ASPECTS ON SEPSIS: TREATMENT AND MARKERS
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TREATMENT AND MARKERS

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*Science is organized knowledge. Wisdom is organized life.*

Immanuel Kant
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ABSTRACT

Sepsis is one of the greatest challenges in critical care medicine today, while the treatment of sepsis and evaluation of its severity is complicated. The first part of this thesis presents two approaches on the use of antimicrobial peptides in sepsis treatment, relying on both soluble and immobilized peptides. All peptides tested, truncated from human and non-human antimicrobial peptides, did neutralize LPS activity in a dose-dependent manner. Immobilization of the peptides did not inhibit their ability to bind LPS, therefore, the peptides can be considered for extracorporeal LPS removal in sepsis therapy. Interestingly, the soluble peptides inhibited LPS induced cytokine production but potentiated LTA induced cytokine production in human blood. Consequently, care should be taken when considering these peptides in treatment of Gram-positive infections. The second part of this thesis evaluates the inflammatory marker soluble urokinase plasminogen activator receptor (suPAR) in sepsis prognosis. Also, an investigation whether suPAR can be detected in human saliva was undertaken. The results indicate that plasma levels of suPAR are increased in sepsis patients compared to controls, but there was no significant difference between survivors and non-survivors. Plasma levels of suPAR did not correlate with other inflammatory markers, suggesting that suPAR reflects general activation of the immune system rather than exerting inflammatory actions. Moreover, suPAR can be detected in saliva and the levels are more than 10 times higher than the corresponding plasma levels in healthy individuals.
ABBREVIATIONS

AMP  Antimicrobial peptide
BIAcore  Biomolecular interaction analysis
BPI  Bacterial/permeability-increasing protein
CARS  Compensatory anti-inflammatory reaction
CRP  C-reactive protein
DAMPs  Damage associated molecular patterns
ELISA  Enzyme linked immunosorbent assay
Hb  Hemoglobin
ICU  Intensive care unit
IL  Interleukin
K_D  Equilibrium dissociation constant
LTA  Lipoteichoic acid
LPS  Lipopolysaccharides
PAMPs  Pathogen-associated molecular patterns
PCT  Procalcitonin
PMB  Polymyxin B
RU  Response units
SIRS  Systemic inflammatory response
SOFA  Sequential organ failure assessment
SPR  Surface plasmon resonance
suPAR  Soluble urokinase plasminogen activator receptor
TLR  Toll-like receptor
TNFα  Tumor necrosis factor alpha
uPA  Urokinase plasminogen activator
uPAR  Urokinase plasminogen activator receptor
LIST OF PAPERS

The thesis is based on the following papers, which are referred to in the text by their roman numerals:


III. The prognostic value of suPAR compared to other inflammatory markers in patients with severe sepsis. Gustafsson A, Ljunggren L, Bodelsson M, Berkestedt I. *Biomarker Insights.* 2012;7:39-44


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*Contributions by the respondent*

In paper I, II and IV, I took part in the planning process, was responsible for the experimental work and analysing the data. I measured suPAR and PCT in paper III. All papers are written by me with support from the co-authors.
INTRODUCTION

The early recognition, diagnosis, and treatment of sepsis remain one of the greatest challenges in the field of critical care medicine today. Current therapy is primarily supportive and includes timely administration of antibiotics, source control of infection, aggressive fluid therapy and organ support. Even so, employing current practices and the most up to date treatment strategies, the mortality rate of the most severe form, septic shock, still exceeds 30%.

Bacterial mediators including endotoxin are critical to sepsis pathogenesis and can be targeted with antimicrobial peptides (AMPs) by medical devices. AMPs, in general, show significant sequential variation, suggesting that a specific sequence is not crucial for biological activity. Modification of AMPs is a common strategy for improving their antimicrobial activity. The first part of this thesis will cover two approaches on the use of truncated AMPs in sepsis treatment, using both soluble and immobilized peptides. A biomarker that could pre-select sepsis patients in urgent need of treatment would be of great value to ensure the optimal use of health care resources. The second part of this thesis will elucidate the role of a biomarker called soluble urokinase plasminogen activator receptor (suPAR) in sepsis prognosis. Furthermore, an investigation regarding suPAR detection in human whole saliva of healthy individuals was undertaken.

In the following pages a brief introduction to sepsis and its connection to innate immunity will be presented, and also, AMPs and suPAR will be introduced. Then, an overview of methods, results, discussion and concluding remarks will be presented. For details, the reader is referred to the separate papers at the last section of this thesis.
Sepsis

Sepsis is defined as a clinical syndrome characterized by the presence of both infection and a systemic inflammatory response (SIRS), the sepsis syndrome results in a cascade of clinical events with increasing severity and mortality (figure 1). Severe sepsis occurs when the septic process has become so grave that at least one organ has become dysfunctional. Septic shock is a catastrophic syndrome characterized by severe hypotension and hyperperfusion, which in patients surviving the first hours usually lead to tissue injury and multiple organ failure². Patients with this condition have high mortality rate (30-60%), despite treatment at an intensive care unit (ICU)¹³. Hence, sepsis represents one of the major causes of death in the industrialized countries⁴⁻⁹.

![Figure 1. Criteria for infection, SIRS, sepsis, severe sepsis, and septic shock. The criteria was defined in 1991 at a conference by the American College of Chest Physicians and the Society of Critical Care Medicine¹⁰ and refined a decade later (2001) by the participants of the International Sepsis Definitions conference¹¹.](image-url)
Innate immune system in sepsis

The innate immunity, also called unspecific immunity, is the first line of defence and is activated upon the encounter of a pathogen. The players in innate immunity are neutrophils, macrophages, monocytes, dendritic cells, NK cells, mast cells and the complement system. The cells have pattern recognition receptors (PRRs) that respond immediately to certain pathogen-associated molecular patterns (PAMPs)\(^\text{12}\). The best-studied examples of PRRs are toll-like receptors (TLRs)\(^\text{13}\). Additionally, TLRs, together with other PRRs, like CD36 and C-reactive protein (CRP) recognize host derived epitopes, i.e. damage associated molecular patterns (DAMPs)\(^\text{14,15}\). Injury of host components in sepsis causes cell lysis, and thus many pro-inflammatory DAMPs are released, e.g. high mobility group box 1 (HMGB1) and AMPs\(^\text{16,17}\). Many PRRs are evolutionarily conserved, suggesting that these proteins play essential roles in innate immunity\(^\text{18,19}\). Signalling through PRRs results in the production of cytokines, type I interferons, AMPs and chemokines, all of which contribute to the host inflammatory response\(^\text{20}\). Cytokines are soluble proteins that mediate immune and inflammatory reactions. Chemokines are cytokines that induce chemotaxis, or directed migration, of leucocytes to the sites of inflammation.

The sepsis process begins when PAMPs, e.g. lipopolysaccharide (LPS) from Gram-negative bacteria or lipoteichoic acid (LTA) from Gram-positive bacteria, are sensed by the host immune cells. The host microbial interaction initiates the production of inflammatory cytokines that contribute to the innate immune response. When a limited number of bacteria invade, the local responses are sufficient to clear the pathogens. Sepsis develops when this initial, appropriate inflammatory response becomes deregulated and rises to such a level that it may injure the host\(^\text{21}\). The inflammatory cytokines activate lipid mediators and reactive oxygen species, as well as upregulating cell adhesion molecules resulting in the initiation of inflammatory cell migration into tissues\(^\text{22}\). PAMPs and cytokines can also induce excessive production of nitric oxide, resulting in vasodilation, contributing to the hypotension during sepsis\(^\text{23,24}\). Cytokines are also important in inducing a procoagulant effect in sepsis. The natural balance of coagulation gets disturbed because of the increased coagulant and diminished fibrinolytic activity. Then, small clots form faster than they can be dissolved, and the clots can inhibit the microcirculation, causing tissue hypoxia and may contribute to the multiorgan failure in septic patients\(^\text{25}\). The key elements in the sepsis syndrome are summarized in figure 2.
Figure 2. A representation of the microvasculature showing key elements of the innate immune response to bacteria causing sepsis. Pathogen-associated molecular patterns (PAMPs) simultaneously activate several parallel cascades that contribute to circulatory collapse and multiple organ failure.
If a pathogen can surpass innate immunity and invade the body, it will be recognized by cells in the adaptive immunity. The adaptive, specific, immunity includes cell mediated and humoral responses and leads to recognition of an extensive diversity in pathogen epitopes continuously throughout life. Cells of both the innate and adaptive immunity will then cooperate, with the ultimate goal of eliminating the invading bacteria.

**The cytokine cascade in sepsis**

Sepsis is characterized by simultaneous release of both pro-inflammatory and anti-inflammatory mediators and if the cytokine balance cannot be established, a massive pro-inflammatory reaction (SIRS) or a compensatory anti-inflammatory reaction (CARS) will arise. Both SIRS and CARS can ultimately lead to shock and death either by excessive inflammation, or indirectly through immune dysfunction. Four pro-inflammatory cytokines, tumor necrosis factor-α (TNFα), interleukin (IL)-1β, IL-6, IL-8, have been most strongly associated with sepsis. It has been suggested that IL-10, transforming growth factor-β (TGF-β), IL-1 receptor agonist (IL-1Ra), and soluble TNF receptor serve to counteract the pro-inflammatory cytokines. However, there are further cytokines released during sepsis, which in addition to those mentioned above, interact in a complex network involving several interaction points and feedback loops. Achieving the correct balance of SIRS and CARS, as well as the intensity of these responses, should greatly influence host survival. However, in contrast to animal data, attempts to modulate the inflammatory response in sepsis patients have failed to improve survival. Perhaps a better definition of sepsis patients for targeted therapy is necessary, as these populations might respond differently to pro-inflammatory or anti-inflammatory agents.

**Pathogen-associated molecular patterns**

Considering that PAMPs are a major cause of the myriad of physiological changes seen in the septic patient, targeting bacterial PAMPs, in theory remains a very attractive approach in sepsis therapy. Here, only LPS and LTA will be described because they are included in the papers of this thesis, other examples of PAMPs are peptidoglycans, bacterial DNA, and double stranded RNA.
Lipopolysaccharide

The cell envelope of a typical Gram-negative bacterium is composed of a thin layer of peptidoglycan, an outer membrane, and LPS and phospholipids. The LPS molecule consists of three parts: an outer variable O polysaccharide side chain, a relatively conserved core region, and a highly conserved lipid A component. The LPS molecule is embedded in the outer membrane of Gram-negative bacteria and the lipid A tail of the molecule serves to anchor LPS in the bacterial cell wall. Upon cell division, death, or antibiotic treatment, LPS is released in the circulation and can trigger an immune response in the patient; LPS is therefore also known as endotoxin. LPS can enter the blood in two ways: a) through a local or systemic infection by Gram-negative bacteria and b) by translocation of Gram-negative bacteria across the intestinal membrane during various disease processes.

In human blood and other body fluids, LPS binds to the LPS-binding protein (LBP), an acute-phase reactant. LBP transfers LPS to CD14, a glycosyl phosphatidylinositol-linked molecule on the surface of myeloid cells. Although CD14 is membrane bound it is unable to generate a transmembrane signal; this is achieved by the activation of the TLR4 on the cell surface and mediated through the adaptor protein known as myeloid differentiation factor-2 (MD-2). A pro-inflammatory response is then set in motion with cytokine production and activation of the complement and coagulation cascade.

It is generally believed that LPS triggers early SIRS in Gram-negative sepsis. Moreover, in patients with severe sepsis, increased concentrations of plasma LPS is correlated to higher mortality compared to patients without measurable LPS.

Lipoteichoic acid

In recent years, the proportion of sepsis induced by Gram-positive bacteria has increased and currently, between one-third and one-half of all cases are caused by Gram-positive organisms. As there are no LPS in Gram-positive bacteria, other microbial cell wall components must be responsible for the pro-inflammatory activity. A widely accepted hypothesis is that LTA and peptidoglycans serve as PAMPs during Gram-positive sepsis.
Gram-positive bacterial cell walls are composed of multiple peptidoglycan layers, wall teichoic acids linked to the peptidoglycan and LTA linked to the cytoplasmic membrane. The amphiphilic LTA from most Gram-positive bacterial strains is generally made up of a hydrophilic backbone with repetitive glycerophosphate units and D-alanine or N-acetylglucosamine substituents, and a lipophilic glycolipid anchor. LTA is shed during bacterial replication, and after antibiotic administration.

Publications regarding the biological activity of LTA, such as induction of inflammatory mediators in various types of cells, are contradictory. In most of the published studies, commercial phenol extracted LTA preparations are used. It has been demonstrated that these preparations have a high degree of compositional heterogeneity and also contain significant amounts of LPS or other immunostimulatory substances. Furthermore, purification of LTA by standard methods using phenol extraction results in loss of D-alanine substituents, which are important to maintain the LTA pro-inflammatory activity. Taken together, the heterogeneity of the LTA preparations, contamination by endotoxin, and inappropriate methods of purification might explain the contradictory results in the literature. In paper II of this thesis LTA were purified using butanol/water extraction to isolate highly pure and biologically active LTA from *Staphylococcus aureus* (*S. aureus*). Recent studies using highly purified LTA prepared from *S. aureus* have shown that staphylococcal LTA can efficiently stimulate cytokine production through TLR2.

**Antimicrobial peptides**

The presence of antimicrobial components in blood and other body fluids, leukocytes and tissues has been known since the end of the 1800s and many were reported in the 1900s. For example, in the 1950s Hirsch reported the presence of a bactericidal protein in rabbit granulocytes that he called phagocytin and then, in the 1960s, Zeya and Spitznagel found cationic proteins in leukocytes, isolated from rabbit and guinea pig, to be antimicrobial. Today, more than 1950 AMPs have been observed in virtually all species, including bacteria, fungi, insects, amphibians, birds, fish, mammals, and humans.

AMPs are generally defined as having less than 50 amino acid residues, a net positive charge and contain around 50% hydrophobic amino acids. In
mammals, AMPs are mainly expressed in the epithelia and in the blood cells. Secretion of biologically active peptides is induced by factors such as bacterial products, injury, and/or inflammatory stimuli. 

Most AMPs are membrane active and lyse the target cell by disrupting the integrity of the membrane. They fold into a variety of secondary structures (often after they insert into membrane bilayers) in which the charged and polar, and hydrophobic residues form patches on the surface of the molecule. Due to differences in the microbe and mammalian membranes the peptides preferentially attack microorganisms. The cationic peptides are more attracted to the negatively charged membranes of bacteria than to the neutral mammalian. This difference in membrane charge is due to a higher level of acidic phospholipids in the outer leaflet of bacterial membranes, while eukaryotic membranes have more cholesterol and zwitterionic phospholipids facing the extracellular space, and the acidic phospholipids at the cytoplasmic side.

Despite the similar general physical properties of AMPs, individual peptides have very limited sequence homologies and a wide range of secondary structures with at least four major themes. The most prominent structures are 1) amphiphilic peptides with two to four β-strands, including the defensins 2) amphipathic linear α-helical peptides such as the cecropins and melittins 3) peptides that form loop structures with one or more disulfide bridges, like bacterenecin 4) extended peptides which often have a single amino acid dominating such as histidine, glycine, proline or tryptophan.

Although many AMPs demonstrate direct antimicrobial activity against bacteria, fungi, eukaryotic parasites and/or viruses, recently it has become evident that they also have a key modulatory role in the innate immune response. An overall scheme of separate effects is presented in figure 3.
Figure 3. Function of AMPs in immunity. Besides from direct killing of bacteria AMPs interact with host cells to indirectly eliminate pathogens, like induce cytokine and chemokine release, neutralize PAMPs and, promote apoptosis and wound healing. The overall scheme presented is a composite of separate effects. LPS, lipopolysaccharide; LTA, lipoteichoic acid; MCP-1, monocyte chemoattractant protein-1.
Below the peptide origins of the truncated peptides used in this thesis are described (see table 1, experimental procedures).

**hCAP18/LL-37**

Cathelicidins are characterized by a conserved N-terminal (the cathelin domain) that is proteolytically cleaved to generate the mature, active peptide contained within the C-terminus. In some mammals, multiple cathelicidins are found. The only human cathelicidin identified to date is called human cationic antimicrobial protein (hCAP18), the name refers to the mass of the full-length polypeptide (approximately 18 kDa) and the cationic character of the C-terminal sequence. hCAP18 is predominantly produced in the specific granules of neutrophils, where it has been detected at extremely high concentrations (~0.63 µg per 10^6 cells). The hCAP18 precursor is cleaved upon neutrophil degranulation by proteinase 3 to yield the 37 amino acid AMP LL-37. During infection and inflammation high concentrations of LL-37 will be released at the sites of neutrophil accumulation. As a result of proteolytic cleavage, LL-37 derivatives KR-20, RK-31 and KS-30 are generated. These three peptides exhibit stronger antimicrobial activity compared to full length LL-37. hCAP18 is also found in various blood cell populations that are involved in immune responses, including NK cells, B cells, monocytes and mast cells, and in different tissues such as the squamous epithelia of the skin, airways, mouth, tongue, esophagus, intestine, cervix, vagina, and epididymis.

LL-37 is potent against Gram-negative and Gram-positive bacteria, the bactericidal activity involves membrane disruption (pore formation, change of lipid packing and organization) following interaction with negatively charged bacterial molecules and insertion into the membrane. Considering that the antibacterial activity of LL-37 is inhibited by apolipoprotein A-1, other factors in human plasma, and physiologically relevant salt concentrations, the primary function of LL-37 might not be to kill bacteria directly but instead modulate immune responses. In this regard, several other functions have been described for LL-37. It acts as a chemotactic factor for monocytes, neutrophils, eosinophils, T cells, and mast cells and is capable of neutralizing the pro-inflammatory response to the TLR4 ligand LPS. Some published data shows that LL-37 can neutralize the TLR2 ligand lipoteichoic acid, however these experiments are made with high concentrations of commercial...
available LTA which might indicate that it is rather a neutralization of the contaminated LTA induced cytokine production. Paper II in this thesis demonstrated that LL-37 potentiates highly purified LTA induced cytokine production in human whole blood. LL-37 has also been shown to stimulate wound healing and angiogenesis\textsuperscript{92,93}, induce apoptosis\textsuperscript{94,95}, and increase the expression of costimulatory molecules on dendritic cells\textsuperscript{96}. Overall, LL-37 plays a critical role in selectively balancing responses to inflammatory stimuli, such as bacterial endotoxin, in human immune cells. The amino acid sequence of LL-37 is NH2-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-COOH\textsuperscript{90}. In this thesis two derivatives of LL-37 were used, LL33 (peptide 1-32) and IG23 (peptide 13-35), with proven LPS neutralization activity\textsuperscript{97}.

**Bacterial/permeability-increasing protein**

Bacterial/permeability-increasing protein (BPI) is a cationic protein with a molecular weight of 55 kDa, present in the azurophil granules of human neutrophils and the specific granules of eosinophils\textsuperscript{98,99}. It can also be detected on the surface of neutrophils and monocytes\textsuperscript{100,101}. The highly cationic N-terminal half of BPI has high affinity for the conserved lipid A region of LPS\textsuperscript{102}. Binding of BPI to LPS results in increased permeability of the outer membrane of Gram negative bacteria, inhibition of cell division, and also to potent neutralization of endotoxin activity\textsuperscript{103,104}. In animal models, BPI is protective against lethal and sub-lethal challenges with Gram negative bacteria and endotoxin\textsuperscript{105-109}.

BPI has a β-turn with alternating cationic and hydrophobic residues in its LPS-binding domain. A peptide, β-pep-25, is a designed peptide with 9 residues of the LPS-binding domain of BPI flanked by β-turn–inducing elements from IL-8\textsuperscript{110,111}. A series of dodecapeptides (SC1-SC8), which “walk through” the amino acid sequence of β-pep-25 has been investigated for the ability to kill bacteria and to neutralize LPS\textsuperscript{112}. One of these peptides, SC4 (KLFKRHLKWKIC-NH\textsubscript{2}), had the best bactericidal activity and displayed bactericidal activity at nanomolar concentrations against Gram-negative bacteria and at submicromolar concentrations against Gram-positive bacteria. SC4 also effectively neutralized LPS and showed no haemolytic activity below 100 µM\textsuperscript{112}. In papers I and II of this thesis cysteine terminated SC4 (KL12) was investigated for LPS binding and neutralization of LPS/LTA induced cytokine production in whole blood.
Factor C protein of the Limulus (horseshoe crab)

LPS can activate the coagulation cascade found in Limulus amebocyte lysate (LAL). This response underlies the important defence mechanism of Limulus against invasion of Gram-negative bacteria. In the presence of LPS, the LPS-sensitive Factor C, serine protease zymogen, is autocatalytically activated. The active Factor C then activates zymogen Factor B to active Factor B, which subsequently activates proclotting enzyme to clotting enzyme. The resulting clotting enzyme converts soluble coagulogen, an invertebrate fibrinogen-like substance, to an insoluble coagulin gel. This mechanism results in entrapment of the invading Gram-negative bacteria. Being the initial activator of the clotting cascade, Factor C functions as a biosensor that responds to subnanogram levels of LPS or lipid A. LPS binds with high sensitivity and avidity to at least two domains, Sushi 1 and Sushi 3, within the Factor C molecule, and four peptides with high affinity for LPS and antimicrobial activity have been designed based on these domains, of which S3Δ (NH2-HAEHKVKIKVKQKYGQFPQGTEVTYTCSGNFMYLM-COOH) is one example. HA27 in this thesis is composed of peptide 1-27 of S3Δ.

CEME

CEME is α-helical peptide derived from a hybrid of silk moth cecropin and bee melittin. A series of CEME variants with small amino acid changes have been designed to gain insight into peptide characteristics that are important for activity. No significant correlation was found between the length, charge, or hydrophobicity of the peptides and antimicrobial activity. The most active peptides had good antimicrobial and antiendotoxin activities, as well as higher LPS-binding affinity. One of the most active peptides called CP207 (NH2-KWKSFIKKLTSVLKKVVTTAKPLISS-COOH) had good antimicrobial activity against Gram-negative bacteria, high LPS binding affinity and, slightly better ability to neutralize LPS induced cytokine production in RAW macrophage cells compared to CEME. Therefore CP207 (KW27) was selected for further investigations in this thesis.

Polymyxin B

Polymyxin B (PMB) is a bacterium-derived cyclic AMP that is bactericidal and able to permeabilize the bacterial outer membrane. PMB is considered the “golden standard” of AMPs and it was used as a model substance in papers I
and II. Due to the fact that PMB is toxic to eukaryotic cells in therapeutic doses it is unsuitable as a systemic drug\textsuperscript{120}.

**Antimicrobial peptides in treatment of sepsis**

In principle, AMPs represent almost ideal candidate drugs in treatment of sepsis; they can be used as single anti-infective agents or in combination with conventional antibiotics as well as immunostimulatory agents that enhance innate immunity. AMPs have excellent antimicrobial activity \textit{in vitro} and, there is some evidence that this good \textit{in vitro} activity can translate to \textit{in vivo} activity. For example, in mice models, LL-37 protects against the lethal effects of LPS injection through neutralization of LPS and suppression of the cytokine production\textsuperscript{86,121-123}. Unfortunately, only a few AMPs have entered clinical trials based on promising data from \textit{in vitro} and animal studies. Two bacterium-derived AMPs, gramicidin S and PMB\textsuperscript{63} have found use in topical creams and solutions. However, these molecules tend to be toxic and this characteristic limits their potential for systemic use\textsuperscript{120}. Another reason for poor or incomplete \textit{in vivo} activity is lack of stability due to the action of host proteases. Understanding the full clinical potential of AMPs in sepsis therapy will require much more research.

**Antimicrobial peptides in extracorporeal removal of endotoxin**

In general, the host has developed innate mechanisms that orchestrate a rapid response to eliminate pathogenic bacteria, and blockade of these pathways may lead to disastrous consequences. The mediators of response to endotoxins such as cytokines and activated complement products play important role in the pathogenesis of sepsis; instead of removing these mediators a better solution would be to remove the reason of the immune response. Removal of PAMPs would inhibit the activation of cells, which in turn inhibits the release of inflammatory cytokines, controlling the inflammatory response and stabilizing the circulation. The removal of endotoxins from solution is well established but selective removal from blood is more difficult and requires the development of adsorbents capable of retaining high endotoxin selectivity under physiological conditions.

In 1983 Toray Industries Inc. developed a blood endotoxin removal cartridge with PMB-immobilized fibers which could be clinically applied by direct hemoperfusion (Toramyxin)\textsuperscript{124}. Studies have shown that Toramyxin clearly
reduces the amount of circulating endotoxin without releasing any PMB to the circulation\textsuperscript{125,126}. Treatment of severe abdominal sepsis with extracorporeal PMB adsorption was recently reported to significantly improve blood pressure, vasopressor requirement, and organ dysfunction and reduce 28-day mortality in a targeted population with severe sepsis and/or septic shock from intra-abdominal Gram-negative infections\textsuperscript{127}. In Japan, this device has been widely used for the treatment of sepsis since 1994, when Toraymyxin was accepted by the national health insurance scheme. However, despite widespread use in Japan, PMB devices have not been fully adopted elsewhere.

The Alteco LPS adsorber (Alteco Medical, Lund, Sweden) is a newly developed LPS adsorber based on a synthetic AMP bound to porous polyethylene discs. It received its European Approval of Conformity certificate in 2005. There are some limited human data that shows that the Alteco LPS adsorber can be used safely\textsuperscript{128} but it failed to show a significant reduction in circulating endotoxin concentrations\textsuperscript{129}. An observational study from Finland with nine septic shock patients showed that treatment with the Alteco LPS adsorber was associated with a decrease in endotoxin activity and sequential organ failure assessment (SOFA) scores\textsuperscript{130}. Another study comparing the Alteco LPS Adsorber with Toraymyxin in patients with Gram-negative sepsis showed improvement in clinical and laboratory variables with both devices. However, the 28-day mortality was 69% (9 of 13 patients), with only two survivors in each group\textsuperscript{131}.

**Biomarkers in diagnosis and prognosis of sepsis**

Biomarkers are molecules that are correlated with disease states or states of altered physiology. Biomarkers may not actually cause the disease, but they do represent a marker of biological process. Considering the complexity of the septic response, there is a great interest in finding biomarkers to accurately diagnose sepsis and to monitor critically ill patients. A desirable biomarker for identifying patients that need more intense monitoring and treatment would be an accurate marker that is easily and quickly obtained bedside. The systemic nature of sepsis and the large numbers of cell types, tissues and organs involved expand the number of potential biomarker candidates, compared with disease processes that involve individual organs or are more localized. Amongst the many biomarkers evaluated in sepsis none has sufficient specificity or sensitivity to be routinely employed in clinical practice\textsuperscript{132}.  

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The two biomarkers that have been most widely studied and used in patients with severe sepsis are CRP and procalcitonin (PCT)\textsuperscript{133-135}. Compared to CRP, PCT is more specific for bacterial infections and may help to distinguish bacterial infections from viral illnesses\textsuperscript{136-138}. In addition, it has been proposed that PCT is a better prognostic marker in sepsis than CRP\textsuperscript{139}.

Cytokine levels are notoriously variable in the blood and have proven rather difficult to assess from routine blood samples. The most reliable and widely utilized cytokine measure in septic patients is IL-6. However, its measurement has proven to be of limited clinical relevance as a biomarker for severe sepsis\textsuperscript{140,141}.

### soluble urokinase Plasminogen Activator Receptor

The urokinase-type plasminogen activator receptor (uPAR) is present on various immunologically active cells including monocytes, neutrophils, activated T lymphocytes, and macrophages, and also on endothelial cells, keratinocytes, fibroblasts, smooth muscle cells, megakaryocytes, and certain tumor cells\textsuperscript{142}. uPAR consists of three homologous domains (D\textsubscript{I}, D\textsubscript{II} and D\textsubscript{III}) and is linked to the cell surface by a glycosyl phosphatidylinositol (GPI) anchor\textsuperscript{143}. Domain D\textsubscript{I} carries the main binding site for the urokinase-type plasminogen activator (uPA)\textsuperscript{143}. The binding of uPA to its receptor uPAR mediates a variety of cellular activities like migration, adhesion, differentiation, and proliferation\textsuperscript{142}. A soluble bioactive form of uPAR (suPAR) is shedded or cleaved from the cell surface\textsuperscript{142}. Full-length suPAR (suPAR\textsubscript{full}) consists of all three domains but lacks the GPI anchor and can be cleaved into two soluble forms, suPAR\textsubscript{II-III} and suPAR\textsubscript{I}. The soluble receptor has similar extracellular functions as uPAR\textsuperscript{144} and has been detected in various body fluids, including blood, plasma, serum, urine, ovarian cystic fluid, and cerebrospinal fluid\textsuperscript{142,145-147}. Paper IV of this thesis demonstrates that suPAR also can be detected in human saliva.

suPAR is a rather new biomarker. Two research groups headed by K Danø, and F Blasi cloned uPAR in 1990\textsuperscript{148}, and identified suPAR in 1991\textsuperscript{149}. Since then intensive research regarding suPAR as a risk marker in various diseases has been carried out. In healthy individuals, suPAR levels are low and quite stable while the concentration increases in conditions that involve immune activation\textsuperscript{150}. Additionally, suPAR levels increase with age and are slightly higher in plasma from females when compared to males\textsuperscript{151}. In 1997 it was shown that
plasma suPAR levels were elevated in breast and colon cancer patients\textsuperscript{152} and in 2000 it was demonstrated that suPAR had strong prognostic value in HIV infected patients\textsuperscript{153}. Moreover, it has been suggested that plasma suPAR may be a general marker of low grade inflammatory processes and that elevated suPAR levels correlate with risks of developing cancer, diabetes, and cardiovascular disease in the general population\textsuperscript{151,154,155}.

As early as 1995, elevated plasma suPAR levels were reported in a small group of septic ICU patients\textsuperscript{156}. In 2004 it was shown that suPAR levels are elevated in patients with pneumococcal sepsis and predicts mortality in these patients\textsuperscript{157}. Though, it was primary in 2011 that several studies indicate that an elevated suPAR level in plasma is associated with a negative outcome in conditions of SIRS, bacteremia, sepsis, and septic shock\textsuperscript{150,158-163}. However, suPAR is more a severity marker than a sepsis marker considering that high suPAR levels are associated with increased mortality in both septic and non-septic populations.

**Saliva biomarkers**

Saliva has multiple roles: it is important not only for protection of the oral cavity, but also for general health, digestion and wellbeing. Saliva is secreted by exocrine glands; the two main sources are the major gland and the minor saliva glands. Saliva can be considered as gland-specific saliva and whole saliva. Whole saliva is a mixture of glandular secretions and gingival crevicular fluid. Human saliva contains many kinds of proteins and peptides; each of them carries several significant biological functions. Saliva, as a clinical tool, has become a more and more attractive option because of its ability to mirror both oral and systemic health conditions\textsuperscript{164}. A thin layer of epithelial cells separating the saliva ducts from the systemic circulation enables the transfer of substances to the saliva by means of active carriage, diffusion through the cell membrane, or passive diffusion via a concentration gradient. Although some molecules are transported into saliva from blood, others are synthesized by the saliva glands\textsuperscript{165}.

There is a great interest in exploring the utility of biomarkers in saliva, since compared to blood drawing, saliva collection is simple and non-invasive and does not carry any of the inconveniences or risks of drawing blood. However, saliva volume, viscosity, content of mucins, abundance of particulate matter,
and bacterial load vary considerably between individuals. These factors have the potential to influence the reliability and validity of the measurements of saliva biomarkers. Also, the most important limitation of saliva sampling in the ICU is the difficulty in collecting the samples in intubated and dehydrated patients, as shown in a previous study\textsuperscript{166}.

Both CRP and PCT have been detected in saliva\textsuperscript{167-169}, however, neither of these markers has been investigated as saliva biomarkers in sepsis. It has been demonstrated that saliva cortisol levels can be used as a surrogate of free serum cortisol level in patients with septic shock with very good correlation\textsuperscript{170,171}.
AIMS OF THE THESIS

The specific aims of each individual paper were:

Paper I
To investigate LPS neutralization/binding of soluble and immobilized truncated AMPs. Moreover, to examine the ability of these peptides to inhibit LPS-induced cytokine production in human whole blood.

Paper II
To analyse the effect of LL-37 and its truncated derivatives on human whole blood responses to LTA.

Paper III
To analyse plasma levels of suPAR in patients with severe sepsis and correlate it to the level of inflammatory activation, severity and mortality.

Paper IV
To investigate whether suPAR can be detected in saliva and if it correlates to plasma suPAR in healthy young individuals.
EXPERIMENTAL PROCEDURES

Cationic peptides (papers I and II)
The peptides used in this thesis were truncated derivatives of known AMPs (see table 1) with proven capacity to bind LPS. In paper II intact LL-37 was used. The cationic peptides were obtained from Innovagen (Lund, Sweden) and all were terminated with cysteine in the carboxy end in order to specifically immobilize them onto a solid matrix in an equal manner (for sequence see table 2). The molecular weight of the peptides was confirmed by mass spectral analysis. The 95 % purity was determined by HPLC.

Table 1. Peptide origin of the peptides investigated

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Based on amino acid sequence from</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>KL12</td>
<td>SC412</td>
<td>β-pep-25 peptide/BPI</td>
</tr>
<tr>
<td>IG23</td>
<td>LL-37 (peptide 13-35)97</td>
<td>hCAP18</td>
</tr>
<tr>
<td>KW27</td>
<td>CEME (CP207)172</td>
<td>Hybrid of silk moth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cecropin and bee melittin</td>
</tr>
<tr>
<td>HA27</td>
<td>S3Δ (peptide 1-27)114</td>
<td>Factor C protein of</td>
</tr>
<tr>
<td></td>
<td></td>
<td>the horseshoe crab</td>
</tr>
<tr>
<td>LL33</td>
<td>LL-37 (peptide 1-32)97</td>
<td>hCAP18</td>
</tr>
</tbody>
</table>

Table 2. Sequences of the truncated peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KL12</td>
<td>NH2-KLFKRHLKWKIIC-COOH</td>
</tr>
<tr>
<td>IG23</td>
<td>NH2-IGKEFKRIVQR1KDFLRNLVPRTC-COOH</td>
</tr>
<tr>
<td>KW27</td>
<td>NH2-KWKSFIKKLTSVLKKVVTTAKPLISSC-COOH</td>
</tr>
<tr>
<td>HA27</td>
<td>NH2-HAEHKVK1KVQKYQFGQGTEVYTEC-COOH</td>
</tr>
<tr>
<td>LL33</td>
<td>NH2-LLGDFRKSKEKIGKEFKRIVQR1KDFLRNLVC-COOH</td>
</tr>
</tbody>
</table>
Limulus amoebocyte test (paper I)

Endotoxin neutralization was performed using a kinetic chromogenic Limulus amoebocyte test (LAL) test, an indicator of the presence of free, non-neutralized LPS\textsuperscript{173}. LPS catalyses the activation of a Limulus proenzyme into an enzyme which cleaves a colourless substrate. When the enzyme cleaves the substrate, a coloured compound is produced and the developing colour was measured automatically every minute during 1 h at 37°C in a Bio-Tek Elx808 microplate reader. Data was transferred and analysed using the Endoscan-V software (Charles River Endosafe, Charleston, USA). The peptide concentration required to inhibit half of the maximum LPS-induced Limulus proenzyme activation (IC\textsubscript{50}) was calculated using a linear interpolation between adjacent peptide concentrations.

Biomolecular interaction analysis (paper I)

In order to monitor binding kinetics between the cationic peptides and LPS surface plasmon resonance (SPR) were analysed using a biomolecular interaction analysis (BIA) core 2000 instrument (BIAcore, Uppsala, Sweden). The SPR technique is an optical method for measuring the refractive index of very thin layers of material adsorbed on a metal. The protocol involves one interacting molecule (in our case the peptide) that is immobilized to a gold sensor chip and its counterpart (LPS) injected into a continuous buffer flow. If binding occurs to the immobilized target, the reflected light intensity changes. The change in angle is proportional to the mass of bound material and is recorded in a sensorgram. The interaction is monitored in real time, thereby offering the possibility of calculating the kinetic rate constants for the association and dissociation phases of the reaction. The ratio of these values gives the apparent equilibrium constant (affinity).

Whole blood incubations (papers I and II)

Heparinized blood was obtained from volunteers. Stock solutions of the different cationic peptides were prepared in endotoxin free water. Each peptide was mixed with LTA or LPS and diluted with 0.9 % physiological saline prior to addition of blood. The mixtures were incubated for 1, 3, 6, 12, 16 or 24, hours respectively at 37 °C and 5% CO\textsubscript{2} in 1.5 ml polypropylene reaction vials. After incubation, samples were resuspended and spun down (2 min at
1 000 g) and supernatants were analysed immediately or stored at -80 °C until cytokine measurement.

**Isolation of human leukocytes (paper II)**

Mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) were isolated from whole human peripheral blood by centrifugation with a density gradient medium (Polymorphprep™, Nycomed Pharma AS, Majorstua, Norway). After centrifugation, two leukocyte bands (PBMCs in the top band and PMNs in the lower one) were obtained. The yield and purity of both types of leukocytes are approximately 95 % according to the manufacturer. The autologous plasma on top of the PBMCs was also collected. To obtain a mix of leukocytes, both PBMCs and PMNs were resuspended in the autologous plasma.

**Alamar blue (paper II)**

The impact on cell viability of the cationic peptides and LTA was evaluated using the one step Alamar Blue assay. The oxidized form of Alamar Blue is a dark blue colour, when taken into cells the dye becomes reduced and turns red. This reduced form of Alamar Blue is highly fluorescent. The extent of this conversion, which is a reflection of cell viability, can be quantified by fluorescence measurement. The fluorescence values were normalized by untreated cells and expressed as per cent viability.

**Immunoassays (papers I-IV)**

**Enzyme linked immunosorbent assay (ELISA)**

In order to measure the cytokine concentrations in cell culture medium, plasma, serum or saliva, different ELISAs were used. ELISA methods are immunoassay techniques used for detection or quantification of a substance based on an immunological reaction. The cytokine analysis kits are constructed as 96 well plates were each well is coated with specific capture antibodies. Plasma, serum, cell culture medium or saliva was added to the wells and cytokines therein attached to the capture antibody. Different kinds of detection system were used. ELISA is an equilibrium method, and the results are correlated with the expression levels of the cytokine.
Luminescence immunoassay (LIA)
The PCT measurements were made using a LIA by BRAHMS (Henningsdorf, Germany). The serum was added to tubes that were coated with a monoclonal antibody against the katacalcin sequence. Then, PCT was detected with an anti-calcitonin monoclonal antibody labelled with a luminescent acridine derivative. The tubes were placed in a luminometer where hydrogen peroxide and sodium hydroxide reacted with the acridine derivative, which emits light as it transforms into acridone. The emitted light intensity is directly proportional to the PCT concentration.

Subjects
Paper III
The local research ethics committee approved the project and informed consent was obtained from patients or next of kin of the unconscious patients. Sepsis patients admitted to the ICU of Lund University Hospital, Sweden, between 23 March 2004 and 7 December 2007 were included. All patients fulfilled at least 2 out of 4 criteria for SIRS, had a suspected or verified underlying infection and respiratory and/or circulatory dysfunctions requiring intensive care. Therefore, they fulfilled the criteria for severe sepsis or septic shock. Severity of organ dysfunction was defined with the SOFA score on the basis of measurements during the first 24 h of admission. The median age of the septic patients was 65 years (range 28-87 years; n= 27); 10 males and 17 females. The mortality rate within 90 days after admission was registered. Survival of the septic patients in 90 days post submission to the ICU was 56 % (15/27 patients). Controls consisted of patients scheduled for neurosurgery. Informed consent was obtained. Sex distribution and age were similar in sepsis and control patients.

Paper IV
The study included a total of 20 healthy volunteers (10 female and 10 male) with a median age of 28 years (range 21-41 years). All participants gave written consent and the study was conducted in accordance with the sixth revision of the Declaration of Helsinki. Participants were students recruited at Malmö University.
Saliva collection (paper IV)

Unstimulated whole saliva was collected from the individuals using an oral swab (5001.02, Salimetrics, PA, USA). Participants rinsed their mouth with water and then the swab was placed under the tongue on the floor of the mouth for 1-2 minutes. After collection, the swab was centrifuged at 1500×g for 15 minutes. Saliva aliquots were immediately frozen at -20 °C.

Statistical analysis

Papers I and II

The differences between induced/inhibited cytokine productions by LPS/LTA/AMPs were analysed with the paired t-test. Differences were considered significant when P < 0.05 (*, 0.01 < P < 0.05; **, 0.001 < P < 0.01; *** P < 0.001).

Paper III

The differences in the plasma levels of suPAR between patients and controls, men and women, and between survivors and nonsurvivors were assessed using the Wilcoxon rank sum test. The difference between levels at admission compared to the four-day samples was assessed with the Wilcoxon signed rank test. Correlations between suPAR and the other inflammatory markers or age were calculated with linear regression on log-transformed data. Differences and correlations were considered as statistically significant when P < 0.05.

Paper IV

The differences in the suPAR levels between plasma and saliva and between males and females were evaluated by using Pearson’s correlation coefficient (r). The significance was tested using the paired t-test. Differences and correlations were considered as statistically significant when P < 0.05.
RESULTS

This section summarizes the most important findings from each paper. The complete version of each paper is present in the last section of this thesis.

Papers I and II

Biological questions addressed

Can the truncated peptides interact with LPS in solution? Does immobilization of the peptides change their ability to bind LPS? Do the peptides have an effect in whole blood stimulated with PAMPs like LPS and LTA?

Most important findings

Peptides truncated from the known antimicrobial peptides LL-37, SC4, S3Δ and CEME neutralized LPS activity in a dose-dependent manner in solution. Immobilization of these cationic peptides did not inhibit their ability to bind LPS (typical sensorgram see figure 4). Interestingly, some of the truncated peptides inhibited LPS induced cytokine production (IL-1β, IL-6 and TNFα) but potentiated LTA induced cytokine production in human whole blood (figure 5) however; on isolated monocytes the peptides inhibited both LPS and LTA induced cytokine production.

Figure 4. The BIAcore plot shows the LPS binding (8-125 nM) to peptide IG23, immobilized at the sensorchip surface. Injection of LPS starts and stops at the arrows. $K_D$ = equilibrium constant. RU = response unit.
Impact to the field
Since immobilization of these peptides did not inhibit their ability to bind LPS, these peptides can be considered for extracorporeal LPS removal in sepsis therapy. Considering that these peptides stimulated LTA induced pro-inflammatory cytokine production rather than inhibit it, care should be taken when considering cationic peptides, especially originated from humans, in treatment of Gram-positive infections.

Figure 5. Cytokine potentiation/inhibition. Human whole blood was stimulated with LTA (A) or LPS (B) in combination with cationic peptides (A: 20 µM B: 0.02-20 µM) for 16 h, 37 °C. After centrifugation plasma samples were analysed with ELISA to determine the concentration of cytokines. 20 µM LL33 and IG23, originating from human LL-37, in combination with LTA potentiated IL-1β secretion 4-fold and TNFα secretion 30-fold. Pure peptides (20 µM) showed no detectable cytokine production (not shown). Note the log scale on the x-axis. Results are presented as means ± SD of at least three independent experiments. (*) indicates a significant difference compared to LTA induced cytokine production.
Papers III and IV

Biological questions addressed

Is suPAR the super biomarker that can be used as a prognostic mortality marker in patients with severe sepsis? Is suPAR correlated with the level of inflammatory activation in patients with severe sepsis? Furthermore, can suPAR be detected in human whole saliva of healthy individuals?

Most important findings

Plasma levels of suPAR were increased in sepsis patients upon admission to the ICU and remained elevated during the first days of treatment (figure 6). The level of suPAR did however not significantly correlate with mortality, but the mean suPAR values were higher in nonsurvivors compared to survivors. Levels of suPAR did correlate with admission SOFA scores and the neutrophil granule protein myeloperoxidase (MPO) but not with the inflammatory markers CRP, PCT, IL-6 or IL-10 in patients with severe sepsis.

suPAR can be detected in saliva and levels are more than 10 times higher than the corresponding plasma levels in healthy young individuals (figure 7).

Impact to the field

Considering the fact that suPAR correlated with MPO but not inflammatory cytokines might suggest that suPAR reflects activation of the cellular immune system rather than exerting pro-inflammatory or anti-inflammatory actions. Nevertheless, since the suPAR level had almost equal prognostic value as the admission SOFA score perhaps a simple suPAR test can be included in clinical practice in the ICUs as an early assessment for low or high risk for developing organ failure.

The advantages of using saliva as a medium for non-invasive sampling compared to blood sampling has increased the interest for the use of saliva as a tool within systemic disease screening and diagnostics. Our detection of suPAR in saliva is clearly just an observation and future studies have to focus on the use of saliva suPAR as a marker for diagnosis or prognosis of any disease.
Figure 6. Plasma levels of suPAR in patients with severe sepsis and controls. P-values refer to statistically significant differences in mean levels of sepsis patients compared to controls, between deceased and alive sepsis patients at 90 days after admission or of sepsis patients at day 4 compared to the day of admission. The circles represent individual patients and the means is indicated with a horizontal line.

Figure 7. suPAR levels in plasma and saliva, healthy females and males. The circles represent individuals and the solid bars indicate mean values.
ONGOING STUDIES AND DISCUSSION

**Cationic peptide interactions with lipopolysaccharide**

LPS derived from invading bacteria can generate a significant inflammatory host response, which in some cases can cause significant tissue damage to the host itself. Therefore, removal of LPS has therapeutic potential. The use of immobilized AMPs in order to eliminate LPS is one such clinical application. Paper I in this thesis is an *in vitro* study that examines the degree of LPS binding by five peptide fragments derived from naturally occurring and synthetic AMPs.

LPS binding was compared between immobilized peptides and peptides in solution. The results indicated that the peptides LL33 and IG23 derived from LL-37 were the best LPS-neutralizers in solution with IC_{50} values below 1 µM, just as good as PMB. The SPR-based system BIACore was used to analyse binding capacity and stability of the interactions between LPS and the peptide fragments. The association (k_a) and dissociation (k_d) rate constants were determined simultaneously using a local curve fit to the equation for 1:1 Langmuir binding in the BIACore evaluation 4.1 software (BIAcore). From these values, the equilibrium dissociation constants (K_D) were calculated. The result showed high association between LPS and KL12, IG23 and KW27 respectively, while the peptides HA27 and LL33 showed lower association. However, it is not just high association that is necessary for a peptide to be useful in removal of circulating LPS by extracorporeal therapy, it is also important that the peptide show very little tendency to release LPS with time. The interaction between LPS and IG23, KW27, LL33 respectively was quite strong (K_D 0.6-1.5 nM). After the LPS injection stopped and LPS solution was replaced by running buffer, only minor amount of LPS dissociated from these peptides. This was
not the case for the peptides KL12 and HA27 ($K_D$ 5 nM). KL12 showed high association but LPS desorbed readily from the peptide when LPS solution was replaced with running buffer, hence KL12 showed no potential to be a candidate for extracorporeal LPS removal in comparison to the other peptides tested.

To further examine the potential utility of these peptides as a valuable tool in sepsis therapy, a key issue is whether the peptides can effectively interact with LPS present in authentic settings, and hence whole blood experiments were made. Stimulation with IG23, KW27, and LL33 significantly inhibited LPS induced pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) in human whole blood. The LPS concentration of 80 pg/ml used for stimulation of leucocytes mirrors a clinically relevant blood concentration in the early phase of Gram-negative sepsis$^{175}$.

Over all, the peptides IG23, KW27 and LL33 were more efficient in LPS binding/neutralization both in solution, in whole blood, and when immobilized to a solid matrix compared to KL12 and HA27. HA27 could not be evaluated in solution or in whole blood considering that HA27 induced a strong Limulus proenzyme activation and cytokine activation independently of LPS. This phenomenon has not been previously described. It is known that a number of proteolytic enzymes, peptidoglycans, simple polysaccharides, and dithiols have the ability to activate the Limulus assay$^{115}$. Further investigations are needed to explain this unknown activation, one could speculate that HA27 possesses an allosteric effect on Factor B.

Cationic peptide interactions with lipoteichoic acid

The interactions of bacterial and host components in activating the innate immune system is an important area of work. The role of LTA in these interactions is particularly important to address because it is less well studied in comparison to other factors like LPS. The prevalence of Gram-positive sepsis is nearly similar to that of Gram-negative$^{40}$ so there is definitely a need for efficient treatment regimens and the use of AMPs could have a great impact on this area. Though, the clinical development of therapeutic agents against LTA has been frustrated, maybe because there are no good assays with which to demonstrate the presence of LTA in the circulation.
There are many studies evaluating the inhibitory effect of LL-37 on LPS induced cell stimulation however, to date, there have been relatively few studies on the effect of LL-37 on responses to LTA by human leukocytes. Scott et al. showed that LL-37 inhibited TNFα production by LTA in the RAW 264.7 macrophage cell line. Furthermore Kandler et al. proved that LL-37 inhibited the production of IL-6, TNFα and IL-12 induced by LTA on dendritic cells. Nell et al. demonstrated that LL-37 inhibited IL-8 production in whole blood stimulated with LTA. All these results are based on commercially available LTA. Paper II of this thesis showed the contradictory result that LL-37 and its derivatives potentiated highly purified LTA induced TNFα and IL-1β production in human whole blood. However, IG23 and LL33 inhibited LTA induced TNFα production on monocytes. These results indicate that a whole blood system, i.e. interactions between the leukocytes, rather than isolated monocytes is essential for the enhanced LTA induced cytokine secretion by LL-37. Accumulated data suggest that platelets play a role in inflammation. A few experiments on platelet depleted whole blood demonstrated that LL37 inhibited LTA induced TNFα secretion (not shown). In addition, experiments with flow cytometry showed that platelets form complexes with other leukocytes in whole blood incubated with LL-37 and LTA (not shown). The ability of platelets to store, produce and release several pro-inflammatory and anti-inflammatory factors, make it an important modulator of the regulation of other immune cells.

Bowdish et al. demonstrated that LL-37 promotes the activation of ERK1/2 and p38 in monocytes at concentrations likely to be found at sites of acute inflammation (20-25 µg/ml) and that in the presence of GM-CSF, activation of these kinases occurred at concentrations of LL-37 as low as 5-10 µg/ml. They hypothesized that low concentrations of LL-37 (< 5 µg/ml), as predicted to be present at the onset of inflammation, are homeostatic and do not activate the effector cells of the innate immune response unless they exist in the presence of inflammatory signals such as pro-inflammatory cytokines. GM-CSF is produced by macrophages and T lymphocytes upon stimulation with TLR agonists and pro-inflammatory cytokines. In addition, Mookherjee et al. demonstrated that LL-37 enhanced IL-6, IL-8, and TNFα by PMBC stimulated with IL-1β.
Our results demonstrate the importance of whole blood experiments due to the complex interactions of LL-37 with leukocytes and platelets. The peptide concentrations used in paper II was rather high, but recent results show that LL-37 in low concentrations also potentiates whole blood cytokine production induced by LTA (figure 8). In summary, the results indicate that the cytokines produced by leukocytes and platelets stimulated with LTA may interact with LL-37 and promote increased TNFα and IL-1β production.

Hasty et al\textsuperscript{181,182} have observed that butanol extracted LTA forms complexes and synergizes with hemoglobin (Hb) to potently increase the amount of IL-6 and TNFα secreted by macrophages and monocytes in a whole blood preparation. Their hypothesis was that LTA forms a complex with Hb in a way that facilitates the presentation of LTA to the TLRs. In the present study, hemolysis is likely to contribute to the effect in whole blood considering the hemolytic effect of LL33 and IG23. However, paper II shows that stimulation of leukocytes by LTA is enhanced by IG23 both with and without Hb. Moreover it is well known that the hemolytic effect of cationic peptides is drastically reduced in the presence of plasma\textsuperscript{79}.

To determine whether the synergistic effect of the LTA-peptide mixtures could be due to physical interactions between the two molecules a non-denaturating gel electrophoresis was performed followed by silver staining. Although LTA migrates on a SDS-PAGE gel at \( \sim 8 \) to10 kDa, it migrates much slower and in
a diffuse manner on a nondenaturating gel. This migration pattern may be explained by the ability of LTA to form micelles in aqueous solution. Paper II shows that when incubated with LL33 or IG23, at a weight ratio of 1:1, LTA disaggregates and migrates faster and more diffusely. It has been suggested that the bioactivity of LPS decreases with the increased size of LPS aggregates and that this can be explained by the fact that the binding sites for mammalian proteins such as LBP, CD14, and others are hidden in multilamellar aggregates rather than in cubic aggregates. Maybe the LTA activity increases as the size of LTA aggregates decrease, making LTA more attractive for activation of human leukocytes. On the other hand, another possible explanation to the migration in gel electrophoresis might be that the LTA and peptide form complexes and that the complexes may more efficiently bind to TLRs, which may result in an enhanced response.

Considering that the peptides and LTA physically interact with each other, as shown by their interaction by nondenaturing gel electrophoresis it would be very interesting to see how immobilized peptides interact with LTA and therefore we have tried to analyse the binding capacity and stability of the interactions between LTA and the peptide fragments with BIAcore. Surprisingly, the results showed that LTA was not able to bind to immobilized peptides (data not shown).

To further investigate the relationship between LL-37 and LTA, the effects of LL-37 on cellular responses to LTA stimulation in combination with different PRRs were analysed. The production of both TNFα (not shown) and IL-1β (figure 9) in whole blood stimulated with LTA and LTA/LL-37 was significantly inhibited by anti-CD14, anti-TLR2, and a combination of anti-TLR2/TLR6. As expected, pre-treatment with antibodies against TLR4 did not affect the LTA induced cytokine production since TLR4 is the main receptor for LPS. CD14 is well known to mediate activation of monocytes by LPS. Here, CD14 was also shown to mediate LTA induced activation, which has previously been reported. TLR2 has been shown to form functional heterodimer complexes with TLR6 to recognize LTA. This can be seen in the current experiment since the combination of antibodies against TLR2 and TLR6 were more efficient in blocking the cytokine production induced by LTA compared to anti-TLR2 alone. An antibody against only TLR6 had low blocking effect. According to several lines of evidence we conclude that
activation of cellular responses by highly purified LTA is mediated by TLR2 and CD14, but is clearly independent of TLR4. However, the results did not add any information about the cytokine production induced by LL-37/LTA compared to LTA since there was no difference in cytokine inhibition by PRRs on LL-37 and LTA/LL-37 challenge. It is not entirely clear whether LL-37 initiates its immunomodulatory activities in a specific (i.e. receptor-mediated) or nonspecific manner. Studies have indicated that there are receptors on different cell types that interact with LL-37, e.g. formyl peptide receptor-like 1 for chemoattraction and the transactivator P2X7 for IL-1β processing, respectively. It would be informative and interesting to block these receptors and find out if the cytokine production by LL-37/LTA is changed.

Figure 9. Effect of anti-CD14, anti-TLR2, anti-TLR4 and anti-TLR6 antibodies on LTA/LL-37 induced cytokine production. Whole blood was incubated with antibodies 20 minutes before LTA (5 µg/ml)/LL-37 (20 µg/ml) challenge. All antibodies were used at 5 µg/ml. Release of IL-1β was quantified after 16 hours of incubation. Results are presented as cytokine concentrations (pg/ml), means ± SD of three independent experiments. Pure LL-37 (20 µg/ml) did not induce any detectable cytokine production. The differences between cytokine release were analysed with paired t-test, values marked (*) indicate a significant difference to LTA or LTA/LL-37 induced IL-1β (*, 0.01 < P < 0.05; **, 0.001 < P < 0.01; *** P < 0.001).
Prognostic value of suPAR in severe sepsis

More than two decades of research has shown that suPAR participates in a range of immunological effector functions including cell adhesion, migration, chemotaxis, immune activation, and the activity of these events may all add to the systemic suPAR concentration. When a pathogen invades the bloodstream, all of these effector functions are activated and the suPAR level may reflect the severity of the infection. To date, the main part of the published results regarding the role of suPAR in sepsis indicates that suPAR is more a prognostic marker than a diagnostic marker.

suPAR is still a rather new risk marker and when we analysed plasma suPAR in sepsis patients in 2010 there were only two articles published regarding this topic. In the first one, a study of 141 adult patients with Streptococcus pneumoniae bacteremia, Wittenhagen et al. found that suPAR levels were higher in the 17% of patients who died from the infection than in those who survived and that suPAR levels above 10 ng/ml independently predicted mortality. In the other study, Kofoed et al. compared the prognostic value of suPAR in 151 sepsis patients to that of other inflammatory markers and of the Simplified Acute Physiology Score (SAPS) II and the SOFA score. Their results showed that suPAR levels had a better prognostic value than PCT and CRP, equal to that of the admission SOFA score and almost as good as the SAPS II score.

Then, in 2011 four large cohort (55-200 sepsis patients) studies were published on the prognostic value of suPAR in bacteremia and sepsis. All four studies found that plasma suPAR concentrations were increased in infected patients compared to non-infected patients and that high levels of suPAR were associated with increased mortality. Despite the relatively low number of patients included in our study, the results are concordant with the larger cohort studies. We found significantly higher levels of plasma suPAR in patients with severe sepsis upon admission to the ICU compared to non-sepsis patients. However, the plasma level of suPAR did not significantly correlate with mortality, only with severity, although the mean suPAR values were higher in non-survivors compared to survivors (figure 6). A probable explanation to the lack of correlation between suPAR and mortality might be the low number of patients included in our study (n=27, ICU mortality = 44 %). Still, on the basis of the results in paper III and other studies, increased suPAR
levels in sepsis patients could be seen as an early warning sign, and higher levels could imply a worse prognosis. Moreover, the independent value of suPAR suggests it may also add value to risk algorithms.

At present, it is unclear whether plasma suPAR actually exerts pro-inflammatory actions or if it just reflects general inflammation. Further studies are needed for a satisfactory understanding of the regulatory mechanisms of suPAR in order to evaluate whether suPAR could be a potential novel therapeutic target in critically ill patients. In paper III suPAR levels were not correlated with the non-specific inflammatory markers CRP and PCT in sepsis patients. Previous reports have also showed no correlation or weak correlation between suPAR and CRP. suPAR did neither correlate with the pro-inflammatory cytokine IL-6, nor the anti-inflammatory cytokine IL-10. The lack of correlation observed between suPAR and other inflammatory markers might suggest that suPAR reflects a different aspect of inflammation. The correlation between suPAR and MPO might suggest that cleavage of suPAR depends on leukocyte activation, i.e. reflects activation of the cellular immune system. MPO is the most abundant component of primary azurophilic granules of granulocytes, and is secreted following activation of these leukocytes.

We measured suPAR plasma concentrations upon admission to the ICU, at the start of intensive care treatment, and in six patients after four days. In contrast to CRP, PCT, MPO, IL-6, and IL-10, plasma suPAR concentrations did not significantly differ within the first four days of ICU treatment. This suggests that the clearance of suPAR is low and/or that the release persists over a longer time compared to the other inflammatory markers. It has been demonstrated that suPAR plasma concentrations are correlated to renal function, suggesting that failing renal clearance might additionally contribute to elevated circulatory suPAR. In paper III no correlation between renal SOFA and suPAR levels was found. Our study did not include follow-up measurement after full recovery but it has been demonstrated that effective treatment of infectious diseases resulted in a decrease in suPAR levels after full recovery.

**Saliva suPAR**

suPAR levels may be measured in other settings than blood, for example the soluble receptor has been detected in urine, ovarian cystic fluid, cerebrospinal fluid, and in lung lavage fluid. To our knowledge, paper IV of this
thesis is the first to show that suPAR can be detected in saliva. The results indicated that suPAR levels are more than 10 times higher in saliva than plasma in healthy young adults with normal plasma suPAR levels. The high levels of saliva suPAR and the fact that saliva and plasma suPAR were not correlated argues against a simple reflection of plasma suPAR in the saliva. However, studies have shown that the prediction of serum CRP levels from saliva CRP levels is more accurate at higher serum CRP concentrations\textsuperscript{197,198}. In paper IV all the subjects had normal plasma suPAR concentrations. It would be of great value to measure saliva suPAR in another population with expected high or low plasma suPAR.

The mode of entry of suPAR into saliva is not yet investigated, however the high concentrations of suPAR are not likely obtained without high expression of uPAR in the oral cavity. Information about uPAR expression in the gingival tissue or saliva glands is limited, but its ligand, uPA, is expressed in gingival tissues\textsuperscript{197,198}. Moreover, Virtanen et al\textsuperscript{199} showed that uPA is expressed in the secretory cells of the saliva glands and secreted at low levels in saliva. Considering the high levels of saliva suPAR found in paper IV and the fact that full-length suPAR is able to bind uPA\textsuperscript{200}, an explanation for the low saliva uPA levels might be that most of the uPA is bound to suPAR and that the suPAR-uPA complex cannot be detected by the assay used. The role of suPAR in saliva is still unknown and further investigations are needed to establish the reasons for the high levels of saliva suPAR. It is worth stressing that an elevated suPAR concentration in saliva may be a consequence of periodontal inflammation. Oral health status was not investigated in paper IV; it would be of substantial value to examine if periodontal conditions affect the saliva suPAR level. If saliva levels of suPAR can be postulated as a saliva biomarker for periodontal disease activity, its reduction with treatment could be used as an objective end-point and therapeutic goal for guided intervention.
CONCLUDING REMARKS

Regarding the question if the AMPs investigated in papers I and II could play a role as therapeutic agents in sepsis, somewhat contradictory results are presented in this thesis. In the treatment of Gram-negative sepsis they can be useful in combination with conventional antibiotics to neutralize endotoxins. Regarding treatment of Gram-positive sepsis the outcome is not that clear. The results presented in this thesis demonstrate that LL-37 and its truncated derivatives enhanced the pro-inflammatory cytokine production induced by LTA. Despite our clear observations, repeated attempts and exact identical experimental conditions, this observation seems inexplicable, but needs to be addressed.

The general hypothesis is that AMPs can bind and neutralize the effect of LTA and therefore, the result in this thesis goes against several other reports. However, a finding is not necessarily true just because a majority of reports support it and alternative hypotheses may be more valid although with less power. On the other hand, although our result is interesting, the effect found in paper II may have a methodological explanation and there could be many errors in our study design. Some examples are listed below.

Using an *ex vivo* whole blood model as compared to the use of purified leukocytes might mimic the *in vivo* situation more closely. Our model might therefore provide a more physiological approach to the study of cytokine promoting effects of LTA and LL-37. However, it is a complex system and it should be kept in mind that the presence of platelets, plasma proteins, such as lipoproteins may also influence the plasma cytokine concentrations in a whole blood model. Also, heparinized blood was used. It has been showed that
heparin may bind cationic peptides and interfere with the signaling\textsuperscript{202,203}. To ensure that heparin did not interfere with the signalling, EDTA blood and citrate were also tested. Those experiments showed the same pattern as heparinized blood although not resulting in such high cytokine levels.

IG23 and LL33 have a cysteine at the C-terminal end (this was done in order to specifically immobilize them onto a solid matrix in an equal manner and initially to evaluate the interaction with LPS). It is well known that cysteine residues may lead to formation of disulfide bonds that in this case could alter the interaction affinity for LTA. However, regarding the potentiation of pro-inflammatory cytokine release similar effects was observed for LL-37 without the presence of cysteine.

Considering that the above circumstances do not seem to interfere with the peptide’s interaction with LPS, perhaps the explanation is found in the LTA preparation. We claim that our LTA preparation, obtained from Dr. von Au-lock, is "highly purified" because endotoxin contamination was excluded by negative LAL assay (less than 0.005 EU per mg LTA). As explained in the introduction, it has been controversial as to whether the LTA molecule exhibits immunostimulating activities. Papers by Hashimoto\textit{ et al}\textsuperscript{204-206} have shown that not LTA but lipoproteins are the dominant immune active compounds in \textit{S. aureus}. One could expect that the butanol extracted LTA preparation is contaminated with lipoproteins. Therefore, it remains unknown, which is the stimulatory component within the LTA preparation.

A major difference between LPS and LTA is that the injection of purified LPS alone can result in shock in an animal model, whereas the injection of purified LTA alone has never been shown to induce shock. Recently, researchers have begun to define molecules of both human and bacterial origin that synergize with LTA and greatly enhance its ability to stimulate cells of the immune system. Muramyl dipeptide, the minimal structural unit of peptidoglycan that retains immunostimulating activity, synergizes with LTA to enhance the induction of inflammatory cytokines in human monocytes\textsuperscript{207}. In addition it has been shown that both Hb and glycosphingolipids synergize with butanol extracted LTA resulting in a profound immune modulation\textsuperscript{181,208}. Perhaps it is also quite possible that the human cathelicidin LL-37 could synergize with LTA to increase pro-inflammatory cytokine secretion.
Successful monitoring of sepsis, especially in its early stage, may reduce any severe impacts on a patient’s health or help to prevent and/or delay succeeding complications. This thesis shows that high levels of suPAR may indicate high risk for developing organ failure. However, perhaps suPAR doesn’t have a future role in the ICUs considering that patients admitted there are severely ill and needs intense monitoring regardless of a low or high suPAR value. Instead suPAR can be used at the emergency departments to discriminate between patients that are critically ill and those that can be admitted to a general ward in hospitals. Then, suPAR can be a biomarker that is capable of saving hospital costs while at the same time improve patient triaging.

The ability to evaluate physiological conditions, trace disease progression, and monitor post-treatment therapeutic results through a non-invasive method is one of the primary objectives in the field of healthcare research. Considering that saliva can be collected through non-invasive means, saliva biomarkers are of great importance. We have shown that suPAR can be detected in saliva and considering that plasma suPAR is a promising marker of inflammation it may also serve as saliva biomarker. Clearly this is just an observation and now we have to evaluate whether saliva suPAR can serve as a marker for diagnosis or prognosis of any diseases.
Vårt medfödda immunförsvar uppfattar yttre hot, så kallade patogener, till exempel bakterier, genom att känna igen molekylära mönster på patogenernas yta. Vid en invasion av mikroorganismer aktiveras snabbt ett system av immunologiska celler (t.ex. monocyter, neutrofila granulocyter och epitcelceller) och signalsubstanter som angriper patogenerna. Vårt medfödda immunförsvar är mycket betydelsefullt för kroppens möjligheter att bekämpa infektioner. Paradoxalt nog är det när detta mycket potenta immunförsvar överreagerar som allvarliga komplikationer till infektioner inträffar, som exempelvis vid sepsis, blodförgiftning. Sepsis är ett potentiellt allvarligt tillstånd där bakterier eller deras toxi in aktiverar immunsystemet i hela kroppen vilket leder till att stora mängder inflammatoriska molekyler produceras. Dessa påverkar i sin tur andra viktiga system i kroppen som koagulations- fibrinolys- och komple mentsystemet. När sepsis orsakar rubbningar i blodcirkulationen, med otill räcklig syretransport ut i vävnaderna som följd, talar man om septisk chock. Septisk chock är en av de vanligaste orsakerna till att patienter dör på våra intensivvårdsavdelningar. Cirka 200 per 100 000 invånare i Sverige drabbas årligen av svår sepsis. Dödligheten för patienter med sepsis varierar i olika undersökningar mellan 30 och 50 % och ända upp till 60 % för patienter med septisk chock.

I alla våra slemhinnor och kroppsvätskor finns proteiner och peptider som hjälper till att skydda oss från patogena bakterier. Dessa kroppsegna antibiotika kallas antimikrobiella peptider (AMP). AMP är små, positivt laddade peptider som produceras av blod- och epitcelceller. På grund av den positiva laddningen har AMP en förmåga att binda till bakteriemembran, som oftast är

POPULÄRVETENSKAPLIG SAMMANFATTNING

Sepsis är ett stort problem inom sjukvården som kräver nya behandlingsmetoder, men också relevanta prognostiska markörer. Denna avhandling fokuserar på AMPs förmåga att integrera med olika toxin (LPS och LTA) från bakterier med avsikt att hindra deras inflammatoriska respons (artikel I och II). Fem olika peptider undersöktes, dessa peptider är korta versioner av kända naturligt förekommande eller syntetiska AMP. Avhandlingen beskriver också ett protein, soluble urokinase plasminogen activator receptor (suPAR), ur två aspekter dels som en prognostisk markör i sepsis dels metodologiskt att kunna bestämma halten av proteinet i saliv (artikel III och IV).

Resultaten från artikel I och II visade att tre av de fem peptiderna kunde binda till LPS både när de var i lösning och när de var fästa på en fast yta. Eftersom de kunde binda till LPS även om de var bundna till en yta skulle dessa peptider kunna användas för att ta bort LPS från blodbanan i ett system som behandlar blodet utanför kroppen. För att studera hur dessa peptider fungerar i kroppen gjordes även försök med blod från friska individer. Blodet behandlades med bakterietoxiner och vi undersökte om peptiderna kunde hämma det inflammatoriska svaret som toxinerna bildade. Resultaten visade att peptiderna kunde dämpa immunförsvarsvares reaktion på toxiner från Gram-negativa bakterier (LPS), men att de förstärkte immunförsvarsvares reaktion på toxiner från Gram-positiva bakterier (LTA). Hypotetiskt skulle detta innebära att en peptidbehandling av Gram-positiv sepsis skulle kunna förvärra inflammationen.

Proteinet suPAR är en relativt ny inflammatorisk markör som har förknippats med både svåra infektioner och låggradig inflammation. Artikel III är en klinisk studie där suPAR i plasma från patienter som vårdades för svår sepsis på intensivvårdsavdelningen i Lund undersöktes. Resultaten visade att koncentrationsnivån av suPAR var signifikant högre hos sepsispatienter jämfört med icke-sepsispatienter och något lägre (dock ej signifikant) hos patienter som överlevde jämfört med de som avled. Halten av suPAR korrelerade med SOFA score,
ett mått på organsvikt. Studien visade också att suPAR inte korrelerade med de vanliga inflammatoriska markörerna CRP och procalcitonin, det korrelerade inte heller med inflammatoriska eller anti-inflammatoriska cytokiner. Däremot korrelerade suPAR med ett protein som frisätts vid aktivering av vita blodceller, vilket skulle kunna tyda på att suPAR inte har en aktiv roll i inflammationen utan att koncentration av suPAR i blodet framförallt beror på aktivering av vita blodceller.

Artikel IV visade att suPAR även går att mäta i saliv hos friska individer och att koncentrationen är cirka 10 gånger högre jämfört med koncentrationen i blod. Idag vet vi att saliv speglar blodets sammansättning, denna kunskap samt fördelen med att använda salivprov i stället för blodprov har ökat intresse att använda saliv för sjukdomsspårning och diagnostisk. Ett stort område för biomarker i saliv är molekyler som kan indikera eventuella sjukliga tillstånd hos individen. Eftersom suPAR i blodet kan påvisa cancer, diabetes och hjärt- och kärlsjukdomar kanske även suPAR i saliv kan vara en bra markör. Salivmarkörer är av stort värde vid screeningundersökningar eftersom provtagning av saliv kan ske utan besvär för patienten och enkelt kan tas i hemmet. Sjukdomsscreening i saliv är ett relativt nytt område, men till exempel mätning av hormonnivåer i saliv har länge använts som komplement i kliniken vid utredning av endokrinologiska funktioner.

Sammanfattningsvis så visar denna avhandling tre peptider som skulle kunna användas i vidare studier för utveckling av nya behandlingsterapier mot Gram-negativ sepsis. Våra resultat understryker även vioken av att förska mer om AMPs interaktioner med toxiner från Gram-positiva bakterier. Denna avhandling visar också att suPAR kan vara en bra markör för att tidigt identifiera potentiellt kritiska fall bland sepsispatienter eftersom höga värden indikerar risk för organsvikt lika bra som SOFA score, som är ett betydligt mer komplikerat och svårberäknat test.
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