Nuclear factor-κB activation in relation to intramammary *Escherichia coli* infections in cows and mice

by

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Dissertation submitted in fulfillment of the requirements for the degree of Doctor (Ph.D.) in Veterinary Sciences

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TABLE OF CONTENTS

Abbreviation key **1**

General introduction **5**

Hypotheses and objectives **69**

Hypotheses 71

Objectives 73

Part I ***In vitro* study of NF-κB activation in bovine neutrophils** **77**

Chapter 1 NF-κB activity in resting bovine neutrophils 79

Chapter 2 Pro- and anti-inflammatory response in bovine neutrophils upon activation by *E. coli* 101

Part II ***In vivo* study of NF-κB activation in a mouse model of intramammary *E. coli* infection** **125**

Chapter 3 Pro-inflammatory response in intramammary *E. coli* infections 127

Chapter 4 *In vivo* imaging of NF-κB activity in intramammary *E. coli* infections 151

General discussion and conclusions **177**

General discussion 179

Conclusions 189

Summary - Samenvatting **199**

Summary 201

Samenvatting 204

Dankwoord **207**

Curriculum Vitae **213**

ABBREVIATION KEY

A	adenine
aa	amino acids
Apaf-1	apoptotic protease activating factor-1
BAFF	B cell activating factor
Bcl	B cell lymphoma
bp	base pairs
C	cytosine
C3a	complement factor 3a
C5a	complement factor 5a
CD	cluster of differentiation
cDNA	complementary DNA
CFU	colony-forming unit
Cl^-	chloride ion
CNS	coagulase-negative staphylococci
CO_2	carbon dioxide
CR	complement receptor
Da	dalton
DD	death domain
DED	death effector domain
DEVD	Asp-Glu-Val-Asp
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
FADD	Fas-associated death domain
FITC	fluorescein isothiocyanate
G	guanine
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRR	glycine-rich region
h	hours
H_2O_2	hydrogen peroxide

HOCl	hypochlorous acid
HRP	horseradisch peroxidase
IAP	inhibitor of apoptosis
ICAM	intracellular adhesion molecule
ICE	IL-1β converting enzyme
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
IL-1R	interleukin-1 receptor
IRAK	IL-1 receptor-associated kinase
IRAP	IL-1 receptor accessory protein
IκB	inhibitor of NF-κB
LBP	LPS binding protein
LPS	lipopolysaccharide
LRR	leucin-rich region
LT	lymphotoxin
MAA	milk amyloid A
Mac-1	macrophage-1
mCD14	membrane CD14
Mcl	myeloid cell leukemia
MD	myeloid differentiation protein
MHC	major histocompatibility
min	minutes
MPO	myeloperoxidase
mRNA	messenger RNA
MyD88	myeloid differentiation factor 88
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NEMO	NF-κB essential modulator
NES	nuclear export signal
NET	neutrophil extracellular trap
NF-κB	nuclear factor-κB
NIK	NF-κB inducing kinase

NLS	nuclear localization signal
$NO^{\cdot}$	nitric oxide
NP-40	Nonidet P-40
O_2	oxygen
O_2^-	superoxide
$OH^{\cdot}$	hydroxyl radical
$ONOO^-$	peroxynitrate
P	phosphate
P	probability
PAMP	pathogen-associated microbial pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
PID	processing inhibitory domain
PKA	protein kinase A
PRR	pathogen recognition receptor
PS	phosphatidylserine
r	correlation
RANKL	receptor activator of NF-κB ligand
RHD	Rel homology domain
RIP	receptor-interacting protein
RLU	relative light units
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
s	second
S. agalactiae	*Streptococcus agalactiae*
S. aureus	*Staphylococcus aureus*
S. uberis	*Streptococcus uberis*
SAA	serum amyloid A
SARM	sterile α- and armadillo-motif-containing protein
SAS	Statistical Analysis Software
SCC	somatic cell count

sCD14	soluble CD14
SCV	small-colony variant
SEM	standard error of the mean
SOD	superoxide dismutase
SPSS	Statistical Package for the Social Sciences
STAT	signal transducer and activator of transcription
T	thymine
TAD	transactivation domain
TBS	Tris buffered saline
TBS-T	TBS with Tween 20
TICAM	TIR-containing adaptor molecule
TIR	Toll/IL-1 receptor
TIRAP	TIR-containing adaptor protein
TLR	Toll-like receptor
TNFR	TNF receptor
TNF-α	tumor necrosis factor-α
TRADD	TNF receptor-associated death domain protein
TRAF	TNF receptor-associated factor
U	units
Ub	ubiquitin
UV	ultraviolet
VCAM	vascular cellular adhesion molecule

GENERAL INTRODUCTION

Adapted from

Notebaert S. and Meyer E. Mouse models to study the pathogenesis and control of bovine mastitis. A review. Veterinary Quarterly (2006) 28: 2-13.

TABLE OF CONTENTS

1. The transcription factor NF-κB 7

1.1. NF-κB family 7

1.2. NF-κB activation 8

1.3. NF-κB function in innate immunity 13

2. Bovine intramammary infections 19

2.1. Biology of the bovine mammary gland 19

2.2. Innate immunity of the bovine mammary gland 20

2.3. Bovine intramammary infections 29

3. Mouse model of intramammary infections 34

3.1. Biology of the mouse mammary gland 34

3.2. Mouse model of intramammary infections 35

1. The transcription factor NF-κB

1.1. NF-κB family

Nuclear factor (NF)-κB was discovered in 1986 as a transcription factor regulating the formation of the kappa variety of the immunoglobulin light chain in B lymphocytes (Sen and Baltimore, 1986). Nowadays, it is known to be present in nearly all eukaryotic cell types where it regulates the expression of a numerous amount of genes, in particular those of which the products are involved in immune processes and inflammatory diseases (Liou, 2002).

The NF-κB family comprises proteins which are structurally and functionally related (May and Ghosh, 1997; Hayden and Ghosh, 2004; Hayden and Ghosh, 2008). Each member contains a conserved amino-terminal region called the Rel homology domain (RHD). This motif is responsible for dimerization with other NF-κB family members and for the interaction with the inhibitor proteins of NF-κB (IκB). Furthermore, it contains the nuclear localization signal (NLS) and controls binding to the κB elements on the DNA.

To date, five NF-κB family members have been identified in mammalian cells: RelA (also known as p65), RelB, c-Rel, NF-κB1 (p50) and NF-κB2 (p52) (Figure 1). RelA, RelB and c-Rel are produced as transcriptionally active proteins. They contain one or more carboxy-terminal transactivation domains (TADs). NF-κB1 and NF-κB2 are synthesized as longer precursor molecules of 105 and 100 kDa, known as p105 and p100, respectively, which are further processed to the smaller, transcriptionally active forms.

NF-κB dimers are sequestered in the cytosol of unstimulated cells via interactions with their inhibitors. The IκB family also comprises structurally and functionally related proteins. To date, eight members have been identified: IκB-α, IκB-β, IκB-γ (which corresponds to the carboxy-terminal part of p105), IκB-ε, IκB-ζ, Bcl-3 and the NF-κB precursor molecules p105 and p100 (Figure 1). They share ankyrin repeats which interact with the NLS of the RHD of NF-κB family members, thereby preventing their nuclear translocation. As an exception, IκB-ζ is a nuclear protein of which the inhibitory action lies within the prevention of NF-κB binding to the DNA. Finally, Bcl-3 is not an inhibitor, but rather an activator of NF-κB. It delivers TAD to transcriptionally inactive NF-κB complexes, such as p50 or p52 homodimers,

or removes such complexes from the DNA in order to allow active dimers to bind to the κB elements to initiate transcription.

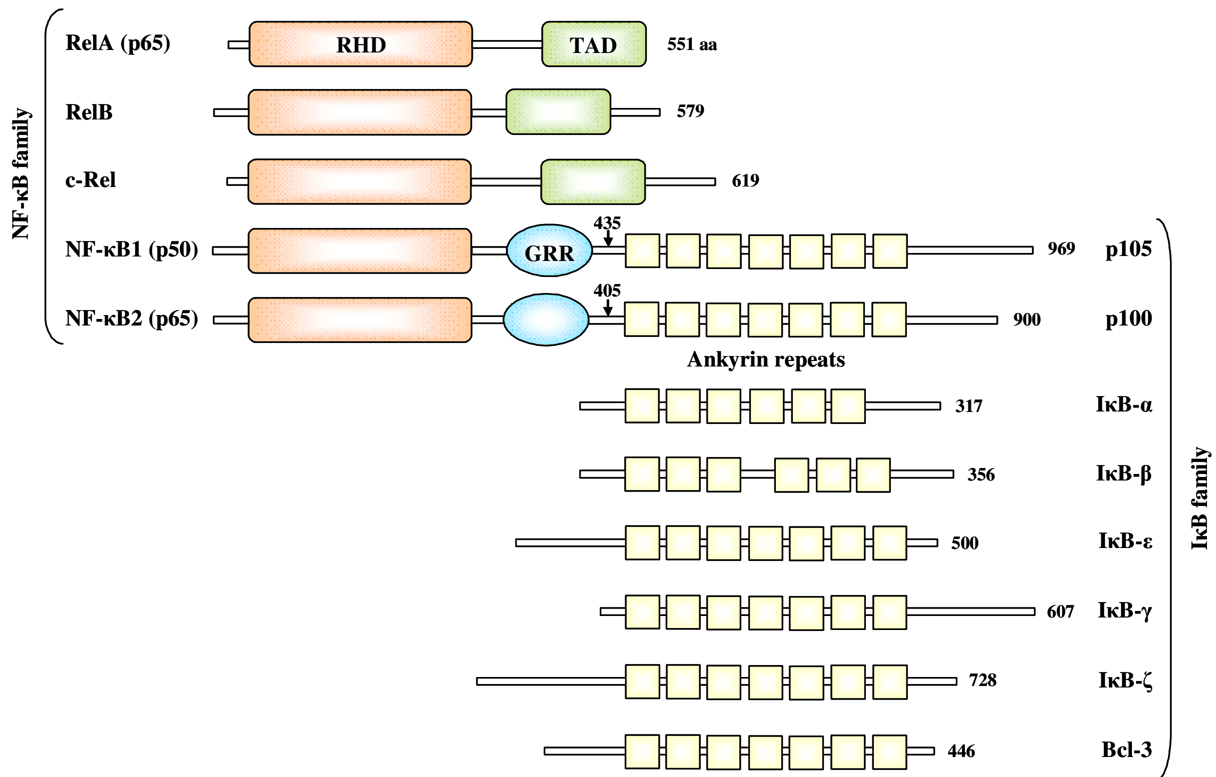


Figure 1: Structures of the NF-κB and IκB protein family members. RHD: Rel homology domain, TAD: transactivation domain, GRR: glycine-rich region, aa: amino acids.

1.2. NF-κB activation

1.2.1. Classical NF-κB activation pathway

NF-κB activation occurs very rapidly upon cell activation mediated by extracellular signals (Karin and Ben-Neriah, 2000). It mainly involves the degradation of IκB so that the NLS is exposed and the NF-κB dimer translocates to the nucleus to initiate transcription (Figure 2). In the classical NF-κB activation pathway, the degradation of the inhibitor is preceded by the phosphorylation of two conserved amino-terminal serine residues (Ser32 and Ser36 for IκB-α). These modifications trigger polyubiquitination (of Lys21 and Lys22 from IκB-α) which leads to the degradation by the 26S proteasome (May and Ghosh, 1998).

The phosphorylation of IκB is mediated by the IκB kinase (IKK) complex, which is activated by different stimuli (Rothwarf and Karin, 1999; Karin and Delhase, 2000) (Figure 2). This complex consists of three important subunits: two catalytic subunits IKK-α (IKK-1) and IKK-β (IKK-2) and a regulatory subunit IKK-γ which is also called NF-κB essential modulator (NEMO). A fully active IKK complex is composed of the IKK-α/IKK-β heterodimer associated with one or several IKK-γ molecules. The presence of the regulatory subunit is indispensable for the activity of IKK. IKK activation depends on phosphorylation of its catalytic subunits, which might be mediated by the NF-κB inducing kinase (NIK) (Hayden and Ghosh, 2008).

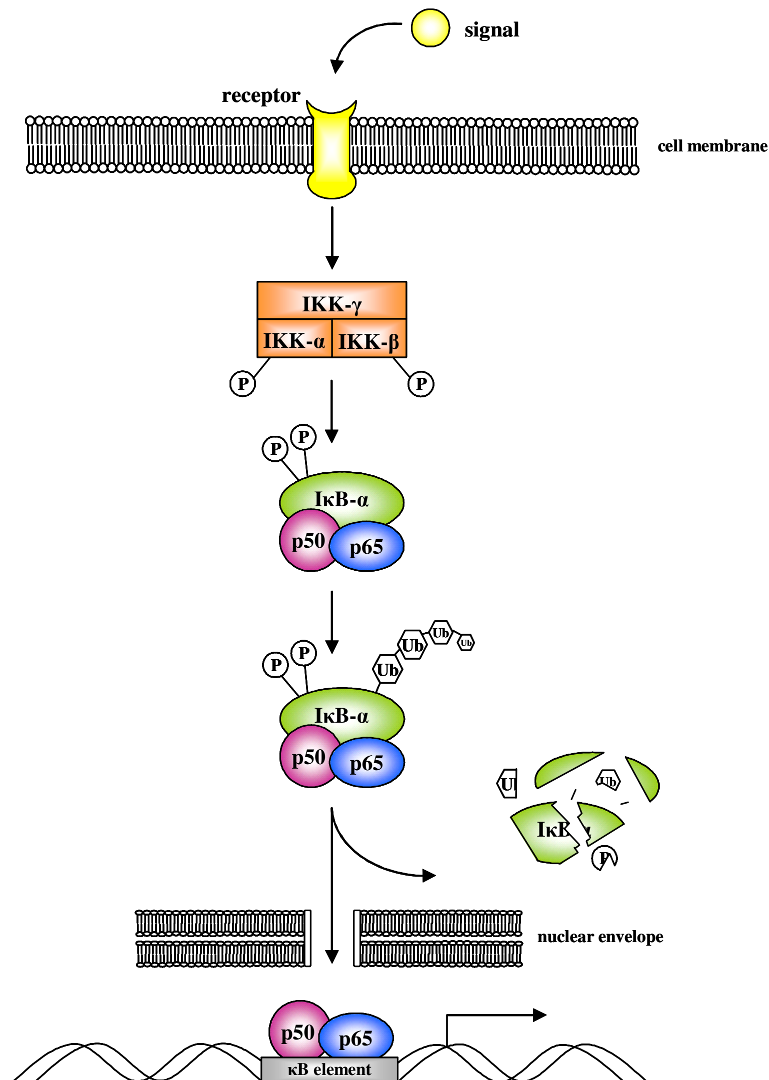


Figure 2: The classical NF-κB activation pathway. The IKK complex becomes activated in response to different extracellular stimuli. This leads to the phosphorylation of IκB-α and its subsequent polyubiquitination. Following the degradation of the inhibitor by the 26S proteasome, the NF-κB p65/p50 heterodimer translocates to the nucleus where it initiates transcription. IKK: IκB kinase, IκB: inhibitor of NF-κB, P: phosphate, Ub: ubiquitin.

The most abundant and best studied NF-κB complex is the p65/p50 heterodimer. This complex is continuously shuttling between the nucleus and the cytoplasm upon interaction with IκB-α (Birbach et al., 2002). Due to the nuclear export signal (NES) of IκB-α, nuclear NF-κB complexes immediately return to the cytoplasm (Huang et al., 2000). Since its rate of nuclear export exceeds the rate of its nuclear import, this complex is generally cytoplasmic. This nucleo-cytoplasmic shuttling is only feasible upon association with IκB-α. IκB-β preserves the cytoplasmic localization of NF-κB by a more efficient blockage of the NLS of NF-κB (Malek et al., 2001). In addition, IκB-β can not export active NF-κB complexes to the cytoplasm. On the contrary, IκB-α binds to NF-κB in the nucleus, which then dissociates from the DNA and returns to the cytoplasm (Tam and Sen, 2001). Newly synthesized IκB-α, of which the expression is under control of NF-κB itself, therefore plays an important role in the termination of NF-κB activation.

1.2.2. Atypical NF-κB activation pathways

Phosphorylation by IκB-α of other amino acids than the serine residues, such as Tyr42, has been described (May and Ghosh, 1998). This modification results in dissociation of the inhibitor from the NF-κB complex without its subsequent degradation by the proteasome. Without its inhibitor, NF-κB enters the nucleus to initiate transcription. Remarkably, it has also been demonstrated that tyrosine phosphorylation of IκB-α is not coupled to NF-κB activation. This modification then prevents phosphorylation of the serine residues, perhaps by altering the substrate sequence that is recognized by IKK, and thereby abolishes NF-κB activation in response to classical stimuli. A second atypical NF-κB activation pathway is characterized by the degradation of IκB-α by the proteasome following its ubiquitination, but in the absence of IKK activation or phosphorylation of its serine or tyrosine residues (Bender et al., 1998; Li and Karin, 1998).

1.2.3. Alternative NF-κB activation pathway

Both NF-κB1 and NF-κB2 are synthesized as larger precursor molecules. The precursor p105 mainly undergoes constitutive processing, which is reported to be co-translational (Lin et al., 1998), while the degradation of the precursor p100 is a tightly regulated event, which generally occurs post-translational (Heusch et al., 1999). Processing requires proteolysis by the proteasome and is therefore comparable to the IκB degradation. Unprocessed p105 and

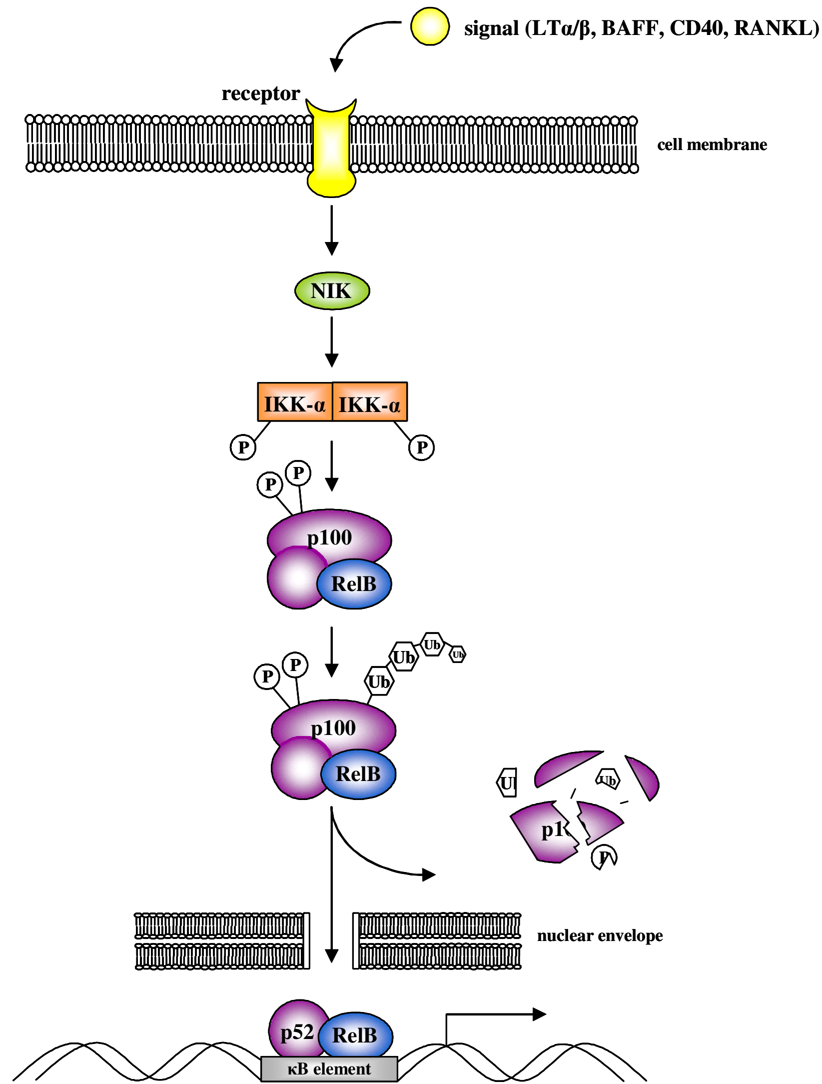


Figure 3: The alternative NF-κB activation pathway. NIK phosphorylates and activates IKK-α in response to specific extracellular stimuli. This leads to the phosphorylation of p100 and its subsequent polyubiquitination. Following the processing of p100 into p52, the NF-κB p52/RelB heterodimer translocates to the nucleus where it initiates transcription. LT: lymphotoxin, BAFF: B cell activating factor, RANKL: receptor activator of NF-κB ligand, NIK: NF-κB inducing kinase, IKK: IκB kinase, P: phosphate, Ub: ubiquitin.

p100 form stable dimers with other NF-κB family members which are retained in the cytoplasm due to their ankyrin repeats. Following phosphorylation and ubiquitination, the carboxy-terminal domain of the precursor is degraded by the 26S proteasome. Complete destruction of NF-κB1 is prohibited by a glycine-rich region (GRR) which acts as a physical barrier preventing entry of p50 into the cavity of the proteasome. This GRR might also be a recognition site for an endoproteolytic enzyme, which separates the N-terminus, p50, from the ubiquitinated C-terminus, which is then degraded (Hayden and Ghosh, 2004). Processing of

p100 into p52 probably occurs by similar mechanisms (Dejardin, 2006) (Figure 3). The presence of a processing inhibitory domain (PID) in p100 ensures the inducible degradation instead of a constitutive processing. A role for NIK and IKK-α, but not for IKK-β or IKK-γ, is apparent in this process (Senftleben et al., 2001). In contrast to the classical NF-κB pathway, the activation of this alternative pathway appears to be restricted to a limited number of inducers such as lymphotoxin (LT) α/β, B cell activating factor (BAFF), CD40 and receptor activator of NF-κB ligand (RANKL) (Dejardin, 2006).

1.2.4. NF-κB transactivation

NF-κB translocation to the nucleus is dependent upon IκB degradation. Additionally, the activity of NF-κB is regulated by post-translational modifications. They enhance the efficiency of DNA binding and ensure recruitment of co-activators to the enhancer regions. Stimulus-dependent phosphorylation of p65 was the first regulatory event that was recognized to occur downstream from IκB degradation (Naumann and Scheidereit, 1994). Protein kinase A (PKA) is the classical example of an enzyme able to phosphorylate p65 (Zhong et al., 1998). Its catalytic subunit is associated with cytosolic complexes of NF-κB and IκB. Degradation of IκB upon stimulation activates PKA, which then phosphorylates Ser276 of p65. This modification prevents the intramolecular association of the N- and C-terminus of p65, thereby facilitating the interaction with co-activators and DNA. Many other protein kinases are known to modulate NF-κB transcriptional activity through the phosphorylation of different sites on NF-κB complexes (Li and Verma, 2002; Hayden and Ghosh, 2004; Perkins, 2006). In addition, other modifications such as acetylation of NF-κB might also regulate its activity (Hayden and Ghosh, 2004; Perkins, 2006).

1.2.5. Specificity in NF-κB activation

NF-κB is activated in response to numerous stimuli and regulates the expression of a large amount of target genes which are involved in a broad range of cellular and physiological processes. A tight regulation of a specific response towards a specific stimulus is therefore crucial. Some stimuli favor the degradation of a certain IκB molecule over another. Not all inhibitors are as efficiently phosphorylated by the IKKs. In addition, some inhibitors more strongly inhibit specific NF-κB dimers than others. Within various cell types, different NF-κB dimers are present. Different NF-κB complexes display distinct affinities for different κB sites

present in promoters of various NF-κB-regulated genes. Finally, in response to a certain stimulus, only specific post-translational modifications and interactions with other transcription factors or signaling pathways occur which influence the transcription of only a subset of genes. Clearly, the specificity of a particular response is regulated at various levels within the NF-κB activation pathway (Perkins, 1997; Perkins, 2007).

1.3. NF-κB function in innate immunity

NF-κB plays an important role in the regulation of innate and adaptive immune responses during infection (Bonizzi and Karin, 2004; Liang et al., 2004). The innate immunity typically comprises cells and general mechanisms that provide immediate defense against pathogens, while the adaptive immune response is composed of highly specialized cells and processes which recognize, eliminate and remember specific invaders via antigens (Vivier and Malissen, 2005). The classical NF-κB activation pathway is mostly involved in innate immunity whereas the alternative pathway may be involved in adaptive immune responses.

1.3.1. NF-κB activators in innate immunity

To date, a large number of NF-κB inducers is identified (Pahl, 1999). In the context of innate immunity, NF-κB is activated by bacteria and bacterial products, such as lipopolysaccharide (LPS), cytokines like interleukin (IL)-1 and tumor necrosis factor (TNF)-α, and stress molecules such as oxygen radicals. The major signaling pathways in response to LPS, IL-1 and TNF-α converge on the activation of IKK, which leads to the degradation of IκB, the subsequent nuclear translocation of NF-κB and the initiation of target gene transcription (Hayden and Ghosh, 2004) (Figure 4).

1.3.1.1. Toll-like receptor signaling to NF-κB

NF-κB plays an essential role in early events of innate immune responses through Toll-like receptor (TLR) signaling pathways (Carmody and Chen, 2007). TLRs are evolutionarily conserved pattern recognition receptors (PRRs) that recognize conserved pathogen-associated microbial patterns (PAMPs) present on various microbes (Janeway and Medzhitov, 2002). To date, 13 mammalian TLRs have been identified and each of these receptors signal to NF-κB (Albiger et al., 2007) (Table 1). TLRs are transmembrane proteins that are characterized by an

extracellular leucin-rich region (LRR) and an intracellular domain which is homologous to the IL-1 receptor (IL-1R) and is therefore called Toll/IL-1 receptor (TIR) domain. The LRR is responsible for the recognition of PAMPs. At least one bacterial ligand has been described for most TLRs (Akira and Takeda, 2004; Takeda and Akira, 2005) (Table 1).

Table 1: TLRs and their species-dependent expression, subcellular location and most important ligands

Receptor	Species	Location	Ligand (origin)
TLR1	Human & mouse	Cell surface	Triacyl lipopeptides (Bacteria & Mycobacteria)
TLR2	Human & mouse	Cell surface	Peptidoglycan (Gram-positive bacteria), lipoteichoic acid (Gram-positive bacteria), porins (Gram-negative bacteria), atypical LPS (Gram-negative bacteria) and zymosan (Fungi)
TLR3	Human & mouse	Cell compartment	Double-stranded RNA (Viruses)
TLR4	Human & mouse	Cell surface	LPS (Gram-negative bacteria), fusion & envelope proteins (Viruses) and taxol (Plants)
TLR5	Human & mouse	Cell surface	Flagellin (Bacteria)
TLR6	Human & mouse	Cell surface	Diacyl lipopeptides (Mycobacteria), lipoteichoic acid (Gram-positive bacteria) and zymosan (Fungi)
TLR7	Human & mouse	Cell compartment	Single-stranded RNA (Viruses)
TLR8	Human & mouse	Cell compartment	Single-stranded RNA (Viruses)
TLR9	Human & mouse	Cell compartment	CpG-containing DNA (Bacteria & Viruses)
TLR10	Human	Cell surface	Unknown
TLR11	Mouse	Cell surface	Profilin (Protozoa)
TLR12	Mouse	Unknown	Unknown
TLR13	Mouse	Unknown	Unknown

TLR signaling is initiated by the recruitment of adapter molecules that all share the TIR domain. To date, 4 adapter proteins have been identified: myeloid differentiation factor 88 (MyD88), TIR-containing adaptor protein (TIRAP), TIR-containing adaptor molecule-1 (TICAM-1) and TICAM-2. Recently, a fifth TIR domain-containing protein known as sterile α- and armadillo-motif-containing protein (SARM) has been shown to negatively regulate TLR signaling via TICAM-1 (O'Neill and Bowie, 2007). Differential recruitment of the

adapter molecules by different TLRs forms the basis for the specificity in gene expression profiles.

TLR4 is the most extensively studied PRR and is best known as the LPS receptor. LPS is a main component of the outer membrane of Gram-negative bacteria. LPS first binds CD14, which is optimally achieved in the presence of LPS binding protein (LBP) (Wright et al., 1990). This complex is then presented to TLR4 of which the extracellular portion is associated with myeloid differentiation protein (MD)-2. Upon ligation, receptor dimerization occurs. The formation of this multiprotein complex induces two distinct signaling pathways, based on the requirement of MyD88 (Figure 4). This adaptor protein is recruited to TLR4 via TIRAP through interactions betweens TIR domains. IL-1 receptor-associated kinase-4 (IRAK-4) is then activated through the interaction with MyD88 via death domains (DDs). IRAK-4 phosphorylates IRAK-1 which then associates with TNF receptor-associated factor-6 (TRAF-6). Both IRAK-4 and IRAK-1 are essential for MyD88-dependent signaling, but whether or not their kinase activity is required for TLR signaling is still a matter of debate. Activated TRAF-6 initiates a kinase cascade involving IKK. This leads to the activation of NF-κB and the up-regulation of the expression of pro-inflammatory cytokines. TRAF-6 is required in MyD88-dependent signaling since its deficiency leads to a complete loss of the NF-κB-mediated cytokine response towards TLR4 (Takeda and Akira, 2005; Carmody and Chen, 2007).

In response to LPS, a MyD88-independent signaling pathway leading to NF-κB activation also exists (Figure 4). Again as a result, the expression of pro-inflammatory mediators is induced, which occurs after the up-regulation of cytokine expression via the MyD88-dependent signaling pathway, since the kinetics of NF-κB activation in response to LPS are delayed in MyD88-deficient cells. However, when both MyD88 and TICAM-1 are lacking, NF-κB activation is completely abrogated, indicating that the latter adapter protein is indispensable for the MyD88-independent signaling pathway (Akira and Takeda, 2004; Takeda and Akira, 2005). TICAM-1 is recruited to TLR4 via TICAM-2. NF-κB activation is induced by TRAF-6 and/or receptor-interacting protein (RIP) (Barton and Medzhitov, 2003; Carmody and Chen, 2007).

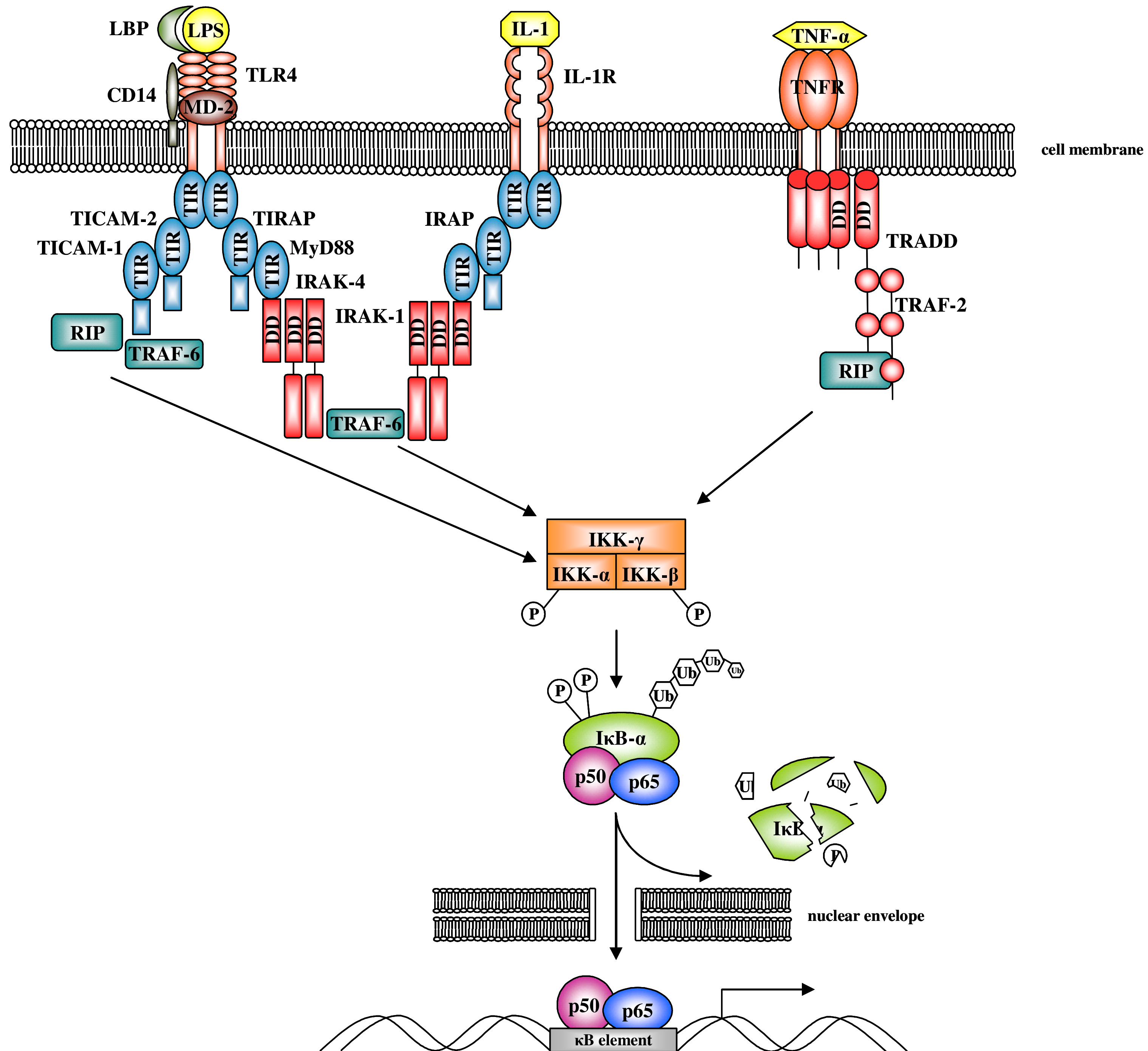

LBP
LPS
TLR4
CD14
MD-2
IL-1
IL-1R
TNF-α
TNFR
cell membrane
TIR
TICAM-2
TICAM-1
TIRAP
MyD88
IRAK-4
IRAK-1
IRAP
DD
TRADD
TRAF-2
RIP
TRAF-6
IKK-γ
IKK-α
IKK-β
P
Ub
IκB-α
p50
p65
nuclear envelope
κB element

Figure 4: Toll/IL-1 and TNF signaling pathways. LPS binds to LPB and CD14 to form a complex that interacts with TLR4 and MD-2. Both a MyD88-dependent and -independent pathway trigger the activation of the IKK complex in response to LPS. The MyD88-dependent pathway involves IRAK-4 and -1 and TRAF-6. The MyD88-independent pathway engages TICAM-1 and -2, RIP and TRAF-6. Upon binding of IL-1 to its receptor, IRAP and MyD88 are recruited. Again, IRAK molecules as well as TRAF-6 are involved in IL-1 signaling towards NF-κB. Finally, upon binding of TNF to its receptor, TRADD and TRAF-2 mediate the activation of RIP. In response to all three stimuli, IκB is degraded and NF-κB translocates to the nucleus to initiate transcription. LPS: lipopolysaccharide, LBP: LPS binding protein, TLR: Toll-like receptor, MD: myeloid differentiation protein, TIR: Toll/IL-1 receptor, TICAM: TIR-containing adaptor molecule, TIRAP: TIR-containing adaptor protein, MyD88: myeloid differentiation factor 88, DD: death domain, IRAK: IL-1 receptor-associated kinase, RIP: receptor-interacting protein, TRAF: TNF receptor-associated factor, IL: interleukin, IL-1R: IL-1 receptor, IRAP: IL-1 receptor accessory protein, TNF-α: tumor necrosis factor-α, TNFR: TNF receptor, TRADD: TNF receptor-associated death domain protein, IKK: IκB kinase, IκB: inhibitor of NF-κB, P: phosphate, Ub: ubiquitin.

1.3.1.2. IL-1 receptor signaling to NF-κB

NF-κB is activated in response to the pro-inflammatory cytokine IL-1. This interleukin binds to the extracellular immunoglobulin-like structure of the IL-1R. Since the intracellular domain of IL-1R is strongly homologous to the intracellular domain of TLRs, the downstream signaling pathway that leads to NF-κB activation is very similar to the one described above (Figure 4). IL-1R is associated with the IL-1R accessory protein (IRAP) and upon ligation, MyD88 is recruited and IRAK molecules are activated and phosphorylated. Again, TRAF-6 is recruited and IKK is activated, leading to NF-κB activation (Dunne and O'Neill, 2003).

1.3.1.3. TNF receptor signaling to NF-κB

TNF-α-mediated NF-κB activation has been studied intensively (Chen and Goeddel, 2002; Aggarwal, 2003, Wajant et al., 2003). The TNF receptor (TNFR) family consists of more than 20 members. As many ligands were identified of which TNF-α performs multiple roles in innate immunity. Upon ligation with TNF-α, the TNFR undergoes trimerization and recruits TNFR-associated death domain protein (TRADD) as an adaptor molecule via interaction between DDs. TRADD subsequently interacts with TRAF-2 and RIP. Both proteins are able to recruit IKK, which is probably activated by additional kinases. As a result, NF-κB is activated (Figure 4).

1.3.2. NF-κB target genes in innate immunity

Numerous genes of which the transcription is under control of NF-κB are nowadays identified (Pahl, 1999). Within the innate immunity, the activation of NF-κB results in the increased expression of proteins regulating host immune responses. They include cytokines and chemokines, immunoreceptors and cell adhesion molecules, acute phase and stress-related proteins, apoptosis regulators and inducible enzymes and antimicrobial peptides (Liou, 2002) (Table 2).

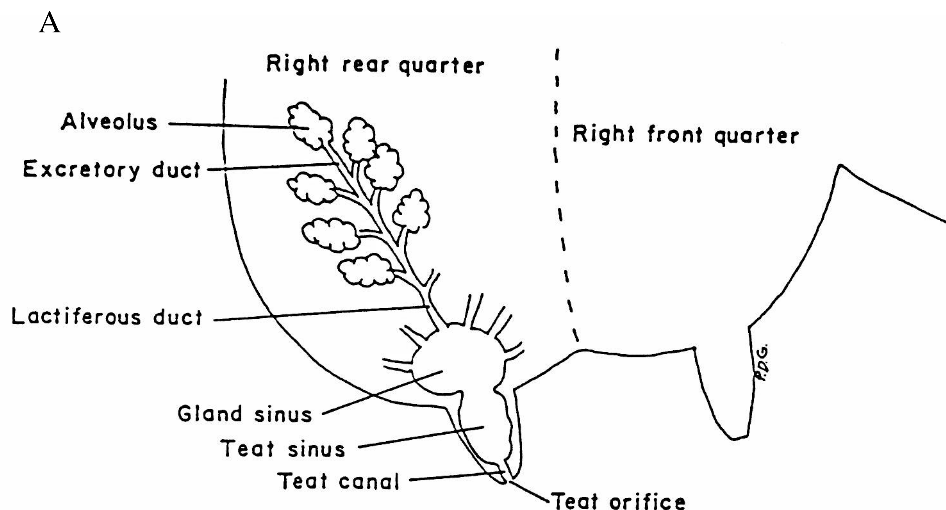
Table 2: NF-κB target genes involved in innate immunity

Class of gene	**Target genes**
Cytokines and chemokines	TNF-α, IL-1α and β, IL-6, IL-8
Growth factors	macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF)
Receptors	TNFR, TLR
Adhesion molecules	E-selectin, intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1
Acute phase proteins	LBP, C-reactive protein (CRP), serum amyloid A (SAA)
Stress molecules	Cyclooxygenase (COX)-2, nitric oxide synthase (NOS)

2. Bovine intramammary infections

2.1. Biology of the bovine mammary gland

The udder of a cow consists of four separate glands each with a single teat. The right and left side of the udder are devided by a median ligament, while the front and hind quarters are more diffusely separated. A mammary gland consists of secreting tissue and connective tissue, which is fibrous and adipose. The amount of secreting tissue determines the milk producing capacity of the cow. The milk is synthesized in secretory epithelial cells that are arranged as a single layer on a basal membrane and surrounded by myoepithelial cells in a spherical structure called the alveolus (Figure 5B). Several alveoli together form a lobule. Each lobule has a milk duct that opens into the gland cistern. The teat consists of a teat cistern, which is continuous with the gland cistern, and a teat canal. Prior to secretion via the teat canal, milk is stored in the alveoli, the milk ducts, the gland and teat cistern (Figure 5A). Upon pressure developed by the accumulation of milk, mucosal folds of the teat cistern close the way to the teat canal. In addition, sphincter muscles keep the teat canal itself closed. This canal is also the main physical barrier against infection. The mammary gland is densely innervated, especially in the teat. The udder also contains a vascular and lymphatic system (Sandholm, 1995).



B

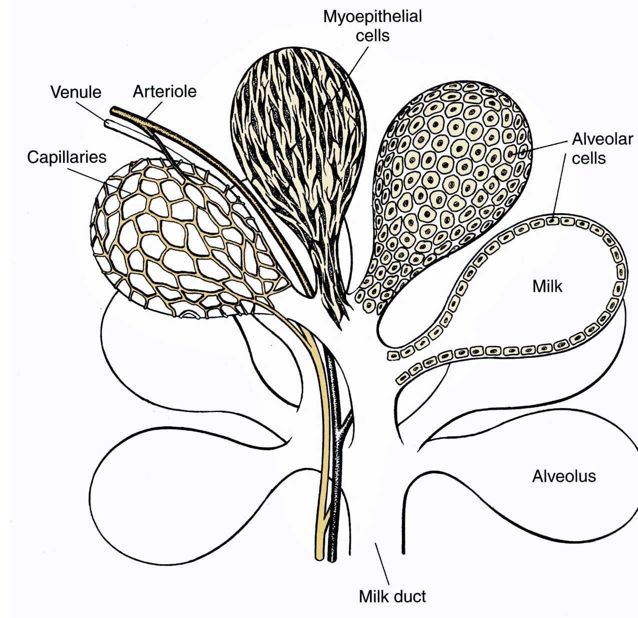


Figure 5A: Diagram of the right half of the bovine udder (from Garrett, 1988) and B: Diagram of a cluster of alveoli in the mammary gland (from Cunningham and Klein, 2007).

2.2. Innate immunity of the bovine mammary gland

2.2.1. Initiation of innate immunity

The first line of defense against invading pathogens is considered to be the teat canal since this is the route by which micro-organisms gain entrance to the mammary gland (Rainard and Riollet, 2006). Besides the sphincter muscles, the teat end also contains keratin, a protein derived from the epithelium that enables trapping of invading bacteria (Hibbitt et al., 1969). Pathogens that are able to traverse the teat end, must escape the antibacterial activities of the mammary gland micro-environment in order to establish an intramammary infection.

In milk and tissue of healthy lactating mammary glands, macrophages represent the dominant cell type (Concha, 1986). These leukocytes are able to destroy bacteria following phagocytosis (Jensen and Eberhart, 1975). However, their ability to secrete pro-inflammatory cytokines, such as TNF-α and IL-1, that facilitate the migration and enhance the bactericidal activities of neutrophils, is believed to be of greater importance to the defense of the mammary gland than their bactericidal competence (Paape et al., 2000). In addition, cytokines

are known to elicit the acute phase response during mastitis. Activated macrophages are also triggered to release prostaglandins and leukotrienes, which increase the local inflammatory reaction (Riollet et al., 2000). Finally, they play a role in the development of adaptive immune responses through antigen processing and presentation in association with major histocompatibility (MHC) class II molecules (Rainard and Riollet, 2006; Oviedo-Boyso et al., 2007).

Besides macrophages, also mammary epithelial cells are in direct contact with invading bacteria. Synthesis of TNF-α, IL-6 and the chemokine IL-8 by epithelial cells encountering bacteria or their toxins additionally leads to the recruitment and activation of neutrophils (Persson et al., 1993). Furthermore, activated macrophages and epithelial cells are able to induce the expression of cellular adhesion molecules on vascular endothelial cells, such as E-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) (Burton and Erskine, 2003; Rainard and Riollet, 2006). This causes the binding of blood neutrophils to the endothelium, their migration through the epithelial and subepithelial matrix and their localization in milk.

Neutrophils are the principal cell type found in mammary tissues and secretions during the early stage of the inflammatory process associated with bacterial infection (Oviedo-Boyso et al., 2007). During mastitis, their numbers can constitute more than 90 % of the total mammary leukocyte population (Paape et al., 1981). Their main function at the site of infection is to ingest and kill bacterial pathogens. In addition, by producing cytokines, they are able to recruit and activate neutrophils themselves (Rainard and Riollet, 2003). If the invading bacteria survive, neutrophil infiltration is replaced by an influx of T and B lymphocytes that are key components of the adaptive immune response towards infection (Oviedo-Boyso et al., 2007). However, neutrophils remain the most important cell type found in chronic intramammary infections (Rainard and Riollet, 2003). Following elimination of bacteria, neutrophil influx subsides and these cells are replaced by macrophages, which also actively participate in their removal. The rapid elimination of neutrophils is essential to minimize inflammatory-derived injury to the mammary tissue (Paape et al., 2003).

2.2.2. Neutrophil function in innate immunity

2.2.2.1. Neutrophil migration to the mammary gland

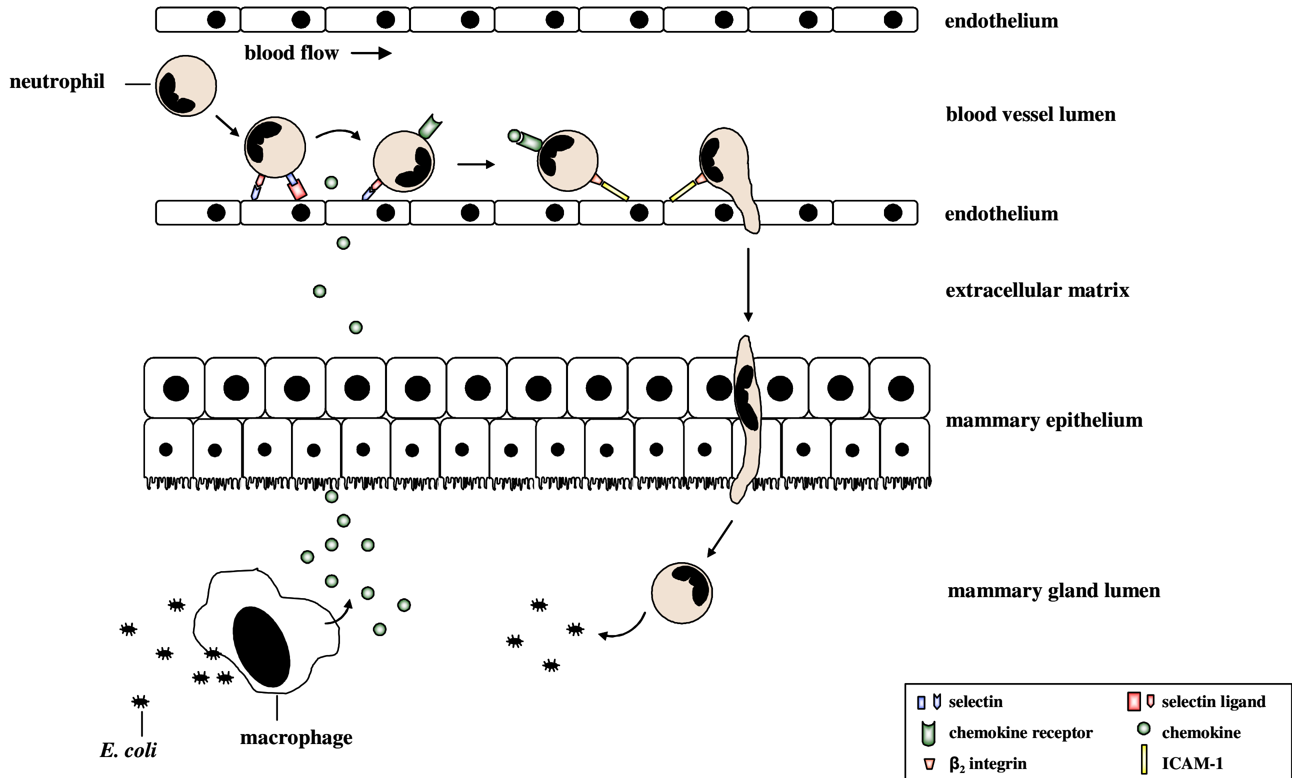


Figure 6: Migration of neutrophils from blood into the mammary gland upon infection. The binding of selectins with their ligands mediates the initial interaction between neutrophils and endothelial cells. In response to signs of infection, a more adhesive contact involving β_2-integrins and ICAM-1 molecules originates which allows neutrophils to transmigrate across the endothelium into the mammary gland. E. coli: Escherichia coli, ICAM-1: intracellular adhesion molecule-1.

Neutrophil migration from blood into the infected mammary gland proceeds through a series of sequential steps (Adams and Shaw, 1994) (Figure 6). The initial neutrophil-endothelial interaction is mainly mediated by the low-affinity binding of L- and E-selectins with their ligands (Kansas, 1996). This slows the neutrophil down thereby creating a marginating pool of cells at the endothelium. After this first contact, neutrophils roll along vascular endothelia as they survey the vessel wall for signs of infection. Signals obtained during rolling lead to the expression of a neutrophil adhesion molecule called Mac-1 or CD11b/CD18, which is a member of the β_2-integrin family of leukocyte adhesion molecules (Kishimoto and Rothlein, 1994). When the expression of Mac-1 increases, L-selectin is proteolytically shed from the

neutrophil surface. The Mac-1 molecule allows neutrophils to bind tightly to activated endothelium via ICAM-1. This adhesive interaction allows neutrophils to transmigrate across the endothelial surface into mammary tissues along a concentration gradient of chemoattractants. The most important mediators in this process are the complement components C5a and C3a, the cytokines TNF-α, IL-1 and IL-6, the chemokine IL-8 and also LPS (Burton and Erskine, 2003; Paape et al., 2003).

2.2.2.2. Neutrophil phagocytosis

Upon arrival at the mammary gland, neutrophils encounter both opsonized and non-opsonized bacteria (Ohman et al., 1988; Burton and Erskine, 2003). Without opsonization, bacteria are recognized by their PAMPs. LPS for example, is bound to CD14 molecules which are stored in granules and externalized to the cell surface of bovine neutrophils upon migration to the infected gland. Other PRRs such as TLRs might also play a role in phagocytosis of non-opsonized bacteria (Burton and Erskine, 2003). Opsonization greatly facilitates recognition and phagocytosis of the pathogen. It comprises binding of immunoglobulins (Igs) or complement components to the surface of the pathogen. Fcγ receptors on neutrophils interact with IgG antibodies, while complement receptors (CRs) recognize complement factors (Figure 7). A special receptor called the $Fc\gamma_2$ receptor is present on bovine neutrophils and is able to recognize IgG_2, one of the main opsonizing antibodies in cattle (Miller et al., 1988; Zhang et al., 1995). The expression of CR1, CR3 and Fc receptors is up-regulated in bovine neutrophils in response to pro-inflammatory signals encountered upon migration to the udder (Paape et al., 2000). Receptor binding to the pathogen leads to internalization, which is mostly initiated by the vigorous extension of pseudopods that surround and ultimately entrap the bacterium in the so called phagosome (Burton and Erskine, 2003) (Figure 7).

2.2.2.3. Neutrophil killing

The antimicrobial activity of bovine neutrophils against ingested pathogens represents a collaboration of multiple agents, including those prefabricated during neutrophil development in the bone marrow and those generated *de novo* following activation. Antimicrobial events are conventionally distinguished as being oxygen-dependent or oxygen-independent, although both systems typically operate simultaneously (Segal, 2005).

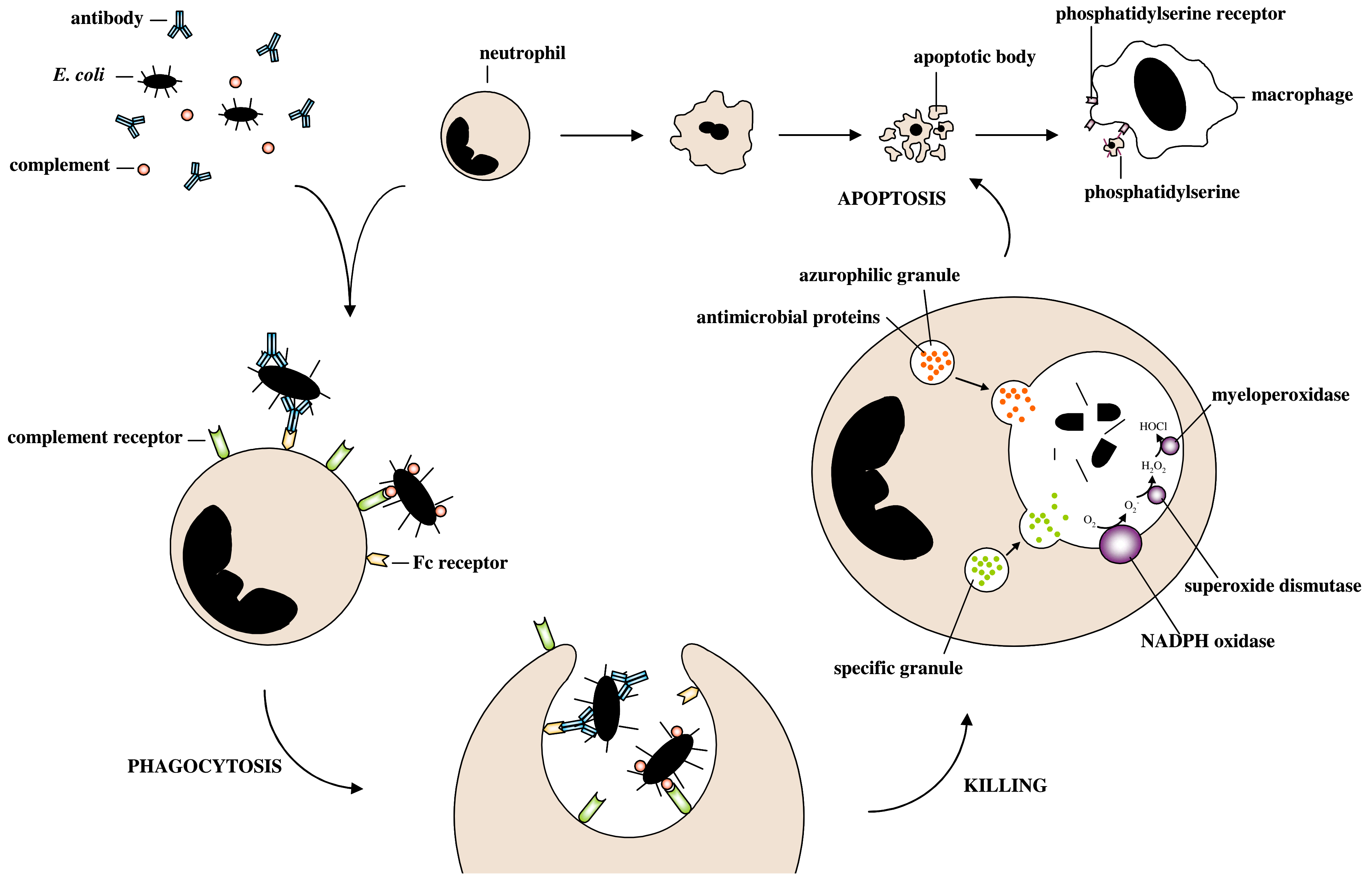

antibody
E. coli
complement
neutrophil
apoptotic body
phosphatidylserine receptor
macrophage
phosphatidylserine
APOPTOSIS
azurophilic granule
antimicrobial proteins
myeloperoxidase
HOCl
H_2O_2
O_2^-
O_2
superoxide dismutase
NADPH oxidase
specific granule
complement receptor
Fc receptor
PHAGOCYTOSIS
KILLING

Figure 7: Neutrophil phagocytosis, killing and apoptosis. E. coli bacteria are opsonized by Igs and complement components. Specific receptors at the neutrophil surface recognize the opsonized bacteria which are internalized through the formation of a phagosome. Both oxygen-dependent and -independent antimicrobial events occur that kill the bacteria. Finally, the neutrophil undergoes an apoptotic cell death and is then removed by macrophages. E. coli: Escherichia coli, O_2*: oxygen,* O_2^-*: superoxide,* H_2O_2*: hydrogen peroxide, HOCl: hypochlorous acid.*

Following phagocytosis, cytoplasmic granules fuse with the nascent phagosome and release their contents into the phagosome, thereby exposing the ingested microbe to an array of toxic agents (Hirsch and Cohn, 1960) (Figure 7). In neutrophils, various granule subsets have been identified and they mainly differ in protein content (Borregaard and Cowland, 1997; Faurschou and Borregaard, 2003). Proteins synthesized at a given stage of myelopoiesis typically localize within the same type of granules (Borregaard and Cowland, 1997; Gullberg et al., 1999; Faurschou and Borregaard, 2003). The primary or azurophilic granules and the secondary or specific granules participate in the antimicrobial activity of neutrophils by mobilization of their arsenal of toxic compounds to the phagosome and to the exterior of the cell. The tertiary or gelatinase granules and the secretory vesicles are important primarily as a reservoir of matrix-degrading enzymes and membrane receptors needed during neutrophil extravasation and diapedesis. Logically, these last two types of granules are mobilized more rapidly than the first two (Faurschou and Borregaard, 2003).

Coincident with degranulation, stimulated neutrophils exhibit an abrupt increase in oxygen consumption. This phagocyte respiratory burst is caused by an NADPH oxidase complex which assembles at the phagosomal membrane (Babior et al., 2002). As a result, reactive oxygen species (ROS) are formed which are highly antimicrobial (Figure 7). They include oxygen ions and radicals as well as peroxides. First, the NADPH oxidase complex generates superoxide (O_2^-) out of molecular oxygen (O_2) (Vignais, 2002). This anion is used by superoxide dismutase (SOD) to produce hydrogen peroxide (H_2O_2). The hydroxyl radical ($OH^{\cdot}$) is formed by combining O_2^- with H_2O_2. Alternatively, H_2O_2 is consumed by myeloperoxidase (MPO), an enzyme present in the azurophilic granules, to form different halogenated products. For example, in combination with the chloride ion (Cl^-), hypochlorous acid (HOCl) is produced, which is considered to be the most bactericidal oxidant in neutrophils. Different ROS are generated in different amounts and have distinct reactivities and toxicities. In addition, their radius of action varies, and so does their life time (Hampton

et al., 1998). Interestingly, reactive nitrogen species (RNS), such as nitric oxide ($NO^{\cdot}$) and peroxynitrate ($ONOO^{-}$) might also be potent antimicrobial agents (Fialkow et al., 2007). Besides their role in the bactericidal activity of neutrophils, both ROS and RNS participate as signaling molecules that regulate diverse physiological responses in neutrophils (Fialkow et al., 2007).

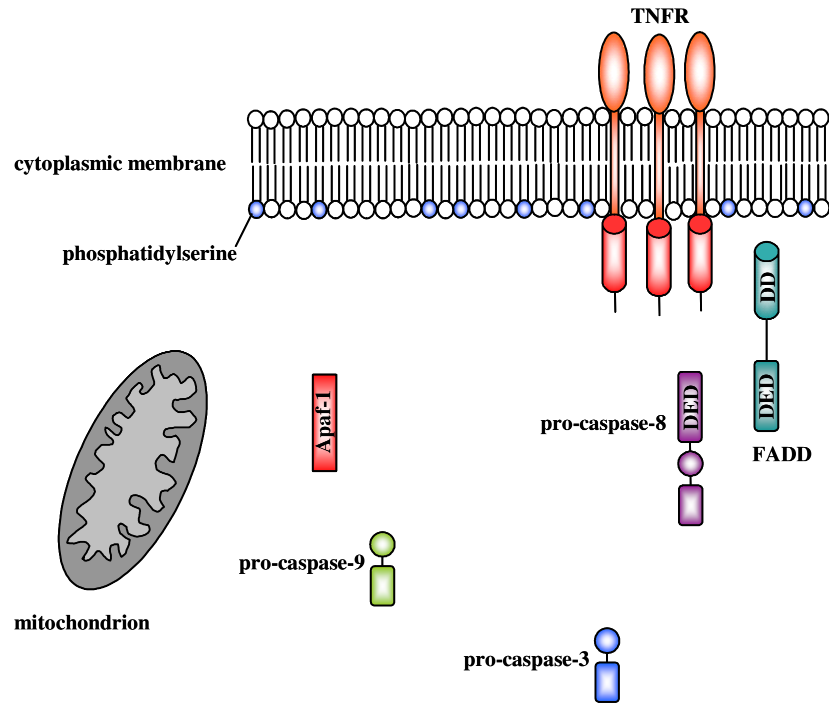
Recently, an alternative way of killing pathogens has been described for human neutrophils. Upon activation, they release granule proteins and chromatin, which together form extracellular fibers that bind bacteria (Brinkmann et al., 2004). These neutrophil extracellular traps (NETs) prevent the pathogens from spreading and ensure a high local concentration of antimicrobial agents that degrade virulence factors and kill bacteria even before they are engulfed. Clearly, sequestering the granule proteins into NETs may also keep these toxic compounds from diffusing and thereby inducing damage to host tissues (Brinkmann and Zychlinsky, 2007).

2.2.2.4. Neutrophil apoptosis

Under normal physiological conditions, neutrophils die to guarantee cellular homeostasis. This constitutive or spontaneous cell death shares many features of apoptosis, such as exteriorization of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane, vacuolated cytoplasm, nuclear condensation and cell body shrinkage (Luo and Loison, 2007). Apoptotic changes are accompanied by the downregulation of cellular functions. Apoptotic neutrophils of the human and bovine species lose their ability of chemotaxis, phagocytosis, respiratory burst and degranulation (Whyte et al., 1993; Haslett et al., 1994; Van Oostveldt et al., 2002). Cell surface receptors and adhesion molecules are downregulated as well (Jones and Morgan, 1995). Apoptotic cells are easily recognized and cleared by tissue macrophages and fibroblasts, as they express PS receptors (Savill et al., 1989; Savill et al., 1993) (Figure 7).

Many proteins are involved in the control of neutrophil apoptosis (Edwards et al., 2003; Maianski et al., 2004). Caspase activation is a well documented event during neutrophil cell death. These cysteine-aspartic acid proteases are activated by either the extrinsic or the intrinsic apoptosis pathway (Creagh et al., 2003) (Figure 8). The first pathway is death receptor-mediated and might be triggered by TNF-α. Besides its anti-apoptotic action, which

HEALTHY CELL **APOPTOTIC CELL**



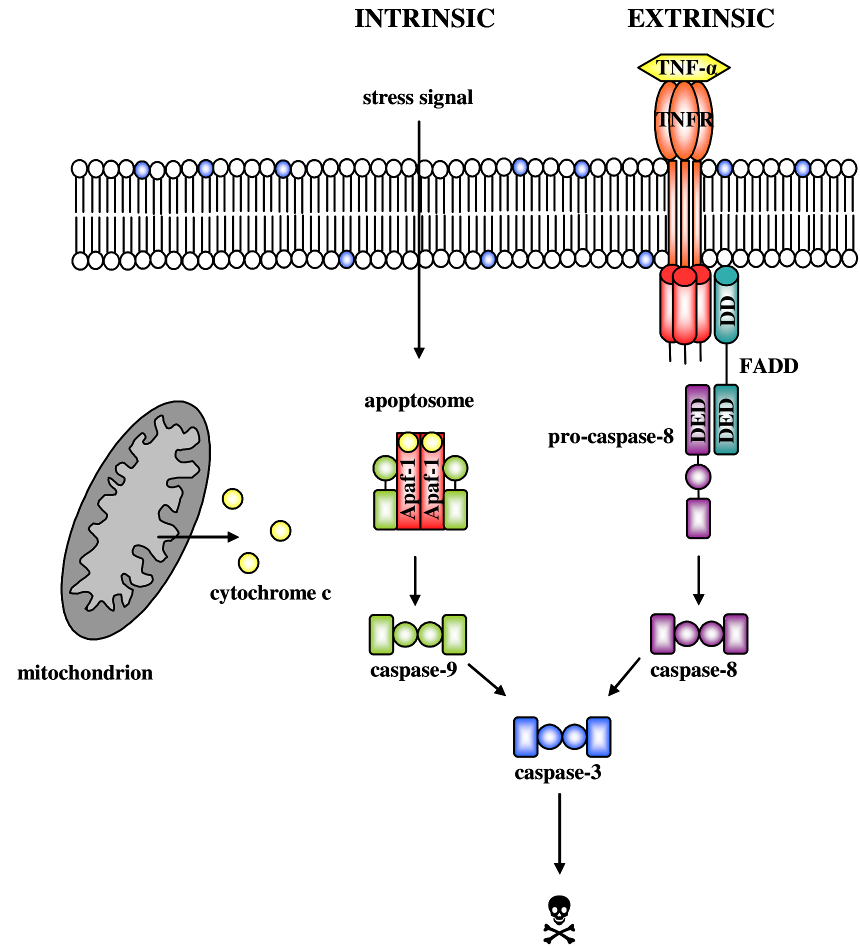


Figure 8: Molecular events occuring during apoptosis. The intrinsic pathway of apoptosis is triggered in response to stress. Following its release from the mitochondria, cytochrome c interacts with Apaf-1 and caspase-9 to promote the activation of caspase-3. The extrinsic pathway of apoptosis is initiated by death receptor engagement. Caspase-8 is recruited and activated through the interaction with FADD. Again, caspase-3 is activated. As a result, phosphatidylserine is exposed on the outer leaflet of the cytoplasmic membrane. Apaf-1: apoptotic protease activating factor-1, DD: death domain, DED: death effector domain, FADD: Fas-associated death domain, TNF-α: tumor necrosis factor-α, TNFR: TNF receptor.

is in part dependent upon NF-κB activation, this cytokine possesses a pro-apoptotic activity, which is demonstrated in both human and bovine neutrophils (van den Berg et al., 2001; Gupta, 2002; Mak and Yeh, 2002; Van Oostveldt et al., 2002). Upon ligation with TNF-α, Fas-associated death domain (FADD) is recruited through the interaction between DDs. This adaptor molecule subsequently binds to the initiator caspase-8 via death effector domains (DEDs). This leads to the activation of caspase-8, which in turn activates effector caspases such as caspase-3 that ultimately results into the breakdown of cellular components. The intrinsic pathway occurs in response to stress and is based upon the release of cytochrome c by the mitochondria and the subsequent activation of the initiator caspase-9 in the apoptosome. This large protein complex also contains apoptotic protease activating factor-1 (Apaf-1). The integrity of the mitochondrial membrane is thought to be regulated by members of the pro-apoptotic subfamily of Bcl-2 proteins. As the Bcl-2 protein family also comprises a subfamily of anti-apoptotic members, mainly changes in expression levels of members of both subgroups determines the control over the cell's fate (Moulding et al., 2001).

Neutrophil cell death is also an essential event during infection. Upon migration to infected tissues, neutrophils encounter various pro-inflammatory cytokines and chemokines which significantly prolong their life span (Lee et al., 1993). In addition, LPS suppresses apoptosis of neutrophils through the activation of TLR4. This effect has also been demonstrated for bovine neutrophils in mammary glands injected with LPS (Sládek and Rysánek, 2001). Signaling via TLRs leads to increased levels of Mcl-1 and A1, the most important pro-survival Bcl-2 family members in human neutrophils (François et al., 2005). A delay in apoptosis coincides with preservation of neutrophil functions (Lee et al., 1993). However, the antimicrobial compounds and ROS released by neutrophils to kill invading pathogens, may also damage tissues. Activated neutrophils thus become apoptotic. Their subsequent removal is essential for the resolution of inflammation in both humans and cows. Prolonged inflammation results in injury of bovine mammary epithelium (Ward, 1983; Zhao and

Lacasse, 2007). Clearly, the balance between the bactericidal function and the elimination of neutrophils needs to be well controlled in order to overcome the infection and simultaneously prevent unwanted and exaggerated inflammation (Maianski et al., 2004).

2.3. Bovine intramammary infections

2.3.1. Bovine mastitis pathogens

Intramammary infections in dairy cows have received a lot of attention because of their major economic impact on the dairy farm through decreases in the quality and quantity of milk production, increases in the cost of treatment and veterinary services, and animal waste (Halasa et al., 2007). In addition, animal welfare has become increasingly important, especially for clinical mastitis. Furthermore, potential harms of antibiotic treatment are significant animal and human health issues as well (Hillerton and Berry, 2005).

Mastitis is mainly caused by bacteria, although fungi, viruses or spores can induce intramammary infections as well. Bacteria causing mastitis are divided into three different groups. The contagious pathogens are transmitted from cow to cow and exposure mainly occurs during the milking process (Fox and Gay, 1993). The best known representative of this class is *Staphylococcus aureus* (*S. aureus*). This agent is responsible for the largest share of all ruminant intramammary infections. Additionally, these staphylococcal infections are of particular interest as they are the most difficult ones to cure. Antimicrobial treatment is often ineffective so that the subclinical infections tend to become chronic.

A second group of bacteria causing intramammary infections are the environmental pathogens (Smith and Hogan, 1993). As opposed to contagious bacteria, these agents can survive and multiply outside the cow’s body. Therefore, infection with an environmental pathogen can occur at any time during the animal’s life by chance entrance of the bacterium. Rather than subclinical infections, these bacteria mainly cause clinical mastitis in cattle. *Escherichia coli (E. coli)* is an important bacterium causing coliform mastitis (Hogan and Smith, 2003).

The common distinction between contagious and environmental mastitis pathogens knows many exceptions and therefore has become less appropriate. Contagious transmission during the milking process has been suggested for some strains of *Streptococcus uberis* (*S. uberis*),

which previously has been categorized as an environmental pathogen (Zadoks et al., 2003). For *E. coli*, transmission between cows has not been documented, but some strains are able to adapt to survival in the mammary gland, leading to transmissions within cows and chronic infections (Bradley and Green, 2001). Furthermore, contagious pathogens such as *S. aureus* and *Streptococcus agalactiae* (*S. agalactiae*) can on rare occasions also originate from environmental sources (Sukhnanand et al., 2005). Instead of the well known dichotomous classification, a sliding scale should therefore be used that accounts for niche adaptation of mastitis pathogens (Zadoks and Schukken, 2006).

A last and third group consists of the so-called minor pathogens such as *Corynebacterium bovis* and the coagulase-negative staphylococci (CNS) (Rainard, 1987). The presence of these pathogens in the mammary gland does not elicit a strong inflammatory reaction like the one that is observed with major pathogens such as *S. aureus* and *E. coli*. In fact, it has even been postulated that these bacteria may provide protection against infection by other pathogens (Matthews et al., 1991; Nickerson and Boddie, 1994; Lam et al., 1997). They may either stimulate a limited cellular response in the udder which is beneficial to the udder health, or protection could arise from competitive exclusion (Woodward et al., 1987) or from the production of bacteriocins (dos Santos Nascimento et al., 2005). Occasionally, some CNS infections do elicit severe clinical signs or become persistent, and therefore not all CNS should be classified as minor pathogens (Taponen et al., 2006).

2.3.2. *E. coli* intramammary infections in cows

2.3.2.1. *Escherichia coli*

E. coli is a Gram-negative, facultative anaerobic, non-spore-forming rod, which belongs to the family of *Enterobacteriaceae*. Gram-negative bacteria have a cell envelope which consists of three layers: the cytoplasmic membrane, the periplasmic space and the outer membrane (Bos et al., 2007) (Figure 9A). A thin peptidoglycan layer is present in the periplasmic space. The peptidoglycan is connected to the outer membrane via lipoprotein molecules. The outer membrane contains phospholipids, membrane proteins and LPS. The latter comprises a hydrophobic region, which is called lipid A, and a hydrophilic polysaccharide region, with the core polysaccharide and the O-antigen repeats (Seydel et al., 2000) (Figure 9B). Lipid A is the toxic component of LPS. Porins also exist in the outer membrane, which act like pores for

particular molecules. Both fimbriae and flagellae may protrude from the cell envelope. Finally, the surface may also be covered with a thick polysaccharide layer, called a capsule (Bos et al., 2007).

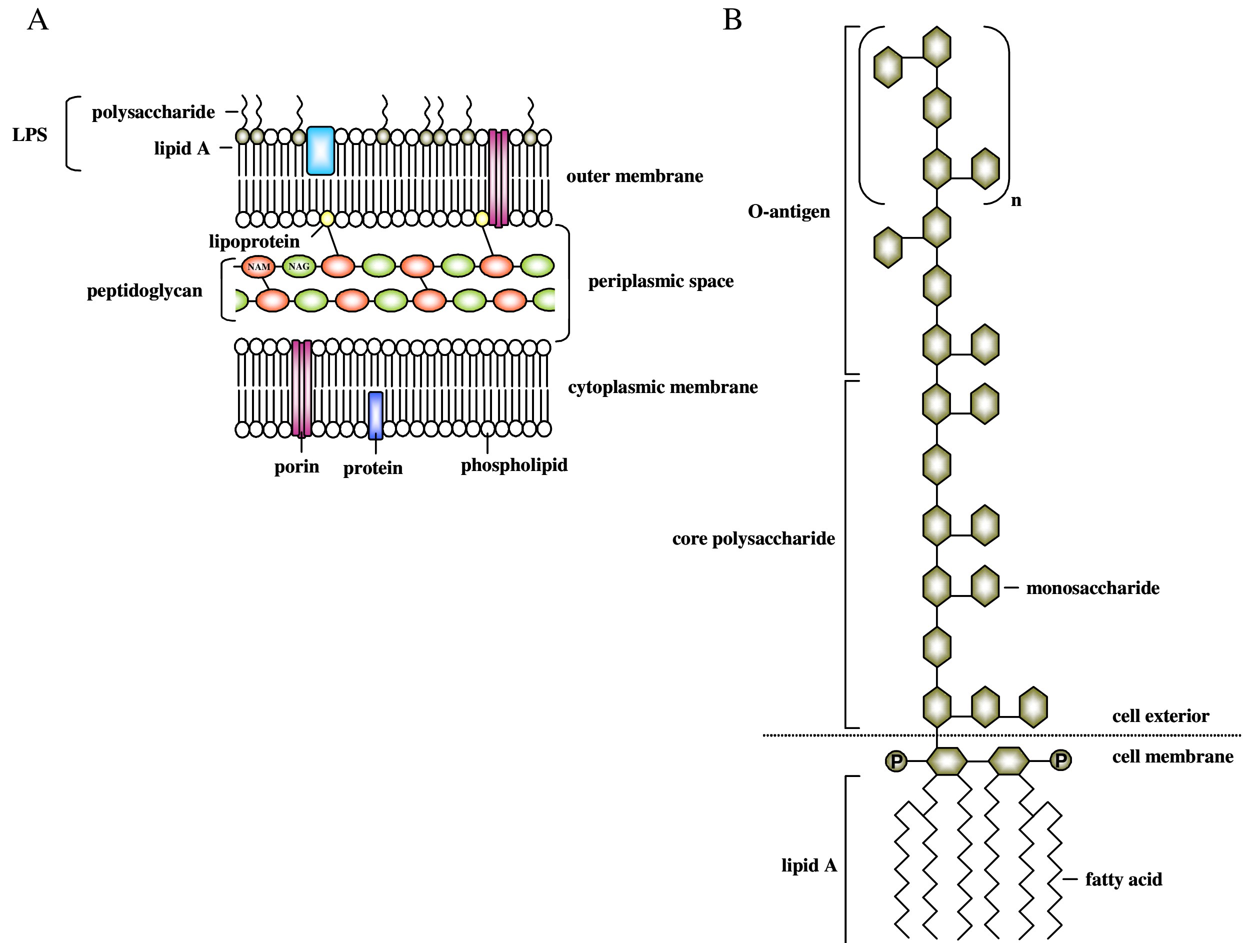


Figure 9A: Structure of the cell envelope of Gram-negative bacteria and B: Structure of LPS of E. coli. NAM: N-acetylmuramic acid, NAG: N-acetylglucosamine, P: phosphate.

E. coli bacteria are normal colonists of the intestinal tracts of humans and animals. They are constantly excreted in the faeces to the environment. Still, *E. coli* bacteria are also responsible for several diseases. This pathogen is known to cause gastroenteritis (Vallance et al., 2002) and is involved in urinary tract infections. *E. coli* might even cause more invasive infections, such as bacteraemia and meningitis (Smith et al., 2007).

2.3.2.2. *E. coli* intramammary infections in cows

E. coli is one of the most important environmental bacteria causing bovine mastitis (Burvenich et al., 2003; Hogan and Smith, 2003). Intramammary infections caused by this bacterium are characterized by a warm, swollen and sensitive quarter or udder which secretes abnormal or sometimes watery milk with flakes or clots. Infected cows may suffer from fever, a rapid pulse, loss of appetite, dehydration, depression and weakness. Death might also occur. Until recently, intramammary infections with *E. coli* were generally considered to be acute and of short duration. Recurrence of clinical signs was believed to be the result of reinfection. However, multiple clinical episodes might also be explained by intramammary persistence of the causative strain (Döpfer et al., 2000; Bradley and Green, 2001). Nowadays, the existence of chronic *E. coli* intramammary infections is well accepted. Even single cases of clinical *E. coli* mastitis have been shown to be manifestations of pre-existing chronic sub-clinical infections (Bradley and Green, 2000).

In order to initiate an intramammary infection, *E. coli* bacteria must invade the udder through the teat canal. They multiply inside the gland cistern and then spread all over the mammary tissue, where an inflammatory reaction originates (Hogan and Smith, 2003). It is generally believed that *E. coli* bacteria do not adhere to and subsequently invade mammary epithelium. Instead, this micro-organism remains in the lumen of the gland and mutiplies in milk secretions. However, recent studies show the formation of intracellular bacterial microcolonies following invasion of mammary epithelial cells (Döpfer et al., 2000; Döpfer et al., 2001; Gonen et al., 2007). These observations might partially explain the pathogenesis of chronic *E. coli* mastitis.

The role of LPS as a key molecule in the pathogenesis of *E. coli* intramammary infections is well defined (Burvenich et al., 2003; De Schepper et al., 2008). LPS is released as a result of bacteriolysis and growth of *E. coli*. This toxin accounts for many pathophysiological signs observed during intramammary infections in ruminants. It initiates the acute phase reponse and causes fever. It also activates the complement system and stimulates immune cells to synthesize and release pro-inflammatory mediators. It increases venular permeability and influences the number of circulating leukocytes. These responses are initiated upon recognition of LPS by the host as a danger signal. LPS interacts with membrane CD14 (mCD14) and soluble CD14 (sCD14) molecules that are expressed in and secreted by bovine

macrophages and neutrophils (Paape et al., 1996). In contrast, mammary epithelial and endothelial cells are not able to synthesize CD14. Still, they are believed to recognize the complex of LPS, LBP and sCD14 (Van Amersfoort et al., 2003). Bovine mammary tissue contains mRNA for TLR4 and its expression is increased in infected quarters (Goldammer et al., 2004). Even though the expression of TLR4 remains to be documented at the protein level, its presence in the membrane is very likely since bovine mammary epithelial cells are able to produce several important cytokines and chemokines upon stimulation with LPS (Srandberg et al., 2005). Human neutrophils are known to express TLR4, but at lower levels than macrophages (Hayashi et al., 2003). TLR4 agonists regulate neutrophil migration and function (Sabroe et al., 2005). Upon LPS stimulation of neutrophils, a wide range of transcriptional responses has also been demonstrated such as the up-regulation of cytokines, chemokines and proteins important for cell growth and survival (Sabroe et al., 2005).

The role of neutrophils in the defense of the mammary gland against *E. coli* is crucial (Paape et al., 2003; Rainard and Riollet, 2006). The time point of neutrophil recruitment and the amount of engaged cells determines the outcome of the infection (Burvenich et al., 2003). Neutrophil phagocytosis and their bactericidal activity are enhanced upon stimulation with pro-inflammatory mediators present at the site of infection (Sample and Czuprynski, 1991; Sordillo and Babiuk, 1991). Still, milk neutrophils as well as milk macrophages tend to be less functional than their circulating counterparts (Mullan et al., 1985). The ingestion of milk fat globules and milk proteins results in a loss of cytoplasmic granules and the subsequent reduction in antimicrobial activity. In addition, pseudopods needed for phagocytosis are thereby eliminated (Paape et al., 1975; Paape and Guidry, 1977). Moreover, it has been shown that diapedesis of neutrophils across the mammary epithelium reduces their bactericidal activity (Smits et al., 1999). In addition, the functional competences of neutrophils are reduced around parturition and during early lactation (Kehrli et al., 1989; Cai et al., 1994). Bovine intramammary infections caused by *E. coli* mainly occur in these time periods (Vangroenweghe et al., 2005). The underlying mechanisms involved in this immunosuppression are still unclear but hormonal and metabolic changes associated with pregnancy, parturition and the onset of lactation might influence the inflammatory reaction (Paape et al., 2002).

3. Mouse model of intramammary infections

3.1. Biology of the mouse mammary gland

Mice have 5 pairs of mammary glands, each with a single nipple. They are typically numbered according to their position on the body, starting from anterior to posterior (Figure 10A). Similar to cows, two pairs of glands are located in the inguinal region, while three additional pairs which are not present in cattle, are lying within the thoracic body area (Hummel et al., 1966). Mouse mammary glands are functionally and anatomically independent from each other, like bovine mammary glands. The only externally visible portion of the mammary gland is the nipple. The rest of the gland extends dorsolaterally as flat subcutaneous sheets of secreting and fibroadipose tissue. One major duct connects the teat to the mammary tissue, which is mainly a branching of smaller ducts. The smallest ducts terminate in alveoli, of which the epithelial cells are responsible for milk secretion in lactating glands (Daniel and Silberstein, 1987) (Figure 10B).

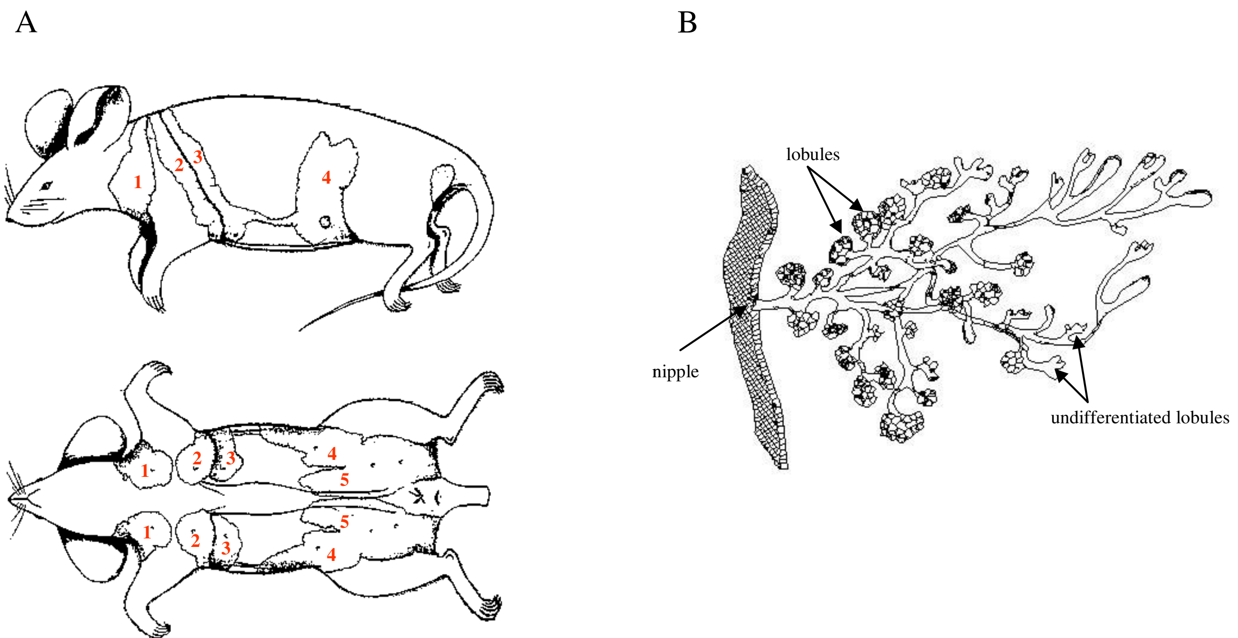


Figure 10A: Anatomical location of mouse mammary glands and their numbering (adapted from Popesko et al., 1990) and B: Anatomical structure of a rodent mammary gland (adapted from Russo and Russo, 1980).

3.2. Mouse model of intramammary infections

3.2.1. Introduction

When intramammary infections are studied in cows, major costs and management problems are inevitable. For this reason, the experimental mouse model of mastitis was developed and characterized already in the 1970s (Chandler, 1969; Chandler, 1970a). This alternative is relatively inexpensive as it requires only standard animal care facilities and basic laboratory animal experience. Currently, the mouse is still considered to be a very suitable tool for research focusing on the pathogenesis and control of bovine intramammary infections. The anatomical structure of the mammary gland of both species is very similar. As in cows, the mouse mastitis model also provides the unique pathogen growth environment of milk. It allows interaction of the micro-organism with the host cells and immune components, besides offering physical factors such as suckling. In *in vitro* models, these features are not taken into account. Finally, observations in mice concerning bacterial counts, neutrophil numbers and histological changes during intramammary infections are similar to those in cows (Chandler, 1970a).

Despite the similarities, some differences also exist between the mammary glands of both species. For example, the bovine udder contains far more resident phagocytic cells than the mouse mammary gland (Anderson and Heneghan, 1979) and milk composition differs significantly in terms of protein, fat and carbohydrate shares (Johnson, 1974). Additionally, another approach for the study of murine mastitis is necessary. As opposed to cows, somatic cell count (SCC) analysis is typically not perfomed since milk collection is a difficult procedure in mice. Nevertheless, milking devices for small animals do exist and milk is then mainly collected following administration of oxytocin (Temple and Kon, 1973). Still, in mouse the intensity of inflammation is primarily assessed indirectly, by observing the resulting tissue damage. However, neutrophil quantification based on the use of mammary gland sections has been described (Brouillette et al., 2005a). The small size of the mouse mammary gland allows it to be removed completely and fixed intact. Alternatively, semi-quantitative observations to evaluate neutrophil infiltration have also been reported (Owens et al., 1992; Sanchez et al., 1994; Guhad et al., 2000). Finally, since the incidence of mastitis is high in early lactating mammals, this disease is well correlated with the animal's reproductive system which is very diverse between cows and mice.

Because of these differences, results obtained from experiments with mice are not necessarily applicable to cattle. Several authors underline the importance of the final assessment in cows following studies with the mouse model of intramammary infections. Still, results obtained in the latter species are highly suggestive for similar findings in cattle. Furthermore, the murine infection model offers additional and very attractive perspectives for the study of mastitis. The availability of a much larger number of animals permits screening of numerous potential antimicrobial and immunomodulatory compounds. In cows such experiments would be very expensive and time-consuming. In addition, to get valid statistics the minimal number of animals is readily accessible using mice. Another critical issue is that promising agents are sometimes only available in limited quantities and can therefore never be appropriately tested in cows. New technologies like real-time *in vivo* imaging are also only applicable to small laboratory animals. Most importantly, the generation of mutant, transgenic or knock-out mice has now made profound mastitis research possible (Kerr et al., 2001). Compared to their wild-type counterparts, these mice offer the unique opportunity to study the contribution of specific host genes to inflammatory diseases. Moreover, short-term delivery of genes to mouse mammary cells holds great promise for both research and therapeutic purposes (Russell et al., 2003).

3.2.2. Methodology

3.2.2.1. Intramammary inoculation techniques in mice

For the induction of mastitis in mice, the pathogen is inoculated via the intramammary route. Inoculation is performed under general anaesthesia of the animals, which is typically obtained by single intraperitoneal injection. Certainly, gas anaesthesia is a safer and more controllable alternative but an investment in specific equipment is then required.

Several variations in the intramammary inoculation technique are described in literature. A first method is to deposit the pathogen on the tip of the teat (Anderson, 1979b). A drop of a highly concentrated bacterial suspension is then needed since only a very small number of organisms are likely to enter the primary duct. In addition, the incidence of mastitis is particularly variable as infections depend on the chance of entrance. Infection also varies with the type of agent inoculated and whether or not the nipples are damaged and suckling by the pups is allowed.

A second method consists of inserting the pathogen through a micropipette of which the tip has entered the primary duct (Nguyen et al., 2000; Lee et al., 2003). Hence, a diameter of less than 75 micrometer is required. The correct positioning of the micropipette and the insertion of its tip into the duct orifice is guided by a micromanipulator and carried out under minor magnification with a dissecting microscope and fibre-optic illumination. For lactating mice, the opening of the primary duct should be apparent but in non-lactating mice it is ofter covered with a layer of dead skin which must be removed prior to injection.

A third possibility is to cut the very near end of the teat with small scissors before injecting the inoculum with a syringe and a needle with a diameter not bigger than 30 gauge (Brouillette and Malouin, 2005) (Figure 11). The insertion depth does not exceed a few millimeters and the use of a blunt needle is recommended in order to assure that injection is directly into the primary duct without penetration of the mammary tissue. For the last two techniques challenge numbers as low as 10 colony-forming units (CFUs) can be used to induce mastitis while the incidence easily reaches a hundred percent (Anderson, 1979b; Lee et al., 2003). Still, the reduction in inoculation numbers also results in a broader spectrum of immune responses. Therefore, higher numbers, up to 10^5 CFU, are applied as well (Anderson, 1983; Brouillette et al., 2003a). However, it should be pointed out that administration of low bacterial numbers better represents what happens *in vivo* in cattle.

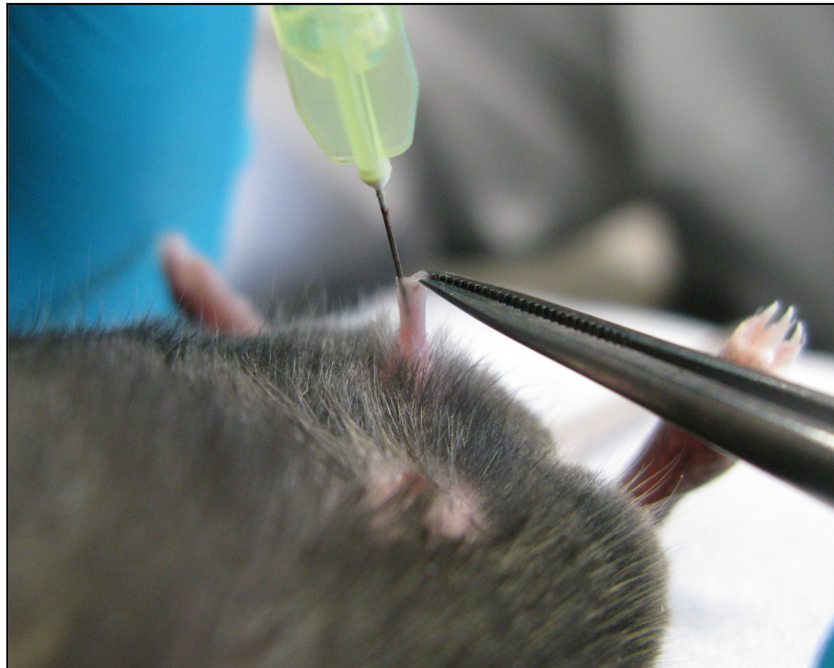


Figure 11: Photograph showing the entrance of a small needle in the teat canal of the right fourth mammary gland of an anaesthetised mouse after the teat end removal and before the injection of the bacterial inoculum.

Small quantities of pathogens are also used in the last and fourth alternative inoculation technique. In this case the gland is surgically exposed and the pathogen is injected into the primary duct by means of a needle, again with a diameter smaller than 30 gauge (Nguyen et al., 2000). This method can not be performed on pregnant or lactating animals as the duct becomes invisible due to extensive proliferation of mammary tissue.

As mentioned above, five pairs of mammary glands are defined on mice. The fourth pair is commonly used for infection studies because these glands are the biggest and therefore the easiest to observe and harvest after exposure. Inoculated organisms are preferably isolates from cases of bovine intramammary infections, but bacteria from other animals such as infected sheep (Buddle et al., 1984; Watkins et al., 1992) and even isolates from milk of nursing women have been used (Thomsen et al., 1985). Generally, only one type of pathogen is inoculated, but the possibility exists to inject different strains into separate glands on one mouse (Anderson, 1974a). Besides mammary glands receiving the pathogen, control glands are also required in the experimental set-up. Injection of heat-killed bacteria, culture-filtrate, medium or saline solution has been reported (Chandler, 1970a). The volume tolerated by the mouse mammary gland varies with the age of the animal and the extent of gland development (Nguyen et al., 2000), but does normally not exceed one hundred microliters. A variety of mouse lineages has been utilised in mastitis studies and generally lactating mice are employed. Inoculation is typically carried out within the first two weeks of lactation. At necropsy, the success rate of infusion can be confirmed by the addition of sterile India ink to the inoculum (Kerr et al., 2001; Lee et al., 2003).

3.2.2.2. Mouse mastitis pathogens

As mastitis in cows is mainly caused by bacteria, these pathogens are also mostly inoculated in mice. One research group has used fungi for the induction of mastitis in mouse mammary glands (Guhad et al., 1995). However, it should be remarked that in this mycotic study the mouse mastitis model was utilized as a suitable tool for the investigation of candidiasis. Table 3 gives an overview of the pathogens that have been employed to induce mastitis in the mouse.

S. aureus is responsible for the largest share of all ruminant intramammary infections and this observation is reflected in the number of mouse mastitis studies involving this contagious

pathogen. Staphylococcal infections are the most difficult ones to cure since antimicrobial treatment is often ineffective. Some authors suggest that this phenomenon might be due to the ability of *S. aureus* to invade and persist within certain types of host cells such as bovine mammary epithelial cells (Almeida et al., 1996). The maintenance of an intracellular pool of bacteria is likely since antibiotics do not always penetrate the gland and host cells completely and if they do, their concentration might be too low to kill the pathogen (Sanchez et al., 1988; Gruet et al., 2001). Lately, more interest goes into the so-called small-colony variants (SCVs) of *S. aureus*. This phenotype is defined as a slow-growing or quiescent, non-toxigenic pathogen with a reduced susceptibility towards antimicrobial agents. The reversion into the virulent phenotype might explain the reappearance of inflammation. SCVs of *S. aureus* have been isolated from the bovine udder (Sompolinsky et al., 1974) and the role of these variants in the persistence of infectious mastitis has been studied in the murine model (Brouillette et al., 2004b). In addition, the localization of bacteria in epithelial cells, neutrophils and macrophages in the mouse mammary glands has been reported (Brouillette et al., 2003a). Interestingly, mouse mastitis caused by *S. aureus* is mainly acute as animals typically die within a few days post-infection because of the presence of bacterial toxins such as the α-toxin, the best characterized and most potent staphylococcal toxin (Anderson, 1979b). In order to stimulate the chronic infection observed in cows, LPS can be injected before bacterial inoculation in mice (Anderson, 1976). The subsequent mammary gland involution and phagocyte infiltration will reduce colonisation leading to a more chronic course of the infection. Likewise, neutrophil infiltration can be induced by the presence of a pathogen of low virulence such as *Staphylococcus epidermidis* (Anderson, 1978a). Another well-known contagious mastitis pathogen is *S. agalactiae*. In contrast to *S. aureus*, this pathogen is very sensitive to antibiotic treatment and can thus be more controlled. Consequently, only a few studies in mice used *S. agalactiae* as mastitis pathogen (Chandler, 1970a/b; Chandler, 1973; Anderson, 1978b; Smith and Chandler, 1978; Anderson and Craven, 1984). Finally, some *Mycoplasma* species can also induce mastitis in cattle and these infections rapidly spread in herds. Treatment is generally ineffective as most antibiotics are useless due to the absence of a cell wall in Mycoplasma bacteria. Since these contagious pathogens are difficult to identify, many infections may remain undiagnosed and research in this field thus deserves more attention. A few reports present data on mastitis induced by *Mycoplasma* in mice (Anderson et al., 1976; Thorns and Boughton, 1980; Buddle et al., 1984).

Table 3: Pathogens used in the mouse mastitis model (in alphabetical order)

Group		Genus	Species	References
Bacteria	Contagious pathogens	*Acholeplasma*	*laidlawii*	Anderson et al., 1976
		Mycoplasma	*agalactiae, bovigenitalium, bovirhinis, dispar, mycoides*	Anderson et al., 1976
			argini, ovipneumoniae	Buddle et al., 1984
			bovis	Thorns and Boughton, 1980
		Pasteurella	*haemolytica*	Watkins et al., 1992
		Staphylococcus	*aureus*	Anderson, 1974, 1975, 1978, 1979a & b, 1982, 1987; Anderson and Chandler, 1975; Anderson and Craven, 1984; Anderson and Holmberg, 1977; Bramley and Foster, 1990; Brouillette et al., 2002, 2003, 2004a & b, 2005 a & b; Buddle et al., 1977; Buzzola et al., 2006; Chandler, 1970 & 1971; Chandler et al., 1976; Chesbro et al., 1972; Chew et al., 1984 & 1985; Cooray and Jonsson, 1990; Craven and Anderson, 1979, 1981 & 1983; Craven et al., 1982 & 1983; Diarra et al., 2003; Garcia et al., 1996; Gomez et al., 1998 & 2002; Haraldsson and Jonsson, 1984; Honkanen-Buzalski et al., 1985; Jonsson et al., 1985; Kerr et al., 2001; Malouin et al., 2005; Mamo et al., 1994a & b, 1995 & 2000; Odierno et al., 1994; Owens and Nickerson, 1989; Owens et al., 1992; Reid et al., 1976; Sanchez et al., 1994; Yancey et al., 1985 & 1987
		Streptococcus	*agalactiae*	Anderson, 1978; Anderson and Craven, 1984; Chandler, 1970a & b; Smith and Chandler, 1978
		Ureaplasma		Howard et al., 1975
	Environmental pathogens	*Campylobacter*	*coli*	Diker et al., 1992
		Escherichia	*coli*	Anderson, 1978, 1979b & 1986; Anderson et al., 1977; Anderson and Craven, 1984; Chandler 1970 & 1971; Chandler and Anger, 1977; Cooray and Jonsson, 1990; Gonen et al., 2007; Lee et al., 2003; Yancey et al., 1985
		Pseudomonas	*aeruginosa*	Anderson and Craven, 1984; Chandler, 1970 & 1971
		Streptococcus	*uberis*	Anderson and Craven, 1984; Sordillo and Nickerson, 1986
	Minor pathogens	*Corynebacterium*	*bovis*	Anderson et al., 1985; Honkanen-Buzalski et al.,1985
			pyogenes	Chandler, 1970
		Staphylococcus	*epidermidis*	Anderson, 1978a & b; Anderson and Holmberg, 1977; Thomsen et al., 1985
			saprophyticus	Thomsen et al., 1985
Fungi		*Candida*	*albicans, krusei*	Guhad et al., 1995, 1998a & b, 1999 & 2000

Of the environmental pathogens, *E. coli* is often inoculated in mouse mammary glands. In contrast to contagious mastitis, the characteristics of this type of intramammary infection very closely resemble those in cattle and thus do not require any modification (Chandler, 1970a). In addition, of importance are the systemic symptoms caused by LPS. Intraperitoneal injection of this toxin has also been reported but appeared to be lethal in mice (Lee et al., 2003). Interestingly, this severe endotoxin shock usually does not occur when challenging mice with the bacterium itself since the mammary gland probably detoxifies the coliform toxin before it can enter the circulation (Dosogne et al., 2002).

The pathogenesis and virulence of different minor pathogens, which appear to provide protection against intramammary infections caused by other micro-organisms, have also been tested in mice (Chandler, 1970a; Anderson and Holmberg, 1977; Anderson, 1978b; Anderson et al., 1985; Honkanen-Buzalski, 1985; Thomsen et al., 1985). Furthermore, these agents are used to convert an acute reaction into a chronic one by inoculating them prior to the major pathogen (Anderson, 1978a).

3.2.3. Areas of application

3.2.3.1. Study of the pathogenesis of mastitis

The mouse mastitis model was originally designed to study the pathogenesis of this infectious disease. Mastitis in mice can be assesed in terms of clinical appearance, macroscopic and microscopic changes and the recovery of the inoculated organisms (Chandler, 1970a). Clinical symptoms arise in local responses, such as darkening of the skin of the nipple and the obvious swelling of the mammary gland, and generalized reactions, which include a ruffled coat, a hunched appearance, weakness and sometimes death. At necropsy, infected glands are characterized by enlargement, discolouration and exudation or abscess formation. Histopathological changes such as the presence of neutrophils in the alveoli and ducts, detached epithelial cells, lesions and necrosis of the mammary tissue can also be observed. A large number of pathogens are recovered from the mammary gland and occasionally, dissemination of bacteria to visceral organs is detected as well (Anderson, 1987; Brouillette et al., 2004a). Nowadays, a more molecular approach to the study of the induced inflammatory reaction by intramammary pathogens is also applied (Gonen et al., 2007).

In the establishment of intramammary infections, not only the entrance in the gland, but also the adhesion of the organism to the mammary epithelium and the subsequent bacterial multiplication are essential. For the study of bacterial adherence and virulence mechanisms, a novel approach within the murine mastitis model has been used extensively. Mutants of pathogens lacking specific adhesion proteins were designed and inoculated in mice to evaluate their significance in the onset of infection. By these means, it could be demonstrated that the adhesion antigen K88 does not play a significant role in the potential of *E. coli* to cause mastitis (Anderson et al., 1977). In contrast, the contribution of fibronectin-binding proteins as members of the class of adhesins to the colonisation of murine mammary glands by *S. aureus* was unequivocally established (Brouillette et al., 2003b). The virulence of mutants which fail to express α-toxin, β-toxin or both was also assessed in mice (Bramley et al., 1989). The absence of these staphylococcal toxins delayed the colonisation by *S. aureus* bacteria and the subsequent infection of the mammary gland. The pathogenicity of other factors as the coagulase enzyme and the cell wall constituent protein A was evaluated as well (Haraldsson and Jonsson, 1984; Jonsson et al., 1985). Whereas the first factor was identified as an important virulent recombinant, the latter one is likely not essential for mastitis infections in mice. In addition, exoprotein synthesis is only vaguely correlated with pathogen virulence (Odierno et al., 1994). Finally, by the use of mutant strains two virulence factors were determined in the mycotic mouse mastitis model (Guhad et al., 1998a/b). Importantly, not only mutant pathogen strains are employed to study the relevance of bacterial proteins in the pathogenesis of intramammary infections, also single proteins can be inoculated (Anderson, 1974b; Anderson and Mason, 1974; Calvinho et al., 1993). As an example, the injection of partially purified β-toxin was reported (Calvinho et al., 1993). This protein was found not to be of major importance since only mild pathological effects were observed. As this result is contradictory to the abovementioned conclusion of the study with β-toxin deficient *S. aureus*, care must be taken with the interpretation of observations from molecular approaches used in pathogenesis studies.

3.2.3.2. Strategies to control mastitis

Besides its suitability for the study of the pathogenesis process, the mouse model of intramammary infections is also a very elegant tool to screen the capacity of many compounds to overcome infection. An overview of control strategies is given in Figure 12.

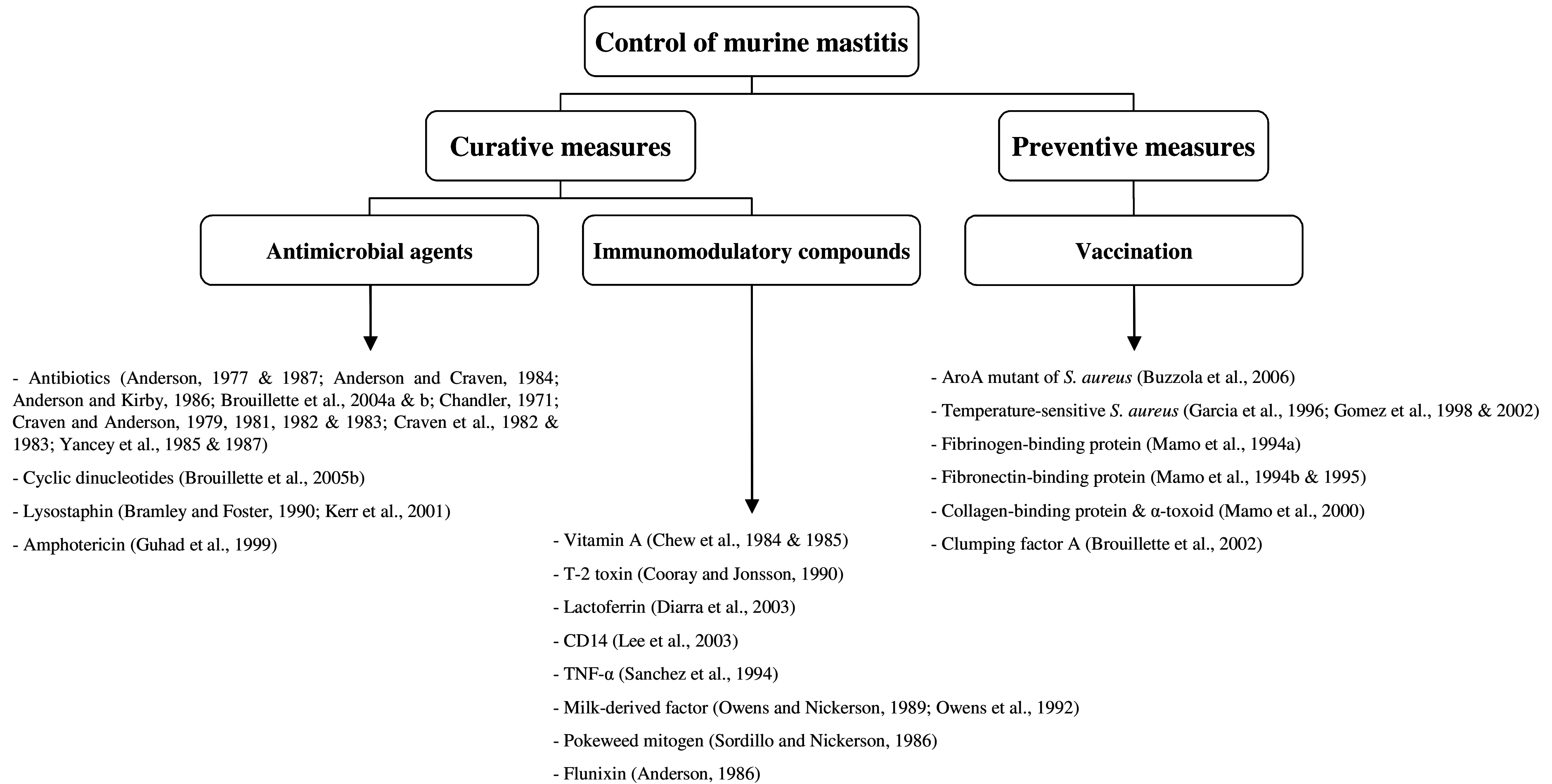

Control of murine mastitis
Curative measures
Preventive measures
Antimicrobial agents
Immunomodulatory compounds
Vaccination
- Antibiotics (Anderson, 1977 & 1987; Anderson and Craven, 1984; Anderson and Kirby, 1986; Brouillette et al., 2004a & b; Chandler, 1971; Craven and Anderson, 1979, 1981, 1982 & 1983; Craven et al., 1982 & 1983; Yancey et al., 1985 & 1987)
- Cyclic dinucleotides (Brouillette et al., 2005b)
- Lysostaphin (Bramley and Foster, 1990; Kerr et al., 2001)
- Amphotericin (Guhad et al., 1999)
- Vitamin A (Chew et al., 1984 & 1985)
- T-2 toxin (Cooray and Jonsson, 1990)
- Lactoferrin (Diarra et al., 2003)
- CD14 (Lee et al., 2003)
- TNF-α (Sanchez et al., 1994)
- Milk-derived factor (Owens and Nickerson, 1989; Owens et al., 1992)
- Pokeweed mitogen (Sordillo and Nickerson, 1986)
- Flunixin (Anderson, 1986)
- AroA mutant of S. aureus (Buzzola et al., 2006)
- Temperature-sensitive S. aureus (Garcia et al., 1996; Gomez et al., 1998 & 2002)
- Fibrinogen-binding protein (Mamo et al., 1994a)
- Fibronectin-binding protein (Mamo et al., 1994b & 1995)
- Collagen-binding protein & α-toxoid (Mamo et al., 2000)
- Clumping factor A (Brouillette et al., 2002)

Figure 12: Curative and preventive stategies employed to control mouse mastitis. S. aureus: Staphylococcus aureus, TNF-α: tumor necrosis factor-α.

The treatment of mouse mastitis started by the use of antibiotics. Bactericidal penicillins and cephalosporins are often employed as inhibitors of bacterial cell wall synthesis (Chandler, 1971; Anderson, 1977; Craven and Anderson, 1979; Craven and Anderson, 1981; Craven and Anderson, 1982; Craven et al., 1982; Craven et al., 1983; Anderson and Craven, 1984; Anderson and Kirby, 1986; Anderson, 1987; Yancey et al., 1987; Brouillette et al., 2004a/b). Occasionally, bacteriostatic agents such as lincosamide and macrolide antibiotics, which restrain general protein synthesis, are tested in mice (Craven and Anderson, 1981; Craven and Anderson, 1983; Yancey et al., 1985). In several studies, the application of these therapeutic compounds by intramammary injection led to a significant reduction in bacterial numbers, in the subsequent neutrophil infiltration and the ultimate inflammation. As mentioned before, this is not the case for intracellular staphylococci, which are not susceptible to the action of inhibitors of cell wall synthesis since they are metabolically dormant (Brouillette et al., 2004b). Hence, intensive research is now performed to discover new agents having bactericidal activity against these intracellular bacteria (Malouin et al., 2005). In addition, novel formulations that allow uptake of active components by cells with an intracellular reservoir of *S. aureus* are tested (Anderson and Kirby, 1986). Next to the intramammary route of administration, other routes such as intravenous, intramuscular and intraperitoneal injection have also been applied since they provide information about the capacity of the compound to reach the mammary gland (Brouillette et al., 2004a). These alternatives are promising since intramammary administration can be complicated due to the presence of keratin in the mammary duct. Moreover, injections in the mammary gland increase the risk of contamination. Hence, drugs with strong affinity for the mammary gland as well as those with a long retention period in the gland are searched for (Yancey et al., 1985). In addition to variations in the route of administration, antibiotics were also applied at different time points following bacterial inoculation (Chandler, 1971). From these kinetic studies, it could be concluded that treatment should be initiated as soon as possible after the onset of the infection process since antibiotics may not be effective if administration is delayed until 24 hours post-infection. In this context, it should be remarked that the detection and diagnosis of ruminant mastitis, which includes also the identification of the causative organism, typically exceeds one day.

Besides antibiotics, other bactericidal agents and even one with antifungal properties have been tested in the murine model of intramammary infections (Guhad et al., 1999). A recent paper describes effective treatment by cyclic dinucleotides such as 3',5'-cyclic diguanylic acid which are small molecules intervening with important signaling pathways in bacteria (Brouillette et al., 2005b). Very innovative research has been carried out on lysostaphin, a natural compound produced by *Staphylococcus simulans* which seems to have considerable potential for the therapeutic control of staphylococcal mastitis (Bramley and Foster, 1990). This enzyme specifically hydrolyses the peptidoglycan that is found in the cell wall of staphylococci. Intramammary infusion of lysostaphin reduced the number of bacteria recovered from the mouse mammary gland as well as the concomitant pathological changes. Furthermore, the secretion of lysostaphin in milk of transgenic mice protects them against intramammary challenge with *S. aureus* (Kerr et al., 2001). Remarkably, even cows expressing lysostaphin in their milk are now generated and again increased resistance to *S. aureus* has been observed (Wall et al., 2005). These studies show the benefits of the murine mastitis model for genetic-engineering and the potential of lactation-specific transgenesis of antibacterial proteins to obtain enhanced mastitis resistance in different target species.

In the mouse mastitis model not only compounds which suppress pathogen growth and survival have been tested, but also agents that enhance host resistance through their immunomodulatory activity. Retinoid metabolites are proposed to positively influence host defense mechanisms. Mice fed a diet supplemented with vitamin A showed less severe mammary gland inflammation after intramammary inoculation (Chew et al., 1984). In addition, vitamin A-deficient animals had increased pathological damage to the mammary gland (Chew et al., 1985). Another modulator could be the T-2 toxin, naturally produced by *Fusarium* species. Treatment of mice with this mycotoxin reduced the virulence of both *S. aureus* and *E. coli* and limited the inflammatory reaction (Cooray and Jonsson, 1990). The authors suggest an activation of macrophages as the mechanism behind the increased resistance to mastitis. Enhancement of phagocytosis and intracellular killing might also be a possible feature of lactoferrin, next to a decreased capacity of adherence to extracellular matrix proteins and the subsequent invasiveness of *S. aureus* into mammary epithelial cells (Diara et al., 2003). This important protein can be found in milk and in neutrophil granules and its bactericidal activity was already well-established before the investigation of its possible immunomodulating properties. Remarkably, mice expressing the lactoferrin transgene in milk were engineered but challenge studies are not yet reported (Platenburg et

al., 1994). Another interesting approach is the inoculation of *E. coli* together with the soluble form of the LPS receptor CD14. The reduced mammary inflammation observed in the latter study is likely the result of the early infiltration of neutrophils (Lee et al., 2003). A similar enhanced recruitment was observed after single treatment with the cytokine TNF-α. Subsequent injection of *S. aureus* also led to a faster elimination of the bacteria (Sanchez et al., 1994). Interestingly, the species-specific recombinant cytokine proved to be more powerful in this control than the human protein. Moreover, combining TNF-α with various antibiotics resulted in an even greater reduction of bacterial counts. An improved resistance and reduced inflammation was reported after challenge with *S. aureus* and infusion of a yet to be identified milk-derived factor (Owens and Nickerson, 1989; Owens et al., 1992). Another paper describes the enhanced antimicrobial defense after injection of pokeweed mitogen (Sordillo and Nickerson, 1986). Mammary gland susceptibility towards *S. uberis* infection seemed to be beneficially modified by this plant lectin. Finally, the anti-inflammatory drug flunixin was tested without success in the mouse model of *S. aureus* mastitis (Anderson, 1986).

A third approach of controlling mastitis is prevention by vaccination. Commercially available vaccines for cattle have shown limited efficacy, especially against *S. aureus* intramammary infections. Instead of preventing the disease, these vaccines are mostly only capable of improving cure rates and diminishing the severity of infection. The mouse mastitis model has become a feasible tool also in this field of mastitis control. Intramammary immunization of a temperature-sensitive mutant of *S. aureus* has led to a decreased severity of mammary gland infection by the wild-type bacterium (Garcia et al., 1996; Gomez et al., 1998). Significantly lower numbers of pathogens in the gland were observed as well as an increase of specific milk and serum antibodies. A T cell-mediated immune response is also induced which might be indispensable in combating *S. aureus* mastitis due to the possible inefficient humoral immunity towards these intracellular bacteria (Gomez et al., 2002). The best results were obtained when immunization occurred in late pregnant mice or during early lactation, but not before gestation (Gomez et al., 1998). In a more recent report, similar results were shown with a different mutant of *S. aureus* (Buzzola et al., 2006). Other studies describe the immunization with the complete fibrinogen-binding protein or domains of the fibronectin-binding protein, both present on the surface of *S. aureus*, and the subsequent protection obtained in the mouse model (Mamo et al., 1994a/b). Furthermore, inoculation of bacteria opsonised with fibronectin-binding protein antisera of immunised mice also led to milder

forms of mastitis (Mamo et al., 1995). In addition, the same group tested vaccines with combinations of proteins such as collagen-binding protein and α-toxoid, again with some success (Mamo et al., 2000). Besides injection of proteins, a promising DNA vaccine which contains the adhesin clumping factor A of *S. aureus* has recently been developed (Brouillette et al., 2002). Less severe mastitis was observed in mice inoculated with bacteria opsonised with sera of immunized animals. Induction of mastitis in these vaccinated mice would be an interesting next step to perform. In conclusion, despite these remarkable reports, vaccination studies carried out in mice are still in their infancy since prevention, which is the ultimate goal of vaccination, remains to be achieved.

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HYPOTHESES AND OBJECTIVES

HYPOTHESES

Neutrophils are the most important cells within innate immune responses towards bovine intramammary *E. coli* infections (Rainard and Riollet, 2006). These leukocytes are terminally differentiated, short-lived cells with an extensive array of surface receptors for response to inflammatory stimuli (Burton and Erskine, 2003) and an arsenal of antimicrobial compounds in their granules to combat invading pathogens (Segal, 2005). Until recently, it was widely assumed that these phagocytes are transcriptionally inactive since they seem to be fully equipped upon their release from the bone marrow into the blood in order to perform their function. Nowadays, complex changes in gene and protein expression profiles within activated neutrophils have been identified (Borregaard et al., 2007). These findings support the emerging concept of an active regulatory role of neutrophils besides their defense function, in the progression of the innate immune response.

Upon invasion of *E. coli* bacteria in the bovine mammary gland, neutrophils are recruited from blood into the milk. Mammary epithelial cells and milk macrophages most likely initiate this event, since they represent the two main cell types that invading pathogens encounter at first (Rainard and Riollet, 2006). To date, several pro-inflammatory cytokines and chemokines have been identified as major regulators of bovine neutrophil migration (Burton and Erskine, 2003; Paape et al., 2003). Moreover, some of these proteins are known to be secreted by bovine mammary epithelial cells and macrophages upon their exposure to bacteria or bacterial toxins (Politis et al., 1991; Boudjellab et al., 1998; Okada et al., 1999; Wellnitz and Kerr, 2004; Pareek et al., 2005).

Gene expression within innate immune responses is highly mediated by the transcription factor NF-κB (Liou, 2002). NF-κB activation has been demonstrated in bovine mammary epithelial cells following *in vitro* stimulation (Safieh-Garabedian et al., 2004; Strandberg et al., 2005; Yang et al., 2006; Fitzgerald et al., 2007). Studies regarding the role of NF-κB in neutrophils have been mainly carried out with human cells. NF-κB is known to regulate the neutrophil's life span (Akgul et al., 2001; Ward et al., 2004) and its contribution in neutrophils under *in vitro* inflammatory conditions is identified as well (McDonald et al., 1997; McDonald and Cassatella, 1997; Kettritz et al., 2004). One *ex vivo* study illustrates NF-κB activation in milk cells of bovine mammary glands infected with contagious mastitis pathogens such as *S. aureus* (Boulanger et al., 2003). However, no reports exist on the role of

this transcription factor in bovine coliform intramammary infections. It is therefore suggested that NF-κB activation in the mammary gland contributes to the development of intramammary innate immune responses towards *E. coli* bacteria. Additional experimental work is necessary to conclusively support this attractive hypothesis.

In this doctoral thesis, the assumption that NF-κB plays a key role in both the regulation of the bovine neutrophil life span and in mediating its immune response is investigated. Additionally, NF-κB activation is suggested to be crucial to the establishment of the local inflammatory environment and the systemic acute phase response during intramammary infection of *E. coli* in mice.

OBJECTIVES

The general objective of this doctoral thesis was to investigate the activation of the transcription factor NF-κB in relation to intramammary *E. coli* infections in cows and mice. A first part covers the *in vitro* experimental studies performed on bovine blood neutrophils. The possible role of NF-κB in bovine neutrophil survival and in the regulation of the inflammatory response mediated by activated neutrophils through cytokine production was revealed. The second part deals with the involvement of NF-κB in the pathogenesis of *E. coli* intramammary infections in mice. The contribution of NF-κB to the establishment of the inflammatory response in the mammary gland and the systemic acute phase response was examined. Throughout this thesis, a number of specific objectives can be distinguished (Figure 1):

- To demonstrate the presence of the important NF-κB subunits p65 and p50 in bovine neutrophils (①).

- To study active NF-κB complexes in bovine neutrophils under normal (②) and inflammatory conditions following incubation with *E. coli* bacteria (③).

- To gain insight in the mechanisms by which NF-κB mediates the host immune response in mice following intramammary infection with *E. coli* bacteria (❹).

- To determine the cellular localization of the induced NF-κB activity in mouse mammary glands upon infection with *E. coli* bacteria (❺).

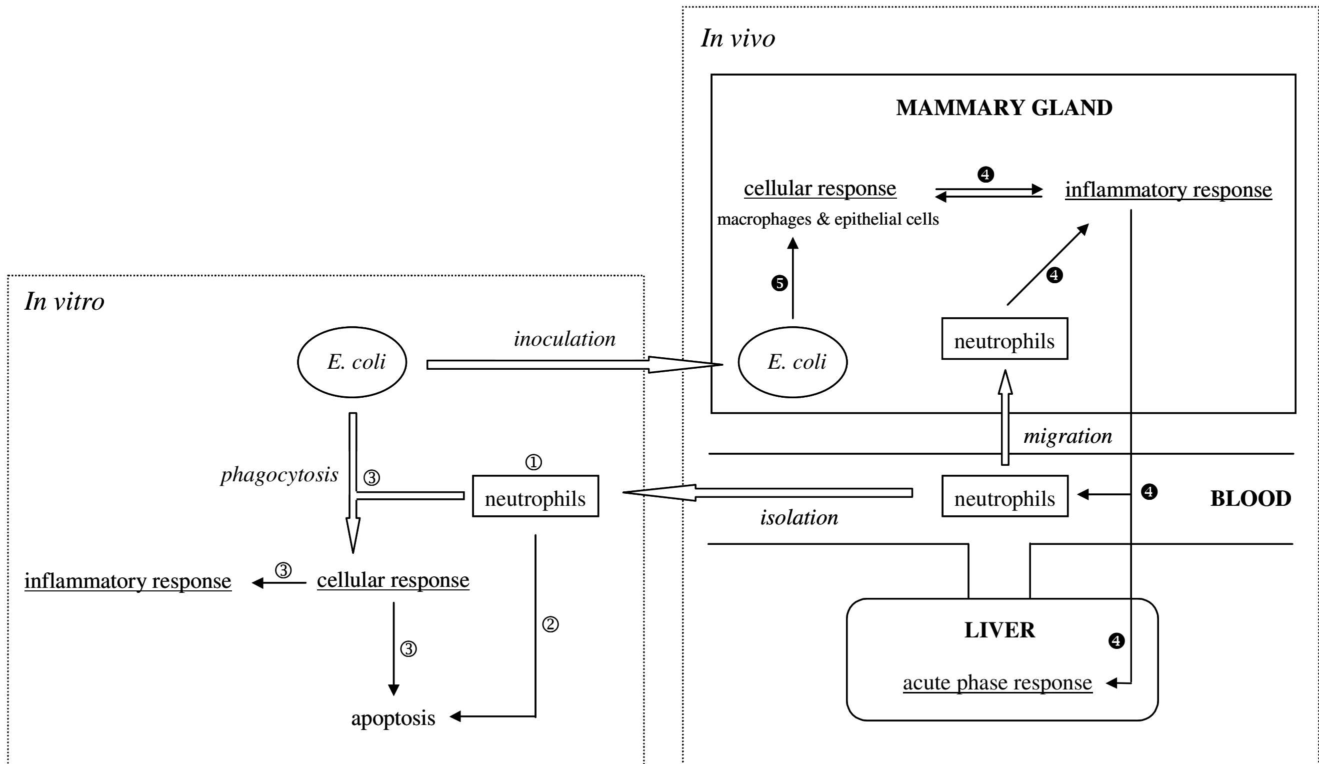

Figure 1: Diagram representing the hypotheses of the thesis. Black numbers on white background are the objectives within the bovine species, while white numbers on black background represent the objectives for the mouse as a host.

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PART 1

IN VITRO STUDY OF NF-κB ACTIVATION IN BOVINE NEUTROPHILS

CHAPTER 1

NF-κB ACTIVITY IN RESTING BOVINE NEUTROPHILS

Adapted from

Notebaert S., Duchateau L. and Meyer E. NF-κB inhibition accelerates apoptosis of bovine neutrophils. Veterinary Research (2005) 36:229-240.

Abstract

Apoptosis is one of the major events that contribute to the regulation of the immune system. For human neutrophils, evidence has been produced that the nuclear transcription factor (NF)-κB is critical in influencing the ultimate outcome of a cell's fate. However, such research has not yet been performed on bovine neutrophils. This urged us to examine the possible involvement of NF-κB in apoptosis of these cells. At first, we investigated whether p65 and p50, the most important members of the NF-κB family, are expressed in isolated blood neutrophils. The presence of both members was demonstrated on the mRNA and protein level. Then the effect on bovine neutrophil apoptosis of gliotoxin, a potent and specific inhibitor of NF-κB, was examined. The rate of constitutive apoptosis was found to be greatly accelerated by inhibition of NF-κB. Furthermore, gliotoxin dramatically augmented the limited pro-apoptotic effect of tumor necrosis factor (TNF)-α, an important inflammatory mediator. Results were obtained in 6 cows by annexin-V-FITC staining of externalized phosphatidylserine (PS) and subsequent flow cytometric analysis. Additional measurement of caspase-3/7 activity and evaluation of morphological criteria confirmed the outcome of this experiment. Finally, NF-κB activity was assessed under these conditions. The activity of p50 was found to be minimally affected by gliotoxin, while significantly lower active p65 values were observed. Still, the highest percentage of apoptosis, which was caused by incubation with both gliotoxin and TNF-α, did not correspond to the lowest activity of p65. We conclude that NF-κB p65 promotes survival of bovine neutrophils by delaying initiation of apoptosis.

1. Introduction

Neutrophils play a critical role in the first line of defense against invading micro-organisms. These cells circulate in the blood vessels until they are attracted to inflamed tissue, where the neutrophil life span is extended by inflammatory mediators in order to destroy the pathogen. Once the infection is cleared, residual neutrophils die by apoptosis ensuring their removal by phagocytes and the rapid resolution of inflammation. Neutrophil apoptosis minimizes the risk of loss of toxic cell contents to the surrounding tissues and thus clearly controls the duration and intensity of an inflammatory response (Kobayashi et al., 2003).

Apoptotic cell death represents a complex sequential process involving different cellular events to which many factors contribute. If apoptosis is defined by morphological features, nuclear condensation (Rogalinska, 2002) and cell shrinking (Bortner and Cidlowski, 2002) are mentioned in particular. However, characterisation of the molecular events makes a more valuable contribution to the understanding of this kind of programmed cell death. Many studies have reported caspases as being central regulators of apoptosis (Denault and Salvesen, 2002). Following proteolysis of pro-caspases, specific substrates are cleaved and thereby activated or inactivated, resulting in a proper execution of the apoptotic program.

Several cellular proteins are capable of inhibiting apoptosis. Since NF-κB controls the expression of many survival genes (Barkett and Gilmore, 1999; Karin and Lin, 2002), this transcription factor is known to prevent the induction of programmed cell death. In resting cells, NF-κB family members are sequestered in the cytoplasm, bound to their inhibitors which belong to the IκB family. Following cell activation, IκB proteins become phosphorylated, ubiquitinated and degraded by the proteasome. Subsequently, NF-κB can enter the nucleus and bind to the DNA in order to promote transcription (Rothwarf and Karin, 1999).

In vitro studies with human neutrophils have shown that a range of inflammatory mediators known to be present at inflamed sites inhibit the apoptosis process in these cells (Brach et al. 1992; Colotta et al., 1992; Lee et al., 1993; Pericle et al., 1994). It has also been reported that incubation with some of these agents leads to the activation of NF-κB (McDonald et al., 1997). Furthermore, a major induction of human neutrophil apoptosis was seen after

inhibiting NF-κB (Ward et al., 1999; Niwa et al., 2000; Nolan et al., 2000; Liu et al., 2003). These data strongly suggest that NF-κB is a major regulator of the human neutrophil life span.

There is accumulating evidence that, also in neutrophils, the anti-apoptotic activity of NF-κB would depend on gene induction. More specifically, the expression of A1 (Bfl-1) and Mcl-1, two anti-apoptotic members of the Bcl-2 family of proteins (Borner, 2003; Burlacu, 2003), seems to be up-regulated by anti-apoptotic stimuli and down-regulated when apoptosis is induced (Chuang et al., 1998; Leuenroth et al., 2000; Moulding et al., 1998 and 2001). At least the expression of A1 has been shown to be dependent upon activation of NF-κB (Grumont et al., 1999; Zong et al., 1999). In addition, the use of gliotoxin as a specific inhibitor of NF-κB (Pahl et al., 1996) leads to depletion of A1 and Mcl-1 (Moulding et al., 2001), even though the expression of the latter protein is not NF-κB regulated (Akgul et al., 2000). Furthermore, the loss of the expression of inhibitor of apoptosis (IAP) may facilitate the induction of neutrophil apoptosis (O'Neill et al., 2004). The transcription of IAP is also under control of NF-κB (You et al., 1997; Stehlik et al., 1998).

A key role for bovine neutrophil apoptosis in the resolution of an inflammatory response has been documented in a growing number of reports (Paape et al., 2003). Still, in contrast to human, research into the role of NF-κB in regulating the life span of bovine neutrophils has not yet been carried out. Therefore, we first investigated whether bovine blood neutrophils express p65 and p50, the most important members of the NF-κB family. Still, the major purpose of the present study was to examine the *in vitro* effect of gliotoxin, a potent and specific inhibitor of NF-κB (Pahl et al., 1996), on bovine neutrophil apoptosis and on the NF-κB activity of p65 and p50.

2. Experimental procedures

2.1. Isolation and culture of bovine blood neutrophils

In this study, 6 healthy heifers of the Holstein-Friesian breed were selected from the Ghent University dairy farm (Biocentrum Agri-Vet, Melle, Belgium). Bovine peripheral blood was collected using sterile polypropylene tubes (IMI, Montegrotto Terme, Italy) pre-filled with an equal volume of Alsever's as an anticoagulans solution (3 mM citric acid monohydrate, 27 mM trisodium citrate dehydrate, 72 mM sodium chloride and 125 mM D-glucose, pH 6.1). After centrifugation (300 g, 15 min) plasma and the buffy coat were removed. Red blood cells were lysed during incubation with an ice-cold isotonic ammonium chloride solution (138 mM ammonium chloride and 21 mM Tris, pH 7.4) for 10 min. The remaining cell suspension was washed twice with phosphate buffered saline (PBS) (200 g, 10 min). Contaminating mononuclear cells were then removed by density gradient centrifugation (1,000 g; 20 min) over Percoll with a specific gravity of 1.094 g/ml, prepared according to the manufacturer's protocol (Sigma-Aldrich, Bornem, Belgium). The remaining cell pellet was again washed twice with PBS and used for further applications. Over 98 % of the isolated cells were granulocytes, of which less than 5 % were eosinophils.

Isolated neutrophils were resuspended at a final concentration of 3 x 10^6 cells/ml in RPMI 1640 with 10 % fetal calf serum (both from Invitrogen, Merelbeke, Belgium). Cultures were supplemented with 0.1 μg/ml gliotoxin (Sigma-Aldrich), 10 ng/ml rhTNF-α (Alexis, Zandhoven, Belgium) or with both effectors. Incubation was performed at 37 °C in a 5 % CO_2 incubator (Binder, Tuttlingen, Germany) for 6 h.

2.2. NF-κB p65 and p50 mRNA detection following polymerase chain reaction (PCR)

Total cellular RNA was extracted from 1 x 10^6 neutrophils after cell lysis with Trizol® reagent (Invitrogen). First, mRNA was converted into cDNA by reverse transcriptase (60 min, 42 °C) and then the cDNA was screened with specific primer pairs (Eurogentec, Seraing, Belgium). The primer sequences used were: for p65: GGACTTCTCAGCCCTTCT and CCATCAGTGTGTGCTTTG; for p50: CTTGCTGCTAAATGCTGCTC and CCAGGTTCTGTAGGACTGTATCTTC. For amplification, 40 cycles were applied (30 s at 94 °C, 45 s at 59 °C for p50 or 60 °C for p65 and 45 s at 72 °C) on a Mastercycler

(Eppendorf, Hamburg, Germany). Finally, amplification products were loaded on a 2.5 % agarose gel (Helena Biosciences, Sunderland, UK) and visualised after electrophoresis under UV-light using ethidium bromide (Sigma-Aldrich). A 100 base pairs (bp) marker (Genecraft, Münster, Germany) was used for sizing the fragments.

2.3. NF-κB p65 and p50 protein detection following western blotting

Total cell extracts were prepared by resuspending 5 x 10^6 neutrophils in 500 µl Laemmli buffer (62.5 mM Tris HCl, pH 6.8; 50 mM dithiotreitol, 10 % glycerol, 2 % sodium dodecyl sulphate and 0.1 % bromophenol blue) supplemented with a cocktail of protease inhibitors (Calbiochem, La Jolla, USA). Samples were sonicated and boiled for 5 min at 95 °C and 20 µl was loaded on a 10 % denaturing polyacrylamide gel followed by blotting on a nitrocellulose membrane (both from Bio-Rad Life Science, Nazareth, Belgium). After blocking in Tris buffered saline with 1 % Tween 20 (TBS-T) and 5 % milk powder for 1 h, overnight incubation with primary anti-p65 (sc-372) or anti-p50 (sc-8414) antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) was carried out. Membranes were then washed and a secondary antibody conjugated to horseradish peroxidase (HRP) was applied for 1 h. Finally, proteins were detected with a ChemiDoc™ System (Bio-Rad Life Science) using Supersignal® West Dura (Perbio Science, Erembodegem, Belgium). A molecular weight ladder with bands from 10 to 200 kDa (Westburg, Leusden, The Netherlands) was used for sizing the proteins.

2.4. Phosphatidylserine translocation assay

Flow cytometric detection of exposed PS was used to quantify the percentage of apoptotic neutrophils. After centrifugation (200 g, 10 min) of 1 x 10^6 neutrophils, the cell pellet was resuspended in 100 µl incubation buffer (10 mM Hepes, 140 mM sodium chloride and 5 mM calcium chloride, pH 7.4) containing 2 µl annexin-V-Fluos labeling reagent (Roche Diagnostics, Vilvoorde, Belgium) and 2 µl propidium iodide (PI) (50 µg/ml; Sigma-Aldrich). Following 10 min incubation in the dark, 400 µl PBS was added and samples were immediately analysed using a FACScan flow cytometer (Becton Dickinson Biosciences, Erembodegem, Belgium). Cells positive for annexin-V-FITC and negative for PI were defined as apoptotic.

2.5. Morphological cell death assessment

After cytocentrifugation (55 g, 5 min) of 2 x 10^5 neutrophils, slides were air dried, fixed with pure methanol and stained with Hemacolor® (Merck, Darmstadt, Germany) according to Pappenheim. Evaluation of apoptotic morphology was based on cell shrinking and chromatin condensation characteristics.

2.6. Caspase-3/7 activation assay

For the assessment of the caspases-3 and -7 activity, the Caspase-Glo™ Assay (Promega, Leiden, The Netherlands) was used according to the manufacturer's protocol. Briefly, 2 x 10^6 neutrophils were lysed on ice during an incubation of 30 min with 100 µl PBS supplemented with 1 % saponin (Sigma-Aldrich) and a cocktail of protease inhibitors (Calbiochem). After centrifugation (8,000 g; 10 min), the supernatant was frozen at -80 °C until further analysis. In a 96-well plate, 5 µl of freshly thawn protein lysate was diluted with lysis buffer to a final volume of 100 µl. Subsequently, the same volume of Caspase-Glo™ 3/7 reagent was added. After an incubation of 1 h at room temperature, chemiluminescence was measured with a Microlumat LB96P luminometer (Berthold Technologies, Vilvoorde, Belgium) and expressed in relative light units per second (RLU/s).

2.7. NF-κB activation assay

For the detection and quantification of the activity of NF-κB p65 and p50, the TransAM™ Transcription Factor Assay Kits (Active Motif, Rixensart, Belgium) were used according to the manufacturer's protocol. In brief, 2 x 10^6 neutrophils were lysed on ice during an incubation of 30 min with 100 µl PBS supplemented with 1 % saponin (Sigma-Aldrich) and a cocktail of protease inhibitors (Calbiochem). After centrifugation (8,000 g; 10 min), the supernatant was frozen at -80 °C until further analysis. For each sample, 5 µl of freshly thawn protein lysate was incubated for 1 h in wells of a 96-well plate containing immobilized NF-κB consensus oligonucleotide. After extensive washing, the NF-κB complex bound to the oligonucleotide was identified using the supplied anti-p65 or anti-p50 antibody. Addition of a secondary antibody conjugated to HRP allowed a chemiluminescent readout on a Microlumat LB96P luminometer (Berthold Technologies) and an expression in RLU/s.

2.8. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). For the percentage of apoptosis, the caspase activity and the NF-κB activity, pairwise comparisons between the control group and the three other treatment groups were made after 6 h of incubation using a mixed model with cow as random effect and treatment as fixed effect. The Bonferroni's multiple comparisons adjustment technique was used with a global confidence level of 95 %. The correlation study was conducted according to Pearson. The SAS System software (SAS Institute GmbH, Heidelberg, Germany) was used for all analyses.

3. Results

3.1. Constitutive expression of NF-κB p65 and p50 in bovine neutrophils

In freshly isolated bovine neutrophils of 3 cows, the presence of p65 and p50 as important members of the NF-κB family was investigated on the mRNA as well as on the protein level. Using specific primer pairs in PCR, bands of 111 bp and 104 bp were detected, corresponding to mRNA from p65 and p50 respectively (Figure 1). After western blotting analysis, both p65 and p50 could also be detected as protein bands at their corresponding molecular weight (Figure 2). When using the anti-p50 antibody, an additional protein band was visualised as this antibody also recognizes the p50 precursor p105. Taken together, these experiments demonstrate the presence of p65 and p50 mRNA and protein in bovine neutrophils.

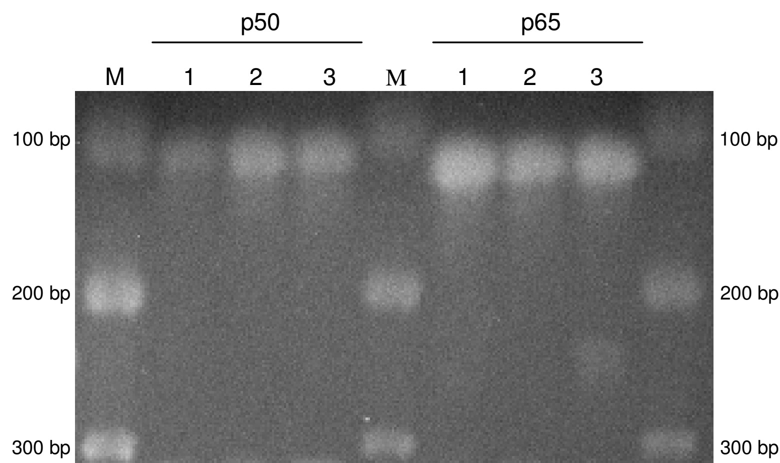

Figure 1: PCR analysis of neutrophils of 3 cows. mRNA of p65 and p50 are seen as bands of 111 and 104 bp, respectively. M represents the 100 bp DNA marker.

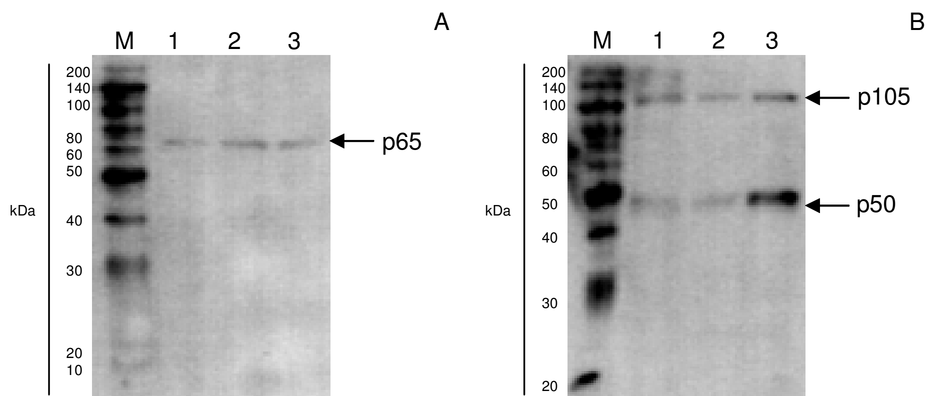

Figure 2: Western blotting analysis of neutrophils of 3 cows for A: the p65 protein and B: the p50 and p105 protein. M represents the molecular weight marker with bands from 10 to 200 kDa.

3.2. Effect of gliotoxin on phosphatidylserine exposure in bovine neutrophils

PS exposure in bovine neutrophils was measured directly after isolation and at different time points (2, 4 and 6 h) following incubation with 0.1 µg/ml gliotoxin and/or 10 ng/ml TNF-α (Figure 3). A time-dependent increase of the percentage of annexin-V-FITC-positive/PI-negative neutrophils was observed in all samples. The constitutive rate of apoptosis was greatly increased when gliotoxin was added. After 6 h of incubation, a significant difference in the percentage of apoptotic cells between control (on average 6.34 %, n = 6) and gliotoxin (41.05 %) was seen (P < 0.01). A remarkably higher percentage of apoptosis was observed in neutrophils incubated with a combination of gliotoxin and TNF-α (83.73 %; P < 0.01). TNF-α alone did not cause a significant increase in neutrophil apoptosis as compared to control (9.25 %). Annexin-V-FITC-positive/PI-positive values were less than 2 % in all samples.

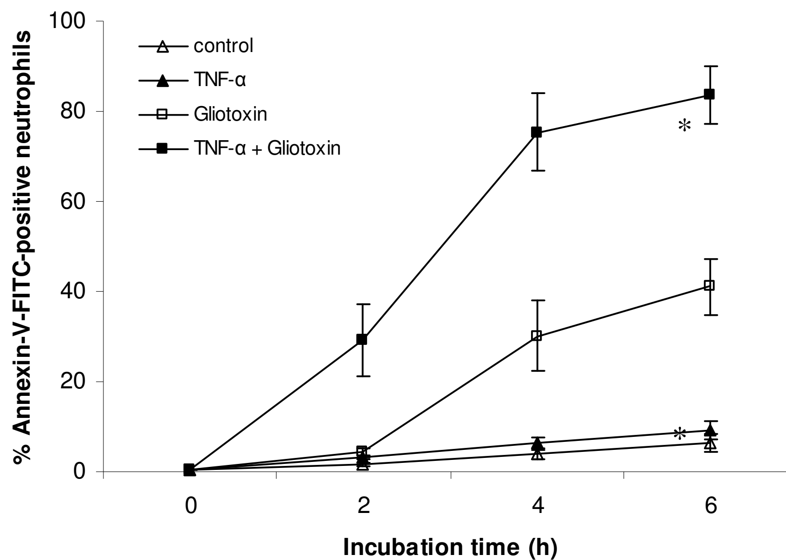

Figure 3: Effect of gliotoxin and/or TNF-α on cell surface exposure of PS in bovine neutrophils after isolation and after 2, 4 and 6 h of culture. Data are means ± SEM of 6 cows. Statistically significant differences compared to the control at 6 h are indicated with an asterisk.

3.3. Effect of gliotoxin on bovine neutrophil morphology

Cytospin slides of neutrophils incubated for 6 h in control medium were compared to slides of cells cultured in medium supplemented with gliotoxin in combination with TNF-α for the same period of time. Changes from normal cell morphology to apoptotic morphology were

clearly seen (Figure 4). Whereas non-apoptotic neutrophils were characterised by a polysegmented nucleus, apoptotic cells had a shrunken appearance with condensed nuclei. These qualitative results were in accordance with the flow cytometric data of PS exposure.

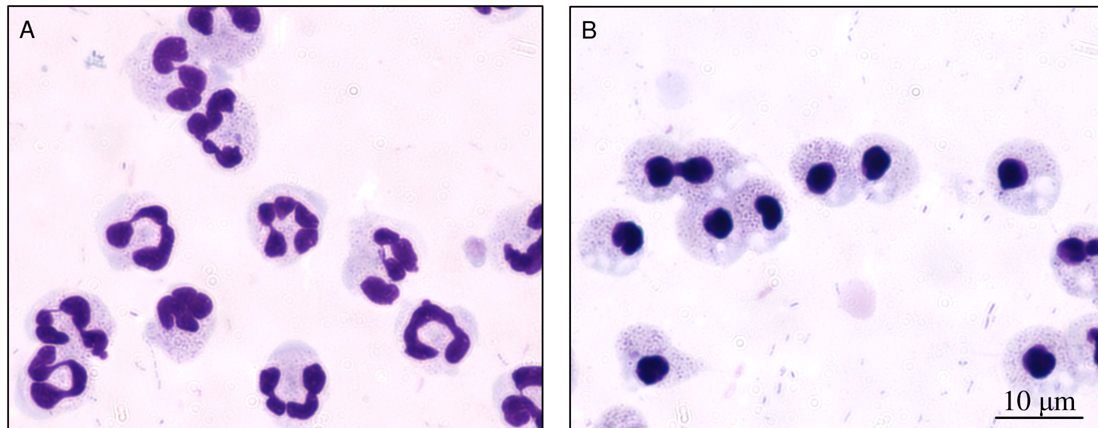

Figure 4: Cytocentrifuge preparations of neutrophils from one representative cow after 6 h of culture (magnification: 1,000 x). A: neutrophils incubated in control medium, B: neutrophils incubated in medium supplemented with gliotoxin and TNF-α.

3.4. Effect of gliotoxin on caspase-3/7 activity in bovine neutrophils

In all samples of freshly isolated neutrophils, caspase activity was minimal (Figure 5). This result was in accordance with the flow cytometric data of PS exposure which showed that an average of more than 98 % of the cells were viable at that time point. After 6 h of incubation, caspase activity increased and cells incubated in control medium showed a 10-fold higher activity as compared to freshly isolated neutrophils. The highest values of caspase activity were obtained when cells were incubated with gliotoxin in combination with TNF-α ($n = 6$, $P < 0.01$). Treatment with gliotoxin alone also significantly induced caspase activity ($P < 0.01$). Treatment with TNF-α resulted in minimal caspase activity with values being comparable to the control at the same time point. In general, these data again fit well with the PS observations. Indeed, a striking correlation ($r = 0.9326$) was obtained between both apoptosis parameters.

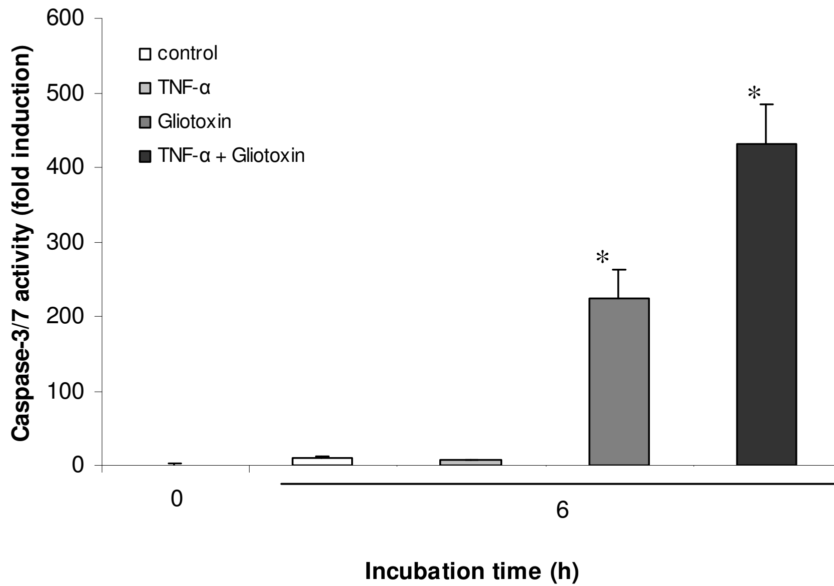

Figure 5: Caspase-3/7 activity of bovine neutrophils after isolation and after 6 h of culture with gliotoxin and/or TNF-α. Data are means ± SEM of 6 cows. Statistically significant differences compared to the control at 6 h are indicated with an asterisk.

3.5. Effect of gliotoxin on NF-κB p65 and p50 activity

In all cell extracts of freshly isolated neutrophils and of neutrophils incubated for 6 h, NF-κB p65 and p50 activities were measured. The highest p65 activity was seen in freshly isolated neutrophils (Figure 6). This activity decreased after 6 h of incubation, with the lowest values obtained in extracts of cells incubated in the presence of gliotoxin ($n = 6$, $P < 0.01$). Treatment with TNF-α alone did not result in a significantly lower p65 activity in comparison with the control at 6 h. Addition of TNF-α to gliotoxin abrogated the inhibition of the p65 activity by gliotoxin alone. This latter effect is not in accordance with the results obtained for the apoptosis parameters. No significant changes in p50 activity were found after 6 h of incubation in the different culture conditions. Values were even systematically comparable to those of the blank (data not shown).

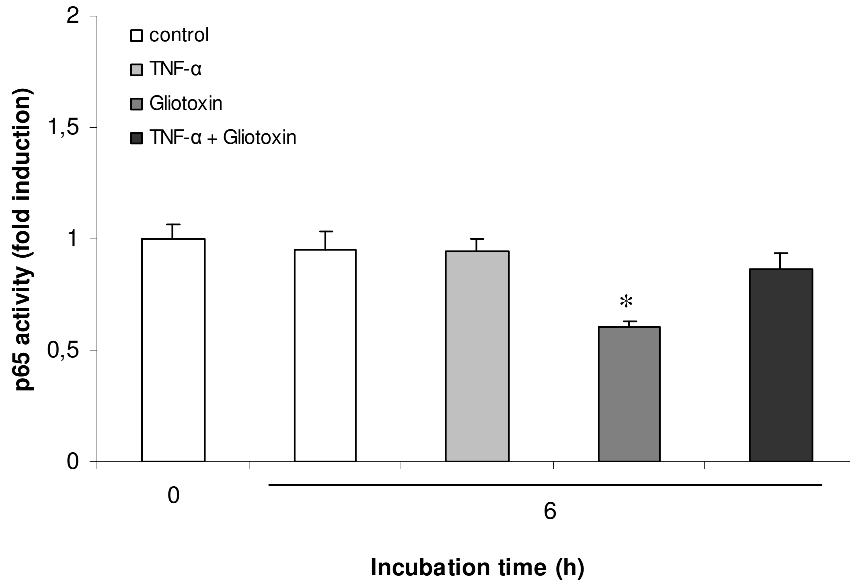

Figure 6: NF-κB activity of p65 in bovine neutrophils after isolation and after 6 h of culture with gliotoxin and/or TNF-α. Data are means ± SEM of 6 cows. Statistically significant differences compared to the control at 6 h are indicated with an asterisk.

4. Discussion

In neutrophils the major inducible NF-κB protein complex has been shown to consist of p65 and p50 (McDonald et al., 1997). At least one transactivation domain is present in p65 which makes this heterodimer responsible for gene induction (Moore et al., 1993). As these observations were obtained in the human species, the need exists to demonstrate the presence of both proteins in their bovine counterpart. In this study, we report that p65 and p50 are constitutively present in bovine blood neutrophils on mRNA level as well as on protein level. Subsequently, we set out to examine their function in these cells.

Since gliotoxin is a potent and specific inhibitor of NF-κB (Pahl et al., 1996), it was used as a pharmacological tool to investigate the involvement of the transcription factor in the regulation of bovine neutrophil apoptosis. PS exposure on the outer leaflet of the plasma membrane is a feature characteristic of apoptotic cells that can be flow cytometrically detected with annexin-V-FITC which binds the phospholipid in a calcium-dependent way (van Engeland et al., 1998). Our data obtained with this assay show that at least 98 % of freshly isolated neutrophils were viable and that incubation gave rise to spontaneous apoptosis. Gliotoxin significantly increased this constitutive rate of apoptosis over a time course of 6 h. Moreover, gliotoxin greatly enhanced the limited pro-apoptotic effect of TNF-α. These observations are consistent with results of similar experiments in human neutrophils (Ward et al., 1999; Liu et al., 2003; Renshaw et al., 2003). In addition to annexin-V-FITC, PI was used as a measure of necrosis in our flow cytometric analysis. Less than 2 % of neutrophils were PI-positive. Therefore we can conclude that treatment induced a purely apoptotic form of cell death.

As an additional apoptosis parameter, caspase activity was measured in all samples made after 6 h of incubation. Because inhibition of caspases-3 and -7 blocks human neutrophil apoptosis (Lee et al., 2000 and 2001; Daigle and Simon, 2001), the combined activity of both these effector caspases was assessed. The obtained results were in agreement with our flow cytometric PS exposure data: gliotoxin increased caspase activity compared to control at 6 h and together with TNF-α maximal activity was achieved. This demonstrates that apoptosis induced in the different culture conditions is dependent on caspase activation. The morphological study also confirmed that gliotoxin acts synergistically with TNF-α to

stimulate bovine neutrophil apoptosis. Taken together, our investigation of multiple complementary apoptosis parameters yielded indisputable results.

Various studies with human neutrophils have shown that NF-κB plays a crucial protective role against apoptosis (Ward et al., 1999; Niwa et al., 2000; Nolan et al., 2000; Liu et al., 2003). In the next part of the current study, p65 and p50 activities were therefore examined to evaluate whether this also applies to bovine neutrophils. We used a novel ELISA-based assay (TransAM^{TM} Transcription Factor Assay Kit), which is known to correlate well with the traditional electrophoretic mobility shift assay (EMSA) (Renard et al., 2001). Based on this assay, it was demonstrated that the activity of p65 was highest in freshly isolated bovine neutrophils and decreased after 6 h of incubation. Values of p65 activity were lowest in neutrophils cultured with gliotoxin. Treatment with TNF-α did not result in a significanlty lower p65 activity. These results are in agreement with our apoptosis data and support the working hypothesis that NF-κB promotes survival in bovine neutrophils. In contrast, following incubation with gliotoxin and TNF-α the p65 activity increased, an observation which is not in accordance with our apoptosis data. As for the p50 activity, no changes were found between the different culture conditions after 6 h of incubation. It was pointed out that p50 values were comparable to those of the blank. Interpretation of these data must be performed with care as the anti-human p50 antibody might not have recognized the active p50 protein of bovine origin in our samples. Indeed, this option can not be excluded as no cross-reactivity has been reported so far for the bovine species.

Gliotoxin belongs to the continuously growing group of selective NF-κB inhibitors. Although widely used, the precise mechanism by which this fungal metabolite inhibits NF-κB is still largely unknown. Some reports suggest the inhibition of the proteasome-mediated IκB degradation and the blockage of NF-κB DNA binding activity (Pahl et al., 1996; Kroll et al., 1999). In our study, administration of gliotoxin induced apoptosis in bovine neutrophils and decreased their basal p65 activity. In human neutrophils, inhibition of the basal NF-κB activity by gliotoxin also seems to be responsible for the induction of apoptosis (Ward et al., 1999). Because of the agreement between our observations and those in human neutrophils, the same conclusion can be drawn, namely that NF-κB promotes neutrophil survival. To the best of our knowledge, the identification of the family members composing the basal NF-κB complex in human neutrophils based on supershift experiments has remained unsuccessful

(McDonald et al., 1997; McDonald and Cassatella, 1997). According to our results obtained with the ELISA-based assay, at least p65 is part of the bovine basal NF-κB complex.

TNF-α is one of the most important orchestrators of inflammation and therefore a logical choice for the study of mediators in the context of neutrophil apoptosis. When evaluating the role of this cytokine on the neutrophil life span one should be aware that this cytokine can exert either an anti- or a pro-apoptotic effect, depending upon the concentration used and the time of exposure (Murray et al., 1997; van den Berg et al., 2001). Interestingly, the anti-apoptotic effect of TNF-α is partly ascribed to the stimulation of a survival pathway involving activation of NF-κB (Liu et al., 1996). In the current study, TNF-α was not found to promote bovine neutrophil apoptosis. Nevertheless, the dose and incubation time chosen have been previously reported to be pro-apoptotic. Furthermore, TNF-α did not seem to decrease p65 activity in our study. However, in comparison with gliotoxin alone, the increase in p65 activity following incubation with gliotoxin and TNF-α seems contradictory to the maximal percentage of apoptosis observed under these conditions. Indeed, in human neutrophils, the synergistic effect of gliotoxin and TNF-α on apoptosis was correlated with a major reduction of NF-κB activity (Ward et al., 1999). The authors even suggest that gliotoxin enhances TNF-α-induced apoptosis by inhibiting the production of a survival factor possibly mediated by NF-κB. Remarkably, in this report only the expression of an inducible form of NF-κB was down-regulated. This inducible NF-κB complex arose following TNF-α administration, but as in our study TNF-α alone did not induce p65 activity, it would appear that the combined effect of gliotoxin and TNF-α can not be visible on the level of NF-κB activity.

In conclusion, our data suggest that NF-κB p65 is active and promotes survival in bovine neutrophils. Still, is should be kept in mind that neutrophils in circulation will only survive for a few hours. Indeed, blood neutrophils are programmed to die spontaneously. We believe that a decrease in p65 activity probably represents an essential trigger for the onset of constitutive apoptosis. In contrast, tissue neutrophils can temporarily loose their susceptibility to apoptosis under inflammatory conditions. In human neutrophils, this loss seems to be associated with the appearance of an inducible NF-κB complex (Hotta et al., 2001). Importantly, NF-κB activity was shown to be increased in environments of inflammation (Sandersen et al., 2001; Boulanger et al., 2003). As we have now observed and partly characterised the basal activity of NF-κB in bovine neutrophils, our future prospect is to investigate the existence of inducible NF-κB complexes in these innate defense cells.

Acknowledgments

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CHAPTER 2

PRO- AND ANTI-INFLAMMATORY RESPONSE IN BOVINE NEUTROPHILS UPON ACTIVATION BY *E. COLI*

Adapted from

Notebaert S., Demeyere K. and Meyer E. Pro-and anti-inflammatory response of bovine neutrophils following phagocytosis of *Escherichia coli*. (2008) In preparation.

Abstract

Neutrophils are imperative to the innate immune response against bacterial pathogens. Upon activation they are stimulated to initiate and execute the acute inflammatory reaction. In this process, the nuclear transcription factor (NF)-κB plays a central role through the induction of the expression of numerous pro-inflammatory mediators. Nevertheless, an apoptosis differentiating program is additionally initiated to facilitate the resolution of inflammation. For bovine neutrophils, research in relation to these two opposing stages within the cell's life remains to be performed. We therefore investigated both pro- and anti-inflammatory events following incubation of bovine neutrophils with *Escherichia coli* (*E. coli*) bacteria within the same *in vitro* study. The activity of the NF-κB p65 subunit increased within the first half hour of culture with *E. coli*, while no changes in activity were seen for control cells. High concentrations of interleukin (IL)-1 and IL-6 were secreted by phagocytic neutrophils from early time points on. These important pro-inflammatory cytokines were not detected in cultures of resting cells. At later time points, phagocytosis induced cell death since neutrophils incubated with *E. coli* displayed significantly more phosphatidylserine (PS) than control cells. Remarkably, caspase-3/7 activity was only observed in resting neutrophils and not in cells incubated with bacteria. Interestingly, phagocytosis was also associated with major morphological changes that were clearly different from the characteristic features observed in neutrophils undergoing spontaneous apoptosis. In conclusion, this kinetic study on the early pro-inflammatory capacity and the subsequent apoptosis induction program of bovine neutrophils following phagocytosis of *E. coli*, contributes to the general understanding of the establishment and resolution of acute inflammatory reactions to coliform infections.

1. Introduction

Neutrophils play an essential role in the first line of defense against invading bacterial pathogens. These innate immune cells survive only a few days since they are destined to rapidly undergo spontaneous apoptosis. Still, their limited life span is highly susceptible to modulation. During an inflammatory response, the apoptotic process is delayed, thereby promoting neutrophil combat towards pathogens. On the other hand, neutrophil apoptosis needs to be accelerated in order to resolve the inflammatory process properly. Activation-induced cell death prohibits leakage of high amounts of toxic compounds thereby reducing damage to the surrounding tissues (Marshall et al., 2007).

Upon bacterial challenge, neutrophils are recruited to the site of infection and become activated. Several host- and pathogen-derived stimuli including pro-inflammatory cytokines and chemokines, growth factors and bacterial molecules like lipopolysaccharide (LPS) are known to trigger neutrophil recruitment and activation (Sabroe et al., 2005; Kato and Kitagawa, 2006). As a result, neutrophil functions such as chemotaxis, phagocytosis and bacterial killing via oxygen-dependent and -independent mechanisms are increased and a remarkably high number of genes are expressed (Subrahmanyam et al., 2001; Fessler et al., 2002; Tsukahara et al., 2003; Zhang et al., 2004; Kobayashi et al., 2002 and 2003b; Kobayashi and Deleo, 2004). Incubation of these phagocytes with some of the abovementioned stimuli or with whole bacteria results in the synthesis of numerous inflammatory cell-surface receptors and mediators, as well as stress and signaling molecules and proteins promoting neutrophil survival.

The alteration in gene expression profiles in response to inflammation is mediated in part by the transcription factor NF-κB (Li and Verma, 2002; Cloutier and McDonald, 2003). In resting cells, NF-κB family members are retained in the cytoplasm through binding with their inhibitors, which belong to the IκB family. Upon cell activation, IκB proteins become phosphorylated, ubiquitinated and degraded by the proteasome. Subsequently, NF-κB can enter the nucleus and bind to the DNA in order to promote transcription (Hayden and Ghosh, 2004). The most intensively studied NF-κB complex is the p65/p50 heterodimer. In neutrophils, NF-κB activation has been reported upon exposure to pro-inflammatory cytokines and chemokines as well as to LPS and whole bacteria (McDonald and Cassatella, 1997; McDonald et al., 1997; Vollebregt et al., 1998; Kettritz et al., 2004). In addition, the

expression of pro-inflammatory and anti-apoptotic proteins is increased in response to these stimuli, and transcription of most of these factors is NF-κB dependent (Pahl, 1999).

Following the establishment of neutrophil-mediated inflammation as an early response to bacterial infection, the resolution of the inflammatory process is initiated. Due to phagocytosis, mechanisms which induce cell death are turned on. Recent studies show an acceleration of neutrophil apoptosis following ingestion of different pathogens (Watson et al., 1996; Rotstein et al., 2000; Engelich et al., 2001; Perskvist et al., 2002; Sim et al., 2005). The onset of neutrophil programmed cell death coincides well with functional impairment (Whyte et al., 1993) and with a decrease in the expression of pro-inflammatory and anti-apoptotic molecules induced in the early stages of inflammation. Moreover, factors that are able to participate in the initiation and execution of apoptosis are up-regulated (Kobayashi et al., 2002, 2003a/c, 2004).

The molecular mechanisms of neutrophil activation-induced cell death are currently intensively investigated. It is well established that reactive oxygen species generated upon phagocytosis promote the onset of neutrophil apoptosis by triggering cell surface changes that result in macrophage recognition and subsequent engulfment of the phagocytic cells (Watson et al., 1996; Fadeel et al., 1998, Wilkie et al., 2007). However, in activation-induced cell death, the contribution of caspases, generally accepted as the key executors of the apoptosis program, is still a matter of debate (Fadeel et al., 1998; Perskvist et al., 2002; Zhang et al., 2003; Wilkie et al., 2007).

Pro- and anti-inflammatory events following neutrophil activation and phagocytosis have been mainly studied in human neutrophils. Bovine neutrophils are entailed in the host defense mechanisms of several important infectious diseases in cattle. Increased bovine neutrophil functions were observed following *in vitro* incubation with pro-inflammatory stimuli (Sample and Czuprynski, 1991; Persson, et al., 1993; Swain et al., 1998; Galligan and Coomber, 2000; Rainard et al., 2000; Borgquist et al., 2002) and bacterial toxins (Czuprynski et al., 1991; Maheswaran et al., 1992; Narayanan et al., 2002). Enhanced expression of the CD18 subunit of β2 integrin inflammatory receptors is described as well (Rainard et al., 2000; Leite et al., 2002). However, NF-κB activation in bovine neutrophils within these inflammatory conditions has not yet been reported. In previous work, we showed a basal NF-κB p65 activity in freshly isolated bovine neutrophils which promotes survival until the initiation of

spontaneous apoptosis (Notebaert et al., 2005). In agreement with observations in human cells, apoptosis in bovine neutrophils leads to functional impairment (Van Oostveldt et al., 2002). To the best of our knowledge, reports on induced apoptotic cell death in bovine neutrophils following phagocytosis are not available. Clearly, imperative information on the molecular effects of bovine neutrophil activation and phagocytosis are still missing. In the current study, both the pro-inflammatory response and the subsequently induced cell death of activated bovine neutrophils were examined. An important role in the regulation of the bovine innate immune response following *E. coli* phagocytosis is apparent for the transcription factor NF-κB but not for the effector caspases-3 and -7.

2. Experimental procedures

2.1. Isolation of bovine neutrophils

Healthy heifers of the Holstein-Friesian breed were selected from the Ghent University dairy farm (Biocentrum Agri-Vet, Melle, Belgium). Bovine neutrophils were isolated as previously described (Notebaert et al., 2005). Peripheral blood was collected from the jugular vein using sterile tubes (IMI, Montegrotto Terme, Italy) pre-filled with an equal volume of Alsever as an anticoagulant solution (3 mM citric acid monohydrate, 27 mM trisodium citrate dihydrate, 72 mM sodium chloride and 125 mM D-glucose, pH 6.1). Plasma and the buffy coat were removed following centrifugation at 300 g for 15 min. Erythrocytes were lysed during incubation with an ice-cold isotonic ammonium chloride solution (138 mM ammonium chloride and 21 mM Tris, pH 7.4) for 10 min. The remaining cell suspension was washed twice in phosphate buffered saline (PBS) at 200 g for 10 min. Contaminating mononuclear cells were then removed by density gradient centrifugation at 1,000 g for 20 min using 1.094 g/ml Percoll (Sigma-Aldrich, Bornem, Belgium). The remaining cells were again washed twice with PBS. Over 98 % of the isolated cells were granulocytes with less than 5 % eosinophils.

2.2. Phagocytosis of *E. coli* by bovine neutrophils

Isolated neutrophils were resuspended in sterile RPMI 1640 with 10 % fetal calf serum (both from Invitrogen, Merelbeke, Belgium) at a final concentration of 5 x 10^6 cells/ml. Living *E. coli* P4:O32 bacteria were supplemented to the neutrophil cultures at a ratio of 5:1 following overnight growth in Brain Heart Infusion broth (Oxoid, Drongen, Belgium). Incubation was performed at 37 °C with mild rotation. Phagocytosis was stopped by placing the cells on ice. Samples were analysed immediately afterwards.

2.3. NF-κB activation in bovine neutrophils

Cultured neutrophils were centrifuged at 200 g for 10 min and lysed on ice in RIPA lysis buffer (150 mM sodium chloride, 20 mM Hepes, 2 mM EDTA, 0.5 % sodium deoxycholate and 1 % NP-40, pH 7) with a cocktail of protease inhibitors (Calbiochem, La Jolla, USA). After centrifugation at 8,000 g for 10 min, the supernatant was collected and the protein

concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Life Science, Nazareth, Belgium), based on the method of Bradford. To determine NF-κB p65 activity, the TransAM^{TM} Transcription Factor Assay Kit (Active Motif, Rixensart, Belgium) was used according to the manufacturer's protocol. Ten µg of each protein lysate was incubated for 1 h in a 96-well plate containing an immobilized NF-κB consensus oligonucleotide. After washing, the NF-κB complex bound to the oligonucleotide was identified using the supplied anti-p65 antibody. Addition of a secondary antibody conjugated to horseradisch peroxidase (HRP) allowed a chemiluminescent readout (Fluoroskan Ascent FL; Thermo Fisher Scientific, Zellik, Belgium) expressed in relative light units per second (RLU/s).

2.4. Cytokine secretion by bovine neutrophils

2.4.1. IL-6 secretion

Supernatants obtained after pelleting cultured neutrophils were used to determine secreted cytokine levels. IL-6 was measured in a bio-assay as a hybridoma growth factor for mouse 7TD1 cells (Landegren, 1984). Briefly, cells were cultured for 72 h in medium with different dilutions of the samples. A colorimetric hexosaminidase reaction reflects the number of cells which is related to the amount of IL-6 in the media. As no recombinant bovine IL-6 is commercialy available, human reference standards were included in the assay. Analysis was based on the half-maximal proliferation of the cells.

2.4.2. IL-1 secretion

To determine IL-1 concentrations in the supernatants, a bio-assay using growth factor-dependent murine D10(N4)M cells was employed (Helle et al., 1988). In brief, cells were cultured for 48 h in medium with different dilutions of the human standard and the samples. Addition of [^{3}H]-thymidine allowed incorporation following proliferation. The scintillation counts reflect the number of cells which is correlated to the amount of IL-1. Analysis was based on the half-maximal proliferation of the cells.

2.4.3. TNF-α secretion

TNF-α levels were determined in the supernatants by using a bio-assay with TNF-α-sensitive mouse L929 cells (Mosmann, 1983). Briefly, cells were cultured for 24 h in medium with different dilutions of the standard and the samples together with actinomycin D. A colorimetric reaction with MTT reflects the number of cells which is inversily correlated to the amount of TNF-α in the media. Analysis was based on the half-maximal cell death.

2.5. Apoptosis of bovine neutrophils

2.5.1. PS exposure

Following culture, 1 x 10^6 neutrophils were centrifuged at 200 g for 10 min and resuspended in incubation buffer (10 mM Hepes, 140 mM sodium chloride and 5 mM calcium chloride, pH 7.4) with annexin-V-fluos labeling reagent (Roche Diagnostics, Vilvoorde, Belgium) and propidium iodide (PI, 50 µg/ml; Sigma-Aldrich). After a 10 min incubation in the dark, PBS was added and samples were analysed using a FACSCanto™ flow cytometer (Becton Dickinson Biosciences, Erembodegem, Belgium). Ten thousand events were collected and analysed using the FACSDiva software (Becton Dickinson Biosciences). Debris was gated out before analysis. Cells positive for annexin-V were defined as apoptotic. Early apoptosis was seen in annexin-V-positive but PI-negative cells, while late apoptotic cells were characterized by combined annexin-V- and PI-positivity.

2.5.2. Caspase-3/7 activation

Neutrophils were centrifuged at 200 g for 10 min and lysed on ice in a caspase lysis buffer (200 mM sodium chloride, 10 mM Tris, 5 mM EDTA, 10 % glycerol and 1 % NP-40, pH 7.5) supplemented with protease inhibitors (0.15 µM aprotinin, 2.1 µM leupeptin and 100 nM phenylmethylsulfonyl fluoride; all from Sigma-Aldrich) and glutathione (1 mM, Sigma-Aldrich). After centrifugation at 8,000 g for 10 min, the supernatant was collected and the protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Life Science). To determine caspase-3/7 activity, the Caspase-Glo® Assay (Promega, Leiden, The Netherlands) was used according to the manufacturer's protocol. In a 96-well plate, 10 µg of the protein lysate was diluted with lysis buffer to a final volume of 100 µl and an equal

amount of Caspase-Glo reagent containing DEVD-aminoluciferin as a substrate for caspases-3 and -7 was added. After 1 h incubation at room temperature, chemiluminescence was measured with a luminometer (Fluoroskan Ascent FL, Thermo Fisher Scientific) and expressed in RLU/s.

2.6. Morphological analysis of bovine neutrophils

At different time points during incubation, 2 x 10^5 neutrophils were harvested and cytospins were prepared by centrifugation at 55 g for 5 min. Slides were air dried, fixed in methanol and stained with Hemacolor (Merck, Darmstadt, Germany).

2.7. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). The SPSS Software (SPSS Belux, Brussels, Belgium) was used to perform the non-parametric Friedman's test for repeated measures. The Dunett's test for multiple comparisons with the control group was also employed (Zar, 1984).

3. Results

3.1. NF-κB activation following *E. coli* phagocytosis

The activity of the transcription factor NF-κB p65 was analysed in bovine neutrophils at different time points (0, 10, 20, 30, 60 and 180 min) following culture with or without *E. coli* bacteria (Figure 1). No changes in activity were seen for neutrophils cultured without bacteria, while for cells incubated with *E. coli* bacteria a time-dependent activation was observed. At 20 min of culture, the p65 activity was significantly increased for phagocytic neutrophils as compared to resting cells.

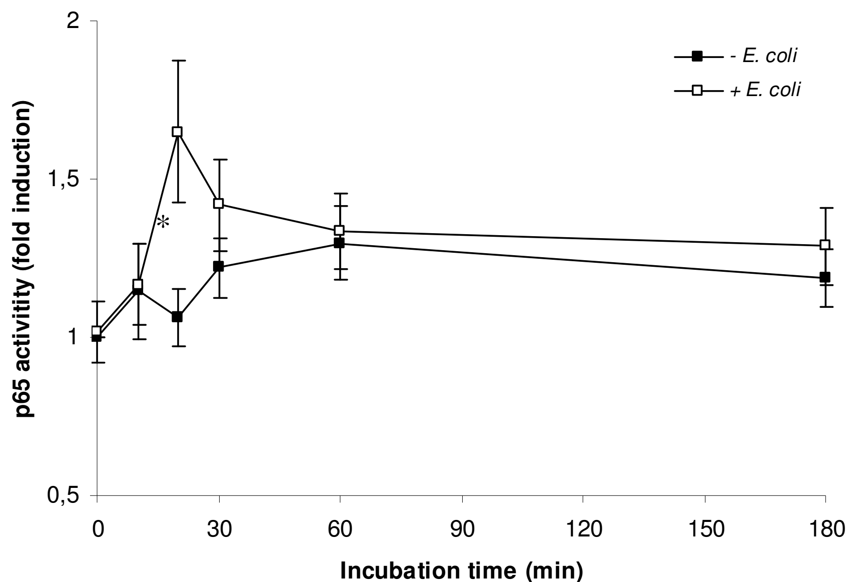

Figure 1: NF-κB activity of p65 in bovine neutrophils after isolation and following 10, 20, 30, 60 and 180 min of incubation with or without E. coli bacteria. Data are means ± SEM of 10 cows. Statistically significant differences are indicated with an asterisk.

3.2. Cytokine secretion following *E. coli* phagocytosis

3.2.1. IL-6 secretion

At different early and later time points (0, 20, 60 and 180 min; 0, 6 and 24 h) IL-6 concentrations were measured in the supernatants of bovine neutrophil cultures supplemented with or without *E. coli* bacteria (Figure 2A). For control cells, values remained undetectable,

while for neutrophils incubated with bacteria, IL-6 concentrations in the medium gradually increased from the start up to 24 h of incubation.

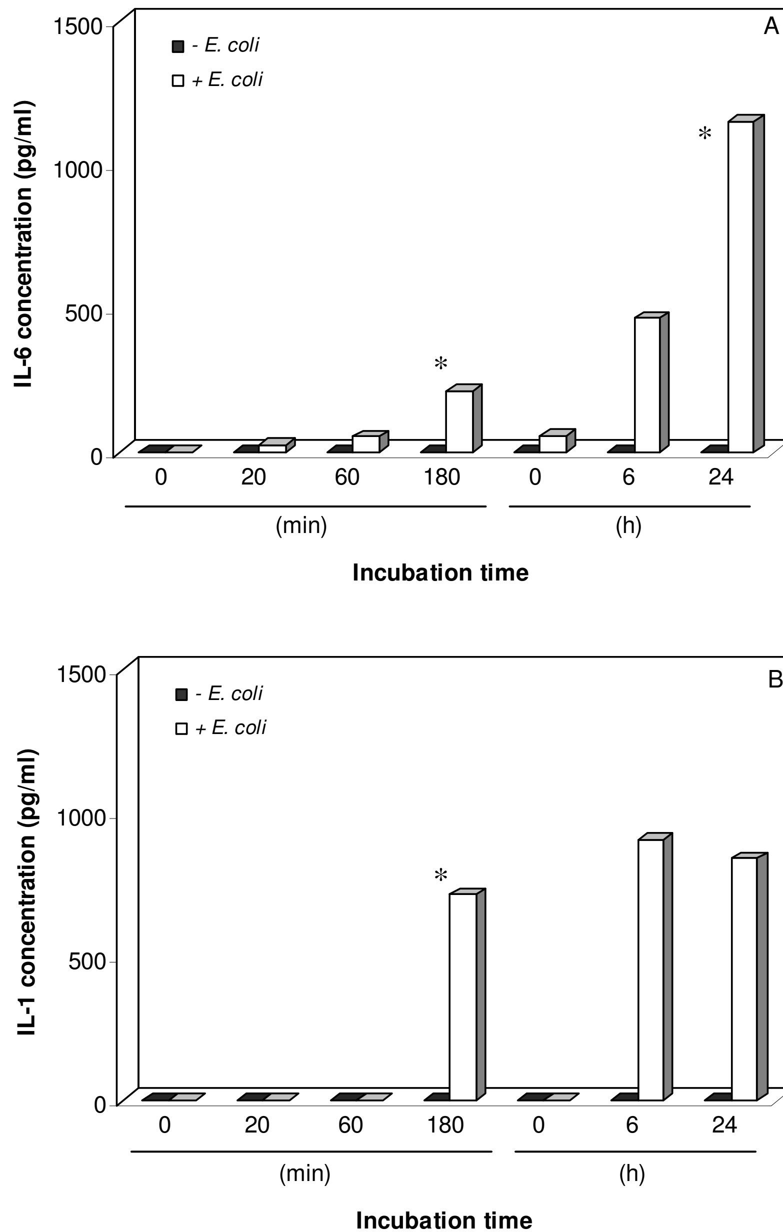

Figure 2: Cytokine concentrations of IL-6 (panel A) and IL-1 (panel B) in supernatants of bovine neutrophil cultures with or without E. coli bacteria. Data are means of 10 (after isolation and following 20, 60 and 180 min of incubation) and 6 cows (after isolation and following 6 and 24 h of incubation). Statistically significant differences are indicated with an asterisk.

3.2.2. IL-1 secretion

At the same time points, supernatants were also analysed for the presence of IL-1 (Figure 2B). Again, this cytokine was not detected in the medium of control cells. For neutrophils incubated with bacteria, secreted values started to increase after 3 h and reached a maximum at 6 h of incubation. At 24 h of culture, no additional secretion was observed.

3.2.3. TNF-α secretion

At the early and later time points, TNF-α concentrations were also measured in the supernatants. For both control cells and cells incubated with *E. coli* bacteria, values remained undetectable.

3.3. Apoptosis following *E. coli* phagocytosis

3.3.1. PS exposure

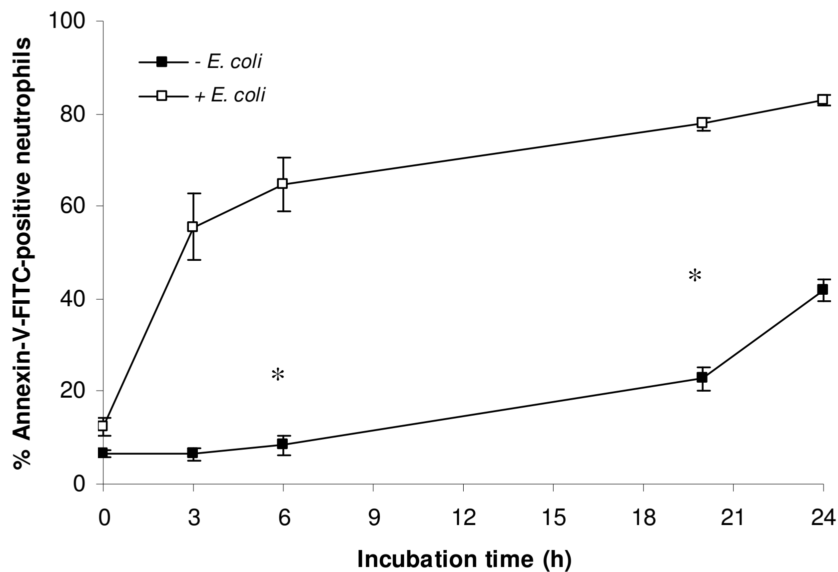

Figure 3: Percentage of cell surface exposure of phosphatidylserine in bovine neutrophils after isolation and following 3, 6, 20 and 24 h of culture with or without E. coli bacteria. Data are means ± SEM of 6 cows. Statistically significant differences are indicated with an asterisk..

PS exposure in bovine neutrophils was measured directly after isolation and at different time points (3, 6, 20 and 24 h) following incubation with or without *E. coli* bacteria (Figure 3). In both conditions, the percentage of annexin-V-positive neutrophils increased over time up to 24 h of incubation. However, from 3 h on, the rate of PS exposure was significantly higher in the presence of *E. coli* bacteria as compared to control.

3.3.2. Caspase-3/7 activation

Caspase-3/7 activity was measured in bovine neutrophils after isolation and at 6 and 24 h following incubation in the presence or absence of *E. coli* bacteria (Figure 4). In all samples of freshly isolated neutrophils, caspase activity was minimal. Cells incubated with bacteria maintained basal levels up to 24 h of culture, while control cells had a significantly increased caspase activity at 6 h which was even more pronounced at 24 h of culture.

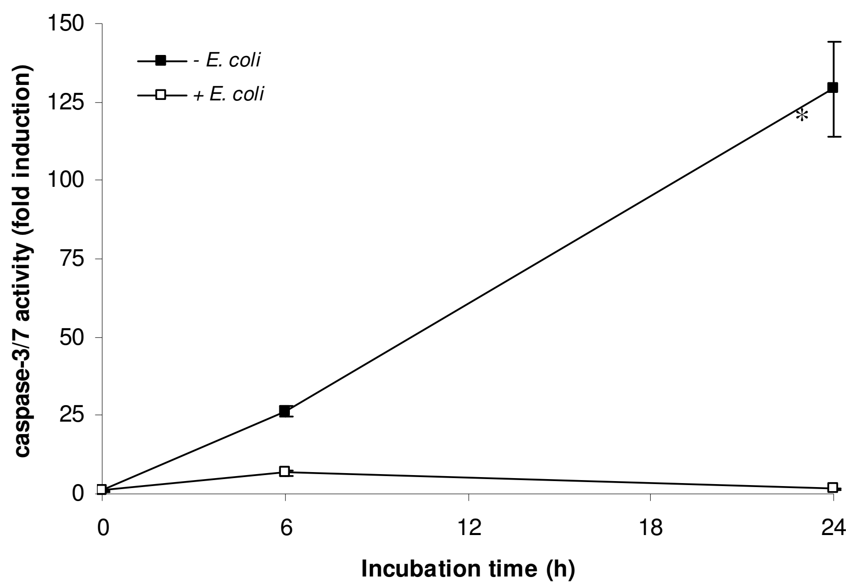

Figure 4: Caspase-3/7 activity of bovine neutrophils after isolation and following 6 and 24 h of incubation with or without E. coli bacteria. Data are means ± SEM of 6 cows. Statistically significant differences are indicated with an asterisk.

3.4. Morphological analysis following *E. coli* phagocytosis

The morphology of neutrophils was studied following incubation with or without *E. coli* bacteria (Figure 5). After 30 min of culture, phagocytic neutrophils appeared as freshly isolated cells with bacteria present at the cell surface or in phagosomes (panel A and B). Following 2 h of incubation with *E. coli*, many neutrophils had lost their characteristic multilobulated nuclei and instead displayed nuclei that were swollen and irregular in shape (panel C). These features became apparent in all cells up to 6 h of culture with bacteria (data not shown), while at that time point most control cells still had a normal cell morphology and some showed condensed nuclei (panel D).

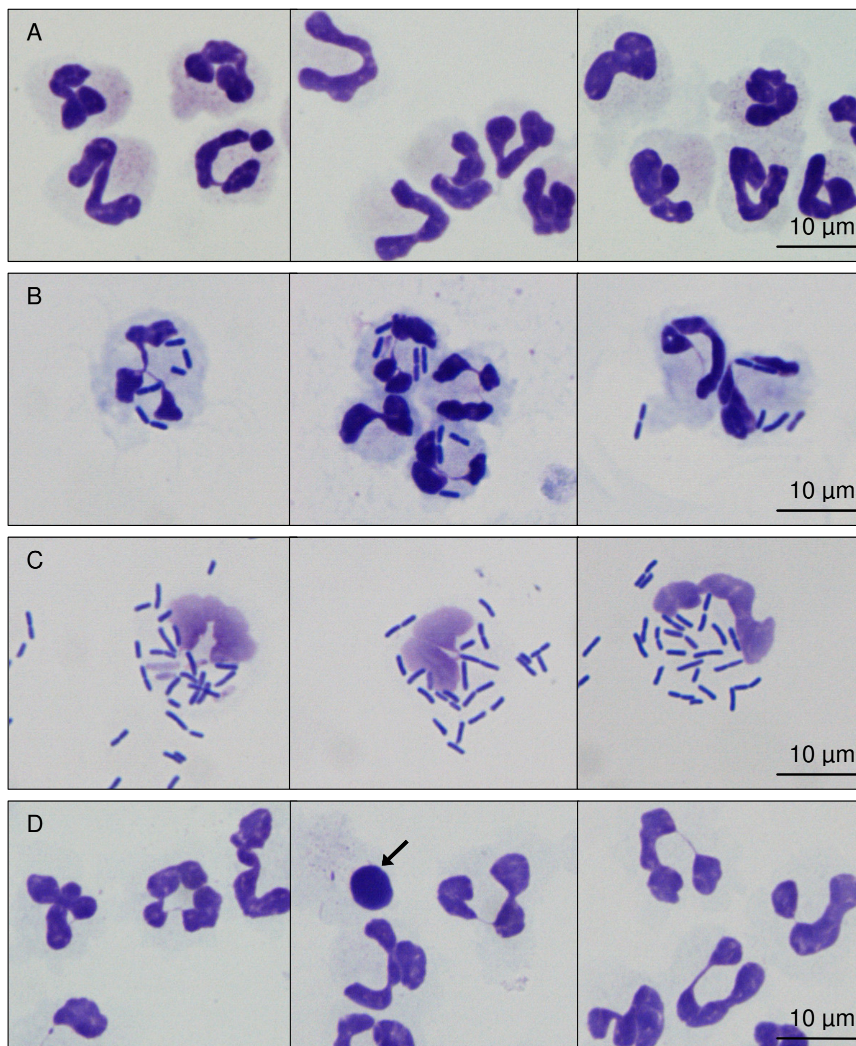

Figure 5: Representative cytocentrifuge preparations at a magnification of 1,000 x of freshly isolated bovine neutrophils (A), neutrophils after 30 min (B) and 2 h (C) of culture with E. coli and following 6 h of culture without bacteria (D). The arrow indicates an apoptotic cell.

4. Discussion

Neutrophil phagocytosis leads to a number of molecular events which are currently intensively investigated. Our present findings characterize both a pro- and anti-inflammatory response of bovine neutrophils following phagocytosis of *E. coli* bacteria. Coliform pathogens are known to be involved in several important diseases in cattle such as enteritis, endometritis and mastitis (Mouricout, 1991; Bradley, 2002; Singh et al., 2007). Bovine neutrophils play a role in the pathogenesis of these infections. In the current *in vitro* study, *E. coli* bacteria were supplemented to bovine neutrophil cultures at a ratio of 5:1. Ratios employed in other *in vitro* studies ranged from 2 up to 50 bacteria, yeast particles or latex beads per neutrophil. Since only a few living bacteria are required to start an *in vivo* infection, a relatively low ratio was also chosen in our study.

At first, the activation of the p65 subunit of NF-κB was observed as a very fast and transient response in bovine neutrophils following incubation with *E. coli* bacteria. This finding has not yet been reported either for bovine phagocytes or for their counterparts of human origin. Still, NF-κB was also rapidly and temporary activated in human neutrophils in response to LPS or following incubation with *Staphylococcus aureus* (*S. aureus*) bacteria or yeast particles (McDonald and Cassatella, 1997; Vollebregt et al., 1998). In these studies, the induced NF-κB complex mainly consisted of p65/p50 dimers as determined by supershift experiments with the electrophoretic mobility shift assay (EMSA). Besides the p65 subunit, the presence of NF-κB p50 was also examined in the activated NF-κB complexes observed in our study using the ELISA-based assay. No significant difference in p50 activity between bovine neutrophils incubated with or without *E. coli* bacteria was apparent (data not shown). It should be remarked that as p50 could thus not be detected in the basal (Notebaert et al., 2005) nor in the inducible NF-κB complexes, it is now likely that the anti-human p50 antibody does not recognize the protein of bovine origin. Therefore, we conclude that at least p65 is involved in activated NF-κB complexes from bovine neutrophils.

The NF-κB activation turned out to be significant albeit modest. This observation is in agreement with a report in which the authors suggest that the limited activation is either due to a substantial background activity as a consequence of the cell preparation or to a response of only a subset of cells (Vollebregt et al., 1998). We rather underline the importance of the regulation of NF-κB activity by post-translational modifications (Perkins, 2006; Neumann

and Naumann, 2007), which can not be measured by using either the ELISA-based kit or EMSA. Nevertheless, the rapid and transient NF-κB activation observed in our study is consistent with its role in the regulation of the innate immune response. It leads to NF-κB mediated gene expression and the subsequent production of pro-inflammatory and anti-apoptotic proteins. Neutrophils thereby initiate and execute the acute inflammatory reaction towards bacterial invasion. NF-κB activation in response to *E. coli* can be mediated by several interactions between neutrophil's receptors and bacterial components. The recognition of LPS by the major endotoxin receptor Toll-like receptor 4 is probably the best known example (Sabroe et al., 2005).

Secondly, we observed an increase in concentration of IL-6 and IL-1 in the medium of bovine neutrophils following incubation with *E. coli* as compared to control cells. However, no TNF-α production was observed following phagocytosis. Neutrophils might avoid the production of TNF-α which is able to promote neutrophil apoptosis and then does not stimulate the immune response towards infection. On the contrary, IL-6 and IL-1 clearly exert a pro-inflammatory function. Both products are expressed under the control of NF-κB, which was also activated in our study. Remarkably, *in vitro* cytokine production by neutrophils in response to *E. coli* bacteria has not yet been reported. The up-regulation of the genes encoding for IL-1α and β has been demonstrated for human neutrophils (Subrahmanyam et al., 2001; Hochegger et al., 2007). In both studies, these changes in mRNA expression level were observed within the first hours of culture with *E. coli* bacteria. In response to activators such as LPS (Cicco et al., 1990; Marucha et al., 1990) or following phagocytosis of latex beads (Kobayashi et al., 2003c), genes encoding both cytokines were also up-regulated from early time points on. Furthermore, the secreted proteins were detected following exposure to LPS (Cicco et al., 1990; Marucha et al., 1990). In the current study, IL-6 concentrations were measured within the first half hour while IL-l levels increased only after a few hours of culture. Since IL-1β is initially transcribed as a proform which still remains to be processed before the active protein can be released (Matsushima et al., 1986), this time delay in secretion of both interleukins can at least be partially explained. Between 6 and 24 h of culture, IL-6 is still produced and secreted while IL-1 is no longer synthesized. Interestingly, anti-inflammatory effects mediated by interleukins such as IL-6 have been reported and include for example the inhibition of NF-κB (Ahmed and Ivashkiv, 2000). In any case, the finding that cytokines are still produced after 24 h of *in vitro* culture of isolated neutrophils is

also physiologically irrelevant, given that apoptotic neutrophils would already be recognized and cleared by macrophages at that time point *in vivo*.

Thirdly, bovine neutrophil apoptosis is accelerated following phagocytosis of *E. coli* bacteria since the rate of PS exposure in activated cells is significantly increased as compared to control cells undergoing spontaneous apoptosis. This observation is in agreement with recent studies on neutrophil apoptosis following ingestion of different pathogens (Watson et al., 1996; Rotstein et al., 2000; Engelich et al., 2001; Perskvist et al., 2002; Sim et al., 2005). Clearly, by this means the resolution of the inflammatory process is initiated. Interestingly, this phagocytosis-induced cell death occured independently from the activity of caspases-3 and -7. On the contrary, both effector caspases were as expected involved in the constitutive apoptosis process observed in about half of the control cells after 24 h of culture. These findings do not corroborate those from human neutrophils incubated with *E. coli*, where caspase-3 activation was demonstrated (Zhang et al., 2003). However, caspase-independent apoptosis has been reported in human neutrophils following activation with phorbol 12-myristate 13-acetate (Fadeel et al., 1998). In addition, a recent report on neutrophils cultured with *S. aureus* also describes a caspase-independent cell death and their subsequent clearance by macrophages (Wilkie et al., 2007). The authors suggest that the apparent discrepancy between their observations and those from Zhang et al. (2003) might be related to the nature of the micro-organism used to activate the neutrophils. Since for *E. coli*, both a caspase-dependent (Zhang et al., 2003) as well as an -independent neutrophil cell death are now reported, the abovementioned explanation can be ruled out. We therefore conclude that additional experiments are required in order to identify the pathways orchestrating apoptosis following phagocytosis.

Finally, bovine phagocytic neutrophils lost their characteristic multilobulated nuclei and instead displayed nuclei that were swollen and irregular in shape. This nuclear morphology is very distinct from the one of neutrophils undergoing spontaneous apoptosis, which is characterized by the appearance of condensed and shrunken nuclei. Still, similar morphological changes were reported for human neutrophils cultured with *S. aureus* (Wilkie et al., 2007). On the contrary, nuclear condensation was observed in bovine neutrophils following phagocytosis of *S. aureus* during *in vivo* infection (Rysanek and Sladek, 2006) as well as in human neutrophils incubated with *E. coli* (Watson et al., 1996). In these studies, morphology was assessed at much later time points. The observed morphology in our study

and the one of Wilkie et al. (2007) might therefore be preceding the characteristic apoptotic appearance. In any case, due to this apparent inconsistency, it has been suggested not to use changes in nuclear morphology to assess apoptosis in phagocytic neutrophils (Wilkie et al., 2007).

In summary, complementary to our former observation and characterization of the basal NF-κB activity in resting bovine neutrophils (Notebaert et al., 2005), we now demonstrate the existence of an inducible p65-containing NF-κB complex which most likely accounts for the early pro-inflammatory response to phagocytosis of *E. coli*, in part by producing and secreting the major signaling cytokines IL-1 and IL-6. In addition, as a later event, when NF-κB activity is no longer apparent, a caspase-independent cell death is induced, ensuring an anti-inflammatory response. Kinetic studies such as the current one in which both stages of the phagocyte's life are subsequently investigated, provide insight in the molecular pathways that are turned on following neutrophil phagocytosis.

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PART 2

IN VIVO STUDY OF NF-κB ACTIVATION IN A MOUSE MODEL OF INTRAMAMMARY *E. COLI* INFECTION

CHAPTER 3

PRO-INFLAMMATORY RESPONSE
IN INTRAMAMMARY *E. COLI* INFECTIONS

Adapted from

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Abstract

Escherichia coli (*E. coli*) infections in mouse mammary glands are rarely described and poorly characterized. In order to investigate the host immune response during coliform mastitis, several inflammatory parameters were evaluated at 24 and 48 h following inoculation of mouse mammary glands with *E. coli*. Successfully challenged mice showed high values of the acute phase protein serum amyloid A (SAA) in blood. Systemic concentrations of the major inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 were also increased as compared to control mice, while IL-1 levels remained negligible. Infected mammary glands showed a significant increase of all cytokine levels as compared to control glands. In accordance, mammary expression of the biologically inactive proform of IL-1β was strongly up-regulated. Remarkably, data obtained in wild type as well as caspase-1 knockout mice showed that IL-1β maturation seemed to occur independently from caspase-1. Finally, *E. coli* infection also triggered activation of the nuclear transcription factor (NF)-κB in the mammary gland. In conclusion, the current study provides novel insights on the contribution of major regulatory proteins to the acute inflammatory host response at the local and systemic level during *E. coli* mastitis in mice.

1. Introduction

Mastitis is an infectious disease of the mammary gland, which is frequently diagnosed among breastfeeding women and causes considerable economic losses in the dairy industry. This intramammary inflammation can be provoked by various microbial pathogens such as *Staphylococcus aureus* (*S. aureus*) and *E. coli*. Even though mastitis is well-studied in cows, performing experiments in cattle has become less attractive due to major costs and management problems. Therefore, mouse models are increasingly used as an alternative for the study of intramammary infections.

A mouse model of infectious mastitis was first described by Chandler in the 1970s (1969 and 1970). Since then, this model has been used with a variety of pathogens to gain knowledge about different aspects of the disease. These include not only the pathogenesis of mastitis and the pathogen's mode of action, but also the control of intramammary infections by new drugs and vaccines (Notebaert and Meyer, 2006).

With the mouse model of mastitis, mainly intramammary infections caused by *S. aureus* have been studied (Brouillette and Malouin, 2005). Among the environmental pathogens, *E. coli* has been used occasionally. Clinical signs and observations as well as general aspects of coliform mastitis in mice such as bacterial growth, neutrophil response and histological changes in the infected mammary gland, have been demonstrated long ago (Chandler, 1970; Anderson, 1979 and 1983). Furthermore, the model has been used to clarify bacterial adherence and virulence mechanisms of the pathogen (Anderson et al., 1977; Anderson, 1978). Finally, control strategies such as the application of antibiotics (Chandler, 1971; Anderson and Craven, 1984; Yancey et al., 1987) and immunomodulating agents (Cooray and Jonsson, 1990; Lee et al., 2003) were also tested in mice inoculated with *E. coli*. In these few reports dealing with *E. coli* mastitis in mice, imperative information involving inflammatory mediators characterizing the host immune response is lacking.

The present study focuses on the local and systemic host defense mechanisms during acute murine mastitis experimentally induced by *E. coli*. The inflammatory response is evaluated by the production of the acute phase protein amyloid A and the major inflammatory cytokines, TNF-α, IL-6 and IL-1. Since IL-1β is processed in the recently discovered and currently intensively studied inflammasome (Mariathasan and Monack, 2007), the role of caspase-1 or

IL-1β converting enzyme (ICE) in its maturation was also studied by using both wild type and caspase-1 knockout mice. In addition, the contribution of NF-κB, a transcription factor known to play a key role in the inflammatory response to several diseases, was assessed through its most relevant family members p65 and p50. In conclusion, the characterization of the early inflammatory response upon *E. coli* mouse mastitis no longer remains unaddressed and the obtained knowledge may contribute to the field of mastitis research.

2. Experimental procedures

2.1. Bacteria

For the intramammary inoculation, *E. coli* strain P4:O32 was used, which was originally isolated from a clinical case of bovine mastitis (Bramley, 1976) and which has been used in various studies of induced mammary infection in both cows (Vangroenweghe et al., 2004) and mice (Lee et al., 2003). Bacteria were grown overnight at 37 °C in Brain Heart Infusion medium (Oxoid Limited, Hampshire, United Kingdom). To verify their purity, bacteria were streaked onto a blood agar plate (Oxoid Limited). Bacterial concentration of the culture was determined using a standard curve plotting colony forming units (CFUs) as a function of the absorbance at 550 nm. Sterile phosphate buffered saline (PBS) (Invitrogen, Merelbeke, Belgium) was applied to further dilute the culture until the desired concentration of 2×10^4 CFU/ml. The actual number of CFU injected was confirmed by spreading the inoculum onto an agar plate and counting the colonies after overnight incubation. The number of bacteria recovered from mammary glands 24 or 48 h after inoculation was determined similarly after preparing serial dilutions of the mammary gland homogenates which were then also spread onto agar plates.

2.2. Intramammary inoculation

Ten lactating C57BL/6 mice were challenged 5 to 10 days after parturition. Mammary glands of two additional mice were used for western blot analysis and one extra female mouse was inoculated for histological studies. The control group consisted of four lactating females. In addition to the wild type animals, caspase-1 knockout mice in C57BL/6 background were also used.

The intramammary inoculation technique was based on the method described by Brouillette and Malouin (2005). The offspring was removed at the time of inoculation. Mice were anaesthetised using a mixture of ketamin (80-100 mg/kg mouse) (Eurovet, Heusden-Zolder, Belgium) and xylazin (10 mg/kg mouse) (VMD, Arendonk, Belgium). After disinfecting the teats of the fourth pair of mammary glands, found from head to tail, the very near end of the teats was cut with small scissors. A 32 G needle (Dyna Medical Corporation, London, Canada) with a blunt end was then inserted into the teat canal and a volume of 50 µl was

slowly injected. Challenged mice received 10^3 CFU of *E. coli* in the right mammary gland (R4) and an equal volume of sterile PBS (Invitrogen) in the left mammary gland (L4) while control mice were inoculated with PBS in both glands. Mice were allowed to recover in their cage. The protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium.

2.3. Clinical observations

Inoculated mice were examined twice a day for generalized and local reactions. The latter included redness of the teat, swelling of the mammary gland and the appearance of a sunken abdomen. Generalized reactions such as awareness of the environment, activity and grooming, weakness and mortality were observed as well. In addition, food and water uptake and body weight were monitored with the same frequency.

2.4. Blood sampling and plasma preparation

At 24 (n = 5 for challenged mice and n = 2 for control mice) or 48 h (n = 5 for challenged mice and n = 2 for control mice) after inoculation, mice were anaesthetised using ketamin and xylazin. Following cardiac puncture with a 26 G needle (Terumo, Medical Corporation, Leuven, Belgium), blood was slowly withdrawn into a syringe filled with approximately 50 U of heparin (LEO Pharma, Wilrijk, Belgium). Subsequently, mice were killed by cervical dislocation. Plasma was prepared by centrifugation of the blood at 2,300 g and 4 °C for 15 min.

2.5. Preparation of mammary gland homogenates

After sacrificing, mice were dissected to expose the mammary glands. The fourth pair of glands was examined for signs of infection such as swelling, discolouration, rigor and abscess formation. Mammary glands were then removed, weighed and homogenized in sterile PBS (Invitrogen) supplemented with a cocktail of protease inhibitors (Calbiochem, La Jolla, USA). To make serial dilutions for CFU determination, a small volume of the sample was used. The remaining homogenate was centrifuged at 16,000 g and 4 °C for 30 min. After removing the creamy top layer, the supernatant was frozen at -80 °C until further analysis and the cell pellet was resuspended in RIPA lysis buffer (20 mM Hepes, 150 mM sodium chloride, 2 mM

EDTA, 0.5 % sodium deoxycholate and 1 % Nonidet P-40, pH 7) with protease inhibitors (Calbiochem). After a 10 min incubation period on ice and a 10 min centrifugation step at 16,000 g and 4 °C, the supernatant was frozen as a protein lysate until further use.

2.6. Preparation of histological sections

Dissected mammary glands were fixed in 3.5 % phosphate-buffered formaldehyde (VWR International, Leuven, Belgium) immediately after necropsy. Histological paraffin sections with a thickness of 8 µm were made and stained with haematoxylin and eosin.

2.7. Serum amyloid A (SAA) quantification

SAA proteins were quantified by means of an ELISA (Tridelta Development Limited, Maynooth, Ireland) according to the manufacturer's protocol. Briefly, a 96-well plate was used with an antibody for mouse SAA coated onto the surface. Standards with a known SAA concentration and plasma samples or mammary gland supernatants were incubated for 1 h together with a secondary antibody labeled with horseradish peroxidase (HRP). After extensive washing, a HRP substrate was added and a coloured product was formed which allowed a colorimetric measurement at 450 nm.

2.8. Cytokine analysis

TNF-α was detected and quantified using a sandwich ELISA (eBioscience, San Diego, USA) according to the manufacturer's protocol. In brief, 100 µl of standards and samples were incubated overnight at 4 °C in a 96-well plate containing a capture anti-mouse antibody for TNF-α. Then, a biotin-conjugated detection antibody was added for 1 h. After incubation with avidin-bound HRP for 30 min, the addition of a HRP substrate solution allowed a colorimetric read-out at 450 nm.

IL-6 was measured in a bio-assay (Landegren, 1984) as a hybridoma growth factor for mouse 7TD1 cells. Briefly, cells were cultured for 72 h in medium with different dilutions of the samples. A colorimetric hexosaminidase enzyme reaction reflects the number of cells which is related to the amount of IL-6 in the media. Reference standards were included in the assay. Analysis was based on the half-maximal proliferation of the cells.

IL-1 was quantified in a bio-assay (Helle et al., 1988) using growth factor-dependent murine D10(N4)M cells. In brief, cells were cultured for 48 h in medium with different dilutions of the standard and samples. Addition of [^{3}H] thymidine allowed incorporation following proliferation. The scintillation counts reflect the number of cells which is correlated to the amount of IL-1. Analysis was based on the half-maximal proliferation of the cells.

2.9. Western blotting

For the detection of IL-1β and the proteolytic activation of caspase-1, mouse mammary glands were homogenized directly into lysis buffer (10 mM Tris HCl pH 7.4, 200 mM sodium chloride, 5 mM EDTA, 1mM oxidized glutathion, 10 % glycerol and 1 % NP-40) supplemented with protease inhibitors (0.15 µM aprotinin, 2.1 µM leupeptin and 100 nM phenylmethylsulphonyl fluoride; Sigma-Aldrich, Bornem, Belgium). The remaining lysate was centrifuged at 20,000 g and 4 °C for 10 min. Laemmli buffer (final concentration of 62.5 mM Tris HCl pH 6.8, 100 mM β-mercaptoethanol, 2 % sodium dodecyl sulphate, 0.1 % bromophenolblue and 10 % glycerol) was added to the supernatant after determination of the protein concentration using the Bio-Rad Protein Assay (Bio-Rad Life Science, Nazareth, Belgium), based on the method of Bradford. Samples were boiled for 15 min and loaded onto a polyacrylamide gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, ‘s-Hertogenbosch, The Netherlands) by semi-dry blotting. Membranes were blocked and then incubated with a primary antibody against IL-1β (R&D Systems, Abingdon, United Kingdom) and caspase-1 (Centre d’Economie Rurale, Marloie, Belgium). The anti-β-actin antibody was purchased from ICN Biomedicals (Costa Mesa, USA). A secondary antibody conjugated to HRP was applied and immuno-reactive proteins were visualised using chemiluminescence reagent (PerkinElmer, Massachusetts, USA).

2.10. NF-κB activation assay

For the detection and quantification of the NF-κB p65 and p50 subunit activities in the RIPA protein lysates of the mouse mammary gland homogenates, the TransAM^{TM} Transcription Factor Assay Kits (Active Motif, Rixensart, Belgium) were used according to the manufacturer’s protocol. Beforehand, protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Life Science). For each sample, 40 µg was incubated for 1 h in a 96-well plate containing immobilized NF-κB consensus oligonucleotide. After extensive

washing, the NF-κB complex bound to the oligonucleotide was identified using the supplied anti-p65 antibody. For the detection of activated p50, an alternative anti-p50 antibody (sc-8414) (Santa Cruz Biotechnology, Heidelberg, Germany) was used since the antibody provided by Active Motif is not known to recognize the mouse protein. For both antibodies used, reactivity towards mouse NF-κB was tested with mouse cell-extracts (Active Motif) before further analysis (data not shown). Addition of a secondary antibody conjugated to HRP allowed a chemiluminescent readout (Fluoroskan Ascent FL, Thermo Labsystems) expressed in relative light unit per second (RLU/s).

2.11. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Comparisons between the control (left, L4) and infected (right, R4) mammary gland samples were made using the non-parametric Mann-Whitney U test. Differences between challenged and control mice and mice killed at 24 or 48 h post-infection were analysed similarly. Changes in body weight were statistically examined by the non-parametric Wilcoxon matched pairs test. The correlation studies were conducted according to Spearman. The SPSS Software (SPSS Belux, Brussels, Belgium) was used for all analyses.

3. Results

3.1. Clinical and post-mortem observations

Following challenge with 10^3 CFU of *E. coli*, mice showed minor indications of illness at 12 h post-inoculation. Obvious signs of infection became apparent at 24 h after injection: animals were inactive and weak, some mice had a hunched back and a ruffled coat, many were not able to walk on their right hind leg. Food and water uptake were diminished and a concomitant loss in body weight was observed (on average 14.3 ± 2.1 %, n = 9, P < 0.01). Macroscopically, the inoculated mammary glands (R4) were swollen, whereas the control glands (L4) showed a normal appearance. Most of these symptoms continued to be visible at 48 h after inoculation. One mouse was excluded from subsequent analyses since no disease symptoms were evident. Clinical signs of infection were also absent in the control group of mice at 24 or 48 h post-inoculation.

Upon necropsy at either 24 (n = 4) or 48 h (n = 5) post-inoculation, R4 glands were enlarged, hardened and darkened or even bloody in appearance. L4 glands were normal in size, smooth and coloured like normal lactating glands (Figure 1). When isolated, infected glands also weighed significantly more than control glands with an average difference between R4 and L4 of 201.2 ± 22.3 mg (n = 9, P < 0.01). Control mice had two normal appearing lactating glands which were not significantly different in weight.

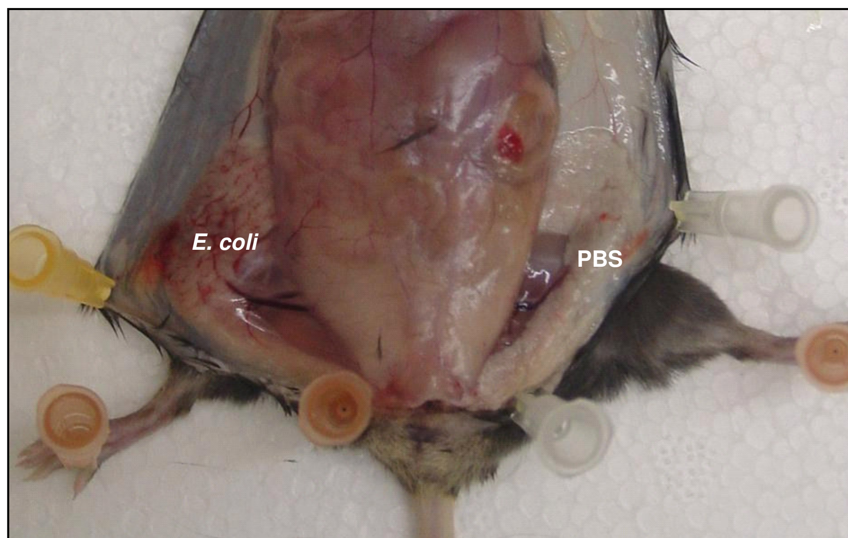

Figure 1: Macroscopic appearance of the fourth pair of mammary glands of a representative mouse upon necropsy 24 h after challenge with E. coli in R4 and PBS in L4.

3.2. Bacteriology

Both at 24 and 48 h post-inoculation, significantly large numbers of bacteria were recovered from the mammary glands inoculated with *E. coli*, with on average 3.3 ± 1.2 x 10^9 CFU/gland (n = 9). None of the control gland homogenates revealed bacterial growth after overnight incubation, nor did the homogenates of the control group of mice.

3.3. Histology

The mammary glands of one additional challenged mouse were utilized at 48 h post-inoculation for histological analysis. Microscopically, massive neutrophil infiltration surrounding and inside the alveoli was seen throughout the infected gland (R4) together with cell fragments possibly due to apoptosis (Figure 2B). In marked contrast, strong dilated alveoli filled with milk protein and lipid droplets and the presence of only a few immune cells characterized the control gland (L4) (Figure 2A).

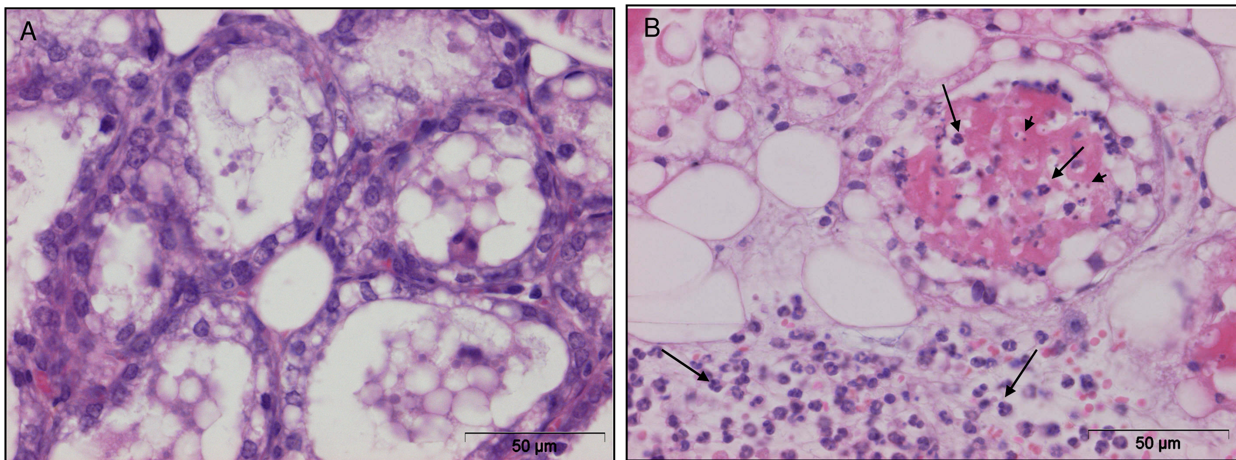

Figure 2: Microscopic images of mammary glands of a representative mouse at 48 h of infection (magnification: 600 x): panel A represents the control gland and panel B shows the infected gland. Arrows indicate infiltrated neutrophils and cell death fragments are marked by arrow heads.

3.4. Inflammatory response

3.4.1. Acute phase protein amyloid A

The acute phase protein SAA was quantified in plasma samples and in the mammary gland homogenate supernatants of the infected mice at 24 (n = 4) and 48 h (n = 5) post-inoculation. Systemic concentrations were also determined in the control group. For all challenged mice, very high concentrations were detected in plasma (on average 9.8 ± 2.9 mg/ml, n = 9) in comparison with values for control mice (89.0 ± 43.1 µg/ml, n = 4). Low levels were also observed in all supernatants (on average 0.9 ± 0.1 µg/ml for R4 and 1.1 ± 0.1 µg/ml for L4, n = 9). Consequently, no significant difference in local concentrations between the infected and the control glands was visible.

3.4.2. Cytokines

Plasma samples and mammary gland homogenate supernatants were also analysed for the presence of cytokines. In challenged mice, TNF-α was detected in plasma at an average concentration of 70.1 ± 22.1 pg/ml (n = 9) while this cytokine was nearly absent in blood of control mice (3.3 ± 3.3 pg/ml, n = 4). Furthermore, TNF-α levels were significantly increased in the supernatants of infected glands as compared to those of control glands (on average 9.1 ± 2.1 ng/ml for R4 and 0.9 ± 0.1 ng/ml for L4, n = 9, $P < 0.01$).

Regarding IL-6, elevated concentrations (on average 1.6 ± 0.7 ng/ml, n = 9) were measured in all plasma samples of challenged mice as compared to control mice (below detection limit, n = 4). In addition, high concentrations were detected in the R4 mammary glands (on average 103.6 ± 34.7 ng/ml, n = 9), while the L4 glands did not contain detectable amounts of this cytokine (n = 9). A significant correlation was obtained between IL-6 levels in plasma and R4 glands of infected animals ($r = 0.803$, $P < 0.01$).

IL-1 concentrations in plasma of challenged (n = 9) and control mice (n = 4) were undetectable. Levels were significantly increased in the supernatants of infected glands as compared to those of control glands (24.3 ± 5.4 ng/ml for R4 and below detection limit for L4, n = 9, $P < 0.01$).

3.4.3. Cytokine processing

Protein lysates of infected and control mammary glands of two mice killed at 24 h post-inoculation were used to determine the expression levels of pro IL-1β. In contrast to the control glands, the infected glands showed high amounts of pro IL-1β and also small quantities of the biologically active mature cytokine (Figure 3A). To investigate the mechanism of IL-1β maturation, protein lysates were examined for the presence of the p20 and p10 active subforms of caspase-1. As only pro caspase-1 was detected, no activation could be demonstrated in the challenged glands (Figure 3B). Interestingly, increased expression levels of pro IL-1β and its mature form were also observed in caspase-1 knockout mice following intramammary inoculation with *E. coli* (Figure 3B).

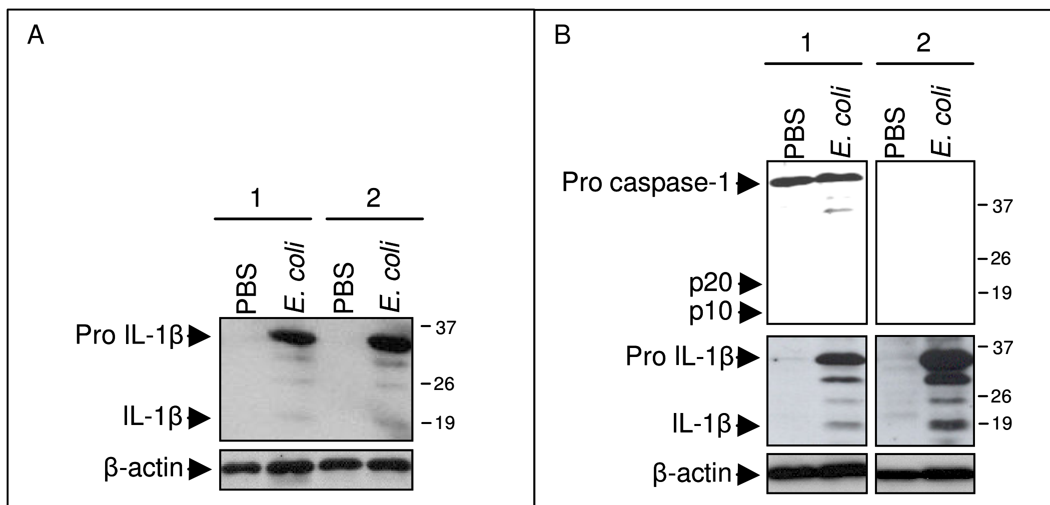

Figure 3: Expression of the proform and the mature form of IL-1β (panel A) and the proform of caspase-1 (panel B) in the fourth pair of mammary glands following inoculation with E. coli or PBS. Both panels show results of two representative mice at 24 h post-infection. All animals are wild type mice except mouse number two of panel B, which is a representative caspase-1 knockout mouse. β-Actin protein levels were determined to provide a loading control.

3.5. NF-κB activation

NF-κB p65 and p50 activities were measured in the RIPA protein lysates of the fourth pair of mammary glands of infected mice isolated at necropsy. As shown in figure 4, both NF-κB

proteins were significantly more activated in the infected mammary glands than in the control glands (n = 9, $P < 0.01$ for p65 and $P < 0.05$ for p50).

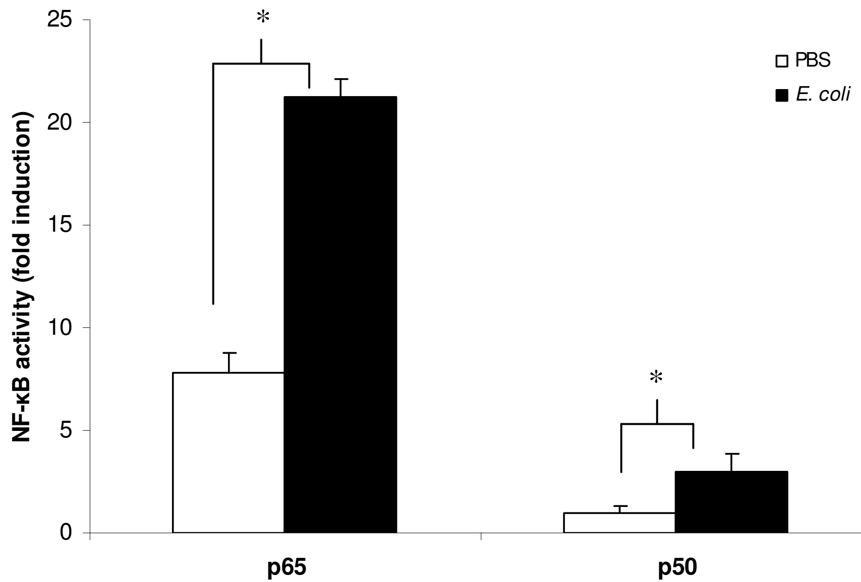

Figure 4: NF-κB p65 and p50 activities in the infected and control mammary glands. Data are means ± SEM of 9 mice. Statistically significant differences are indicated with an asterisk.

3.6. Kinetics of inflammation

Changes in parameters of challenged mice sacrificed at 24 h (n = 4) were compared to those of mice killed 48 h post-inoculation (n = 5). Loss in body weight and increases in bacterial counts, cytokine concentrations and NF-κB activity in the infected glands in comparison with the control glands, were not significantly different between mice killed at the two different time points.

4. Discussion

The mouse model of induced mastitis is commonly used as a practical approach for the study of bovine intramammary infections (Brouillette and Malouin, 2005; Notebaert and Meyer, 2006). *S. aureus* is most frequently inoculated in the mouse mammary gland, while *E. coli* is relatively rarely used in the mouse model, even though this agent causes severe clinical mastitis in cattle, making it an important and well-studied disease in cows (Burvenich et al., 2003). In addition to clinical signs of illness, inflammation is generally characterized by high bacterial counts, increased neutrophil numbers and histological changes. In the present study, these features were also examined following inoculation of the R4 mammary gland of lactating mice with *E. coli*. From 24 h after bacterial inoculation on, challenged mice showed obvious disease symptoms such as the appearance of a hunched back and a ruffled coat, weakness and inactivity, loss of appetite and a concomitant decrease in body weight. These severe clinical signs persisted at least up to 48 h post-infection. At necropsy, clear indications of inflammation such as swelling, rigor and discolouration were observed in the inoculated mammary gland. Our bacteriology data demonstrated the presence of high numbers of *E. coli*, while massive neutrophil infiltration additionally confirmed the successful induction of acute mastitis in mice.

Our major objective was to characterize the host inflammatory response during the acute phase reaction of intramammary infections in mice caused by *E. coli*. First, we demonstrated high plasma concentrations of SAA, a major positive acute phase protein in mice, at 24 and 48 h post-infection. It is well documented that the hepatic biosynthesis of this protein is dramatically up-regulated during inflammation, mainly in response to pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Uhlar and Whitehead, 1999). SAA is involved in repair processes minimizing tissue damage and thereby permitting rapid restoration of normal physiological function (Jensen and Whitehead, 1998). SAA has never been studied in a mouse model of mastitis. Still, in other mouse infection models, SAA concentrations exceeding 1 mg/ml are frequently observed (Uhlar and Whitehead, 1999; Cho et al., 2004; Martinez et al., 2005). In addition, large increases of SAA concentrations in blood following experimentally induced or naturally occurring *E. coli* intramammary infections in cattle have been published (Pyörälä, 2003; Petersen et al., 2004).

Even though the liver is the major site of acute phase protein synthesis, extra-hepatic production of these inflammatory mediators is documented as well (Uhlar and Whitehead, 1999). For cattle, it has been suggested that SAA may be produced locally within the mammary gland and should therefore be called milk amyloid A (MAA) (Eckersall et al., 2001). Alternatively, increased amyloid A levels in the bovine gland may also result from leakage of SAA through the blood-milk barrier due to its increased permeability (Jacobsen et al., 2005). Our data in mice revealed no significant difference in the amyloid A concentrations from the *E. coli* inoculated mammary gland as compared to the control gland. This observation does not corroborate previous observations in cows, where milk of infected quarters contained higher levels of MAA than milk of uninfected quarters (Lehtolainen et al., 2004; Nielsen et al., 2004; Jacobsen et al., 2005). Furthermore, in mouse mammary glands receiving LPS, a major up-regulation in mRNA expression for amyloid A was observed (Zheng et al., 2006). A possible explanation for these apparently contrasting data lies within the reactivity of the antibody used. In contrast to the anti-bovine antibody, the anti-mouse SAA antibody of the ELISA kit probably does not cross react with the locally produced amyloid A isoform. Detection of mouse MAA at the protein level therefore remains to be demonstrated.

Secondly, major inflammatory cytokines such as TNF-α, IL-1 and IL-6 were studied at the local and systemic level in the *E. coli* mouse mastitis model. The concentrations of TNF-α and IL-6 were increased in blood at 24 and 48 h post-inoculation as compared to control mice. Negligible values were obtained for unchallenged mammary glands whereas both inflammatory mediators were clearly present in infected glands. For IL-1, no increase in blood levels was observed but bacterial inoculation also led to high concentrations in the mammary gland. Cytokines are produced and released by immune cells at the *E. coli* infection site following activation by LPS or other inflammatory mediators. Our histological data confirmed the attendance of neutrophils in the infected gland. In addition, leakage of these molecules into circulation might elicit the observed acute phase response. In accordance with our results, one group has previously shown elevated TNF-α levels in *E. coli* infected glands of mice (Lee et al., 2003). Following intramammary challenge with LPS, mRNA levels of the interleukins were also up-regulated (Zheng et al., 2006). No reports on systemic concentrations in mastitic mice are available so far. Still, values within the same order of magnitude were obtained for the selected cytokines in other mouse infection models (Abram

et al., 2000). In addition, similar observations were made in studies on bovine mastitis (Nakajima et al., 1997; Hagiwara et al., 2001; Bannerman et al., 2004; Lee et al., 2006).

Of the cytokines analysed in the current study, only IL-1 is known to be expressed as a biologically inactive proform. Significantly increased local levels of this protein were demonstrated following bacterial inoculation. It is well accepted that the generation of mature IL-1β requires the activity of ICE or caspase-1 (Thornberry and Molineaux, 1995). Caspases are expressed as inactive zymogens which are cleaved upon activation into p20 and p10 subunits thereby forming a catalytic tetramer (Shi, 2004). In the infected mouse mammary glands, caspase-1 activation could not be demonstrated. Our results therefore do not corroborate those obtained for bovine mammary glands inoculated with *E. coli* at the transcription level (Long et al., 2001). Still, elevated mRNA levels of ICE do not necessarily implicate higher activity at the protein level. Since caspase-1 knockout mice also showed high levels of pro IL-1β and maturation to the active cytokine, a caspase-1 independent processing of the proform is apparent. Cleavage of pro IL-1β by other enzymes than ICE, such as neutrophil elastase and cathepsin, has been reported (Black et al., 1991; Coeshott et al., 1999). In addition, caspase-1 independent IL-1β maturation has been suggested in other infection models in mice (Rowe et al., 2002).

Finally, in the mouse mammary gland, activation of NF-κB was demonstrated through its most important family members p65 and p50 following inoculation with *E. coli*. Increased NF-κB activity was also demonstrated in isolated milk cells of cows infected with other mastitis pathogens (Boulanger et al., 2003). Pathogen perception leads to activation of this transcription factor via the well-known Toll-like receptors (TLRs), of which TLR4 recognizes the LPS cell wall component of *E. coli* (Akira and Takeda, 2004). Next to pathogen recognition, activation of NF-κB is also triggered by pathogen engulfment or phagocytosis (McDonald and Cassatella, 1997; Vollebregt et al., 1998). In addition, active complexes are found in immune cells exposed to pro-inflammatory mediators released at the infection site (McDonald et al., 1997; Hotta et al., 2001). In the context of inflammation, NF-κB regulates gene expression of cytokines and chemokines, immunoreceptors and cell adhesion molecules, acute phase and stress-related proteins and apoptosis regulators (Pahl, 1999). In our interest, the transcription of TNF-α and IL-6 are under control of NF-κB. Elevated cytokine levels observed in infected glands can therefore be explained by locally increased NF-κB activity. Furthermore, the synthesis of the inactive precursor of IL-1β requires NF-κB activity. The

elevated IL-1 concentrations following inoculation with *E. coli* are thus explained by both an increase in expression of the precursor by NF-κB as well as an enhanced cleavage by other enzymes than caspase-1.

In conclusion, we characterized the host immune response during *E. coli* intramammary infection in mice by providing new information on the participation of acute phase proteins, cytokines, inflammatory enzymes and transcription factors in this mouse model of inflammation. These key proteins can serve as markers which can easily be monitored in future mouse mastitis studies evaluating treatments with antimicrobial or novel immunomodulatory compounds. Still, our study also reveals potential differences between mouse and cow, thereby emphasizing that the extrapolation of findings in mice to other species should be made with caution. However, by using genetically modified mice, we have demonstrated the great potential of the rodent model to elucidate molecular mechanisms and cellular processes involved in the host immune response towards mammary gland infections.

Acknowledgments

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CHAPTER 4

IN VIVO IMAGING OF NF-κB ACTIVITY IN INTRAMAMMARY *E. COLI* INFECTIONS

Adapted from

Notebaert S., Carlsen H., Janssen D., Vandenabeele P., Blomhoff R. and Meyer E. *In vivo* imaging of NF-κB activity during *Escherichia coli*-induced mammary gland infection. Cellular Microbiology (2008) In press.

Abstract

In mammary gland infections, the contribution of nuclear factor-κB (NF-κB) is not well defined and was therefore investigated following intramammary inoculation of *Escherichia coli* (*E. coli*). Non-invasive real-time *in vivo* imaging of the transcription factor activation was performed in mammary glands of transgenic mice expressing luciferase under the control of NF-κB. Bacterial inoculation resulted in a major increase in luminescence as compared to control glands. This activation was confirmed by immunohistochemical nuclear staining of the NF-κB p65 subunit in mammary epithelium of infected glands, while nuclear p50 was not detected. The systemic response to the intramammary inoculation of *E. coli* was also studied. NF-κB activation in the liver increased over time and a relatively mild but longer lasting response was observed as compared to the acute hepatic response of mice receiving lipopolysaccharide (LPS). This systemic reaction was confirmed by increased circulating levels of the acute phase protein serum amyloid A (SAA), tumor necrosis factor (TNF)-α and interleukin (IL)-6. In addition, high concentrations of both cytokines in the mammary gland inoculated with bacteria showed that the infection was also well established at the local level. These results indicate that *in vivo* monitoring of NF-κB activation is an attractive novel approach to study mammary gland inflammation and that this transcription factor is imperative in the early stages of the host immune response towards coliform intramammary infections, both at the local and systemic level.

1. Introduction

Immune and inflammatory responses are often orchestrated by the transcription factor NF-κB (Li and Verma, 2002). In resting cells, NF-κB family members are sequestered in the cytoplasm, bound to their inhibitors, which belong to the IκB family. Upon cell activation, IκB proteins become phosphorylated, ubiquitinated and degraded by the proteasome. Subsequently, NF-κB can enter the nucleus and bind to the DNA in order to promote transcription (Baeuerle and Baltimore, 1996). NF-κB can be activated by many distinct stimuli, including pro-inflammatory cytokines, bacteria and bacterial toxins such as LPS. NF-κB alters the transcription of a large number of target genes of which the proteins are involved in host response processes to foreign challenge or tissue injury (Pahl, 1999).

Mastitis is defined as an inflammation of the mammary gland mainly caused by microbial pathogens, such as *Staphylococcus aureus* (*S. aureus*) and *E. coli* (Watts, 1988). In breastfeeding women, mastitis is quite common (Fetherston, 2001) and in the dairy industry, intramammary infections are of great economical importance (Hillerton and Berry, 2005). Consequently, the control and pathogenesis of this infectious disease has been studied intensively. However, molecular studies regarding the role of NF-κB in mastitis are scarce. Only a few *in vitro* studies demonstrate NF-κB activation in murine or bovine mammary gland cells following incubation with *E. coli*, LPS or TNF-α (Safieh-Garabedian et al., 2004; Strandberg et al., 2005; Szperka et al., 2006; Yang et al., 2006; Fitzgerald et al., 2007). In addition, isolated milk cells of cows with mastitis mainly caused by *S. aureus*, contained increased levels of activated NF-κB (Boulanger et al., 2003). These active NF-κB complexes frequently consisted of the p65 and p50 subunits, which are considered to be the most relevant NF-κB family members.

To assess NF-κB activity, various methods have been used covering the measurement of mRNA levels of genes containing NF-κB binding elements in their promotor or *in vitro* reporter gene expression under the control of NF-κB, the determination of free NF-κB complexes able to bind a specific oligonucleotide using the classical electrophoretic mobility shift assay (EMSA) or enzyme-linked immunosorbent assay (ELISA)-based methods, and the quantification of phosphorylated proteins in nuclear extracts by western blot or immunohistochemical analysis (Carlsen et al., 2004).

For the first time, real-time *in vivo* imaging of the NF-κB activity was performed in mammary glands of intact animals following challenge with *E. coli* bacteria. Transgenic mice expressing luciferase under the control of NF-κB were used for this purpose. This novel approach to detect mammary gland NF-κB activity *in vivo* allowed us to investigate the kinetics of inflammation during bacterial challenge. The results thus obtained underline the importance of this transcription factor in the pathogenesis of *E. coli* induced mammary gland infections.

2. Experimental procedures

2.1. Bacteria

For the intramammary inoculation, *E. coli* strain P4:O32 was used as previously described (Notebaert et al., 2008). Bacteria were grown overnight at 37 °C in Brain Heart Infusion medium (Oxoid Limited, Hampshire, United Kingdom). To verify their purity, bacteria were streaked onto a blood agar plate (Oxoid Limited). Bacterial concentration of the culture was determined using a standard curve plotting colony forming units (CFUs) as a function of the absorbance at 550 nm. Sterile phosphate buffered saline (PBS) (Invitrogen, Merelbeke, Belgium) was applied to further dilute the culture until the desired concentration of 2×10^5 CFU/ml. The actual number of CFU injected was $2.2 \pm 0.6 \times 10^4$ as determined by spreading the inoculum onto a blood agar plate and counting the colonies after overnight incubation.

2.2. Intramammary inoculation of *E. coli* in transgenic mice

All animal experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium. The 3x-κB-luc transgenic mice were generated as previously described (Carlsen et al., 2002). Mice were housed in a controlled light, humidity and temperature environment and were provided food and water *ad libitum*. Female mice were challenged 5 to 10 days after parturition. The intramammary inoculation technique was carried out as previously described for wild type mice (Notebaert et al., 2008). In brief, mice were anaesthetised using a mixture of ketamin (80-100 mg/kg mouse) (Eurovet, Heusden-Zolder, Belgium) and xylazin (10 mg/kg mouse) (VMD, Arendonk, Belgium). After disinfecting the teats of the fourth pair of mammary glands, the very near end of the teats was cut with small scissors. A 32 G needle with a blunt end was then inserted into the teat canal and a volume of 50 µl was slowly injected. Challenged mice received 10^4 CFU *E. coli* in the right fourth mammary gland (R4) and an equal volume of sterile PBS in the left fourth mammary gland (L4). As an alternative, mice received LPS from *E. coli* (5 µg in 50 µl; Sigma-Aldrich, Bornem, Belgium) in the right mammary gland. The offspring was removed before mice returned to their cage to recover.

2.3. *In vivo* and *ex vivo* imaging of NF-κB activity

Before imaging, mice were anaesthetised by inhalation of isoflurane (Abbott Laboratories, Kent, United Kingdom) and shaved on their ventral and lateral sides. Mice were injected intraperitoneally with D-luciferin (Biosynth, Staad, Switzerland; 120 mg/kg dissolved in PBS, pH 7.4) (Figure 1B) and placed in a light-sealed imaging chamber connected to an ultra sensitive CCD camera of the IVIS imaging device (Caliper Life Sciences, Hopkinton, USA) (Figure 1A and C). Gray scale images were obtained before luminescence imaging to localize the fourth pair of mammary glands. A small fraction of the gland is observed at the ventral side of the body while the largest part is visible at the lateral sides. Luminescence was recorded 5 min after the injection of D-luciferin. Exposed or excised mammary glands were imaged in the same way immediately after dissection. Images were analysed with the Living Image software (Caliper Life Sciences).

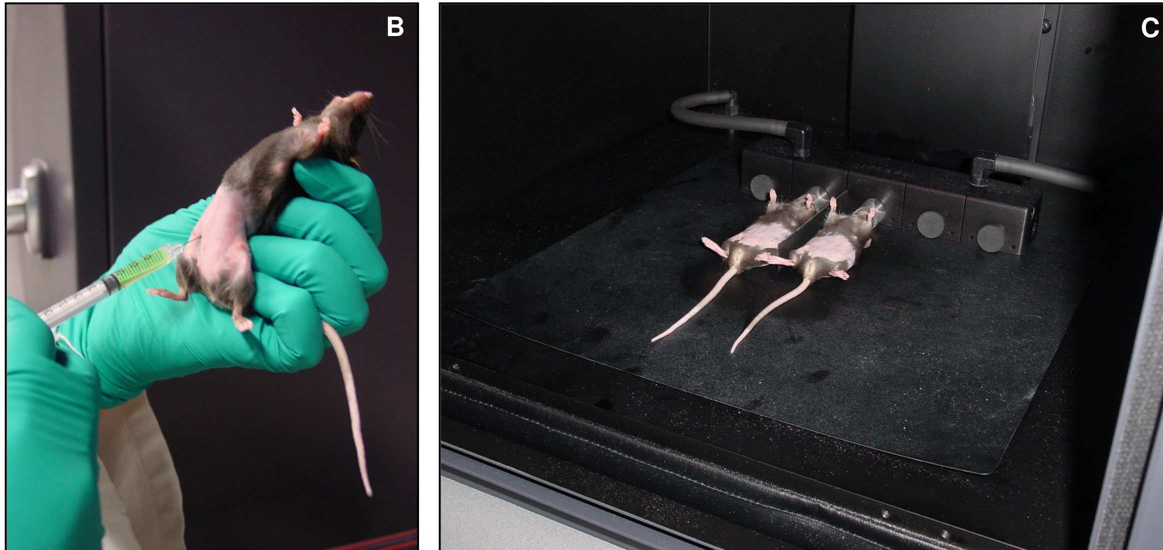

Figure 1A: The IVIS imaging device of Caliper Life Sciences, B: The intraperitoneal injection of D-luciferin in an anaesthetised mouse before imaging and C: Anaesthetised mice in the light-sealed imaging chamber of the device.

2.4. Immunohistochemical analysis of NF-κB activity

Excised mammary glands were fixed in 3.5 % phosphate-buffered formaldehyde (VWR International, Leuven, Belgium) immediately after imaging. Histological paraffin sections with a thickness of 5 µm were prepared. After deparaffinization and hydration, antigens were unmasked using a pressure cooker and a microwave oven. Endogenous peroxidase activity was quenched with hydrogen peroxide in methanol for 5 min. After rinsing in Tris buffered saline (TBS), non-specific binding sites were blocked by goat serum for 30 min. The sections were then incubated overnight at 4 °C with rabbit anti-p65 (sc-372; Santa Cruz, Heidelberg, Germany) or rabbit anti-p105/p50 (ab7971; Abcam, Cambridge, United Kingdom) diluted in TBS with bovine serum albumin. The samples were washed with TBS and then incubated with a biotinylated goat anti-rabbit secondary antibody (Dako, Heverlee, Belgium) for 1 h. After another wash, a mixture of avidin and biotinylated horseradish peroxidase (HRP) or streptavidin coupled to HRP (both from Dako) was used for the detection of p65 and p105/p50 respectively. Subsequently, the samples were stained with diaminobenzidine solution (Dako) for 5 min and rinsed with distilled water. After counterstaining with hematoxylin, the slides were again washed, dehydrated and mounted. Inactive NF-κB

complexes were visualized by brown staining of the cytoplasm while brown nuclei reflected active complexes.

2.5. Clinical and post-mortem observations

Following intramammary inoculation, mice were examined for generalized and local reactions. The latter included redness of the teat, swelling of the mammary gland and the appearance of a sunken abdomen. Generalized reactions such as awareness of the environment, activity and grooming, weakness and mortality were observed as well. In addition, food and water uptake and body weight were monitored with the same frequency. After sacrificing, mice were dissected to expose their mammary glands. The fourth pair of glands was examined for signs of infection such as swelling, discolouration, rigor and abscess formation.

2.6. Blood sampling and plasma preparation

Mice were anaesthetised using ketamin and xylazin. Following cardiac puncture with a 26 G needle (Terumo, Medical Corporation, Leuven, Belgium), blood was slowly withdrawn into a syringe filled with approximately 50 U of heparin (LEO Pharma, Wilrijk, Belgium). Subsequently, mice were killed by cervical dislocation. Plasma was prepared by centrifugation of the blood at 2,300 g and 4 °C for 15 min.

2.7. Preparation of mammary gland samples

After sacrificing, mice were dissected and mammary glands were removed, weighed and homogenized in PBS (Invitrogen) supplemented with a cocktail of protease inhibitors (Calbiochem, La Jolla, USA). The homogenate was then centrifuged at 16,000 g and 4 °C for 30 min. After removing the creamy top layer, the supernatant was frozen at -80 °C until further analysis.

2.8. Serum amyloid A quantification

SAA proteins were quantified by means of an ELISA (Tridelta Development Limited, Maynooth, Ireland) according to the manufacturer's protocol. Briefly, a 96-well plate coated

with an anti-mouse SAA was used. Standards with a known SAA concentration and plasma samples were incubated for 1 h together with a secondary antibody labeled with HRP. After extensive washing, an HRP substrate was added and a coloured product was formed which allowed a colorimetric measurement at 450 nm.

2.9. Cytokine analysis

TNF-α was detected and quantified using a sandwich ELISA (eBioscience, San Diego, USA) according to the manufacturer's protocol. In brief, 100 µl of standards and plasma and mammary gland samples were incubated overnight at 4 °C in a 96-well plate containing a capture anti-mouse antibody for TNF-α. Then, a biotin-conjugated detection antibody was added for 1 h. After incubation with avidin-bound HRP for 30 min, the addition of a HRP substrate solution allowed a colorimetric read-out at 450 nm.

IL-6 was measured in a bio-assay (Landegren, 1984) as a hybridoma growth factor for mouse 7TD1 cells. Briefly, cells were cultured for 72 h in medium with different dilutions of the plasma and mammary gland samples. A colorimetric hexosaminidase enzyme reaction reflects the number of cells which is related to the amount of IL-6 in the media. Reference standards were included in the assay. Analysis was based on the half-maximal proliferation of the cells.

2.10. Statistical analyses

Data are expressed as the mean ± SEM. By using the non-parametric Wilcoxon matched pairs test, comparisons between the *E. coli* inoculated gland (R4) and the control gland receiving PBS (L4) were made and changes in weight were analysed. Differences between challenged and control mice were statistically examined by the non-parametric Mann-Whitney U test. The SPSS Software (SPSS Belux, Brussels, Belgium) was used for all analyses.

3. Results

3.1. *In vivo* NF-κB activity during intramammary *E. coli* challenge

3.1.1. Time course in mouse mammary glands

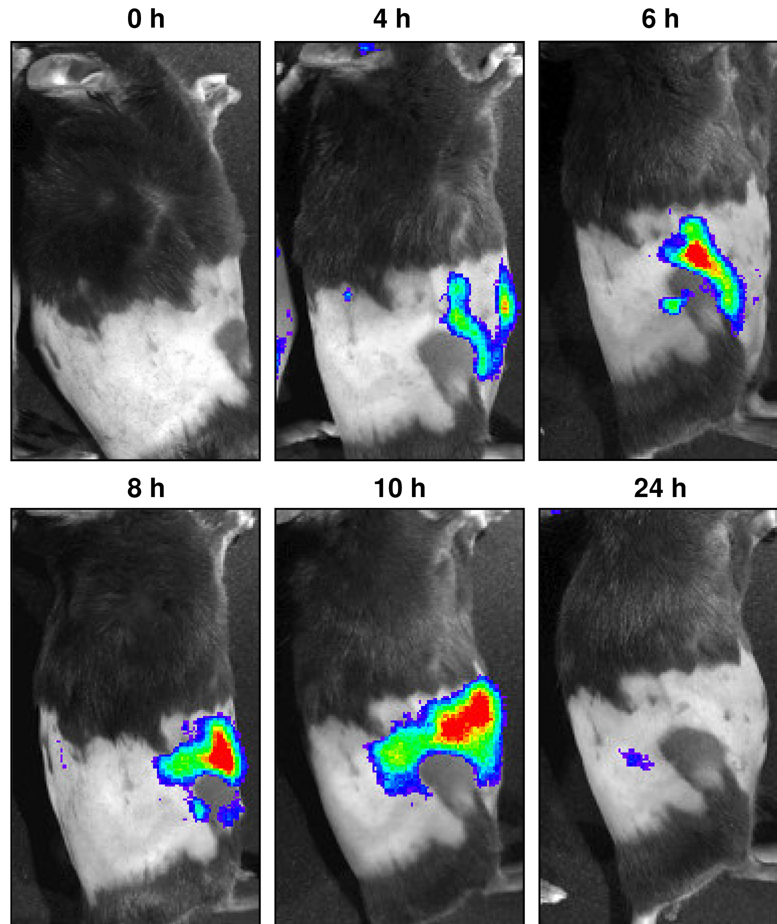

Figure 2: In vivo imaging of NF-κB activity following intramammary inoculation. Transgenic mice received 10^4 CFU E. coli in the R4 mammary gland and were then positioned on their left lateral side to image luciferase activity at different time points (0, 4, 6, 8, 10 and 24 h) following challenge. A transient increase in luminescence was located in the infected gland. Images are representative for all challenged mice.

To study the intramammary kinetics of NF-κB activation, 6 transgenic female mice were challenged with 10^4 CFU *E. coli* in the right fourth mammary gland (R4) and PBS in the left fourth mammary gland (L4). Inoculated mice were imaged at the ventral and lateral sides at different time points during the course of infection (0, 4, 6, 8, 10 and 24 h post-inoculation). At the time of inoculation, low activities were found in both the right and the left mammary

gland. At later time points, luminescence of glands challenged with *E. coli* was significantly increased as compared to control glands receiving PBS ($P < 0.05$ for 4, 6, 8 and 10 h post-inoculation, n = 6). Maximal activities were seen either at 6 or at 8 h post-inoculation. Luminescence recorded in ventral view was higher than the activity observed at the lateral sides of the body. At 24 h following inoculation, NF-κB activity of the R4 glands nearly regained basal levels, thereby approaching the activity of the L4 control glands (Figure 2 and Figure 3A and B).

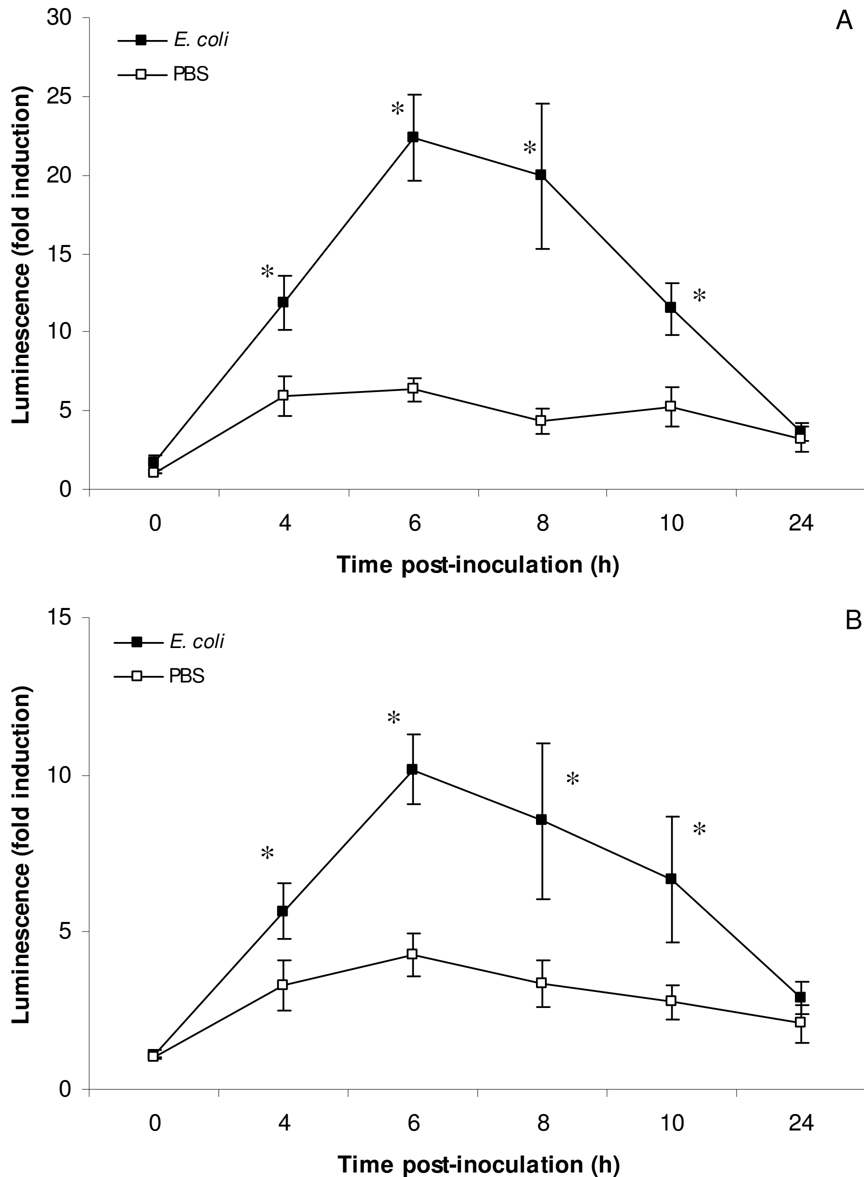

Figure 3: Average luminescence obtained during in vivo imaging of NF-κB activity of the mammary glands following intramammary inoculation. Six transgenic mice received 10^4 CFU E. coli in the R4 mammary gland and PBS in the L4 mammary gland. They were positioned dorsally (panel A) and on their lateral sides (panel B) to image luciferase activity at different time points (0, 4, 6, 8, 10 and 24 h) following challenge. Luminescence of the infected gland was transiently increased as compared to the control gland. An asterisk indicates the significant difference between both glands.

3.1.2. Time course in the liver

To study the reaction of the liver towards bacterial inoculation, 2 mice were challenged with 10^4 CFU *E. coli* and chemiluminescence originated in the liver was determined at the indicated time points. As an alternative, 2 additional mice received 5 µg LPS in the R4 mammary gland. At the time of inoculation, low NF-κB activities were found in the liver of both groups of mice. From 4 h post-inoculation on, luminescence increased for all mice. Mice challenged with LPS showed a stronger response than mice receiving *E. coli*. Inoculation with endotoxin resulted in a maximal liver chemiluminescence at 6 h post-inoculation, returning to basal levels at 24 h (Figure 4). NF-κB activity following bacterial challenge reached maximal levels at 8 h post-inoculation and remained high up to 24 h post-inoculation (Figure 5).

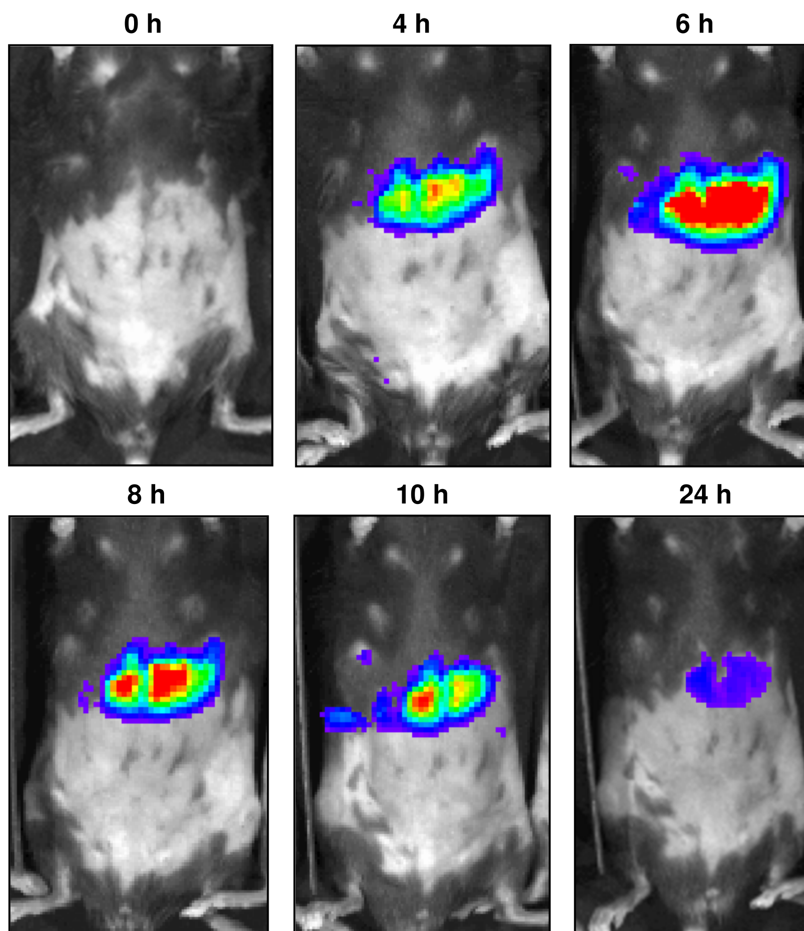

Figure 4: In vivo imaging of NF-κB activity following intramammary inoculation. Transgenic mice received 5 µg of LPS in the R4 mammary gland and were then positioned dorsally to image luciferase activity at different time points (0, 4, 6, 8, 10 and 24 h) following challenge. A transient increase in luminescence was located in the liver. Images are representative for all challenged mice.

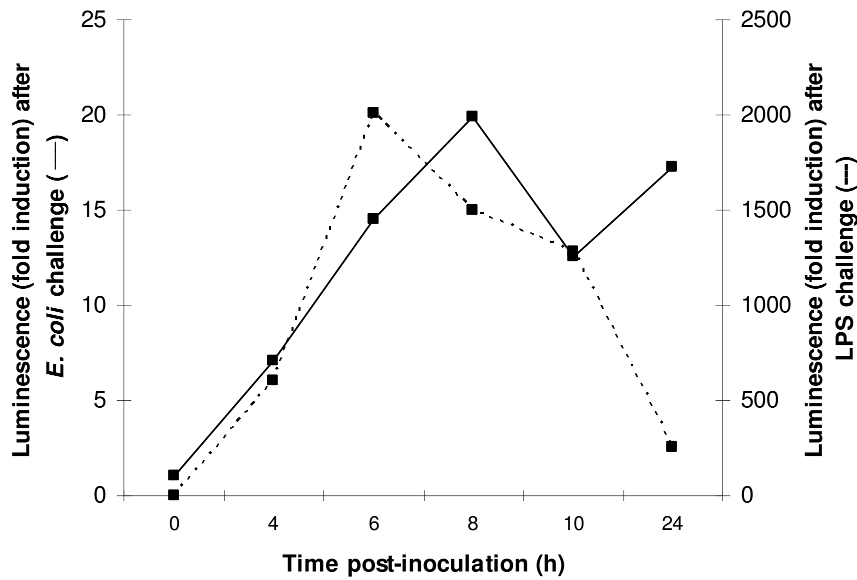

Figure 5: Average luminescence obtained during in vivo imaging of NF-κB activity of the liver following intramammary inoculation. Two transgenic mice received 10^4 CFU E. coli in the R4 mammary gland (solid line) while two other transgenic mice were inoculated with 5 µg of LPS (dashed line). They were positioned dorsally to image luciferase activity at different time points (0, 4, 6, 8, 10 and 24 h) following challenge. A very high and transient activity was seen in the liver following LPS challenge while a more modest and longer lasting increase was apparent after inoculation of E. coli.

3.2. *Ex vivo* NF-κB activity during intramammary *E. coli* challenge

3.2.1. Chemiluminescence in mouse mammary glands

In order to evaluate the NF-κB activity in exposed and isolated mammary glands following *E. coli* challenge, 6 transgenic female mice were inoculated and *in vivo* luminescence was followed over time until maximal levels of activity were reached. Similar observations as the time course in figure 3 were made up to 8 h post-inoculation. At that time point, glands challenged with bacteria showed significantly higher NF-κB activities as compared to control glands upon necropsy or excision ($P < 0.05$, $n = 6$) (Figure 6A and B).

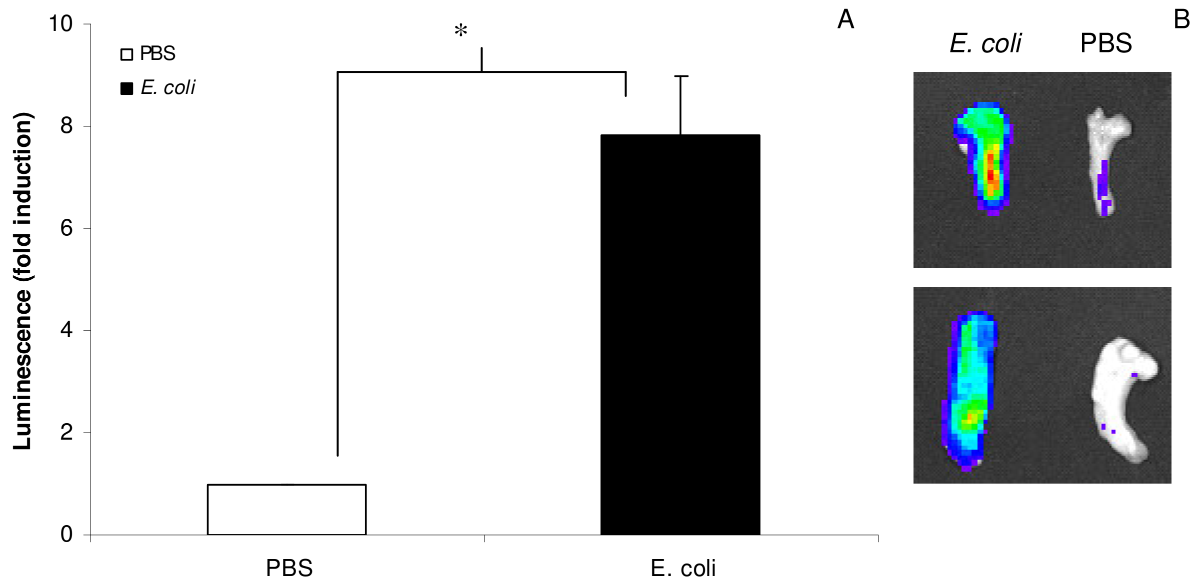

Figure 6: Ex vivo imaging of NF-κB activity following intramammary inoculation. Six transgenic mice received 10^4 CFU E. coli in the R4 mammary gland and PBS in the L4 mammary gland. At 8 h post-inoculation, they were sacrificed and dissected to expose their mammary glands. A: Average luminescence obtained during ex vivo imaging of NF-κB activity of the mammary glands upon necropsy. Mice were positioned dorsally to image luciferase activity following challenge. Luminescence of the infected gland was increased as compared to the control gland. An asterisk indicates the significant difference between both glands ($P < 0.05$). B: Ex vivo imaging of NF-κB activity of two representative pairs of mammary glands upon excision. Luminescence of the infected gland was increased as compared to the control gland.

3.2.2. Immunohistochemical analysis of p65 and p50 subunits in mouse mammary glands

In order to identify cells with activated NF-κB, sections of mammary glands isolated at 8 h post-inoculation were analysed histologically for the presence of p65 and p50 as well as the precursor of the latter, p105. Activated NF-κB was detected by the nuclear localization of p65 only in mammary epithelial cells of the gland challenged with bacteria, while no nuclear signal was obtained for cells in control glands (Figure 7). In contrast, p50 was not observed in the nucleus of cells in either gland (Figure 8).

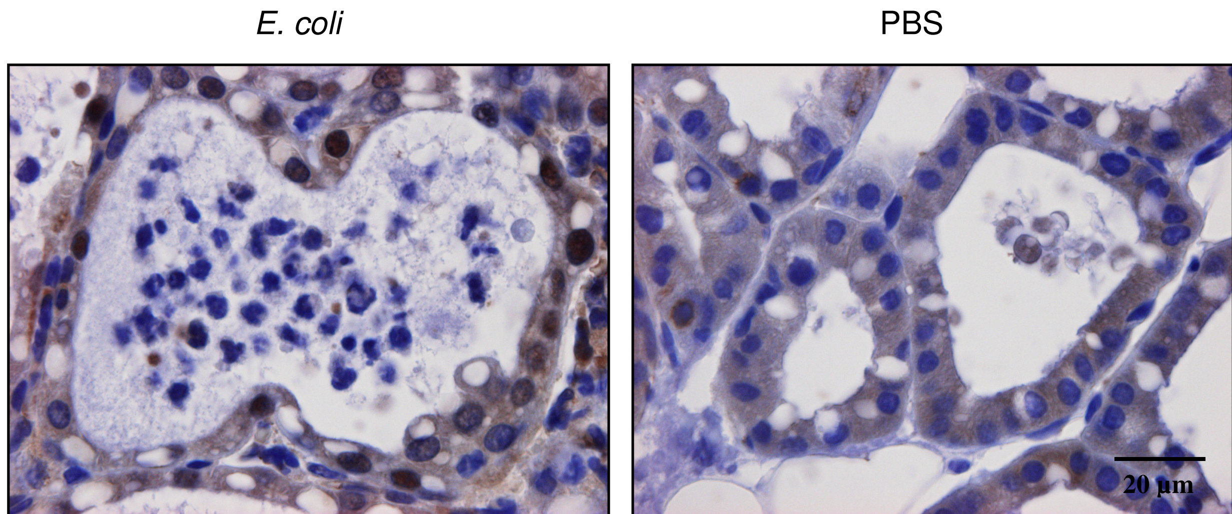

Figure 7: Immunohistochemical staining of the NF-κB p65 subunit following intramammary inoculation. Transgenic mice received 10^4 CFU E. coli in the R4 mammary gland and PBS in the L4 mammary gland. At 8 h post-inoculation, they were sacrificed and mammary glands were harvested. Control glands showed no signs of inflammation while in infected glands, neutrophil recruitment was apparent in many alveolar spaces which are lined up by the epithelial cells. Inactive NF-κB p65 was visualized in the cytoplasm of all cells of both glands while a strong activity was only observed in the nuclei of mammary epithelial cells of the infected gland. Images are representative for all challenged mice (magnification: 1,000 x).

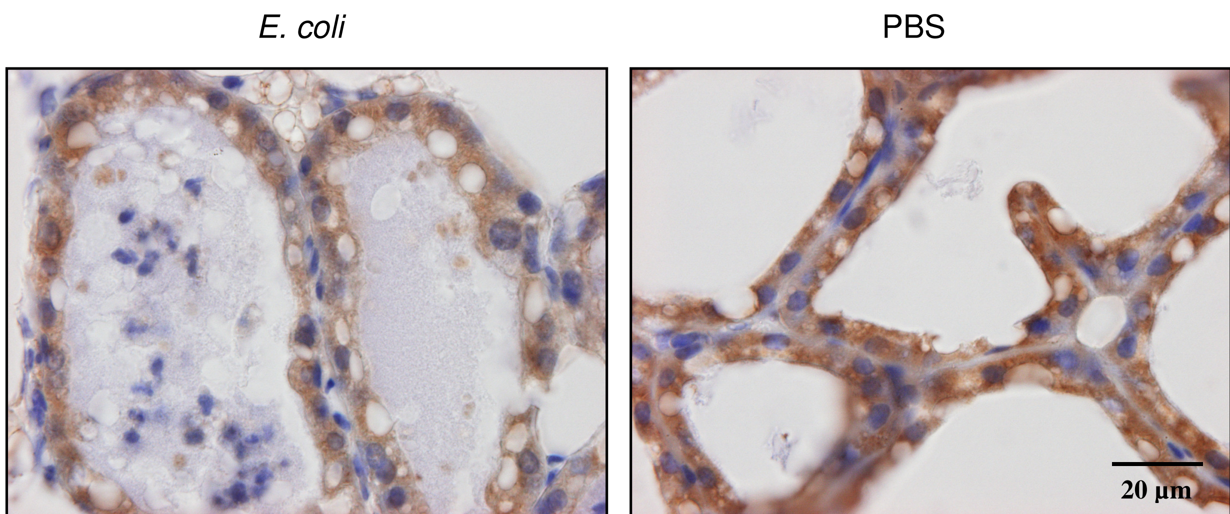

Figure 8: Immunohistochemical staining of the p105/p50 subunit following intramammary inoculation. Transgenic mice received 10^4 CFU E. coli in the R4 mammary gland and PBS in the L4 mammary gland. At 8 h post-inoculation, they were sacrificed and mammary glands were harvested. Control glands showed no signs of inflammation while limited neutrophil recruitment was apparent in infected glands. Inactive NF-κB p105/p50 was detected in the cytoplasm of all cells of both glands. In addition, activity was absent in the nuclei of all cells of both infected and control glands. Images are representative for all challenged mice (magnification: 1,000 x).

3.3. Acute phase reaction

To confirm the systemic reaction to the intramammary inoculation of *E. coli* bacteria, the acute phase protein SAA was quantified in plasma samples of the 6 challenged mice sacrificed at 8 h post-inoculation. Plasma concentrations were significantly increased (on average 387 ± 148 µg/ml, n = 6) as compared to pre-challenge values (on average 10.03 ± 0.61 µg/ml, n = 4; P = 0.01).

3.4. Cytokine production

To additionally confirm the inflammatory reaction, plasma samples and mammary gland homogenate supernatants were also analysed for the presence of cytokines. In challenged mice sacrificed at 8 h post-inoculation, TNF-α was detected in plasma at an average concentration of 32.24 ± 5.31 pg/ml (n = 6), while this cytokine was nearly absent in blood of control mice (3.52 ± 1.33 pg/ml, n = 4; P = 0.01). Furthermore, TNF-α levels were significantly increased in the supernatants of infected glands as compared to those of control glands (on average 3.18 ± 0.36 ng/ml for R4 and 1.28 ± 0.13 ng/ml for L4, n = 6, $P < 0.05$).

Regarding IL-6, elevated concentrations (on average 1.98 ± 0.24 ng/ml, n = 6) were measured in all plasma samples of challenged mice as compared to pre-challenge values (on average 0.25 ± 0.25 ng/ml, n = 4; P = 0.01). In addition, high concentrations were detected in the R4 mammary glands (on average 81.76 ± 18.46 ng/ml, n = 6), while amounts of IL-6 as low as 0.59 ± 0.29 ng/ml were detected in the L4 glands (n = 6, $P < 0.05$).

3.5. Clinical and post-mortem observations

The 6 mice killed at 8 h post-inoculation, showed no or only minor indications of illness: animals were active but a significant loss in body weight was observed (on average 7.0 ± 2.0 %, $P < 0.05$, n = 6). Upon necropsy, early indications of inflammation were present: the right mammary glands inoculated with *E. coli* were pink and mildly hardened, while the left control glands were white, soft and full of milk. Infected glands also weighed more than control glands with an average difference of 57.0 ± 21.1 mg ($0.1 < P < 0.05$, n = 6).

The 6 mice followed up in time until 24 h post-inoculation, showed obvious signs of infection: they were less active and had slightly swollen inoculated glands. Body weights were significantly decreased (on average 12.0 ± 1.7 %, $P < 0.05$, $n = 6$). Following dissection of the mice, challenged glands were enlarged, hardened, darkened and sometimes bloody in appearance. Again, control glands looked normal in size, color and morphology. The average difference in weight between both glands was 121 ± 47.1 mg ($P < 0.05$, $n = 6$).

4. Discussion

In the present study, a clear activation of the transcription factor NF-κB has been demonstrated under *in vivo* conditions in mouse mammary glands inoculated with *E. coli* bacteria. The transgenic mice used in our experiments have already proved their utility in the assessment of NF-κB activity in various tissues and under diverse physiological and pathological conditions (Carlsen et al., 2002). For the first time, we now show that this model is undoubtedly valuable to the study of mammary glands and intramammary infections as well. The major advantage of this non-invasive molecular imaging technique is the visualization of both the temporal and spatial regulation of NF-κB activity within the natural environment of an intact living animal, as it allows repetitive assessments within the same mouse. In addition, light generated upon injection of D-luciferin is directly correlated to the amount of transcriptionally active NF-κB. Alternative methods such as the traditionally used EMSA are not able to prove transcription, since modifications of NF-κB such as phosphorylation and acetylation, additionally regulate transcription after binding to the DNA (Carlsen et al., 2004).

The importance of the transcription factor NF-κB in the early phase of the pathogenesis of mastitis is clearly established with this *in vivo* imaging approach. Until now, indications on its critical role were only obtained from *in vitro* experiments focusing on murine or bovine mammary tissue cells incubated with *E. coli*, LPS or TNF-α (Safieh-Garabedian et al., 2004; Strandberg et al., 2005; Szperka et al., 2006; Yang et al., 2006; Fitzgerald et al., 2007). Importantly, NF-κB activation was apparent within the first hours of culture. In addition, one *ex vivo* study demonstrated increased levels of activated NF-κB in milk cells isolated from natural cases of ruminant mastitis mainly caused by *S. aureus* (Boulanger et al., 2003). These results were obtained when the infection was already well established, as demonstrated by the culmination of neutrophil infiltration. In the current *in vivo* study, maximal NF-κB activity was achieved at 6 to 8 h post-inoculation. The contribution of neutrophils to this activity is minimal since their influx into the mammary gland this early in the inflammation process is still limited. In fact, the immunohistochemical analysis of infected mouse mammary glands showed that particularly epithelial cells are responsible for the high NF-κB activity observed by imaging. These findings are in agreement with the early NF-κB activation in bovine mammary epithelial cells following *in vitro* stimulation (Safieh-Garabedian et al., 2004; Strandberg et al., 2005; Yang et al., 2006; Fitzgerald et al., 2007). Still, NF-κB activation in

other cell types at later time points, as observed in milk neutrophils by Boulanger et al. (2003), can not be excluded. However, we believe this activation is very minimal in comparison with the strong activation observed in epithelial cells, as also been demonstrated in our previous work (Notebaert et al., 2008).

Kinetic studies of experimentally induced mastitis in cows show that the inflammatory response is not initiated until bacterial concentrations reach a certain level in milk (Shuster et al., 1996). Bacterial growth is accompanied by the release of microbial products that can be recognized by the host as a danger signal. The LPS membrane component of Gram-negative bacteria such as *E. coli*, is known to be recognized by Toll-like receptor 4 (TLR4) (Miyake, 2004). Mammary tissue expresses mRNA for TLR4 and this expression is increased in udders of cows suffering from infections (Goldammer et al., 2004). Signaling mediated by TLR4 leads to the activation of NF-κB which results in the expression of several proteins involved in inflammatory processes (Doyle and O'Neill, 2006). Interestingly, a recent report showed that LPS and TLR4 signaling in mammary epithelium are not required to elicit the inflammatory response towards *E. coli* intramammary infections (Gonen et al., 2007). These authors suggest that other components of *E. coli* bacteria might play a role in the pathogenesis of coliform mastitis, such as lipoproteins, peptidoglycans, flagellins and CpG DNA, which are recognized by TLR1, -2, -5 and -9 respectively (Albiger et al., 2007). Although the specific molecular interaction between *E. coli* and the mammary epithelium remains to be elucidated, the NF-κB signaling pathway is activated in response to all TLRs thereby resulting in the expression of inflammatory mediators.

Mammary epithelial cells thus generate cytokines and chemokines targeting neutrophils and mononuclear cells (Rainard and Riollet, 2006). These secondary mediators include TNF-α and IL-6, of which the expression is known to be dependent upon NF-κB (Hayden et al., 2006). In the current study, both cytokines are clearly up-regulated in infected glands as compared to control glands. We previously also observed high local concentrations of TNF-α and IL-6 upon *E. coli* intramammary infection in wild type mice (Notebaert et al., 2008). Following *in vitro* culture with LPS or *E. coli*, several cytokines and chemokines are produced by bovine mammary epithelial cells, as shown at the mRNA and protein level (Boudjellab et al., 1998; Okada et al., 1999; Wellnitz and Kerr, 2004; Pareek et al., 2005; Strandberg et al., 2005, Lahouassa et al., 2007). In mammary epithelial cells from cows experimentally infected with *E. coli*, these inflammatory proteins are increased (McClenahan

et al., 2006) and secreted in milk as well (Riollet et al., 2000). In addition, NF-κB activation seemed to be essential for the production of bactericidal peptides following *in vitro* culture of bovine and murine mammary epithelial cells with *E. coli* (Yang et al., 2006).

Interestingly, in the current study only the NF-κB p65 subunit was activated in epithelial cells of infected mouse mammary glands, while p50 did not seem to contribute to the observed NF-κB activation. We suggest an induction of p65 homodimers in mouse mammary gland infections instead of an involvement of the classical p65/p50 heterodimers. This hypothesis is in agreement with previous findings in epithelial cells of different origins and derived from various species. In bovine mammary epithelial cells, p65 homodimers were induced in response to TNF-α (Fitzgerald et al., 2007). In addition, bronchial epithelial cells from heaves-affected horses contained p65 homodimers as activated NF-κB complexes (Bureau et al., 2000). In human gastric epithelial cells infected with *Helicobacter pylori* (Wada et al., 2001) and in colon or cervical epithelium incubated with enteropathogenic bacteria or their toxins (Philpott et al., 2000; Schulte et al., 2000; Kim et al., 2006), these homodimers were observed as well. This uncommon NF-κB complex is thought to be crucial for the transcription of a limited number of genes, such as those encoding for IL-8 and the intracellular adhesion molecule-1 or ICAM-1 (Kunsch and Rosen, 1993; Ledebur and Parks, 1995). These two molecules are known to participate in neutrophil chemotaxis, diapedesis and transmigration (Kunsch and Rosen, 1993; Rahman et al., 1999). In early inflammatory conditions arising from bacterial infections, p65 homodimers thus seem to be imperative for subsequent neutrophil participation.

Our clinical and post-mortem observations show that the intramammary infection was well established in the transgenic mice. To investigate the severity of infection, NF-κB activity was additionally examined in the liver by *in vivo* imaging. A clear reaction of the liver following *E. coli* intramammary inoculation was observed. It is generally suggested that cytokines produced during the inflammatory process are the primary cause of the systemic effects observed during intramammary infections. We demonstrated increased concentrations of IL-6 and TNF-α in blood of infected mice in comparison with control mice. In addition, the same observation was previously made for wild type animals (Notebaert et al., 2008). As a potent inducer of NF-κB, at least TNF-α can target this transcription factor in cells throughout the body. Elevated plasma levels of the positive acute phase protein SAA were also demonstrated in the present study in analogy with *E. coli* infected wild type mice (Notebaert

et al., 2008). NF-κB controls the biosynthesis of SAA, which is dramatically up-regulated in the liver during inflammation (Uhlar and Whitehead, 1999). Interestingly, the liver response towards *E. coli* challenge was relatively mild, slow and longer lasting as compared to the very strong reaction in mice inoculated with LPS. The LPS dose chosen is of the same order of magnitude as used in other studies with mice (Gonen et al., 2007). In cows, the intramammary challenge with LPS is frequently employed to study inflammation of the bovine mammary gland due to its resemblance to *E. coli* challenge (Lohuis et al., 1988a and 1988b). Still, the more dynamic release of LPS during bacterial growth and killing in the *E. coli* mastitis model is in clear contrast with the single dose administration during endotoxin challenge and might explain the observed differences in the liver response curves.

In conclusion, we demonstrate the elegant possibility to monitor NF-κB activity in mammary glands in real time. During the early hours of bacterial infection, activation of this transcription factor was apparent and was localized within the mammary epithelium. We identified p65 as being the key player in NF-κB mediated transcription. In addition, a systemic reaction was confirmed through the molecular imaging of the NF-κB activity within the liver and by measurement of circulating levels of NF-κB dependent acute phase proteins and cytokines. This first *in vivo* study regarding the role of NF-κB in the pathogenesis of mastitis emphasizes the importance of investigating early events in infected mammary glands. As most research within the field of mammary gland infections has been carried out upon diagnosis, it has mainly focused on the role of neutrophils thereby relatively underestimating the active contribution of mammary epithelial cells. We demonstrate that mammary epithelium is not just a passive physical barrier, but a critical regulator within the establishment of the immune response towards mastitis, via major activation of the transcription factor NF-κB.

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GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION

The transcription factor NF-κB regulates many physiological processes, including innate and adaptive immune responses, inflammation, cell death and proliferation (Liou, 2002). Because of the diversity in its function, a tightly controlled NF-κB activation is mandatory. Moreover, aberrant regulation is associated with the development of chronic inflammation, autoimmune disorders and different types of cancer (Yamamoto and Gaynor, 2001; Garg and Aggarwal, 2002; Kumar et al., 2004). In particular, an increased and sustained NF-κB activation is mainly involved in the pathogenesis of diseases like asthma, rheumatoid arthritis, diabetes and multiple sclerosis, and in the onset of malignancies. Therefore, the inhibition of NF-κB has been of major interest in pharmacological interventions (Garg and Aggarwal, 2002; Calzado et al., 2007). However, in order to treat diseases more effectively, an extensive understanding of the role of NF-κB in health and disease is indispensable (Campbell and Perkins, 2006).

The pathophysiological function of NF-κB has been studied mainly in the context of human diseases. As a practical approach, cell culture systems and animal models have been used besides individual patients (Kumar et al., 2004). However, some diseases like intramammary infections have received little attention in man, even though the incidence and complications of mastitis among breastfeeding women should not be underestimated (Fetherston, 2001; Michie et al., 2003). In contrast and not unexpectedly, intramammary infections have been a major concern in the dairy industry (Bradley, 2002). Intensive research has therefore been carried out mainly during lactation, which is the final stage of the mammalian reproductive cycle. Still, molecular approaches to the study of veterinary medicine are not yet fully exploited (Binns, 1993). Consequently, very limited research has been conducted to reveal the contribution of NF-κB to the pathogenesis of clinical mastitis. This Ph.D. work examined specific molecular aspects of the relationship between pathophysiological conditions and NF-κB activation in the mammary gland. Because an in-depth discussion focusing on a particular facet of our research is presented in each chapter, the following paragraphs will merely deal with some of the more general findings that became apparent during our experimental work.

In vitro studies carried out to examine NF-κB activation in bovine neutrophils necessitated the purification of these cells from peripheral blood (**chapters 1 and 2**). In this way, mechanisms involved in neutrophil activation and apoptosis could be elucidated, while influences of contaminating cells on these processes were eliminated. For example, it is well known that the

presence of mononuclear cells, particularly monocytes, delays neutrophil apoptosis (Sabroe et al., 2005). Various neutrophil isolation procedures with differences in the average amount of harvested cells and in their viability and activation status have been described in literature (Grisham et al., 1985). Our choice was based on reports describing purification methods for bovine cells that yielded populations of more than 95 % of highly viable and functional neutrophils (Roth and Kaeberle, 1981; Chambers et al., 1983). The use of the selected density gradient based purification, however, imposes a considerable sample preparation time prior to the specific neutrophil experiments. Also, our preference to use blood neutrophils of heifers instead of circulating cells from lactating cows is motivated by the absence of variables such as stage of lactation, parity and the hormonal and metabolic status of the animal that might influence neutrophil responses (Burvenich et al., 2003).

Bovine neutrophils represent the first line of defense against intramammary *E. coli* bacteria (Paape et al., 2003; Burvenich et al., 2004). Their proliferation and maturation occur in the bone marrow prior to their release into circulation. In the absence of an invading pathogen, neutrophils migrate into tissues such as the liver, lungs and spleen where they rapidly undergo spontaneous apoptosis (Paape et al., 2003). We found that this programmed form of cell death can be induced in resting bovine neutrophils through the application of gliotoxin as an NF-κB inhibitor (**chapter 1**). Characteristic apoptotic features such as plasma membrane exposure of phosphatidylserine, effector caspase activation and stereotypical morphological changes involving cell shrinkage and nuclear chromatin condensation were observed. In order to confirm the process of apoptosis, a range of techniques was required since some of its markers are not entirely specific as they also occur to various degrees in other types of cell death (Savill et al., 2002). Apoptosis in resting neutrophils is thus prevented by a constitutive NF-κB activition. Cell viability is essential for leukocytes in circulation where they must be perceptive for signs of infection. However, in the absence of a trigger that mediates migration into infected tissues, neutrophils probably lose this basal NF-κB activity. Since this transcription factor is involved in the expression of anti-apoptotic proteins of the Bcl-2 family (Akgul et al., 2001; Dutta et al., 2006), pro-apoptotic members are able to take over control when NF-κB is no longer involved. Consequently, caspase activation occurs which then leads to the execution of the apoptotic process, that probably also includes caspase-mediated cleavage of NF-κB (Kucharczak et al., 2003).

In human neutrophils, apoptosis is similarly induced by agents known to inhibit NF-κB (Ward et al., 1999). A wide variety of both natural and synthetic compounds exists which act at different levels of the NF-κB activation pathway (Gilmore and Herscovitch, 2006). NF-κB activation can be inhibited by blocking its nuclear translocation or its binding to specific κB sites on the DNA. More upstream strategies include the interference with IKK and the proteasome thereby preventing IκB phosphorylation and its degradation, respectively. Gliotoxin is reported to block both the proteasome-mediated degradation of IκB (Kroll et al., 1999) and the NF-κB DNA binding activity (Pahl et al., 1996). However, it has also been suggested that the redox-cycling of gliotoxin accounts for its immunosuppressive activity (Gardiner et al., 2005). Consequently, one might argue that other proteins than NF-κB could also be affected by gliotoxin and the use of more specific NF-κB inhibitors is therefore recommended. As mentioned before, targeting the NF-κB activation pathway is recognized as a potential therapy for many inflammatory diseases and cancer (Garg and Aggarwal, 2002; Calzado et al., 2007). It is noteworthy that some NF-κB inhibitors are currently tested on patients in phase II clinical trials, like NF-κB decoys which are double-stranded oligonucleotides that compete for binding of the transcription factor with κB sites of target genes thereby attenuating NF-κB-mediated transcription (Isomura and Morita, 2006). However, for many potential drug molecules, clinical side effects might become a subject of concern. Clearly, complete or prolonged inhibition of NF-κB is detrimental since this transcription factor is required to maintain normal functioning of the body (Fraser, 2006). Furthermore, interfering with the NF-κB activation pathway undoubtedly affects major alternative signaling cascades due to their intricate crosstalk. Cooperative interactions of NF-κB with other DNA-bound transcription factors are well described. In addition, IκB was found to associate with several other proteins than NF-κB in order to modulate their function. Finally, other substrates than the NF-κB inhibitors are now identified for IKK. Remarkably, this also implies that therapeutic effects of blocking IKK are not exclusively assigned to the inhibition of NF-κB activation. For a more detailed discussion on the mechanisms that link the NF-κB pathway to other signaling cascades, we would like to refer to the latest review written by Perkins (2007).

Complementary to the observation of a basal NF-κB activity in resting bovine neutrophils, an inducible NF-κB complex was demonstrated in additional *in vitro* work with activated cells (**chapter 2**). This NF-κB activation has not been described before for bovine neutrophils incubated with *E. coli* bacteria. Until recently, it has been assumed that the main functions of

neutrophils are phagocytosis and killing of invading bacteria. However, our results indicate that neutrophils might also actively regulate the early pro-inflammatory response through NF-κB-mediated gene transcription. In human neutrophils, NF-κB activation has been demonstrated in response to several phagocytic and pro-inflammatory stimuli (McDonald et al., 1997; McDonald and Cassatella, 1997). Moreover, the involvement of alternative transcription factors in activated neutrophils supports the concept of their regulatory role other than their defense function (Cloutier and McDonald, 2003; McDonald, 2004). Among those factors, the signal transducer and activator of transcription (STAT) family members have been studied in bovine neutrophils as well (Boutet et al., 2004). Following *in vitro* incubation with granulocyte-macrophage colony-stimulating factor (GM-CSF), a delay in apoptosis was observed in these cells as a result from the STAT-mediated expression of anti-apoptotic Bcl-2 family members. Interestingly, the contribution of both NF-κB and STAT signaling pathways to the pathogenesis of bovine mastitis caused by bacteria other than *E. coli* has already been investigated (Boulanger et al., 2003; Boutet et al., 2004). Active STAT and NF-κB complexes found in milk cells of infected cows were suggested to be of importance to the establishment of the local inflammatory reaction mediated by neutrophils.

The expression of the major cytokines IL-1 and IL-6 is known to be dependent upon NF-κB (Libermann and Baltimore, 1990; Hiscott et al., 1993; Mori and Prager, 1996) and their pro-inflammatory activities are well described (Feghali and Wright, 1997). These interleukins are likely to be involved in the NF-κB-mediated regulation of the pro-inflammatory response of bovine neutrophils towards *E. coli* bacteria. In our *in vitro* study, NF-κB activation appeared to be a very fast event in bovine neutrophils exposed to *E. coli* bacteria. Consequently, IL-1 and IL-6 could also be detected at early time points following NF-κB activation (**chapter 2**). This immediate neutrophil response is required in *in vivo* situations where neutrophils that sense the presence of a pathogen need to quickly inform and prepare other inflammatory cells by transducing signals before they are removed by macrophages. The induced NF-κB complex also disappears very rapidly. Persistent NF-κB activation could lead to the development of chronic inflammatory diseases and cancer and must therefore be avoided (Kumar et al., 2004). This transient NF-κB activation might be regulated by IκB-α, which is rapidly degraded in response to stimuli but also quickly resynthesized under control of NF-κB itself (Hayden and Ghosh, 2004; Perkins and Gilmore, 2006). Resynthesized IκB-α can enter the nucleus and transport DNA-bound NF-κB back to the cytoplasm. Interestingly, IκB-β can also interact with NF-κB complexes bound to κB sites of target genes but, unlike IκB-α, does

not displace them, leading to a sustained NF-κB activation. As an alternative, NF-κB-mediated transcription might also be terminated by specific NF-κB modifications that decrease DNA binding activity or target the transcription factor for degradation (Perkins, 2006).

The NF-κB family member p65 has been identified as part of both the constitutively active and the inducible NF-κB complex present in resting and activated bovine neutrophils, respectively (**chapters 1 and 2**). Provided that transient NF-κB activation is regulated by IκB-α, one might expect the contribution of p50 as well, at least to the inducible NF-κB complex, since IκB-α preferentially binds p65/p50 heterodimers (Hayden and Ghosh, 2004). Unfortunately, we were unable to detect p50 in bovine neutrophils with an ELISA-based method and could therefore not demonstrate its involvement. One of the major practical complications of molecular research in the bovine species is the lack of specific anti-bovine antibodies. Still, cross-reactivity of anti-human antibodies might occur since the bovine genome is fairly homologous to human DNA. Indeed, more than 90 % identity is apparent between the primary protein sequences of the precursor p105 of both species. Nevertheless, cross-reactivity implies the use of relative instead of absolute values, as the antibody might bind the bovine protein with a lower affinity than the human antigen. Clearly, by using polyclonal antibodies, a greater chance of antigen detection is likely since more than one epitope can be recognized. The anti-p50 antibody provided by the manufacturer however is monoclonal and is reported to strongly bind human p50 and weakly the mouse and rat protein. Cross-reactivity with the bovine protein has not been described but seemed likely since the antibody recognizes an epitope that lies within the Rel homology domain which is highly conserved among vertebrates (Hayden and Ghosh, 2004). As the results were poor, an extra attempt was made with another antibody that has similar characteristics and worked well for western blot detection of p50 in resting bovine cells. Again, ELISA results were not satisfactory and were therefore not included in this Ph.D. thesis. Interestingly, the use of the latter antibody is reported to be effective in several techniques like flow cytometry, immunofluorescence and of course western blot analysis but not in ELISA. Indeed, antibodies frequently may not perform in all immunoassays. It is well realized that an unequivocal detection of p50 is also quite difficult to obtain in other cells and thus remains a major challenge in bovine neutrophils too.

Next to the early NF-κB activation and cytokine production in response to *E. coli* bacteria, an activation-induced neutrophil cell death occurred as a late event in our *in vitro* study (**chapter 2**). Even though we showed exposure of phosphatidylserine following phagocytosis, caspase activation could not be demonstrated and morphological features were very distinct from those typical for apoptotic cells. It thus remains to be investigated which type of cell death takes place in activated bovine neutrophils. However, in a study with similar findings for human neutrophils, apoptosis is still suggested to occur (Wilkie et al., 2007). The diagnosis in the latter paper is based on the demonstration of neutrophil recognition and engulfment by macrophages, which are both key features of the apoptotic process (Savill et al., 2002). Indeed, in the assessment of apoptosis, classical morphological changes and caspase activation may have become less important than initially postulated. For the latter, this might be due to the fact that also caspase-independent apoptosis pathways have been increasingly described (Hail et al., 2006). Moreover, for activated neutrophils it has been suggested that caspase activation is prevented by ROS (Fadeel et al., 1998; Wilkie et al., 2007). Following phagocytosis, these antimicrobial molecules are produced by neutrophils in order to kill the pathogen. Furthermore, both ROS and RNS seem to participate as key regulators of diverse signaling pathways. In our interest, ROS are able to modulate Fc receptor function and regulate signaling events downstream of this receptor (Fialkow et al., 2007). In addition, oxidants influence NF-κB activation since many proteins of its signaling pathway are redox-sensitive. For more detailed discussions on this topic, we would like to refer to reviews written by Gloire et al. (2006) and Pantano et al. (2006). However, it is worth mentioning that ROS activity is also under control of NF-κB (Bubici et al., 2006). Finally, ROS participate in the regulation of neutrophil apoptosis (Fialkow et al., 2007).

The observed activation-induced neutrophil cell death is essential to ensure the appropriate termination of the inflammatory response (Marshall et al., 2007). Signaling pathways and transcription factors that regulate the initiation of inflammation need to be repressed while other mechanisms mediating its resolution are turned on. However, for some key players within innate immunity both a pro- and anti-inflammatory function has been revealed. Even though NF-κB is known to mediate the expression of pro-inflammatory genes during the onset of inflammation (Liou, 2002), a role for this transcription factor within the resolution of the inflammatory response has been described as well (Rossi et al., 2007). Results of both *in vitro* and *in vivo* experiments suggest that there are at least two important waves of NF-κB activation in inflammatory loci (Ward et al., 2004). While the first wave leads to the

production of pro-inflammatory mediators and the promotion of neutrophil survival, the second one causes the synthesis of anti-inflammatory proteins that induce granulocyte apoptosis. It is most likely that different NF-κB complexes are responsible for the diversity in gene expression profile during the two stages of inflammation. Homodimers of p50 are reported to predominate during the resolution of inflammation (Lawrence et al., 2001). This complex is known to repress pro-inflammatory gene transcription (Bohuslav et al., 1998), but might also enhance gene expression in the presence of Bcl-3 (Bours et al., 1993; Fujita et al., 1993). In addition, homodimers of p50 are suggested to support other transcription factors in the expression of anti-inflammatory proteins that induce apoptosis (Lawrence et al., 2001). As the inhibition of NF-κB during the resolution of inflammation prevents apoptosis and thus protracts the inflammatory response, the timing of any therapeutic intervention involving NF-κB inhibition is very crucial for successful treatment of inflammatory diseases. Furthermore, the elucidation of anti-inflammatory pathways might be useful to the understanding of chronic inflammatory conditions. In relation to our study, it would be of interest to investigate whether NF-κB also exerts an anti-inflammatory function in activated bovine neutrophils besides its observed pro-inflammatory role.

Even though *in vitro* studies are useful to investigate specific cellular responses, cell culture conditions remain artificial and thus can not replicate all biological processes that occur in intact animals. In addition, findings obtained under simplified *in vitro* conditions can not be extrapolated directly to the more complex *in vivo* situation. It is therefore necessary to perform *in vivo* experiments both to validate *in vitro* data and to extend the knowledge relevant to *in vivo* conditions. In our *in vivo* experiments, the experimental mouse model of mastitis was employed to study the role of NF-κB in the pathogenesis of *E. coli* intramammary infections (**chapters 3 and 4**). This model is considered to be a very suitable tool for the investigation of bovine mastitis because of major similarities between both species (Brouillette and Malouin, 2005; Notebaert and Meyer, 2006). Besides anatomical and functional properties of the mammary gland, these also include basic observations during intramammary infections. Moreover, one of the main advantages of a rodent model is the possibility to utilize genetically modified animals as an alternative strategy within research. For many years now, the use of these animals has been shown to be a powerful tool to unravel the pathogenesis of several diseases and to develop novel therapies (Bhogal and Combes, 2006).

In our first *in vivo* study, we investigated how NF-κB might be involved in the establishment of the local inflammatory environment and the systemic acute phase response following inoculation of *E. coli* bacteria in the mouse mammary gland (**chapter 3**). At 24 and 48 h post-inoculation, NF-κB activation and the NF-κB-mediated expression of important cytokines were clearly demonstrated for infected mammary glands. Next to IL-1 and IL-6, which were both analyzed in our *in vitro* experiments as well, TNF-α was also evaluated. Additionally, the infection resulted in an acute phase response, demonstrated by high circulating levels of SAA. The synthesis of SAA is mainly localized within the liver and is also under control of NF-κB. Systemic TNF-α might cause the increased expression of SAA as this cytokine is a potent inducer of NF-κB. Our findings regarding the cytokine and acute phase protein up-regulation are in accordance with those of studies in cows with *E. coli* intramammary infections (Hirvonen et al., 1999; Burvenich et al., 2003; Van Merris et al., 2004). Still, the contribution of NF-κB to the acute inflammatory response has never been studied in these animals. Only one *ex vivo* report deals with the role of NF-κB in the pathogenesis of natural cases of bovine mastitis caused by bacteria other than *E. coli* (Boulanger et al., 2003).

As an acute inflammatory reaction develops within the first days following bacterial invasion, we choose the 24 and 48 h time points for sampling in the mouse model of *E. coli* mastitis. The main cell types present in infected mouse mammary glands in these stages of infection are epithelial cells, macrophages and infiltrated neutrophils. As demonstrated in clinically infected cows, the migration of lymphocytes, especially T cells, to the mammary gland might then also have started (Mehrzad et al., 2008). T helper cells are imperative during mastitis as they activate other leukocytes through cytokine production (Sordillo and Streicher, 2002). A well documented example is their ability to promote neutrophil function (Riollet et al., 2000). Cytotoxic T lymphocytes may also play a role as they remove old or damaged cells that increase the susceptibility of the mammary gland to infections (Taylor et al., 1994). Finally, B lymphocytes are of importance due to their antibody production and their role as antigen-presenting cells (Sordillo and Streicher, 2002). All of the abovementioned cells are able to produce pro-inflammatory cytokines like IL-1, IL-6 and TNF-α. Moreover, they all may account for the observed local NF-κB activation. Further studies need to be conducted in order to identify the nature and contribution of these cell-specific responses. In addition, it would be of interest to focus on both earlier and later time points of the infection process.

Among the evaluated cytokines, only IL-1β is known to be expressed as a biologically inactive proform. We showed that an increased expression of the proform and its subsequent processing accounts for elevated levels of the mature form in infected mouse mammary glands. It is well accepted that the generation of mature IL-1β requires the activity of IL-1 converting enzyme or caspase-1 (Thornberry and Molineaux, 1995). Indeed, while most caspases are involved in the apoptotic process, some members of this family like caspase-1, also play a role in other biological processes such as inflammation (Martinon and Tschopp, 2007). We used caspase-1 knockout mice to demonstrate that IL-1β processing occurs independently from this enzyme (**chapter 3**). It has been described that other proteins might mediate the cleavage of the IL-1β proform (Black et al., 1991; Coeshott et al., 1999). How this processing is regulated in the mouse model of *E. coli* mastitis remains to be investigated. Interestingly, caspase-1 has been reported to regulate other pro-inflammatory events than the IL-1β cleavageas well, such as the activation of NF-κB (Lamkanfi et al., 2004). Remarkably, the proteolytic activity of caspase-1 is not required for this activation. Further studies in this research domain are thus an exciting prospect too. In any case, our experiments with the caspase-1 knockout mice are a clear example of the use of genetically modified animals as an elegant tool to elucidate molecular events that occur upon infection.

The knockout technology has been applied to individual components of the NF-κB signaling pathway too. Not only conventional knockouts but also conditional gene targeting has been indispensable to the current understanding of the diverse functions of NF-κB. Conditional knockouts are particularly useful when the classical knockout results in early lethality or in very complex phenotypes involving more than one tissue or cell type. The contribution of a certain protein to the NF-κB signaling pathway can also be studied through the application of specific modifications to its gene by means of the knock-in technology. Furthermore, transgenic overexpression of NF-κB regulators leading to a permanent NF-κB inhibition or constitutively activation has provided invaluable information as well. A more detailed description of these technologies is given by Pasparakis et al. (2006). Finally, transgenesis also offers the attractive possibility to visualize NF-κB activation by placing a reporter gene under the control of NF-κB. Various proteins have been used to report for NF-κB activation, like β-galactosidase (Bhakar et al., 2002), green fluorescent protein (Magness et al., 2004) and luciferase (Carlsen et al., 2002).

In order to study kinetics of NF-κB activation during the acute inflammatory reaction towards intramammary inoculation of *E. coli* bacteria, transgenic mice expressing luciferase under control of NF-κB were used in an additional *in vivo* experiment (**chapter 4**). Indeed, while wild type animals need to be sacrificed to obtain samples for NF-κB analysis at a certain time point, the use of transgenic mice allow repetitive measurements of NF-κB activation within the same animal over a long period of time (Carlsen et al., 2002). In addition, their exploitation enables the simultaneous visualisation of the transcription factor activation in different tissues of one animal. In our study, a gradual increase in NF-κB activation was observed both within the mammary gland and the liver upon the induction of *E. coli* mastitis. Maximal activation in the infected gland was achieved at 6 to 8 h post-inoculation, when the infection was not yet well established. NF-κB-dependent cytokine and acute phase protein expression was demonstrated for these early hours too. The evaluation of the NF-κB activation at 24 h post-inoculation revealed an important signal reduction. This finding coincides well with the observation of a minor NF-κB activation at that time point in our first *in vivo* study with wild type animals.

Immunohistochemical analysis of infected glands at 8 h post-inoculation revealed that mouse mammary epithelial cells are responsible for the observed NF-κB activation. Remarkably, only p65 and not p50 could be identified as a key role player in this event. We suggest that activated p65 homodimers mediate the epithelial expression of proteins that primarily induce neutrophil infiltration into infected mammary glands. Since NF-κB activation in epithelial cells decreases once neutrophil influx has begun, we suggest that infiltrated leukocytes take over their role to further establish the inflammatory reaction. In this event, NF-κB undoubtedly plays a role as well, but its activation will probably never be that strong as in mammary epithelium given the modest transcription factor activation observed both in bovine neutrophils following phagocytosis of *E. coli* and in infected mouse mammary glands from 24 h on. Indeed, even though neutrophils are capable of performing gene transcription, the degree of transcriptional activity is much lower than that of other cells (Cassatella et al., 1991). Still, it should also be remarked that the comparison of quantitative data obtained with the ELISA-based method with those of the *in vivo* imaging technique must be made with caution. In addition, species differences are to be taken into account.

CONCLUSIONS

In our *in vitro* studies, we first demonstrated that NF-κB inhibition induces bovine neutrophil apoptosis. We suggest that a basal NF-κB activity contributes to cell viability in circulation. However, the possibility that the transcription factor promotes neutrophil survival via the expression of anti-apoptotic Bcl-2 family members, remains to be examined. In addition, when bovine neutrophils encounter *E. coli* bacteria, an inducible NF-κB complex seems to be responsible for the initiation of a pro-inflammatory response via the expression of important cytokines. Nevertheless, activated neutrophils also rapidly undergo apoptosis in order to be eliminated by macrophages. We conclude that both the activation of NF-κB and the induction of apoptosis in bovine neutrophils are essential in order to obtain a clear establishment and proper resolution of the acute inflammatory reaction towards *E. coli* bacteria.

By using the *in vivo* mouse model of *E. coli* mastitis, we found clear indications that NF-κB activation is crucial to the establishment of the inflammatory response and the acute phase reaction in infected mammary glands and the liver, respectively. Even more so than infiltrating neutrophils, mammary epithelial cells are undoubtedly involved in the initiation of the local inflammation. It has recently been shown that this inflammatory process also occurs in the absence of LPS/TLR4 signaling (Gonen et al., 2007). Therefore, it would be of interest to further investigate what kind of molecular interaction between *E. coli* bacteria and cells present in the mammary gland leads to the activation of NF-κB. Furthermore, in addition to the inflammatory proteins in our studies, the identification and role of other gene products transcribed by NF-κB in infected mammary glands need to be elucidated. Finally, the NF-κB-mediated reaction of the liver should not be underestimated as local inhibition of NF-κB was previously shown to result in high susceptibility to bacterial infection (Lavon et al., 2000).

Finally, we have exploited the possibility of using genetically modified mouse strains to gain insight in molecular mechanisms regulating the inflammatory reaction during *E. coli* mastitis. Importantly, the mouse model of intramammary infections also offers the unique opportunity to screen multiple potential drug molecules for the treatment of mastitis. Nowadays, treatment of bovine mastitis almost completely relies on the use of antibiotics, which is often criticized due to possible implications for animal and human health (Hillerton and Berry, 2005). Alternative approaches are therefore mandatory. Recently, one research group demonstrated the effectiveness of an NF-κB inhibitor to reduce severity of intramammary *S. aureus*

infections in mice (Boulanger et al., 2007). Clearly, strategies that modulate NF-κB activation should also be tested in the intramammary *E. coli* infection model as a first step to reveal if some of these inhibitors hold promise to be implemented in the treatment of bovine coliform mastitis as well.

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SUMMARY - SAMENVATTING

SUMMARY

Nuclear factor (NF)-κB plays a central role in the host immune response towards bacterial infections. The transcription factor regulates the expression of numerous genes of which the products are involved in innate immunity and inflammation. Consequently, NF-κB activation has been implicated in many infectious diseases. However, its involvement in the pathogenesis of intramammary *Escherichia coli* (*E. coli*) infections remains to be elucidated. Therefore, a detailed investigation into the role of NF-κB is essential in mammary gland cells and neutrophils, which are the most important leukocytes within innate immune responses towards *E. coli* mastitis.

Throughout this doctoral thesis, several specific objectives were considered. First, the presence of NF-κB in bovine blood neutrophils and its function under normal conditions and during *in vitro* incubation with *E. coli* bacteria were investigated. Additionally, *in vivo* experiments were performed to gain insight in the mechanisms by which NF-κB mediates the host immune response in mice with *E. coli* mastitis. By using NF-κB reporter mice, kinetics of NF-κB activation were also studied so that critical time points within the infection process could be defined. Finally, the cellular localization of NF-κB activation was determined in order to identify key role players in the innate immune response towards intramammary *E. coli* infections.

The *in vitro* part of the work covers the investigation of NF-κB activation in bovine neutrophils. For the purpose of these experimental studies, an adequate isolation procedure was developed in order to obtain highly purified blood leukocytes. For this cell population, the presence of the important NF-κB subunits p65 and p50 was demonstrated at the mRNA and the protein level with PCR and Western blot analysis, respectively. A possible role for NF-κB in bovine neutrophil survival was also examined in the **first chapter**. The apoptotic process was clearly accelerated upon inhibition of NF-κB. Apoptosis was assessed by means of a dual-color flow cytometric method with staining of exposed phosphatidylserine by annexin V-FITC and of cellular DNA by propidium iodide. In addition, enzyme activity assays for the effector caspases-3 and -7 were employed and morphological analysis by light microscopy was also carried out. It was concluded that a constitutive NF-κB p65 activition in resting bovine neutrophils promotes their survival.

In the **second chapter**, a role for NF-κB in activated bovine blood neutrophils was explored. Upon exposure to *E. coli* bacteria, a fast and transient NF-κB p65 activation was measured using an ELISA in which only active NF-κB complexes are detected with anti-p65 and anti-p50 antibodies, that bind to immobilized oligonucleotide sequences with κB sites. In addition, by means of biological assays it was demonstrated that the NF-κB-dependent expression of the major cytokines IL-1 and IL-6 by bovine neutrophils was increased following phagocytosis of *E. coli* bacteria. It was thus suggested that NF-κB regulates the pro-inflammatory response of activated neutrophils, in part through cytokine production. In marked contrast, an acceleration of neutrophil cell death also occurred as a late event following phagocytosis. Again, phosphatidylserine exposure was demonstrated, while no activity of caspases-3 and -7 was apparent and morphological features were atypical of apoptosis. It was suggested that the early pro-inflammatory capacity and the subsequent apoptosis induction program of bovine neutrophils are both necessary in order to obtain a clear establishment and proper resolution of the acute inflammatory reaction towards *E. coli* bacteria.

The *in vivo* part deals with the study of the involvement of NF-κB in the pathogenesis of intramammary *E. coli* infections in mice. For these experiments, the intramammary inoculation technique was established for mice. In the **third chapter**, NF-κB p65 and p50 activation was observed in mouse mammary glands at 24 and 48 h following inoculation with *E. coli* bacteria. Furthermore, an increased expression of the abovementioned interleukins and TNF-α was found for infected glands. High concentrations of IL-6, TNF-α and the positive acute phase protein SAA were also measured in blood of infected animals. In conclusion, NF-κB seems to mediate both the local pro-inflammatory response and the systemic acute phase reaction towards *E. coli* mastitis in mice. Importantly, the mouse model of intramammary infections also offers the elegant opportunity of using genetically modified animals for the molecular study of inflammatory mediators. As also reported in the third chapter, caspase-1 knockout mice were helpful to the understanding of IL-1β maturation in infected mouse mammary glands. The processing of its proform clearly occurs independently from caspase-1, which is classified as an inflammatory instead of a cell death caspase.

In the **fourth chapter**, *in vivo* experiments with transgenic mice expressing luciferase under control of NF-κB are described. These mice were valuable to explore NF-κB kinetics in order to determine critical time points within the early hours of infection. The *in vivo* imaging

technique has not been applied before in the context of mastitis. NF-κB reporter mice showed a gradual increase in NF-κB activation in mammary glands following intramammary inoculation of *E. coli* bacteria. Maximal activation was achieved at 6 to 8 h post-inoculation, which is much earlier than the time points that were focused on in the first *in vivo* study with wild type animals. Within these early hours, the infection was not yet well established as shown by minor neutrophil influx. Still, the expression of the pro-inflammatory cytokines IL-6 and TNF-α was again increased locally. Immunohistochemical analysis of infected glands utilizing anti-p65 and anti-p50 antibodies was carried out to obtain the cellular localization of the NF-κB activation. By these means, mammary epithelial cells were revealed to be key role players in the establishment of the local pro-inflammatory reaction towards *E. coli* mastitis. Furthermore, p65 appeared to be the major NF-κB family member in this process. During real-time *in vivo* imaging, NF-κB proteins in the liver were found to be activated as well. The observed increased systemic levels of SAA are likely to be explained by this liver NF-κB activation. These results indicate that NF-κB activation is imperative also in the early stages of inflammation both at the local and systemic level.

Taken together, NF-κB plays an important role in the regulation of the bovine neutrophil life span and its mediated immune response towards *E. coli* bacteria. In addition, NF-κB activation is crucial to the establishment of the local inflammatory environment and the systemic acute phase response during intramammary infection with *E. coli* bacteria in mice. Besides neutrophils, an even more prominent role for mammary epithelial cells in local immune responses has been revealed. Furthermore, the observed NF-κB activity in the liver indicates significant events in organs other than the infection hearth. The results from these *in vitro* and *in vivo* studies form a basis for future research concerning the role of NF-κB in the pathogenesis of intramammary *E. coli* infections. This transcription factor might also hold promise as a potential therapeutic target in the treatment and control of mastitis.

SAMENVATTING

De nucleaire factor (NF)-κB speelt een cruciale rol in de immuunrespons van de gastheer tegenover bacteriële infecties. Deze transcriptiefactor reguleert de expressie van een groot aantal genen waarvan de genproducten bijdragen tot inflammatie en de aangeboren immuniteit. NF-κB activatie is dan ook betrokken bij vele infectieuze aandoeningen. Toch is zijn rol in de pathogenese van intramammaire *Escherichia coli* (*E. coli*) infecties nauwelijks bestudeerd. Een grondig onderzoek naar de functie van NF-κB in melkkliercellen en in neutrofielen als de belangrijkste leukocyten binnen de aangeboren immuunrespons tegenover *E. coli* mastitis, is derhalve aangewezen.

In dit doctoraal proefschrift werden verschillende specifieke doelstellingen nagestreefd. Vooreerst werd de expressie van NF-κB in boviene bloedneutrofielen nagegaan en werd de functie ervan bepaald in deze cellen zowel in afwezigheid van een stimulus als bij *in vitro* incubatie met *E. coli* bacteriën. Vervolgens werden *in vivo* experimenten uitgevoerd om inzicht te verwerven in de mechanismen waarmee NF-κB de immuunrespons in muizen met *E. coli* mastitis reguleert. Door gebruik te maken van NF-κB reporter muizen werd de kinetiek van NF-κB activatie bestudeerd, waardoor kritische tijdstippen binnen het infectieproces konden worden gedefiniëerd. Tenslotte werd ook de cellulaire localisatie van de NF-κB activatie bepaald zodat celtypes met een belangrijke functie in de aangeboren immuunrespons tegenover intramammaire *E. coli* infecties werden geïdentificeerd.

In het *in vitro* deel van dit proefschrift wordt het onderzoek rond NF-κB activatie in boviene neutrofielen beschreven. Voor deze studies werd een geschikte isolatieprocedure ontwikkeld om een sterk opgezuiverde neutrofielpopulatie te verkrijgen. In deze geïsoleerde bloedneutrofielen werd de aanwezigheid van de voornaamste NF-κB familieleden p65 en p50 aangetoond op mRNA- en eiwitniveau met respectievelijk PCR en Western blot analyse. In het **eerste hoofdstuk** werd ook een mogelijke rol voor NF-κB in de overleving van boviene neutrofielen bestudeerd. Bij inhibitie van NF-κB werd het apoptose proces onmiskenbaar versneld. Apoptose werd geëvalueerd aan de hand van een flowcytometrische techniek waarbij getransloceerd fosfatidylserine wordt gedetecteerd met behulp van FITC gelabeld annexine V en cellulair DNA met propidium jodide. Daarnaast werden ook testen gebruikt die de enzymactiviteit van de effector caspasen-3 en -7 bepalen terwijl ook een morfologische

analyse werd uitgevoerd. Deze studie leidde tot het besluit dat een constitutieve NF-κB p65 activatie essentieel is voor de overleving van ongestimuleerde boviene neutrofielen.

In een **tweede hoofdstuk** werd de rol van NF-κB in geactiveerde boviene bloedneutrofielen nagegaan. In aanwezigheid van *E. coli* bacteriën werd een snelle en tijdelijke NF-κB p65 activatie gemeten met een ELISA die enkel actieve NF-κB complexen detecteert via anti-p65 en anti-p50 antilichamen, die binden op geïmmobiliseerde oligonucleotidesequenties met κB sites. Verder werd door middel van biologische assays aangetoond dat de NF-κB gemediëerde expressie van de belangrijke cytokines IL-1 en IL-6 toeneemt in boviene neutrofielen na fagocytose van *E. coli* bacteriën. Bijgevolg werd verondersteld dat NF-κB de pro-inflammatoire respons van geactiveerde neutrofielen reguleert, gedeeltelijk door cytokineproductie. Er werd daarentegen ook een versnelling in celdood opgemerkt als een laat gevolg van fagocytose. Opnieuw werd de translocatie van fosfatidylserine aangetoond, ditmaal echter niet in combinatie met caspase-3 en -7 activiteit. Ook de morfologische kenmerken van de cellen waren niet karakteristiek voor het apoptose proces. Zodoende werd gesuggereerd dat de vroege pro-inflammatoire capaciteit en het daaropvolgende apoptose-inductieproces van boviene neutrofielen onmisbaar zijn voor zowel de inductie als de efficiënte beëindiging van de acute inflammatoire reactie tegenover *E. coli* mastitis.

Het *in vivo* deel handelt over de betrokkenheid van NF-κB in de pathogenese van intramammaire *E. coli* infecties bij de muis. Voor deze proeven werd de intramammaire inoculatietechniek op punt gesteld voor muizen. In het **derde hoofdstuk** werd de activatie van NF-κB p65 en p50 vastgesteld in melkklieren van muizen op 24 en 48 uur na inoculatie van *E. coli* bacteriën. Een verhoogde expressie van de bovengenoemde interleukines en TNF-α werd ook aangetoond voor geïnfecteerde klieren. Daarnaast werden hoge waarden voor IL-6, TNF-α en het positieve acute fase eiwit SAA gemeten in bloed van geïnfecteerde dieren. Hieruit werd geconcludeerd dat NF-κB zowel de lokale pro-inflammatoire respons als de systemische acute fase reactie tegenover *E. coli* mastitis in muizen reguleert. Van belang is ook dat het muismodel van intramammaire infecties de elegante mogelijkheid biedt om gebruik te maken van genetisch gemodificeerde dieren voor de moleculaire studie van inflammatoire mediatoren. Zoals ook beschreven werd in het derde hoofdstuk waren de caspase-1 knockout muizen geschikt om inzicht te verwerven in de IL-1β maturatie in geïnfecteerde melkklieren van de muis. De verknipping van de IL-1β provorm gebeurt zonder

twijfel onafhankelijk van caspase-1, een signaalproteïne dat eerder als een inflammatoir dan als een celdood caspase wordt beschouwd.

In het **vierde hoofdstuk** worden *in vivo* experimenten beschreven met transgene muizen die luciferase onder de controle van NF-κB tot expressie brengen. Deze muizen waren van nut in de opheldering van de kinetiek van NF-κB activatie waarbij kritische tijdstippen binnen de vroege fase van het infectieproces konden worden bepaald. De *in vivo* imaging techniek werd nog niet toegepast in de context van mastitis. NF-κB reporter muizen vertoonden een geleidelijke lokale toename in NF-κB activatie na intramammaire inoculatie van *E. coli* bacteriën. Maximale activatie werd bekomen op 6 tot 8 uur na inoculatie, veel vroeger dan de tijdstippen waarop er in de eerste *in vivo* studie met wild-type dieren werden gemeten. In deze eerste uren is de infectie nog beperkt, zoals blijkt uit de nog geringe neutrofielinflux. Toch was de expressie van de pro-inflammatoire cytokines IL-6 en TNF-α opnieuw lokaal toegenomen. Een immunohistochemische analyse waarbij gebruik gemaakt wordt van anti-p65 en anti-p50 antilichamen werd uitgevoerd op geïnfecteerde melklieren om de NF-κB activatie cellulair te lokaliseren. Hierdoor konden mammaire epitheelcellen worden aangeduid als hoofdrolspelers in de inductie van de lokale pro-inflammatoire reactie tegenover *E. coli* mastitis. Bovendien bleek p65 het belangrijkste NF-κB familielid betrokken bij dit proces. Tijdens de real-time *in vivo* imaging waren NF-κB proteïnen in de lever ook duidelijk geactiveerd. De toegenomen systemische waarden van SAA kunnen waarschijnlijk worden verklaard door deze NF-κB activatie in de lever. Deze resultaten wijzen erop dat NF-κB activatie zowel lokaal als systemisch ook vereist is in de vroege stadia van inflammatie.

Algemeen beschouwd kan gesteld worden dat NF-κB een belangrijke rol speelt in de regulatie van de levensduur van boviene neutrofielen en in de immuunrespons van deze cellen tegenover *E. coli* bacteriën. Daarenboven is NF-κB activatie essentieel voor de ontwikkeling van de lokale inflammatoire omgeving en de systemische acute fase respons tijdens intramammaire infecties van *E. coli* bacteriën in de muis. Meer zelfs dan de neutrofielen treden hier de mammaire epitheelcellen op de voorgrond. Bovendien wijst de NF-κB activatie in de lever op een belangrijke invloed op andere organen dan de infectiehaard. De resultaten die in deze *in vitro* en *in vivo* studies werden bekomen, vormen een basis voor verder onderzoek naar de rol van NF-κB in de pathogenese van intramammaire *E. coli* infecties. Ook in de controle en behandeling van mastitis is deze transcriptiefactor mogelijks beloftevol als potentieel therapeutisch doelwit.

DANKWOORD

DANKWOORD

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Sofie

CURRICULUM VITAE

CURRICULUM VITAE

1. Personalia

Family name:	Notebaert
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2. Academic education

2002-2008	Ph.D. training, Faculty of Veterinary Sciences, Ghent University
1999-2001	Licentiate in Biochemistry, Faculty of Sciences, Ghent University
1997-1999	Candidate in Biology, Faculty of Sciences, Ghent University
1991-1997	High School, Sint-Bavohumaniora, Gent (Latin-Mathematics)

3. Professional activities

01/03/2002 - 29/02/2008	Academic assistant and Ph.D. student at Ghent University, Faculty of Veterinary Sciences, Department of Pharmacology, Toxicology, Biochemistry and Organ Physiology
01/01/2002 – 01/03/2002	Scientific researcher at Flanders Institute for Biotechnology and Ghent University, Faculty of Sciences, Department for Molecular and Biomedical Research

4. Bibliography

4.1. Scientific papers

- Regulation of the transcriptional activity of the nuclear factor-κB p65 subunit
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- Pro- and anti-inflammatory response of bovine neutrophils following phagocytosis of *E. coli*
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4.2. Scientific abstracts

- Cell death of bovine granulocytes is induced by selective NF-κB inhibition but is not augmented by TNF-α
  S. Notebaert, E. Meyer
  FEBS Forum for Young Scientists (01-03/07/2003, Brussels, Belgium)
- NF-κB and apoptosis in bovine neutrophils
  S. Notebaert, E. Meyer
  Apoptosis in myeloid cells: Molecular Insights into Disease Processes (19-20/11/2003, Edinburgh, Scotland)
- Retinoids as regulators of the bovine neutrophil life span
  S. Notebaert, E. Meyer
  4th IDF International Mastitis Conference (12-15/06/2005, Maastricht, The Netherlands)
- Multi-color labeling of quarter milk neutrophils from heifers in early lactation for the flow cytometric assessment of functional quality changes
  S. Piepers, S. De Vliegher, S. Notebaert, E. Meyer, G. Opsomer
  BVAC/ABCA: Imaging technology in microbiology: cytometric and molecular approaches (18/11/2005, Brussels, Belgium)
- Preliminary results of a multi-color flow cytometric protocol to determine the viability of bovine neutrophils in milk
  S. Piepers, S. De Vliegher, S. Notebaert, E. Meyer, G. Opsomer
  NMC 45th Annual Meeting (22-25/01/2006, Tampa, Florida, USA)
- Retinol binding protein in urine of cats
  I. Van Hoek, E. Meyer, S. Notebaert, I. Janssens, S. Daminet
  16th European Congress of Veterinary Internal Medicine (14-16/09/2006, Amsterdam, The Netherlands)
- A routine flow cytometric method to assess the viability, apoptosis and necrosis of isolated bovine blood and milk neutrophils
  S. Piepers, S. De Vliegher, S. Notebaert, K. Demeyere, E. Meyer, G. Opsomer
  Cytométrie 2006 (18-20/10/2006, Bruges, Belgium)
- Pro- and anti- inflammatory effects of *in vitro Escherichia coli* phagocytosis by bovine neutrophils
  S. Notebaert, E. Meyer
  Heifer Mastitis Conference 2007 (24-26/06/2007, Ghent, Belgium)

4.3. Scientific events

- International NF-κB symposium: NF-κB regulation, gene expression and disease
  (04-08/07/2001, Ghent, Belgium)
- Mammary Gland, Health and Disease: Immune cells and bioactive substances in function of susceptibility and spreading of infections in humans and animals
  (13-14/09/2002, Ghent, Belgium)
- FEBS Forum for Young Scientists
  (01-03/07/2003, Brussels, Belgium)
- FEBS Meeting on Signal Transduction: from membrane to gene expression, from structure to disease
  (03-08/07/2003, Brussels, Belgium)
- Biochemical Society: Apoptosis in Myeloid Cells: Molecular Insights into Disease Processes
  (19-20/11/2003, Edinburgh, Scotland)
- 4th IDF International Mastitis Conference
  (12-15/06/2005, Maastricht, The Netherlands)
- Heifer Mastitis Conference 2007
  (24-26/06/2007, Ghent, Belgium)

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