swedish Friesian (SLB) are the most common breeds amongst dairy cows (Växa, 2018). Since their domestication, cattle have provided human societies with draught power, milk, meat and hides (Ajmone-Marsan *et al.*, 2010). The influence of cattle on human culture is attested by their frequent depiction in art and appearance in religion. The depictions of bull sports in Minoan art, of milking in Ancient Egyptian art and the cow Audumla, of Norse Mythology, who gave her milk to Ymir, the first being, are but a handful of examples of the influence that this animal species has had on human civilisation (Loughlin, 2000; Encyclopaedia Britannica). In this thesis, the term bovine will be used to refer to cattle.

1.3.2 The Composition of Milk

Milk is a complex biological emulsion of water and fat along with two other major milk constituent classes: proteins and sugars (lactose). It also contains many other substances, e.g., minerals and vitamins. Milk is the main source of nutrition for the mammalian neonate. Hence, it must contain all the nutrients required for growth. Milk fats released into the liquid as membrane-enclosed globules, and lactose, the disaccharide of glucose and galactose, act as sources of energy. Milk proteins, caseins and whey proteins, provide the amino acids required for the growth of tissues. The exact proportions of the different components vary considerably between different mammalian species. For example, the milk of marine mammals and polar bears contain more fat than the milk from other mammals. Human and bovine milk, as relevant examples, vary in the content of lactose, total protein, and the ratios of casein and whey, fats and minerals (Björnhag, 2004; Fox *et al.*, 2015) (Table 5).

Table 5. *Comparison of the composition of human and bovine milk in terms of lactose, protein, fat and minerals.*

Component	**Human**	**Bovine**
Lactose (g/100 g)	6,3 – 7,0	4,4 – 5,6
Protein (g/100 g)	0,9 – 1,9	3,0 – 4,0
Approximate casein:whey ratio	40:60	80:20
Fat (g/100 g)	2,1 – 4,0	3,3 – 6,4
--Saturated (%)	36 – 45	55 – 73
--Monounsaturated (%)	44 – 45	22 – 30
--Polyunsaturated (%)	8 – 19	2,4 – 6,3
Minerals (g/100 g)	0,2 – 0,3	0,7 – 0,8

References: Fox *et al.*, 2015; Gantner *et al.*, 2015

1.3.3 A Brief Overview of the Mammary Secretory Tissue

The bovine udder is quartered. Each quarter is functionally distinct with no direct connections. The right and left side of the udder are separated by thick connective tissue bands. The front and rear quarter on one side are only separated by a thin connective tissue septum. The whole udder is supported by a number of connective tissue bands, including the bands separating the two udder halves. The secretory tissue of one quarter is divided into the following components: (1) alveoli (singular: alveolus), (2) milk ducts and (3) connective tissue. The function of the gland connective tissue is to protect the more delicate alveoli (Nickerson & Akers, 2011).

The globular alveoli are the functional units of the secretory tissue. An alveolus consists of a single layer of specialised epithelial cells surrounding a hollow cavity. These epithelial cells, also called mammary epithelial cells (MECs) or simply mammocytes, absorb compounds from the blood and convert them into milk components. The milk is secreted into the hollow cavity. The mammary epithelial layer is surrounded by myoepithelial cells, a type of smooth muscle cell. Capillaries connect each alveolus to the general circulation. Milk accumulates inside an alveolus and, upon contraction of the myoepithelial cells, is forced out through a single duct – an opening providing egress from the alveolus. Alveoli are clustered together, and the duct of each alveolus connects them into a larger duct system. This system drains into the gland cistern. The gland cistern is in turn connected to the teat cistern. Milk is drained from the teat cistern through the teat canal. A sphincter[6] closes the teat canal and prevents leakage.

The udder is supplied with a very plentiful blood flow by arteries entering both halves of the udder near the rear quarters. Blood is drained from the mammary gland primarily through veins exiting from the front and rear of the udder. The udder possesses two large lymph nodes, one in each udder half. Milk synthesis is controlled by hormones, the release of which is controlled by the nervous system. The nervous system is otherwise not directly involved in the control of milk synthesis (Björnhag, 2004; Nickerson & Akers, 2011; Fox *et al.*, 2015).

1.3.4 The Innate Immune System of the Mammary Gland

The innate immune system of the mammary gland is divided into: (1) resident defences, (2) inducible defences and (3) cellular defences[7]. The resident

6. Circular smooth muscle

7. Cellular defences are considered to be a part of the resident defences. I have chosen to detail them separately for the sake of clarity

defences are constitutively present in the udder, whereas the inducible defences must be mobilised in response to an infection. Cellular defences are represented both by the resident population of leucocytes and by leucocytes that are recruited into the gland during inflammation (Rainard & Riollet, 2006).

Resident Defences. Resident defences are partly composed of anatomical barriers and partly of humoral defences. Between milkings, the teat canal is blocked by a keratin plug generated from the epithelial cells of the teat cistern. It constitutes a simple anatomical barrier to pathogen entry into the mammary tissue. The humoral defences are composed of a series of proteins present in the milk, including components of the complement system (C3b, C5a), lactoferrin (iron-chelator), transferrin (iron-chelator), lysozyme (an enzyme targeting PGN) and opsonic antibodies produced in the absence of antigenic stimulation (IgM).

Inducible Defences. Numerous genes are activated in mammary cells in response to infection, including nitric oxide synthase (iNOS; catalyses the formation of nitric oxide), host defence peptides (short proteins with antibacterial activity) and lactoferrin (concentration in milk increases dramatically upon inflammation) (Rainard & Riollet, 2006).

Cellular Defence. Three cell types are important for the innate immune system in the mammary gland: (1) MECs, (2) neutrophils and (3) macrophages (Rainard & Riollet, 2006; Ezzat Alnakip *et al*., 2014). Mast cells have also been demonstrated to be present in the udder (Nielsen, 1975).

Cellular Defences of the Innate Immune System of the Mammary Gland

Leucocytes involved in the mammary immune system are primarily macrophages and neutrophils (Rainard & Riollet, 2006; Ezzat Alnakip *et al.*, 2014). Both MECs and macrophages have been identified as possible activators of inflammation in the mammary tissue in response to bacterial infection (Elazar *et al.*, 2010; Brenaut *et al*., 2014).

Mammary Epithelial Cells. Aside from their milk synthesis function, MECs synthesise a wide range of inflammatory mediators following exposure to bacterial stimuli. In several *in vitro* experiments, MECs have been shown to express several cytokines in response to stimuli with purified bacterial components, conditioned media or heat-inactivated bacterial cells – e.g., CCL2, TNF-α, IL-1β, IL-6 and IL-8 (Strandberg *et al.*, 2005; Fu *et al.*, 2013; Gilbert *et al.*, 2013). In an ovine *in vivo* infection model of *S. aureus* mastitis, MECs orchestrated the early stages of the inflammatory response based on a mechanism that was dependent on IL-8 (Brenaut *et al*., 2014). Hence it is possible that bovine MECs share a similar 'activator of inflammation role' during bacterial infection.

Neutrophils. The function of the resident mammary neutrophil population is not clear. However, the recruited neutrophil population becomes the predominant leucocyte in the mammary gland during inflammation (Rainard & Riollet, 2006). In a healthy gland, neutrophils make up 3 – 26% of the total leucocytes in milk. In an inflamed gland, they reach up to 90% of the total leucocytes (Ezzat Alnakip *et al*., 2014). Once inside the mammary gland, these neutrophils have the same function as they do elsewhere in the host – to neutralise invading pathogens. A distinguishing feature of the mammary neutrophils is their reduced antimicrobial activity compared with neutrophils in other tissues. Upon entry into the mammary gland, neutrophils engulf milk fat globules and proteins, leading to a reduction in the number of granules available for killing ingested microbes (Paape *et al.*, 2003). However, they remain able to deal with invading microbes, possibly due to their sheer numbers (Blowey & Edmondson, 2010).

Macrophages. As in other tissues, the resident mammary macrophage population represents the potential initiators of the inflammatory response (Rainard & Riollet, 2006). In a mouse model of mastitis, the mammary macrophage population was demonstrated to be essential in the recruitment of neutrophils into the mammary gland in response to lipopolysaccharide (LPS) infusion. The mechanism was dependent on TNF-α and TLR4 signalling (Elazar *et al.*, 2010). Bovine macrophages have been shown to be able to phagocytose mastitis pathogens *in vitro* and release chemoattractants in response to *S. aureus* (Politis *et al.*, 1991; Grant & Finch, 1997). As in other tissues, monocytes are recruited into the mammary gland during inflammation. They differentiate into macrophages and are essential in the resolution of inflammation by clearing away neutrophils, as described elsewhere (see *Inflammation*) (Paape *et al.*, 2003). Macrophages are the predominant leucocyte in healthy glands, constituting up to 79% of the total leucocyte content in milk. In an inflamed gland, this proportion falls to 9 – 32% (Ezzat Alnakip *et al.*, 2014).

The Bovine Mast Cell

Mast cells are present in the udder, where they are the primary source of histamine (Nielsen, 1975; Maslinski *et al.*, 1993; Beaudry *et al.*, 2016). The bovine mast cell is a little studied area in comparison with those of mice and humans. A few studies have attempted to elucidate bovine mast cell heterogeneity and tissue distribution. Like human mast cells, bovine mast cells are divided into three subtypes based on their protease content: M_T, M_{TC} and M_C. Tryptase-positive mast cells are present in all sections of studied tissues, whereas chymase status varies (Küther *et al.*, 1998; Jolly *et al.*, 1999). Chymase-positive mast cells are more numerous in connective tissues than in mucosal

tissues (Jolly *et al.*, 2000). It remains to be determined whether bovine mast cells express more than one type of tryptase or chymase, but methodological discrepancies suggest that the proteases are heterogenous as in mice and humans (Jolly *et al.*, 1999; Jolly *et al.*, 2000).

1.3.5 Mastitis

Mastitis is an inflammation of the mammary tissue (Adelsköld *et al.*, 1923; Erskine, 2016). It typically arises as a response to an intramammary infection (IMI). Though commonly of bacterial origin, IMIs can also be caused by algae and fungi. Viruses are also possible mastitis pathogens (Dion, 1982; Watts, 1988; Bradley, 2002; Wellenberg, van der Poel & Van Oirschot, 2002). Mastitis is marked by the five cardinal signs of inflammation: reddening of the tissue, swelling of the tissue, pain in the inflamed tissue, an increased temperature in the tissue and impaired function. The latter is manifested as a reduced milk yield (Erskine, 2016).

Activation of Inflammation in the Mammary Gland

As noted previously, both MECs and the resident population of mammary macrophages are potential activators of the inflammatory response in the bovine mammary gland (Elazar *et al.*, 2010; Brenaut *et al.*, 2014). Cows, like other mammals, express the repertoire of TLRs 1 – 10 (Menzies & Ingham, 2006). Minimally, TLR2 and TLR4 agonists are detected by bovine MECs, e.g., LPS and lipoteichoic acid (LTA) (Gilbert *et al.*, 2013).

Effect of Mastitis on Milk & Economic Impact

Mastitis has numerous effects on milk apart from the reduced yield. The proportions of proteins, fats, ion concentrations, pH and concentrations of enzymes are altered in milk from an inflamed gland (Kitchen, 1981). These changes will, in turn, affect the taste of the milk and the possibility of further processing, e.g., the manufacturing yield is lower from mastitic milk due to the reduction of casein levels (Blowey & Edmondson, 2010). The recruitment of leucocytes in the mammary gland is also measurable in milk as an increase in milk somatic cell counts (SCCs). SCCs are primarily composed of neutrophils and macrophages that are recruited into the udder (Ezzat Alnakip *et al.*, 2014). SCCs are given in cells/ml and are used to measure the quality of the milk. High levels are considered to indicate poor milk quality. Additionally, increased levels are directly associated with reduced milk yield (Schukken *et al.*, 2003; Hagnestam-Nielsen *et al.*, 2009). SCCs are measured on the herd level or on

individual level. Herd level SCCs are performed using the bulk tank milk. Apart from determining the total quality of the milk from one dairy herd, bulk tank SCCs (BTSCCs) are also used to follow changes in SCCs over time. High BTSCCs typically indicate the presence of subclinical mastitis cases (Blowey & Edmondson, 2010). National standards for BTSCCs vary considerably. Within the European Union as well as Australia, New Zealand and Canada the levels are <400,000 cells/ml. In the United States, the level is 750,000 cells/ml (USDA, 2016). Individual cow SCCs (ICSCCs) can be used to identify specific individuals with high SCCs (Blowey & Edmondson, 2010). On the individual level, uninfected quarters have been found to have a mean SCCs of 70,000 cells/ml (Schukken *et al*., 2003).

Taken together, mastitis incurs economic costs in terms of a reduced milk yield and quality, veterinary costs (diagnostics and treatment) and culling of incurable cases. Mastitis is considered to be one of the most economically destructive diseases in the dairy industry worldwide (Halasa *et al.*, 2007; Hogeveen, Huijps & Lam, 2011) and it is reported in dairy herds on a global scale (Persson Waller *et al.*, 2009; Östensson *et al.*, 2013; Abebe *et al.*, 2016; Levison *et al.*, 2016; Busanello *et al.*, 2017; Gao *et al.*, 2017).

Clinical Forms of Mastitis

Mastitis manifests itself in two forms: (1) clinical mastitis and (2) subclinical mastitis. Clinical mastitis is characterised by visually distinguishable symptoms in both the udder and the milk. Visible alterations in the milk include a change in colour (white to yellow) and the appearance of clots. Clinical mastitis cases restricted to local symptoms are termed mild. Cases with systemic symptoms, such as fever or anorexia, are termed severe. Cases with rapidly developing symptoms are termed acute. In subclinical mastitis visible changes in the udder and the milk are at most transient. However, a reduction in milk yield and increased SCCs are still present. Subclinical mastitis cases that persist for at least two months are termed chronic. Due to the lack of easily recognisable symptoms, subclinical mastitis cases are difficult to detect (Erskine, 2016).

Mastitis Bacterial Pathogens & Differential Immune Response

Mastitis pathogens are traditionally divided into two general types: (1) contagious pathogens and (2) environmental pathogens. Contagious mastitis pathogens are present as reservoirs inside the mammary gland and can spread from one animal to another. Environmental pathogens are opportunistic and originate from outside the animal. In general, contagious mastitis pathogens cause subclinical mastitis whereas environmental pathogens cause clinical

mastitis (Blowey & Edmondson, 2010). However, although microbial species are typically categorised as one or the other of these two classes, the distinctions are less apparent on the strain level (Bradley, 2002).

Common mastitis bacterial pathogens are streptococci, staphylococci and coliforms (Blowey & Edmondson, 2010). In Sweden, two common bacterial mastitis pathogens are *S. aureus* and *E. coli* (Ericsson Unnerstad *et al*., 2009). These two species also represent the two types of mastitis pathogens: *S. aureus* is generally considered a contagious pathogen and *E. coli* an environmental pathogen. They also reflect the imperfect distinction between the two classes. Some *E. coli* strains can cause persistent infection, whereas some *S. aureus* strains cause acute infections. Virulence factors enabling iron acquisition, mobility and adherence are often found in *E. coli* strains that are able to cause persistent rather than transient infections with which the species is normally associated with (Dogan *et al*., 2006; Lippolis *et al.*, 2014; Fairbrother *et al.*, 2015). In *S. aureus*, genes encoding superantigens and antibiotic resistance mechanisms are typically found in persistent strains (Haveri *et al.*, 2007). *E. coli* and *S. aureus* are also good examples of the contrasting immune responses that different species can provoke (Schukken *et al.*, 2011). *E. coli* typically elicit a rapid cytokine response, including IL-1β, IL-8 and TNF-α. *S. aureus* elicits a cytokine profile including IL-1β and interferon (IFN)-γ. This response is slower and yields lower cytokine concentrations (Bannerman, 2009) (Table 6).

Treatments & Diagnostics

The diagnosis of mastitis is based on both clinical examination of the affected animals and an examination of the milk (Duarte, Freitas & Bexiga, 2015; Erskine, 2016). The milk can be examined for visible changes as well as with more refined methodology. An increase in SCCs is the gold standard for detecting cases of subclinical mastitis (Duarte, Freitas & Bexiga, 2015). Monitoring SCCs over time is one method used to detect suspected mastitis cases. However, many other factors influence SCCs apart from mastitis, including age, stage of lactation, stress and season. Hence, SCCs cannot be solely relied on and other factors, such as the history of a dairy herd, must be taken into consideration to determine the likelihood of mastitis (Blowey & Edmondson, 2010). Identification of a mastitis pathogen is based on genotypic (PCR) and phenotypic (milk culture) methods (Duarte, Freitas & Bexiga, 2015). Identification of the causative organism is a prerequisite to determine treatment options or other course of action (Blowey & Edmondson, 2010).

Mastitis is typically treated with antibiotics and symptomatic treatment, e.g., nonsteroidal anti-inflammatory drugs (NSAIDs) (Erskine, 2016). Not surprisingly, antibiotic resistant strains of bacterial mastitis pathogens have been

isolated worldwide (Persson, Nyman & Grönlund-Andersson, 2011; Gao *et al.*, 2012; Saini *et al.*, 2012). In Sweden, the use of antibiotics is limited to specific cases of acute clinical mastitis (Persson Waller, 2018). Other courses of action include milking infected cows last (to reduce the spread of infection) and culling (Blowey & Edmondson, 2010).

Table 6. *Comparison of* Escherichia coli (E. coli) *and* Staphylococcus aureus (S. aureus) *as mastitis pathogens in terms of pathogen type, mastitis type, immunogenic component(s), cytokine profile (protein level unless otherwise stated), rectal temperature and milk somatic cell counts (SCCs). Cytokine level comparisons are relative. (-): no response. (+): response. (++): stronger response.*

	E. coli	*S. aureus*	Reference
Pathogen Type[a]	Environmental	Contagious	Blowey & Edmondson, 2010
Mastitis Type[a]	Clinical	Subclinical	Blowey & Edmondson, 2010
Immunogenic Component(s)	LPS	LTA Lipoproteins	Mehrzad *et al*., 2008; Gilbert *et al*., 2013
Immune Response[b]	16 hours	24 – 32 hours	Bannerman *et al.*, 2004
Cytokine Profile			
IL-6[c]	+	++	Lee *et al*., 2006
IL-8	+	-	Bannerman *et al.*, 2004
TNF-α	+	-	Bannerman *et al.*, 2004
IL-1β	++	+	Bannerman *et al.*, 2004
IFNγ	++	+	Bannerman *et al.*, 2004
Rectal Temperature (°C)	40,5	39 – 39,5	Bannerman *et al.*, 2004
SCC (10^6 cells/ml)	>40	~30	Bannerman *et al.*, 2004

[a]Species level

[b]Time point where changes in cytokine expression and/or secretion, rectal temperature and SCCs levels first become significant

[c]Gene expression

Mastitis & Metabolomics

Metabolomics, also called metabonomics, is the study of changes in metabolites on a system-wide scale under certain conditions (e.g., cells in a culture) (Rochfort, 2005). Metabolites are molecules generated by an organism's metabolism, the life-sustaining chemical processes of a living organism (Lazar & Birnbaum, 2012). Metabolomic studies are undertaken using techniques such as nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS) (Rochfort, 2005). In the context of bovines and mastitis, the metabolic profiles of milk have been studied both in naturally occurring cases of disease and in experimentally induced disease. Such studies have found that mastitis

induces distinct metabolite profiles. For example, animals classified as healthy, clinical and subclinical have distinct milk profiles, milk classified as low or high SCC vary significantly with regard to a number of metabolites, and metabolite profiles vary over time in animals where mastitis was experimentally induced by infusion of bacteria (Sundekilde *et al.*, 2013; Moyes *et al.*, 2014; Thomas *et al.*, 2016; Xi *et al.*, 2017).

In Vivo *Mastitis Models*

Both mouse and bovine *in vivo* models are used to study mastitis. Mouse models include both mammary and non-mammary models (Bogni *et al.*; 1998; Leitner, Lubashevsky & Trainin, 2003; Elazar *et al.*, 2010). Bovine *in vivo* models include intramammary infusion or injection of purified bacterial components (e.g., LPS or LTA) and whole or inactivated bacteria (Yagi *et al.*, 2002; Leitner *et al.*, 2003; Rainard *et al.*, 2008; Pellegrino *et al.*, 2010). Although these bovine models have some practical disadvantages in comparison with the mouse models, they offer the possibility of studying mastitis in the relevant species and tissue.

2 Present Investigations

2.1 Aims of the Present Studies

The objective of these studies was to investigate the inflammatory mechanisms in bacterial infections, with a special focus on mast cells and bovine mastitis.

- **Paper I:** Investigate the induction of vascular endothelial growth factor (VEGF) by *S. aureus* in mast cells *in vitro*
- **Paper II:** Investigate the activation of mast cells *in vitro* by *S. aureus* and the role of mast cells in *S. aureus* infection *in vivo*
- **Paper III:** Investigate and differentiate the virulence of a set of acute mastitis bacterial isolates of bovine origin using an *in vivo* mouse infection model
- **Paper IV:** Investigate the clinical, immunological and metabolic changes that occur during mastitis using an *in vivo* bovine model of LPS-induced acute mastitis

2.2 Paper I

VEGF is essential for promotion of the survival, proliferation and migration of endothelial cells. In addition to these pro-angiogenic effects, VEGF also acts as a chemoattractant for immune cells. Mast cells produce and secrete VEGF and thus are thought to be involved in angiogenesis. In a previous study (**Paper II**), a gene array revealed that VEGF was highly upregulated in mast cells stimulated with live *Staphylococcus aureus in vitro*. This phenomenon provided a hitherto unknown link between bacteria and the induction of VEGF in mast cells. The aim of this study was to further investigate this finding. As many previous studies had used immature mast cell models, we decided to use fully mature

mouse mast cells derived from the peritoneum of adult mice (peritoneal cell-derived mast cells; PCMCs).

To investigate whether mature mast cells could synthesise and release VEGF, PCMCs were co-cultured with *S. aureus*. VEGF gene expression was analysed by quantitative PCR. VEGF secretion was analysed by enzyme-linked immunosorbent assay (ELISA). We found that VEGF expression was upregulated after six hours of co-cultivation and a significant increase in the concentration of VEGF in the co-culture supernatant was seen after just two hours. Next, we investigated whether live whole bacteria were required to induce VEGF expression and secretion. Purified bacterial components LPS, LTA, Pam3CSK4 (PAM3), PGN or a mixture of all four), heat inactivated bacteria and conditioned media (bacterial growth media or PCMC media conditioned with bacterial growth followed by sterile filtering) failed to elicit any response at the gene expression level. Subsequently, we investigated whether the induction of VEGF expression required direct contact between the mast cell and the bacteria. To achieve this goal, we utilised a Transwell system. Here, the PCMCs and bacteria were separated by a thin membrane that allowed the diffusion of soluble products, but not the migration of whole bacteria. Using this system, we found that direct contact was not required for the induction of VEGF expression. Finally, we investigated which cell-signalling pathway might be involved in this induction. PCMCs were pre-treated with inhibitors for the adaptor molecule Myd88 (involved in most TLR signalling pathways), nuclear factor of activated T-cells (NFAT; signalling molecule involved in the induction of pro-inflammatory genes in mast cells) and NF-κB (transcription factor involved in the regulation of genes related to the immune system) before co-cultivation with *S. aureus*. We observed only a partial inhibitory effect on VEGF upregulation in response to NF-κB inhibition.

Summary of **Paper I**:

- Induction of VEGF expression and secretion in fully mature mast cells requires whole live bacteria
- This induction is seemingly independent of TLRs but partly dependent on the NF-κB pathway
- VEGF induction is not dependent on direct contact between the mast cell and bacteria, but the soluble factor(s) involved could not be identified

2.3 Paper II

Many studies have focused on elucidating the role of mast cells in bacterial infections, primarily using immature mast cells *in vitro* and Kit-dependent mast

cell knockout mice *in vivo*. In this study, the role of the mast cell in bacterial infections was studied using mature mast cells (PCMCs) *in vitro* and Kit-independent mast cell knockout mice *in vivo* (Mcpt5-Cre^{+} x R-DTA with mast cell competent Mcpt5-Cre^{-} x R-DTA littermates used as controls).

PCMCs were co-cultivated with *S. aureus in vitro*. Gene expression was comprehensively studied using an Affymetrix microarray system. This revealed a profound upregulation of several genes encoding pro-inflammatory cytokines, including IL-3, IL-13 and TNF-α. Release of these cytokines into the cell culture supernatant was confirmed by ELISA.

Mice were infected with *S. aureus* by intraperitoneal injection. Controls were injected with an equivalent volume of sterile bacterial growth medium. Infection was allowed to proceed for four hours, one day or three days. The impact of the infection was monitored by weighing the mice, bacterial burden by determining the colony-forming unit per ml (CFU/ml) in the peritoneal lavage fluid, the infiltration of cells into the peritoneum by cell counting and the effect on the peritoneal cell population by differential cell counting. No differences were observed between mast cell-deficient and competent mice.

The influence of the mast cells on the production of cytokines in the peritoneum was investigated using a cytokine array. Cytokines detected at high concentrations in this assay (IL-6, MCP-1) and cytokines from the *in vitro* experiments (IL-3, IL-13) were further investigated by ELISA. No differences were observed between mast cell-deficient and competent mice.

Summary of **Paper II**:

- Mast cells are activated to release cytokines in response to *S. aureus in vitro*
- Mast cells do not influence the course of *S. aureus* infection *in vivo* nor do they contribute to the peritoneal cytokine profile

2.4 Paper III

The aim of this study was to use a non-mammary mouse infection model to differentiate the virulence of a selection of acute clinical mastitis bacterial isolates of bovine origin. Non-mammary mouse models have previously been used in mastitis research. In the present study, isolates of the species *S. aureus* (8325-4, 556, 392) and *E. coli* (MG1655, 676, 127) were used. The laboratory strains 8325-4 and MG1655 were used as controls. The generation time for all of the strains was determined and did not significantly differ in a species-based comparison.

The mice were infected by intraperitoneal injection, and the infection was allowed to proceed for 24 hours. The infection was assessed by weighing the

mice, scoring the severity of infection, and weighing lymph nodes and spleens. Clearance of bacteria from the peritoneum was assessed by determining the CFU/ml in the peritoneal lavage fluid. Effects on the recruitment of immune cells to the peritoneum and the peritoneal cell population were determined by cell counting. The pro-inflammatory cytokine profile in the peritoneum was investigated using a cytokine array.

E. coli strain 127 caused strikingly consistent and high clinical scores, but it had no effect on the lymphoid organs. Strain 127 also persisted at greater numbers in the peritoneum. All strains induced neutrophil recruitment. The concentrations of three cytokines (G-CSF, CXCL1 and CCL2) were particularly highly increased based on a cytokine array in mice infected with strain 127 compared with the other strains. The concentrations of these cytokines were subsequently measured by ELISA in peritoneal lavage fluid (local) and plasma (systemic). The concentrations of these cytokines were higher in mice infected with 127 than any of the other strains, and correlated significantly with both the clinical score and bacterial burden. Hence, strain 127 caused both a local and systemic cytokine response.

Summary of **Paper III**:

- *E. coli* 127 strain consistently caused more severe infections as judged by clinical scoring and persisted at greater number in the peritoneum 24 hours after infection, but did not affect the lymphoid organs
- *E. coli* 127 strain generated a distinct cytokine profile (G-CSF, CXCL1, CCL2) in both the peritoneal lavage fluid (local response) and plasma (systemic response)
- The concentrations of these cytokines correlated with both disease severity and bacterial burden in the peritoneum

2.5 Paper IV

The objective of this study was to investigate the clinical and molecular changes that occur during acute bovine mastitis. A kinetic approach was applied, and changes were registered in terms of clinical parameters (clinical score, milk changes, rectal temperature), concentrations of cytokines in milk and plasma, and changes in the metabolome. Additionally, a role for mast cells in this model was investigated by analysing histamine levels in milk and plasma. Mastitis was induced by an intramammary infusion of LPS dissolved in physiological saline solution. Controls received an equivalent volume of physiological saline solution. Only healthy primiparous lactating cows were enrolled for the purposes of this study. Samples were collected in the form of milk and blood.

Clinical signs of mastitis in the udder (heat, pain, swelling, temperature) were visible two hours post-infusion and persisted up to 24 or 72 hours. Visible changes in the milk and a significant increase in the milk SCCs were delayed and appeared only after four hours. These changes also persisted for a longer period of time, up to 120 hours post-infusion, than did the other clinical parameters. Increases in the concentrations of the cytokines IL-6, TNF-α and CCL2 in milk were significant at two hours post-infusion. G-CSF and CXCL1 did not significantly increase in milk. In plasma, only a transient increase in IL-6 was detectible at four hours post-infusion. Histamine did not increase significantly in either milk or plasma, indicating that mast cells did not have a major role in the response to LPS.

The metabolomics profiles of the milk and plasma were analysed using a targeted (plasma) and untargeted (milk) NMR-based analysis approach. In milk, major changes occurred 24 hours post-infusion, including a reduction of lactose levels. Hence, these changes were preceded by a significant degree of time by the other local changes in the udder, judged in terms of clinical parameters and cytokine concentrations. Changes in the plasma required less time to develop, where an increase in lactose together with concurrent reductions in the levels of ketone bodies and short-chain fatty acids were detectible four hours post-infusion. The systemic changes in the plasma coincided with the transient IL-6 increase in plasma. The progression of these changes can be summarised as follows: (1) signs of inflammation in the udder and increases in milk cytokine concentrations (two hours), (2) visible changes in the milk and increases in milk SCCs (four hours), (3) changes in the plasma metabolome (four hours) and (4) changes in the milk metabolome (24 hours)

Summary of **Paper IV**:

- Intramammary infusion led to visible signs of acute mastitis two hours post-infusion, returning to normal levels within 24 to 72 hours post-infusion
- Changes in the milk were delayed compared with general and systemic changes, but persist for longer periods of time
- Concentrations of IL-6, TNF-α and CCL2 increased in milk; only a transient increase in levels of IL-6 was detectible in plasma
- The absence of increased histamine levels in milk and plasma suggests that mast cells have no role in LPS-induced acute mastitis, but activation independent of degranulation cannot be excluded
- Changes in the metabolome were registered in both the plasma and the milk, developing at four hours post-infusion before returning to normal within 72 hours

3 Concluding Remarks & Future Perspectives

Inflammation is an unspecific response of the immune system to trauma and invasion by foreign particles such as for example bacterial pathogens. Inflammation, due to its unspecific nature, causes collateral damage to the host tissue in addition to the beneficial effects of eliminating or limiting the spread of an infectious agent. Two common examples in which the negative impact of inflammation causes extensive damage to the host are mastitis (inflammation of the mammary tissue) and IgE-mediated hypersensitivity (activation of mast cells). Mast cells have also been suggested to have a role in bacterial infections, as a sentinel cell that is ideally positioned to respond quickly and early to the presence of bacteria. The inflammatory mechanisms that are operative during bacterial infection, in the context of mastitis and the contribution of mast cells, were studied using a four-pronged approach. (1) Mast cell mechanisms were studied *in vitro* using mature primary mouse mast cells and (2) *in vivo* using Kit-independent mast cell-deficient mice. This strategy is in contrast to many previous studies in which immature mast cells and Kit-dependent mast cell-deficient mice have been used. Mastitis was investigated: (1) *in vivo* using a mouse non-mammary infection model and (2) an LPS-induced acute bovine *in vivo* mastitis model.

The conditions required for the synthesis and release of VEGF by fully mature mast cells stimulated with *S. aureus* were comprehensively studied *in vitro*. At this stage, the identity of the soluble factor(s), which only seemed to be produced during co-cultivation of mast cells and bacteria, could not be elucidated. These factor(s) could potentially be identified by analysing media conditioned with both *S. aureus* and mast cells using mass spectrometry. VEGF is involved in tumour angiogenesis. Several species of bacteria are known to infiltrate and colonise tumours. Likewise, mast cells frequently populate tumours. It is thus possible that bacterial stimuli of tumour-populating mast cells

yield the VEGF required for tumour angiogenesis. If a dual cancer-bacterial infection *in vivo* model could be developed, this hypothesis could be investigated using strains of tumour-colonising bacteria and mast cell-deficient mice. Prior to any such investigation, the upregulation of VEGF in response both to different strains of *S. aureus* and to other species of bacteria would be required to establish whether VEGF induction is species specific.

A similar approach could be used to investigate the role of the mast cell in general bacterial infections. In our study, a laboratory strain of *S. aureus* was used and no differences were found between mast cell competent and deficient mice. Optimally, by using different strains of *S. aureus* and different bacterial species, as well as different strains of those species, and alternate routes of infection, more general conclusions regarding the mast cell in infection could be made. Ideally, wildtype strains derived from diseased animals could be used as an alternative to the laboratory strains.

A similar approach was used in our non-mammary mouse mastitis model. Mice were infected by intraperitoneal injection with a number of different bacterial strains representing two major mastitis pathogen species, *E. coli* and *S. aureus*. We found that one particular strain of *E. coli*, strain 127, caused consistently more severe infections and generated a distinct cytokine profile (CCL2, G-CSF, CXCL1). The concentrations of these cytokines correlated with both disease severity and bacterial burden. It is imperative that these strains, and potentially additional strains and other species of common mastitis bacterial pathogens, are used in further investigations with a bovine model system rather than a mouse system. In lieu of an *in vivo* bovine infection system, which would be prohibitively difficult both from a practical and ethical standpoint, a model using bovine mammary tissue could be employed. Tissue could be sourced from recently slaughtered animals. Though bovine cell lines are available, the bovine mammary immune response is likely better modelled using whole tissue rather than individual cell types.

The clinical, immunological and metabolic changes that occur during mastitis were studied using a kinetic approach applied to an *in vivo* LPS-induced acute bovine mastitis model. We found that metabolic changes occurred earlier in the plasma than in the milk, which was also reflected in the clinical parameters, where changes in the general condition occurred earlier than changes in the milk. No role for mast cells in mastitis was found with this LPS-induced model, at least not in a role involving degranulation. An ideal continuation of this study would be to use a similar intramammary infusion model substituting LPS with live whole bacteria, such as the bacterial strains used in our mouse non-mammary infection model. This approach would enable the investigation of changes in clinical parameters and molecular and metabolic

profiles over time in response to both different species of bacteria and different strains. Such a strategy would better reflect the actual changes in bovine physiology that operate during mastitis than the response to a purified bacterial component. However, as noted previously, such a model would be prohibitively difficult to use due to both practical and ethical reasons. An alternative would be to infuse cows with inactivated bacteria. Whether any response could be elicited to such stimuli could be determined by performing pilot studies using a bovine tissue mammary *in vitro* model similar to the one outlined above.

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Popular Science Summary

The signs of inflammation are easily recognisable: heat, swelling, redness and pain. Inflammation is a broad response of the immune system to exposure to trauma or harmful microorganisms such as bacteria. Inflammation damages both invading microbes and host tissue alike. This thesis is focused on the mechanisms involved in inflammation caused by bacterial infection, in the context of mast cells and mastitis.

The mast cell is a white blood cell, a component of the immune system. These cells are found in high numbers in the skin, intestine, lungs and other tissues that are directly exposed to the environment. Mast cells store large numbers of pro-inflammatory compounds (so called 'mediators'). Such mediators include histamine and cytokines (proteins used to signal between cells). These substances can be released within seconds of mast cell activation. Mast cells also produce mediators in response to activation, which are released over time (minutes to hours). Such mediators include antimicrobial peptides (peptide that directly kill microbes) and many additional cytokines. Mast cells are believed to assist in the response to bacterial infection by, for example, recruiting other immune cells to an infected tissue by releasing cytokines.

Mastitis is an inflammation of the milk-producing tissue in the mammary glands of mammals. The inflammation is usually caused by a bacterial infection. Mastitis is one of the most economically destructive diseases in the dairy industry worldwide. It reduces milk yield and quality and incurs high veterinary costs.

Mast cell responses to live bacteria were studied by culturing them together (**Paper I**) or by using a mouse infection model (**Paper II**). In **Paper I**, mast cell production and the release of vascular endothelial growth factor (VEGF) were studied. VEGF is a potent promoter of blood vessel growth (angiogenesis). We found that live *Staphylococcus aureus* (*S. aureus*) was required to activate the release of VEGF from mast cells. Individual purified bacterial components (e.g., bacterial cell wall components) did not active mast cells to produce VEGF. Mast

cells have long been thought to be involved in angiogenesis due to their ability to produce VEGF, but this bacterial induction of VEGF has not been previously reported. It could possibly be involved in tumour processes. Both mast cells and bacteria have been observed to populate certain tumours. It is possible that bacteria promote blood vessel growth in tumours by inducing mast cells to produce VEGF.

In **Paper II**, using a mouse *S. aureus* infection model, comparing normal wildtype mice with genetically modified mice lacking mast cells, we observed that the lack of mast cells did not affect the progress of the infection. This was measured by weighing the mice, measuring the clearance of bacteria (how well the immune systems of the mice could kill the bacteria), measuring the concentrations of pro-inflammatory cytokines and counting the inflammatory cells recruited to the infected site. Previous mouse infection studies have used mast cell-deficient mice that are dependent on mutations in a receptor called c-Kit. c-Kit is a receptor for a growth factor that is essential for mast cells. Defects in this receptor cause a loss of mast cells. However, because c-Kit is required by many other cells, mutations also cause many other defects. It is therefore difficult to draw conclusions from these models because the observed effects in the mast cell-deficient mice could have been caused by other defects. In contrast, we used a new model in which the mast cells were removed using a targeted genetic modification independent of the c-Kit receptor (Mcpt5-Cre mice). These mice have fewer non-mast cell-related defects.

Mastitis was investigated using a live mouse model (**Paper III**) and a live bovine model (**Paper IV**). In **Paper III**, the live mouse model was used to study the virulence (the ability of a microbe to infect or damage a host) of a selection of *Escherichia coli* (*E. coli*) and *S. aureus* strains originally isolated from the udders of cows afflicted with acute mastitis. The aim was to assess whether the virulence of a strain could be linked to the immune response they provoked. The response to infection was judged by a clinical score, by estimating bacterial clearance and by measuring the release of cytokines. We found that one strain of *E. coli* (strain 127) caused consistently more severe infections and elicited a distinct profile of cytokines (CXCL1, G-CSF, CCL2). We also found that the concentration of these cytokines correlated to the clinical score and the extent of bacterial clearance.

In **Paper IV**, a live bovine mastitis model was used to study the local and systemic effects of the inflammatory response over time. Mastitis was provoked by infusing lipopolysaccharide (LPS; a bacterial component) into udders. The effects on the animals were observed as clinical parameters (signs of inflammation in the udder), signs of mastitis in the milk (change in colour and appearance of clots), recruitment of immune cells into the milk and release of

cytokines into the milk as well as blood. We also measured changes in the levels of metabolites in the milk and the blood. Measurement in the blood allowed an estimation of the effects in whole animals (systemic). Metabolites are molecules that are generated by a living organism's metabolism. We found that local and systemic effects occurred at different time points after LPS infusion. We observed that the changes occurred in the following order: (1) signs of inflammation in the udder and increases in milk cytokine concentrations (two hours), (2) visible changes in the milk and increased numbers of immune cells in the milk (four hours), (3) changes in the levels of plasma metabolites (four hours) and (4) changes in the levels of milk metabolites (24 hours).

Conclusions:

- **Paper I:** Mast cell synthesis and release of VEGF required live whole *S. aureus*.
- **Paper II:** Mast cells do not influence the course of *S. aureus* infection in mice, with regards to weight loss, bacterial clearance, recruitment of immune cells and the release of cytokines.
- **Paper III:** The release of cytokines in response to a highly virulent *E. coli* strain can be correlated to the severity of infection and bacterial clearance in a mouse mastitis model.
- **Paper IV:** Local (udder) and systemic (blood) changes in response to acute mastitis occur at different time points after induction of inflammation. Changes in the udder occur earlier than the systemic changes.

Populärvetenskaplig Sammanfattning

Inflammation är ett ospecifikt immunsvar mot exempelvis invasion av sjukdomsalstrande organismer (bakterier, virus, svampar och parasiter). Inflammation kännetecknas av svullnad, hetta, smärta och rodnad.

Immunförsvaret består både av anatomiska barriärer (exempelvis huden) och ett antal specialiserade celler (vita blodkroppar) vars funktion är att förhindra och bekämpa angrepp av mikroorganismer. Mastcellen är en vit blodkropp som återfinns i särskilt stora antal i vävnader som är i direkt kontakt med miljön såsom huden, lungor och tarmar. Mastceller lagrar en rad olika substanser som har en pro-inflammatorisk effekt på kroppen (så kallade mediatorer). Dessa substanser kan mastcellen frisätta som svar på olika slags stimuli. Frisättningen, även kallad degranulering, sker inom loppet av sekunder. Mastcellen kan även nyproducera substanser som den avger över tid (timmar). Mångårig forskning har identifierat mastcellen som en slags ’vaktcell’ mot sjukdomsalstrande mikroorganismer. Mastit är en av de mest förlustbringande sjukdomarna inom mjölkindustrin. I denna avhandling studerades inflammation i sammanhanget bakterieinfektion med särskilt fokus på mastcellen och mastit.

Det är känt sedan tidigare att mastceller produceras vaskulär endotelcelltillväxtfaktor (förkortat VEGF), en tillväxtfaktor inblandad i nybildningen av blodkärl (angiogenes). Vi studerade mastcellens produktion av VEGF i samband med bakterieinfektion. Detta då en tidigare studie påvisat att det genetiska uttrycket av VEGF ökade markant som svar mot stimulans med *Staphylococcus aureus* (*S.* aureus; en vanlig bakterieart). Vi studerade detta tidigare okända samband närmare genom att odla mastceller och levande *S. aureus* under olika odlingsförhållanden. Vi stimulerade även mastceller med renade bakteriekomponenteter. Vi fann att uttrycket av VEGF i mastceller var beroende av närvaron av levande hela bakterier, då inaktiverade bakterier och enskilda bakteriekomponenter inte aktiverade mastcellens produktion av VEGF (**Artikel I**).

Mastcellens roll i bakterieinfektion studerades med en levande musmodell. Många tidigare studier inom detta område har använt sig av så kallade c-Kit beroende mastcellsdefekta möss. Tillväxtfaktorn stamcellfaktor (förkortat SCF) är vital för utvecklingen av mastceller. Omogna mastceller binder till sig SCF genom en receptor kallad c-Kit. I c-Kit beroende mastcellsdefekta möss är receptorn c-Kit muterad. Mutationen innebär att den inte kan binda till SCF. Konsekvensen är att dessa möss inte kan bilda några mastceller (det vill säga, ”mastcellsdefekta”). Problemet med dessa möss är att c-Kit även är en viktig receptor för många andra celler. Vid sidan av bristen på mastceller lider dessa möss även av andra defekter, något som kan påverka resultaten som dessa möss ger i studier. Vi använde oss av en ny mastcellsdefekt musmodell där frånvaron av mastceller inte är beroende av mutationer i c-Kit, så kallade Mcpt5-Cre^{+} x R-DTA möss. Olikt många tidigare studier, där c-Kit muterade möss använts, fann vi att mastcellen inte spelade någon roll i immunsvaret mot *S. aureus* infektion av bukhålan. Vi såg inga skillnad mellan de mastcellsdefekta mössen och de normala kontrollmössen. Mössens viktförändring, deras immunförsvars förmåga att döda bakterier (”clearance”), rekrytering av vita blodkroppar och produktion av cytokiner (signalmolekyler viktiga för immunförsvaret) studerades (**Artikel II**).

Med en musmodell studerade vi hur immunsvaret varierade mot olika stammar av ett par vanliga mastit orsakande bakterier, *Escherichia coli* (*E. coli*) och *S. aureus*. Olika stammar av samma art kan ha olika förmåga att orsaka sjukdomar. Urvalet av stammar i denna studie isolerades ursprungligen från kor med akut klinisk mastit. Med denna musmodell fann vi att en av *E. coli* stammarna – stam 127 – orsakade allvarligare infektioner än de övriga stammarna. Immunsvaret mot denna stam ledde även till en distinkt profil av cytokiner som korrelerade med sjukdomstillståndet (det vill säga, högre mängd cytokin utvecklades i mer sjuka djur). Dessa resultat skulle kunna följas upp i en komodell, exempelvis i en modell där juvervävnad från slaktade djur stimuleras med bakterier (**Artikel III**).

Vi studerade förloppet av akut klinisk mastit med en levande komodell, där mastit framkallades med en juverinfusion av *E. coli* endotoxin. Sjukdomsförloppet studerades i termer av kliniska förändringar (bedömdes med en klinisk score och mjölkförändringar), förändringar av mängden cytokiner i mjölk och blod, samt förändringar i koncentrationen av metaboliter i mjölk och blod. Metaboliter är molekyler som genereras av en levande organisms metabolism. Sjukdomsförloppet följdes över tid. Vi fann att dessa förändringar skedde i en specifik ordning: (1) tecken på inflammation i juvret och förhöjd mängd cytokiner i mjölken (två timmar), (2) synliga förändringar i mjölken och förhöjda somatiska cellantal i mjölken (SCCs) (fyra timmar), (3) förändringar i

mängden metaboliter i blodet (fyra timmar) och (4) förändringar i mängden metaboliter i mjölken (24 timmar) (**Artikel IV**).

Slutsatser:

- **Artikel I:** Uttryck av VEGF i mastcellen krävde stimuli med levande *S. aureus.*
- **Artikel II:** Mastcellen påverkar inte förloppet av *S. aureus* infektion i möss med avseende på viktförlust, ”clearance”, rekrytering av vita blodkroppar och frisättningen av cytokiner.
- **Artikel III:** Frisättningen av cytokiner som sker som svar mot en hög virulent *E. coli* stam korrelerade mot graden infektion och ”clearance” i en musmodell av mastit.
- **Artikel IV:** Lokala och systemiska förändringar under akut mastit sker vid olika tidpunkter efter infusion med endotoxin.

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