Multiplex Flow Cytometric Assays for Markers of Inflammation

Development and Application in Bovine Samples

Johanna Dernfalk
Faculty of Veterinary Medicine and Animal Science
Department of Anatomy, Physiology and Biochemistry
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2008
Abstract


The aim of this thesis was to develop new techniques for quantification of bovine pro-inflammatory markers, with emphasis on cytokines and acute phase proteins, and to apply the techniques, using mastitis as disease model.

Singleplex, duplex and triplex xMAP assays were developed for the bovine cytokines tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and the bovine acute phase proteins serum amyloid A (SAA) and lipopolysaccharide binding protein (LBP). Detection limits, linear ranges and intra- and inter-assay variations differed between assays, but generally, lower detection limits and wider linear ranges were observed in singleplex assays than in duplex and triplex assays. The detection limits for TNF-α, IL-1β and IL-6 were satisfactory in the singleplex assays. Further development work is required before the multiplex formats can be used in assays where very low cytokine concentrations are of interest. In the assays for acute phase proteins the detection limits and linear ranges were satisfactory, but could probably be further improved.

All xMAP assays could be used for quantification of the analytes in milk and plasma samples from cows with experimentally induced Escherichia coli or Staphylococcus aureus mastitis. Simultaneous detection of IL-1β, SAA and LBP was performed in plasma. LBP is secreted solely during bacterial infections, and cytokines and acute phase proteins are secreted at different phases of the inflammation. Thus a time perspective and information whether the infection is of bacterial origin or not is provided by measuring IL-1β, SAA and LBP simultaneously.

In one of the studies, ten cows were grouped as high or low responders for TNF-α, IL-1β and IL-6, based on their cytokine response in an ex vivo whole blood stimulation assay (WBA) with lipopolysaccharide (LPS) and E. coli. After the WBA, the cows were intramammary inoculated with E. coli, and the WBA was evaluated for its usefulness as a predictive tool of the severity of E. coli mastitis. The pre-inoculation WBA with used stimulation doses and incubation times could not predict the severity of an E. coli induced mastitis.

Keywords: bovine, multiplex particle based flow cytometry, immunoassay, suspension array, biomarkers, pro-inflammatory cytokines, acute phase proteins, mastitis, whole blood stimulation assay, milk, blood

Authors’ address: Johanna Dernfalk, Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, P.O. Box 7011, SE-750 07 Uppsala, Sweden. Johanna.Dernfalk@afb.slu.se
The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" but "Hm... that's funny..."

~ Isaac Asimov
## Contents

**Appendix** 7

**Abbreviations** 8

**Introduction** 9
- Bovine infectious diseases 9
  - Mastitis 10
- Innate immunity and innate immune responses 11
- Innate immune responses to bacterial mastitis 16
- Methods to study innate immune responses 17
  - Quantification of cytokines and APPs 18
  - Particle based flow cytometry 19
  - The *ex vivo* whole blood stimulation assay 21

**Aims** 23

**Materials and methods** 24
- Animals and infection models 24
  - Paper I 24
  - Papers II – III 24
  - Paper IV 24
- Laboratory analyses 25
  - Bacterial growth and SCC in milk 25
  - The *x*MAP assays 25
    - Microspheres 25
    - Antibodies and recombinant proteins 25
    - Coupling of antibodies to microspheres 25
    - The Luminex100 System 25
    - Validation 26
      - Quantification of TNF-α, IL-1β, IL-6, SAA and LBP 26
  - The *ex vivo* WBAs 26
  - ELISA assay 27
- Statistical analyses 27
  - Paper I 27
  - Papers II and IV 27
  - Paper III 28

**Results** 29
- Development of *x*MAP assays 29
  - Antibody clones cross-reacting between species 29
  - Singleplex and multiplex *x*MAP assays 29
- Experimental *E. coli* mastitis 32
- Concentrations of TNF-α, IL-1β and IL-6 in milk and plasma 32
- The *ex vivo* WBAs 32
Experimental LPS and PGN mastitis 32

**Discussion** 33
Methodological considerations 33
  *Antibody clones cross-reacting between species* 33
*The xMAP assays* 34
  *Sensitivity* 34
  *Linear range* 35
  *Intra- and inter-assay variation* 35
  *Cross-reactivity between reagents* 36
  *Matrix effects* 36
The WBA as a predictive tool 37
  *Experimental infection designs* 38
Cytokines and APPs in bovine milk and plasma 38

**Conclusions** 41

**Future perspectives** 42

**Populärvetenskaplig sammanfattning** 44

**References** 46

**Acknowledgements** 58
Appendix

Papers I-IV

This thesis is based on the following papers which will be referred to in the text by their Roman numerals:


II. Dernfalk, J., Persson Waller, K., Johannisson, A. 2007. The xMAP™ technique can be used for detection of the inflammatory cytokines IL-1β, IL-6 and TNF-α in bovine samples. *Veterinary Immunology and Immunopathology* 118, 40-49.

III. Dernfalk, J., Persson Waller, K., Johannisson, A., Røntved, C.M. TNF-α, IL-1β and IL-6 production *ex vivo* after *Escherichia coli* and LPS stimulation, and its associations with *Escherichia coli* mastitis and cytokine production in dairy cows. *In manuscript.*

IV. Dernfalk, J., Persson Waller, K., Johannisson, A. Simultaneous detection of interleukin-1β, serum amyloid A and lipopolysaccharide binding protein in bovine plasma using multiplex xMAP technology. *In manuscript.*

Papers I and II are reproduced with kind permission from the journals concerned.
## Abbreviations

The following abbreviations will be used in the text:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANCOVA</td>
<td>Analysis of covariance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APP</td>
<td>Acute phase protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GLM</td>
<td>General linear model</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS-binding protein</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pattern-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>pi</td>
<td>Post inoculation</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>rboIL-6</td>
<td>Recombinant bovine IL-6</td>
</tr>
<tr>
<td>rboSAA</td>
<td>Recombinant bovine SAA</td>
</tr>
<tr>
<td>rboTNF-α</td>
<td>Recombinant bovine TNF-α</td>
</tr>
<tr>
<td>rhuLBP</td>
<td>Recombinant human LBP</td>
</tr>
<tr>
<td>rhuTNF-α</td>
<td>Recombinant human TNF-α</td>
</tr>
<tr>
<td>rovIL-1β</td>
<td>Recombinant ovine IL-1β</td>
</tr>
<tr>
<td>rovTNF-α</td>
<td>Recombinant ovine TNF-α</td>
</tr>
<tr>
<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>SAA</td>
<td>Serum Amyloid A</td>
</tr>
<tr>
<td>SCC</td>
<td>Somatic cell count</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Sulfo-NHS</td>
<td>N-hydroxysulfosuccinimide</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>WBA</td>
<td>Whole blood stimulation assay</td>
</tr>
</tbody>
</table>
Introduction

Bovine infectious diseases, especially mastitis, generate considerable animal welfare problems and economical losses in the dairy industry worldwide and further investigations on preventive measures, diagnostic methods and alternatives for treatment are therefore needed. Knowledge of the innate immune response in general and of the early host response to intramammary pathogen invasion is essential. By mapping different inflammatory markers, the progress of an infection can be studied in detail. The ability to characterize and quantify cytokines and acute phase proteins (APPs) is fundamental to understand the inflammatory responses to infectious diseases.

Bovine infectious diseases

The term infectious diseases did not exist in early human communities (Last, 1998). Although fatal diseases, such as smallpox and plague, spread through the world and people died en masse, connections were not drawn between an individual’s survival of a disease and resistance to the same disease during the next outbreak. It was not until the tenth century that Chinese physicians observed that a patient obtained immunity after survival of a smallpox infection and used that knowledge for a first vaccination attempt (Gross & Sepkowitz, 1998). They developed a method, variolation, where they dried material from smallpox scabs and induced a mild smallpox infection. The technique spread and it had reached the whole civilized world by the 1750s (Feery, 1976; Barquet & Domingo, 1997).

Inspired by the progress in human immunology, veterinarians in 1754 started to inoculate cows with mild forms of cattle plague successfully inducing resistance (Tizard, 2004). During the nineteenth century, epochal advances in immunological research in both animal and man were achieved by e.g. Edward Jenner, Louis Pasteur and Daniel Elmer Salmon constituting the fundamental knowledge about infectious diseases and the immunological response to them (Gross & Sepkowitz, 1998; Last, 1998).

Although research in the fields of immunology, microbiology, hygiene and nutrition has led to eradication of several dangerous diseases, infectious diseases are still a large problem (BSE Inquiry: The Report, 2000). Infectious pneumonia with bovine respiratory syncytial virus (BRSV) and parainfluenza type 3 virus (PI-3 virus) as important pathogens cause major fatalities in dairy calves worldwide (Bryson, 1985: Ames, 1997). Another important health problem in cattle is different diarrhoeal diseases, for example winter dysentery and diarrhoeas caused by bovine viral diarrhoea virus (BVDV) (Trävén, 1993; Kalaycioglu, 2007).

In Swedish cattle, diseases that are common in other parts of Europe, e.g. brucellosis, Mycobacterium bovis, tuberculosis, foot-and-mouth disease and Johne’s disease are presently not found (Herlin, Hultgren & Ekman, 2007). With continuously increasing travelling and more flexible export- and import laws for animals within Europe, the risks of transfer of unwanted disease pathogens to Sweden increase. Improved methods for early detection of such diseases are...
therefore important so that programmes to prevent spreading of the pathogens can be effective.

**Mastitis**

Most cases of bovine mastitis are of infectious origin, but trauma, physical injury and hyper-active lymphocytes have also been reported as reasons for mastitis (Tournant, 1995; Bradely, 2002). It is found in a clinical form, characterized by local and systemic signs of inflammation and infection, and in a sub-clinical form, where the milk quality and quantity are deteriorated but clinical symptoms are absent. Most clinical cases of mastitis are acute, while the sub-clinical form of the disease frequently becomes chronic (Barkema, Schukken & Zadoks, 2006). Depending on the degree of local and systemic signs during acute clinical forms of mastitis, the disease is ranked as mild, moderate or severe (Wenz et al., 2001). Severe cases of acute clinical mastitis can be fatal if left untreated (Eberhart, 1984). Despite extensive research on preventive strategies, control programs and treatments of mastitis during the last decades (Eberhart, 1986; Ziv, 1992; du Preez, 2000), it is still considered the most important production disease in cattle of developed countries. That is because of the economic consequences for the farmers, due to unacceptable milk quality and low quantity, veterinary costs, and culling (Rajala-Schultz & Gröhn, 1999; Seegers, Fourichon & Beaudeau, 2003).

Henceforth, attention will be focused on different forms of infectious mastitis. Bovine infectious mastitis can be caused by innumerable pathogens. Since the late 1800s, *streptococci* have been associated with mastitis (Jones, 2005), and by the mid-1900s it was well-established among veterinarians that infectious mastitis could be caused by e.g. *staphylococci*, *streptococci*, coliform bacteria and mycobacteria (Norcross & Stark, 1970; Schultze, Stroud & Brasso, 1985). Since then the list has grown to include mycoplasma, virus, yeast and algae. However, bacterial infections are still the major cause (Bradley, 2002; Wellenberg, van der Poel & van Oirschot, 2002).

Contagious pathogenic bacteria live and multiply in infected mammary glands and can spread from one udder quarter to another within an udder, or from cow to cow. This group of bacteria includes the Gram-positive *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* and several mycoplasma and *Arcanobacterium species* (Bradley, 2002; Kerro-Dego, van Dijk & Nederbragt, 2002). Another group of pathogenic bacteria is present in the animals’ environment and they usually invade the mammary gland by teat contamination. This group of bacteria includes the Gram-negative *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae*, the Gram-positive *Streptococcus dysgalactiae* (*S. dysgalactiae*), *Streptococcus uberis*, and different Bacillus species (Kerro-Dego, van Dijk & Nederbragt, 2002; Persson Waller & Unnerstad, 2004). In Sweden, the yearly incidence of infectious mastitis is about 60%, with most cases being sub-clinical (Swedish Dairy Association, 2007). The most prevalent pathogens, together explaining about half of all reported mastitis cases, are *S. aureus* and *S. dysgalactiae*. Another important pathogen, responsible for the most severe cases of clinical mastitis is *E. coli*.
Udder infections can occur at any time during a cows’ lactation cycle, but higher prevalence of infections with environmental pathogens such as *E. coli* are observed around drying-off and calving (Burvenich *et al.*, 2003; Rajala-Schultz, Hogan & Smith, 2005; Burvenich *et al.*, 2007). Also, the parity number seems to be associated with the susceptibility and severity of clinical mastitis (Gilbert *et al.*, 1993; Vangroenweghe, Lamote & Burvenich, 2005). The variation in susceptibility to udder pathogens is associated with the status of the cow and her immune system. Pregnancy, calving, induction of milk production and drying-off are periods of considerable challenge to the body. These periods involve tissue remodelling and changed nutritional demands which affect the functions of the immune system, making it less capable of fighting infections (Burvenich, *et al.*, 2003, 2007; de Schepper *et al.*, 2008).

**Innate immunity and innate immune responses**

The primary task of the immune system is to protect the host from environmental microbes that threatens it. In general, the immune system is characterized by its capacity to recognize and discriminate between self and non-self (Tizard, 2004). The host is protected by two forms of immune defence mechanisms, the innate and the acquired immunity, that interact closely to eliminate foreign invaders and protect the host. In the innate immunity, non-specific mechanisms recognise and kill microorganisms, while responses of the acquired immunity are specific, leading to selective elimination of pathogens. In addition, repeated exposure to the same antigen amplifies the immune responses of the acquired immune system, *i.e.* the responses are adaptive. Innate immune mechanisms dominate the early stages of an infection while later stages are characterized by actions of the acquired immunity. In this thesis, attention is focused on the induction of the innate immune response.

The first obstacle microbial invaders have to conquer before they can settle in a host is the anatomical and physical barriers of the body (Tizard, 2004). The skin and the mucosal membranes of the respiratory and gastro-intestinal tract constitute the basic physical defence barriers. The individual tries to remove the intruder by coughing, sneezing, vomiting and diarrhoea, but given time a persistent invader often overcomes the physical hindrances. If microorganisms manage to enter the body, the first line of immunological defence, the innate immune system, becomes activated (Chinen & Shearer, 2007; Opitz *et al.*, 2007).

In the innate immune system, phagocytic cells (*e.g.* neutrophils, monocytes and macrophages) together with natural killer (NK) cells, act as constant surveyors of the body, scanning for invaders (Tracey, 2002). When an attack is encountered, a localized inflammatory response is initiated. Inflammation is a local defence mechanism aiming to remove invading agents or initiate a healing process of damaged tissue. That is performed by enhanced blood flow to the site of invasion, locally increasing the quantity of phagocytic cells that can attack and destroy the invaders. Increased blood flow to the inflammatory site also accumulates other antimicrobial factors such as complement components and antibodies at the site of inflammation (Goldsby *et al.*, 2003). Neutrophils and macrophages are the major
phagocytic cells of the body, whereas NK-cells recognise and lyse cells without major histocompatibility complex (MHC) class I molecules on their surface (Tizard, 2004). Actions of the acquired immune system are initiated by antigen presenting cells (APC) displaying parts of invading organisms on their cell-surface receptors. Three major cell types act as APC, macrophages, dendritic cells and B-lymphocytes. The antigen presentation activates the functional cells of the acquired immunity, the lymphocytes. B-lymphocytes produce antibodies against invading microorganisms, helper-T-cells regulate the immune response and cytotoxic T-cells kill virally infected cells and cells without MHC class I on their surface (Tizard, 2004).

The alterations observed during acute inflammatory responses are mediated through release of various inflammatory mediators, foremost cytokines (Koj, 1996; Ebersole & Cappelli, 2000; Tracey, 2002). Cytokines involved in the inflammatory response can roughly be divided into three subgroups: (i) pro-inflammatory cytokines, that initiate and enhance the cascade of events; (ii) interleukin (IL)-6-type cytokines, that induce the systemic actions; (iii) anti-inflammatory cytokines, that down-regulate the inflammatory response. Important mediators in the group of pro-inflammatory cytokines are tumour necrosis factor (TNF)-α, IL-1β and IL-8. Among the IL-6-type cytokines, IL-6 is a prominent actor, but leukaemia inhibitory factor, IL-11, oncostatin M and cardiotrophin-1 are also important members of this group. In the set of down-regulating cytokines IL-4, IL-10, IL-13 and transforming growth factor (TGF)-β have prominent roles.

A wide variety of cells produce cytokines when activated by different stimuli, but macrophages are the dominant producers of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (Tizard, 2004). The main functions of TNF-α, IL-1β and IL-6 are to induce the acute phase response, i.e. starting inflammatory processes such as production of APPs in the liver, attracting neutrophils to the site of inflammation, and activating B-, T- and NK-cells (Moshage, 1997; Goldsby et al., 2003; Petersen, Nielsen & Heegaard, 2004). The initiation of the acute phase response is described in Figure 1.
APPs are produced by hepatocytes, and their blood plasma concentration increases (positive APPs) or decreases (negative APPs) by at least 25% during inflammatory responses (Morley & Kushner, 1982). The positive APPs are produced within a few hours of injury and aid in eradication of pathogens and in wound healing by acting as components in clotting factors, as well as protease inhibitors and metal-binding proteins (Ramadori & Armbrust, 2001). Circulating IL-6 is believed to play the most important role in induction of APP production and the IL-6 synthesis is regulated by TNF-α and IL-1β. Thus, APP production could be considered a synergistic action of those cytokines (Heinrich, Castell & Andus, 1990; Akira, Taga & Kishimoto, 1993). The major functions of the pro-inflammatory cytokines and APPs in early innate immune responses are listed in Table 1.

The concentrations of the major APPs, C-reactive protein (CRP), serum amyloid A (SAA) and haptoglobin increase massively after cytokine stimulation of hepatocytes in many species (Gruys et al., 2005; Ceciliani, Giordani & Spagnolo, 2002). The expression levels of the APPs differ, however, from species to species, proteins considered major APPs in one species might not be relevant in another.
In cows, haptoglobin, SAA and α₁-acid glycoprotein are major APPs, while CRP concentrations not are correlated to inflammatory responses in cows (Petersen, Nielsen & Heegaard, 2004).

Table 1. Origin and some functions performed by a few important mediators during early innate immune responses to infections. Some of the functions described are performed synergistically by more than one cytokine. Information is compiled from Petersen, Nielsen & Heegaard (2004); Tizard (2004 and Horst Ibelgaufs’ COPE with cytokines (2008).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Major source</th>
<th>Important activities in inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour necrosis factor-α (TNF-α)</td>
<td>Macrophages</td>
<td>Chemoattractant for neutrophils</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>Inducing IL-1β and IL-6</td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td>Enhancing B- and T-cell proliferation</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td></td>
</tr>
<tr>
<td>Interleukin-1β (IL-1β)</td>
<td>Monocytes</td>
<td>Chemoattractant for neutrophils</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>Inducing IL-6, IL-8 and APP</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>Stimulating B-, T- and NK-cells</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
<td>Inducing APP production</td>
</tr>
<tr>
<td></td>
<td>Endothelial cells</td>
<td>Aiding in B-cell differentiation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stimulating B- and T-cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibiting TNF-α, IL-1β, IL-6</td>
</tr>
<tr>
<td>Interleukin-6 (IL-6)</td>
<td>Monocytes</td>
<td>Inducing APP production</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
<td>Aiding in B-cell differentiation</td>
</tr>
<tr>
<td></td>
<td>Endothelial cells</td>
<td>Stimulating B- and T-cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibiting TNF-α, IL-1β, IL-6</td>
</tr>
<tr>
<td>Interleukin-8 (IL-8)</td>
<td>Monocytes</td>
<td>Activating neutrophil oxidative burst</td>
</tr>
<tr>
<td></td>
<td>Macrophages</td>
<td>Chemoattractant for all known migratory immune cells</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
<td></td>
</tr>
</tbody>
</table>

The innate immune system recognizes a great number of different pathogens by locating highly conserved motifs shared by several pathogens. These motifs are commonly referred to as pathogen-associated molecular patterns (PAMPs), and include e.g. mannose, double-stranded RNA, cytosine-guanine-rich DNA, and the bacterial cell wall constituents lipopolysaccharide (LPS), peptidoglycan (PGN), and lipoteichoic acid (LTA) (Aderem & Ulevitch, 2000; Zeytun et al., 2007). Cells of the immune system utilize evolutionary conserved pattern recognition.
receptors, Toll-like receptors (TLRs), for recognition of PAMPs. At least ten functional TLRs have been identified in mammals, where each receptor has specificity for PAMPs in bacteria, viruses, fungi or parasites (Chen et al., 2007). Interaction between PAMPs and TLRs on the immune cells induces production of cytokines and other immunological mediators that are important in elimination of pathogenic microorganisms.

A component of the Gram-negative bacterial cell wall is LPS, a component not found in Gram-positive bacteria, where instead PGN is the major component. PGN can also be found to a lesser extent in the walls of Gram-negative bacteria. (Trent et al., 2006) (Figure 2). LPS is recognized by the host mainly through TLR-4, and PGN is detected by TLR-2 (Underhill, 2003). The TLRs are however not capable of inducing inflammatory responses on their own, but need help from other immune-recognition proteins. Both LPS and PGN recognition is associated with the soluble protein lipopolysaccharide binding protein (LBP), and the membrane bound proteins CD14 and MD-2 (Dziarski, R., Tapping, R.I. & Tobias 1998; Dziarski & Gupta, 2000; Beutler, 2000, Weber et al., 2003). Although TLR-4 and TLR-2 elicit different intracellular signalling cascades and therefore different immune responses, the production of pro-inflammatory cytokines is induced by both TLRs (Werling & Jungi, 2003; Bannerman et al., 2004).

Figure 2. Construction of the cell walls of Gram-negative (left) and Gram-positive (right) bacteria. The peptidoglycan layer is thicker in walls of Gram-positive bacteria than in walls of Gram-negative bacteria, where instead lipopolysaccharide (LPS) is found in the outer membrane (Modified from www.gsbs.utmb.edu/microbook, Jan-11, 2008).
Innate immune responses to bacterial mastitis

During clinical mastitis the cardinal symptoms of inflammation i.e. redness, heat and swelling can be observed locally in the udder. The animals often show signs of pain, loss of appetite, fever, increased heart- and respiration rates, diarrhoea, slow reticulo-rumen motility and weakened skin turgor can be present (Lohuis et al., 1988; Vangroenweghe et al., 2005). Other important clinical manifestations are considerably reduced milk yield and changed milk composition (Shuster et al., 1991; Rajala-Schultz et al., 1999). The clinical symptoms during mastitis have mainly been explained by the immune mediated shock and the extensive inflammatory process, and not so much by the invading pathogen (Dosogne et al., 2002; Hoeben et al., 2000; Burvenich et al., 2007).

Ehrlich (1892) was first to report presence of antibodies in milk. He suggested that the mammary gland functioned as a reservoir for antibodies, but had no capacity to produce them. Today it is well established that the mammary gland has substantial immunological capacity, which is of cardinal importance in the defence against pathogenic udder invasion. The basic defence mechanisms include anatomical, cellular and soluble factors that act in coordination and are crucial for mammary gland resistance to bacterial invasion (reviewed by Oviedo-Boyso et al., 2007). During lactating periods, the flushing of milk physically hinders infection and in addition, the milk contains antimicrobial substances such as complement components, lactoferrin, lactoperoxidase and thiocyanate ions, together with different immune cells (Tizard, 2004).

Macrophages, lymphocytes, neutrophils and epithelial cells are the cell types found in healthy milk. The macrophages are the dominant celltype, guarding the gland against intruders (Paape et al., 2000 & 2003). In addition, the antibody subsets IgA and IgG1 are present in milk. IgA is synthesized locally in the udder and IgG1 is actively transferred there from serum (Tizard, 2004). The concentrations of IgA and IgG1 in milk are, due to the continuous milk flow, unfortunately not high enough to exert adequate protective influence.

When bacteria colonize the udder and start proliferating, PAMPs, e.g. LPS, PGN and LTA, are released from dead bacteria. These PAMPs are caught on TLRs on macrophages in the mammary gland. The macrophages internally process their PAMPs, become activated and start to launch the acute mammary inflammatory response as well as activating the adapted immunity. The macrophages perform this by secreting e.g. complement factors, TNF-α, IL-1β, IL-6, IL-8, and arachidonic acid metabolites such as leukotrienes, prostaglandins and platelet activating factor (Tizard, 2004). These mediators guide leukocytes, mainly neutrophils, from blood to the affected mammary gland (Rainard & Riollet, 2006).

In healthy mammary glands, the milk usually contains < 100000 cells/ml, a number that within hours of bacterial infection of the udder can increase to > 1million cells/ml (Harmon, 1994).

Neutrophils and macrophages in the udder ingest and kill pathogens by different means e.g. by production of reactive oxygen intermediates, reactive nitrogen intermediates, hydrolytic enzymes and defensins that all are toxic for devoured pathogens (Paape et al., 1979; Tizard, 2004). Neutrophils are the major effector
cells in bovine innate and adaptive immunity and have been extensively studied (reviews by Paape et al., 2000, 2003; Rainard & Riollet, 2006, Lun et al., 2007).

In order to be effective during bovine mastitis, neutrophils have to migrate rapidly to the site of infection and start to phagocyte and kill bacteria immediately (Paape et al., 2003). Neutrophils can only ingest and kill a limited number of pathogens before they undergo apoptosis, while macrophages can ingest and kill numerous particles during their longer life-span (Paape et al., 2000).

The release of pro-inflammatory cytokines during bacterial mammary gland infections also affects the acquired immune system by activating B- and T-lymphocytes. Also, as mentioned before TNF-α, IL-1β and IL-6 are responsible for the systemic symptoms such as fever, lethargy, malaise, loss of appetite and weight loss seen during some forms of mastitis, in addition with the induction of APP production (Tizard, 2004). (Figure 3).

Methods to study innate immune responses

Different methods to describe, quantify and grade immune responses have been developed (Masseyeff, 1991; Ferré, 1994; Eckersall et al., 1999; Sachdeva & Asthana, 2007). The first mediator in the group later called cytokines was described in the 1950s (Isaacs & Lindenmann, 1957). Since then, molecular characterization of many cytokines has been performed and their functions have been described (Ryan & Majno, 1977; Sikora, 1980; Dinarello, 2000). The cytokine family is continuously expanding and different cytokines have become useful tools for diagnosis and treatment of bovine immunological diseases (Lothhouse et al., 1996; Moore, 1996; Wood & Jones, 2001). Also, APPs frequently serve as markers of inflammation and infection in various species and diseases (reviewed by e.g. Petersen et al., 2004). In cattle, quantification of APPs
in blood plasma, especially SAA and haptoglobin, have been shown to discriminate between acute and chronic inflammatory conditions (Horadagoda et al., 1999; Eckersall et al., 2001).

Quantification of cytokines and APPs

Under normal circumstances cytokines are undetectable or found in very low levels in body fluids and tissues (Sachdeva & Asthana, 2007), but high levels can be found early in the inflammatory process. Since different cytokines can be related to specific groups of pathogens it is valuable to quantify them as early as possible. A wide range of assays for detection of cytokines in different biological samples are available. Depending on the type of information required, different ways to detect the cytokines are used, some methods assess the biological activity, others the mRNA expression, and yet others the secretion of a cytokine or its soluble receptor.

Cytokines and their soluble receptors are usually analysed in body fluids and cell-supernatants with Enzyme-Linked Immunosorbent Assay (ELISA), Radioimmunoassays (RIA), chemiluminescence or bioassays. When the cytokine production from specific cell populations or individual cells are of interest, methods such as multi-parametric flow cytometry, different mRNA-based assays (reverse-transcriptase linked polymerase chain reaction (RT-PCR), Northern blotting, In situ hybridisation (ISH), RNA-protection assays), intra-cytoplasmic cytokine staining, and Enzyme-Linked Immunospot (ELISPOT) can be used. To detect presence of cytokines in tissues, immunostaining and different mRNA based assays usually are performed. Each assay has its advantages and limitations. Standard ELISA is for instance not suited for high throughput analyses since it only measures one analyte at the time, and RT-PCR, which can be performed in multiplex style, measure gene expression and not the proteins.

Coming technologies for cytokine quantification seem to be in multiplex formats, i.e. several cytokines are analysed simultaneously in the same sample. Examples of multiplex technologies are multiplex ELISA, DNA- and protein micro-arrays and microsphere-based multiplex flow cytometric assays (Grøndahl-Hansen, et al., 2003; Wilson et al., 2005; Andersson et al., 2007). Simultaneous assessment of several cytokines in a biological sample is probably more valuable than measuring the absolute concentrations of a single component, since they act together (Ebersole & Cappelli, 2000; Sachdeva & Asthana, 2007).

Unlike the cytokines, APPs can often be found in low levels in body fluids from healthy individuals of different species (Bannerman et al., 2003; Ledeu & Rifai, 2003). The feature that makes them interesting as markers of inflammation and infection is their rapid elevation in blood plasma when the immune system is activated (Blackburn, 1994; Malle & DeBeer, 1996; Jensen & Whitehead, 1998; Heegard, et al., 2000). In cows with mastitis, hundredfold higher SAA plasma concentrations have been recorded compared to concentrations in healthy cows (Eckersall et al., 2001). Most frequently used assays for detection of bovine APPs are ELISA, mRNA based assays, bioassays and biosensor assays (Eckersall et al., 1999, Åkerstedt et al., 2006).
Particle based flow cytometry

Flow cytometry is a powerful and fast technique where fluorescent and light scattering properties of cells or particles are analyzed (Shapiro, 2003). Particles are lead through an illuminating beam of coherent light, usually originating from a laser. The scattered laser light and emitted fluorescence is translated to information about the cell. Flow cytometry can be used to analyse intracellular and surface properties of cells and particles, distinction between live and dead cells can be made and physical sorting of cells with interesting properties can be performed.

In 1975 Knapp, et al. introduced antigen-coated microspheres with fluorescent labels as solid phase for capture and quantification of antigen-antibody reactions. That technique has been continuously improved and new applications have been found (Phillips et al., 1980; Wanda & Smith, 1982; MacCrindle, Schwenzer & Jolley 1985; Frengen et al., 1994). For instance, in 1982, Lisi et al. described a fluorescence immunoassay where diametrically different microspheres were used, enabling detection of different analytes in the same sample. In 1997 Fulton et al. presented the LabMAP technology (Luminex Corporation, Austin, TX, USA), presently called the xMAP technology. The new technique combines microsphere immunoassays, flow cytometry, rapid digital signal processing and multiplexing. Multiple analytes can individually and quantitatively be detected simultaneously in small volumes of samples with reliable statistics, as data are collected from at least 100 microspheres for each analyte.

Like in conventional immunoassays, the xMAP technique utilizes a solid and a soluble phase, where polystyrene microspheres function as the solid phase. Capture antibodies, often monoclonal, are covalently coupled to the microspheres, and the amount of bound analyte is determined with the use of fluorophore-coupled detection antibodies. In order to translate fluorescence intensities to quantitative data, the microspheres are analysed in a dedicated flow cytometer. To allow multiplexing, different microsphere subsets are internally dyed with a mix of far red and infrared fluorophores, giving them unique identities (Figure 4). By coupling different monoclonal antibodies to different microsphere subsets, multiplexing is possible. The dedicated flow cytometer used for xMAP assays is equipped with two lasers, one for classification of microspheres and one for quantification of the amount of analyte in the sample. For classification, a red laser exciting the red and infrared dyes inside the microspheres is used, and for quantification, a green laser exciting the molecules attached to the reporter antibody, resulting in orange fluorescence, is used.
Figure 4. Basic principles of the xMAP technique. To the left, an example of a sandwich immunoassay performed on a microsphere is shown. Capture antibodies are covalently coupled to microspheres, the analyte of interest binds to the capture antibody and is detected by a fluorophore-coupled reporter antibody. Note the proportionally different scales of the microsphere and the antibodies bound to it, the antibodies should be considerably smaller. In the middle, the 100 unique subsets of internally dyed microspheres are illustrated and to the right the two lasers in the flow cytometer, one for classification of the microsphere and one for quantification of analyte are depicted (With permission from Breackmans et al., 2003).

The microsphere subsets can either be purchased antibody-conjugated or conjugation-ready i.e. covered with avidin, carboxyl groups or oligonucleotide adapters. When microspheres with carboxyl groups are utilized, covalent coupling is usually performed using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS). EDC reacts with the carboxyl groups on the microspheres, forming amine-reactive intermediates which sulfo-NHS stabilizes by converting them to amine-reactive sulfo-NHS esters, thus increasing the efficiency of the coupling reaction (Staros, Wright & Swingle, 1986).

Other multiplex particle based flow cytometric assays are available on the market. BD Biosciences (San Jose, CA, USA) has for instance developed the cytometric bead array (CBA), where microspheres with different fluorescence intensities from a single fluorophore are used for capture and quantification of soluble particles (Morgan et al., 2004). Like the xMAP technique, an immunological sandwich assay is performed in the CBA. The main difference between the techniques, apart from the different internal colouring system, is that a dedicated flow cytometer is not necessary to carry out the CBA. Instead, it can be performed with any multi-use flow cytometer.

The xMAP technique comes with conjugation-ready microspheres and commercially available kits containing antibody-coupled microspheres and detection antibodies. The CBA technique only operates with commercially available kits. Kits are available for e.g. cytokines, phosphoproteins, cancer markers, lipoproteins and endocrine markers in human, mouse and rat samples.

Since Carson & Vignali (1999) reported simultaneous detection of 15 murine cytokines in a single sample by the use of the xMAP technique, numerous studies on cytokines, inflammatory mediators, antibodies, growth factors and apoptotic
markers in various biological fluids in health and disease, have been performed with particle based flow cytometric assays (Cook et al., 2001; Kuller et al., 2005; Elshal & McCoy, 2006). Most studies have been focused on cytokines in human samples (Camilla et al., 2001; de Jager et al., 2005), but measurements have also, apart from the work in this thesis, been conducted in pigs, rodent and horses (Johannisson et al., 2006; Zhong et al., 2007; Go et al., 2008).

The technique has become more versatile since the microspheres can be purchased carrying avidin, carboxyl groups or oligonucleotides. Studies on for instance genotyping, endocrine markers, allergens, enzymes and tumor markers in different species have been reported (Thrailkill et al., 2005; Dolezalova et al., 2007; Haasnot & de Pré, 2007; Sun et al., 2007; Zhu & Salmeron, 2007).

All new laboratory techniques must be evaluated on the reproducibility, precision and accuracy. This is usually performed by comparison to existing methods. Microsphere based flow cytometric assays have been compared to ELISA (Prabhakar, Eirikis & Davis, 2002; Thrailkill et al., 2005; Pang et al., 2005; Elshal & McCoy, 2006; Lash et al., 2006). Conclusions from those studies are good correlation but poor concurrence of quantitative values between the assays. This is probably due to different antibody clones, blocking agents and detection systems in the different assays.

The ex vivo whole blood stimulation assay (WBA)

Cytokines are potent mediators of the immune system, and they are often only secreted transiently in low concentrations even during inflammatory states (Eskay, Grino & Chen, 1990; Bemelmans, van Tits & Buurman, 1996; Kelso, 1998). This makes detection of them in body fluids difficult. By stimulating whole blood samples with specific pathogens or fragments of pathogens ex vivo the immunological responsiveness in an individual can be monitored (Finch-Arietta & Cochran, 1991; Wouters et al., 2002).

In whole blood stimulation assays (WBA), leukocytes are used in an environment mimicking their natural one. This has advantages compared to both in vitro and in vivo studies. Monocytes extracted from blood to be used in studies in vitro tend to become activated by handling (Desch et al., 1989; Allen et al., 1992). Also, when monocytes are cultured in a medium, cytokines, receptors, hormones, metabolites and other components in the blood that naturally interact with the monocytes are lost. In the ex vivo assays, repeated challenges with different doses of endotoxin in samples from the same individual are possible, something that cannot be performed as easily during in vivo studies.

Ex vivo WBA with LPS as the stimuli have been useful for measurements of TNF-α, IL-1β, and IL-6 responsiveness in healthy individuals of several species (Finch-Arietta & Cochran, 1991; Zangerle et al., 1992; Wouters et al., 2002; Carstensen, Røntved & Nielsen, 2005; Røntved et al., 2005). Several studies suggest that the severity of clinical symptoms during E. coli mastitis is correlated to the cows’ capacity of pro-inflammatory mediator production (reviewed by e.g. Burvenich et al., 2007). Investigations of the pro-inflammatory cytokine responsiveness to LPS could be valuable in terms of susceptibility analyses to Gram-negative bacterial infections.
During acute infections and active inflammatory diseases the production of TNF-α, IL-1β and IL-6 has a pivotal role in initiating the immunological protection processes. It is however important that the production is closely regulated. High blood plasma concentrations of TNF-α have been associated with severe clinical mastitis and septic shock (Sordillo & Peel, 1992; Sordillo, Pighetti & Davis, 1995). Cows with a balanced immune system stand a better chance to successfully eliminate pathogen invasion, for instance in the udder. Whether the differences in capacity to produce TNF-α after various stimuli in healthy individuals have a connection to the genetic background has been studied. Louis et al. (1998) showed that an individuals’ degree of TNF-α production is predisposed by genetic background, while de Jong et al. (2002) on the contrary showed that it is not. In a study performed on cows by Elsasser, Blum & Kahl (2005), the degree of TNF-α production after LPS stimulation was shown to be genetically regulated and also inheritable.

Bovine ex vivo studies on TNF-α responsiveness to LPS have strengthened the WBAs’ usefulness for monitoring of the in vivo innate immune response (Elsasser, Blum & Kahl, 2005; Røntved et al., 2005). Therefore extended information about the immune function connected to mammary E. coli infections in cows could be gained by quantifying the responsiveness of TNF-α, IL-1β and IL-6 to LPS and E. coli. Also, if correlations between the responsiveness to TNF-α, IL-1β or IL-6 ex vivo and the severity of clinical E. coli mastitis could be established, the WBA may be used as a predictive tool for the severity of clinical mastitis in cows. The ability to predict the intensity of a response to different immunological challenges in livestock could also improve breeding programs, where TNF-α response data could be used to identify a genetic predisposition for a problem that could develop in calves of specific breeding lines.
Aims

The overall aim of the present study was to develop new techniques for quantification of bovine pro-inflammatory markers, with emphasis on cytokines and APPs, and to apply the techniques, using mastitis as a disease model.

The specific aims were:

- to investigate the ability of commercially available antibodies against human TNF-α to cross-react with bovine TNF-α using the xMAP technique.
- to investigate if the xMAP technique is suitable for quantification of the cytokines TNF-α, IL-1β and IL-6 in singleplex and multiplex assays in bovine samples.
- to apply the xMAP technique for TNF-α, IL-1β and IL-6 in studies on bovine milk and blood plasma samples from cows with mastitis.
- to evaluate if \textit{ex vivo} LPS and \textit{E. coli} induced production of TNF-α, IL-1β and IL-6 in blood samples from healthy cows can predict their immunological response after intramammary \textit{E. coli} inoculation.
- to evaluate if concentrations of TNF-α, IL-1β and IL-6 in milk and blood plasma after intramammary \textit{E. coli} inoculation are correlated to each other and to the severity of clinical signs.
- to investigate if the xMAP-technology is applicable for multiplex detection of the cytokine IL-1β and the acute phase proteins SAA and LBP in bovine biological samples.
Materials and methods

In this section of the thesis material, methods and experimental procedures are summarized and commented. Details about the materials and methods used in Papers I-IV are described in each paper, respectively.

Animals and infection models

All experimental handling with animals were approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden or by the Danish Animal Experiments Inspectorate. Milk somatic cell count (SCC) and milk yield was observed daily during all the experimental protocols. Systemic and local clinical signs were monitored in the cows. Systemic signs included general attitude, appetite, saliva secretion, rectal temperature, heart rate, respiration frequency, rumen contraction frequency, and signs of diarrhoea. The udder was inspected for signs of inflammation and the milk composition was analysed. Fresh milk was aseptically collected for bacterial examinations and SCC determination. Milk samples for analyses of APPs and cytokines were collected in test tubes, aliquoted and frozen in -20°C until use. Blood samples used for analyses of APPs and cytokines were aseptically aspirated in EDTA vacuettes from the jugular veins of the cows and centrifuged at 4°C so plasma could be collected, aliquoted and frozen in -80°C.

Paper I

Samples with known concentrations of TNF-α from two different experimental studies were included in Paper I. Milk samples were obtained from a study in which six clinically healthy cows of the Swedish Red and White, and Swedish Holstein breeds had been intramammary inoculated with S. aureus (Grönlund et al., 2003). Blood samples were obtained from another study, in which five Danish Holstein Friesian cows had been intravenously challenged with LPS from E. coli (Røntved et al., 2005).

Paper II – III

Milk and blood samples from ten high-yielding clinically healthy Danish Holstein cows in the midst of their first or second lactation, intramammary inoculated with live E. coli were used. The cows had a daily mean ± SD milk yield of 35 ± 5 kg and a SCC < 84000 cells/ml at the start of the experiment. In Paper II, blood and milk from six of the cows were included, while blood and milk samples from all ten cows were included in Paper III.

Paper IV

Blood plasma samples from two Danish Black and White cows were used. The udder of one cow had been inoculated with LPS from E. coli first and PGN from S. aureus two weeks later. The other cow was treated in the reverse order. The cows had a daily milk yield of 30 – 35 kg and a SCC < 110000 cells/ml at the start of the experiment.
Laboratory analyses

Bacterial growth and SCC in milk (Papers I – III)

Bacterial analyses of milk were performed according to standard procedures at the Section of Mastitis, National Veterinary Institute, Uppsala, Sweden, and according to standard procedures at the Department of Animal Health, Welfare and Nutrition, Research Centre Foulum, Tjele, Denmark. SCC was determined in fresh milk samples by analysis with a fluorooptical method on a Fossomatic instrument (Foss Electric Ltd, Hillerød, Denmark).

The xMAP assays

Microspheres

Different subsets of polystyrene microspheres (Ø 5.6 μm) with carboxyl groups on the surfaces, internally dyed with far red and infrared fluorophores, were used in all xMAP assays. In Papers I-II the microspheres were purchased from Luminex Corporation (Austin, TX, USA) and in Papers III - IV the microspheres originated from Bio-Rad Laboratories (Hercules, CA, USA).

Antibodies and recombinant proteins

In the xMAP assays, monoclonal antibodies against human TNF-α (Paper I), bovine TNF-α (Papers II – IV), ovine IL-1β (Papers II – IV), bovine IL-6 (Papers II – III), bovine SAA (Paper IV) and bovine LBP (Paper IV) were used. The polyclonal antibodies were directed against the same species except for IL-6 which was directed against ovine IL-6. As standards were recombinant bovine TNF-α, ovine IL-1β, bovine IL-6, bovine SAA and human LBP applied.

The anti-bovine LBP antibodies used in Paper IV are validated by the manufacturer (HyCult Biotechnology, Uden, The Netherlands) to cross-react with the recombinant human LBP used as standard. In addition, the same antibody clones have previously been employed in sandwich-format for detection of LBP in bovine samples (Bannerman et al., 2003). The antibodies against ovine IL-1β are guaranteed by the manufacturer (AbD Serotec, Oxford, England) to detect bovine IL-1β. The recombinant ovine IL-1β has earlier worked successfully as standard for IL-1β detection in bovine samples (Persson Waller et al., 2003).

Coupling of antibodies to microspheres

Before an xMAP assay was performed, antibodies were coupled to microspheres. The coupling was performed in two steps: (1) activation of the carboxyl groups on the microspheres using EDC and sulfo-NHS in sodium phosphate buffer (2) covalent coupling of antibodies to the microspheres. The antibody-coupled microspheres were kept protected from light at 2 – 8 ºC, until use.

The Luminex 100 System

In all xMAP assays, the Luminex 100 instrument and the Luminex XY platform from Luminex Corporation were utilized. The XY platform allows analyses to be performed in 96-well-plates instead of in single tubes. The settings for the
classification and quantification lasers were calibrated daily, using special calibration microspheres of known fluorescent light intensities (Bio-Rad Laboratories), when the laboratory work in Papers I – IV was performed. Validation of the optics, fluidics, reporter- and classification systems was performed every third month, as suggested by the manufacturer, also with special validation microspheres bought from Bio-Rad Laboratories.

In Paper I, the software Luminex Data Collector 1.7 (Luminex Corporation) was used, while BioPlex Manager 4.0 (Bio-Rad Laboratories) was employed in Paper II - IV. Both softwares provide information on mean and median fluorescence intensity, standard deviations and number of microspheres of each subset in combination with data regression analysis. Analyte concentrations were obtained by interpolation from standard curves calculated with a five parametric regression model.

Validation

All singleplex and multiplex (duplex and triplex) xMAP assays used in the studies were optimised and validated before use. The concentrations of capture antibodies on the microspheres and the antibodies used for detection- and reporting were determined by titration. Limits of detections (LOD), linear ranges of the standard curves and intra- and inter-assay variations were calculated for the assays. Also, the recovery percentage of an analyte in milk and plasma as compared to buffer and cross-reactivity between the different analytes in the multiplex assays were studied.

Quantification of TNF-α, IL-1β, IL-6, SAA and LBP (Paper I - IV)

In Paper I, four milk and four serum samples from the *S. aureus* mastitis study and the LPS challenge study, respectively, were used. They were integrated as standards in a commercially available Fluorokine MAP kit (R&D Systems, Minneapolis, MN, USA) directed towards human TNF-α. In addition, the samples were used to test cross-reactivity with antibodies against human TNF-α in our lab.

Milk and plasma samples collected from six cows at one occasion before, and one occasion after inoculation were used to validate singleplex and multiplex xMAP assays for TNF-α, IL-1β and IL-6 in Paper II. In Paper III, the singleplex assays developed in Paper II were used for quantification of IL-1β and IL-6 in the samples collected before and after inoculation of *E. coli*. In Paper IV, the plasma samples were analysed for their content of IL-1β, SAA and LBP using a triplex xMAP assay developed and validated in our lab.

The *ex vivo* WBAs (Paper III)

The WBA for bovine blood was developed and described by Røntved *et al.* (2005). In Paper III, a modified version where duplicate blood samples were incubated with Dulbecco’s Modified Eagle’s Medium (DMEM), phosphate buffered saline (PBS), LPS, heat-killed or live *E. coli* for 3.5 or 24 hours, was conducted. The WBA was carried out on two consecutive days before the start of the *E. coli* mastitis experiment and mean cytokine concentrations of the two days were calculated. Based on the concentrations of TNF-α, IL-1β and SAA after stimulation with LPS and live *E. coli*, the cows were grouped as high or low
responders for each cytokine. The cows were ranked 1-10, and the cow with the highest cytokine response was ranked nr 1. For each cytokine, the cows ranked 1-5 were grouped as high responders and the cows ranked 6-10 were grouped as low responders. The groups were used for further evaluation of differences in susceptibility to \( E. \text{coli} \) infection, severity of clinical symptoms, and cytokine concentrations in milk and plasma between the high and low responders.

**ELISA assay (Paper III)**

The ELISA used for quantification of TNF-\( \alpha \) in blood samples from the \( \text{ex vivo} \) WBA and in all milk and plasma samples from the \( E. \text{coli} \) mastitis study was originally described by Ellis et al. (1993) and we used it with the modifications described by Røntved et al. (2005).

**Statistical analyses**

Data obtained from the Luminex 100 were evaluated with BioPlex Manager 4.0 (Bio-Rad Laboratories) and data are presented as mean value ± standard deviation (SD) as interpolated from standard curves calculated with a five parametric regression model. The statistical calculations of obtained results were performed with Minitab 15 (Minitab Inc., Coventry, England) and Statistical Analysis Systems (SAS) 9.1 (SAS Institute Inc., Cary, NC, USA).

A p-value < 0.05 was regarded as statistically significant in all studies, and all results shown are significant, unless something else is stated.

**Paper I**

The limit of detection (LOD), defined as the lowest concentration of each analyte that can be detected, was established for the different antibody clones in the xMAP and Fluorokine MAP assays. The mean fluorescence intensity (MFI) for six replicates containing microspheres coupled with capture antibodies against each analyte, detection antibodies against each analyte, and assay buffer was calculated, and LOD was defined as average MFI + 3 SD, as suggested by the International Conference on Harmonization (1994).

Recovery percentages in serum and milk were determined by setting the MFI from experiments conducted in buffer to 100% and calculating the results from analyses of the same analytes in serum and milk as percentages ± SD.

**Papers II and IV**

LOD was established for all analytes in singleplex and multiplex xMAP assays, as described for Paper I with the modification that eight replicates was used. Differences between the LODs for each analyte were evaluated by paired Student’s t-test. Intra- and inter-assay coefficients of variation (CV’s) were calculated for each analyte by the formula: SD/mean x 100.

Cross-reactivity between reagents included in the multiplex xMAP assays were evaluated by assays where different antibody-coupled microspheres directed against the different analytes in the multiplex assay were incubated with only one
antibody at the time. Paired Student’s t-tests were applied on these data. In Paper II, paired Student’s t-test was also applied on the data to examine whether equivalent results were achieved using singleplex and triplex xMAP assays.

**Paper III**

In the WBA, the effects of different stimuli *ex vivo* on the cytokine concentrations in blood samples were analysed with a non-parametric multiple comparison test with randomized block design, the Friedman’s test. Differences in cytokine concentrations due to incubation time and different *E. coli* doses were evaluated with a non-parametric two-sample rank test, a Mann-Whitney U test in the WBA. Based on the results obtained in the WBA, the cows were grouped as high (H) and low (L) responders for each cytokine, and differences in severity of clinical symptoms and concentrations of cytokines in milk or plasma were analysed with Mann-Whitney’s U test.

Pre-inoculation mean values ± SD was determined for heart rate, respiration, milk yield and SCC. The pre-inoculation mean value ± 2 SD was considered normal and values above or below were considered abnormal. The effect of time post inoculation (pi) on concentrations of milk and blood cytokine concentrations were analysed with the Friedman test. A general linear model analysis of covariance (ANCOVA) where time pi and within-cow covariance were compensated for was used for determination of associations between cytokine concentrations in milk and plasma pi and clinical signs, and between cytokine concentrations in milk and plasma, respectively. In order to do statistical analyses of the data, which not was normally distributed, data was transformed to a Poisson distribution prior to analysis.
Results

Development of xMAP assays (Papers I, II and IV)

Singleplex xMAP assays for TNF-α, IL-1β, IL-6, SAA and LBP were developed and successfully applied for detection of the analytes in milk and blood samples from cows with mastitis, using ovine and bovine reagents. In addition, duplex assays for simultaneous detection of IL-1β and IL-6, TNF-α and IL-1β, or TNF-α and IL-6 as well as triplex assays for simultaneous detection of TNF-α, IL-1β and IL-6 or IL-1β, SAA and LBP were developed and used for detection of the analytes in bovine milk and blood samples.

Antibody clones cross-reacting between species

All antibody clones directed against human TNF-α evaluated in Paper I could detect recombinant human TNF-α (rhuTNF-α) using the xMAP assay. Two antibody clones, Mab11 (BD Pharmingen, San Diego, CA, USA) and 6401.1111 (BD Biosciences, San Diego, CA, USA), could also detect recombinant ovine TNF-α (rovTNF-α) in concentrations greater than 2.5 ng/ml. None of the antibody clones tested could, however, detect TNF-α in bovine milk or serum samples. The Fluorokine MAP-kit from R&D Systems detected very low concentrations (about 4 pg/ml) of rhuTNF-α from two different sources, R&D Systems and AbD Serotec. RovTNF-α could, however, not be detected with the Fluorokine MAP-kit, nor could TNF-α in the bovine milk and serum samples with known concentrations of TNF-α.

Singleplex and multiplex xMAP assays

In Paper II singleplex, duplex and triplex xMAP assays for TNF-α, IL-1β and IL-6 were developed, validated and used for detection of cytokines in milk and plasma from cows with mastitis. In singleplex assays the LODs for TNF-α, IL-1β and IL-6 were 0.5, 0.08 and 0.2 ng/ml, respectively. Corresponding LODs in the triplex assay were 3.5, 2.0 and 6.5 ng/ml.

In Paper IV, singleplex and triplex xMAP assays were designed, validated and applied for detection of IL-1β, SAA and LBP in plasma samples from cows with mastitis. LODs were for IL-1β, SAA and LBP 0.1, 2.0 and 1.0 ng/ml, respectively, in the singleplex assays, and 0.4, 3.8 and 0.8 ng/ml in the triplex assay. A selection of the intra- and inter-assay coefficients of variation (CV’s) obtained for the different xMAP assays developed in Paper II and IV are shown in Table 2.
Table 2. Intra- and inter-assay variations of singleplex (A) and triplex (B) xMAP assays of rovIL-1β, rboIL-6, rboTNF-α, rboSAA and rhuLBP. Samples with 25, 100 or 200 ng/ml of recombinant analyte were analysed.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Intra-assay Mean (ng/ml)</th>
<th>SD</th>
<th>CV (%)</th>
<th>Inter-assay Mean (ng/ml)</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rboTNF-α</td>
<td>114.2</td>
<td>1.7</td>
<td>1.5</td>
<td>218.2</td>
<td>11.1</td>
<td>5.1</td>
</tr>
<tr>
<td>rovIL-1β¹</td>
<td>123.1</td>
<td>4.8</td>
<td>3.9</td>
<td>24.6</td>
<td>3.5</td>
<td>14.2</td>
</tr>
<tr>
<td>rovIL-1β²</td>
<td>23.7</td>
<td>1.0</td>
<td>4.1</td>
<td>28.3</td>
<td>4.1</td>
<td>14.6</td>
</tr>
<tr>
<td>rboIL-6</td>
<td>86.9</td>
<td>9.3</td>
<td>10.7</td>
<td>185.0</td>
<td>37.2</td>
<td>20.1</td>
</tr>
<tr>
<td>rboSAA</td>
<td>29.1</td>
<td>5.3</td>
<td>18.0</td>
<td>24.4</td>
<td>5.2</td>
<td>21.4</td>
</tr>
<tr>
<td>rhuLBP</td>
<td>25.7</td>
<td>4.0</td>
<td>15.5</td>
<td>31.5</td>
<td>6.4</td>
<td>20.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Intra-assay Mean (ng/ml)</th>
<th>SD</th>
<th>CV (%)</th>
<th>Inter-assay Mean (ng/ml)</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rboTNF-α</td>
<td>127.4</td>
<td>7.9</td>
<td>6.2</td>
<td>195.3</td>
<td>17.2</td>
<td>8.8</td>
</tr>
<tr>
<td>rovIL-1β¹</td>
<td>131.1</td>
<td>27.8</td>
<td>21.2</td>
<td>21.5</td>
<td>8.5</td>
<td>39.6</td>
</tr>
<tr>
<td>rovIL-1β²</td>
<td>25.6</td>
<td>0.8</td>
<td>3.2</td>
<td>23.9</td>
<td>1.6</td>
<td>6.6</td>
</tr>
<tr>
<td>rboIL-6</td>
<td>92.6</td>
<td>21.5</td>
<td>23.2</td>
<td>174.2</td>
<td>66.2</td>
<td>38.0</td>
</tr>
<tr>
<td>rboSAA</td>
<td>24.7</td>
<td>1.5</td>
<td>6.0</td>
<td>25.8</td>
<td>5.1</td>
<td>19.8</td>
</tr>
<tr>
<td>rhuLBP</td>
<td>27.7</td>
<td>2.1</td>
<td>7.7</td>
<td>20.1</td>
<td>4.2</td>
<td>20.7</td>
</tr>
</tbody>
</table>

¹IL-1β evaluated in Paper II, ²IL-1β evaluated in Paper IV.

In Paper I, rhuTNF-α was added to bovine milk and serum samples and the recovery could be detected using the Fluorokine MAP-kit. In milk, the recovery was 59.8 ± 2.0% while it in plasma was 36.4 ± 1.6%. That suggests that components of the matrix interfere with the antibody assay. Also during the development work for Papers II and IV interference due to matrices was observed.

Reliable standard curves could be produced for all the above mentioned analytes in both singleplex and multiplex assays. The linear ranges of the curves varied between the analytes and for all of them the range was wider in singleplex assays than in multiplex assays. Standard curves from the two triplex assays (TNF-α, IL-1β, IL-6 and IL-1β, SAA, LBP, respectively) are shown in Figure 5.
Figure 5. Mean fluorescence intensities of representative standard curves of triplex xMAP assays for recombinant bovine TNF-α, recombinant ovine IL-1β, recombinant bovine IL-6 at the top. At the bottom are standard curves for recombinant ovine IL-1β, recombinant bovine SAA, and recombinant human LBP shown.
**Experimental E. coli mastitis (Paper III)**

Six of ten cows became infected and developed moderate clinical mastitis after the *E. coli* inoculation. Detectable concentrations of *E. coli* bacteria were found in all infected mammary gland quarters pi. Fever and reduced daily milk yield were observed in all infected cows. Looser faeces consistency was observed at least once in all cows, both infected and non-infected. Local symptoms of the udder, such as redness, swelling, elevated SCC, changed milk colour and composition, were recorded in all infected cows from 18 hours pi until the end of the study. Also the non-infected cows showed sporadically elevated SCC pi.

**Concentrations of TNF-α, IL-1β and IL-6 in milk and plasma**

The variation in cytokine response after the *E. coli* inoculation was large, but significantly elevated concentrations of TNF-α, IL-1β and IL-6 were found in milk and plasma from all the infected cows. Except for the mean peak concentration of IL-1β in milk (72 hours pi), the mean peak concentrations of all cytokines were observed within the first 24 hours pi in both milk and plasma. High concentrations of TNF-α, IL-1β and IL-6 in plasma, and TNF-α in milk during the *E. coli* mastitis were associated with more severe clinical signs.

**The ex vivo WBAs (Paper III)**

Incubation with LPS and *E. coli* in the *ex vivo* WBA stimulated the leukocytes to produce TNF-α, IL-1β and IL-6. Elevated levels were observed in blood samples after both 3.5 and 24 hours, with highest concentrations of all cytokines at 3.5 hours. Two cows were ranked high responders for all three cytokines, two were ranked low responders for all three cytokines and six were ranked differently for the different cytokines.

Two cows ranked low for all cytokines, one cow ranked high for all cytokines and three cows ranked differently for the different cytokines became infected by the *E. coli* inoculation. One cow ranked high for all cytokines, and three cows ranked differently depending on cytokine were not infected by the *E. coli* inoculation. No significant differences between the pre-inoculation high and low responders and the TNF-α, IL-1β and IL-6 production in milk and plasma pi were observed. The diarrhoea score and the heart rate pi differed between the pre-inoculation high and low groups for IL-1β and IL-6, respectively.

**Experimental E. coli LPS and S. aureus PGN mastitis (Paper IV)**

Detectable concentrations of IL-1β, SAA and LBP were found in all plasma samples analysed. In both cows the LPS inoculation induced higher concentrations of SAA and LBP than the PGN inoculation. IL-1β was found in highest plasma concentrations after LPS inoculation in one cow and after PGN inoculation in the other cow. LPS induced a peak in the LBP plasma concentration in both cows while PGN only induced LBP production in one cow and not the other.
Discussion

This thesis is focused on development of microsphere based flow cytometric assays for detection of bovine markers of inflammation and infection. The reasons for this was that well-functioning immunoassays was lacking for immunologically interesting markers in different domestic animal species, and that flow cytometric microsphere assays previously had been proven successful in studies on rodents and humans. In this thesis, the xMAP technique was shown to function in bovine samples, allowing simultaneous detection of cytokines and APPs. The usefulness of the assays designed for bovine samples would be increased with further work on assay development. With the continuously expanding range of commercially available reagents, this technique has great potential of replacing less versatile techniques used today.

Methodological considerations

Antibody clones cross-reacting between species

In the field of veterinary immunology, an important problem is the lack of reagents for immunoassays. In several cytokine and APP studies on cattle, antibodies against ovine or human homologues of the analytes are utilized, and human recombinant proteins are often applied as standards (Horadagoda et al., 1993; Sluster, Kehrl & Stevens, 1993; Bannerman et al., 2003; Grönlund et al., 2003; Lahouassa et al., 2007; Sohn et al., 2007). Biological cross-reactivity between antibodies and cytokines or APPs of different species is evident, for instance several commercially available assays are validated for use in multiple species (SAA-ELISA, Tridelta Ltd, Maynooth, Ireland; LBP-ELISA, HyCult Biotechnology, Uden, Netherlands). The cross-reactivity between antibodies against a protein homologue in another species is often not as strong as within a species. Therefore, absolute concentrations achieved in such assays should be interpreted with caution. The sensitivity is, however, often enough for illustration of large concentration changes within animals.

In Paper I, all antibodies tested against human TNF-α could detect rhuTNF-α when applied in the xMAP technique, indicating that the technique worked satisfactorily. Other studies conducted in our laboratory have proven that the xMAP method can be used with reliable results. Successful cytokine measurements have been performed in human blood plasma and serum, brain tissue from mice and in porcine blood serum and lung lavage (Stricklin & Arvidsson, 2004; Johannisson et al., 2006; Tyvold et al., 2008).

Two of the antibody clones in Paper I identified rovTNF-α while none of the evaluated clones detected TNF-α in bovine samples. The detection level of rovTNF-α using anti-human TNF-α antibodies was, however, not satisfactory, given that cytokines are powerful signal molecules that induce effects at low concentrations (Kelso, 1998). The results from Paper I are probably explained by inability of the antibody clones to cross-react between species. Antibodies against
bovine TNF-α have become available, but this is not yet the case for antibodies against many other animal proteins.

Another study where cross-species reactivity of antibodies was investigated was conducted by Pedersen et al. (2002). In that study, monoclonal antibodies against different human, ovine or bovine cytokines were investigated for their ability to detect homologous cytokines produced by blood cells from different species. Antibodies directed against ovine TNF-α detected not only ovine TNF-α but also TNF-α produced by bovine, caprine, porcine, mink and human cells. The antibody clone against IL-6 also cross-reacted between species and could detect ovine, bovine, caprine, porcine, canine, mink and human IL-6, although to lesser extent than the anti-ovine TNF-α clone.

The reason that cross-species reactivity between antibodies and homologous immunological proteins can occur depends on their high conservation through evolution, thus they have similar structures in different species (Syversen et al., 1994; Scheerlinck, 1999; Soller et al., 2007). In the two latter reports, the origin of cytokines in human, mouse, sheep, cow, pig, rat, dog, cat and horse were investigated. The conclusions were that more than 60% amino acid homology between cytokine homologues usually allowed for cross-reactivity between species, and that cross-reactivity was seen more frequently with higher amino acid homology. When the coding sequences of IL-1β were compared between the species mentioned above, about 70 to 84% identity was observed. Corresponding values for TNF-α were 80 to 94%. In the report by Syversen et al. (1994), a high degree of homology between SAA from humans, sheep, cow, horse, dog, cat and mink, was found. Within the different cytokine and APP genes, some sequences are more well-preserved through evolution than others. If these well-preserved regions function as active site for antibody binding, proteins with low amino acid homology can still have a strong tendency to cross-react.

The xMAP assays

Sensitivity
LODs of the singleplex assays for TNF-α, IL-1β, SAA and LBP, the duplex assay format for IL-1β and the triplex assay for IL-1β, SAA and LBP were lower or comparable with other immunoassays used for detection of these analytes in bovine samples (Goto et al., 1997; Bannerman et al., 2003; Grönlund et al., 2003; Rambeaud et al., 2003; Lehtolainen, Røntved & Pyörälä, 2004). Other assays with even lower detection limits for TNF-α, IL-1β and IL-6 have also been reported (Rainard & Paape, 1997; Whelan et al., 2003; Molina, 2005).

During inflammatory responses the pro-inflammatory cytokines are secreted transiently and in low concentrations (pg-ng/ml). Therefore, sensitive immunoassays with low LODs are necessary. The singleplex assays for TNF-α, IL-1β and IL-6 developed in Paper II had satisfactory LODs, but that was not the case for the duplex and the triplex assays. One reason for this could be that the singleplex assays for TNF-α, IL-1β and IL-6 had to be modified in order to design a functional multiplex assay. For instance, MFI signals generally increase with longer incubation time but at the same time the background noise increases. In the
duplex and triplex analyses the incubation time for IL-6 had to be shortened and this meant that the sensitivity for TNF-α and IL-1β were lost due to proportionally higher background fluorescence.

APPs can, unlike cytokines, be found in low but detectable levels (ng-μg/ml) in milk and blood plasma from healthy cows (Bannerman et al., 2003; Lehtolainen, Røntved & Pyörälä, 2004). In this study the LODs of the APP assays were satisfactory and we could show that the triplex xMAP assay for IL-1β, SAA and LBP was useful for detailed studies on APPs during inflammatory responses.

Linear range
During our studies we generally observed wider linear ranges in singleplex assays than in duplex or triplex assays, a phenomenon earlier reported for xMAP assays (Carson & Vignali, 1999). The linearity of the standard curves for IL-1β was similar in the different assays developed in Paper II and IV, supporting the robustness and reproducibility reported for the xMAP technique. Multiplex flow cytometric assays usually have broader linear ranges than ELISAs (Kellar & Iannone, 2002; Jennalm et al., 2003). Working ranges of ELISAs normally spread over one or two orders of magnitude while xMAP assays can have working ranges over three or four orders of magnitude. The linearity of the standard curves in the different singleplex assays agreed with those findings, although the linear ranges for the singleplex assays in Paper IV were not fully explored due to lack of sufficient quantities of recombinant proteins.

In multiplex assays where cytokine and APP detection is combined, the linear ranges of the individual assays are important. APPs can be abundant in biological fluids and would allow large dilution while cytokines are found only in low concentrations. To avoid dilution problems the sensitivity of the different assays included in the multiplex assay could be altered. The sensitivity of the APP assays could be lowered by coupling fewer antibodies to each microsphere (Dasso et al., 2002; Sun et al., 2007), making it possible to analyse them together with cytokines.

Intra- and inter-assay variation
Intra- and inter-assay variations of an assay are important measures of the precision and reproducibility. Low intra-assay variation allows for less replicates on the same plate, which is very valuable when the sample volume is small, and also saves time and consumption of reagents. Low inter-assay variation is important for comparisons of analysis results performed on different days and in studies where repeated analyses are performed over a long time span.

In the xMAP assays validated here, most CV’s were comparable with values in guidelines for analytical procedures and other reports on assay development for soluble analytes (Validation of analytical procedures, 1996; Wadhwa & Thorpe, 1998; Morgan et al., 2004). In some of the multiplex assays, the inter-assay CV’s was however higher than recommended. Problems due to high intra- and inter-assay variation can be reduced by running samples in duplicates and by including internal control samples and standard curves in each assay.
Cross-reactivity between reagents

Significant cross-reactivity was not found between any reagents in the multiplex xMAP assays developed in this thesis. This allows for reliable quantification of several analytes in the same sample, with low risk of false positive results. Cross-reactivity is often a problem in assays where several antibodies and antigens are combined in the same solution (Rosner, Grassman & Haas, 1991). This problem is especially prominent in veterinary immunology where reagents developed for specific species are rare (Shuster, Kehrli & Stevens, 1993; Bannerman et al., 2003; Lahouassa et al., 2007; Sohn et al., 2007). Yet, the choice of antibody clones is most important. In addition, assay buffers, incubation times and temperatures, blocking components and sample diluents also have a large influence (de Jager & Rijkers, 2006). In a sandwich immunoassay, the specific binding of antibodies to an analyte is utilized. Monoclonal antibodies are often used for the detection of an analyte as distortions due to other molecules become minimized. The concentration of bound analyte is often determined by polyclonal antibodies, as their broad range of epitopes increases the chances of detecting all analytes bound to the monoclonal antibodies.

Matrix effects

In the recovery experiment performed in Paper I, rhuTNF-α added to bovine milk or serum samples was detected to a lower degree compared to quantifications in buffer, indicating a quenching effect of the milk and serum. In Paper II, a general reduction of MFI was observed when samples were analysed in multiplex assays compared with singleplex assays. Similar findings were seen in Paper IV, but to a lesser extent than in Paper II. Those findings concur with results acquired e.g. by Carson & Vignali (1999). The reduced MFI is probably due to cross-reactivity between different host derived antibodies, the recombinant proteins and other components in the biological samples.

Quenching of fluorescent signals has previously been observed in immunoassays (Mire-Sluis, Gaines-Das & Thorpe., 1995; Selby, 1999; Phillips et al., 2006). When immunoassays are performed in e.g. milk and blood plasma samples, endogenous antibodies, soluble receptors and anti-cytokine antibodies may interfere and disturb the results of the assay, leading to both false positive and false negative values. The composition of milk from cows with mastitis may diverge depending on the phase of the inflammation (Pyörälä, 2003; Berglund et al., 2007), which means that recovery of analytes may differ widely. This could be one explanation why the recovery of rhuTNF-α was low in Paper I.

Recovery of recombinant human cytokines added to blood serum have been found to be concentration dependent, and high non-physiological concentrations were recovered to a larger extent than concentrations in a physiologically expected range (Prabhakar et al., 2004). These and many other data show the importance of validating each assay using the intended matrix. During mastitis, the composition of milk becomes strongly altered, and validation of a general assay for use in milk from cows with mastitis can be difficult.
False positive signals are often caused by heterophile antibodies making bridges between capture and detection antibodies (Phillips et al., 2006). When an analyte binds to soluble receptors or auto-antibodies in biological samples the concentration of an analyte is underestimated (Boscato & Stuart, 1988; Phillips et al., 2006). Such false negative results have been reported for TNF-α, IL-1β and IL-6 in human blood plasma and serum, and in synovial fluid (Svensson et al., 1993; de Jager & Rijkers, 2006), but could also be expected to occur in bovine samples. Since physical barriers between vessel walls and the surrounding tissue are destroyed during many inflammatory responses, components of plasma can be found for instance in milk during mastitis (Rainard, 2003). Problems with soluble receptors and heterophile antibodies could therefore be expected in milk samples from cows with mastitis. Problems with matrix effects can to some extent be reduced by dilution.

The WBA as a predictive tool

The looser consistency of faeces and higher heart rate found in the pre-inoculation low responders for IL-1β and IL-6, were not enough to suggest that those groups were more susceptible to severe forms of E. coli mastitis than the high responders for IL-1β and IL-6. Differences in kinetics of bacterial clearance in milk, other clinical symptoms and in vivo cytokine concentrations were not observed between any of the pre-inoculation high and low groups, thus the WBAs, performed in their present form, could not be considered useful predictive tools for the clinical outcome of a following E. coli mastitis.

A reason that the TNF-α, IL-1β and IL-6 WBAs did not work as predictive tools of the severity of E. coli mastitis could be the LPS doses and incubation times which were used. In previous WBA studies, the aim has been to induce maximum response of the cytokines, most prevalently TNF-α, ex vivo, for comparisons with processes in vivo (Hutchinson et al., 1999; Wurfel et al., 2005). In order to determine the maximum response, studies on doses have been performed, and the TNF-α response seems to be dose dependent up to the level where the maximum response is reached (Finch-Arietta and Cochran, 1991; Foster et al., 1993; Nakamura, Nitta & Ishikawa, 2004; Røntved et al., 2005). In human, bovine and porcine blood samples, the maximum TNF-α response to LPS has been observed after 2 to 8 hours stimulation (Allen et al., 1992; Carstensen et al., 2005; Røntved et al., 2005). In the WBA performed in Paper III, a LPS dose previously determined to induce maximum TNF-α response in bovine blood (Røntved et al., 2005) was used, and our results corroborate the earlier studies, as higher TNF-α concentrations were found after 3.5 hours than after 24 hours stimulation. In order to determine the time point and LPS concentrations for maximum IL-1β and IL-6 responsiveness to LPS, further studies are needed. Thereafter, customized studies using the maximum response of IL-1β and IL-6 in the WBA method might be used as predictive tools for the immunological response to an infection.

The cytokine responsiveness may also be influenced by physiological status of the cow. Parturition, metabolic status, and lactation phase have been observed to affect the production of pro-inflammatory cytokines (Doherty et al., 1994; Sordillo, Pighetti & Davis, 1995). Impaired immunological changes in cattle
around parturition have been demonstrated in both in vivo studies (Mallard et al., 1997) and ex vivo TNF-α responsiveness studies (Røntved et al., 2005). The latter study implicates that results from ex vivo WBAs can be useful for monitoring the in vivo innate immune system of cows if the right conditions and design is used.

Although ex vivo WBAs are designed to mimic the natural environment for the immune system as whole blood is used, data from studies performed outside an animal should always be interpreted and translated to in vivo situations with caution. The regulation of immunological responses is complicated and can not solely be explained by the cytokine levels in blood samples. The production rate and source of the cytokines, clearance dynamics, presence of circulating soluble receptors for cytokines and LBP, and membrane receptor distribution and their activity all contribute to the impact, shape, magnitude and duration of cytokine responses to LPS (Koj, 1996; Krishnaswamy et al., 1999).

**Experimental infection designs**

To reach the aims of this thesis, milk and blood samples from cows with clinical mastitis were needed, and samples from three different experimental mastitis studies have been utilized. In Papers I-II and IV, the focus was on development and application of the xMAP technique in bovine samples, therefore those experimental designs will not be discussed further.

In Paper III, however, the focus was on monitoring the effects of the experimentally induced E. coli infection in the cow in relation to the WBAs performed before the infection was induced. However, only six of ten cows became infected by the E. coli inoculation and developed clinical mastitis. With that small number of cows it is difficult to draw conclusions from the results of the ex vivo WBA experiments as a predictive tool of severity of the E. coli infection.

**Cytokines and APPs in bovine milk and plasma**

Secretion of pro-inflammatory cytokines in biological fluids has been reported in a range of bovine mastitis studies, and TNF-α, IL-1β and IL-6 have been suggested as important mediators (Shuster, Kehrli & Baumrucker, 1995; Shuster et al., 1997; Riollet, Rainard & Poutrel, 2000; Bannerman et al., 2004; Sohn et al., 2007). Major APPs and other inflammatory markers, e.g. SAA, haptoglobin, LBP, ITIH4 (inter-alpha-trypsin inhibitor heavy chain 4) and C5a, have also been detected in biological fluids in cows with mastitis (Shuster et al., 1997; Hirvonen et al., 1999; Grönlund et al., 2003; Bannerman et al., 2004; Lehtolainen, Røntved & Pyörälä, 2004; Piñeiro et al., 2004). Our results from the TNF-α, IL-1β, IL-6, SAA and LBP analyses in Paper II – IV corroborate earlier studies as all used analytes could be detected in milk or blood from cows with mastitis.

As mentioned earlier, the production of the cytokines and APPs vary between inflammatory responses and the physiological status of the host. More severe cases of E. coli mastitis are often reported around calving, in early lactation and at drying-off (reviewed by Burvenich et al., 2003). High concentrations of TNF-α in serum were correlated with severe mastitis, sepsis and death in some studies...
(Sordillo & Peel, 1992; Hirvonen et al., 1999; Blum et al., 2000; Hisaeda et al., 2001), while other studies did not report such associations (Nakajima et al., 1997; Hoeben et al., 2000). Our results from Paper III agree with the latter studies as the cows which developed moderate mastitis had elevated TNF-α levels in both milk and plasma.

Other factors than the physiological status of a cow influence the cytokine and APP concentrations in milk and blood during experimentally induced mastitis. The choice and dose of stimuli is important. The clearance of structural components of bacteria, e.g. LPS, PGN or LTA, would presumably be easier than clearance of proliferating bacteria in the udder. Thus, live pathogens are expected to induce longer lasting immunological responses than bacterial components, which were supported by a comparative study on *E. coli* and LPS mastitis performed by Blum et al. (2000).

When bacteria were used to induce mastitis, increased mRNA expression or secretion of TNF-α, IL-1β, IL-6, SAA and LBP in milk and blood have been reported (Shuster et al., 1997; Eckersall et al., 2001; Bannerman et al., 2004; Vangroenweghe et al., 2005; Hyvönen et al., 2006; Lahouassa et al., 2007). In studies where *E. coli* LPS, *S. aureus* LTA or α-toxin were used to induce mastitis, pro-inflammatory cytokines and APPs were frequently detected in milk but seldom in blood (Shuster, Kehrli & Stevens, 1993; Rainard & Paape, 1997; Lehtolainen, Røntved & Pyörälä, 2004). The TNF-α response in milk and blood during LPS mastitis has been shown to be local and regulated within the mammary gland (Rainard & Paape, 1997; Paape et al., 2002; Lehtolainen, Røntved & Pyörälä, 2004). Taken together, the results suggest that the immunological response is systemically activated and regulated to a larger extent during bacterial udder invasion than when only fragments of bacteria are present.

In samples from the experimental *E. coli* mastitis study in Papers II and III, IL-1β and IL-6 were detected at least once in milk and plasma samples from all six infected cows. TNF-α concentrations were found at least once in milk samples from all the infected cows and in plasma from four of the infected cows. Our data from Papers II-III indicate that TNF-α, IL-1β and IL-6 are involved in the local inflammatory response to *E. coli* bacteria in the udder, as well as in the systemic immune response to the invading pathogen, which concur with the results discussed above. In Paper IV, plasma from only two cows were analysed, and conclusions are difficult to draw. However, in accordance with previous studies, SAA and LBP were detected in plasma samples collected after endotoxin inoculation (Bannerman et al., 2003; Lehtolainen, Røntved & Pyörälä, 2004). Also the PGN inoculation induced elevated SAA and LBP levels. The SAA plasma concentration increased two- to fiftyfold during mastitis, while LBP concentrations became doubled after both inoculations.

The initiation and regulation of early immune responses are not fully explored but the production of TNF-α, IL-1β and IL-6 seems to be closely related, and the cytokines can probably influence the production of each other (Moshage, 1997; Gruys et al., 2005). Our results agree with that theory.
In Paper III, we saw that high plasma concentrations of IL-1β were associated with high IL-6 and TNF-α plasma concentrations within the cow. In addition, high concentrations of TNF-α in milk and plasma tended to be associated with each other. The mean peak concentrations of TNF-α, IL-1β and IL-6 in plasma and the mean peak concentration of TNF-α in milk were observed at the same time point as fever, elevated heart rate and changes in milk colour occurred in the infected cows. Other *E. coli* mastitis studies have been reported where peak concentrations of the pro-inflammatory cytokines in milk and plasma were concurrent with onset of clinically visible symptoms (Sordillo & Peel, 1992; Bannerman *et al.*, 2004). In a study by Lehtolainen, Røntved & Pyörälä (2004), the milk concentrations of TNF-α and SAA after intramammary LPS inoculation seemed to be associated with each other and with the clinical signs. The results in Paper III concur with results from that study, where elevated milk TNF-α concentrations were found to be associated with local udder symptoms and changed milk parameters. In addition, high plasma levels of TNF-α, IL-1β and IL-6 were associated with fever, elevated heart rate, looser faeces consistency, reduced milk yield, changed milk colour and swollen and red udder.
Conclusions

- Commercially available antibodies against human TNF-α did not cross-react with bovine TNF-α when applied in the xMAP assay. Two clones of anti-human TNF-α antibodies cross-reacted with recombinant ovine TNF-α in concentrations above 2.5 ng/ml.

- TNF-α, IL-1β and IL-6 could be detected in bovine milk and plasma with singleplex, duplex and triplex xMAP assays using ovine and bovine reagents. The singleplex xMAP assays for TNF-α, IL-1β and IL-6 could be applied to monitor cytokines in milk and plasma from cows with *E. coli* mastitis. In order to be a valuable tool in studies where low concentrations of TNF-α, IL-1β and IL-6 are of interest, the triplex xMAP assay need further development work.

- *Ex vivo* WBAs using LPS and *E. coli* induced production of TNF-α, IL-1β and IL-6. The cytokine concentrations in the pre-inoculation blood samples, induced by the LPS and *E. coli* doses and incubation times used, could not predict the severity of an *E. coli* induced mastitis.

- In cows, which had developed mastitis after intramammary inoculation of *E. coli*, high blood plasma concentrations of TNF-α, IL-1β and IL-6, and high milk TNF-α concentrations were associated with more severe clinical symptoms.

- The cytokine IL-1β and the acute phase proteins SAA and LBP could be detected in bovine plasma from cows inoculated with LPS and PGN using singleplex and triplex xMAP assays.
Future perspectives

The development of microsphere based flow cytometric assays for multiplex analysis of human analytes has been rapid. From being able to analyze six cytokines simultaneously in 1999 (Chen et al., 1999), more than 50 cytokines were detected simultaneously seven years later (Elshal & McCoy, 2006). During the last year an equine anti-virus antibody xMAP assay has been reported (Go et al., 2008). With this progress, it is plausible to believe that microsphere based flow cytometric assays will be applied in new areas of veterinary medicine during the next years.

Predicting in which areas of veterinary medicine the xMAP technique will be applied is difficult mainly because the accessibility to reagents is limited. Below, a few thoughts on how our existing xMAP panels could be expanded, and some examples on where development of xMAP panels would be valuable are presented and discussed.

In Paper IV, the triplex assay for IL-1\(\beta\), SAA and LBP was validated for blood plasma but with further validation work, the assay can be applied in any biological fluid, e.g. saliva, lung lavage, blood or faeces, depending on the type of information wanted. LBP is only present in high concentrations during bacterial infections and therefore serves as a good marker of such infections. By adding a marker for virus, our assay would contribute more information about the origin of a disease. Production of Type 1 interferons (IFN-\(\alpha\) and IFN-\(\beta\)) is induced as a response to presence of virus or double-stranded DNA in a host (Sandberg, Mattson & Alm, 1990; Haller, Kochs & Weber, 2006), making them good markers of viral infections. For example, serum concentrations of IFN-\(\alpha\) and IL-6 were used as markers of ongoing viral and bacterial infections in pigs (Le Bonnardiere & Laude, 1981; Fossum et al., 1998). Thus it is feasible to use the xMAP panel in other domestic species than cows. Other options for detection of viral infections could be IFN-\(\gamma\) inducible protein (IP-10/CXCL10), which is directly induced by IFN-\(\alpha\) and IFN-\(\beta\) (Gangur, Birmingham & Thanesvorakul, 2002), or Mx proteins which are produced in cells stimulated with IFN-\(\alpha\) (Horisberger & Gunst, 1991). Simultaneous detection of IL-1\(\beta\), SAA and IFN-\(\alpha\) can be performed in our laboratory (Dernfalk, Persson Waller & Johannisson, 2006), but at present the lack of IFN-\(\alpha\) reagents makes further work with that panel impossible.

Bacterial and viral infections traditionally elicit different immunological responses, but cross-talk between immunological pathways occurs and therefore immunological markers used to differentiate between viral and bacterial infections should be regarded with caution.

Health problems and economical losses due to infections are evident in many animal species. An example is bacterial dysentery infections which convey high mortality in growing pigs (Raynaud, Brunault & Philippe, 1980). In order to develop prophylactic measures and vaccination programmes for dysentery in pigs, an understanding of the host immune responses during the disease is essential. Johannisson et al. (2006) reported of development of a triplex xMAP assay for
simultaneous detection of TNF-α, IL-1β and IL-8 in porcine blood plasma and serum, which could provide useful information if applied in biological samples from pigs with dysentery. If this xMAP assay for porcine cytokines was expanded with IL-6 and SAA, also information about the acute phase response would be achieved.

Pigs are used as model animals in comparative studies with humans, for instance in the research on lung diseases. The porcine cytokine xMAP assay developed by Johannisson et al. (2006) was used to continuously monitor cytokines in bronchial epithelial fluid collected by microdialysis in pigs (Tolvold et al., 2008). It is planned to expand the xMAP panel to include the pro-inflammatory cytokine IL-6, the anti-inflammatory cytokine IL-10 and the modulating cytokine IFN-γ to increase the understanding of the inflammatory responses to lung injury or infection.

The majority of reports with xMAP assays are published within the field of immunology, although applications many other areas are possible (Morgan et al., 2004; Elshal & McCoy 2006). An area attracting considerable interest in the human research field is at the moment obesity and its connections to development of type-2 diabetes, cardiovascular disease and correlations to increased risk of cancer (Lawrence & Kopelman, 2004). Studies on the interactions between nutrition, endocrinology and metabolism also become increasingly important in our domestic animals. For instance, race horses and dairy cows demand optimal nutrition, and obesity among pets is an increasing problem. Therefore an xMAP assay for simultaneous detection of proteins involved in hormonal metabolic signalling at circulation and at tissue levels could be designed for different animal species. Metabolic xMAP analyses are available for many human metabolic steroids and proteins (Yurkovetsky, 2007).
Swedish summary

Populärvetenskaplig sammanfattning

Infektioner hos nötkreatur är ett stort problem i hela världen. Mastit (juverinflammation) orsakas oftast av att juvret invaderas av bakterier och trots att det har gjorts otaliga försök att kartlägga orsakerna till och förloppet av mastit är sjukdomen fortfarande ett allvarligt problem. Studier för att förjupa kunskaperna om mastit, utveckla förebyggande åtgärder, diagnostiska metoder eller hitta alternativa behandlingsvägar är därför ständigt aktuella.


Ett exempel på hur man kan mäta immunologiskt viktiga protein är med den flödescytometribaserade xMAP-tekniken. Flödescytometri är en teknik där partiklars egenskaper bestäms via det ljus de sänder ut efter att ha blivit belysta av en laser. I xMAP-analyser fångas den analyt man vill mäta mellan två antikroppar riktade mot olika delar av analyten. De antikroppar som ska fånga in analyten kopplas till små plastkulor (mikrosfärer) som blandas med det biologiska provet. Om det finns analyter av intresse i provet binder de in till antikropparna på kulorna. Ett mått på mängden inh bunden analyt fås genom att fluorescensinmärkta antikroppar sätts till lösningen innan proverna analyseras i en flödescytometer. För att kunna mäta flera olika analyter samtidigt i samma prov används mikrosfärer med unika färgkombinationer. xMAP-tekniken har visat sig användbar inom många områden, bland annat för analys av cytokiner, antikroppar, ämnesomsättningssenzynm, intracellulära proteiner och cancermarkörer.

Syftet med den här avhandlingen var att utveckla xMAP-analysen för inflammatoriska markörer vars koncentrationer ökar vid inflammationer i kor. Fokus låg på utveckling av analyser för cytokiner och akutfasproteiner i mjölk och blod från kor med mastit.

För att uppnå syftet designades xMAP-analysen för att studera en (singleplex), två (duplex), eller tre (triplex) analyter samtidigt. Analysen för cytokiner tumour necrosis factor (TNF)-α, interleukin (IL)-1β och IL-6 samt för akutfasproteinerne serum amyloid A (SAA) och lipopolysaccharide binding protein (LBP) utvecklades. Metoderna validerades enligt riktlinjer för immunologiska metoder.
För varje xMAP-analys bestämdes lägsta detektionsgräns, inom vilka koncentrationer analyserna var tillförlitliga samt variation inom och mellan olika analysställen. I de multiplexa (duplexa och triplexa) analyserna kontrollerades även eventuell korsreaktivitet mellan reagenser, dvs om detektering av en analyt ledde till att en ökad koncentration av en annan analyt felaktigt registrerades.

Att en analys har låg detektionsgräns är främst viktigt i cytokinstudier eftersom de är mycket kraftfulla mediatorer och därför bara utsöndras i låga koncentrationer under inflammatoriska svar. De singleplexa cytokinanalyserna hade tillfredsställande känslighet, men inte de multiplexa analyserna. När det gäller akutfasproteinaanalyser krävs oftast inte lika låga detektionsnivåer, istället är analysens räckvidd viktigare eftersom akutfasproteiner kan förekomma i både låga och mycket höga koncentrationer. Räckvidden för tillförlitlig koncentrationsbestämning av SAA och LB P var tillfredsställande, men bör kunna förbättras ytterligare. Korsreaktivitet noterades inte mellan några av reagenserna i de duplexa och triplexa xMAP-analyserna.

De singleplexa och multiplexa analyserna utvecklade i den här avhandlingen kunde användas för detektering av analyterna i mjölk- och blodplasmaprover från kor med mastit. Komponenter i mjölk och plasma påverkade detektering av analyter vilket är ett mycket vanligt problem som till viss del går att åtgärda med spädning av provet. I en av studierna visade vi att xMAP-tekniken kunde användas för att mäta ett cytokin och två akutfasproteiner i samma prov, något som kan ge ett tidsperspektiv på ett inflammationsförlopp, eftersom cytokiner främst utsöndras i början av en inflammation medan förhöjda koncentrationer av akutfasproteiner kan mätas under längre tid.

I en av avhandlingens studier inokulerades tio kor med bakterien *Escherichia (E.) coli* i juvret, vilket ledde till att sex av djuren fick mastit. Innan den experimentella mastitstudien genomfördes togs blodprover från korna. Dessa stimulerades med *E. coli* och en komponent som finns i bakteriens vägg kallad lipopolysackarid. När blodprover stimulerades kallas det för *ex vivo* stimulering. Efter *ex vivo* stimuleringen kunde TNF-α, IL-1β och IL-6 mätas i blodet. Korns förmåga att producera cytokiner *ex vivo* jämfördes med deras immunförsvars förmåga att hantera inokulationen av *E. coli* i juvret. Vi såg att kor som producerade låga koncentrationer av IL-1β eller IL-6 *ex vivo* hade högre tendens att utveckla diarré respektive hade högre puls än vad grupperna som producerade höga nivåer av IL-1β eller IL-6 hade. Inga andra skillnader i kliniska symptombland infektionen sågs mellan kor som *ex vivo* hade producerat höga och låga koncentrationer av cytokinerna. *Ex vivo* stimuleringen utförd på det här sättet kunde därför inte anses vara ett värdefullt verktyg för att förutsäga svårighetsgraden av *E. coli*-mastiter.

Vid analyser av cytokiner i mjölk och plasma samlad från korna efter *E. coli* inokuleringen observerades att höga plasmanivåer av IL-1β hade samband med höga plasmakoncentrationer av TNF-α och IL-6. Dessutom kunde höga TNF-α, IL-1β och IL-6 plasmakoncentrationer och höga TNF-α koncentrationer i mjölk associeras med kliniska symptom både lokalt och systemiskt. Dessa resultat stämmer väl överens med tidigare cytokinstudier på kor med mastit och visar xMAP-metodernas användbarhet.
References


Elshal, M.F. & McCoy, J.P. 2006. Multiplex bead array assays: performance evaluation and
collection comparison to ELISA. Methods 38, 317 – 323.
Eskay, R.L., Grino, M. & Chen, H.T. 1990. Interleukins, signal transduction, and
the immune system-mediated stress response. Advances in Experimental Medicine and
Biology 274, 331 – 341.
Ferré, F. 1994. Polymerase chain reaction and HIV. Clinical Laboratory Medicine 14,
313 – 333.
Agents and Actions. 34, 49 – 52.
various cytokines (IL-6, IFN-alpha, IFN-gamma, TNF-alpha) as markers of acute
bacterial infection in swine – a possible role for serum interleukin-6. Veterinary
Immunology and Immunopathology 64, 161 – 172.
by LPS-stimulated murine, rat and human blood and its pharmacological modulation.
Agents and Actions 38, Spec no: C77-79.
minimization of serum interface in flow cytometric two-site immunoassays. Clinical
Chemistry 40, 420 – 425.
Gangur, V., Birmingham, N.P. & Thanesvorakul, S. 2002. Chemokines in health and
Gilbert, R.O., Gröhn, Y.T., Miller, P.M., Hoffman, D.J. 1993. Effect of parity on
periparturient neutrophil function in dairy cows. Veterinary Immunology and
Immunopathology 36, 75 – 82.
fluorescent-microsphere immunoassay for detection of antibodies specific to equine
arteritis virus and comparison with the virus neutralization test. Clinical and Vaccine
Immunology 15, 76 – 87.
Detection of interleukin-1β in sera and colostrums of dairy cattle and in sera of neonates.
Journal of Veterinary Medical Science 59, 437 – 441.
Gross, C.P. & Sepkowitz, K.A. 1998. The myth of the medical breakthrough: smallpox,
vaccination, and Jenner reconsidered. International Journal of Infectious Diseases 3,
54 – 60.
Gründahl-Hansen, J., Barfod, K., Klausen, J., Andresen, L-O., Heegard, P.M.H., Sørensen,
V. 2003. Development and evaluation of a mixed long-chain lipopolysaccharide based
ELISA for serological surveillance of infection with Actinobacillus pleuroneumoniae
serotypes 2, 6 and 12 in pig herds. Veterinary Microbiology 96, 41 -45.


Svensson, M., Hansen, M. B., Heegaard, P., Abell, K., Bendtzen K., 1993. Specific binding of interleukin 1 (IL-1) beta and IL-1 receptor antagonist (IL-1ra) to human serum. High-affinity binding of IL-1ra to soluble IL-1 receptor type I. *Cytokine* 5, 427-35.


Validation of analytical procedures: methodology. 1996. ICH Harmonised Guideline, National Institute of Health Sciences, Bethesda, MD, USA.


Acknowledgements

My Ph.D. studies were carried out at the Department of Anatomy, Physiology, and Biochemistry, Faculty of Veterinary Medicine and Animal Sciences, SLU, Uppsala, Sweden, in co-operation with the Department of Animal Health, Welfare and Nutrition, Research Centre Foulum, Faculty of Agricultural Sciences, University of Aarhus, Tjele, Denmark. Financial support was provided by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), and by the Swedish Research Council (Vetenskapsrådet).

Writing a thesis is nothing you do on your own…and I would like to express my genuine gratitude to everyone that have helped and encouraged me through the years, and especially to:

Professor Stig Drevemo, the head of the Department of Anatomy, Physiology and Biochemistry, for letting me perform my studies there. And for giving me the opportunity to teach anatomy, a subject I didn’t even know I liked until I got the opportunity to really “dig into it”… Professor Klaus Lønne Ingvartsen, the head of the Department of Animal Health, Welfare and Nutrition, for letting me conduct parts of my studies there.

Associate Professor Anders Johannisson, my main supervisor, for being my guide on this expedition! For sharing your knowledge about anything from flow cytometric applications and method validation to where to find the best restaurants in Uppsala, for always willingly discuss new possibilities, analyse ideas and problems with me, for reading and answering mails late (late!) nights, for sharing my optimism, and for persistently encouraging me when things didn’t go our way in the lab…

Professor Karin Persson Waller, my highly appreciated associate supervisor, for your wholehearted engagement in my work! Your broad knowledge has been invaluable in discussions about project-planning, dairy cows and strange results, and your ambitious, creative and constructive help during different writing processes has without doubt improved the quality (and markedly reduced the babbling) of our papers!

Ph.D. Christine Røntved, my dear co-author at the Research Centre in Foulum, Denmark, for welcoming me as a member of your experimental-trial-team, for sending me milk- and blood- samples whenever I was in need, for teaching me so much about immunological methodology and the immune system in cows. And for making me ENJOY (!) two weeks without sleep working-around-the-clock in the dairy barn and the lab…
Professor Kerstin Olsson, for your fantastic and highly appreciated help during the final writing steps of this thesis! With your experienced eyes, many questions, constructive criticism and wise ideas, the content and thoughts of this book became considerably clearer. (And in this section, I don’t listen to your recommendations about not using too many ‘big’ adjectives... here they are justified!)

Professor Katsuro Hagiwara, Rakuno Gakuen University, Japan, Ph.D. Ian Colditz, Australian Commonwealth Scientific and Research Organization (CSIRO), Armidale, Australia, Ph.D. Ulrika Grönlund Andersson, Department of Antibiotics, National Veterinary Institute, Uppsala; and Ph.D. Charina Gånheim, Department of Clinical Sciences, SLU, Uppsala, for generously providing me with reagents and biological samples for assay development work during these years.

Lars Berglund and Tomas Thierfelter, for invaluable help with finding proper statistical analyses for my data, especially those from the experimental mastitis studies.

All my colleagues at the Department of Anatomy, Physiology and Biochemistry, for making it joyful to go to work every day! For nice chats about anything from gardening, which gympa-classes to attend, cooking, to discussions about current happenings at SLU. Also, you should have full credit for your enthusiastic participation and engagement in the Pub FysmysAH nights and other social activities we have put you through during the last years! ☺

Ph.D. students, former and present, at the Department of Anatomy, Physiology and Biochemistry, for making the days, lunches and coffee breaks MORE than pleasant! My room-mates, Cecilia, for, entertaining me with crazy dog stories and enlightening our office with a big smile! Anna, for always being in a cheery mood, buying flowers to the office and dragging me along on the doggiewalks - writing truly benefits from a pinch of fresh air (and from laughing at the dogs)! Helena, for filling our spacious office with chats, pep-talks and lovely kardemummaskorpor. Marie, for sharing the horror and joys of writing a thesis…

Staff and Ph.D. students at the Departments of Veterinary Immunology, Clinical Sciences and Biomedical Sciences and Veterinary Publich Health, for discussions about immunology, cows, pigs, horses, writing, food, travelling, children, dogs… and for lovely trips to Quebec and Paris!

Maria Åkerstedt and Åse Sternesjö, my dear colleagues at the Department of Food Science, for being good friends, great discussion partners and excellent travel company around Europe!

Szabi Nagy, Joan Ballester, Robert Jonasson, Ann-Sofi Bergqvist and Fernando Saravia, for keeping me company in the lab!
Ann Hammarberg, my friend and former colleague, for introducing me to the work as a teacher in the field of anatomy. For always believing in my capacity, cheering me on, allowing me to gain experience of course management (which hopefully made me a better teacher), and for being our cat Yoqko’s private veterinarian!

Anna Olofsson, Marie Mörk, Malin Hagberg and Jonas Wensman, for sharing hard work and laughing trying to organise a functional Ph.D. council when the Faculty of Veterinary and Animal Science was formed.

My blogfriends out there on the www, Annapanna, Anna & Niklas, Fiddeli, Felicitas, Familjen Sjöström, Linda K, Lindarling, Lisa, Nina, Pinglan... for sharing stories, thoughts and pictures of your every-day-life, connecting my days in front of the computer to the spinning world out there!

Cecilia my darling kibbutz-friend, for making my social life existent when I moved to Uppsala! Never stop to “dance the iron!” Anna, my lovely “kombo” for making it fun to come home from work! Pharmen-gänget, former and present, for shortening the work week by making Wednesdays a part of the weekend!

My “Uppsala-family”, Alexandra, Tommy, Daniel, Julia and Mattias, for many crazy days and nights together! Alexandra and Julia, for endless hours contemplating the necessities and non-necessities of life, both at work over a cup of coffee, and at home with a glass of wine. Alexandra, for accompanying (and pushing) me to bike to Stallet even when it was dark and snowy, for cheering me on during this writing, and for loving our cats - ready to cat-sit when needed! Julia, for growing tomatoes, making kolonilotts-gardening much more fun, and for being great travel company to the Icehotel! Daniel, for always having a laugh waiting around the corner, for never saying no to a cup of coffee, and for creating Klurendrejeriet® - so I got to think about other things than work! Mattias, for initiating the nice going-away-for-lunch-tradition at work, for bringing appreciated meat and company to dinner, and for letting me whirl around on the dance floor every now and then although I don’t know how to bugga…

Marie, Ulrika, Sofie with families, my precious friends! For sharing the process of (trying to) become a grown up, for always being proud of me and somehow believing that being a Ph.D. student is “cool” (although you all have much cooler jobs!) I’m expecting us to continue with our traditional taco-dinners with wine, chocolate and gossiping regularly until we’re very, very old! Ok?

Sara and Petra with families, my best friends! For always being close despite the physical distances, for thinking like me, enthusiastically discussing everything-and-nothing, always making the best of your present situation, but also dreaming and planning the future together. That in combination with splendid dinners, weekends, trips and vacations together is unbeatable! The Sunshine Tour RULED, Sara! And driving through L.A. is piece of a cake, right, Petra?
Hans, Rietje, Karen, Eelco, Joke en Ben, my “family-in-law”, for immediately opening your arms for me, and from that moment on support your Swedish wing of the family in every possible way! Nu heb ik tijd, nu zal ik ordentelijk nederlands leren! (meer dan “Laffe herder”)

My family, my mother and father Kicki and Christer, my sister Emma with family Daniel, Ida and Elin and my brother Andreas with family Malena, Tyra and Stina, for making family occasions special and truly joyful! And for always supporting, and being proud of me whatever stupid ideas I’ve come up with during the years! You all are the foundation of my life. Emma, for never being further than a phone call away, and for being the best sister anyone ever could wish for, I’m very happy to be a daily part of your life. I think we are not only biological, but also soul twins!

Joris, my “dutch guy with the wobbly knee”, for sharing each and every day with me - both the boring working-non-stop-do-we-really-have-to-fix-dinner-ones and those spent in the sofa watching movies, or eating ice cream in Rättvik 😊 For being my personal computer nerd, helping me when frustrated, for always taking care of me, fixing boring things, and delivering small presents to my office… and for always, always finding a way to make me smile! Älsklingentje! ♥