GRAM-POSITIVE ENDOTOXEMIA AND MODULATION OF THE INNATE IMMUNE RESPONSE

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Anders E. Myhre
Oslo, August 2007

CONTENTS

ABBREVIATIONS

cAMP	cyclic adenosine 3',5'-monophosphate
CD	cluster of differentiation
CpG	cytosine-phosphate-guanosine
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EMD	enamel matrix derivative
ERK	extracellular signal-related kinase
$G^{+/-}$	Gram-positive/negative
HDL	high-density lipoprotein
IL	interleukin
IRF	interferon-regulatory factor
LAMP	lysosome associated membrane protein
LBP	lipopolysaccharide-binding protein
LDL	low-density lipoprotein
LPS	lipopolysaccharide
LXR	liver x receptor
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MDP	muramyl dipeptide
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MODS	multiple organ dysfunction syndrome
MyD	myeloid differentiation protein
NF	nuclear factor
NOD	nucleotide-binding oligomerization domain
PBMC	peripheral blood mononuclear cells
PepG	peptidoglycan
PGLYRP	peptidoglycan recognition protein
PI3K	phosphatidylinositol 3-kinase
PMN	polymorphonuclear cells
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
RT-PCR	reverse transcriptase polymerase chain reaction
TGF	tumor growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	toll-interleukin-1 receptor-related adaptor protein inducing interferon

LIST OF PAPERS

Paper I

Myhre AE, Stuestøl JF, Dahle MK, Øverland G, Thiemermann C, Foster SJ, Lilleaasen P, Aasen AO, Wang JE. Organ injury and cytokine release caused by peptidoglycan are dependent on the structural integrity of the glycan chain. *Infect Immun*. 2004 Mar;72(3):1311-7.

Paper II

Wang YY, **Myhre AE**, Pettersen SJ, Dahle MK, Foster SJ, Thiemermann C, Bjørnland K, Aasen AO, Wang JE. Peptidoglycan of *Staphylococcus aureus* induces enhanced levels of matrix metalloproteinase-9 in human blood originating from neutrophils. *Shock*. 2005 Sep;24(3):214-8.

Paper III

Myhre AE, Dahle MK, Foster SJ, Thiemermann C, Aasen AO, Wang JE. Peptidoglycan of *Staphylococcus aureus* enhances release of IL-8 and expression of NOD2 in human monocytes and neutrophilic granulocytes. 2007. Manuscript.

Paper IV

Myhre AE, Lyngstadaas SP, Dahle MK, Stuestøl JF, Foster SJ, Thiemermann C, Lilleaasen P, Wang JE, Aasen AO. Anti-inflammatory properties of enamel matrix derivative in human blood. *J Periodontal Res*. 2006 Jun;41(3):208-13.

Paper V

Myhre AE, Ågren J, Dahle MK, Tamburstuen MV, Lyngstadaas SP, Collins JL, Foster SJ, Thiemermann C, Aasen AO, Wang JE. Liver X receptor is a key regulator of cytokine release in human monocytes. *Shock*. 2007. In press.

INTRODUCTION

Throughout the last couple of decades the scientific community has steadily warmed to the thought of the innate immune system as an appropriate object of study. Most immunologists have traditionally thought of innate immunity as a very crude and primitive host defense system, subordinate to the complexity, specificity and efficacy of the adaptive immune response. The discovery of toll-like receptors (TLRs) with inherited specificity for microbiological motifs, and the realisation that an efficient host response (innate and adaptive) is under the control of TLR signaling, have dramatically widened our understanding of infectious immunology and inflammatory disease. Most investigators now believe the two paradigms of immunity to be entwined and appreciate the intricate relationship between the two. This thesis is limited to attend to a few concepts of innate immunity and inflammation, most importantly the interactions of some conserved bacterial motifs with circulating human leukocytes, and ways in which the subsequent inflammation can be modulated.

Endotoxins

Endotoxins are molecules closely associated with the cytoplasm or cell wall of certain micro-organism. They are biologically characterized by their ability to cause fever and in sufficient doses - shock and death. Another defining feature is that they induce inflammation through interaction with specific high-affinity receptors on leukocytes (reviewed in [1]). Endotoxins have been the focus of extensive research for more than a century, and have for most of these years been synonymous with lipopolysaccharide (LPS) of Gram-negative ($G^{\div}$) bacteria [1]. LPS is an important structural component of the outer leaflet of the outer membrane of $G^{\div}$ bacteria (Figure 1), containing a polysaccharide and a lipid component, lipid A, which serves to anchor LPS in the bacterial cell wall and also accounts for the interactions of LPS with the host's immune system.

In recent years, other components of bacterial cell walls, most prominently lipoteichoic acid and peptidoglycan (PepG), have been recognized to have endotoxic properties (reviewed in [2]). PepG is an integral structural part of both G^{+} and $G^{\div}$ bacterial cell walls (Figure 1); although it makes up a much larger part of G^{+}s (the layer is 20-80

nm vs. 7-8 nm thick). PepG consists of a glycan backbone with alternating moieties of N-acetylglucosamine acid and N-acetyl muramic acid, where the muramic acid acts as the linking substance between peptides and sugars. The peptide is composed of alternating L- and D-amino acids; the first is always L-alanine and the second and fourth are invariably D-glutamine and D-alanine. The PepG that makes up the thin layer in Gram-negative bacteria is characterized by a mesodiaminopimelic acid in the third position. Different types of cross-linkage between the peptides occur (Figure 2).

The emergence of alternative bacterial motifs has widened the scope of endotoxin research and is a natural consequence of the fact that the incidence of G^+ sepsis is increasing, accounting for 33% of incidences in Norway in 1999 [3] and 52% in the United States in 2000 [4]. The prevalence pattern of nosocomial infections have changed the most, in part due to the outburst of methicillin-resistant *Staphylococcus aureus* strains.

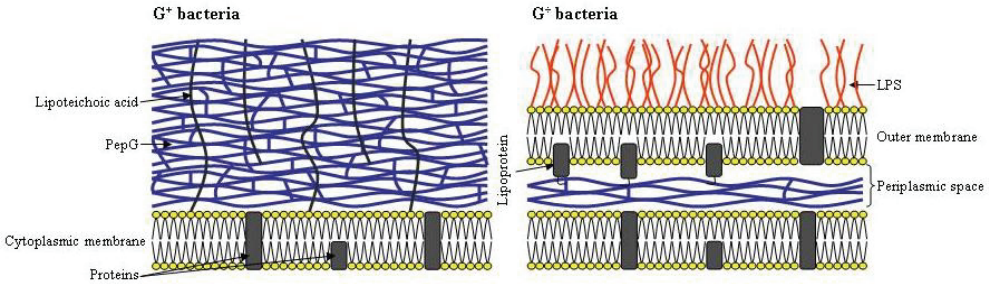

Figure 1. Prototypical cell walls of G^+ and G^- bacteria. The peptidoglycan layer (blue) is thicker in G^+ than in G^- bacteria (20-80 nm vs. 7-8 nm).

Bioavailability and interactions with the host

In order to elicit the pathophysiological changes of endotoxemia, endotoxins need to escape their embedment in the bacterial cell walls and interact with the host. During normal growth, LPS is continuously shed [5], and lipid A is subsequently exposed to the host environment, where it ignites signaling cascades which will be reviewed later on. PepG is also shed in significant amounts during bacterial growth, although this might be restricted to certain strains (reviewed in [6]). Both LPS and PepG are released from bacteria during an infection, as the consequence of bacterial lysis, and they can reach the circulation through translocation from the intestine, although it is uncertain whether this is mainly an indicator of bacterial translocation or direct absorption of endotoxins across the

intestinal wall [7;8]. In addition, some antibiotics (especially β-lactams) potently enhance the release of endotoxins from both G^+ and G^- bacteria (reviewed in [9]), although this is of unknown clinical relevance.

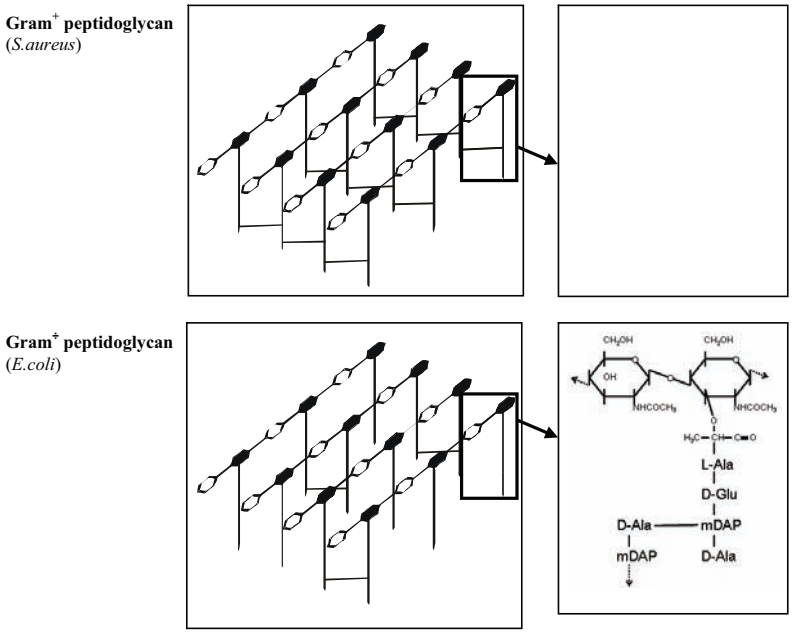

Figure 2. Organization and structure of PepG. There are two main structural differences between G^+ and G^- PepG: Cross-linkage is more widespread in G^+ PepG, and PepG from G^- bacteria is characterized by the mesodiaminopimelic acid in the third position of the stem peptide. D/L-Ala, D/L-alanine; D-Glu, D-glutamine; Gly, Glycine, L-Lys, L-lysine; m-DAP, mesodiaminopimelic acid.

The cellular interactions of endotoxins with the host are mainly mediated by tissue macrophages and blood leukocytes. LPS and PepG are members of a broader class of conserved microbiological motifs, called microbe-associated molecular patterns (MAMPs), which activate the innate immune system. In contrast with other cell types which are generally relatively indifferent to MAMPs, cells of the hematopoietic lineage express a host of pattern recognition receptors (PRRs) that recognize MAMPs and these are of primary importance for the in vivo response to LPS [10]. Increasing knowledge about PRRs has emerged in the last decade and has revolutionized the way we think of the innate immune system. Most notably the TLRs have been discovered to play instrumental roles in integrated immunity by conferring responsiveness to a number of different bacterial motifs (reviewed in [11]), TLR4 being the cell-activating receptor for LPS [12].

The nucleotide-binding oligomerization domains (NODs) are another class of MAMP receptors, binding muropeptides, the building blocks of PepG [13;14].

LPS engages in a complex with LPS-binding protein (LBP) which binds to the CD14 receptor [15]. Subsequently, a signal is relayed across the cell membrane by TLR4, which requires the cell surface molecule MD-2 for activation [12;16]. TLR2 has for some time been believed to relay PepG signaling [17-20], but there is evidence that the intracellularly located NOD1 and NOD2 are the true cell activating receptors for PepG [13;14;21;22], through detection of its minimal bioactive substructures muramyl dipeptide (MDP, part of both G^+ and G^- PepG) and diaminopimelic acid-containing tripeptides (only in G^- PepG). In addition cryoporin/NALP3 has been identified as yet another cell activating receptor for MDP [23;24], although other studies have not reached similar conclusions [25]. The PepG recognition proteins (PGRP, named PGLYRP in mammals) are a set of newly discovered pattern recognition receptors. They do not confer cell activation in mammals, but serve bactericidal functions, partaking in neutrophil killing of intra- and extracellular bacteria [26;27], and enzymatically digesting PepG molecules (Figure 3) [28].

Host response

Engagement of TLR4 by LPS leads to recruitment of adaptor molecules (myeloid differentiation protein-88 [MyD88], Mal, toll-interleukin-1 receptor-related adaptor protein inducing interferon [TRIF] and TRIF-related adaptor molecule [TRAM]) to its intracellular domain. Two major inflammatory pathways - nuclear factor-κB (NFκB) [29] and interferon-regulatory factor 3 (IRF3) [30], are activated via MyD88/Mal and TRIF/TRAM, respectively (reviewed in [31]) (Figure 3). Whereas activation and nuclear translocation of NFκB leads to tumor necrosis factor (TNF)α transcription, IRF3 activation typically induces interferons implicated in anti-viral defense. The mitogen-associated protein kinase (MAPK) family (ERK1/2, JNK, p38) also participate in signaling events downstream of TLR4 (reviewed in [32]). In total, activation of TLR4 turns on more than 1.000 genes, of which 75% are MyD88-independent [33]. They include pro-inflammatory cytokines (e.g. interleukin [IL]-1, IL-6, IL-12, TNFα, macrophage migration inhibitory factor, high-mobility group B-1), anti-inflammatory cytokines (e.g. tumor growth factor (TGF)β, IL-10), interferons, colony-stimulating factors, chemokines (IL-8, monocyte chemoattractant proteins [MCP], macrophage

inflammatory proteins [MIP]), receptors (tissue factor, TLR4, NOD2), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and others [10;34-37].

When NOD proteins are activated by PepG fragments they associate with RICK/RIP2 [38] to activate downstream signaling pathways, most notably NFκB pathway and MAPKs (reviewed in [39]). In addition, PepG seems to activate caspase-1 through the cryopyrin/NALP3 inflammasome, thereby stimulating the processing and secretion of IL-1β and IL-18 [23;24]. In summary PepG induces a variety of functionally different cytokines in monocytic cells, of which the chemokines have been demonstrated to be particularly strongly induced [40]. Among these IL-8 (CXCL8) represents the prototypical chemokine, recruiting neutrophilic granulocytes to a site of inflammation and inducing release of lysosomal enzymes and reactive oxygen species from activated neutrophils [41] (reviewed in [42]).

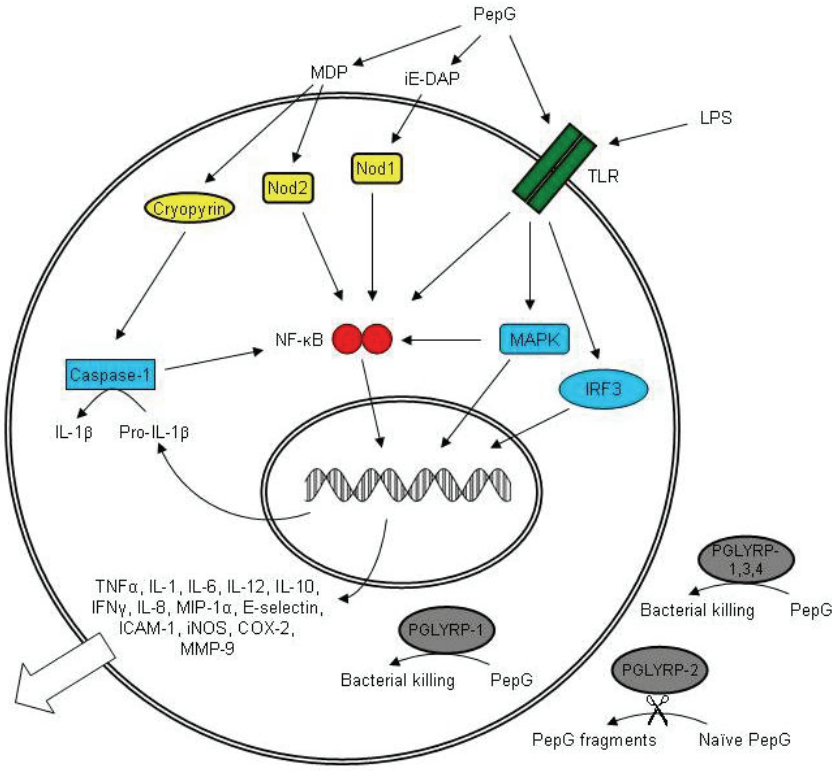

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Figure 3. The cellular response to LPS and PepG. The complexity of intracellular signaling pathways is substantially simplified.

Both LPS and PepG have been shown to enhance the release of matrix metalloproteinases (MMPs) from whole blood and neutrophilic granulocytes [43;44] in vitro, as well as in vivo endotoxemia [43;45]. MMPs are enzymes which are primarily involved in degradation and remodelling of extracellular matrix. More recently they have been heavily implicated in innate immune responses, and especially MMP-9 is believed to be an important inflammatory mediator (reviewed in [46]). This notion is supported by the fact that MMP-9-null mice are protected against LPS-induced shock [47] and experimental models of arthritis [48], bullous pemphigoid [49], and autoimmune encephalomyelitis [50]. MMP-9 plays a significant part in host defense, partly by inactivating serine protease inhibitor α1-proteinase [51], which is an inhibitor of neutrophil elastase, an important mediator in the defense against G^{+} infections [52].

The traditional view of sepsis has been that of an uncontrolled inflammatory response, or as Lewis Thomas put it: "Our arsenals for fighting off bacteria are so powerful...that we are more in danger from them than the invaders."[53]. In line with this is the notion that the morbidity of sepsis or endotoxemia is largely mediated by an unrestrained cytokine storm. The prototypic inflammatory cytokines IL-1 and TNFα are quickly released from monocytes/macrophages and induce the secretion of a host of other cytokines from different leukocyte populations, as well as prostaglandins, leukotriens, nitric oxide, reactive oxygen species and cell adhesion molecules (reviewed in [54]). This systemic inflammatory response is associated with circulatory hypoxia, disseminated intravascular coagulation, microcirculatory collapse and massive infiltration of phagocytes, which initiate adult respiratory distress syndrome, renal failure, myocardial depression and multiple organ dysfunction syndrome (MODS) (reviewed in [55]). Some investigators have suggested that MODS is caused by metabolic shutdown, mediated by cytokine- or hormone-mediated depression of mitochondrial function, and that this is an adaptive response to severe or prolonged cellular stress [56]. Others argue that uncorrected, persisting microcirculatory dysfunction is the driving force in the pathophysiology of sepsis, leading to hypoxia, cellular distress and organ failure (reviewed in [57]). However, partly because of improvements in intensive care, few patients die from circulatory collapse, hypoxemia or renal failure. They rather die because care is given up when prolonged treatment is considered futile [58].

The morbidity of sepsis may not solely be attributed to an excessive inflammatory response (reviewed in [58]). There is substantial evidence to suggest that most septic patients eventually become immunosuppressed, unable to eradicate the infectious agent

and predisposed to nosocomial infections [59] (reviewed in [60]). Potential mechanisms of immunosuppression are a shift from a Th1 to a Th2 response [61] (reviewed in [62]), T-cell anergy [63], lymphocyte apoptosis [64;65] and inability of macrophages to activate T-cells (reviewed in [58]). Endotoxin tolerance, the inability of leukocytes to respond to a second endotoxic challenge, is a well described phenomenon [66] (reviewed in [67]) It is associated with poorer clinical outcomes in ICU patients [68] and might be employed as a tool to identify and differentiate patients with sepsis from those with systemic inflammatory response syndrome [69;70].

These phenomena may contribute to failure to clear a primary infection and a tendency to acquire secondary infections. The true picture is probably one that takes into account both the exaggerated inflammatory response and the compromised immune system. It must also be noted that septic patients are a highly heterogeneous group. While fulminant meningococcemia is characterized by a rapid progression of disease and high circulating levels of pro-inflammatory cytokines [71], an immunosuppressed patient contracting a nosocomial infection post-operatively is bound to be a different story.

Metabolism in sepsis and the liver X receptor

Sepsis is associated with a multitude of endocrine and metabolic alterations. It has been observed that patients admitted to intensive care units (ICUs) for both infectious and non-infectious causes have markedly reduced values of both total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) [72]. Low cholesterol levels are strongly associated with exacerbation of disease and adverse outcome in several patient groups [73-75]. This may be due to the severity of the underlying disease (e.g. cancer, sepsis) which represents a catabolic driving force, but lipoproteins, especially HDL, are also known to interact with and neutralize LPS, and attenuate inflammation and MODS in experimental models [76;77]. Hyperglycemia and insulin resistance are common in critical illness, and not related to glucose homeostasis at baseline [78]. There is now substantial evidence to suggest that strict glucose control in the ICU significantly reduces morbidity and mortality [79;80].

The altered metabolism in critical illness and its potentially important role in the pathology of disease, has led investigators to target mechanisms governing lipid and glucose metabolism. The liver X receptor (LXR) belongs to the nuclear receptor superfamily (reviewed in [81]) and is a whole body master regulator of cholesterol

uptake, .transport and metabolism [82;83] and is also involved in the regulation of apoptosis and glucose sensitivity [84;85] (Figure 4). Its physiological ligands are oxysterols, oxidized metabolites of cholesterol, and in macrophages ligand binding initiates transcription of genes controlling reverse cholesterol transport, thereby protecting cells from lipid overload [86;87]. It has been demonstrated that LXR signaling has anti-inflammatory effects on rodent macrophages, suppressing LPS-induced genes including iNOS, COX-2, IL-1β, IL-6, MCP, and MMP-9. In addition, LXR-null mice have enhanced cytokine responses to LPS challenge [88;89]. On this background our laboratory has recently introduced the concept that LXR may be a node in the pathophysiology of sepsis, and that impaired LXR signaling may, at least in part, underlie development of septic complications. We have demonstrated that pre-treatment with a synthetic LXR ligand protects against hepatic injury in a rat model of endotoxemia, by suppressing TNFα release from Kupffer cells [90], and that liver injury is aggravated in LXRα-deficient mice after cecal ligation and puncture, compared with wild type mice (unpublished results). A study in human monocytes, exploring the anti-inflammatory potential of LXR agonism is presented in the thesis (paper V)

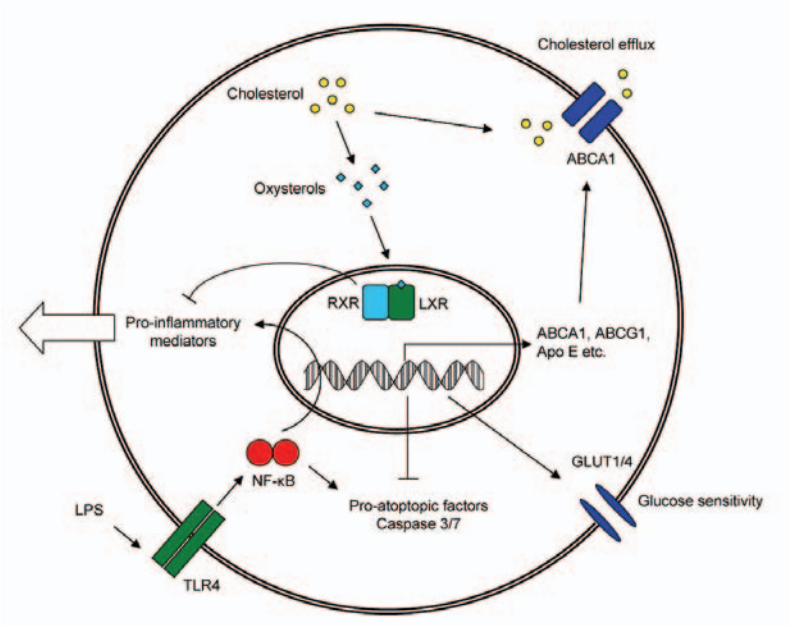

Figure 4. Cellular effects of LXR activation. Activation of LXR by its endogenous ligands, oxysterols, leads to dimerization with RXR and initiation of gene transcription. The results are promotion of cholesterol efflux and glucose sensitivity and inhibition of transcription of pro-atoptopic factors and release of pro-inflammatory mediators

Enamel matrix derivative

Enamel matrix derivative (EMD) is a substance extracted from developing porcine tooth buds. It primarily contains amelogenins, which are the major proteins of developing tooth enamel matrix and are predominantly involved in formation of enamel and dental cementum. There is substantial evidence that enamel matrix derivative (EMD) improves the clinical outcome of periodontal treatment in patients with severe periodontitis [91;92] and promotes healing of venous leg ulcers [93]. One observes an attenuation of gingival inflammation, enhancement of periodontal ligament and gingival fibroblast proliferation and promotion of vessel formation, thereby promoting healing of soft tissue wounds [92;94-97]. A local increase in the synthesis and secretion of growth factors and cytokines, including TGFβ and IL-6, is involved [95], but the precise mechanisms of EMD-cell interactions are largely unknown. Lysosome associated membrane protein (LAMP)-1 and CD63 have recently been implicated in the recognition and internalization of amelogenins [98;99], but there are no reports of LAMP-1 or CD63 regulating signal transduction pathways.

The available literature suggests that EMD has immunomodulatory properties, but the potential effects of EMD on inflammatory responses, such as cytokine formation, remain mostly unexplored. However, it has been shown that cAMP is elevated in periodontal ligament and epithelial cells after exposure to EMD [95]. Factors that initiate intracellular formation of the second messenger cAMP down-regulate the monocyte/macrophage-driven cytokine burst [100;101] and cAMP is known to inhibit activation of nuclear factor-κB and particularly the release of TNFα, as well as regulating several other mechanisms of innate and adaptive immunity [102;103] (reviewed in [104]).

In paper IV we aimed to elucidate the impact of EMD on endotoxin-induced inflammatory cytokine burst and whether this could be attributed to cAMP accumulation.

AIMS OF STUDY

- To explore the structural requirements of PepG to interact with the innate immune system to induce cytokines in human blood and organ injury in rats.

- To study the capacity of PepG to elicit the release of matrix metalloproteinase-9 and interleukin-8 and to alter the expression of NOD2 in human blood, monocytes and neutrophil granulocytes.

- To explore the potential of amelogenin and an LXR agonist to modulate inflammation, assessed by the synthesis of pro- and anti-inflammatory cytokines, in human blood and monocytes.

METHODS

Whole blood model

A whole blood model was used in papers I-V as developed and described previously by our laboratory [105]. Its main advantage is that it is a fast and easy way to explore inflammatory responses in a human cell system with complex leukocyte interactions. Venous blood is drawn from healthy volunteers, anticoagulated with heparin and incubated in plastic tubes at 37˚C with slow rotation. At different time points plasma is removed by centrifugation and frozen for later analyses. In other experiments $CD14^+$ cells (monocytes, macrophages and a subset of granulocytes) are isolated using magnetic beads attached to a CD14 antibody (Dynabeads). RNA is thereafter isolated.

We have previously characterized this whole blood model [105] and have shown that pro-inflammatory cytokines (IL-1β, IL-6, TNFα) are readily released into plasma upon exposure to LPS and that corresponding mRNA accumulates in $CD14^+$ cells. Leukocyte viability is relatively stable (93,5 % at 24 h), pO_2 decreases from 5.7 to 3.4 kPa in 24 h, while pCO_2 is markedly increased, from 5.7 to 21 kPa. The rise in pCO_2 is accompanied by a decrease in pH from 7.1 to 6.6. There is evidence that hypoxia can increase leukocyte responsiveness to LPS [106] and that cytokine release is augmented in macrophages exposed to high levels of CO_2 [107;108]. We can therefore not exclude that altered gas tensions influence the inflammatory responses in this model.

Primary cultures of human monocytes and neutrophilic granulocytes

In papers II, III and V monocytes and neutrophils were isolated from venous blood drawn from healthy volunteers. The blood was subjected to density gradient centrifugation, according to principles described by Arne Bøyum [109]. In this procedure, blood is applied on top of a layer of Polymorphprep (AxisShield). Two distinct bands are formed by centrifugation, one containing polymorphonuclear cells (>95 % neutrophilic granulocytes, as determined by flow cytometry) and one containing mononuclear cells. To isolate monocytes from the mononuclear cell suspension, adherent monocytes are allowed to adhere to plastic plates, and lymphocytes and non-adherent cells are removed by

washing. By using this method we probably select a sub-population of blood monocytes, most likely those that are most differentiated towards macrophages.

Quality control of PepG

In endotoxin research the purity of bacterial products is of paramount importance. A number of important findings in the last couple of decades have to be interpreted with caution because it is difficult to exclude contamination or co-purification of the bacterial cell wall products with other bacterial components. Professor Simon J. Foster has provided us with highly purified PepG from *S.aureus*, *Bacillus subtilis* and *Curtobacterium flaccumfaciens*. PepG was purified as described for *B.subtilis* by professor Foster in 1992 [110]. In short, proteins were removed by pronase treatment and anionic polymers were removed from purified cell walls by treatment with hydrofluoric acid. The PepG was then washed numerous times and sterilized by autoclaving. It was subjected to gel electrophoresis with no evidence of protein, and the PepG was also enzymatically digested, obtaining the expected reverse-phase high-pressure liquid chromotography muropeptide profile with no spurious products. The PepG preparations were analysed for the presence of LPS by Limulus Amebocyte Lysate test, and was shown to contain 35 pg LPS per mg PepG. Moreover, pre-treatment with the LPS inhibitor polymyxin B or the CD14 antibody 18D11 (which specifically blocks LPS-induced signaling) had no effect on the production of TNFα induced by PepG [111]. Prior to experiments PepG was dispersed by sonication (3,000 Hz, 3 × 10 s).

In vivo endotoxemia

In paper I, in vivo experiments were performed as described by Wang et al. [112]. First, male Wistar rats were anaesthetized with thiopentone sodium. Then, the trachea, right carotid artery and left jugular vein were cannulated to facilitate respiration, register mean arterial pressure and heart rate, and provide an access for administration of compounds. After surgery, heart rate and blood pressure were allowed to stabilize for 10–15 minutes, followed by slow injection of saline or PepG into the jugular vein over a 10-min period. The rectal temperature was kept at 37˚C throughout the experiment. The rats were euthanized at 6 h and plasma was collected for analyses of markers of hepatic injury/dysfunction (AST, ALT, γGT, bilirubin) and renal failure (urea, creatinine).

Assays

Enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR), and multiplex antibody bead analysis are standardized and commonly used methods of analysis and will not be described in further detail. Zymography was performed by Yun Yong Wang and Solveig Pettersen and I will therefore not elaborate on this method. Procedures are described in the methods section of each paper and are generally performed according to manufacturer's instructions.

SUMMARY OF RESULTS

Paper I

In two models of PepG endotoxemia we showed that *Staphylococcus aureus* PepG induced markers of hepatic injury/dysfunction (AST, ALT, γGT, bilirubin) and renal failure (urea and creatinine) in the rat, and the release of functionally different cytokines (IL-6, IL-8, IL-10, and TNFα) in human blood. These effects were abrogated by enzymatically hydrolyzing the glycan backbone of PepG, but only weakly reduced by breaking the peptide cross-bridges, suggesting that the integrity of the glycan backbone of PepG is crucial for its endotoxic properties. However, the PepG fragments retained their ability to synergize with LPS. PepG from bacteria with different peptide side chains (*S.aureus*, *C.flaccumfaciens*, *and B.subtilis*) had similar immunostimulatory potentials.

Paper II

In this paper we demonstrated that *S.aureus* PepG induced the accumulation of MMP-9 in human blood. MMP-9 release was significantly enhanced in neutrophil cultures, and seemed to be the result of degranulation and not de novo synthesis, as the combined amount of intra- and extracellular MMP-9 was not increased by PepG. Inhibition of p38 MAPK attenuated MMP-9 release, suggesting a role for p38 MAPK in neutrophil degranulation caused by PepG.

Paper III

S.aureus PepG dose-dependently triggered prolonged release of IL-8 in primary cultures of human monocytes and neutrophilic granulocytes, as well as in whole blood. Inhibition of p38 MAPK and Src family kinases reduced IL-8 release in both cell types, whereas ERK1/2 and PI3K inhibition affected monocytes only. This suggests that differing signaling pathways are triggered by PepG in these cells. The basal level of NOD2 expression was higher in monocytes than in neutrophils and was up-regulated by both PepG and LPS.

Paper IV

We revealed that enamel matrix derivative (EMD) containing amelogenins of different sizes attenuated the release of pro-inflammatory (TNFα, IL-8), but not anti-inflammatory (IL-10) cytokines in human blood challenged by endotoxins (PepG or LPS). The effect was most pronounced as pre-treatment. The anti-inflammatory effect of EMD was accompanied by cAMP accumulation in blood mononuclear cells, and as a cAMP analogue mimics the actions of EMD, we suggested that EMD's anti-inflammatory potential can at least partly be attributed to increased intracellular cAMP.

Paper V

We showed that LXRα was up-regulated by LPS in human monocytes, suggesting a role for this receptor in inflammation. Furthermore, a synthetic LXR agonist attenuated the release of a multitude of cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12, TNFα, MIP-1α/β and MCP-1) induced by LPS, and to some extent PepG. This effect on cytokine release was neither accompanied by a down-regulation of endotoxin receptor expression, nor by attenuation of cytokine mRNA levels, suggesting that the LXR effect occurred via posttranscriptional mechanisms.

DISCUSSION

This thesis aimed to explore the pathophysiology of endotoxemia from different points of view. First, from a microbiological point of view, with regard to the structure of PepG, secondly, from a cellular point of view, elucidating the cellular response to PepG, and ultimately from an interventionist point of view, modulating potentially important signaling events in order to re-establish homeostasis.

In Paper I we explored the biological activity of different PepG structures by cutting the molecule with two different enzymes. It has been known for some time that the integrity of the glycan backbone is of importance for PepG's inflammatory potential [113-115]. Our study was the first to report that PepG-induced organ injury in the endotoxemic rat is strongly attenuated by prior digestion of the glycan backbone. We also reported that cytokine formation and mRNA accumulation in an ex vivo whole human blood model were attenuated by loss of backbone integrity.

Human lysozyme digests glycan chains and is a major secretory molecule of both macrophages and neutrophilic granulocytes [116;117]. The importance of inactivating peptidoglycan is pin-pointed by the fact that lysozyme-deficient mice develop more severe inflammatory lesions after PepG exposure than their wild-type counterparts [118]. However, lysozyme-mediated bacterial killing might release large quantities of PepG into the bloodstream, augmenting the inflammation in sepsis and endotoxemia. There is also evidence that highly inflammatory substructures might be released upon degradation of PepG, approximating the potency of LPS [119].

This thesis highlights the fact that endotoxins cause the release of a multitude of functionally different cytokines and inflammatory mediators from human leukocytes. This is a well known feature of LPS, and we believe it is of great interest that also PepG induced the release of TNFα, IL-1β, IL-6, IL-8, IL-10, IL-12, MIP-1α, MIP-1β, MCP-1 and MMP-9 in our studies. In Paper II we focused on MMP-9, a matrix metalloproteinase involved in remodelling of extracellular matrix [46], and found that it is readily released from human neutrophils by degranulation in response to PepG. Degradation of tissue matrix might be beneficial in combating infections, making the site of infection more accessible for granulocytes and lymphocytes. In the context of sepsis and endotoxemia the

same properties of matrix metalloproteinases could be detrimental to the host, contributing to organ damage and circulatory collapse. Besides, MMP-9 has additional roles in inflammation, modulating the activity of pro-inflammatory cytokines (IL-1β) [120] and chemokines (IL-8) [121]. In Paper III we focused on IL-8, which is a prototypical chemokine, mainly promoting attraction of neutrophils to a site of inflammation and subsequent release of lysosomal enzymes and respiratory oxygen species [42;122;123]. Serum levels of IL-8 correlate with severity of disease in sepsis patients [124;125] and is associated with the development of nosocomial pneumonia in multitrauma patients [126]. However, the significance of this remains uncertain, as high concentrations of IL-8 actually may reduce recruitment of neutrophils and inhibit neutrophil-endothelium interactions [127]. In summary, paper II and III provide further evidence in support of peptidoglycan as an important endotoxin.

The two last studies included in this thesis explore ways of curbing the inflammatory potential of endotoxins. Paper IV aimed to elucidate the properties of a xenobiotic material, EMD, a substance mainly containing amelogenin proteins. Amelogenin has recently been shown to be expressed by a number of cell types, including cells of hematopoietic linage [128], implying that it might serve other functions than enamel and dental cementum formation. Basic and clinical research has demonstrated that EMD plays an important role in tissue regeneration [91-94], but although the literature suggests that EMD has immunomodulatory properties, this area is mostly unexplored. Our study is the first to demonstrate the effects of EMD on the inflammatory response of human blood leukocytes. We showed that EMD curbed the pro-inflammatory cytokine burst, but did not affect the anti-inflammatory response (IL-10), and that this pattern is almost similar to the effects of an increase in cAMP formation, which has been demonstrated to suppress TNFα [129] and increase IL-10 [130]. EMD has been shown to enhance cAMP levels in periodontal ligament and epithelial cells [95], and cAMP accumulation was indeed induced by EMD in our study. Apart from this the cellular mechanisms behind the anti-inflammatory effect are largely unknown. Some investigators have presented data demonstrating that amelogenin negatively regulates receptor activator of nuclear factor-κB (RANK) ligand [131;132], which controls osteoclast formation and initiates a broad range of inflammatory cellular events in osteoclasts through RANK (reviewed in [133]). However, the cAMP/PKA pathway is known to enhance, rather than suppress, RANK ligand expression [134;135].

EMD consists mainly of amelogenin, and when separated by gel electrophoresis a complex mixture of gene splice products are revealed [136]. These peptides have differing bioactivity [137], and it is unlikely that the whole protein (M180) is required to produce the observed effects. We have used recombinant amelogenin as well as smaller sequences in the whole blood model, without producing conclusive results, and this work should be taken further, not least because amelogenin fragments have differing solubility [138]. The hydrophobic properties became evident in a recent study from our lab, where EMD aggregated in capillary beds of pigs and caused a rise in systemic and pulmonary blood pressure [139]. There should be taken steps to isolate bioactive fragments which are applicable for systemic administration.

In Paper V, we utilized a synthetic ligand to activate a well-known endogenous receptor, LXR. We found that the LXR agonist worked as a general suppressor of cytokine release in monocytes. When we went further to elucidate the cellular mechanisms, we were surprised to find that cytokine mRNA levels were not affected. Neither was there evidence of a diminished transport of mRNA out of the nucleus or a decreased cellular release. This is in line with studies we have done in rat Kupffer cells (unpublished results). Further work is underway to explore the underlying mechanisms of LXR's effects, which could, according to preliminary data, involve mechanisms governing mRNA stability or translation, possibly thorough inhibition of p38 MAPK phosphorylation.

LXR is a whole body master regulator of reverse cholesterol metabolism, insulin sensitivity and apoptosis and partakes in several aspects of macrophage biology [81;82;84;88]. Low circulating levels of cholesterol [75], decreased glucose tolerance [79] and increased leukocyte apoptosis [64] are common in critically ill patients and predict a poor outcome. LXR potentially counteracts all these conditions, and hence, it is an extremely attractive molecule to target. We have previously demonstrated that an LXR ligand attenuates liver injury and Kupffer cell TNFα release in endotoxemic rats, and more recent work has shown that liver injury is aggravated in LXR deficient mice subjected to cecal ligation and puncture (unpublished results). Sepsis is a complex disease warranting complex approaches, and LXR has the potential to counteract sepsis at multiple levels. Therefore, studies are therefore underway to investigate the role and function of LXR in monocytes of septic patients.

Merely placing a lid on endotoxin-induced pro-inflammatory mediator release is probably a too simplistic way of handling the pathophysiology of sepsis [140].

Controlling excessive inflammation in the early phase of disease is certainly beneficial, but in the clinical setting one seldom gets the chance to apply a drug prior to the onset of controlled endotoxemia, and the measured cytokines might have come and gone and elicited late-onset responses (reviewed in [141]). In addition, cytokine formation is a crucial part of competent innate and adaptive immune responses [141], and abrogating them in the context of an out-of-control infection might be detrimental to the eradication of the infectious agent. Antibodies against TNFα or IL-1 have in several large phase III studies been ineffective and in some settings harmful, and anti-cytokine monotherapy is considered to be of limited value (reviewed in [142]). It is becoming increasingly clear that sepsis is an extensively complex process that affects various metabolic, endocrine, neurological and immunological factors [55;64;75;143]. What we preferably should try to do is restricting the excessive systemic inflammation, while at the same time addressing the whole picture with regard to metabolic dysfunction, apoptosis, immunological anergy and more. In this context, LXR agonism seems to provide an especially intriguing possibility.

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