Inflammatory responses in stress and trauma

Impact on Toll-like receptor 4 signalling

Bård S. Lundeland

Institute of Clinical Medicine
Faculty of Medicine
University of Oslo
Oslo

and

Protection Division
Norwegian Defence Research Establishment
Kjeller

and

Department of Anaesthesiology
Akershus University Hospital
Lørenskog
Norway

2012

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1458

ISBN 978-82-8264-492-1

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

Produced in co-operation with Akademika publishing.
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Acknowledgements

The present thesis is based on cooperation between the University of Oslo, the Norwegian Defence Research Establishment (FFI) and Akershus University Hospital (Ahus), and took place in the period from 2007 to 2012.

Dr. med. Per Vaagenes initiated the project and introduced me to a scientific group at FFI dedicated to research related to stress and inflammation. During the first part of this work Per was my main supervisor, and I am very grateful for inspiring discussions, his friendly attitude and irrepressible optimism, even when challenges were encountered.

I am also indebted to my supervisors dr. med. Yngvar Gundersen and dr. med. Per-Kristian Opstad. It has been inspiring to work close with so experienced scientists, and always easy to ask for advice. During the last part of the work Yngvar was the main supervisor, and I am very thankful for his wise guidance and optimistic support. Yngvar also introduced me to international communities dedicated to our research field, and our trips to congresses have been academically interesting and most pleasant.

I would like to thank Ingjerd Thrane for a very nice cooperation and expert technical assistance. Her practical overview and good preparation have been of great importance. Thanks to Anne Myran, Anita Larsen, Laila Drogseth, Marianne Aamodt and Sigrun Sterri for the help during the war surgery course.

Many thanks go to Richard W. Olaussen and Yan Zhang for the close collaboration, particularly regarding flow cytometric analysis. I would also like to express my thanks to Helene Østerholt for a very pleasant cooperation.

I am grateful for the support from my colleagues at the Department of Anaesthesiology at Ahus, especially Tor Martin Hoel and Nils Smith-Erichsen. I would also like to thank Tom Øresland, Janet M. Blatny and Anna B. Frengen for the support, and Øystein H. Horgmo for help with the illustrations.

I am thankful to the cadets from the Norwegian Military Academy who participated in this research project and to Norwegian Defence Medical Service for the cooperation in connection with the war surgery course.

At last, but not least, I am deeply grateful for the patience and support from my wife, Mette, and our lovely children Marte, Elise and Bendik.

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Lundeland B, Gundersen Y, Opstad PK, Thrane I, Zhang Y, Olaussen RW, Vaagenes P. One week of multifactorial high-stress military ranger training affects Gram-negative signalling. *Scandinavian Journal of Clinical & Laboratory Investigation* (In press).

Abbreviations

CD14	cluster of differentiation 14
CLR	C-type lectin receptor
CXCL8	CXC ligand 8 (IL-8)
DAMP	danger-associated molecular pattern
ELISA	enzyme-linked immunosorbent assay
FFA	free fatty acid
FITC	fluorescein isothiocyanate
FSC	forward scatter
HA	hyaluronic acid
HMGB1	High mobility group box 1
HSP	heat shock protein
IFN	interferon
IL-1β, 6, 10	interleukin-1β, 6, 10
IRF3	IFN regulatory factor 3
LBP	LPS-binding protein
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MD-2	myeloid differentiation protein-2
MFI	median fluorescence intensity
MODS	multiple organ dysfunction syndrome
MyD88	myeloid differentiation primary response gene 88
NEFA	non-esterified fatty acid
NF-κB	nuclear factor kappa B
NLR	Nod-like receptor
NOD	nucleotide-binding oligomerisation domain
PAMP	pathogen-associated molecular pattern
PE	phycoerythrin
PRR	pattern-recognition receptor
PUFA	polyunsaturated fatty acid
RIG	retinoic acid-inducible gene
RLR	RIG-I-like receptor
RNS	reactive nitrogen species
ROS	reactive oxygen species
SFA	saturated fatty acid
SIRS	systemic inflammatory response syndrome
SSC	side scatter
TIR	Toll/IL-1 receptor
TIRAP	TIR-associated protein

TLR	Toll-like receptor
TNF-α	tumour necrosis factor-α
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter-inducing IFN-β
UFA	unsaturated fatty acid
URTI	upper respiratory tract infection

Introduction

Inflammation

Inflammation is of vital importance to resist microorganisms and induce repair of damaged tissues, and references to this process can be found in the writings of some thousands of years old cultures. Several medical terms that described red, hot and swollen skin lesions were used, e.g. in writings from Mesopotamia *ummu* described "the hot thing", related to local inflammation or fever, whereas the Greek term for inflammation was *phlegmonè*, "the fiery thing" (Ryan and Majno, 1977). Although ancient medical texts contain references to the inflammatory process, the Roman doctor Cornelius Celsus seems to be the first to define the clinical symptoms (Medzhitov, 2010). In the first century AD he described the four cardinal signs of inflammation: *rubor et tumor cum calore et dolore* (redness and swelling with increased temperature and pain). Much later the fifth sign, *functio laesa* (reduced function), was added by the German scientist Rudolf Virchow in his *Cellular Pathology* (1858).

Inflammation is the body´s initial response to tissue stress or injury caused by microbial, mechanical or chemical stimuli, and at a basic level the main purpose of acute inflammation is to ensure a coordinated delivery of immune cells and blood plasma components to a focus of infection or tissue injury (Davies and Hagen, 1997; Medzhitov, 2008). It involves recognition of danger by specific sensors, recruitment of relevant cells, elimination of the causative agent and repair of damaged tissues with subsequent resolution of inflammation and return to homeostasis (Barton, 2008). However, to be protective to the host, the inflammatory process must be appropriately tuned in order to avoid overstimulation and collateral tissue damage.

Acute major insults, infectious or traumatic, may cause an exaggerated response where mediators produced at a primary focus leak to the systemic circulation. The consequences may be fatal with development of critical illness including shock, organ dysfunction and tissue hypoxia. The systemic inflammatory response syndrome (SIRS), a clinical condition that represents loss of local control of inflammation, was defined at a consensus conference in 1991 (Bone et al., 1992). It describes an acute generalised inflammatory process independent of causative agent, and implies that two or more of the following criteria are met: 1) body temperature > 38 °C or < 36 °C; 2) heart rate > 90 beats/min; 3) hyperventilation, manifested

by respiratory rate > 20 breaths/min or by $PaCO_2$ < 4.3 kPa; 4) white blood cell count > 12 x 10^9 cells/l or < 4 x 10^9 cells/l, or the presence of more than 10 percent immature neutrophils (band forms).

When SIRS is a result of an infectious process, it is termed sepsis. SIRS/sepsis represents a continuum of physiological derangements, and in the more severe end of the spectrum are patients classified to have the multiple organ dysfunction syndrome (MODS). This condition is a major cause of death in patients treated in intensive care units, and is defined as the presence of dysfunction in 2 or more organs or organ systems in an acutely ill or traumatised patient such that homeostasis cannot be maintained without intervention (Baue, 2006; Hietbrink et al., 2006; Wang and Ma, 2008).

However, the pathophysiological picture related to severe trauma and infections is not limited to pro-inflammatory changes. To restore homeostasis an anti-inflammatory response, with release of anti-inflammatory mediators and inhibition of several immune cells functions, is initiated. Compensatory anti-inflammatory response syndrome (CARS), described by Bone et al. (1997), may become an additional threat to the host with increased risk of post-traumatic or secondary infections. Some authors claim that the syndrome follows SIRS/sepsis in a two-wave time-related pattern, whereas others consider the pro- and anti-inflammatory events to occur concomitant (Hietbrink et al., 2006; Adib-Conquy and Cavaillon, 2009; Christaki et al., 2011).

It should be emphasised that the significance of systemic inflammatory activity is not restricted to acute insults. Even low-grade chronic systemic inflammation has been associated with serious conditions, e.g. type 2 diabetes and cardiovascular diseases (Medzhitov, 2008).

Innate immunity

Inflammation is a consequence of activation of the immune system, which traditionally has been divided in two parts. The origin of the first line of defence, the innate branch, probably dates back to the creation of single cell organisms more than 3.5 billion years ago, whereas the adaptive branch, the second line defence system, emerged in vertebrates around 500 million years ago (Cooper and Herrin, 2010).

Previously, innate immunity was considered to be rather unspecific. It encompasses all parts of the immune defenses that lack immunologic memory, including cellular (e.g. monocytes/macrophages, neutrophils) and humoral (e.g. cytokines, complement) factors, but sometimes even physical, chemical and microbiological barriers are included (Delves and Roitt, 2000; Parkin and Cohen, 2001). However, the discovery of germ-line encoded pattern recognition receptors (PRRs) sensing pathogen associated molecular patterns (PAMPs) from groups of microorganisms has unveiled a rather sophisticated and specific system (Kawai and Akira, 2010). In addition, not only exogenous PAMPs are recognized by PRRs, also endogenous molecules liberated by stressed or injured cells or tissues are sensed. As a group these molecules are called alarmins or danger-associated molecular patterns (DAMPs; Christaki et al., 2011).

The response from the innate system is quick and independent of previous exposure, but sometimes causes collateral damage through lack of specificity. In contrast, the action of the antigen-specific adaptive system is precise but delayed, depending on clonal expansion of T and B lymphocytes, and is characterised by memory, which causes a quicker and more vigorous response to repeated exposure (Parkin and Cohen, 2001).

Mononuclear phagocytes and neutrophils are crucial cellular components of the innate immune response. Monocytes are mononuclear phagocytes within the blood stream where they circulate for 1 to 3 days (Cavaillon and Adib-Conquy, 2005). They have retained proliferative capacity and can differentiate into a variety of tissue resident macrophages throughout the body, as well as specialised cells such as dendritic cells and osteoclasts (Gordon and Taylor, 2005; Dale et al., 2008). The cells of the innate immune system serve several important functions, e.g. production of inflammatory mediators, phagocytosis and antigen-presentation.

Immune cells, and especially the monocytes/macrophages, express PRRs on the cell surface and intracellular. Over the last few years several PRR families have been described, of which the Toll-like receptors (TLRs) are most extensively studied and considered to be the primary sensors of pathogens (Kumar et al., 2011). TLRs are present on the cell surface and in the lumen of intracellular vesicles (Kawai and Akira, 2009). Other important PRR families include membrane-bound C-type lectin receptors (CLRs) and cytosolic proteins such as NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) (Kawai and Akira, 2011). The

different PRRs are activated by diverse but overlapping microbial (PAMPs) and non-microbial (alarmins) structures, and down-stream cascades of enzymatic reactions ultimately lead to expression of inflammatory genes.

Cytokines are important protein end-product mediators orchestrating the immune response by autocrine, paracrine and endocrine actions. Important effects of these mediators in a focus of infection or tissue injury are vasodilation, increased vascular permeability and formation of an inflammatory exudate containing plasma proteins and leukocytes, mainly neutrophils (Medzhitov, 2008). Plasma cascades, including the complement system, the coagulation system, the fibrinolytic system and the kallikrein–kinin system, are also important contributors to the innate immune response (Castellheim et al., 2009).

In normal conditions the phagocytes contribute to homeostasis by removal of apoptotic cells, whereas during infection they have high capacity to engulf microbes in vacuoles called phagosomes. Killing and destruction of the microorganisms is facilitated by the fusion of phagosomes with pre-formed lysosomes containing degradative enzymes, and the production of cytotoxic reactive oxygen species (ROS) and reactive nitrogen species (RNS; Smith, 1994). To induce adaptive immune responses antigens from the microbes are presented to lymphocytes via peptide-MHC class II complexes on the cell surface (Schenten and Medzhitov, 2011), representing a close link between the two branches of the immune system.

Toll-like receptors

It was the german scientist Christiane Nüsslein-Volhard who gave name to this PRR family (Anderson et al., 1985). Her group was studying gene mutations in fruit flies, and found weird-looking fly larva with under-developed ventral portion of the body. “Das war ja toll (*that is amazing/great*)!” was her spontaneous comment when seeing the effect on the larva, and she named the mutated gene Toll (Hansson and Edfeldt, 2005). The role of Toll in immune defence was described by Jules Hoffmann´s group (Lemaitre et al., 1996). They found that flies had dramatically reduced survival after fungal infection if they had mutations in the Toll signalling pathway. One year later a human homologue of the *Drosophila toll*

Table 1: *PAMP detection by TLRs.*

Species	PAMPs	TLR Usage
Bacteria, mycobacteria	LPS	TLR4
	Lipoproteins, LTA, PGN, lipoarabinomannan	TLR2/1, TLR2/6
	Flagellin	TLR5
	DNA	TLR9
	RNA	TLR7
Viruses	DNA	TLR9
	RNA	TLR3, TLR7, TLR8
	Structural protein	TLR2, TLR4
Fungi	Zymosan, β-glucan	TLR2, TLR6
	Mannan	TLR2, TLR4
	DNA	TLR9
	RNA	TLR7
Parasites	tGPI-mutin (*Trypanosoma*)	TLR2
	Glycoinositolphospholipids (*Trypanosoma*)	TLR4
	DNA	TLR9
	Hemozoin (*Plasmodium*)	TLR9
	Profilin-like molecule (*Toxoplasma gondii*)	TLR11

Modified from Kawai and Akira (2011), with permission from Elsevier.

protein was cloned and characterised (Medzhitov et al., 1997). A family of proteins structurally related to *Drosophilia toll* has subsequently been described, collectively referred to as TLRs (Takeda et al., 2003).

Until today, 10 and 12 functional TLRs have been described in human and mouse, respectively (Kawai and Akira, 2011; table 1). The TLRs are type I transmembrane proteins composed of ectodomains containing leucine-rich repeats responsible for the recognition of ligands, transmembrane domains and intracellular domains required for downstream signal transduction. Each TLR has a distinct function in terms of PAMP recognition and immune responses, as shown in studies of knock-out mice. They are located on the cell surface where they identify lipids, lipoproteins and proteins (TLR1, 2, 4, 5, 6, 11), or intracellular in membranes of endosome, lysosome and the endoplasmic reticulum predominantly recognising microbial nucleic acids (TLR 3, 7, 8, 9; Kawai and Akira, 2010). TLRs are found primarily on cells of the immune system, but are also expressed on many other cell types, including epithelial cells, endothelial cells, adipocytes, hepatocytes and myocytes (Kariko et al., 2004; Francaux, 2009; Schaffler and Scholmerich, 2010; Foncea et al., 2012).

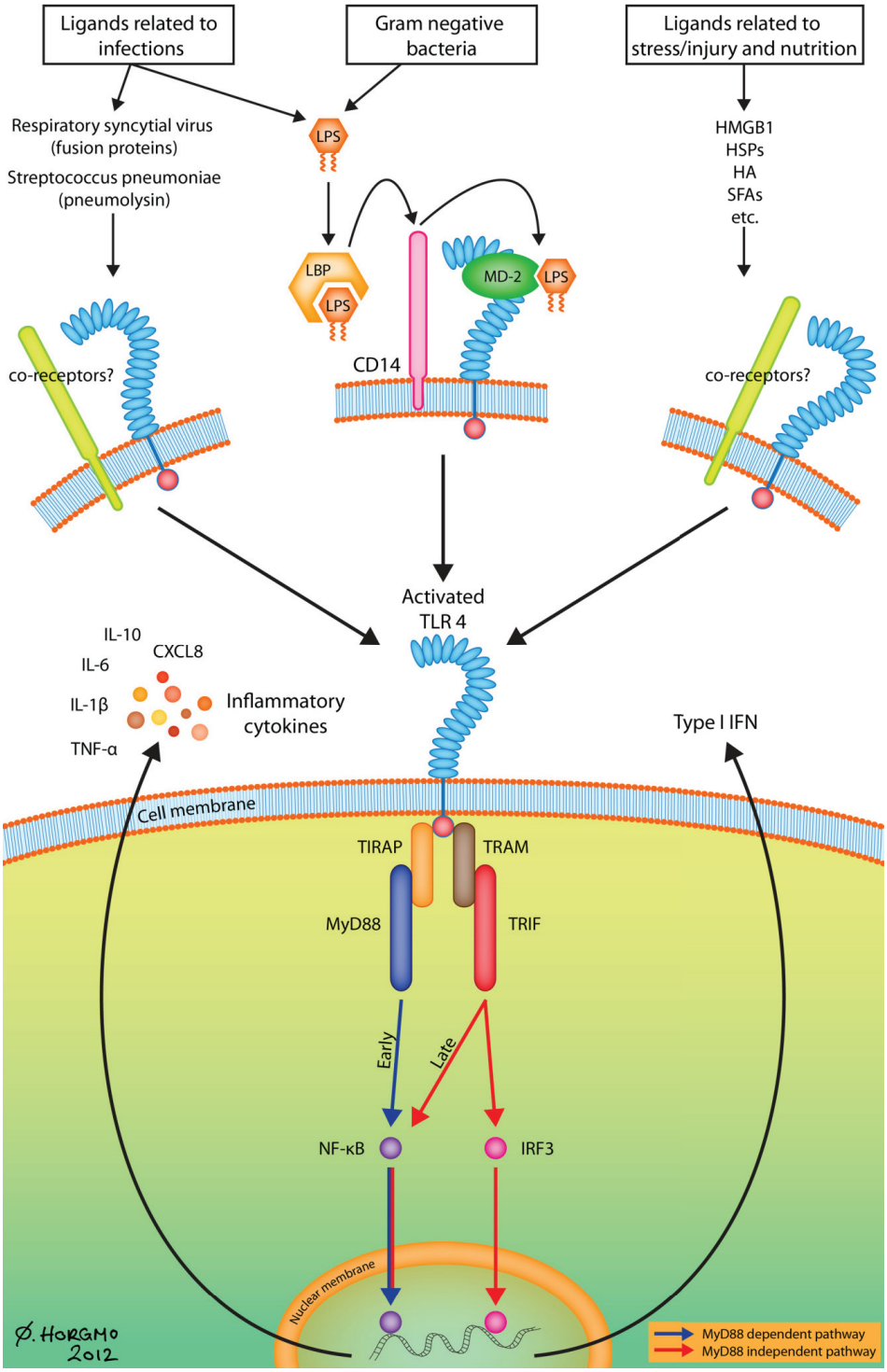
Ligands related to infections
Gram negative bacteria
Ligands related to stress/injury and nutrition
Respiratory syncytial virus (fusion proteins)
Streptococcus pneumoniae (pneumolysin)
LPS
HMGB1
HSPs
HA
SFAs
etc.
LBP
LPS
MD-2
LPS
CD14
co-receptors?
co-receptors?
Activated TLR 4
IL-10
CXCL8
IL-6
IL-1β
TNF-α
Inflammatory cytokines
Type I IFN
Cell membrane
TIRAP
TRAM
MyD88
TRIF
Early
Late
NF-κB
IRF3
Nuclear membrane
Ø. HORGMO 2012
MyD88 dependent pathway
MyD88 independent pathway

Figure 1: *Simplified illustration of TLR4 signalling in monocytes/macrophages. LPS from Gram-negative bacteria are transported in plasma bound to LBP (top middle). CD14 facilitates the transfer of LPS to the TLR4/MD-2 complex. In addition, other ligands related to infections (top left) or stress/injury (top right) are able to activate TLR4. The signal is transmitted into the cell and downstream signalling is induced via two main pathways. Initially, the MyD88 dependent pathway results in early-phase activation of NF-κB with subsequent production of inflammatory cytokines. In addition, a late-phase activation of NF-κB is caused via the MyD88 independent pathway. Also, IRF3 activation, that mediates production of type I interferons, is regulated by the MyD88 independent pathway.*

Abbreviations: TLR4; Toll-like receptor 4, LPS; lipopolysaccharide, LBP; LPS-binding protein, HMGB1; High mobility group box 1, HSPs; heat shock proteins, HA; hyaluronic acid, SFAs; saturated fatty acids, TNF-α; tumour necrosis factor-α, IL-1β, 6, 10; interleukin-1β, 6, 10, CXCL8; CXC ligand 8, IFN; interferon, TIRAP; TIR-associated protein, TRAM; TRIF-related adapter molecule, MyD88; myeloid differentiation primary response gene 88, TRIF; TIR-domain-containing adapter-inducing IFN-β, NF-κB; nuclear factor kappa B, IRF3; IFN regulatory factor 3.

They have been described to have an important role in the linking of innate and adaptive immunity, e.g. in inducing dendritic cell maturation and providing cytokine stimuli for T-lymphocyte activation (Pasare and Medzhitov, 2005; Mills, 2011).

Toll-like receptor 4

Bruce Beutler´s group discovered TLR4 as the lipopolysaccharide (LPS) receptor (Poltorak et al., 1998), and "for discoveries concerning the activation of innate immunity" Beutler and the aforementioned Jules Hoffmann shared half of the Noble Prize in Medicine and Physiology for 2011.

Endotoxin was originally described by Richard Pfeiffer and Robert Koch in 1892, when they showed that heat-killed cholera bacteria retained toxic potential and thus that the toxin was an

integral part of the bacteria (Opal, 2002; Billiar, 2007). Due to the molecular structure the term “lipopolysaccharide” was later applied to this common component of the outer cell wall of Gram-negative bacteria. LPS is made up of hydrophilic polysaccharides and hydrophobic lipid A, of which the latter is crucial for immune activation (Takeda, 2010). It is a potent inducer of innate immune responses, activating various immune cells to liberate mediators, e.g. inflammatory cytokines and ROS, and causes increased phagocytosis and chemotaxis (Tamandl et al., 2003). Endotoxin alone is sufficient to induce a picture of septic shock in human, and it has been speculated if this molecule might be a “motor” of SIRS even in absence of Gram-negative infection (Taveira da Silva et al., 1993; Johnson et al., 2004; Carcillo, 2006).

In plasma LPS is transported in a complex with LPS-binding protein (LBP), which facilitates the association of LPS with another protein: CD14 (Schutt, 1999). This is a glycosylphosphatidylinositol (GPI)-anchored protein that is expressed especially on the surface of peripheral blood monocytes and tissue macrophages (Ziegler-Heitbrock and Ulevitch, 1993; Tamandl et al., 2003). It also exists in a soluble form which can be used to activate cells lacking CD14 (Salomao et al., 2009). Membrane bound CD14 has no cytoplasmic region and therefore cannot transfer the signal into the cell, but facilitates the transfer of LPS to the MD-2 molecule, which in addition to LPS-binding probably is required for surface expression of TLR4 (Akashi-Takamura and Miyake, 2008; Takeda, 2010). Two copies of the TLR4-MD-2-LPS complex create a receptor multimer that initiates intracellular signalling (Kawai and Akira, 2010).

The intracellular part of TLR4, the Toll–interleukin 1 receptor (TIR) domain, is shared with the interleukin (IL)-1 receptor family and binds various adapter proteins inducing activation of two main pathwas (Lin and Yeh, 2005; Kawai and Akira, 2010). The myeloid differentiation factor 88 (MyD88)-dependent pathway generates production of inflammatory cytokines via activation of the transcription factor nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs). Signalling through the alternative MyD88-independent (TRIF-dependent) pathway requires that TLR4 undergoes endocytosis, is trafficked to the endosome and cause activation of the transcription factor interferon (IFN) regulatory factor 3 (IRF3), as well as late-phase activation of NF-κB and MAPK, and subsequently production of type I IFN in addition to inflammatory cytokines.

In addition to LPS signalling, TLR4 is also involved in the recognition of other microbial molecules, e.g. proteins derived from *respiratory syncytial virus* and *Streptococcus pneumonia* (Kawai and Akira, 2010). However, not only pathogens induce immune activation through this receptor. Endogenous host molecules liberated by injury and stress are able to trigger inflammatory responses by means of TLR4. Among these molecules are heat-shock proteins (HSPs), High mobility group box 1 (HMGB1), saturated fatty acids (SFAs) and components of the extracellular matrix (Lee et al., 2001; Mollen et al., 2006). Thus, TLR4 represents a common molecular basis for immune activation caused by infection and stress/injury (Lorne et al., 2010).

Inflammatory cytokines

Cytokines are small proteins that play a key role in orchestrating the immune response. Although the word "cytokine", derived from Greek roots, means "to put cells into motion", these molecules have a more complex bioactivity (Suzuki et al., 2002). They normally act in an autocrine or paracrine manner, but in connection with pathological conditions and stress elevated production may cause spillover into the circulation and give effects at distant sites (Kim and Deutschman, 2000). The target cells are affected through binding to specific receptors initiating intracellular second messenger cascades. Among the most important cytokines are the primary pro-inflammatory mediators tumour necrosis factor-α (TNF-α) and IL-1β, the secondary pro-inflammatory mediators IL-6 and CXC ligand 8 (CXCL8 (IL-8)), and the anti-inflammatory IL-10 (Gogos et al., 2000; Tamandl et al., 2003). These mediators are essential in activation and regulation of the innate immune response, including initiation of coagulation via induction of tissue-factor expression, and involved in the modulation of adaptive immunity (Castellheim et al., 2009; Netea et al., 2010).

TNF-α and IL-1β

TNF-α and IL-1β have a similar function, despite having different structure, receptors and intracellular signalling pathways (Kim and Deutschman, 2000; Tsukamoto et al., 2010). They

have multiple overlapping and synergistic effects, e.g. both are able to induce a septic shock-like state in animals upon intravenous administration (Waage and Espevik, 1988; Hack et al., 1997). Activated monocytes/macrophages are the main producers of these cytokines, but other cells are also involved (Kim and Deutschman, 2000; Gabay et al., 2010). TNF-α is expressed as a membrane protein which is cleaved by a metalloproteinase, whereas IL-1β is produced as a pro-protein that is activated via inflammasome (NLR-activated) or alternative pathways (Kim and Deutschman, 2000; Netea et al., 2010).

These cytokines are the most prominent pro-inflammatory mediators, central in starting off innate immune responses, including production of cytokines, adhesion molecule expression and growth stimulation, and triggers the acute phase response, including fever, acute protein synthesis, anorexia and somnolence (Hehlgans and Mannel, 2002; Gabay et al., 2010). After a challenge with a low dose of LPS, TNF-α is the first cytokine to appear in plasma, with peak levels at about 90 min, followed by IL-1β which reaches maximal levels after 2 to 3 hours (Hack et al., 1997).

In addition to inflammatory characteristics, TNF-α is also involved in the regulation of apoptosis, and, as the name indicates, a tumour limiting effect has been described in some patients with sarcoma, carcinoma and lymphoma (Hehlgans and Mannel, 2002; Bertazza and Mocellin, 2008). TNF-α may also modulate the expression of TLR4 (Lin and Yeh, 2005).

Their important proximal places in acute inflammation have made TNF-α and IL-1β tempting targets for pharmaceutical interventions in connection with sepsis. Even though promising results of novel therapies have been described in animal models of sepsis, the results in clinical trials have been disappointing (Bone, 1996; Dyson and Singer, 2009). Possibly, these results reflect the complexity in this syndrome, with fluctuating pro- or anti-inflammatory dominance, depending on time, microbes, genetics and the patient´s previous diseases. However, in chronic inflammatory diseases, e.g. rheumatoid arthritis and inflammatory bowel diseases, blockers against TNF-α and IL-1β have been shown effective and is in clinical use (Molto and Olive, 2010; Perrier and Rutgeerts, 2011; Thalayasingam and Isaacs, 2011).

IL-6

IL-6 is a cytokine with both pro- and anti-inflammatory properties that is produced by different cell types, e.g. monocytes/macrophages, neutrophils, lymphocytes, endothelial cells and smooth as well as contracting muscle cells (Kim and Deutschman, 2000; Pedersen et al., 2001; Mathur and Pedersen, 2008). It is produced in response to PAMPs as LPS, but also as a secondary cytokine in response to TNF-α and IL-1β. In connection with exercise it is produced locally in contracting skeletal muscles, and the plasma concentration can increase 100-fold after a marathon race (Pedersen et al., 2001). IL-6 is described to affect TLR4 expression, e.g. human monocytes challenged with IL-6 showed up-regulated TLR4 cell surface protein (Tamandl et al., 2003). It is implicated in the induction of the acute-phase response, including production of hepatocyte-derived proteins like CRP, and infusion of IL-6 into humans causes systemic responses as fever, but not shock and capillary-leakeagelike syndrome as observed with TNF-α and IL-1β (Pedersen et al., 2001). Compared with other circulating cytokines, levels of IL-6 best correlate with outcome in patients with the sepsis syndrome, and it has been associated with injury severity and mortality after trauma (Hack et al., 1997; Stensballe et al., 2009).

IL-6 has a dual role in the regulation of inflammation, and among the anti-inflammatory qualities are the inhibitory effect on TNF-α and IL-1β production (Pedersen, 2011). It also favors the release of anti-inflammatory mediators, like IL-10, IL-1 receptor antagonist, soluble TNF receptor and cortisol (Adib-Conquy and Cavaillon, 2009).

CXCL8 (IL-8)

Recruitment of neutrophils and mononuclear cells to a focus of infection or injury is a salient feature of inflammation. This process is controlled by a group of structurally related cytokines known as chemokines, abbreviated from chemotactic cytokines, whereof CXCL8 is an important member (Hack et al., 1997; Suzuki et al., 2002). CXCL8 is mainly a secondary pro-inflammatory cytokine, synthesised by a variety of leukocytic cells (monocytes/macrophages, neutrophils, lymphocytes) and non-leukocytic cells (endothelial cells, fibroblasts, epithelial cells) in response to different stimuli, such as pro-inflammatory cytokines and environmental

changes (hypoxia, reperfusion, hyperoxia; Mukaida, 2003). It facilitates chemotaxis, stimulates the expression of adhesion molecules and several functions of neutrophils, like degranulation and respiratory burst (Kim and Deutschman, 2000; Kobayashi, 2008). In animals exposed to bacteremia and endotoxemia, IL-8 concentrations have been correlated with severity of the insult, and in injured patients elevated values have been described in those who later developed MODS (Van Zee et al., 1991; Kim and Deutschman, 2000).

IL-10

IL-10 is a major anti-inflammatory cytokine. Almost all leukocytes are able to synthesise this protein, but the most important sources in vivo are monocytes/macrophages and T lymphocytes (Sabat et al., 2010). Monocytes/macrophages are also the main target of this cytokine, and it suppresses nearly all the pro-inflammatory qualities of these cell-types, e.g. LPS-induced production of TNF-α, IL-1β, IL-6, CXCL8 etc. In addition, it inhibits antigen-presentation and enhances the release of anti-inflammatory mediators like IL-1 receptor antagonist and soluble TNF-α receptors. IL-10 is produced later than the pro-inflammatory cytokines, involving activation of the MyD88-independent pathway, and its main task is to limit the immune response and minimise collateral damage (Rossol et al., 2011). It is described as the most important cytokine involved in CARS, and high levels have been correlated with multiple organ dysfunctions and mortality (Ward et al., 2008).

Aims of the study

Over the past years detailed knowledge has been achieved regarding recognition of molecules associated with pathogens and sterile cell stress/tissue injury, signalling pathways and mediators involved in the regulation of immune responses. However, diverse stressors, e.g. trauma, hypo- and hyperthermia, strenuous exercise, psychological strain, sleep deprivation or energy deficit, modulate immunological responses (Munford and Pugin, 2001; Lenz et al., 2007). Thus, in the stressed host, the reaction to pathogens and alarmins may be affected and cause increased risk for infections and complications, both locally and systemically (Kurz et al., 1996; Nieman, 1997; Medzhitov, 2008; Lowry, 2009). The aim of the present work was to achieve further knowledge about how different stressors affect expression of and signalling through the LPS-receptor TLR4.

Ad paper I

In this study we focused on TLR4 signalling related to trauma-associated endotoxin tolerance. We used a porcine model of standardised gunshot injury and peri-operative stress. The main purpose of the study was to investigate if trauma affects TLR4 expression on monocytes, and if the development of endotoxin tolerance is regulated on this level. In addition, we investigated if trauma caused release of measurable levels of HMGB1 in the blood circulation, and if this protein was related to trauma-related endotoxin tolerance.

Ad paper II

The focus of this paper was to explore how clinically relevant hypo- and hyperthermia affect Gram-negative immune signalling. We measured the plasma concentrations of selected pro- and anti-inflammatory cytokines and the surface expression of the TLR4 protein on $CD14^+$ monocytes after *ex vivo* incubation of human whole blood at different temperatures. Also, to elucidate if the cytokine profile was affected by altered temperature, the balance between pro- and anti-inflammatory cytokines was calculated.

Ad paper III

Previously, our group has described increased cytokine release by circulating leukocytes after stimulation with LPS during a one week military ranger-training course with heavy physical and psychological challenges and restrictions of sleep and food intake. The aim of this study was, by using the same model, to investigate how prolonged multifactorial stress influences the expression of TLR4 on monocytes, and test the hypothesis that the increased cytokine response to LPS could be regulated by TLR4. In addition, we measured the impact of multifactorial stress on plasma liberation of free fatty acids.

Methodological considerations

Ethical considerations

The studies in the present thesis were conducted in accordance with the World Medical Association´s Declaration of Helsinki.

The animals in study I were handled according to the Animal Welfare Act and statutes from the Norwegian Ministry of Agriculture.

The Regional Committee for Medical Research Ethics approved study II and III. All of the participants gave their informed written consent beforehand and were free to withdraw at any time without negative consequences. A physician without connection to the research program continually followed the participants in study III, and could, if necessary, refuse further course participation.

The course in traumatology and war surgery

A group of senior trauma surgeons have developed this course, which they arrange annually in cooperation with the Norwegian Defence Medical Service (Gaarder et al., 2005). The main focus is to educate surgeons in the principles of damage control surgery, and the Norwegian Medical Association has made the course mandatory for general surgeons. In addition, other health professionals (e.g. anaesthesiologists and specialised nurses) and non-health professionals (e.g. special soldiers and special police officers) participate in the course. A number of studies have been performed in connection with these courses, e.g. by Gundersen et al. (2005; 2007; 2008). Thus, the animals used in the education to improve the treatment of traumatised patients also contribute to increased scientific knowledge.

Experimental description

Before beginning the experiment, the pigs were fully anaesthetised and remained so until they were euthanised at the end of the surgical training session. To ensure absence of pain, the pigs also received epidural anaesthesia, and the adequacy of analgesia was continuously monitored by veterinarians.

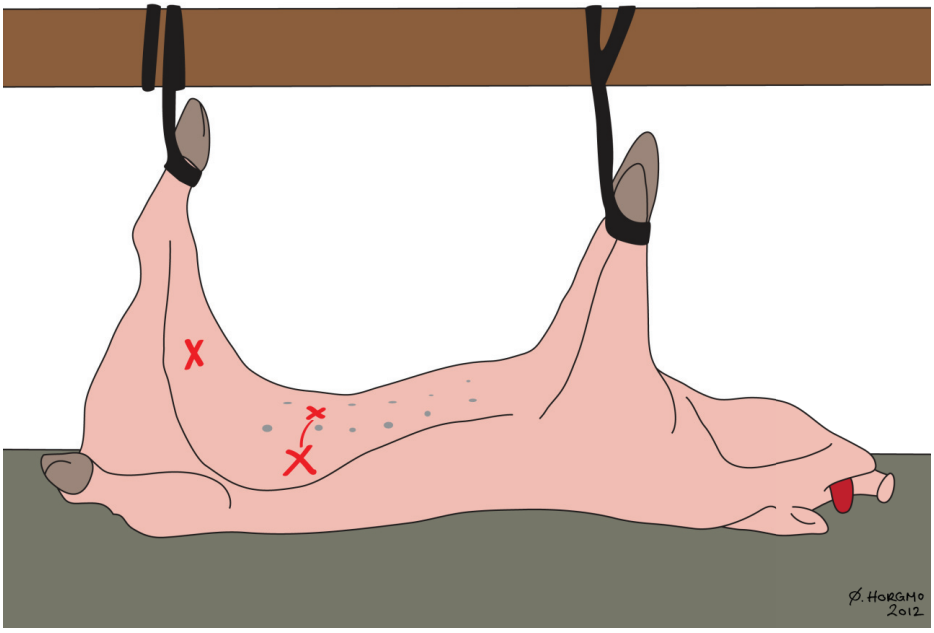

Figure 2: *Anaesthetised pig at the firing range before shooting. Entrance point for the gunshot is marked at the right thigh. On the abdomen marked entrance point and wanted exit point for the pistol shot.*

A detailed description of the experiment is found in paper I. Ear veins were cannulated for fluid administration and medication, and the animals were orally intubated and, if necessary, ventilated with room air. The femoral artery was isolated and cannulated for haemodynamic monitoring and blood sampling. Thereafter, the animals were transported to a nearby firing range, and according to the protocol they received one rifle shot from a distance of 25 m in

the thigh, and one superficial pistol shot from short distance against left upper abdomen. Both entrance points, and also wanted exit point for the abdominal shot, were marked beforehand to ensure a standardised injury and not to injure the liver and large abdominal vessels (Figure 2). First aid treatment was started directly upon shooting, and included dressing of wounds, compression of bleeding, control of ventilation (if necessary) and intravenous fluid resuscitation.

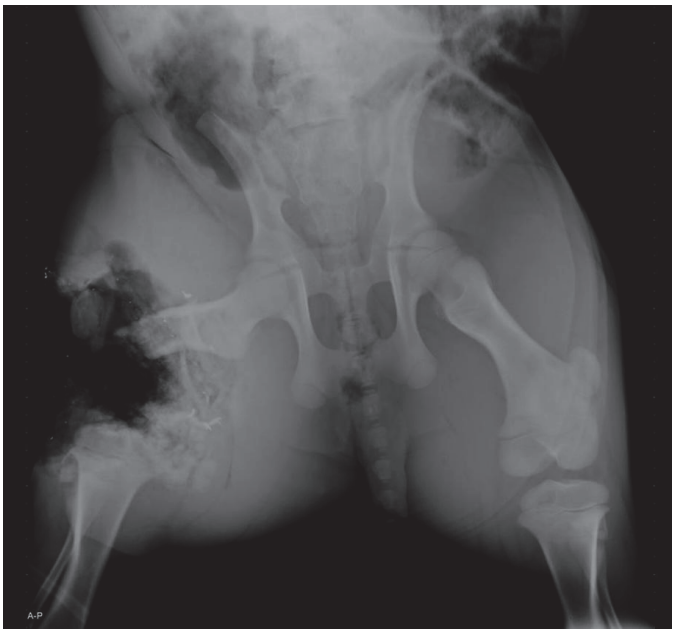

Figure 3: *X-ray (anterior-posterior view) of an animal after shooting. Extensive bone and soft tissue damages in the right thigh and abdominal free gas are seen.*

The animals were then transported to a nearby field hospital where surgeons at first controlled the thigh for on-going bleeding and thereafter, within 10 to 15 min after shooting, proceeded to damage control laparotomy. Intestinal perforations, directly caused by the bullet or indirectly by the pressure wave, were immediately closed. Thereafter, the peritoneal cavity was rinsed with saline solution. NaCl 0.9% and Dextran 70/NaCl were infused as needed to compensate for blood loss and for maintaining systolic blood pressure above 90 mmHg. Transfusions of blood or blood substitutes were not given. The *in vivo* experimental period was terminated after 90 min. Blood was sampled at 0 and 90 min.

The ranger-training course

Figure 4: *Cadets from the Norwegian Military Academy on patrol during the ranger-training course.*

The military ranger-training course is arranged by the Norwegian Military Academy as part of the cadets' obligatory training program. The course has been arranged in Norway from 1967, modelled on American courses, and is arranged annually with only minor modifications. The military purpose of the training is to make the cadet fit as a leader in prolonged and high intensity combat situations, and to survive under extreme conditions in the field. During the course they are exposed to physical and psychological heavy strain combined with sleep deprivation and energy depletion. The course has been extensively studied in a number of papers, especially by Opstad. Several endocrine and metabolic alterations have been described, e.g. increased plasma levels of catecholamines, adrenergic desentisation, decreased levels of thyroid hormones and adrenal and testicular androgens (Opstad, 1995). Over the

years no negative health effects have been reported in the participants, with the exception of minor injuries.

Experimental description

Eight healthy and well-trained cadets were included (paper III), and all of them completed the course. It was arranged in the south-eastern part of Norway in the early summer, lasted 7 days and took place in a forested area at about 500 m altitude. The course contained diverse demanding activities around the clock to test the physical and mental endurance and stamina of the participants, including cross-country runs, long foot marches with heavy packs, combat patrol operations and marksmanship training. In addition, the participants were seriously deprived of sleep (about 1h/24 h) and food (1.5–3.0 MJ/24 h), but had free access to drinking water. Previously, daily energy expenditure has been measured to 26 - 27 MJ and average VO_2 to 35% of max (Opstad, 1995; Hoyt et al., 2006). Peripheral blood was obtained by antecubital vein puncture on days 0, 3, 5 and 7. The procedure took place between 7 and 8 a.m. each day to exclude circadian variation, and the samples were put on ice and processed within 2 h.

The *ex vivo* whole blood model

General

To elucidate how the different stressors affect TLR4 signalling we used an *ex vivo* whole blood model. This model has been described as a suitable tool to evaluate the function of circulating leukocytes (Wang et al., 2000). Selected cytokines were used as markers of overall leukocyte activity, a process depending on complex interactions between different humoral factors as well as cell to cell communication. Thus, the use of whole blood has several advantages over cell cultures, e.g. the cells remain in their natural environment, including all cell types and plasma constituents, and the cell separation process, which can affect activation and induce artifacts, is avoided. In this way the response in whole blood is more physiological as compared with incubated cells. In the study by Wang et al. (2000) incubation with LPS 10

ng/ml for 4 to 6 hours induced high concentrations of the cytokines TNF-α, IL-1β and IL-6, with minor affection of metabolic factors like pH, pO2 and pCO2.

In the studies of this thesis we incubated heparinised whole blood with 10 ng/ml LPS (Escherichia coli serotype 0111:B4, Sigma-Aldrich) or an equivalent amount of saline for 4 h (paper I) or 6 h (paper II + III). During the incubation time the tubes were gently rotated (x 6/min) to avoid cell sedimentation. After centrifugation the supernatant was removed and immediately frozen at –20 °C.

Leukocyte viability

In the study by Wang et al. (2000) leukocyte viability exceeded 98% after 14 h of incubation at normothermia. In paper II we incubated whole blood at different temperatures (33, 37 or 40 °C), and to investigate the possible influence of hypo- and hyperthermia on cell viability we performed the trypan blue exclusion test (Strober, 2001). The viability remained high (> 98 %) after 6 h incubation with LPS at all temperatures.

Laboratory analyses

Enzyme-linked immunosorbent assay (ELISA)

To measure plasma concentrations of inflammatory cytokines and HMGB1 we used commercially available ELISA kits. This method provides a specific and highly sensitive technique for quantification of molecules in a solution, based on the principle of protein binding by specific antibodies combined with an enzyme-induced chromogenic reaction. In short, standards and samples are pipetted into the wells of a microplate precoated with a monoclonal antibody against the protein of interest. The protein is bound to the immobilised antibody, and after washing away any unbound substances, a second, enzyme-linked antibody against the protein, making a sandwich complex, is added to the wells. Subsequently, a

substrate solution is added, and a coloured product is created in proportion to the quantity of protein present in the samples or standards. The absorbance is measured by a microplate reader, and the protein concentrations are obtained by extrapolation from a standard curve.

Flow cytometry

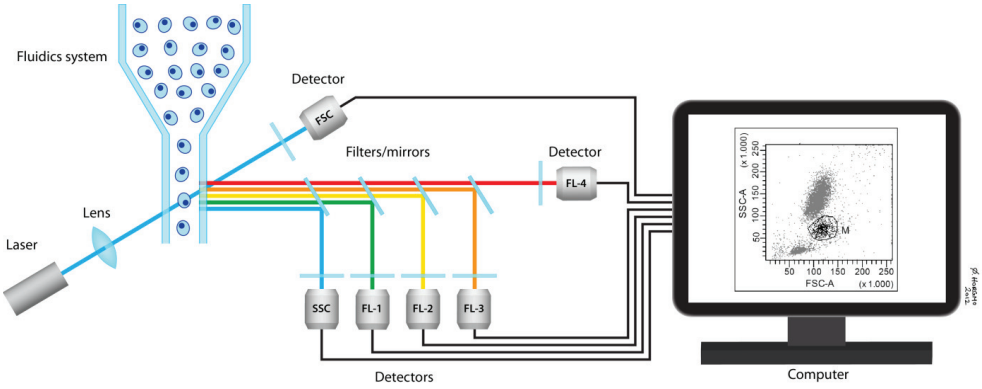

Figure 5: *Schematic overview of a simple flow cytometer.*

To measure the surface expression of the LPS receptor TLR4 on monocytes we performed flow cytometric analysis at Department of Clinical Molecular Biology at Akershus University Hospital. This is a method in which physical and chemical characteristics of single cells, or other biological or non-biological particles of roughly the same size, are measured in a fluid stream (Shapiro, 2003).

The basic building blocks of a flow cytometer are the light source, the flow chamber, the optical system, the light detectors and the computer (Ormerod, 2008; figure 5). Usually, the

light source is a laser, and many instruments have several lasers producing light at different wavelengths. The sample is injected into a stream of sheath fluid, and within the flow chamber the cells are delivered one by one to the laser beam. When the cells pass through the laser intercept, they scatter the laser light according to physical characteristics (like size and granulation), and in addition, any fluorescent molecule bound to the cell will fluoresce. The scattered and fluorescent light is collected by lenses, and according to wavelengths routed to the appropriate detectors by combinations of mirrors and optical filters. Voltage pulses are amplified and converted to digital numbers which can be displayed on data plots. The forward scatter channel (FSC) collects light scattered in the forward direction, and FSC intensity represents an estimate of the cell size. Light scattered approximately at a 90° angel to the excitation line is detected in the side scatter channel (SSC), and the intensity is correlated to the granulation of the cell. Thus, by combining FSC and SSC intensity it is possible to separate different cell types, e.g. blood leukocyte subsets (Figure 6A).

Data about surface receptors, e.g. CD14 and TLR4, or intracellular molecules can be obtained by fluorochrome-labeled antibodies. The principle is that laser light is absorbed by the fluorochrome, exciting its electrons from a resting state to a higher energy level. This state is unstable and the energy is, within nanoseconds, released by heat and emission of a quantum of light (fluorescence). Due to the lower energy, the emitted light will always have longer wavelength than the exciting light. Thus, the intensity of the emitted light, which correlates with the number of antigens binding the fluorochrome-labeled antibody, can be measured separately by use of optical filters. In the papers of this thesis we used monoclonal antibodies (mAbs) against CD14 and TLR4 conjugated with the fluorochromes fluorescein isothiocyanate (FITC) and phycoerythrin (PE), respectively.

We used flow cytometers (FACSCanto II and FACSAria) from BD Biosciences (San Jose, CA), equipped with FACS Diva Software for analysis. Five thousand monocytes were acquired by help of an electronic gate, defined according to their CD14-staining and side scatter light characteristics (Figure 6B). Median fluorescence intensity (MFI) for TLR4 was recorded and corrected for nonspecific antibody binding by subtracting the MFI measured for a matched isotype control sample (Figure 6C, D). The analysis software Cyflogic (version 1.2.1, CyFlo Ltd., Turku, Finland) was used for overlay histograms (Figure 6E).

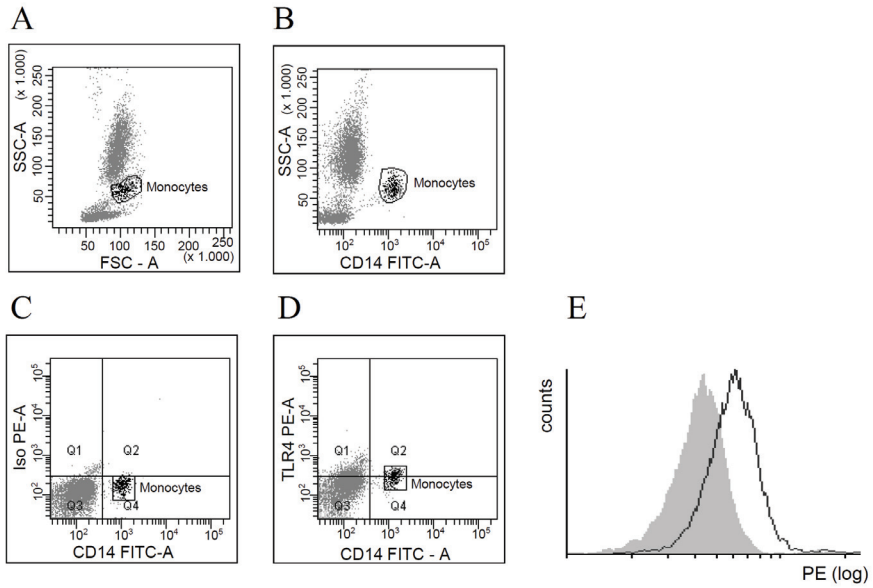

Figure 6: *Flow cytometric figures. Human leukocytes with gatings on monocytes. Forward scatter (FSC) vs. side scatter (SSC) (A) and CD14-fluorescein isothiocyanate (FITC) vs. SSC (B) dot plots. CD14-FITC vs. isotype-control phycoerythrin (PE) and TLR4-PE (C, D). Overlay histogram showing the expression of isotype-control-PE (grey histogram) and TLR4-PE (open histogram) on $CD14^+$ cells (E).*

Gas chromatography

In paper III the plasma concentrations of non-esterified fatty acids (NEFAs, also called free fatty acids (FFAs)) were measured by gas chromatography at an external laboratory (Nofima, Ås, Norway). The analysis involved extraction of the NEFAs from the plasma, transforming of the NEFAs into volatile methyl esters, and finally measurement of the NEFA concentrations by use of a gas chromatograph equipped with a flame ionization detector. The different NEFAs were identified by external standards and quantified by an internal standard. The procedure is described in detail in the method part of paper III.

Statistical analysis

SigmaPlot 11.0 (Systat Software Inc., San Jose, CA) was used for statistical calculations. *p* values less than 0.05 were considered statistically significant. The data were tested for normality and presented as mean ± SEM or median and interquartile ranges, as appropriate.

In paper I TLR4 expression and concentrations of cytokines and HMGB1 were ln-transformed to achieve normal distribution (Altman D.G., 1991).

Depending on testing for normality and equal variance, differences between the time points were estimated by the parametric methods paired t-test/one-way repeated measures ANOVA, or the corresponding non-parametric methods Wilcoxon signed rank test/repeated measures ANOVA on ranks. ANOVA tests were followed by Student Newman Keuls post hoc test. Pearson's product moment correlation (parametric) or Spearman rank order correlation (non-parametric) were used for analysis of correlation.

Summary of results

Paper I

In this study nine land-race pigs were exposed to standardised gunshot injuries and peri-operative stress. All of the included animals survived the study period. Blood was sampled before and 90 min after the trauma and incubated *ex vivo* with and without LPS. Without LPS-stimulation the supernatant concentration of TNF-α significantly increased after trauma, unmasking an activation of the leukocytes. LPS-stimulation caused a fourfold increase (ln-values) before the trauma, whereas the TNF-α concentration after trauma was not significantly affected by LPS. The Post-traumatic attenuated LPS-response, as measured by TNF-α, indicates development of tolerance to LPS. In contrast, there were no significant differences in LPS-stimulated CXCL8 concentrations at the two time points. Trauma caused liberation of HMGB1 to the blood stream in all the animals. The surface expression of the TLR4 protein on $CD14^+$ monocytes was not significantly affected by the trauma. No correlations between cytokine concentrations and TLR4 expression or HMGB1 liberation were found.

Paper II

This paper demonstrates how TLR4 signalling is affected by moderate and clinically relevant alterations in temperature by using the *ex vivo* whole blood model. Blood was drawn from 11 healthy volunteers and incubated with or without LPS at 33, 37 and 40 °C. The production of TNF-α was increased at hyperthermia, whereas the IL-1β concentrations were inversely related to temperature. The IL-10 concentration was reduced at temperature alterations in both directions, but mostly at hypothermia. Thus, hypothermia induced a powerful pro-inflammatory phenotype caused by strongly enhanced TNF/IL-10 and IL-1β/IL-10 ratios, whereas the impact of fever-range hyperthermia was modest, showing a moderate increase in TNF/IL-10 ratio only. Monocyte surface expression of the LPS receptor TLR4 was inversely related to temperature. Cytokine production and TLR4 expression were not correlated.

Paper III

We followed a group of eight cadets engaged in a ranger-training course arranged as part of their education at the Norwegian Military Academy. It involves semi-continuous activity around the clock for one week, and in addition lack of sleep, nutrition and a huge psychological pressure. This multifactorial stress scenario induced leukocytosis and liberation of cortisol and biochemical markers like creatine kinase (CK), aspartate aminotransferase ASAT), alanine aimotransferase (ALAT) and urea in the plasma. LPS stimulation of whole blood unveiled significantly increased values of TNF-α on day 3, and when correcting for monocytes numbers increased concentrations of all the measured cytokines (TNF-α, IL-1β and IL-6) were seen. Later in the course there was a normalisation of the cytokine response. The gentle handling of the cells *ex vivo* unmasked a significantly increased monocyte TLR4 expression at all time points during the course as compared with baseline, either with or without LPS-stimulation. Mobilisation of fat increased plasma concentrations of both SFAs and unsaturated fatty acids (UFAs), with the highest increase among the UFAs. The cytokine production did not correlate with monocyte TLR4 expression or plasma SFAs.

Discussion

TLR4 represents a common receptor for LPS and endogenous ligands to induce innate immune responses. This signalling is of vital importance to protect us from Gram-negative infections and initiate repair of damaged tissues. However, severe complications might occur if the immune activation through this pathway is not tightly regulated, e.g. SIRS and septic shock (Lin and Yeh, 2005). In addition, TLR4 signalling has been linked to development of several other clinical conditions related to inflammation, e.g. insulin resistance and atherosclerosis (Shi et al., 2006; Mann, 2011). Due to the proximal place in the inflammatory pathway TLRs have been considered to be potential targets for pharmaceutical interventions in acute and chronic inflammatory diseases (Zhu and Mohan, 2010).

TLR4 signalling and trauma

Dysregulation of immune system functions after surgery and multiple trauma, whether it leads to hyperinflammation or immunoparalysis, increases the risk of severe complications, like MODS, and remains a serious challenge in critical care management (Menger and Vollmar, 2004; Tschoeke and Ertel, 2007). Previously, our group has described an early reprogramming of circulating leukocytes, with a significant reduction of LPS-induced *ex vivo* TNF-α production from 30 min after standardised gunshot injuries and peri-operative stress in pigs (Gundersen et al., 2005; Gundersen et al., 2008).

This phenomenon, called endotoxin tolerance (or LPS tolerance), may be a part of the body's normal stress responses to prevent systemic inflammation. It was described decades ago when animals pre-exposed to low doses of LPS had reduced fever response when exposed repeatedly, and lower mortality when rechallenged with a lethal dose of LPS (Cavaillon et al., 2003). Similarly, tolerance to LPS has been demonstrated in several clinical studies, e.g. in sepsis patients, healthy volunteers exposed to LPS and after trauma or surgery (Granowitz et al., 1993; Keel et al., 1996; Lemaire et al., 1998; Majetschak et al., 1999; West and Heagy, 2002; Reikeras et al., 2009; Versteeg et al., 2009; Reikeras, 2010).

However, tolerance to LPS might impair resistance to pathogens and has been hypothesised to be a potential mechanism behind the increased infection susceptibility after surgery and trauma (Keel et al., 1996; Munford and Pugin, 2001; Versteeg et al., 2009). Reduced TLR4 expression has been suggested to contribute to the development of endotoxin tolerance. In a study with mouse peritoneal macrophages pre-treated with LPS, Nomura et al. (2000) found reduced TLR4 surface expression which correlated with a decrease in the production of inflammatory cytokines, and they concluded that the down-regulation of TLR4 expression may explain one of the mechanisms for LPS tolerance. Reduced expression of the TLR4 protein has also been described in monocytes from patients after surgery (Ikushima et al., 2004).

In paper I we investigated if the expression of TLR4 on circulating monocytes was affected by standardised gunshot injuries and peri-operative stress in pigs, and searched for an association with LPS-stimulated cytokine production *ex vivo*. As previously described, trauma induced a significantly reduced TNF-α response to LPS, the hallmark of endotoxin tolerance (Cavaillon and Adib-Conquy, 2006). We found the surface expression of TLR4 on $CD14^+$ monocytes to be unaffected, and no correlations with *ex vivo* production of TNF-α or CXCL8 were unveiled. Corresponding findings have been described by Lendemans et al. (2007) in patients exposed to severe blunt trauma. However, other investigators have found increased as well as reduced monocyte surface expression of TLR4 in traumatised patients (Adib-Conquy et al., 2003; Perez-Barcena et al., 2010). As opposed to the reduced TNF-α response to LPS, the CXCL8 production was not affected in our study. One possibility might be that 4 h incubation time was not long enough to demonstrate a tolerance development since the maximal production of this cytokine comes rather late (Van Zee et al., 1991; Cagiola et al., 2006). Our findings indicate that trauma-induced LPS tolerance is not primarily regulated by TLR4 expression on circulating monocytes, and that the molecular explanation for endotoxin tolerance should be sought elsewhere. Several other mechanisms have been suggested to be involved, including the presence of desensitising and neutralising molecules in plasma, inhibition of NF-κB and down-regulation of the TLR4 signalling pathway (Cavaillon and Adib-Conquy, 2006).

HMGB1 is one of the best known endogenous danger signals (Bianchi, 2009). Originally this molecule was thought only to facilitate gene transcription, however, recent studies have

discovered that it occupies a central position in mediating local and systemic responses to necrotic cell death, e.g. in connection with trauma, haemorrhage and sepsis (Lotze and Tracey, 2005). Levy et al. (2007) have shown that the TLR4-HMGB1 pathway plays a critical role in the development of systemic inflammation and remote organ damage after isolated peripheral tissue injury in mice. Interestingly, HMGB1 has also been described to induce endotoxin tolerance, e.g. preconditioning with HMGB1 caused tolerance to LPS in human monocyte-like cells (Aneja et al., 2008). In study I none of the animals had measurable levels of HMGB1 in blood serum at baseline, but the trauma induced a highly significant release of HMGB1 to the blood circulation in all the animals.

Lack of association between *ex vivo* cytokine production and HMGB1 release might be due to the rather complex biological effects of HMGB1 as a modulator of innate immune responses. In addition to the function as a ligand to TLR4, it signals through other receptors, like TLR2 (Lorne et al., 2010). HMGB1 creates pro-inflammatory complexes with PAMPs, cytokines and fragments of RNA and DNA, which are all structures present in the blood circulation after an extensive trauma like in our model, and these complexes might affect a variety of signalling systems (Bianchi, 2009).

TLR4 signalling and temperature stress

During physiological conditions the body temperature is strictly controlled within a few tenths of degrees from 37 °C. However, disturbances are frequently encountered in clinical practice. Altered body temperature is one of the manifestations of systemic inflammation (Bone et al., 1992), and several immune system functions are affected by temperature (Kurz et al., 1996). Changes in body temperature can represent a threat to the host, e.g. hypothermia is associated with increased incidence of post-operative wound infections, and is a risk factor for development of ICU-acquired infection in medical patients (Kurz et al., 1996; Laupland et al., 2012). Also, studies have described increased mortality in hypothermic septic patients and seriously injured patients with accidental hypothermia (Clemmer et al., 1992; Tiruvoipati et al., 2010; Ireland et al., 2011). High fever might also be a risk factor in the non-septic ICU patient (Lee et al., 2012).

On the other hand, a febrile response is protective in patients with infectious diseases and sepsis (Ryan and Levy, 2003; Su et al., 2005; Lee et al., 2012), and in connection with certain pathological conditions reduced body temperature can be organ protective, e.g. after cardiac arrest, ischemic stroke and in newborns with hypoxic-ischaemic encephalopathy (Bernard et al., 2002; Sessler, 2009; Ceulemans et al., 2010; Drury et al., 2010). The mechanisms behind the protective effect of hypothermia are still not fully elucidated, but one important contributing factor is temperature modulation of innate immune responses (Gundersen et al., 2001; Gunn and Thoresen, 2006; Polderman, 2009).

In paper II the production of the pro-inflammatory cytokines IL-1β and TNF-α was affected differently by temperature. The IL-1β concentration was inversely related to temperature, which might represent a negative feedback mechanism involved in the regulation of body temperature (Kappel et al., 1991). The increased TNF-α concentration during hyperthermic incubation could be part of the protective effect of fever in infections.

The production of IL-10 was reduced in both hypo- and hyperthermic incubation of whole blood. This anti-inflammatory cytokine plays a major role in dampening the immune responses, and the finding might indicate a reduced ability to balance the effect of pro-inflammatory mediators during disturbances in body temperature. The relation between pro- and anti-inflammatory cytokines has been considered to be important in inflammatory diseases, and imbalance has been linked to mortality in patients with infections (Girardin et al., 1992; van Dissel et al., 1998; Gogos et al., 2000). In our study, hypothermic incubation increased the ratios of TNF-α/IL-10 and IL-1β/IL-10 seven and nine times, respectively. Similar results have been described in cell cultures of monocytes and peripheral blood mononuclear cells (Matsui et al., 2004; Matsui et al., 2006). Fever, on the other hand, only affected the TNF-α/IL-10 ratio, which was doubled. Thus, fever-range incubation induced a modest pro-inflammatory effect, and we speculate if this represents a more physiological and protective response to LPS in the blood compartment as compared with the exaggerated pro-inflammatory cytokine ratio measured during hypothermia.

In the present study, flow cytometric analysis showed an inverse relation between temperature and TLR4 surface expression on circulating monocytes after *ex vivo* incubation. Few studies have addressed how temperature affects expression of TLR4. Zhou et al. (2005) found increased TLR4 mRNA and total protein expression after heat-shock (42 °C for 1 h, followed

by recovery at 37 °C) in isolated monocytes. However, the surface expression of TLR4 was not significantly affected, indicating an increased protein expression inside the monocytes. Also, Zhao et al. (2007) found up-regulated TLR4 protein expression in macrophages after heat-shock, but the surface expression was not measured in that study.

We found no indications for regulation of cytokine production in response to LPS on monocyte surface level of TLR4. However, we cannot exclude that the increased TLR4 expression during hypothermia might affect the cytokine response in a situation with ligand excess, e.g. after liberation of large amounts of alarmins in connection with severe tissue stress or injury. Elevated levels of circulating pro-inflammatory cytokines have been described in connection with accidental hypothermia and after coronary artery bypass grafting with hypothermic cardiac arrest (Aibiki et al., 1999; Grunenfelder et al., 2000)

TLR4 signalling and multifactorial stress

Studies have indicated that moderate exercise may reduce the risk of upper respiratory tract infections (URTIs) while high level of physical activity is associated with increased risk (Nieman, 1997; Moreira et al., 2009; Gleeson, Bishop, Oliveira et al., 2011). Thus, a "J"-shaped model has been proposed to show the relation between exercise and risk of URTIs, with intensity of physical activity ranging from sedentary lifestyle to high-performance endurance exercise along the x-axis, and risk of infections along the y-axis (Nieman, 1994; Friman and Wesslen, 2000). Post-exercise impaired immune function may represent an "open window" to pathogens (Friman and Wesslen, 2000; Nieman, 2007), and reduced monocyte expression of TLR4 has been described after acute bouts of exercise and might be related to this condition (Lancaster et al., 2005; Simpson et al., 2009; Oliveira and Gleeson, 2010). The time of infection in relation to exercise has been described to affect disease development. In rodents, strenuous exercise immediately before bacterial inoculation with Gram-positive (Streptococcus pneumonia) or Gram-negative (Francisella tularensis) bacteria reduced disease-related mortality, whereas a similar bout of exercise performed after inoculation increased late-phase lethality (Ilback et al., 1991).

In addition to affecting the risk of infections, physical exercise has been associated with anti-inflammatory effects that can protect against chronic diseases, e.g. type II diabetes mellitus and atherosclerosis (Mathur and Pedersen, 2008). Several mechanisms have been suggested to be involved, e.g. reduction of visceral fat mass with subsequently reduced release of pro-inflammatory adipokinines, increased release of anti-inflammatory cytokines (myokines) from contracting skeletal muscles, reduced expression of TLRs on monocytes/macrophages and altered composition of monocyte subpopulations (Flynn and McFarlin, 2006; Timmerman et al., 2008; Gleeson, Bishop, Stensel et al., 2011).

The multifactorial stress scenario in study III affected TLR4 expression and signalling. When the monocytes were stained directly, we found a tendency for increased TLR4 surface expression during the ranger-training course (p = 0.07), but the gentle handling *ex vivo* unveiled significantly increased monocyte TLR4 expression on days 3, 5 and 7 vs. baseline, whether with or without LPS stimulation. Even though most studies describe reduced monocyte TLR4 surface expression after acute bouts of exercise, conflicting results exist. Booth et al. (2010) found increased TLR4 expression on monocytes after an "all out" 60 km trial on an indoor cycling trainer, and they proposed that the discrepancy might be caused by a higher intensity in their study. Our study shows that long-lasting multifactorial stress with semi-continuous strenuous physical activity also increases monocyte TLR4 expression. In the present model psychological stress and depletion of sleep and energy might also affect the results. In mice, chronic restraint stress for 2 days increased the expression of TLR4 mRNA in the spleen (Zhang et al., 2008), and social defeat has been described to increase the surface expression of TLR4 on microglia (Wohleb et al., 2011). In a chronic stress model with rats, including restraint stress and forced swimming, Wang et al. (2011) showed that myocardial TLR4 mRNA and pro-inflammatory cytokines (TNF-α and IL-6) were increased.

Combined stress during intense military training has been showed to cause increased plasma levels of IL-6 (Gomez-Merino et al., 2005; Gundersen et al., 2006), and this cytokine has been described to up-regulate TLR4 cell-surface protein in human monocytes (Tamandl et al., 2003). However, in the cycling study by Booth et al. (2010) post-exercise autologous serum did not affect TLR4 expression on resting monocytes in vitro, which indicates that exercise-induced changes in monocytes TLR4 expression are not caused by soluble serum factors.

Monocytes can be divided into subsets by staining for the surface antigen CD16 in addition to CD14, and most prominent are the "classical" ($CD14^+/CD16^-$) and "pro-inflammatory" ($CD14^+/CD16^+$) subpopulations (Ziegler-Heitbrock, 2007). Pro-inflammatory monocytes have been described to have higher expression of TLR4 and to be released from the marginal pool in preference to the classical subset after acute exercise (Steppich et al., 2000; Simpson et al., 2009). A selective mobilisation of monocyte subsets may therefore contribute to the increased monocyte expression of TLR4 that we found in the present study. However, in the study by Simpson et al. (2009) changes in TLR4 expression were seen within the specific monocyte subpopulations, indicating changes on a cellular level. In addition, in vivo factors like mechanical and circulatory stress could affect cellular TLR4 expression (Booth et al., 2010).

Ex vivo incubation with LPS induced significantly elevated plasma concentrations of TNF-α on day 3 in the course, with subsequent falling values. When correcting for monocyte count a similar significant pattern was seen for all measured cytokines (TNF-α, IL-1β, IL-6). During and after vigorous physical exercise most studies show increased concentrations of pro-inflammatory cytokines in plasma, especially IL-6 that is described to be produced in muscle fibres and is called "the myokine prototype" (Pedersen and Hoffman-Goetz, 2000; Pedersen, 2011). The measured cytokines have a short half-life in plasma, which have been estimated to be 14-18 min for TNF-α, and even shorter for IL-1β and IL-6 (Davies and Hagen, 1997; Toft et al., 2011). Thus, the concentrations measured in this study, after 6 h incubation, reflect the production *ex vivo*. Among circulating leukocytes the monocytes are the main producers of TNF-α, IL-1β and IL-6, especially after LPS stimulation, therefore, the findings in our study indicate that multifactorial stress causes changes in LPS signalling on monocyte level (Andersson and Matsuda, 1989; Xing and Remick, 2003; Xing and Remick, 2004). Previously, increased percentage of monocytes that express intracellular TNF-α, IL-1β and IL-6 after exhausting physical exercise have been reported (Rhind et al., 2001). In addition to strenuous physical activity, psychological stress and sleep deprivation may cause increased liberation of the measured cytokines (Brydon et al., 2005; Irwin et al., 2006; Irwin et al., 2010).

Lack of nutrition, on the other hand, has been associated with impaired immune function, including reduced production of pro-inflammatory cytokines and increased production of anti-inflammatory cytokines (Calder and Jackson, 2000; Gleeson et al., 2004; Gleeson, 2006).

However, the picture is complex, as illustrated in a study by Sun and colleagues (2001). They found that LPS-stimulated peritoneal macrophages from mice under calorie restriction were less responsive than macrophages from controls, as measured by production of inflammatory cytokines (i.e. IL-6), and expressed lower levels of TLR2 and TLR4 mRNA. Nevertheless, after polymicrobial sepsis induced by coecal ligation and puncture (CLP), serum levels of TNF-α and IL-6 were increased in calorie restricted animals, which also died earlier compared with animals fed ad libitum.

Fat is an important source of energy during prolonged exercise, and in a previous study, the cadets lost 3.5 kg fat mass during the ranger-training course (Hoyt et al., 2006). In the present study plasma concentrations of NEFAs at the end of the course were four times higher compared with baseline. SFAs are able to activate TLR4, whereas UFAs, especially n-3 polyunsaturated fatty acids (PUFAs), have inhibitory effect (Lee et al., 2003). The SFA concentrations increased almost three times, but no association with cytokine production was seen. Plasma n-3 PUFAs were below detectable values, but the total concentration of UFAs increased almost seven times, thus, the balance between UFAs and SFAs (U/S ratio) increased. Corresponding results have been seen after acute exercise (Mougios et al., 1995; Nikolaidis and Mougios, 2004).

The multifactorial stress in the present study boosts the monocytes´ cytokine response to LPS in the middle part of the course, which might represent a protective response against Gram-negative bacteria in the blood circulation. Later in the course, however, the response declined. This might be a result of adaption to the stressful conditions and effects of anti-inflammatory mediators, e.g. by cortisol which reached maximal levels at day 5. However, reduced ability to maintain the same high physical activity level throughout the whole week can also be an explanatory factor.

Cytokine production and TLR4 expression

Studies with mice that lack TLR4 have shown the importance of this receptor in activating innate immune responses by LPS, like initiating production of inflammatory cytokines

(Hoshino et al., 1999). Also, subjects with TLR-4 polymorphisms have lower levels of circulating inflammatory mediators, such as IL-6 (Kiechl et al., 2002).

Thus, TLR4 has been considered to be a target for treatment of inflammatory diseases. Promising results have been described in animal sepsis models (Daubeuf et al., 2007), however, results from clinical studies with the TLR4 antagonists Eritoran and TAK-242 in patients with severe sepsis have so far been disappointing, with equal mortality rates in treatment groups compared with placebo (Rice et al., 2010; Tidswell et al., 2010). In addition, TAK-242 did not suppress serum cytokine levels (IL-6, TNF-α, IL-8).

None of the studies in this thesis unveiled associations between monocyte TLR4 expression and cytokine production, even if temperature stress and multifactorial stress induced significant changes in TLR4 expression levels. Thus, it seems to be an abundance of the receptor, which implies that the fine-tuned regulation of the cytokine production must be controlled elsewhere, e.g. by intracellular proteins (Liew et al., 2005; Adib-Conquy and Cavaillon, 2009).

The inflammatory responses are complex, especially in acute severe conditions like sepsis. Individual, time dependent patterns caused by simultaneous release of pro- and anti-inflammatory mediators make immunomodulation difficult. In the future, real-time immunologic monitoring and combinations of target points might improve the treatment of these patients (Christaki et al., 2011).

Conclusions

The studies in this thesis show that monocyte TLR4 expression and/or *ex vivo* cytokine response to LPS stimulation is affected by different stressors.

Gunshot injuries and peri-operative stress in pigs induced tolerance to LPS in *ex vivo* whole blood, as measured by reduced TNF-α response, whereas the surface expression of TLR4 on $CD14^+$ monocytes was unaffected. Trauma resulted in significantly elevated plasma concentrations of the alarmin HMGB1.

In whole blood from healthy volunteers incubated *ex vivo* at normothermia and clinically relevant hypo- and hyperthermia the surface expression of TLR4 on $CD14^+$ monocytes was inversely related to temperature. Compared with normothermia, hypothermic incubation induced a powerful pro-inflammatory phenotype caused by strongly enhanced pro/anti-inflammatory cytokine ratios (TNF-α/IL-10 and IL-1β/IL-10). Hyperthermia only increased the TNF-α/IL-10 ratio, but to a smaller extent.

One week of high-stress ranger training significantly increased *ex vivo* monocyte surface expression of TLR4. LPS-stimulation caused an increased production of pro-inflammatory cytokines on day 3, after which the response declined. Mobilisation of fat increased plasma concentrations of both SFAs and UFAs, with the highest increase among the UFAs.

We found no association between the surface expression of TLR4 on $CD14^+$ monocytes and LPS-stimulated *ex vivo* whole blood production of inflammatory cytokines in any of the studies.

Future perspectives

Over the past few years detailed knowledge about how cells of the immune system recognise danger has been achieved, including the characterisation of different PRRs. These receptors, including the major TLR family, play a pivotal role in initiating and perpetuating inflammation, and have shown that the innate immune system possesses highly developed specificity.

LPS is one of the most powerful immune inducers, and has been described as a motor of SIRS even in absence of Gram-negative infection. Thus, the signalling of this molecule through TLR4 has been extensively studied. In the present thesis we have described how monocyte TLR4 surface expression and TLR4 signalling is differently affected by various stressors. More research is needed to describe in detail how the signalling is modulated during stress, and possibly unveil new potential immune regulatory approaches for acute as well as chronic inflammatory conditions. In this work, stress models, like the war surgery course and the ranger-training course, are suitable tools.

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