Early Inflammatory Response in Periparturient Sows to Experimentally Induced *Escherichia coli* Mastitis

with Special Reference to Cytokine Responses

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Abstract

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The overall objective of the present work was to monitor the induction of some key factors involved in the early inflammatory response in the mammary gland of periparturient sows to experimentally induced *Escherichia coli* (*E. coli*) mastitis. We wanted to gain a better understanding of why some sows develop clinical signs of mastitis, while others remain clinically healthy.

Concentrations of the proinflammatory cytokines interleukin (IL)-6 and tumour necrosis factor-alpha (TNF-α), and the acute-phase protein (APP) serum amyloid A (SAA) in blood increased in sows following intramammary *E. coli* inoculation as measured by enzyme-linked immunosorbent assay (ELISA). Furthermore, concentrations of IL-6 and TNF-α in blood were higher in sows that developed clinical signs of mastitis than in sows that remained clinically healthy after inoculation. Notably, immunohistochemistry (IHC) analysis of biopsy specimen revealed that some baseline production of cytokines took place in normal mammary glands of pregnant sows, and that sows that developed clinical signs of mastitis had significantly lower baseline production of IL-1β than did sows that remained clinically healthy. Twenty-four hours after inoculation, there was an increase in the production of IL-1β, IL-6, IL-8 and TNF-α in the inoculated mammary glands of sows that developed clinical signs of mastitis. By contrast, sows that remained clinically healthy did not show increased production of IL-1β, IL-6 and TNF-α in the inoculated mammary glands. However, at the mRNA level there was an increase in the expression of IL-1β and TNF-α also in these sows. The anti-inflammatory cytokine IL-10 mRNA expression increased in the inoculated mammary glands 24 hours after inoculation, and it was higher in the inoculated mammary glands of sows that developed clinical signs of mastitis compared with sows that remained clinically healthy, while the expression of another anti-inflammatory cytokine, transforming growth factor-beta 1 (TGF-β1) mRNA was unaltered, as was mRNA expression for the IL-1 receptor type I (IL-1R1).

In addition, the pattern-recognition receptor (PRR) Toll-like receptor 2 (TLR2) mRNA expression increased in the inoculated as well as the non-inoculated mammary glands of sows that developed clinical signs of mastitis and of sows that remained clinically healthy. However, TLR2 mRNA expression was higher in the inoculated mammary glands than in the non-inoculated mammary glands of sows that remained clinically healthy.

The findings of the present study suggest that IL-6 and/or TNF-α in blood could be used as markers for identification of periparturient sows with coliform mastitis, and that development of clinical symptoms of coliform mastitis is associated with the degree of local production of regulatory cytokines in response to intramammary *E. coli* inoculation.

Keywords: cytokine, acute-phase protein (APP), Toll-like receptor (TLR), mammary gland, mastitis, pig, *Escherichia coli* (*E. coli*)

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Appendix

Papers I–IV

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

I. Zhu, Y., Österlundh, I., Hultén, F. & Magnusson, U. 2004. Tumor necrosis factor-α, interleukin-6, serum amyloid A, haptoglobin, and cortisol concentrations in sows following intramammary inoculation of *Escherichia coli*. *American Journal of Veterinary Research* 65, 1434–1439.

II. Zhu, Y., Berg, M., Fossum, C. & Magnusson, U. 2007. Proinflammatory cytokine mRNA expression in mammary tissue of sows following intramammary inoculation with *Escherichia coli*. *Veterinary Immunology and Immunopathology* 116, 98–103.

III. Zhu, Y., Fossum, C., Berg, M. & Magnusson, U. 2007. Morphometric analysis of proinflammatory cytokines in mammary glands of sows suggests an association between clinical mastitis and local production of IL-1 beta, IL-6 and TNF-alpha. *Veterinary Research* doi: 10.1051/vetres:2007035, in press.

IV. Zhu, Y., Magnusson, U., Fossum, C. & Berg, M. 2007. *Escherichia coli* inoculation of porcine mammary glands affects local mRNA expression of some Toll-like receptors and regulatory cytokines. (Submitted)

Abbreviations

APC	antigen-presenting cell
APP	acute-phase protein
cDNA	complementary deoxyribonucleic acid
CFU	colony-forming unit
DAB	3, 3'-diaminobenzidine tetrahydrochloride
DC	dendritic cell
DNaseI	deoxyribonuclease I
E	amplification efficiency
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic deoxyribonucleic acid
Hp	haptoglobin
HPA	hypothalamic-pituitary-adrenal
HPRT	hypoxanthine phosphoribosyl-transferase
IHC	immunohistochemistry
IL	interleukin
IL-1R1	type I IL-1 receptor
IL-1RAcP	IL-1 receptor accessory protein
LBP	LPS-binding protein
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MIP2	macrophage inflammatory protein-2
mRNA	messenger RNA
NF-κB	nuclear factor-κB
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PG	prostaglandin
PGN	peptidoglycan
PHS	periparturient hypogalactia syndrome
PRR	pattern-recognition receptor
qRT-PCR	quantitative real-time RT-PCR
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-PCR
SAA	serum amyloid A
SCC	somatic cell count
TGF-β1	transforming growth factor-beta 1
T_H	T helper
T_H1	T helper type 1
TIR	Toll/IL-1R
TLR	Toll-like receptor
TNF-α	tumour necrosis factor-alpha

Introduction

Rationale

Mastitis affects sows in all regions of the world and plays a major role in the periparturient hypogalactia syndrome (PHS) (Bäckström *et al.*, 1984; Hermansson *et al.*, 1978; Martineau, Smith & Doizé, 1992; Ringarp, 1960). Although PHS in the sow has been recognized for years, effective prophylactic and therapeutic management of the problem has frequently remained an elusive goal for the practitioner. Gram-negative pathogens, e.g. *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*) and various species of *Enterobacter* constitute the most common pathogens implicated in coliform mastitis (Bertschinger, 1999; Ross *et al.*, 1981). Most coliform mastitis in sows is caused by *E. coli* and occurs within the first few days postpartum (Hermansson *et al.*, 1978; Ringarp, 1960). Coliform mastitis is acute in nature and rarely becomes chronic (Persson, Pedersen Mörner & Kuhl, 1996b; Ringarp, 1960; Smith & Hogan, 1993). It can be subclinical or exhibit a wide range of symptoms, from mild to severe with general clinical signs, such as fever, anorexia, lethargy, swelling of the mammary glands, milk with clots and an abnormal appearance, and losses in milk production (Persson, Pedersen Mörner & Kuhl, 1996b; Ringarp, 1960; Ross *et al.*, 1975; Smith & Wagner, 1984). It is well known that bacterial, environmental, management, genetic and physiological factors influence mastitis susceptibility in the sow as well as in the cow (Persson *et al.*, 1989; Sordillo & Streicher, 2002; Waller, 2000), but the reason why certain sows develop clinical signs of mastitis when challenged with environmental *E. coli*, while others remain clinically healthy, is still elusive.

An inflammatory response to microbial invasion or injury is a part of the early defence mechanisms. Such a reaction has to be activated as soon as possible to terminate the spread of infection, even at the cost of further tissue damage. The inflammatory response may cause more damage than the microbe itself and must therefore be fine-tuned and regulated precisely. Therefore, excessive responses may cause morbidity and mortality, while insufficient responses in immunodeficient individuals may allow an infection to become established (Cohen, 2002; Nathan, 2002; Tracey, 2002). During the early stages of intramammary infections, macrophages together with epithelial cells initiate the inflammatory response to eliminate invading bacteria; however, an insufficient as well as excessive inflammatory response may lead to mastitis (Oviedo-Boyso *et al.*, 2007; Rainard & Riollet, 2006; Sordillo, Shafer-Weaver & DeRosa, 1997). Hence, studies on early inflammatory responses in periparturient sows to experimentally induced *E. coli* mastitis may improve our understanding of how environmental bacterial components trigger manifestations of acute infectious disease in the host.

Coliform mastitis

Escherichia coli is an organism that is most often identified in either milk samples or infected mammary glands, and that is associated with various forms of subclinical and clinical mastitis in sows (Armstrong, Hooper & Martin, 1968; Persson, Pedersen Mörner & Kuhl, 1996b; Ringarp, 1960; Ross *et al.*, 1981). However, coliform mastitis in sows appears to be non-contagious. An early Swedish study reported an average PHS incidence in sows of 12.8% (signs of mastitis were found in 45–52% of agalactic sows) (Hermansson *et al.*, 1978). Field studies from the United States (Bäckström *et al.*, 1984) and Denmark (Svendsen *et al.*, 1975) on mastitis in sows showed an incidence of mastitis of 6.9% and 9.5%, respectively. A previous study has shown that the mortality rate of piglets nursing multiparous sows with mastitis was 56%, while that of piglets nursing healthy sows was only 17% (Bäckström *et al.*, 1984). Notably, the subclinical form of mastitis is more difficult to diagnose; however, piglets suckling on glands of sows with mastitis have lower mean weights owing to insufficient milk intake (Persson, Pedersen Mörner & Kuhl, 1996b; Ross *et al.*, 1975; Smith & Wagner, 1984).

Studies in cattle have shown that indicators of inflammation can be used in the diagnosis of mastitis (Pyörälä, 2003). Today, the most commonly used indicator of bovine mastitis is somatic cell count (SCC), which reflects the disease-combating response of the animal to the pathogen. Due to the normally higher somatic cell content of sow's milk, with an SCC of between 1 and 4 million cells per millilitre in healthy sows (Hurley & Grieve, 1988; Magnusson, Rodríguez-Martínez & Einarsson, 1991; Persson, Pedersen Mörner & Kuhl, 1996a; Schollenberger *et al.*, 1986), tests developed for use in the cow cannot be recommended. Acute-phase proteins (APPs) such as serum amyloid A (SAA) and haptoglobin (Hp) are potential candidates for bovine mastitis monitoring (Petersen, Nielsen & Heegaard, 2004). A recent review has proposed that the subtle and sensitive changes in the cytokine network in normal and mastitic bovine mammary glands may encourage the use of cytokines in the diagnosis and prognosis of udder health (Alluwaimi, 2004), but their diagnostic value in porcine mastitis and PHS has not yet been confirmed.

General aspects of defence mechanisms in the mammary gland

The mammary gland is protected by the physical barrier of the teat streak canals and a variety of immune defence mechanisms. The teat streak canal is tightly closed by longitudinal folds that come from the teat cistern to the teat canal. Together with the teat skin and the milk flow, the teat canal constitutes the first line of defence against intramammary infections.

The immune system can be divided into two subsystems: innate immunity and adaptive immunity. The bovine mammary gland defence mechanisms, including physical, soluble and cellular factors, have been previously reviewed (Burton & Erskine, 2003; Burvenich *et al.*, 2003; Kehrli & Harp, 2001; Sordillo, Shafer-Weaver & DeRosa, 1997; Sordillo & Streicher, 2002). In brief, the mammary

gland contains chemical substances that are microbicidal or inhibit microbial growth, e.g. lysozyme and the enzyme lactoperoxidase, the antimicrobial proteins lactoferrin and transferrin, and the antimicrobial peptides defensins (Rainard & Riollet, 2006). In addition, the complement system is a group of plasma proteins, with effector functions that include recruitment of phagocytes, and opsonization and killing of pathogens (Rainard, 2003).

Phagocytes such as macrophages and neutrophils can recognize, ingest and eliminate the invading micro-organisms (Burvenich *et al.*, 1999; Paape *et al.*, 2002). Proinflammatory cytokines are known to be important in eliciting the acute-phase response and allowing an accumulation of leukocytes at the site of infection (Riollet, Rainard & Poutrel, 2000a). Taken together, the innate immune response makes an important first line of defence but also has a crucial role in the activation of adaptive immunity.

Inflammatory responses later in an infection also involve lymphocytes. All lymphocyte responses to antigen require not only the signal that results from antigen binding to their receptors, but also, a second signal, which is delivered by another cell. T cells need antigen-receptor engagement plus signals from antigen-presenting cells (APCs). B cells need antigen-receptor engagement plus signals from activated T cells. The antibodies, which bind pathogens or their products in the extracellular space of the body, can prevent the antigen from exerting its actions and amplify the innate immune response (Kehrli & Harp, 2001; Sordillo, Shafer-Weaver & DeRosa, 1997).

With regards to mammary gland defence mechanisms in the sow, an increase in the number of granulocytes, found in blood at parturition, has been reported (Magnusson & Fossum, 1990; Österlundh, Holst & Magnusson, 1998). Likewise, a higher number of granulocytes has been seen in mammary secretions collected at parturition compared with samples collected on days 2 or 3 (Österlundh, Holst & Magnusson, 1998) or 7 postpartum (Magnusson & Greko, 1998), and the phagocytic capacity of granulocytes in blood as well as in mammary secretions did not vary around parturition (Magnusson & Greko, 1998; Österlundh, Holst & Magnusson, 1998). By contrast, a later study showed lower phagocytic capacity of granulocytes in colostrum compared with that in milk collected from 12 to 13 days postpartum (Österlundh, Holst & Magnusson, 2001). Moreover, before intramammary *E. coli* inoculation, no impaired function of granulocytes was found in sows that developed clinical signs of mastitis (Österlundh *et al.*, 2002). On the other hand, after inoculation, the proportion of CD4+ and CD8+ lymphocytes was higher in the mammary glands of sows that developed clinical signs of mastitis than in those that remained clinically healthy in the same experiment (Löving & Magnusson, 2002).

The early inflammatory response

Inflammation is a complex set of interactions among soluble factors and cells, which can arise in any tissue in response to microbial invasion or injury. Inflammatory responses are operationally characterized by pain, redness, heat, and swelling at the site of infection. An acute inflammatory response results in increased local blood flow, increased vascular permeability, and an accumulation of leukocytes and certain soluble factors in the tissue. Initiation of the inflammatory response occurs primarily through activated macrophages, mast cells, together with epithelial cells at the site of infection or injury. On activation, macrophages (Cohen, 2002) and mast cells (Benoist & Mathis, 2002) as well as epithelial cells (Svanborg, Godaly & Hedlund, 1999) rapidly release cytokines and other inflammatory mediators such as histamine, leukotrienes, prostaglandins, nitric oxide and toxic oxygen radicals, which promote further inflammation and tissue injury. Following the induction of the inflammatory response, the liver and other tissues undergo dramatic upregulation and synthesis of a variety of APPs which can mediate or inhibit inflammatory processes, function as transport proteins and participate in tissue repair (Baumann & Gauldie, 1994; Tilg, Dinarello & Mier, 1997).

As the inflammation progresses, leukocytes, lymphocytes and other cells are activated and recruited to the inflamed site by a signalling network involving a great number of cytokines including growth factors and chemokines (Conti *et al.*, 2004; Coussens & Werb, 2002; Dinarello, 2004; Nathan, 2002). Cytokines serve in both autocrine and paracrine manners, affecting the behaviour of the cells at the site of inflammation. Some cytokines can also act as messengers (Havell & Sehgal, 1991) between the local site of infection or injury and other sites in the body, e.g. inducing hepatocytes to synthesize APPs (Baumann & Gauldie, 1994; Steel & Whitehead, 1994; Tilg, Dinarello & Mier, 1997). Another way in which pathogen recognition rapidly triggers an inflammatory response is through activation of the complement cascade (Janeway *et al.*, 2001).

The inflammatory response is usually a healing response, since it is a reaction designed to terminate the spread of infection. However, under certain circumstances, many of the components of the inflammatory response can cause cell and tissue damage, microvascular thrombosis, fever, systemic capillary leakage syndrome, and hence multiple organ failure, sepsis and even death (Cohen, 2002). To avoid detrimental inflammatory reactions, a balance between activation and inhibition is needed throughout the immune response. Therefore, anti-inflammatory mediators are also released that limit the acute inflammatory response and prevent the spread of inflammatory mediators into the bloodstream (Tracey, 2002).

The Toll-like/interleukin-1 receptor superfamily

The initial recognition of infectious agents or parts of the microbes, as they enter the body, is largely based on pattern-recognition receptors (PRRs) that are present on or in various cells in the body (Aderem & Ulevitch, 2000; Hallman, Rämet & Ezekowitz, 2001).

Toll-like receptors (TLRs) constitute an ancient family of PRRs. In the murine system up to 13 TLRs have been identified (Akira, Uematsu & Takeuchi, 2006; Hallman, Rämet & Ezekowitz, 2001; Takeda, Kaisho & Akira, 2003). It seems that many inflammatory processes, both sterile and infectious, may depend on TLR signalling (Akira & Takeda, 2004; Beutler, 2004; Cohen, 2002). Signalling via TLRs may commonly lead to early activation of the transcription factor nuclear factor-κB (NF-κB) and the release of several proinflammatory mediators, including the proinflammatory cytokines interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-alpha (TNF-α) (Akira, Uematsu & Takeuchi, 2006; Pålsson-McDermott & O'Neill, 2004; Raetz & Whitfield, 2002). Toll-like receptors recognize pathogens at either the cell surface or on lysosome/endosome membranes (Akira, Uematsu & Takeuchi, 2006). The variability of bacterial ligands and their innate immune receptors is an important factor in determining the outcome of infectious disease (Miller, Ernst & Bader, 2005).

Pattern-recognition receptors recognize the invading pathogens through identification of conserved components of these microbes. These microbial structures are termed "pathogen-associated molecular patterns (PAMPs)" and include various bacterial cell wall components, such as lipopolysaccharide (LPS), lipopeptides, peptidoglycan (PGN) and lipoteichoic acid (LTA) (Aderem & Ulevitch, 2000). In the case of Gram-negative bacteria, the recognition of LPS from Gram-negative bacteria is mediated by CD14, LPS-binding protein (LBP), MD-2 (Shimazu *et al.*, 1999) and TLR4. This TLR, TLR4, is the main PRR for LPS from Gram-negative bacteria. Another TLR, TLR2, on the other hand, recognizes LTA from Gram-positive bacteria, peptidoglycan and bacterial lipoproteins abundant in the cell wall of Gram-positive and Gram-negative bacteria, and other bacterial products that are distinct from Gram-negative LPS (Brightbill *et al.*, 1999; Grabig *et al.*, 2006; Werts *et al.*, 2001). One study reports when TLR4-/- knockout mice were challenged with LPS, and that they were unable to mount a cellular immune response, with no detectable change in TNF-α expression (Hoshino *et al.*, 1999). In some human diseases, single gene defects that result in the failure of a particular innate immune receptor to be expressed cause general increased susceptibility to infection (Altare *et al.*, 1998; Picard *et al.*, 2003). As regards mastitis, a previous study has shown that both TLR2 and TLR4 mRNA expression increased in infected mammary glands of cows with mastitis caused by either *Staphylococcus aureus* (*S. aureus*) or *E. coli* (Goldammer *et al.*, 2004).

The IL-1 receptor family includes type I IL-1 receptor (IL-1R1), IL-1R2, the IL-1 receptor accessory protein (IL-1RAcP), IL-18R, IL-18RAcP, and the orphan receptor T1/ST2 (Subramaniam, Stansberg & Cunningham, 2004). Type I IL-1

receptor is expressed on endothelial, epithelial, epidermal dendritic cells, fibroblasts and T cells as an 80 kDa protein (Dinarello, 1996; Sims *et al.*, 1988). Interleukin-1 exerts its biological actions by binding to specific receptors including IL-1R1 and IL-1R2 (Allan, Tyrrell & Rothwell, 2005; Liew, Liu & Xu, 2005; Subramaniam, Stansberg & Cunningham, 2004).

Based on the homologous cytosolic Toll/IL-1R (TIR) domain, the TLR/IL-1R superfamily includes TLRs and the IL-1 receptor family subgroups (Bowie & O'Neill, 2000; Dunne & O'Neill, 2003). Except for IL-1R2 and T1/ST2 (Liew, Liu & Xu, 2005), all members of this superfamily activate common signalling pathways, most notably leading to activation of NF-κB and stress-activated protein kinases (Dunne & O'Neill, 2003; Subramaniam, Stansberg & Cunningham, 2004).

Cytokines

Cytokines can be defined as a group of small, soluble proteins (-25 kDa) that at low concentrations are able to regulate the function of cells and tissues (Janeway *et al.*, 2001). They are released by various cells in the body, usually in response to an activating stimulus, and mediate their effects through binding to specific receptors. In addition, to differentiate cytokines from classic hormones, cytokines can be produced by cells not organized in specific glandular organs. Using this very broad definition, cytokines would include interleukins, chemokines, interferons, colony- stimulating factors, peptide growth factors, tumour necrosis factors (Nathan & Sporn, 1991), and other proteins (Phillips *et al.*, 2005) that are often not perceived as immune regulators but interact with the immune cells, including activins and inhibins. Cytokines can act in an autocrine manner that affects the behaviour of the cell that releases cytokines, or in a paracrine manner that affects the behaviour of adjacent cells. Most cytokines act at near distances, but some cytokines can act in an endocrine manner that affects the behaviour of distant cells. The latter ability depends both on their ability to enter the circulation and on their half-life (Janeway *et al.*, 2001).

Monocytes/macrophages, neutrophils, mast cells, dendritic cells (DCs), B cells, various types of T cells, and even fibroblasts and endothelial and epithelial cells are potently triggered to release cytokines through the innate immune system using PRRs during the inflammatory process (Aderem & Underhill, 1999; Akira & Takeda, 2004; Bannerman & Goldblum, 2003; Benoist & Mathis, 2002; Svanborg, Godaly & Hedlund, 1999). Alternatively, processing of antigens as they are taken up by APCs, metabolized, and presented to T helper (T_H) lymphocytes provides one pathway for this class of cytokine production (Borish & Steinke, 2003; Steinke & Borish, 2006). A diverse range of genetic and external factors can affect cytokine production and activity at various stages, including transcription, translation, cleavage and cellular release, or at the level of interaction with receptors, including soluble receptors (Akira & Takeda, 2004; Albee & Perlman, 2006; Allan, Tyrrell & Rothwell, 2005; de Groot *et al.*, 2005).

Cytokines are involved in diverse actions, including antigen presentation, control of immune effectors and inflammation, lymphocyte proliferation and

differentiation, cellular recruitment and activation, and adhesion molecule expression. Some cytokines have behavioural, endocrinological and metabolic effects (Borish & Steinke, 2003; Hanada & Yoshimura, 2002; Mackay, 2001). Cytokines such as IL-1β, IL-6, IL-8, TNF-α, IL-10 and transforming growth factor-beta 1 (TGF-β1) have been proposed to play a physiological role at parturition by being involved in the regulation of parturition (Bowen *et al.*, 2002; Keelan *et al.*, 2003). Furthermore, cytokines appear to be an important component of a paracrine/autocrine communication network in the mammary gland at the different stages of pregnancy and parturition (Hennighausen & Robinson, 2005; Ip, Shoemaker & Darcy, 1992; Rainard & Riollet, 2006; Varela & Ip, 1996). It should be noted that some baseline production of some of these cytokines may take place in clinically healthy individuals (Hagiwara *et al.*, 2000; Hebisch *et al.*, 2004; Vangroenweghe, Lamote & Burvenich, 2005).

The proinflammatory cytokines

The proinflammatory cytokines mediate the early inflammatory response to microbial challenges and play a dual pathophysiological role, which may either promote or limit the processes of inflammation by activating other cells to release anti-inflammatory mediators (Akira, Taga & Kishimoto, 1993; Kodama, Davis & Faustman, 2005; Kofler, Nickel & Weis, 2005; Svanborg, Godaly & Hedlund, 1999; Tracey, 2002). This in turn makes them associate with symptoms of disease (Allan, Tyrrell & Rothwell, 2005; Barber & Yang, 1998; Beutler & Rietschel, 2003; Borish & Steinke, 2003; Fossum *et al.*, 1998; Hirano, 1998). The systemic effects include the induction of hepatic synthesis and secretion of APPs, and changes in body temperature, blood flow and blood pressure through activation of the immune system (Baumann & Gauldie, 1994; Riollet, Rainard & Poutrel, 2000a). One of these symptoms is elevation of the body temperature, which is mainly caused by IL-1β, IL-6 and TNF-α (Conti *et al.*, 2004; Dinarello, 2004; Romanovsky *et al.*, 2005).

In addition to their role in the systemic symptoms of disease, the proinflammatory cytokines are considered to initiate the inflammatory response to microbial invasion, contributing to the establishment of inflammation by altering vascular permeability, and promoting the accumulation of leukocytes and certain soluble factors at the site of infection (Baumann & Gauldie, 1994; Riollet, Rainard & Poutrel, 2000a).

Interleukin-1 is known to influence thymocyte proliferation and B cell growth and differentiation and is necessary to stimulate the production of IL-2, IL-6 and chemokine attractants for neutrophils such as macrophage inflammatory protein-2 (MIP2) and IL-8 (Armstrong *et al.*, 2004). Interleukin-1, which is produced during infection, not only has local effects involving the local inflammatory response; it also has systemic effects including the induction of acute-phase responses and effects on appetite, sleep and body temperature (Allan, Tyrrell & Rothwell, 2005; Dinarello, 1996).

In vitro and *in vivo* studies in several species have clearly shown that the production of proinflammatory cytokines including IL-1β, IL-6, IL-8 and TNF-α is upregulated following challenge with *E. coli* or endotoxin derived from *E. coli* (Bannerman *et al.*, 2003; Burvenich *et al.*, 2003; Dosogne *et al.*, 2002; McClenahan, Sotos & Czuprynski, 2005; Riollet, Rainard & Poutrel, 2000b; Shuster, Kehrli & Stevens, 1993; Vangroenweghe, Duchateau & Burvenich, 2004). Previous studies on mastitis in cattle have shown positive correlations between the concentrations of IL-6 and TNF-α in blood and the severity of mastitis (Hagiwara *et al.*, 2001; Nakajima *et al.*, 1997; Sordillo & Peel, 1992).

The anti-inflammatory cytokines

To avoid detrimental inflammatory reactions, a balance between activation and inhibition is needed throughout the immune response. Therefore, anti-inflammatory mediators such as IL-10 and TGF-β1 are also released to inhibit the further release of proinflammatory mediators, limit the acute inflammatory response and prevent the spread of inflammatory mediators into the bloodstream (Finlay & McFadden, 2006; Navarre & Zychlinsky, 2000; Portnoy, 2005; Tracey, 2002). In humans, the production of IL-10 and TGF-β1 have been found in normal mammary tissues and breast milk (Garofalo *et al.*, 1995; Gomm *et al.*, 1991; Rigotti *et al.*, 2006; Saito *et al.*, 1993). Interleukin-10 prevents the development of immunopathological lesions that result from an exacerbated protective immune response to acute and chronic infections. Overexpression of IL-10 correlates with diminished host cell activity and the failure to control bacterial infections (Mege *et al.*, 2006; Moore *et al.*, 2001). Transforming growth factor-beta can modulate expression of adhesion molecules, provide a chemotactic gradient for leukocytes and other cells participating in an inflammatory response, and inhibit them once they have become activated (Letterio & Roberts, 1998). It has been reported that TGF-β not only suppresses the generation of T helper type 1 (T_H1) and T_H2 cells (Taylor *et al.*, 2006), but also is directly involved in the generation of T_H17 cells that produce IL-17, which in turn promotes a range of immune-mediated inflammatory diseases (Cua & Kastelein, 2006). Studies in cattle have shown that the production of IL-10 (Bannerman *et al.*, 2004), TGF-β1 and TGF-β2 (Chockalingam, Paape & Bannerman, 2005) in milk increases following intramammary inoculation with *E. coli*.

Hormones and acute-phase proteins

The activation of the hypothalamic-pituitary-adrenal (HPA) axis is achieved prior to parturition, leading to increased production of cortisol and prostaglandins (PGs). Production of PGs is stimulated by cortisol. Prostaglandins play a physiological role at parturition by being involved in the drive towards contractile activity and parturition (Britt, Almond & Flowers, 1999; Challis, Lye & Gibb, 1997; Mitchell *et al.*, 1995). However, both cortisol and PGs have immune modulatory effects. Around parturition, high cortisol concentrations in blood may be largely responsible for delaying neutrophil recruitment and activation (Burton & Erskine, 2003). In the sow, it is well established that the concentrations of these hormones vary around parturition with peak plasma concentrations of cortisol up

to approximately two to three times (Baldwin & Stabenfeldt, 1975; Österlundh, Holst & Magnusson, 1998). In addition to their physiological role at parturition, cortisol and the PGs are also involved in the inflammatory response to invading bacteria. In pigs, it has been reported that the concentrations of cortisol and prostaglandin F2-alpha ($PGF_{2\alpha}$) in blood increased following experimental administration of *Salmonella typhimurium* endotoxin (Garcia *et al.*, 1998; Magnusson *et al.*, 1994). Further, increased plasma concentrations of prostaglandin E ($PGF_{2\alpha}$) (Mwanza *et al.*, 2001) and PGE_2 (Wright *et al.*, 2000) have been found after administration of LPS derived from *E. coli*.

The APPs are a group of serum proteins that undergo substantial quantitative changes in response to infection or trauma (Baumann & Gauldie, 1994; Heinrich, Castell & Andus, 1990). Hepatocytes increase their synthesis and secretion of APPs, including SAA, Hp, C-reactive protein (CRP) and fibrinogen when stimulated by IL-1, IL-6 and TNF-α (Baumann & Gauldie, 1994; Suffredini *et al.*, 1999; Taga & Kishimoto, 1997). An inflammatory response can therefore be readily monitored by quantifying the concentration of these serum proteins which are produced in significant amounts by the liver in response to the proinflammatory cytokines. Serum concentrations of individual APPs may increase from two to three times to several hundred times during the acute phase of an inflammation. In addition to hepatocyte synthesis and secretion of Hp and SAA, extrahepatic synthesis of these proteins in the bovine mammary gland has also been reported (Hiss *et al.*, 2004; McDonald *et al.*, 2001). In pigs the concentrations of Hp and SAA in blood increased following experimental infection with *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) (Hall *et al.*, 1992; Heegaard *et al.*, 1998), or *Mycoplasma hyorhinis* (Magnusson *et al.*, 1999). As regards mastitis, concentrations of Hp and SAA in blood have been reported to have increased in dairy cows with naturally occurring mastitis (Conner *et al.*, 1986; Eckersall *et al.*, 2001) or in cows with experimentally induced *E. coli* mastitis (Salonen *et al.*, 1996), but when the present study was initiated the role of soluble mediators of the early inflammatory response in the mammary gland of the sow was largely unknown.

Aims of the study

The overall objective of the present work was to monitor the induction of some key factors involved in the early inflammatory response in the mammary gland of periparturient sows to experimentally induced *E. coli* mastitis. We wanted to gain a better understanding of why some sows develop clinical mastitis when challenged with environmental *E. coli*, while others remain clinically healthy.

The specific aims were to –

- establish a technology that enables the detection and quantification of selected cytokines and cellular receptors in biopsy material from porcine mammary glands;

- test the hypotheses that concentrations of the proinflammatory cytokines TNF-α and IL-6, and the acute-phase proteins SAA and haptoglobin in blood increase in sows following intramammary *E. coli* inoculation, and that sows that develop clinical signs of mastitis have higher concentrations of TNF-α, IL-6, SAA and Hp in blood than do sows that remain clinically healthy;

- investigate whether there is a constitutive as well as an induced proinflammatory cytokine mRNA expression and protein production in mammary glands of sows intramammarily inoculated with *E. coli* at parturition;

- establish whether regulatory cytokine production varies between mammary glands of sows that do and sows that do not develop clinical signs of mastitis;

- evaluate whether mRNA expression of IL-1R1, TLR2 and TLR4 is upregulated in the mammary gland of sows following intramammary *E. coli* inoculation, and whether this expression differs between sows that develop clinical signs of mastitis and sows that remain clinically healthy; and

- investigate whether there is consistency in the variations in cytokine levels between different physiological compartments in sows intramammarily inoculated with *E. coli*.

Methodological considerations

Experimental design and sample collection

The samples analysed in the present study (and summarized in Table 1) originate from previous studies performed by Österlundh et al. (2002) and Löving & Magnusson (2002) on a total of 16 crossbred (Swedish Landrace × Yorkshire) primiparous sows. The animals were obtained from a commercial farm approximately 6–8 weeks before farrowing and used in the present study, 16 sows for Paper I, and twelve inoculated sows for Papers II–IV. Prior to the start of the trial, clinical signs of mastitis or other diseases were not observed in any of the sows. Experimental procedures and housing conditions were approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden.

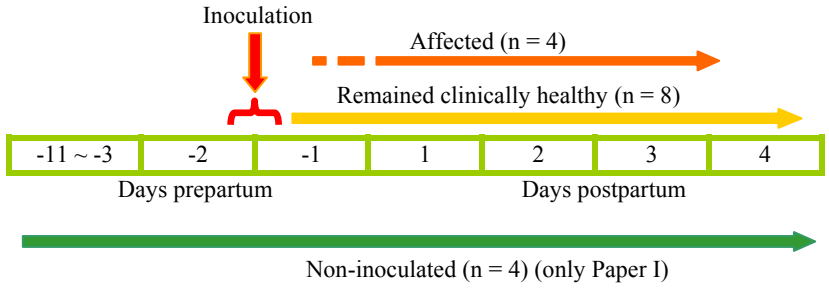


Figure 1. Schematic description of the experimental design.

Experimental model and inoculation

The experimental model and categorization of sows have previously been described in detail (Österlundh *et al.*, 2002). In brief, the sows were randomly assigned to two groups. Sows in the non-inoculated group (n = 4) (Paper I) underwent normal farrowing, while sows in the inoculated group (n = 12) (Papers I–IV) were inoculated with *E. coli* (serotype O127) (Pedersen Mörner, Faris & Krovacek, 1998). Each teat at the right side of the mammary glands (i.e. of inoculated mammary glands) was inoculated with 0.5 mL of bacterial suspension (10^5 colony-forming units (CFUs)/mL) during the 24-hour period preceding parturition (Fig. 1). The concentrations of bacteria in culture solution were confirmed retrospectively using the viable count method (Söderlind, 1973). The contralateral mammary glands were used for sampling from non-inoculated mammary glands.

Table 1. Overview of samples used and analyses performed in the present study

Samples and analyses	Sows		
	Inoculated sows Affected (n = 4) Non-affected (n = 8)		Non-inoculated sows (n = 4)
Serum/plasma ELISA	IL-6, TNF-α, SAA, Hp, cortisol		
Mammary gland biopsies	Inoculated	Non-inoculated	Not analysed
IHC RT-PCR qRT-PCR	IL-1β, IL-6, IL-8, TNF-α IL-1β, IL-6, IL-8, TNF-α IL-1β, IL-6, TNF-α, IL-10, TGF-β1, IL-1R1, TLR2, TLR4		

For an explanation of the abbreviations used, see "Abbreviations".

Clinical examination and categorization of animals

During the experiment, clinical examinations were performed within 48 hours postpartum, including palpation of the mammary glands, and recording of rectal temperature, habitus and appetite as described (Österlundh *et al.*, 2002). Sows that exhibited all the symptoms of clinical mastitis, i.e. fever of > 39.5°C, anorexia, lethargy, and swelling of two or more mammary glands, were categorized as affected. Based on this, four of the inoculated sows were categorized as clinically affected. The other eight animals were categorized as non-affected, some had mild habitus and affection of the appetite or a fever on one or two measurement occasions, and one sow had mild mammary swelling.

Blood samples (Paper I)

Blood samples were collected from non-inoculated and inoculated sows for 3 consecutive days within 3–11 days prepartum, and 0, 24, 48, 72 and 96 hours after inoculation, respectively (Fig. 1). Plasma and serum samples were collected and stored at -80°C until subsequent analysis.

Biopsy samples (Papers II–IV)

The biopsy procedure was performed as previously described (Magnusson, 1999), with some modifications (Löving & Magnusson, 2002). Briefly, two biopsies per mammary gland were carried out using a human Bard® Magnum® Biopsies instrument and a Core Tissue Biopsy Needle (12 G × 10 cm) (CR BARD Inc., Covington, GA, USA). Mammary gland biopsy was performed immediately before inoculation and from the inoculated and the contralateral non-inoculated mammary glands, 24 hours after inoculation. The samples were designated as the pre-inoculation, non-inoculated and inoculated samples, respectively. On each sampling occasion, mammary glands were used that had not been sampled previously. The specimens were immediately frozen in liquid nitrogen and stored at -80°C until used for analyses.

Blood analyses (Paper I)

Plasma IL-6, serum TNF-α and plasma SAA concentrations were assayed (Plasma IL-6, using polyclonal anti-porcine IL-6 antibody; serum TNF-α, using monoclonal anti-porcine TNF-α antibody (R & D Systems Europe Ltd, Abingdon, UK); and plasma SAA, using monoclonal antibody specific for SAA (Tridelta Development Ltd, Bray, Wicklow, Ireland)) by means of commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions.

Serum Hp concentrations were determined using an automated chemistry analyser (Cobas Mira; Hoffman-La Roche Ltd, Basel, Switzerland) for a haemoglobin-binding method (Phase Range haptoglobin assay reagent kit; Tridelta Development Ltd, Bray, Wicklow, Ireland).

Plasma cortisol concentrations were determined by means of a solid-phase radioimmunoassay kit (Coat-A-Count Cortisol; Diagnostic Products Corporation, Los Angeles, CA, USA) validated for use in porcine plasma (Österlundh, Holst & Magnusson, 1998).

Reverse transcriptase-polymerase chain reaction and nested polymerase chain reaction (Paper II)

The extraction of total RNA from tissues with low concentrations of mRNA is the key step in reverse transcriptase-polymerase chain reaction (RT-PCR) from limited tissue samples (e.g. biopsies). The combined use of two commercially available RNA isolation kits that consistently produce high-quality RNA was therefore applied (Papers II and IV). Briefly, total RNA was extracted from frozen mammary biopsy samples using TRIzol Reagent (Invitrogen Ltd, Paisley, UK), followed by additional RNeasy Spin Column purification. The resuspended RNA was further purified through the RNeasy Isolation System (Qiagen Ltd, Crawley, UK) using RNeasy Spin Columns. For each sample, the amount of RNA extracted was determined and its purity (OD260/OD280 absorption ratio > 1.90) verified before storage at -80°C.

An amount of 2 μg of total RNA was reverse-transcribed to synthesize first-strand complementary deoxyribonucleic acid (cDNA) using reverse transcriptase (Invitrogen Ltd, Scotland, UK). Several reverse transcription reactions of each sample were run and pooled at the end in order to minimize the differential synthesis of cDNA in each reaction. The cDNAs were then used as templates for PCR. The products from PCR reactions were used as templates in separate nested PCR reactions. Repeated experiments were carried out in order to determine the optimal cycle number for each cytokine to ensure that the analyses were performed at the exponential phase of amplification, before the saturation level was reached. The PCR products were separated by agarose gel electrophoresis. After staining with ethidium bromide, the band intensities of the PCR products were measured by a ChemiDoc Imager (Bio-Rad Laboratories, Inc, CA, USA).

The intensity of the bands was quantified by densitometry analysis using Quantity One software (Bio-Rad Laboratories, Inc, CA, USA). Cytokine mRNA expression was presented as the ratio of the cytokine band intensity in relation to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) band intensity.

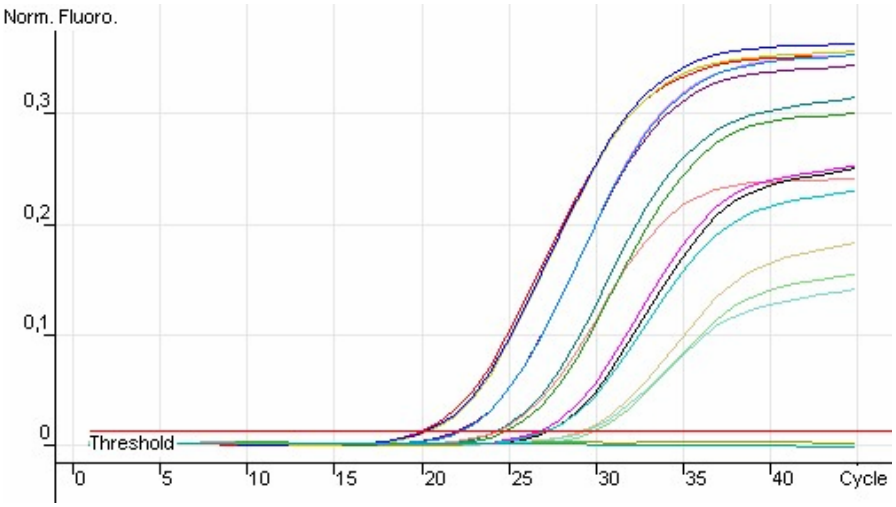
Before the real-time RT-PCR technique was developed, standard RT-PCR was used as a semi-quantitative assessment of gene expression. However that method is undoubtedly less sensitive compared with quantitative real-time RT-PCR, while conventional quantification protocols are labour-intensive, time-consuming and highly expensive. Consequently, standard RT-PCR is mainly applicable to determining the expression or absence of expression of mRNA for a certain gene.

Real-time reverse transcriptase-polymerase chain reaction (Paper IV)

Quantitative real-time RT-PCR (qRT-PCR) has been recognized as an accurate and sensitive method for the detection and quantification of gene expression levels, in particular for low abundance mRNA, in tissues with low concentrations of mRNA. Because real-time RT-PCR can be monitored directly during the PCR process using fluorogenic probes, it offers the opportunity to observe the amplification kinetics of a PCR in "real time" via accumulation and measurement of specific fluorescence signals with each cycle (Bustin, 2000; Heid *et al.*, 1996). Fluorescently labelled probes provide a highly sensitive and specific method of detection, since only the desired PCR product is detected.

The purity and the integrity of RNA are critical elements for the overall success of RNA-based analyses. The integrity of total RNA was analysed by use of the Agilent RNA 6000 Nano Kit, and the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). In addition, some RNA aliquots were treated with deoxyribonuclease I (DNaseI) to eliminate genomic DNA (gDNA), which can influence the interpretation of real-time RT-PCR assay data. The RT-PCR amplification was conducted using the Rotor-Gene™3000 (Corbett Robotics Pty Ltd, Brisbane, Australia). One hundred nanogram of total RNA were added directly to a TaqMan one-step RT-PCR reaction containing rTth DNA polymerase enzyme (Applied Biosystems, Stockholm, Sweden). A no template control of nuclease-free water was included in each run. All reactions were performed in duplicate. In Paper IV, most of the cytokine and receptor real-time RT-PCR assays were adopted from published methods. However, each system was re-evaluated and optimized for the current approach. All reagents were titrated for optimal performance using total RNA isolated from porcine peripheral blood mononuclear cells (PBMCs) that were induced to produce the various cytokines and receptors according to established protocols (Andersson *et al.*, 2007).

a



b

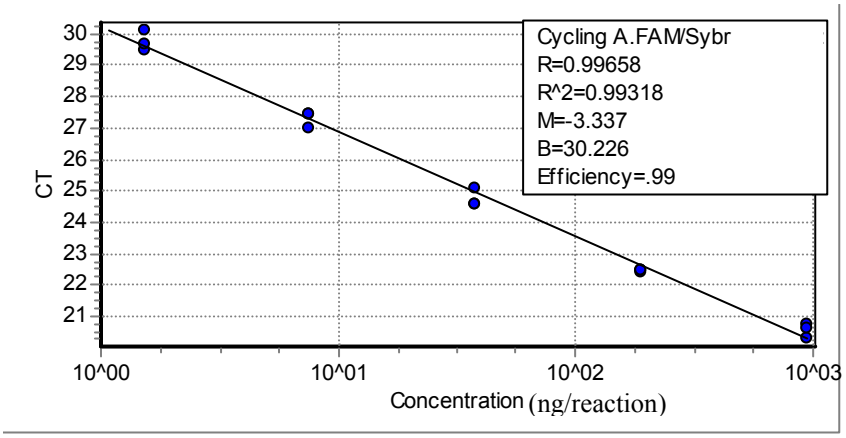


Figure 2. Serial dilutions of total RNA extracted from mammary biopsy samples were amplified by real-time RT-PCR using gene-specific primers to calculate the amplification efficiency (E) of each system (i.e. direct the Rotor-Gene software output). (**a)** Amplification plot for the target gene porcine Toll-like receptor, TLR4, showing 40 cycles of amplification of five dilutions (triplicate) of a fivefold dilution series of template RNA, and two no-template controls. The threshold is shown by the purple line. (**b)** The derived standard curve (a plot of C_T v log of concentration) from the amplification plot. The E of porcine TLR4 was determined using the C_T slope method with five data points (concentrations).

One of the major concerns regarding any real-time RT-PCR assay is the amplification efficiency (E). The E-value is empirically determined from the amplification of serial dilutions of the template RNA according to the following equation:

$$E = [10^{(-1/slope)}] - 1,$$

where the slope is calculated from the standard curve of total RNA concentrations v the C_T values (i.e. the fractional cycle number at which the fluorescence exceeds a fixed threshold) (Bustin & Nolan, 2004). In Paper IV, fivefold serial dilutions (triplicate) of total RNA extracted from mammary biopsy samples were amplified by real-time RT-PCR using gene-specific primers (e.g. Fig. 2a) to calculate the E of each system. The determined E-values were 0.99 for TLR4 (Fig. 2b), 1.02 for hypoxanthine phosphoribosyl-transferase (HPRT), 0.99 for TLR2, 1.06 for IL-1β, and 0.90 for IL-1R1, most of them close to the theoretical efficiency of E = 1.0, but the determined E-value for IL-6 was 0.72.

The use of an endogenous reference gene corrects for variation in RNA content, variation in reverse-transcription efficiency, possible RNA degradation or presence of inhibitors in the RNA sample, variation in nucleic acid recovery, and differences in sample handling. The expression level of the endogenous reference gene should not vary under different experimental conditions or in different states of the tissue; however, an ideal candidate gene which remains unchanged in each situation is not available (Bustin, 2002; Giulietti *et al.*, 2001). It should be noted that different reference genes are suitable for different organs. The most common housekeeping genes used in the relative expression studies of immunologically relevant genes in the pig are β-actin, cyclophilin, GAPDH and HPRT (Andersson *et al.*, 2007; Ledger *et al.*, 2004). The housekeeping genes GAPDH (Duvigneau *et al.*, 2005) and HPRT (Foss, Baarsch & Murtaugh, 1998) are considered to be acceptable as the endogenous reference genes when comparing different porcine tissues and immune cells. In Paper IV the C_T value was determined for each sample. To evaluate the relative mRNA expression, samples were normalized to the housekeeping gene HPRT. However, it has been suggested that the geometric mean of multiple carefully selected reference genes can be used for normalization (Duvigneau *et al.*, 2005; Skovgaard *et al.*, 2007).

Immunohistochemistry and image analysis (Paper III)

The analyses of biopsies collected from mammary glands of sows inoculated with *E. coli* demonstrated the transcription of the genes for a number of proinflammatory cytokines, but the analyses of IL-6 and TNF-α in the same set of sows revealed that variations in concentrations in blood are not reflected in the mRNA expression in mammary tissues (Papers I and II). This observation indicates the need to relate observations at the mRNA level to the protein level in order to understand the biological significance of the mRNA results and possibly disclose mechanisms of regulation. A semi-quantitative assessment of the amount

of cytokine in the mammary gland was therefore established using immunohistochemistry (IHC) analysis combined with image analysis (Paper III).

Pilot studies in our laboratory have shown that the cytokines are located both in the cytoplasmic compartments and scattered throughout the cells in mammary tissues. For such distribution of immunoreactive proteins, computerized image analysis is far more reproducible than manual scoring in assessing positive areas (Elsasser *et al.*, 2004; Huse *et al.*, 2006). Even so, IHC is only a semi-quantitative assessment of the amount of cytokine, and gives a relative measurement implying that quantitative comparisons between the various cytokines are not possible.

The 3, 3'-diaminobenzidine tetrahydrochloride (DAB)-stained areas, i.e. the positively stained areas, in the sections (counterstained with 10% Mayer's haematoxylin and mounted with glycerolgelatin) were quantified using Easy Image 3000 (Tekno Optik AB, Göteborg, Sweden). Five fields (magnification, 200 ×) from each tissue sample (three section slices were mounted per slide) were captured using a Nikon Microphot FXA equipped with a Nikon DS-5M digital camera (Nikon, Tokyo, Japan). The DAB colour-specific staining was determined by defining a threshold for hues and intensities that corresponded to those colour attributes that were assessed as positive in five randomly selected samples within the same assay run, compared with negative controls. This spectral threshold was then used for analysing all images for that particular cytokine within the same IHC run. In this way, unbiased continuity of analysis across samples was achieved. For each sample, a relative value of the amount of cytokine produced was expressed as the average percentage of the positively stained areas in five view fields. These data were accordingly considered semi-quantitative.

Statistical analysis

Statistical evaluation was performed using the SAS statistical software package, Version 9.1 (SAS Institute Inc., Cary, NC, USA).

Data from blood samples (Paper I) were analysed using analysis of variance (ANOVA) for repeated measures. The statistical model included the fixed effect of sow category, time of sample collection, and the interaction between sow category and time of sample collection. To account for repeated measures, the statistical model also included the random effect of sow within sow category. When there was an overall significant effect of sow category or time of sample collection, differences between means were compared using Student's *t*-test.

Data from mammary tissue samples (Papers II–IV) were analysed using PROC MIXED. The statistical model included the fixed effect of sow category, the mammary gland sample and the interaction between sow category and mammary gland sample, and the random effect of sow within sow category. When there was an overall effect of sow category or mammary gland sample ($p < 0.05$), pairwise comparisons between the least-square means were made. P-values < 0.05 were considered statistically significant.

Results and Discussion

More knowledge about the inflammatory response and its regulation following bacterial-induced mastitis is needed to gain a better understanding of why certain sows develop clinical signs of mastitis when challenged with environmental bacteria, and others remain clinically healthy. In the current experiment only one-third of the inoculated sows developed clinical mastitis. Previous studies using the same experimental model but focusing on granulocytes that are crucial for bacterial clearance in the mammary gland, did not indicate any impaired function of these cells before inoculation, in the sows that developed clinical signs of mastitis (Österlundh *et al.*, 2002). The present study of the innate immune system in periparturient sows, responding to experimentally induced *E. coli* mastitis, therefore concentrated on the events preceding the granulocyte response. In particular, soluble factors that may be involved in the early inflammatory response were explored.

The proinflammatory cytokines and the acute-phase proteins as markers for mastitis

The proinflammatory cytokines in blood – markers for coliform mastitis

Increased concentrations of IL-6 and TNF-α in blood were found following intramammary *E. coli* inoculation. Furthermore, the concentrations of IL-6 and TNF-α in blood were significantly higher in sows that developed clinical signs of mastitis than in sows that remained clinically healthy 24 hours after inoculation (Paper I). These data are consistent with previous studies in cattle, in which the levels of IL-6 and TNF-α in blood correlated with the severity of coliform mastitis (Nakajima *et al.*, 1997; Sordillo & Peel, 1992).

The presence of cytokines in blood collected from healthy, pregnant sows (Paper I) is in accordance with findings in pregnant women (Hebisch *et al.*, 2004; Vassiliadis *et al.*, 1998), and appears to be important at different stages of pregnancy and parturition (Alvarez-de-la-Rosa *et al.*, 2000; Denison, Kelly & Calder, 1997). Cytokines such as IL-1β, IL-6, IL-8, TNF-α, IL-10 and TGF-β have also been proposed to play a physiological role at parturition by being involved in the regulation of the parturition process as such (Bowen *et al.*, 2002; Keelan *et al.*, 2003).

It seems that the production and the effect of some of the cytokines that are involved in the regulation of parturition are not reflected in blood, as revealed by the unaffected levels of IL-6, TNF-α (Paper I), IL-1β and IL-8 (Cicarelli *et al.*, 2005) during parturition. We showed a constitutive production of proinflammatory cytokines in blood collected from healthy, pregnant sows, while no increase in the concentrations of IL-6 and TNF-α in blood was found following the onset of parturition. These results are in accordance with a previous study in humans, in which the concentrations of IL-1β and TNF-α in blood were unaltered during all the periods of collection (Cicarelli *et al.*, 2005). By contrast, in another study,

increased serum concentrations of IL-1β and IL-6 were found at normal term delivery (Hebisch *et al.*, 2004).

Obviously, there are contradictions in the literature about the increase in proinflammatory cytokines in blood at normal parturition. It should be mentioned that the frequency of sample collection and the definition of "peripartum" may influence the results. In the present study, we established strict time points to define peripartum. Our data indicate that the concentrations of IL-6 and TNF-α in porcine blood are not affected by normal parturition. Taken together, these findings suggest that IL-6 and/or TNF-α in blood could be used as markers for identification of periparturient sows with coliform mastitis.

The acute-phase proteins and cortisol

Previous studies in cattle have suggested that the concentrations of Hp and SAA in blood may have the potential to be used in detection of the disease, but they could not be used for discriminating the severity of mastitis (Eckersall *et al.*, 2001; Lehtolainen, Rontved & Pyörälä, 2004). Here we found that the levels of Hp and SAA in blood increase 24 and 48 hours after normal parturition, respectively (Paper I). This is why we did not observe a specific increase in serum Hp concentrations following inoculation with *E. coli*. Our data revealed that plasma SAA concentrations increased 24 hours after intramammary *E. coli* inoculation, but we were unable to observe any difference in the levels of SAA and Hp in blood between sows that developed clinical signs of mastitis and sows that remained clinically healthy after inoculation. Although we found increased plasma cortisol concentrations in sows that developed clinical signs of mastitis 24 hours after inoculation, the present (Paper I) and previous studies in pigs showed that the levels of cortisol in blood also increase around normal parturition (Baldwin & Stabenfeldt, 1975; Magnusson *et al.*, 2001; Österlundh, Holst & Magnusson, 1998). These observations suggest that plasma SAA, serum Hp and plasma cortisol are less suitable as markers for coliform mastitis in periparturient sows.

Cytokine responses in the mammary gland to intramammary *Escherichia coli* inoculation

Cytokine production in normal mammary glands

Cytokines appear to be an important component of a paracrine/autocrine communication network in the mammary gland at different stages of pregnancy and parturition (Hennighausen & Robinson, 2005; Rainard & Riollet, 2006). Cytokine production at both the mRNA (Papers II and IV) and the protein (Paper III) level was seen in normal mammary glands of pregnant sows. Our findings are consistent with previous studies in humans (Adams, Rafferty & White, 1991; Basolo *et al.*, 1993; Garofalo *et al.*, 1995; Gomm *et al.*, 1991; Green *et al.*, 1997; Palkowetz *et al.*, 1994) as well as in cattle (Hagiwara *et al.*, 2000; McClenahan *et al.*, 2006; Okada *et al.*, 1997) in which some baseline production of cytokines takes place in normal mammary glands. Indeed, our data demonstrate that cytokines can be produced and secreted in the mammary gland.

The pre-inoculation production of IL-6 in blood was lower in sows that developed clinical signs of mastitis compared with sows that remained clinically healthy (Paper I). By contrast, when studying mammary glands in the same set of sows, no differences were found between the two groups in their pre-inoculation levels of IL-6 measured by RT-PCR (Paper II) and qRT-PCR (Paper IV), or determined by IHC analysis (Paper III). Possible explanations for this inconsistency between systemic and local proinflammatory cytokine levels may be that the proinflammatory cytokines could also be produced elsewhere (Baumann & Gauldie, 1994; Kofler, Nickel & Weis, 2005).

Notably, the pre-inoculation levels of IL-1β (Papers II–IV) were lower in the mammary glands of sows that developed clinical signs of mastitis compared with sows that remained clinically healthy. The underlying mechanisms of this remain unknown, although it has been reported that IL-1 is necessary to stimulate the production of chemokine attractants for neutrophils such as MIP2 and IL-8, which in turn recruit neutrophils to the site of infection, an event required for bacterial clearance (Armstrong *et al.*, 2004; Gamero & Oppenheim, 2006; Miller *et al.*, 2006). Consequently, these earlier data and the data presented here call for further studies to elucidate a possible role of IL-1β in protection against coliform mastitis.

Proinflammatory cytokine responses in the mammary gland

The analyses of proinflammatory cytokines by standard RT-PCR (Paper II) and qRT-PCR assays (Paper IV), as well as IHC analysis (Paper III) indicated an increased production of IL-1β, IL-6 and TNF-α in the inoculated mammary glands of sows that developed clinical signs of mastitis. By contrast, in sows that remained clinically healthy there was no increase in the production of IL-1β, IL-6 (Papers II and III), and TNF-α (Paper III) in the inoculated mammary glands. However, as shown by qRT-PCR assays (Paper IV), there was an increase in the expression of IL-1β and TNF-α mRNAs also in these sows. Moreover, IL-1β mRNA expression was higher in the inoculated mammary glands of sows that developed clinical signs of mastitis compared with those that remained clinically healthy following intramammary inoculation. The discrepancy between the two studies is likely due to the fact that qRT-PCR assays are more sensitive and have a much greater range when comparing different levels of nucleic acid, compared with standard RT-PCR assays.

In accordance with our results, previous studies in cattle have shown a significant increase in the proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α at the level of both mRNA (Lee *et al.*, 2006; McClenahan *et al.*, 2006) and protein (Bannerman *et al.*, 2004; Blum *et al.*, 2000; Chockalingam, Paape & Bannerman, 2005; Hoeben *et al.*, 2000; Lee *et al.*, 2003; Riollet, Rainard & Poutrel, 2000b; Shuster *et al.*, 1997; Vangroenweghe, Duchateau & Burvenich, 2004) in either mammary tissues or milk collected from infected glands following intramammary inoculation with *E. coli*. These data indicate that development of clinical symptoms of coliform mastitis is associated with locally increased proinflammatory cytokine production in response to intramammary *E. coli* inoculation.

Interleukin-1, which is produced during infection not only has local effects, involving the local inflammatory response, but also has systemic effects, including the induction of acute-phase responses and effects on appetite, sleep and body temperature (Allan, Tyrrell & Rothwell, 2005; Dinarello, 1996). Most of the biological actions of IL-1 seem to be mediated through specific receptors including IL-1R1 (Dinarello, 1996). It was somewhat unexpected that no increase in IL-1R1 mRNA expression was found in mammary glands in any of the groups (Paper IV). Interestingly, some studies have indicated that the effects of IL-1β are not only mediated by IL-1R1 (Parker *et al.*, 2002; Touzani *et al.*, 2002). Whether this could be the case in the mammary gland is currently unknown.

As regards IL-8, an increased production at both the mRNA (Paper II) and the protein (Paper III) level was found in the inoculated mammary glands of both sows that did and sows that did not develop clinical signs of mastitis. Likewise, an increase in IL-8 mRNA expression (Paper II) was found in the non-inoculated mammary glands in both groups of sows after inoculation. This may suggest that in the lactating porcine mammary gland, IL-8 has a physiological role to attract neutrophils. This is supported by data in other species and by data showing that colostrum collected from healthy sows contains a high proportion of neutrophils (Magnusson, Rodríguez-Martínez & Einarsson, 1991; Negus, 1996). This physiological increase in local production of IL-8, following the onset of parturition, may explain why we did not observe any difference in IL-8 production in mammary glands between sows that developed clinical signs of mastitis and sows that remained clinically healthy after inoculation (Paper III). By contrast, as shown by standard RT-PCR assays (Paper II), IL-8 mRNA expression was higher in the inoculated mammary glands of sows that developed clinical signs of mastitis than in those that remained clinically healthy. Similarly, a previous study in cows showed that the concentrations of IL-8 in milk in infected quarters were higher in cows that developed moderate clinical signs of mastitis than in those that developed mild clinical signs of mastitis following intramammary *E. coli* inoculation (Vangroenweghe, Duchateau & Burvenich, 2004).

Anti-inflammatory cytokine responses in the mammary gland

Anti-inflammatory cytokines such as TGF-β and IL-10 specifically inhibit the release of TNF and other proinflammatory mediators (Tracey, 2002; Tsunawaki *et al.*, 1988). Interleukin-10 is regarded as a critical anti-inflammatory mediator, while overexpression of IL-10 correlates with diminished host cell activity and the failure to control bacterial infections (Mege *et al.*, 2006; Moore *et al.*, 2001). As shown by qRT-PCR assays, IL-10 mRNA expression increased in the inoculated mammary glands of sows that developed clinical signs of mastitis as well as of those that remained clinically healthy 24 hours after inoculation (Paper IV). This is in accordance with a previous study in cows, in which the increased production of IL-10 was seen in milk collected from infected quarters 24 hours after intramammary inoculation with *E. coli* (Bannerman *et al.*, 2004). Moreover, we showed that IL-10 mRNA expression was higher in the inoculated mammary glands of sows that developed clinical signs of mastitis compared with sows that remained clinically healthy (Paper IV).

Concerning TGF-β1, a recent study has shown increased concentrations in milk collected from infected quarters of cows 32 hours after intramammary inoculation with *E. coli* (Chockalingam, Paape & Bannerman, 2005). By contrast, we did not observe any increase in TGF-β1 mRNA expression 24 hours after inoculation (Paper IV). It cannot be excluded, however, that an increase in TGF-β1 mRNA expression may have been detected later after inoculation.

In general, these findings suggest that development of clinical symptoms of coliform mastitis is associated with the degree of local production of regulatory cytokines in response to intramammary *E. coli* inoculation.

Toll-like receptors and *Escherichia coli* in the mammary gland

As shown by qRT-PCR assays (Paper IV), both TLR4 and TLR2 mRNA expression increased in the inoculated as well as the non-inoculated mammary glands of sows that developed clinical signs of mastitis and of sows that remained clinically healthy. However, TLR2 mRNA expression was higher in the inoculated mammary glands than in the non-inoculated mammary glands of the sows that remained clinically healthy. For TLR4, our data are inconclusive when it comes to discussing effects of *E. coli* inoculation as well as effects of normal parturition. Increased TLR4 and TLR2 mRNA expression in the inoculated mammary glands has previously been shown in cows with mastitis caused by either *S. aureus* or *E. coli* (Goldammer *et al.*, 2004). Toll-like receptor 2 recognizes LTA from Gram-positive bacteria, as well as peptidoglycan and bacterial lipoproteins abundant in the cell wall of Gram-positive and Gram-negative bacteria. Signalling via TLRs may commonly lead to the early activation of NF-κB and the subsequent expression and release of several proinflammatory mediators, including IL-1β, IL-6 and TNF-α (Aderem & Underhill, 1999; Akira & Takeda, 2004). Moreover, the increased expression in the non-inoculated mammary gland shown here (Paper IV) indicates a systemic release of factors that upregulate TLR2 expression, in addition to local induction at the site of infection. Even so, it may be that the expression of TLR2 as well as of TLR4 is normally upregulated in the mammary gland around parturition. This possibility warrants further studies on the expression and regulation of TLRs during pregnancy and parturition.

The discrepancy between systemic and local cytokine responses

The present results also suggest that the increased blood levels of IL-6 and TNF-α are accompanied at least in part by a local response that is likely to be important in the defence against mastitis. We showed here that the concentrations of IL-6 and TNF-α in blood were higher in the sows that developed clinical signs of mastitis compared with sows that remained clinically healthy 24 hours after intramammary inoculation with *E. coli* (Paper I). By contrast, biopsy samples from mammary glands suggested that the levels of IL-6 and TNF-α in inoculated mammary glands did not differ significantly between the two groups 24 h after inoculation, as shown by IHC analysis (Paper III) or by qRT-PCR assays (Paper IV). Possible explanations for this discrepancy between systemic and local proinflammatory

cytokine levels may be that proinflammatory cytokines could also be produced elsewhere, not only at the site of bacterial inoculation (Akira, Uematsu & Takeuchi, 2006; Baumann & Gauldie, 1994; Cohen, 2002). Alternatively, there could be a larger diffusion of cytokines into the blood in the sows with clinical signs of mastitis, which may be attributable to the inflammatory damage of mammary glands. It seems, therefore, that the discrepancy between different read-outs should be taken into account when relating cytokines to infectious diseases.

Conclusions

In the present thesis,

- RT-PCR and qRT-PCR, as well as semi-quantitative IHC analysis combined with image analysis, were applied in the detection and quantification of selected cytokines and cellular receptors in biopsy material from porcine mammary glands.

The data presented in this thesis indicate that –

- concentrations of the proinflammatory cytokines IL-6 and TNF-α, and the acute-phase protein SAA in blood increase in sows following intramammary *E. coli* inoculation. Furthermore, sows that develop clinical signs of mastitis have higher concentrations of IL-6 and TNF-α in blood than do sows that remain clinically healthy. The proinflammatory cytokines IL-6 and/or TNF-α in blood could be used as markers for identification of periparturient sows with coliform mastitis.

- some baseline production of cytokines takes place in normal mammary glands of pregnant sows, and sows that develop clinical signs of mastitis have significantly lower baseline production of IL-1β than do sows that remain clinically healthy.

- development of clinical symptoms of coliform mastitis is associated with the degree of local production of regulatory cytokines in response to intramammary *E. coli* inoculation.

- mRNA expression of TLR2 is upregulated in the mammary gland of sows following intramammary *E. coli* inoculation.

- the discrepancy between different read-outs should be taken into account when relating cytokines to infectious diseases.

Overall, the findings from the present thesis work suggest that development of clinical symptoms of coliform mastitis is associated with the degree of local production of regulatory cytokines.

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