Modulating Organ Dysfunction in Experimental Septic Shock

Effects of Aminoglycosides, Antiendotoxin Measures and Endotoxin Tolerance

MARKUS CASTEGREN
Sepsis is a common diagnose in the intensive care population, burdened with a high mortality. The systemic inflammatory reaction underlying the development of septic organ dysfunction can be modeled using Gram-negative bacterial lipopolysaccharide, endotoxin. This thesis used a porcine endotoxemic experimental sepsis model to address clinical questions difficult to answer in clinical trials; furthermore a model of secondary sepsis was developed.

No additional effect on the development of renal dysfunction by tobramycin was found, indicating that a single dose of tobramycin does not further compromise renal function in inflammatory-induced acute kidney injury.

Antiendotoxin treatment had no measurable effect on TNF-α-mediated toxicity once the inflammatory cascade was activated. There was an effect on the leukocyte response that was associated with improvements in respiratory function and microcirculation, making it impossible to rule out fully the beneficial effect of this strategy. However, the effects were limited in relation to the magnitude of the endotoxin concentration reduction and the very early application of the antiendotoxin measure.

The lungs stood out compared to the other organ systems as having a threshold endotoxin dose for the protective effect of endotoxin tolerance. As to the development of circulatory and renal dysfunction, tolerance to endotoxin was evident regardless of the endotoxin pre-exposure and challenge dose.

There was a temporal variation of endotoxin tolerance that did not follow changes in plasma TNF-α concentrations and maximal tolerance was seen very early in the course. More pronounced endotoxin tolerance at the time of maximum tolerance was associated with a more marked hyperdynamic circulation, reduced oxygen consumption and thrombocytopenia eighteen hours later.

It might be of interest to use the experimental model of long-term endotoxemia followed by a second hit, which has been designed to resemble an intensive care setting, for the study of treatment effects of immunomodulating therapies in secondary sepsis.

Keywords: Lipopolysaccharide, animal model, pig, tobramycin, endotoxin elimination, immunoparalysis, secondary sepsis

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Picture taken by Markus Castegren in Laem Mae Phim, Rayong district, Thailand, February 9, 2011.
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals. Note that the author of this thesis changed his surname from Carlsson to Castegren in 2010.


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Abbreviations

AKI  Acute kidney injury
ALI  Acute lung injury
AMI  Acute myocardial infarction
ANOVA Analysis of variance
ARDS Acute respiratory distress syndrome
ATP  Adenosine 5'-triphosphate
B-   Analysis performed on whole blood
BE   Base excess
CARS Compensatory anti-inflammatory response syndrome
CI   Cardiac index
CLP  Caecal ligation and puncture
CRP  C-reactive protein
CV   Coefficient of variation
CXC-R2 Chemokine receptor 2
DAMP Danger associated molecular pattern
DO₂ Oxygen delivery
EAA  Endotoxin activity assay
ELISA Enzyme-linked immunosorbent assay
eNOS Endothelial-derived nitric oxide syntethase
FasL Fas ligand
FiO₂ Inspired oxygen fraction
HLA-DR Human leukocyte antigen DR
HMGB-1 High mobility group box protein 1
HPA  Hypothalamic-pituitary axis
I:E  Inspiratory:expiratory ratio
i.v. Intravenous
ICU  Intensive care unit
IL   Interleukin
iNOS Inducible nitric oxide syntethase
LAL  Chromogenic limulus amoebocyte lysate assay
LBP  Lipopolysaccharide binding protein
LPS  Lipopolysaccharide
LVSWI Left ventricular stroke work index
MAP  Mean arterial pressure
mCD14 Membrane bound CD14
MIF  Migration inhibitory factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MODS</td>
<td>Multiple organ dysfunction syndrome</td>
</tr>
<tr>
<td>MPAP</td>
<td>Mean pulmonary arterial pressure</td>
</tr>
<tr>
<td>MV</td>
<td>Minute ventilation</td>
</tr>
<tr>
<td>NAG</td>
<td>N-acetyl-β-D-glucosaminidase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>P-</td>
<td>Analysis performed on plasma</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>Arterial carbon dioxide tension</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Arterial oxygen tension</td>
</tr>
<tr>
<td>PaO₂/FiO₂</td>
<td>Ratio of arterial oxygen pressure to inspired oxygen</td>
</tr>
<tr>
<td>PCWP</td>
<td>Pulmonary capillary wedge pressure</td>
</tr>
<tr>
<td>PEEP</td>
<td>Peak end expiratory pressure</td>
</tr>
<tr>
<td>PIM</td>
<td>Pulmonary intravascular macrophage</td>
</tr>
<tr>
<td>Ppause</td>
<td>Pause proximal airway pressure</td>
</tr>
<tr>
<td>Ppeak</td>
<td>Peak proximal airway pressure</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RR</td>
<td>Respiratory rate</td>
</tr>
<tr>
<td>sCD14</td>
<td>Soluble CD14</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SG</td>
<td>Succinylated gelatin solution</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SvO₂</td>
<td>Mixed venous oxygen saturation</td>
</tr>
<tr>
<td>SVRI</td>
<td>Systemic vascular resistance index</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TV</td>
<td>Tidal volume</td>
</tr>
<tr>
<td>VAP</td>
<td>Ventilator-associated pneumonia</td>
</tr>
<tr>
<td>VO₂</td>
<td>Oxygen consumption</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell count</td>
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</table>
Introduction

Sepsis, or the more colloquial blood poisoning, is one of the leading causes of death in intensive care and is a main reason of why infections are potentially lethal. The word sepsis stems from the Greek word σέπειν, which translates “to cause decay”. Lever and Mackenzie define sepsis as a systemic illness caused by microbial invasion of normally sterile parts of the body. This systemic illness encompasses a general inflammatory situation and is the host’s response to invasion of threatening microorganisms, e.g. bacteria.

While the mortality rate in sepsis has decreased from approximately 45% in 1990 to 35% in 2000, there is evidence of the incidence of sepsis increasing. A Swedish investigation reported an overall mortality rate of 25% in sepsis while the intensive care unit mortality rate in septic shock was 57%. In addition to the substantial risk of death, the survivors are at high risk of suffering long-term complications resulting in reduction of their quality of life. The costs to society are also high; in Sweden the mean cost of care per patient admitted to the intensive care unit (ICU) and surviving sepsis was calculated to just below 40,000 Euro. An American survey estimated the economic burden of sepsis to nearly 17 billion US dollars annually.

Martin et al. reported an incidence of sepsis to 240 cases per 100,000 population in the year 2000. This incidence is almost the same as that of acute myocardial infarction (AMI), with an incidence of 287 cases per 100,000 population in the year 2000. The mortality in AMI is however much lower than in sepsis. Yeh et al. reported a mortality rate in AMI of 8% in 2008. In contrast to the treatment of sepsis, during the last decades many new effective therapies as well as the use of new biomarkers for a more rapid diagnose have evolved for AMI. Even though many new therapies for sepsis have been tested in clinical trials, no dramatic improvements have evolved to lower the staggering mortality rates.

Clinical research into novel therapies in sepsis treatment is cumbersome in many ways. Patient heterogeneity, problems with enrollment to clinical trials due to the acuteness of the condition and a small signal-to-noise ratio for the treatment effect in a study population at risk for death from many other conditions are some of the main problems. Even though animal models have numerous imperfections, due to the difficulties mentioned above they still play an important role, especially in basic research into the
pathophysiological basis of sepsis, e.g., development of organ dysfunction following systemic inflammation.

One century ago, the host response in the pathogenesis of sepsis was not regarded as important in comparison to the damage inflicted by bacterial toxins and bacterial virulence. The generalized Schwartzman reaction, i.e. inducing symptoms of sepsis with endotoxin, changed the view to focus more on the role of the host immune response in the development of sepsis\textsuperscript{10}. In the middle of the 1980\textsuperscript{th} decade, the correlation between severe infectious disease and multiple organ failure started to be clear, as well as awareness of the fact that non-infectious conditions such as pancreatitis, burns and systemic inflammatory diseases cause a reaction much alike the one observed in sepsis\textsuperscript{11,12}.

The host can produce a state of systemic inflammation as a response to non-infectious stimuli such as trauma, severe burns or pancreatitis, called the systemic inflammatory response syndrome (SIRS). The innate immune response in SIRS is similar to that in sepsis\textsuperscript{13} why the conditions can be difficult to distinguish in a clinical setting. Indeed, to diagnose a patient with sepsis, at least two criteria of SIRS (increased heart rate, respiratory rate, body temperature or leukocyte count or decreased body temperature or low leukocyte count) in addition to a suspected infection have to be fulfilled\textsuperscript{14}. A septic patient showing signs of hypoperfusion, hypotension or at least one organ dysfunction fulfills the criteria for severe sepsis, while a severe sepsis in addition to hypotension unresponsive to a standardized fluid resuscitation is defined as septic shock.

In a prospective study performed on close to 4000 patients admitted to ICUs, 68 \% met the criteria for SIRS. Among these, 25 \% developed sepsis while only 4 \% developed septic shock. Of the patients with sepsis and positive blood-cultures, 64 \% developed severe sepsis within two weeks, and 25 \% of the patients with severe sepsis progrediated to septic shock within the same time frame. However, the median time of progression from one stage to the next was 24 hours\textsuperscript{15}. The progression from systemic inflammation to multiple organ dysfunction, shock and ultimately death is highly complex, but the basis of understanding this lies within the host inflammatory response.

**Host inflammatory response**

The driving force of the immune system is the need to recognize danger while the goal is to respond to the dangers threatening the organism\textsuperscript{16}. The immune response to danger is inflammation. The immune system can be divided into two arms: the innate or natural immunity and the adaptive immunity. The innate immune response is instant and activates preformed proteins in the plasma cascades and immune competent cells like dendritic
cells, monocytes, macrophages, lymphocytes, and neutrophils. In contrast to the innate immunity, the adaptive immune response, mediated by B and T lymphocytes, can mount a specific immune response, i.e. antibodies and cytotoxic T cells, over a period of several days in a specific manner.

The danger model of inflammation

Matzinger proposed the “danger model” where the innate immune system is activated by molecular patterns derived either from pathogens or from stressed or damaged tissue. Danger associated molecular patterns (DAMPs) are recognized by pattern recognition receptors (PRRs) located either intracellularly or on the cell surface of innate immune cells. Bacteria constitute several DAMPs, e.g. lipopolysaccharide/endotoxin (Gram-negative bacteria), lipoteic acid (Gram-positive bacteria) and bacterial DNA. Endogenous stress DAMPs include, among others, high mobility group box protein-1 (HMGB-1), S-100 proteins and heat shock proteins. There are many PRRs described, among which the toll like receptor family (TLR) with 13 known members in mammals, are extremely important for activation of the innate immune response. The TLRs recognize different ligands, but several TLRs are known to respond to more than one molecular pattern. TLR2 recognizes lipoteic acid in addition to cell-wall components of yeast and mycobacteria but is also activated by lipoproteins and petidoglycans present in Gram-negative bacteria, e.g. Neisseria Meningitidis. TLR4 binds lipopolysaccharide but is also activated by endogenous DAMPs in hemorrhagic shock and following ischemia/reperfusion. Other TLRs important in bacterial activation of the innate immune response are TLR5 recognizing bacterial flagellin and TLR9 responding to both bacterial and viral DNA.

The binding of a ligand to TLRs leads to an intracellular activation of a signalling domain such as toll like/interleukin-1 receptor domain and their corresponding adaptors such as MyD88. Downstream cascade activation of protein kinases, e.g. IL-1R associated kinase, ultimately leads to activation of transcription factors such as NF-κB. The expression of transcription factors subsequently leads to production of cytokines, chemokines, reactive oxygen species (ROS) as well as lipid mediators (e.g. prostaglandins, platelet activating factor and leukotrienes).

Activation of the inflammatory response

The pro-inflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1) activate neutrophilic granulocytes, endothelial cells, epithelial cells in the intestine and lungs, and organ specific cells like hepatocytes. In addition, these cytokines further induce production of other cytokines, lipid mediators, chemokines and reactive oxygen species.
and IL-6 are the primary cytokine mediators together with prostaglandin E2 in the classical induction of fever. Two other central cytokines in SIRS and sepsis are migration inhibitory factor (MIF) and HMGB-1. MIF enhances production of cytokines, nitric oxide (NO), matrix metalloproteinases and prostaglandins while at the same time counteracting the immune suppressive effects of glucocorticoids. HMGB-1 in addition to being an endogenous DAMP induces migration of immune cells to the site of injury as well as their release of cytokines.

Activated neutrophils, through exocytosis of adhesion molecules, are recruited and sequestered to the site of injury and are vital in the elimination of pathogens through phagocytosis secondary to binding microorganisms opsonised by antibodies and complement. To migrate to the site of injury, neutrophils are dependent on a concentration gradient of inflammatory chemotactic mediators. In the case of SIRS and sepsis as opposed to a local infection, the specific migration is hampered because of the general high concentrations of mediators and a reduced chemotactic response of the neutrophils, thus risking unspecific adhesion to activated endothelia and in some cases neutropenia. Intracellular transformation of oxygen to highly reactive oxygen intermediates released into the phagosome or into the extracellular environment is a key element of the neutrophilic microbial killing capacity. Following TLR-mediated activation, neutrophils show prolonged survival and delayed apoptosis. However, phagocytosis of bacteria activates neutrophil apoptosis, a feature that is thought to have a vital impact on the resolution of inflammation.

Activation of endothelium induces increased leukocyte adhesiveness, a procoagulant surface and reduced barrier function. The endothelial production of NO has a significant impact on acute inflammation. NO produced by the constitutive NO-synthase (NOS) isoforms (endothelial-derived NOS and neuronal NOS) in the vascular endothelium and elsewhere acts as a neurotransmitter, an inhibitor of platelet aggregation and a vasodilator. During sepsis, activation of inducible NOS (iNOS) in the lung epithelium and other organs occurs, leading to NO overproduction. The result of excessive circulating NO is enhanced bacterial destruction, but also profound vasodilatation, activation of inflammatory cascades and depression of cardiac function. NO is also implicated in the development of decreased mitochondrial respiration leading to mitochondrial hibernation, which probably is a central mechanism for initiating MODS in critical illness. The concentration of NO, kinetics and localization both inside and outside of the cell are crucial factors determining the regulation and net effect of its presence and are probably the reasons for the double-edged effects seen on e.g. cardiac function, platelet activation and mitochondrial respiration.

Activated hepatocytes synthesize acute phase proteins, e.g. C reactive protein (CRP), fibrinogen, coagulation factor VIII and lipopolysaccharide-binding protein (LBP). It needs to be stressed that the acute phase proteins
all have important mechanisms in inflammatory processes and are not only measurable markers of diseases. One example is the commonly analyzed CRP, which enhances neutrophil phagocytosis by acting as an opsonin as well as being involved in endotoxin-induced complement activation.

Anti-inflammatory response
During acute inflammation, an anti-inflammatory response develops. This response has been given many names, e.g., immunoparalysis or compensatory anti-inflammatory response syndrome (CARS) \(^{32}\). The anti-inflammatory response is regarded as a control element with the purpose of limiting the potentially injurious effects of inflammation. The down-regulation of the pro-inflammatory mediators by anti-inflammatory substances abrogates the excessive and overwhelming inflammatory response \(^{33}\). Some important anti-inflammatory mediators are transforming growth factor β (TGF-β), IL-1 receptor antagonist and IL-10.

Pro- and anti-inflammation is not black or white as some proinflammatory cytokines, e.g. IL-6 display anti-inflammatory mechanisms depending on the plasma concentration \(^{34}\). Apart from production of anti-inflammatory mediators, a leukocyte reprogramming has been proposed as a central feature of the anti-inflammatory response with altered apoptosis-mechanisms and reduced expression of leukocyte cell surface proteins, e.g. human leukocyte antigen DR (HLA-DR) on monocytes and chemokine receptor 2 (CXCR2) on neutrophils. A reduced propensity of monocytes and macrophages to release pro-inflammatory cytokines after endotoxin stimulation is also a prominent feature of the anti-inflammatory response \(^{35}\).

Neuroendocrine regulation of inflammation
The central nervous system regulates innate immune responses through hormonal and neuronal routes. The neuroendocrine stress response and the autonomic nervous system generally inhibit innate immune responses at systemic and regional levels, whereas peripherally the autonomic nervous system tends to amplify local innate immune responses. These systems work together to first activate and amplify local inflammatory responses that contain or eliminate invading pathogens, and subsequently to terminate inflammation and restore host homeostasis \(^{36}\). The hypothalamus-pituitary axis (HPA) provides an important physiological feedback loop of inflammation through the anti-inflammatory effects of glucocorticoids. Glucocorticoids have been proven to induce a general inhibition of cytokine expression in myeloid cells by interference with NF-κB. Peripherally, glucocorticoids induce the production of MIF and thus exert a local negative feed-back mechanism of its own immune suppression \(^{21}\).
Plasma cascade systems involved in inflammation

In addition to the innate cytokine immune response, plasma cascade systems are involved in the inflammatory response and development of organ dysfunction, i.e. the complement system, the coagulation system, the fibrinolytic system and the kallikrein-bradykinin system. These systems are interconnected and there are several reciprocal regulatory mechanisms, e.g. thrombomodulin and activated protein C have marked anti-inflammatory properties; thrombin, tissue factor and factor VIIIa enhances proinflammatory cytokine production; thrombin and kallikrein activates complement through cleaving C3; C4b binding protein inactivates protein S and thus protein C driven anticoagulation; IL-6 promotes tissue factor expression.

Figure 1. Simplified picture of the activation of the innate immune response by endotoxin and the subsequent development of organ dysfunction.
Organ dysfunction and MODS

Multiple organ dysfunction syndrome (MODS) is defined as the progressive, potentially reversible dysfunction of two or more organ systems after acute, life-threatening disruption of systemic homeostasis. Approximately 75% of the MODS-cases are caused by sepsis, while the rest are secondary to trauma, ischemia/reperfusion injuries and major burns

Hypoxemia and tissue hypoperfusion have traditionally been held responsible for the development of MODS in sepsis. A central aspect in tissue hypoperfusion is microcirculatory dysfunction. Blood cell aggregation with formation of microthrombosis, endothelial cell swelling, arteriole vasoconstriction, endothelium injury, and increased microvascular permeability with interstitial edema are all factors proposed to be responsible for capillary flow shutdown. However, several non-hemodynamic mechanisms are proposed to contribute to septic organ dysfunction, e.g. cellular hibernation through bioenergetic failure, inflammation-induced apoptosis/necrosis and oxidative stress.

Cellular hibernation

The association between NO overproduction, antioxidant depletion, decreased ATP concentrations and mitochondrial dysfunction with organ failure and outcome in septic patients has proposed bioenergetic failure as an important pathophysiological mechanism underlying MODS. Mitochondria are responsible for around 95% of body’s production of adenosine 5'-triphosphate (ATP). The mitochondria are also involved in regulating cell death pathways, i.e. apoptosis and necrosis, and intracellular calcium regulation. The production of ATP requires acetyl coenzyme A provided from glucose or from β-oxidation of fats. ATP is produced in the mitochondrial Krebs cycle, however 15 times as many ATP molecules are produced in the electron respiratory chain where the electrons produced in Krebs cycle are invested and together with oxygen provides the energy for the ATP synthesis. Thus, in the order of 95% of the mitochondrial production of ATP is oxygen dependent. Rich calculated that the amount of inner mitochondrial surface area needed to support the ATP production required at rest equates approximately 14000 m², or two soccer fields. During early inflammation, the host’s oxygen consumption is increased, in part explained by increased mitochondrial respiration. A prolonged inflammatory response with tissue hypoxia, decreasing NO levels, sustained production of ROS and decreasing energy availability leads to mitochondrial down-regulation. With the cell’s power plant drastically cutting down on production, the cellular energy-dependent processes by necessity need to slow down, or the cell would die. This has been interpreted as a form of cellular hibernation, i.e. an adaptive mechanism designed to withstand...
overwhelming inflammation. Singer hypothesized MODS to “… not be failure as such, but a potentially protective, reactive mechanism.”

Cell death programs

The association between cell death and organ dysfunction is highly complex. In one sense apoptosis and necrosis are sophisticated processes that protect the host against harmful cells. On the other hand apoptosis has been considered as an underlying mechanism in acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) and MODS. To complicate the matter, evidence is building up challenging the dichotomy of apoptosis and necrosis and suggesting the two routes of cell death to be the two far ends of a continuum of cell death programs. Apoptosis can be triggered by death receptors, belonging to the TNF-superfamily. Apart from TNF-α, Fas ligand (FasL) and TNF-related apoptosis-inducing ligand are important ligands to death receptors of the TNF-superfamily. Apoptosis induced by TNF-α and FasL are implicated in endotoxin-induced ALI. There is also good evidence that renal tubular cells die through activation of TNF-superfamily death receptors in experimental models of acute ischemic and toxic renal failure. TNF-α and FasL are two of the possible culprits involved in septic acute kidney injury (AKI).

Oxidative stress

Reactive oxygen species (ROS), e.g. hydrogen peroxide, the hydroxyl radical, and superoxide, derived by leukocytes, endothelia and platelets have important roles in eliminating microorganisms but are also inducing cytokine expression through activation of NF-κB. ROS are implicated in the development of organ dysfunction and microcirculatory impairment seen during sepsis. During sepsis, production of ROS is increased which is also the case in endotoxin-induced inflammation. ROS have been found to activate platelets, to promote expression of adhesion molecules on endothelia cells and to promote tissue factor expression, resulting in a procoagulant situation with subsequent microthrombosis formation.

In animal models anti-oxidant therapy with for instance N-acetylcysteine or ascorbate, has been able to reduce inflammatory-induced ALI and to prevent microcirculatory plugging in addition to reducing iNOS expression. In a human endotoxemic model, ascorbate treatment was able to prevent vascular endothelial dysfunction and vasodilatation. Anti-oxidant therapy represents a promising way of preventing organ dysfunction, however no large clinical trials have been able to show any beneficial effect during sepsis.
Organ dysfunction induced by intensive care

The measures taken by critical care practitioners to stabilize a patient with severe sepsis and septic shock also influence the development of organ dysfunction. There are numerous obvious risks with intensive care, e.g. ventilator-associated lung injury, ventila
tor associated pneumonia (VAP), anesthesia-induced vasodilatation and negative cardiac inotropy, fluid overloading with subsequent pulmonary edema and intra-abdominal hypertension. Apart from these iatrogenic risk factors for morbidity there are also less evident implications of the role of intensive care in the development of organ dysfunction, e.g., ventilator-induced extra-pulmonary organ dysfunction.

An interesting area supporting non-hemodynamic mechanisms for septic organ dysfunction is organ-cross talk, a theory proposed to at least in part explain the results of the ARDS network study in which reduced mortality was observed in patients with ALI/ARDS that were ventilated with a lung-protective strategy. Imai et al. showed that rabbits with experimental ALI ventilated with an injurious strategy had increased epithelial cell apoptosis in the kidney and small intestine as well as reduced renal function. In this study, renal cells incubated in vitro with plasma from animals ventilated with an injurious strategy showed a markedly higher degree of apoptosis compared with cells exposed to control plasma. Expanding the study, the authors found that FasL blockade attenuated in vitro apoptosis of renal cells. To further confirm the FasL association to renal damage, the investigators used plasma from a clinical study of patients with ARDS and managed to find a correlation between plasma levels of FasL and creatinine.

Drug effects on inflammation and organ function

Many of the drugs used in intensive care have effects on inflammation and organ dysfunction. Catecholamines or catecholamine-increasing drugs such as ephedrine have been found to inhibit TNF-α and potentiate IL-10 production during human endotoxemia. Propofol as well as the anesthetic gas Sevofluorane have been shown to protect against ALI by suppressing proinflammatory mediators in animal endotoxemic models. Several common antibiotics used in the treatment of sepsis have also been attributed effects on inflammation. Ceftazidime and some other β-lactam antibiotics have demonstrated abilities to inactivate ROS. Tobramycin and gentamicin have both been demonstrated to protect lung epithelial cells from oxidative injury through inactivating ROS.

In 2006 Kumar et al. published an important study showing that, in the presence of septic shock, each hour’s delay in administration of effective antibiotic therapy resulted in a 12-％ increase in the risk of mortality. The surviving sepsis guidelines state that empirical antibiotic therapy for all
likely pathogens should be initiated within one hour from diagnosis\textsuperscript{63}. The combination of $\beta$-lactam antibiotics with aminoglycosides results in a broad antimicrobial spectrum with a theoretical rapid bacterial killing and low antibiotic-induced endotoxin release\textsuperscript{64,65}. Aminoglycosides carry the risk of induction of renal damage, especially after long-term use or over-dosage\textsuperscript{66-68}. The risk of suffering kidney injury is very high in the septic population. Around 30\% of all patients in intensive care suffer from AKI, half of which are related to sepsis\textsuperscript{48}. Of the patients with septic shock and positive blood cultures, around 50\% will develop AKI\textsuperscript{69}. The risk of AKI and its possible consequences may hamper enthusiasm for the use of aminoglycosides in the initial phase of septic shock by some intensive care practitioners\textsuperscript{70}.

Even if \textit{in vitro} and animal studies have demonstrated that functional alterations after exposure of kidney cells to high aminoglycoside concentrations may occur after a few hours\textsuperscript{71-73}, aminoglycoside-induced nephrotoxicity is clinically related to the cumulative dose and, in most cases, is evident first after several days\textsuperscript{67}. This experience and the fact that septic AKI is mainly caused by other mechanisms\textsuperscript{48,69} make it less probable that a limited initial aminoglycoside exposure would cause renal impairment of clinical relevance.

A double-blind, randomized, placebo-controlled trial addressing this issue would be of greatest interest but will be difficult to perform because of the limited time frame for enrollment due to the current recommendations and the heterogeneity of patients with sepsis.

**Experimental sepsis models**

Based on the methodological difficulties in clinical sepsis studies mentioned earlier, the experimental sepsis models are important catalysts in the rapidly growing development of knowledge of the inflammatory basis of sepsis. The clinical symptoms and inflammatory reaction seen in human sepsis can be mimicked in a variety of animal models; including intravenous infusion of live bacteria, intra-abdominal, intra-pulmonary, intra-cerebral or soft tissue inoculation of live bacteria, caecal ligation and puncture (CLP) and intravenous infusion of endotoxin\textsuperscript{9}. Regarding the models using live bacteria, the main dichotomy is whether the model has an infectious focus, i.e. lungs, abdomen or brain, or not, i.e. bacteremia.

The most common bacteria used in bacteremia models are \textit{Escherichia Coli}. Considerably high doses of live bacteria have often been used since doses corresponding to clinical sepsis often fail to produce pathophysiological responses\textsuperscript{74}. In most cases a 100- to 1000-fold more bacteria are required in bacteremic models compared to intra-peritoneal models to result in a septic response.
One of the most widely used experimental models is CLP. The caecum is ligated distal to the ileocaecal valve and punctured with two standardized needle punctures. This technique results in a rapid onset of shock and pathophysiological responses resembling those in sepsis 75. CLP however carries a wide variability in pathophysiological responses due to the uncontrolled amount of invading bacteria as well as inter-individual variance in the gastrointestinal microbiological flora.

Abdominal inoculation of live bacteria is a model with lower variability compared to CLP due to the possibility of controlling the bacterial spectrum and amount inoculated. In large animal models using abdominal inoculation, the bacterial inoculum is often embedded in a fibrin clot to reduce the mortality rate. This leads to development of intra-peritoneal abscesses and pathophysiological responses very similar to what is seen in sepsis 76.

The models described above together with intravenous (i.v.) infusion of endotoxin are the most commonly used models of experimental sepsis. All models result in a rapid development of organ dysfunction and shock, a clinical scenario mainly seen in human sepsis occurring in healthy patients with highly virulent bacteria, i.e. *Neisseria meningitidis, Streptococcus pyogenes* or *Streptococcus pneumoniae*. However, models aiming to resemble sepsis occurring in patients with inflammatory mechanisms already activated at the time of the septic hit, e.g. sepsis occurring following trauma, surgery or as a superimposed infection, are uncommon.

**Endotoxin/lipopolysaccharide**

During the late 19th century William B. Coley in New York started to treat patients with sarcomas and carcinomas unreachable for surgery with injections of something that would soon be known as “Coley’s toxins”. This was a mixture of heat-killed *Bacillus prodigiosus* (now called *Serratia marcescens*) and *Streptococcus pyogenes* 77. Administration of Coley’s toxins was reported to lead to tumor remission but also carried with it dramatic side effects, e.g. fever, shock and organ failure. The term endotoxin is a remnant of the century-old conception postulated by Richard Pfeiffer that endotoxins were located inside the bacteria *Vibrio cholera* to distinguish it from the already known exotoxins 78. Today we know that the biological effects of the lysates of heat-killed Gram-negative bacteria used in Coley’s toxins and in the early experiments by Pfeiffer, primarily were caused by a class of substances termed lipopolysaccharides (LPS).

**Molecular structure and inflammatory activation**

Endotoxin/LPS have an inward directed, i.e. directed towards the bacterial cellular membrane, lipophilic region called Lipid A, and an outward-directed
hydrophilic polysaccharide portion. The latter is composed of a core region bound to Lipid A, and the O-specific chain directed outward from the bacterial membrane. LPS, or specifically the membrane-bound Lipid A, constitute about 75% of the total membrane surface area of the outer Gram-negative bacterial cellular membrane, and is released during growth and lysis of the bacterial cell.

The structure of the O-specific chain is characterized by a very high variability even within a bacterial species and is the basis of the serological classification of bacterial strains, e.g. the LPS used in this thesis is derived from the *Escherichia Coli* strain O111. Galanos proved that the immunostimulatory structure of LPS is Lipid A through demonstrating that solubilized free Lipid A triggered an inflammatory and pathophysiological response comparable to LPS preparations. The structure of Lipid A is much more preserved among bacterial species than other parts of LPS. However, Lipid A derived from even single bacterial species exhibit small variations resulting in different biological activities of LPS derived from different bacterial strains.

LPS constitute a powerful danger associated molecular pattern initiating an inflammatory response mainly through activating peripheral monocytes or tissue macrophages, which both constitutively express the membrane-bound form of CD14 (mCD14). The acute phase protein LBP catalyzes transfer of monomerized LPS from aggregated structures to mCD14 that subsequently initiates binding to TLR4 in complex with MD2. Both MD2 and CD14 are thus requisites for an effective LPS action on TLR4. With the aid of soluble CD14 (sCD14), LPS may also activate CD14-negative cells such as endothelial cells and dendritic cells. Expression of mCD14 and secretion of sCD14 by phagocytic cells are increased by pro-inflammatory cytokines. In addition to the TLR4-coupled activation of the cellular innate immune system, LPS induces the complement system via complement factor C1q activation by Lipid A.

**Sensitivity to endotoxin**

A main feature of animal sensitivity to LPS is that it increases with higher phylogenetic maturity, i.e. humans are more sensitive to LPS than primates, which in turn are more sensitive than pigs. Frogs and fish are extremely resistant to LPS and Berzi et al. reported the lethal dose to be around 200 mg x kg\(^{-1}\) (*E.Coli* 078) compared to approximately 1 mg x kg\(^{-1}\) for dogs and rabbits. A dose-response relationship between logarithmic increases in the endotoxin dose and most physiological parameters has been shown in the pig. Because of the significant morbidity and mortality induced by higher doses of endotoxin, naturally no dose-response studies in the higher dose range have been conducted on humans. However, case reports on iatrogenic administration of endotoxin together with the
experiences of administration of Coley’s toxins, show that the human pathophysiological response to higher doses of endotoxin is similar to what is seen in the pig and other species\textsuperscript{77,86}. Administration of lower doses of LPS to healthy humans, i.e. in the order of nanograms x kg\textsuperscript{-1}, produces a very similar response to what is seen in animal models and during mild Gram-negative infection\textsuperscript{87}. In addition to the variation of LPS sensitivity between species, other factors are also important in influencing the pathophysiological response to a certain dose of LPS, e.g. sex hormones\textsuperscript{88}, rate of endotoxin administration\textsuperscript{89}, prior exposure of endotoxin\textsuperscript{35} and even season of the year\textsuperscript{90}.

Quantification of endotoxin in plasma
The quantitative measurement of endotoxin in plasma is most commonly performed using the chromogenic limulus amoebocyte lysates assay (LAL). The LAL-assay is associated with significant technical difficulties and can in addition be triggered by plasma proteins and fungal products rendering the measurement of endotoxin in septic patients very difficult\textsuperscript{91}. Amoebocytes of the American horseshoe crab\textit{Limulus polyphemus}\textsuperscript{†} contain granules containing serine protease zymogens which form a coagulation cascade extremely sensitive to induction by LPS\textsuperscript{93}. Since the LAL-assay can be triggered by extremely small amounts of LPS, endotoxin-free sampling vials should be used and extreme hygienic precautions need to be taken during blood sampling and laboratory practice for endotoxin concentration analysis. The endotoxin activity assay (EAA) is a chemiluminiscence-based analysis for quantifying endotoxin levels in blood, based on complement activation of the luminescence reaction. Marshall et al. showed the EAA to be able to detect endotoxemia in patients with Gram-negative bacteremia in a higher percentage than the LAL-assay\textsuperscript{91}.

Porcine endotoxemia as a model of sepsis
The experimental model of sepsis used in this thesis is a large animal endotoxemic model. The pig is one of the most phylogenetically mature non-primate mammals, thus allowing more translational comparisons to clinical issues than for instance rodent models. The porcine models are

\textsuperscript{†}Dolksvans (swedish), see cover. Belongs to the phylum Arthropoda, subdivided into four classes: Insecta (insects), Arachnida (spiders), Crustacea (shellfish) and Merostomata (horseshoe crabs). While the other three classes of Arthropoda contain a sum of more than a million species, Merostomata are currently represented by merely four species. The evolution of the horseshoe crab extends back far before the dawn of human civilization, before the dinosaurs and before flowering plants which explains its unique immune system used in the LAL-assay\textsuperscript{92}. 

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approximating the human size allowing advanced preparatory procedures and invasive monitoring. In addition, ventilators, ventilator-protocols and fluid regimens that are normally applied on humans are possible to use in porcine models, thus facilitating comparison of results. This together with the fact that the porcine pulmonary, cardiac, renal and gastrointestinal anatomy and physiology are similar to those of humans has made the pig a common model animal 94.

As mentioned earlier, a dose-response relationship between endotoxin dose and pathophysiological responses has been shown 85. In the lower dose range, i.e. <0.25 µg endotoxin x kg\(^{-1}\) x h\(^{-1}\) (E.Coli 0111:B4 Sigma) the response is that of a mild general inflammation with increasing leukocyte counts, mild fever and only slight and transient changes in hypoperfusion and physiological parameters. Above 1 µg endotoxin x kg\(^{-1}\) x h\(^{-1}\) (E.Coli 0111:B4 Sigma) a potent systemic inflammatory reaction occurs with a rapid development of organ dysfunction as to circulation, respiration, hypoperfusion and kidney function. The mortality rate following endotoxin doses between 1 and 4 µg endotoxin x kg\(^{-1}\) x h\(^{-1}\) (E.Coli 0111:B4 Sigma) is usually around 20 %, which also resembles the situation in human sepsis 89.

One limitation of the porcine model is the presence of pulmonary intravascular macrophages (PIMs), which contrasts to findings in mice and humans 95. It has been shown in sheep, a species normally equipped with PIMs, that these cells might increase the sensitivity to endotoxin 96. However, apart from the endotoxin-induced rapidly developing pulmonary hypertension that occurs before the activation of TNF-\(\alpha\) and for which the PIMs and mediators, such as endothelin, have been considered responsible 95,97,98, oxygen exchange and pulmonary compliance deteriorate gradually in a way similar to what is observed in patients. The pulmonary hypertension is usually reaching a peak during the first hour of endotoxin administration followed by a decrease to lower but still increased levels 85. It has been shown that the pulmonary hypertension is attenuated if endotoxin is administered with an initial gradual increase of the infusion rate 89,99.

The initial circulatory response to endotoxin is that of a hypodynamic circulatory state, i.e. decreased cardiac index (CI), oxygen delivery (DO\(_2\)) and increased systemic vascular resistance index (SVRI) 89. During a continuous endotoxin infusion there is a transition to a hyperdynamic circulation after approximately twelve hours with decreasing mean arterial blood pressure (MAP) and SVRI and increasing CI, reaching levels commonly seen during human hyperdynamic sepsis 100.

It must be stressed that the porcine endotoxemic model animals are not suffering from septic shock but from endotoxemic shock, limiting the possibility to extrapolate results to a patient clientele. The inflammatory reaction induced by endotoxin is, however, a very straightforward model of systemic inflammation, unbiased by effects from bacterial toxins 9. The effects of bacterial toxins are possibly of importance in the aforementioned
bacteremia-models using very high bacteria-counts to induce the septic inflammatory reaction.

The endotoxemic models are thus suited for studying and modulating systemic inflammation-induced specific organ injuries. Moreover, endotoxemic models are well suited for studying endotoxin specific therapies, e.g. antiendotoxin strategies, and endotoxin specific phenomena, e.g. endotoxin tolerance.

Antiendotoxin strategies

Endotoxin plays an important role in triggering the inflammatory response underlying the septic syndrome. Well over 70 % of patients with severe sepsis and septic shock have elevated levels of plasma endotoxin and it has also been shown that higher plasma levels predict higher mortality. A large number of clinical studies have investigated the effects of different treatment strategies aimed at reducing the plasma endotoxin concentration. During the 1980th and 1990th decades first polyclonal and later monoclonal antibodies directed to endotoxin were developed and tested. Initial single-center trials reported positive results with reduced mortality and morbidity. The following larger multi-center trials were not able to find any effect of antiendotoxin antibodies and a lack of neutralizing capacity was proposed as a reason for the failures.

Perfusing a column with endotoxin-binding capacity is the basis of the extracorporeal apheresis technique to eliminate endotoxin. Materials such as polymyxin B-immobilized fibres and diethylaminoethyl-modified cellulose have both been used in clinical trials that initially reported positive results. The positive results included improved survival, the mortality rate in the control groups was however very high, e.g. one study reported 89 % 30-day mortality in the standard-therapy group. The following multi-center trials failed in reporting improved survival or even decreased cytokine or plasma endotoxin levels. The latter studies showed some positive effects of the antiendotoxin therapy in secondary variables, e.g. improved DO₂ and CI during the second day after inclusion and less need for renal replacement therapy.

Even though the concept of modulating the inflammatory response and organ dysfunction by removing an important trigger of inflammation represents an interesting and apparently safe approach, the lack of conclusive results after numerous studies raises questions on the entire antiendotoxin concept. A fundamental question that needs an answer is whether antiendotoxin strategies have the ability to modulate the host inflammatory response once a systemic inflammatory response already has been induced.
Endotoxin tolerance

It is known that re-exposure of animals or cells to endotoxin after a previous dose is not accompanied by the profound metabolic and pathophysiological changes that are induced by the first encounter 113. This phenomenon is called endotoxin tolerance and has been widely investigated and recently reviewed 113-115. In several studies on endotoxin tolerance the inflammatory response has been investigated and a reprogramming of leukocytes has been demonstrated with diminished releases of TNF-α and other inflammatory cytokines 116-119. As endotoxin tolerance is defined as a reduced responsiveness to endotoxin, the propensity of blood to release cytokines after *ex vivo* endotoxin exposure is often used to quantify the level of endotoxin tolerance 114. Several authors have reported on the key role of iNOS in modulating endotoxin tolerance 120,121. Dias et al showed that mice deficient of the iNOS gene were not rendered tolerant to endotoxin and that in wild-type endotoxin tolerant mice given the specific iNOS-antagonist aminoguanidine, the normal physiological response to endotoxin returned 121.

Endotoxin tolerance can be regarded as a protective feature of the innate immune system as it has in animal studies been shown to reduce mortality 122,123 and to prevent cardiac, renal and lung injury following ischemia/reperfusion injury 124-126. Endotoxin tolerance has in addition been reported in leukocytes of septic patients, post-operative patients, trauma and pancreatitis patients and patients surviving cardiac arrest and resuscitation 127.

In patients with ARDS, contrary to the general view of organ protection in the presence of endotoxin tolerance, the severity of the lung injury was associated with the level of endotoxin tolerance 129. Hoogerwerf et al. recently demonstrated that alveolar macrophages in the human lung were hypersensitized after instillation of endotoxin as reflected by an increased *ex vivo* endotoxin-induced expression of IL-1β and IL-6 genes six hours after the first exposure 130. Furthermore, in mice it has been demonstrated that bronchoalveolar cells are less likely than splenocytes, peritoneal cells and bone marrow cells to develop endotoxin tolerance 131. One could hypothesize that this discrepancy in results concerning the pulmonary reaction during endotoxin tolerance might be explained by differences between the studies as to the time and dosing of endotoxin exposure.

Endotoxin tolerance is of interest in clinical situations when the anti-inflammatory response has been activated, e.g. post-operative sepsis or super-imposed infections with sepsis. As discussed in the experimental sepsis models section, models resembling the latter clinical situations are, if ever published, scarce. The effects of endotoxin tolerance have as described above been studied extensively, however most studies have focused on specific organ effects rather than the net effect on sepsis manifestations. To
be able to develop an experimental model of sepsis based on endotoxin tolerant subjects, this knowledge is vital.

In experimental models, the time to induce tolerance, i.e. between the first endotoxin dose and the second hit, varies widely between different studies; in the above referred investigations the first hit was given between nine hours to six days before the second hit. Gresiman and Hornick described an early and a late phase of endotoxin tolerance, where the early phase develops and starts to wane within hours and the second phase, associated with high serum levels of anti-endotoxin antibodies, takes several days to evolve. There is not much conclusive work done on the temporal development of the early phase of endotoxin tolerance, as recently discussed by West et al. This naturally raises questions on reproducibility of and comparability between diverging results.
Aims

We aimed to investigate the following issues in our endotoxemic pig model:

- whether a single high dose of an aminoglycoside further deteriorates systemic inflammatory response-induced renal dysfunction.
- whether the antiendotoxin concept has the ability to affect the inflammatory response once a general inflammatory state has been established.
- whether reduced plasma endotoxin concentration has the ability to affect hemodynamics, hypoperfusion and organ dysfunction once a general inflammatory state has been established.
- whether tolerance to endotoxin uniformly affects physiological manifestations of severe sepsis such as hypoperfusion and organ dysfunction.
- whether the effect of endotoxin in endotoxin tolerant animals is dose dependent with respect to preexposure and challenge dose.
- whether endotoxin tolerance, once developed, is constant during a twenty-four hour infusion of endotoxin.
- whether individual levels of endotoxin tolerance correlate to circulatory changes and organ dysfunction.
Methods

In total 77 piglets weighing 27.4 ± 1.4 (mean ± SD) kg were included in the studies. All animals were handled according to the guidelines of the Swedish National Board for Laboratory Animals and the European Convention on Animal Care. The Animal Ethics Board (Permit number: C 215/5) in Uppsala, Sweden, approved the experiment. The animals were between nine and eleven weeks old and were without evidence of illness. Water and food access was ad libitum until one hour before the experiment.

The experiments in paper IV were performed on blood drawn from the animals in paper III during the first twenty-four hours of endotoxemia.

Anesthesia and fluid administration

All animals were given premedication with 50 mg xylazin intramuscularly immediately before transport to the research facility. General anesthesia was induced by injecting a mixture of 6 mg x kg⁻¹ tiletamin-zolazepam, 2.2 mg x kg⁻¹ xylazin and 0.04 mg x kg⁻¹ atropine intramuscularly. A bolus dose of 20 mg morphine and 100 mg ketamine were given i.v. through a catheter placed in a peripheral auricular vein before securing the airway. Xylacín is an α₂-receptor agonist, zolazepam is a benzodiazepine, whereas tiletamin and ketamine are N-methyl-D-aspartate receptor antagonists. Anesthesia was maintained with 8 mg x kg⁻¹ x h⁻¹ sodium pentobarbital, morphine 0.26 mg x kg⁻¹ x h⁻¹ and 0.48 mg x kg⁻¹ x h⁻¹ pancuronium bromide dissolved in 2.5% glucose solution, given as a continuous i.v. infusion. If deepening of the anesthesia was needed during the experiment, 50 mg ketamine was given i.v.

Paper I and II

Fluid was administered with a total infusion rate of 30 mL x kg⁻¹ x h⁻¹ as 8 mL x kg⁻¹ x h⁻¹ of anesthetic infusion and 22 mL x kg⁻¹ x h⁻¹ of 0.9 % sodium chloride solution.

Paper III

Ketamine 1 mg x kg⁻¹ x h⁻¹ was added to the anesthetic infusion. No atropine was given with the induction dose. The anesthetic infusion was supplemented by 2 mL x kg⁻¹ x h⁻¹ of Ringer’s acetate solution, resulting in a total crystalloid infusion rate of 10 mL x kg⁻¹ x h⁻¹. In addition to this, bolus
doses of 4% succinylated gelatin (SG) up to 15 mL x kg\(^{-1}\) x h\(^{-1}\) were given according to the intensive care protocol (*Table 1*).

**Preparations**

In *paper I and II*, the airway was secured by performing a surgical tracheotomy, whereas in *paper III* the animals underwent orotracheal intubation. A branch of the cervical artery was catheterized for pressure monitoring and blood sampling. A central venous line and a 7F Swan-Ganz catheter were inserted through the internal jugular vein into the superior caval vein and the pulmonary artery, respectively. A minor vesicotomy was performed and a cystostomy catheter was introduced into the bladder. As the preparations were completed, a thirty-minute stabilization period was allowed before baseline values were obtained.

**Paper III**

All the preparatory procedures were undertaken under aseptic conditions. Before the start of the preparations, 20 mg x kg\(^{-1}\) cefuroxime sodium was given i.v., thus decreasing the risk of bacterial contamination of the model. In addition to the preparations above, after the minor vesicotomy was performed, a 14 Ch Bülow drainage tube was placed intra-peritoneally before closure of the abdomen. The drainage was applied with the purpose of minimizing ascites fluid from causing intra-abdominal hypertension and subsequent restriction of pulmonary compliance and reduced cardiac preload. As the preparations were completed, the animals were placed in the left lateral position and given a colloid bolus of 10 mL x kg\(^{-1}\) of 4% SG.

**Maintenance of vital functions**

The animals were mechanically ventilated, with a volume-controlled mode, throughout the experiment. Respiratory settings were: respiratory rate (RR) 25 min\(^{-1}\), inspiratory-expiratory ratio (I:E) 1:2, inspired oxygen fraction (FiO\(_2\)) 0.3, positive end-expiratory pressure (PEEP) 5 cmH\(_2\)O, tidal volume (TV) 10 mL x kg\(^{-1}\). TV was adjusted before the start of the protocol to result in PaCO\(_2\) of 5.0-5.5 kPa. In *paper I and II*, no respiratory settings were adjusted once the protocol was started. In *paper III*, the respiratory settings were subject to change according to the intensive care protocol (*Table 1*).

If MAP approximated mean pulmonary arterial pressure (MPAP) during the first hour of the protocol (ninety minutes in *paper III*), a single dose of 0.1 mg adrenalin was given i.v.
Paper III

Atelectasis was prevented and treated by performing an alveolar recruitment manoeuvre every third hour. This was performed by holding a respiratory pause during the inspiratory phase for ten seconds at a Ppeak of ≥30 cmH$_2$O. If the animal had Ppeak of <30 cmH$_2$O prior to alveolar recruitment, PEEP was increased to result in Ppeak of 30 cmH$_2$O and returned to the initial PEEP after the recruitment manoeuvre. In addition, the animals were turned 90° every third hour to the following set positions: left lateral to prone, prone to right lateral, right lateral to prone, and so forth. The goal was to resemble an intensive care setting where the animals were treated according to a strict protocol to vital parameters within pre-set limits. The interventions and the threshold values for intervention are shown in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Threshold value</th>
<th>Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{PaO}_2$</td>
<td>&lt;12 kPa (&lt;90 mmHg)</td>
<td>Increase FiO$_2$ 0.1</td>
</tr>
<tr>
<td>$\text{PaO}_2$</td>
<td>&gt;21 kPa (&gt;157 mmHg) or &gt;18 kPa (&gt;135 mmHg) for 1 h</td>
<td>1. If PEEP &gt;5 cmH$_2$O, decrease PEEP 2 cmH$_2$O. Lowest possible PEEP 5 cmH$_2$O. 2. Decrease FiO$_2$ 0.05. Lowest possible FiO$_2$ 0.3.</td>
</tr>
<tr>
<td>$\text{PaCO}_2$</td>
<td>&lt;4.5 kPa (&lt;33 mmHg) or &lt;5.0 kPa (&lt;38 cmH$_2$O) for 1 h</td>
<td>Decrease TV 10%</td>
</tr>
<tr>
<td>$\text{PaCO}_2$</td>
<td>&gt;6.0 kPa (&gt;45 mmHg) or &gt;5.5 kPa (&gt;41 mmHg) for 1 h</td>
<td>1. Increase TV 10% up to a maximum of 15 mL x kg$^{-1}$. 2. Increase RR to 30 min$^{-1}$ and adjust TV to result in increased MV of 10%.</td>
</tr>
<tr>
<td>$\text{P}_{\text{aw}}$</td>
<td>&gt;30 cmH$_2$O</td>
<td>Increase PEEP to 10 cmH$_2$O</td>
</tr>
<tr>
<td>Ppeak and MAP</td>
<td>&gt;40 cmH$_2$O combined with MAP &lt;50 mmHg</td>
<td>1. Adjust I:E to 1:1. 2. Increase RR to 30 min$^{-1}$ and reduce TV to result in maintained MV.</td>
</tr>
<tr>
<td>MAP and/or CI</td>
<td>MAP &lt;50 mmHg and/or CI &lt;2.0 L x min$^{-1}$</td>
<td>1. 10 mL x kg$^{-1}$ SG. 2. 5 mL x kg$^{-1}$ SG. (total possible fluid bolus 15 mL x kg$^{-1}$ x h$^{-1}$)</td>
</tr>
<tr>
<td>MAP</td>
<td>&lt;40 mmHg or &lt;50 mmHg after maximal fluid bolus</td>
<td>Start noradrenaline i.v. at 0.03 µg x kg$^{-1}$ x min$^{-1}$ with initial bolus of 0.1 mg. If MAP &lt;50 mmHg after 5 min, double infusion rate.</td>
</tr>
<tr>
<td>MAP</td>
<td>&gt;100 mmHg</td>
<td>If noradrenaline infusion, decrease rate 10% every 5 min.</td>
</tr>
<tr>
<td>MAP</td>
<td>MAP=MPAP (first 90 min of the protocol)</td>
<td>Single dose of 0.1 mg adrenaline i.v.</td>
</tr>
<tr>
<td>B-glucose</td>
<td>&lt;4.0 mmol x L$^{-1}$ or &lt;4.3 mmol x L$^{-1}$ for 1 h</td>
<td>100 mg x kg$^{-1}$ glucose i.v.</td>
</tr>
<tr>
<td>B-glucose</td>
<td>&gt;10.5 mmol x L$^{-1}$ or &gt;10.0 mmol x L$^{-1}$ for 1 h</td>
<td>Start i.v. infusion of aspart insulin (Novorapid™) with an infusion rate of 1 U x h$^{-1}$. Check B-glucose 20 min after start of the infusion.</td>
</tr>
<tr>
<td>Core temperature</td>
<td>&lt;37.8 °C</td>
<td>Cover the animal with blankets and adapt a fluid warmer to the insuffusions.</td>
</tr>
<tr>
<td>Core temperature</td>
<td>&gt;40.2 °C</td>
<td>Turn off active heating devices and remove covering blankets.</td>
</tr>
</tbody>
</table>

Table 1. Parameters, threshold values for interventions and interventions during the entire experiment

Measurements, laboratory analyses and calculations

Systemic and pulmonary blood pressures were monitored continuously through intravascular catheters. Cardiac output was measured by the thermodilution method. Pulmonary capillary wedge pressure (PCWP) was
assessed hourly. Proximal airway pressure values and respiratory volumes were recorded from ventilatory readings. Urine output rate was registered hourly; the first reading was made after the first hour had passed. Core temperature was registered with an esophageal thermometer. Blood samples were taken from the arterial line for analysis of arterial blood gases and acid-base balance, blood cells, cytokines and creatinine. A blood sample from the Swan-Ganz catheter was analyzed for mixed venous saturation. All blood samples in paper I-III were taken once an hour, except in paper III between hours -18 to 0, when blood was drawn every third hour to avoid iatrogenic anemia. In paper IV, blood was drawn from the animals in paper III every third hour during the first twenty-four hours of that experiment.

Commercial porcine-specific sandwich enzyme-linked immune-sorbent assay (ELISA) was used for the determination of TNF-α and IL-6 in plasma (DY686 and DY690, R&D Systems, Minneapolis, MN, USA). The ELISAs had intra-assay coefficients of variation (CV) of less than 5% and total CV of less than 10%.

Hemoglobin and blood cell counts, i.e. white blood cells, neutrophilic granulocytes and platelets, were analyzed on a CELL-DYN 4000™ hematology analyzer (Abbott Scandinavia AB, Kista, Sweden).

Blood gases were analyzed on an ABL™ 5 and Hemoximeter™ (Radiometer, Brønhøj, Denmark).

Creatinine measurements were performed using the modified kinetic Jaffe reaction on an Architect Ci8200 analyzer (Abbott Laboratories, Abbott Park, IL, USA).

Paper I

Plasma concentrations of cefuroxime and tobramycin were analyzed as well as plasma (urea, cystatin C and electrolytes) and urine (N-acetyl-β-D-glucosaminidase (NAG), creatinine and electrolytes) renal markers. Plasma concentrations of cefuroxime were determined by a high-pressure liquid chromatography method. The total analytical precision (CV) above a lower limit of 0.5 mg/L was <10%. Tobramycin assays were performed, using a TDx analyzer (Abbott Laboratories) with a precision of <10%. NAG was measured with a colorometric assay (Cat. No. 875 406, Roche Diagnostics, Mannheim, Germany). The total CV of the method was 5 %. Other plasma and urine analyses were performed on an Architect ci8200 (Abbott Laboratories, Abbott Park, IL, USA).

Multiple post-mortem biopsies were taken from standardized locations in the left kidney of each pig immediately after the end of the protocol and subsequent euthanasia of the animal. Renal cortex biopsies from the animal with the lowest creatinine clearance rates in each group were selected for assessment with transmission electron microscopy. From each animal, three
to four randomly obtained tissue samples were analyzed. In each sample, at least ten perpendicularly cut sections of proximal tubuli were examined. Group allocation of the tissue samples was blinded for the examiner.

**Paper II**

Heparinized tubes were used for the endotoxin samples. After centrifugation, the supernatants were transferred to endotoxin-free tubes and stored at -70°C until analysis. Analysis of endotoxin was performed in duplicate with the LAL-assay (Endochrome-KTM; Charles River Endosafe, Charleston, SC).

To reduce inter-animal variation and the number of animals necessary for statistical analysis, all parameters but those logarithmically transformed, were expressed as relative values in relation to that obtained at baseline just before administration of endotoxin.

**Paper IV**

Every third hour throughout the experiment, endotoxin \((E.coli: 0111:B4;\) Sigma Chemical Co., St Louis, MO, USA) was added to a blood sample of 2 ml whole blood to yield a final concentration of 10 ng endotoxin x mL^{-1}. The ex vivo-stimulated samples were subsequently incubated at 39°C for two hours. After centrifugation the supernatants were transferred to plasma tubes and stored at -18°C until analysis of TNF-\(\alpha\).

**Protocol**

Endotoxin was given as an i.v. infusion \((E.coli: 0111:B4;\) Sigma Chemical Co., St Louis, MO, USA) obtained from a single batch, dissolved in physiological saline solution during the entire experiment. Animals not given endotoxin received infusion of sodium chloride with an infusion rate corresponding to the endotoxin infusion rate. The animals were studied for six hours after which time all surviving piglets were killed by an injection of potassium chloride injection i.v. In paper III the animals were studied for six or thirty hours, depending on group allocation. Group allocation in all studies was decided by randomization in blocks, with a block size of ten. When the final group size was achieved, allocation to that group was terminated. Organ dysfunction and hypoperfusion are mainly seen during the last three hours of a six-hour experiment \(^{85}\). Therefore, in paper I, III and IV, only pigs that survived to the end of the experiment were included in the study.

**Paper I**

Groups I and II received endotoxin infusion in the dose of 1 \(\mu\)g x kg^{-1} x h^{-1} throughout the experiment whereas groups III and IV received saline. Groups I and III received tobramycin sulfate infusion in a dose of 7 mg x kg^{-1}
whereas groups II and IV received the corresponding amount of saline. All pigs were given 20 mg x kg\textsuperscript{-1} cefuroxime sodium as an i.v. infusion for twenty minutes, starting twenty minutes after the start of the protocol. An overview of the design is given in Table 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cefuroxime</th>
<th>Endotoxin</th>
<th>Tobramycin</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>8</td>
</tr>
<tr>
<td>II</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>8</td>
</tr>
<tr>
<td>III</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>4</td>
</tr>
<tr>
<td>IV</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 2. The experimental design in paper I.

**Paper II**

The animals were initially allocated to one of two endotoxin infusion rates, 0.063 or 4 µg x kg\textsuperscript{-1} x h\textsuperscript{-1}, referred to as the low and the high-dose group, respectively. At completion of the first hour of the experiment, the animals were randomized a second time to one of three varying durations of the endotoxin infusion of one, two or six hours. Group name, number of animals in each group, infusion rate, and duration of the endotoxin infusion are summarized in Table 3.

<table>
<thead>
<tr>
<th>Group name</th>
<th>Endotoxin dose (µg x kg\textsuperscript{-1} x h\textsuperscript{-1})</th>
<th>Duration of infusion (h)</th>
<th>Group size</th>
</tr>
</thead>
<tbody>
<tr>
<td>High 1h</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>High 2h</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>High 6h</td>
<td>4</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Low 1h</td>
<td>0.063</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Low 2h</td>
<td>0.063</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Low 6h</td>
<td>0.063</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Control</td>
<td>Saline</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3. Group name, number of animals in each group, infusion rate, and duration of the endotoxin infusion in paper II.

**Paper III**

To be able to study the dose-dependency of both the preexposure dose as well as the challenge dose, the animals were randomized to a total of nine groups: eight groups with different endotoxin preexposure and challenge doses and one saline control group. An overview of the study design with group names and different endotoxin pre-exposure and challenge doses are summarized in Figure 2.
Figure 2. Experimental design in paper III. The filled circle marks the point of inclusion in the study. The arrow denotes baseline for all groups.

Paper IV

The blood used for the experiments in paper IV was, as previously stated, drawn from the animals studied for thirty hours in paper III. Nine animals received a continuous infusion of 0.063 µg endotoxin x kg$^{-1}$ x h$^{-1}$ for twenty-four hours whereas nine other animals received 0.25 µg endotoxin x kg$^{-1}$ x h$^{-1}$ for twenty-four hours. Three animals received only saline and served as the control group.

End points and statistics

In this model log concentrations of cytokines and plasma endotoxin approximates to the normal distribution, as well as normal values of blood cell counts, electrolytes, plasma creatinine and blood gases. All physiological variables measured except for urine output approximates to the normal distribution. Variables dependent on urine output are non-normally distributed (plasma cystatin C, creatinine clearance, urine output, urine NAG/creatinine, and urine electrolyte levels). A p-value of <0.05 was considered significant. Statistica™ (StatSoft, Tulsa, OK, USA) was used in the statistical calculations.
Paper I
As cefuroxime is almost exclusively eliminated via the kidneys and tubular excretions account for approximately half of the renal clearance \(^{134,135}\), the elimination rate of cefuroxime was chosen as the primary variable. The elimination rate follows first-order kinetics with the cefuroxime plasma concentration decreasing log-linearly over time. Therefore, the change in log concentration per hour was chosen as the primary end point. Differences in cefuroxime elimination rate were tested with Student’s \(t\) test for independent samples. To detect a maximal difference of 30% in the elimination rate from one hour to the last hour of the experiment with a power of 0.8, an \(\alpha\)-error of 0.05 and a SD of 20%, eight animals in each group were needed. Secondary end points mainly reflecting the glomerular filtration rate were creatinine clearance, plasma cystatin C concentration, and urea whereas electrolytes and NAG mainly mirrored tubular injury. Statistical analysis of these variables was performed using repeated measures analysis of variance (ANOVA) on the values obtained at four to six hours. To detect differences at specific times, a post hoc one-way ANOVA was employed. For the non-normally distributed variables, the Mann-Whitney \(U\) test was performed at specific times.

Paper II
The primary end point was to detect differences in TNF-\(\alpha\) concentration during the last three hours of the experiment in the high-dose group between animals with continued six-hour infusion of endotoxin and those with terminated infusions. The power analysis conducted was based on a detectable difference of 15% at six hours, an \(\alpha\)-error of 0.05, a power of 0.8, and an SD of 10%. This yielded that six evaluable animals were needed in each group. To optimize the number of animals needed for the study and still be able to investigate whether termination of the endotoxin infusion at the time of the TNF-\(\alpha\) peak would differ in effect compared with when a general inflammatory state safely had been established, the one-hour and two-hour infusion groups were motivated. However, the results from the two groups in which endotoxin was terminated were to be combined from three hours, unless there was a trend toward a difference in TNF-\(\alpha\) concentration at three or four hours, defined as a \(p\) value of <0.5 in favor of a higher concentration in the two-hour endotoxin infusion group. If such a trend did exist, each group was to be expanded to six animals. Differences between groups over time from three to six hours were assessed with ANOVA for repeated measures for all variables, except for urine output. For this parameter, the Mann-Whitney \(U\) test was used at each point from three to six hours. For the other variables, a post hoc one-way ANOVA was made at these points.
Paper III
The principal aim was to study organ dysfunction and hypoperfusion in animals with a tolerance-induced reduction in the inflammatory response as expressed by lower TNF-α levels in the groups that had been exposed to endotoxin for twenty-four hours. Thus, the response during the last six hours of animals preexposed to endotoxin was compared to the response of unexposed animals. This comparison was also made for the rest of the parameters. The comparisons between pre- and unexposed animals were analyzed with Mann-Whitney $U$ test. Group size was calculated from a detectable difference in TNF-α of at least 25% with an $\alpha$-error of 0.05, a power of 0.8 and a SD of 10%, which yielded a group size of three. In the individual group analyses, all normally distributed parameters were analyzed with the Student’s $t$ test and creatinine clearance with the Mann-Whitney $U$ test.

Paper IV
Primary endpoint was to investigate the temporal development of the propensity to release TNF-α following ex vivo endotoxin stimulation by comparing the cytokine values before and after ex vivo stimulation. This was done by repeated measures ANOVA, where the group by time interaction analysis was used to determine whether the in vivo and ex vivo stimulated cytokine levels followed different temporal dynamics over time. The effect of the different endotoxin infusion rates was also analyzed by means of repeated measures ANOVA.

The difference between TNF-α values, after and before ex vivo endotoxin stimulation, $\Delta$TNF-α, was employed as the parameter of endotoxin tolerance for individual patients. Because log $\Delta$TNF-α did not clearly approximate to normal distribution, non-parametric tests were used in the analyses and Wilcoxon’s matched pairs test used in the comparison of endotoxin tolerance at different times with that at the start of the experiment. The association between endotoxin tolerance at different time points and organ dysfunction and hypoperfusion parameters in individual animals was analysed by Spearman rank order correlations. Since $\Delta$TNF-α values are inversely related to endotoxin tolerance, negative correlation values, represent a positive correlation to endotoxin tolerance, and *vice versa*.
Results

Of the 89 pigs studied during the experiments, 12 died before the experimental end point. 7 of the animals were initially randomized to receive endotoxin for six hours while 5 of the animals were randomized to receive endotoxin for thirty hours. All of the 12 animals died in association to the early pulmonary hypertension seen in this model, as discussed in the Introduction, section Porcine endotoxemia as a model of sepsis. 11 of the dead animals were excluded and replaced according to the protocol in papers I, III and IV. The baseline parameters of the diseased animals were comparable to the other animals.

Tobramycin and renal dysfunction

Plasma tobramycin levels in group I and III were in the range seen in the clinical setting (Table 4).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Group I (mg x L⁻¹)</th>
<th>Group III (mg x L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>2</td>
<td>9.1 ± 1.5</td>
<td>10.0 ± 1.8</td>
</tr>
<tr>
<td>3</td>
<td>4.7 ± 0.7</td>
<td>5.3 ± 1.2</td>
</tr>
<tr>
<td>4</td>
<td>2.9 ± 0.4</td>
<td>3.6 ± 0.8</td>
</tr>
<tr>
<td>5</td>
<td>2.2 ± 0.5</td>
<td>2.5 ± 0.9</td>
</tr>
<tr>
<td>6</td>
<td>1.8 ± 0.5</td>
<td>1.8 ± 0.6</td>
</tr>
</tbody>
</table>

Table 4. Plasma tobramycin concentration in the tobramycin treated groups. Mean ± SD.

Cefuroxime elimination rate

Plasma cefuroxime levels declined from peak levels at one hour in all four groups (Figure 3). During the first three hours the elimination rate was of similar magnitude in all groups. After the third hour, there were significantly lower cefuroxime elimination rates in the two groups subjected to endotoxin, i.e. I and II, in comparison with the non-endotoxemic groups, i.e. III and IV (Table 5). Because some animals, mainly in the non-endotoxemic groups, had reached concentrations below the detection limit during the last hour, mean elimination rates were also calculated from values obtained at four and
five hours. No difference in elimination rates could be detected in the animals with endotoxin-induced renal injury given tobramycin or not at any of the time points. The same result was obtained also when the elimination rate was calculated from the start of the renal deterioration at three hours. Similarly; non-endotoxemic animals given tobramycin had similar elimination rate as those given saline alone.

![Plasma cefuroxime concentration in plasma over time.](image)

**Figure 3.** Plasma cefuroxime concentration in plasma over time. Circles (black)-Group I; Squares -group II; Diamonds (black)-group III; Triangles -group IV. Mean ± SE

**Table 5.** Renal function expressed as cefuroxime elimination rate ($\Delta \text{Log}_{10}[\text{cefuroxime}] \times t^{-1}$). Mean ± SE.

<table>
<thead>
<tr>
<th>Elimination rate time period</th>
<th>Endotoxemic animals (I-II)</th>
<th>Non-endotoxemic animals (III-IV)</th>
<th>p</th>
<th>Endotoxemic animals</th>
<th>Non-endotoxemic animals</th>
<th>p</th>
</tr>
</thead>
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<td></td>
<td>mg x L$^{-1}$ x h$^{-1}$</td>
<td>mg x L$^{-1}$ x h$^{-1}$</td>
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<td>mg x L$^{-1}$ x h$^{-1}$</td>
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<td></td>
</tr>
<tr>
<td>1-4 h</td>
<td>0.34 ± 0.01</td>
<td>0.38 ± 0.01</td>
<td>0.044</td>
<td>0.34 ± 0.01</td>
<td>0.34 ± 0.03</td>
<td>0.87</td>
</tr>
<tr>
<td>1-5 h</td>
<td>0.36 ± 0.01</td>
<td>0.37 ± 0.01</td>
<td>0.005</td>
<td>0.30 ± 0.02</td>
<td>0.30 ± 0.02</td>
<td>0.99</td>
</tr>
<tr>
<td>1-6 h</td>
<td>0.26 ± 0.01</td>
<td>0.31 ± 0.01</td>
<td>0.004</td>
<td>0.26 ± 0.01</td>
<td>0.26 ± 0.02</td>
<td>0.99</td>
</tr>
<tr>
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<td>0.28 ± 0.03</td>
<td>0.34 ± 0.02</td>
<td>0.003</td>
<td>0.27 ± 0.04</td>
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<tr>
<td>3-5 h</td>
<td>0.32 ± 0.03</td>
<td>0.37 ± 0.03</td>
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<td>0.22 ± 0.04</td>
<td>0.23 ± 0.03</td>
<td>0.86</td>
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<tr>
<td>3-6 h</td>
<td>0.17 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>0.003</td>
<td>0.17 ± 0.03</td>
<td>0.17 ± 0.02</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Secondary renal function analyses

Urine output decreased in endotoxemic groups during the last hours of the experiment and was at four, five and six hours significantly lower than in the non-endotoxemic groups. Urine output did not differ between groups I vs. II or groups III vs. IV. Similarly, creatinine clearance was lower in the endotoxemic groups than in the non-endotoxemic groups, with no demonstrable differences between groups I vs. II or groups III vs. IV. In agreement with this, cystatin C levels were higher in the endotoxemic
animals at four to six hours with no differences between animals treated with tobramycin or not. In urea levels, no differences could be demonstrated between any of the groups. Urine NAG/creatinine ratio, reflecting renal tubular function, did not reveal significant differences between non-endotoxemic vs. endotoxemic groups or groups I vs. II or III vs. IV at four to six hours. In the endotoxemic animals plasma concentrations of sodium, potassium and magnesium were increased, whereas phosphate levels were unchanged and calcium levels decreased at the end of the experiment. No differences between tobramycin treated animals and those in groups II and IV were demonstrable in any of these parameters. Urinary concentrations of sodium in endotoxemic animals were lower, those of magnesium higher, whereas levels of potassium and calcium did not differ in comparison with those observed in animals receiving saline. With the exception of magnesium in the endotoxemic animals, there were no differences between animals given tobramycin or not in any of the electrolytes analyzed.

Transmission electron microscopy
In the biopsies from the three endotoxemic pigs, cells with distorted brush border alternating with cells with normal brush border, numerous lysosomes of various sizes with inclusion bodies and absence of intercellular space between the proximal tubular cells were found. Additionally, partly decomposed mitochondria were seen in the tissue specimen from the pig in group II, while mitochondria appeared to be normal in the specimen from the other animals. No additional changes could be observed in the tobramycin treated animals. In the biopsies from the pig in group IV normal brush borders, scant number of lysosomes, prominent intercellular space between tubular cells, normal mitochondria and abundant vacuoles were seen.
Figure 4. Representative micrographs of the epithelium in proximal tubules. The endothelium from group I (endotoxin + tobramycin, Figure 4A) and group II (endotoxin, Figure 4B) demonstrates similar focal changes. There are no intercellular spaces between the cells (arrows). Both the basal cellular foldings (*) and the apical tubular invaginations (TI) are absent. In these sections, some of the mitochondria (M) are swollen and disintegrated, but the luminal brush border and basal lamina (BL) are preserved. Numerous large secondary lysosomes (SL) with electron-dense inclusions are present. The number and size of secondary lysosomes in groups I and II were similar. The epithelium from control group IV (controls, Figure 4C) shows typical lateral intercellular spaces (arrows), basal foldings, and continuous brush borders. Apical TI and vacuoles (V) are numerous and M are mainly located basally. A basal lamina encircles the proximal tubule. Collagen and a peritubular capillary are seen at the bottom of the micrograph. TL, lumen of tubule; N, nucleus; BB, brush border. Bar =1 μm.
Plasma endotoxin and the inflammatory response

There were no differences in TNF-α concentrations at three hours and four hours between High 1h and High 2h (P=0.97, P=0.95, respectively), and Low 1h and Low 2h (P=0.37 in favor of a lower concentration in the two-hour infusion group, P=0.99, respectively). According to the statistical plan, the results from these groups from three hours onwards were combined to form the groups High 1+2h and Low 1+2h in the further calculations. With exception of the plasma endotoxin data, this combination was also done in the analysis of the other parameters.

Endotoxin levels in the high-dose group decreased rapidly after cessation of the endotoxin infusion and after the third hour the concentration was lower than in animals with continued endotoxin administration (Figure 5). In the low-dose groups no noteworthy increases above baseline or control endotoxin levels could be noted.

![Figure 5. Plasma endotoxin concentration over time in the high-dose group.](image)

The results of the responses in TNF-α, IL-6, leukocytes (WBC) and platelets are shown in Figure 6. No difference was seen in TNF-α concentration between groups High 1+2h and High 6h from three hours to six hours. Similarly, in the low-dose groups TNF-α levels fell rapidly after the peak at one hour and in most animals the detection limit was reached at three hours with no detectable difference between groups Low 1+2h and Low 6h. IL-6 values peaked at three hours in the high-dose groups, after which the concentration fell significantly more rapidly in animals with decreasing plasma endotoxin concentration as compared with those with continued infusion as indicated by the significant group by time interaction.
in the ANOVA. In the low-dose experiment, the difference between groups in the interval of three to six hours did not reach the level of significance but a one-way ANOVA revealed significantly lower levels at four hours and five hours in the Low 1+2h group. WBC dropped rapidly during the first two hours in all animals in the high-dose groups. In the group with continued endotoxin infusion, WBC remained low for the remainder of the experiment, whereas in the animals with decreasing plasma endotoxin concentration, there was a gradual increase in WBC. In the low-dose groups, WBC fell during the first hour, after which there was a return towards baseline. In the animals with continued endotoxin administration the leukocyte count was lower from three hours to six hours than in the low-dose groups, which at that time had nearly normal values. Platelets dropped continuously during the experiment for all groups with no differences between groups, irrespective of continuation or termination of the endotoxin infusion.
Figure 6. Changes in TNF-α, IL-6, white blood cell count (WBC) and platelet count over time. TNF-α and IL-6 are expressed as absolute values and WBC and platelets as relative values. Groups High 1+2h and Low 1+2h are used from three hours. Values are expressed as mean ± SE. * denote level of significancy of p<0.05.
Reduced endotoxin concentration and organ dysfunction

In the high-dose experiment, MAP increased towards baseline during the last hours of the experiment, whereas DO$_2$ and BE remained at lower levels with no significant differences between High 6h and High 1+2h in any of these variables. SvO$_2$ also remained at a low level in the animals with continued endotoxin infusion, whereas both improved recovery and improved values at the end of the experiment were observed in those with decreasing endotoxin concentration. In the low-dose experiment MAP, DO$_2$, SvO$_2$ and BE were somewhat higher in animals with decreasing endotoxin concentration, although these differences did not reach statistically significant levels. The hemoglobin concentration in this model may serve as a marker of the capillary leakage and in all animals increasing values were found during the first hours (Figure 7). In the high-dose group, there was a significantly faster decrease in hemoglobin concentration in the High 1+2h group than in the High 6h group but no statistical difference was observed in the concentrations from three hours to six hours. However, in the low-dose experiment a statistical difference was reached, with lower hemoglobin concentration during the last hours in the animals with decreasing endotoxin concentration. Static pulmonary compliance deteriorated markedly in the high-dose experiment with no significant difference between the groups. However, there was a commencing recovery in the animals with decreasing endotoxin concentration as indicated by the group by time interaction in the ANOVA. This finding is further supported by the corresponding changes in PaO$_2$ and PaCO$_2$, which both resulted in better recovery and values at the end of the experiment for the animals with terminated infusion. Differences in respiratory function together with those in hypoperfusion resulted in improved pH values in the animals with decreasing endotoxin concentration, whereas in the animals with continued infusion no trend towards recovery could be identified. In the low-dose experiment these changes were more evident with significantly better lung compliance, lower PaCO$_2$ at three hours and four hours and higher pH values in the terminated infusion group. Renal function deteriorated in the high-dose group during the last hours of the experiment with decreasing urine production and increasing plasma creatinine concentrations. No differences were found between animals with falling endotoxin concentration and those with continued infusion. Similar results were found in the low-dose experiment.
Figure 7. Relative changes in parameters reflecting hypoperfusion and respiratory dysfunction over time. Groups High 1+2h and Low 1+2h are used from three hours. Values are expressed mean ± SE * denote level of significance p<0.05.

- ● High 1h  ■ High 2h  ▲ High 1+2h  ●●● High 6h
- ○ Low 1h  □ Low 2h  △ Low 1+2h  ●●● Low 6h
Endotoxin tolerance studies

The mortality rate after endotoxin challenge animals unexposed to endotoxin, including those excluded from the experiment, was 3/9. The corresponding mortality rate for those preexposed was 0/12. All parameters for all groups were in the same range at the inclusion of the study. At baseline, there were some minor differences between the groups (Figure 10, Table 6). PaO₂/FiO₂ was lower in the animals pre-exposed to endotoxin, the difference reaching significance in the animals given the low-dose challenge. In addition, slightly lower BE values were noted at baseline in the endotoxin pre-exposure groups. In the SalC group a slight reduction in PaO₂/FiO₂ was found during the first twenty-four hours (p<0.05). The endotoxin pre-exposure groups demonstrated higher CI at baseline than the SalC group (p<0.01), whereas only a trend toward lower values in PaO₂/FiO₂ was seen.

The SalC group showed signs of a mild inflammatory reaction toward the end of the experiment with an increase in leukocytes. No changes were observed in cytokines or any of the other parameters. In the control groups that received unchanged endotoxin infusion rate for thirty hours, i.e. groups LC and HC, the responses were similar to those of the group SalC.

Tolerance and the inflammatory response

Group HH responded with a higher TNF-α surge than group LH during the initial three hours of the experiment, but the opposite was evident following the endotoxin challenge. After baseline, group H reacted with higher TNF-α values than the LH and HH groups (Figure 8, Table 6).

Preexposed animals in comparison with those unexposed to endotoxin demonstrated lower cytokine values and higher leukocyte counts, both at the time of the largest change and at the end of the experiment (Figure 9). Leukocytes were found to decrease in all animals given the high-dose endotoxin challenge, which was less evident in the animals given the high-dose endotoxin pre-exposure (Figure 10). In the animals given the low-dose endotoxin challenge the leukocyte count was unchanged or increased in groups HL and LL, with a pronounced difference at the end of the experiment when compared with group L (Figure 10).
Figure 8. TNF-α levels during the entire experiment in the high-dose endotoxin challenge groups. The arrow indicates baseline. The values are given as mean ± SE on a logarithmic scale. P-values represent the results of repeated measures ANOVAs comparing groups LH and HH at -23 h to -21 h and at 1 h to 3 h.

Effects of tolerance on circulation and renal dysfunction

Preexposed animals compared to unexposed demonstrated tolerance in CI and SvO₂ at the time of maximal response (Figure 9). Hypoperfusion, as expressed by changes in BE, demonstrated a relatively pronounced attenuation at the time of maximal change, which persisted throughout the experiment. In circulatory and hypoperfusion parameters the differences between animals treated with the two pre-exposure endotoxin doses were limited, as were the differences between the high- and low-dose challenges (Table 6). Cardiac function, as determined by LVSWI, demonstrated a tolerance pattern similar to that of hypoperfusion (Figure 9, Table 6).

Animals preexposed to endotoxin demonstrated less pronounced changes in creatinine clearance at the end of the experiment than those unexposed (Figure 9).
Tolerance and pulmonary dysfunction

Preexposed animals in comparison with unexposed demonstrated reduced compliance and PaO$_2$/FiO$_2$ at three hours (Figure 9). At the end of the experiment, these differences were no longer present.

Figure 9. The results of the main tolerance analyses. The response compared to baseline at the time of the largest response and at the end of the experiment. Values are mean ± SE and expressed as index values in per cent compared to the baseline value, except for cytokines and base excess, where the subtracted difference from baseline are given. Creatinine clearance is given as absolute values (mL x min$^{-1}$), median ± interquartile range. P-values are calculated from Mann Whitney U-tests. White bars- Preexposed groups (LL+LH+HL+HH), n=12. Grey bars- Unexposed groups (H+L), n=6.
However, in these respiratory parameters the groups responded markedly different to the high- and low-dose challenges (Figure 10). In the groups given the high endotoxin challenge, i.e. groups HH and LH, PaO₂/FiO₂ continued to decrease during the last three hours of the experiment and at the end of the experiment, PaO₂/FiO₂ was substantially lower in groups HH and LH than in group H (Figure 10). In contrast, this deterioration was not at all observed in the animals given the low-dose endotoxin challenge. A similar pattern was seen in pulmonary compliance, though not as pronounced. The respiratory parameters did not differ between the high- and low-endotoxin preexposure groups. In the animals not preexposed to endotoxin a slight improvement was observed during the last three hours of the experiment.

Figure 10. Leukocytes, PaO₂/FiO₂ index and pulmonary compliance. Values are presented at baseline, at the time of the largest response and at the end of the experiment. * P<0.05, pre-exposed challenge group vs. corresponding challenge group not pre-exposed to endotoxin, i.e. H or L. Values are given as mean ± SE. C_stat=static pulmonary compliance.
Table 6. Values for all groups at baseline, at the time of maximal change and at the end of the experiment for the cytokines, circulatory variables and creatinine clearance. Endotoxin doses are µg x kg⁻¹ x h⁻¹. Values are mean ± SD for all parameters, except for creatinine clearance where values are given as median (range). *p<0.05, preexposed challenge group vs. corresponding group not preexposed to endotoxin, i.e. H or L.

Temporal development of endotoxin tolerance

Endotoxin infusion resulted in an increase in TNF-α concentrations with the highest concentration at three hours after which concentrations gradually returned to normal values (Figure 11). Ex vivo endotoxin stimulation resulted in a significant increase in TNF-α compared to in vivo values (p<0.001) (Figure 11). When comparing the two infusion rates no dose-response could be seen at six hours and later, neither before ex vivo stimulation nor after (p= 0.91 and 0.26, respectively). Therefore, values from the two groups were analyzed together in the following calculations.

The ex vivo TNF-α values showed different temporal dynamics than the in vivo values as indicated by a highly significant group by time interaction analysis of the repeated measures ANOVA comparing the ex vivo and the in vivo values after the TNF-α peak, i.e. from three to twenty-four hours (P=0.002).
In the control animals, the TNF-α concentration was $2.3 \pm 0.2 \log (\text{ng} \times \text{L}^{-1})$ at baseline with no significant changes during the experiment. After \textit{ex vivo} endotoxin stimulation, there was a marked increase that did not change significantly at any time point during the experiment.

The numerical nadir point of $\Delta$TNF-α after the initial \textit{in vivo} TNF-α peak, was at six hours followed by increasing values the following hours (Figure 12). The individual $\Delta$TNF-α values were higher at baseline than at six, nine, twelve and twenty-four hours (P=0.004, 0.01, 0.04 and 0.02, respectively), while no significant differences between baseline values and the values at fifteen, eighteen and twenty-one hours were seen (P=0.1, 0.2 and 0.1 respectively).

Figure 11. TNF-α levels \textit{in vivo} and after \textit{ex vivo} endotoxin stimulation during the entire experiment. The values are logarithmically transformed and given as mean ± SE.

Figure 12. $\Delta$TNF-α values at baseline and after the TNF-α peak, i.e. from six to twenty-four hours. The values are logarithmically transformed and given as mean ± SE. The p-values are calculated the Wilcoxon matched pairs test comparing paired values at baseline to the respective points. * denotes p<0.05.
Association between individual tolerance and outcome

Expressed as values relative to baseline, selected variables are shown in Figure 13. The transition from a hypodynamic to a hyperdynamic circulatory state was evident at 12 h. LVSWI followed the same temporal course as that of CI, whereas DO₂ and oxygen consumption (VO₂) were virtually unchanged. A biphasic development was also true for hemoglobin and leukocyte count. Hemoglobin increased to a peak at three hours followed by a slow return to baseline values towards the end of the experiment. The leukocyte count on the other hand decreased to six hours after which the values increased to the end of the experiment. PaO₂/FiO₂, pulmonary compliance and platelet count showed a continuous decrease during the entire experiment. BE and creatinine clearance showed a limited continuous decrease throughout the experiment.

![Figure 13A](image1.png)  
![Figure 13B](image2.png)

Figure 13. The responses of key circulatory variables and PaO₂/FiO₂ (Figure 13A) and blood cells and hemoglobin (Figure 13B) during the experiment. Values are relative to baseline values (%) and given as mean ± SE.
Please see *Table 7* for the results of the correlation analyses. ΔTNF-α at the end of the experiment, i.e. at twenty-four hours, correlated strongly to ΔTNF-α at baseline, i.e. at baseline and at the nadir point, i.e. at six hours. No physiological parameters correlated to ΔTNF-α at baseline.

On the other hand, ΔTNF-α at the nadir point at six hours and at the end of the experiment correlated to the circulatory variables, negatively to CI and positively to SVRI and MAP, indicating an association between endotoxin tolerance and the hemodynamic pattern. In addition, significant correlations were demonstrated between ΔTNF-α on one side and VO₂ as well as platelet count on the other. Regarding all other parameters, with the exception of the haemoglobin concentration for which there was a marginal correlation, no significant correlations were observed.

<table>
<thead>
<tr>
<th></th>
<th>ΔTNF-α baseline</th>
<th>ΔTNF-α 6 h</th>
<th>ΔTNF-α 24 h</th>
</tr>
</thead>
<tbody>
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<td>0.65*</td>
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<td>ΔTNF-α 6 h</td>
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<td>0.35</td>
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<td>Platelet count</td>
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<td>0.58*</td>
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<td>-0.53*</td>
<td>-0.31</td>
</tr>
<tr>
<td>SVRI</td>
<td>0.44</td>
<td>0.70*</td>
<td>0.78*</td>
</tr>
<tr>
<td>DO₂</td>
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<td>0.05</td>
<td>0.08</td>
</tr>
<tr>
<td>VO₂</td>
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<td>0.50*</td>
<td>0.44</td>
</tr>
<tr>
<td>Base excess</td>
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<td>-0.06</td>
<td>-0.06</td>
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<tr>
<td>LVSWI</td>
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</tr>
<tr>
<td>PaO₂/FiO₂ ratio</td>
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<td>-0.01</td>
<td>0.22</td>
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<tr>
<td>Compliance</td>
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<td>-0.02</td>
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</tr>
<tr>
<td>Creatinine clearance</td>
<td>0.16</td>
<td>0.23</td>
<td>0.33</td>
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*Table 7.* Spearman Rank R correlation coefficients between ΔTNF-α values and physiological parameters. * denotes significant correlation (p<0.05).
Discussion

Endotoxin is one of the best-studied triggers of the inflammatory response that underlies the pathogenesis of Gram-negative sepsis \(^{21}\). The pig is an animal high in phylogenetic order and reacts to endotoxin in many ways similar to the human body \(^{94}\). Modeling sepsis with endotoxin is a way to achieve a precise and reproducible inflammatory reaction with low variability. For these reasons we chose a porcine endotoxin infusion model for the studies. The questions that we aimed to answer would have been impossible to study in human volunteers, because of the very mild inflammation that can be achieved if not putting the subjects at risk. To answer the questions in clinical trials would have been equally difficult because of the extremely short time frame for inclusion in such a study in addition to the numerous difficulties mentioned in the introduction \(^8\).

Lipcsey and co-workers have previously shown that there is a log-linear dose-response relationship to most inflammatory, hypoperfusion and organ dysfunction parameters \(^{85}\). It is therefore known that infusion of 1 µg endotoxin x kg\(^{-1}\) x h\(^{-1}\) produces a powerful inflammatory response together with a rapidly progressive renal failure of moderate magnitude, which in paper \(I\) was manifested by decreased urine output that in the endotoxin groups was <25% of that in the non-endotoxemic animals. To be able to detect even minor injurious effects of tobramycin, the experiment was designed so that the nephrotoxic effect of tobramycin should have the possibility of being relatively marked in relation to that induced by endotoxin, but still with an undisputable inflammatory response-induced progressive acute kidney injury. Because cefuroxime is up to 95% renally excreted, both via glomerular filtration and tubular secretion, concentrations of cefuroxime were chosen as the primary end point in paper \(I\). In comparison with creatinine and cystatin C, plasma concentrations of an exogenous substance without a demonstrated nephrotoxic effect have the advantage of not being dependent of variations in inflow to plasma. However, even if a toxic effect of cefuroxime in the concentrations reached in the present study cannot be totally excluded, cefuroxime or other cephalosporins has often been given in combination with aminoglycosides for the treatment of severe sepsis or septic shock, thus giving additional relevance to the model. In addition, any potential harm inflicted by cefuroxime would have affected all groups equally and would in such case not have influenced the conclusions.
In the endotoxemic animals, the addition of tobramycin did not affect cefuroxime elimination in any way, neither if calculated from the peak concentration nor from the presumed start of the renal dysfunction at three hours after the start of the protocol. Similar results were seen in the more conventional glomerular filtration rate markers creatinine clearance, urea and cystatin C. Sepsis-induced acute oliguria is often associated with a low urinary sodium concentration as was also demonstrated in the present model. Tobramycin did not reduce the resorption of sodium as would be expected if aminoglycoside toxicity was present. Furthermore, with the exception of the urinary levels of magnesium, no differences or trends between endotoxemic animals given tobramycin or not could be demonstrated in the urine or plasma concentration in any of the measured electrolytes. Bonferroni-correction for multiple statistical analyses was not performed since a higher p-value for statistical significance has a higher chance to detect trends. In this case, changes in urinary magnesium concentration were not associated with differences in plasma concentration or hourly excretion. Consequently, the increase in magnesium concentration is most probably explained by the lower urine production in the endotoxemic group treated with tobramycin. Urinary excretion of the lysosomal enzyme NAG has been demonstrated to be a sensitive marker of tubular function and injury in animals and humans and has been used as a marker of aminoglycoside-induced renal toxicity. Even in this parameter, no difference could be demonstrated by the addition of tobramycin in endotoxemic animals. A slight trend that did not reach the significance level was observed but this difference was not more pronounced than in the non-endotoxemic animals.

Aminoglycoside-induced ultra-structural changes have been described early on and already after low doses and consist of enlargement of lysosomes and the development of lysosomal myeloid bodies. Although based on a relatively low quantity of samples where the findings were difficult to quantify and patchy in character, our study did not reveal any marked ultra-structural changes by the addition of tobramycin. Thus, our results are in disagreement with those reported from rodent studies. The discrepant results are most likely explained by the 10- to 50-fold higher aminoglycoside doses used in the rodent studies, since the susceptibility to aminoglycosides among various animal species and humans does not seem to vary appreciably. There are numerous limitations to the possibility to extrapolate the results to other aminoglycosides and to cases with sepsis-induced kidney injury of a very severe nature. Furthermore, tobramycin-induced kidney injuries debuting after the observational period were of course not possible to study. However, the kidneys were exposed to most of the administered tobramycin already after three hours (Table 4) and no progressive injury could be seen during the last three hours. These findings, together with the facts that septic AKI may develop rapidly within hours and
functional and electron microscopic aminoglycoside-induced tubular changes have been shown to occur within two hours, both in \textit{in vitro} and in animal studies\textsuperscript{71-73}, indicate that delayed harmful effects are not likely after one single dose. The late onset of aminoglycoside nephrotoxicity seen in clinical practice and in many animal experiments is most probably caused by the gradual tubular accumulation that occurs after multiple doses\textsuperscript{147,148}.

The study found no additional effect on the development of renal dysfunction by tobramycin. As discussed in an editorial comment by Noble, the study could lay the foundation to future studies to ascertain whether initial high-dose aminoglycosides combined with β-lactam antibiotics can further improve important patient-centered outcomes\textsuperscript{70}.

The concept of neutralizing a trigger of the inflammatory response without direct interaction with the host response itself is theoretically an attractive option in treating patients with severe sepsis or septic shock. One trigger aimed for elimination through hemofiltration in a number of clinical studies, is endotoxin. In the high-dose experiment of \textit{paper II}, the endotoxin levels were in the higher range of those seen in clinical severe sepsis, and a reduction in endotoxin concentration occurred that was considerably greater than that found in previous clinical antiendotoxin studies\textsuperscript{109,110,149}.

The terminated endotoxin infusions corresponded to an effective antiendotoxin strategy eliminating 5/6 and 2/3 of the total endotoxin load in animals whose infusions were stopped after one and two hours, respectively. In this porcine model, the response in TNF-α has been demonstrated to be log–log linearly dependent on the endotoxin dose\textsuperscript{85}, which would be equivalent to a proportionally positive response to an effective antiendotoxin strategy that is applied before or at the start of the endotoxin infusion. In this model, TNF-α concentration peaks at one hour whereupon there is a gradual decrease\textsuperscript{85}. Despite the termination very early in the course after one or two hours, thus close to the TNF-α peak, there was no effect on the TNF-α concentration in the high-dose experiment.

Our findings represent strong evidence that endotoxin elimination applied at the TNF-α peak or later will have very little or no effect on TNF-α–mediated toxicity. Regarding IL-6 concentration, a slight reduction was seen at the end of the experimental period in the groups with decreasing endotoxin concentration that was more evident in the low-infusion rate group. It has been demonstrated that IL-6 concentrations are more related to endotoxin concentration than to ensuing circulatory changes and organ dysfunction\textsuperscript{85}. Therefore, it cannot be concluded that this finding is associated with an overall beneficial effect. On the other hand, the reduction of endotoxin concentration resulted in an improvement in pulmonary compliance and function as determined by PaO$_2$, PaCO$_2$, and pH. With the exception of PaO$_2$, the effect was somewhat more evident in the low-dose endotoxin experiment. These changes were associated with differences in leukocyte levels indicating reduced adherence in the endotoxin decrease.
groups and diminished capillary leakage as reflected by the hemoglobin concentration. Furthermore, in circulatory parameters, there was a slight effect of reduced endotoxin concentration with an increase in SvO₂ at the end of the experiment that, however, was not mirrored by differences in BE within the duration of the experiment.

In total, the biological effect of the endotoxin concentration decrease was small considering the magnitude of the endotoxin load reduction and the early application of the measure. Although not demonstrated in this study, the effect will most likely not be enhanced if the antiendotoxin action is instituted later in the septic course, e.g. after several hours or days. These findings together with the negative results from smaller randomized clinical trials imply that the chance of beneficial effects in large clinical trials must be considered to be limited.

In a recent study by Bengsch et al.¹⁴⁹, in which extracorporeal plasma treatment was instituted after more than two days of severe sepsis in patients not responding to conventional treatment, a significant decrease in endotoxin concentration could be demonstrated. This decrease, however, was followed by a return to almost pretreatment values before the next apheresis treatment that was performed one day later. It may be speculated that in these cases, there is not only the problem of longstanding endotoxin effects but also of a substantial refill from the gastrointestinal tract that may reduce any effect achieved by an antiendotoxin procedure.

The EUPHAS-study, an Italian multi-center study using polymyxin B fibres for endotoxin removal with a blood-apheresis technique, recently reported reduced 28-day mortality in the intervention group. The study also showed improvements in blood pressure coupled with less need for vasopressor support in the intervention group, but not in the control group.¹¹¹ The fact that the control and intervention groups differed in baseline parameters such as age and amount of positive cultures in addition to difficulties in blinding the experiment dims the conclusions of the experiment. A larger multi-center trial, EUPHRATES, has recently been designed representing another opportunity to test for clinical efficacy of endotoxin removal.¹¹²

In *paper II*, the fall in endotoxin concentration was around $2 \log_{10} \text{EU} \times \text{mL}^{-1}$ in the high-dose group. A corresponding increase in the endotoxin dose would have resulted not only in a 100-fold increase in the TNF-α concentration but also in substantial effects on dose-dependent parameters, such as leukocytes, platelets, circulation, and organ function. Consequently, it seems more beneficial to prevent an increase in endotoxin concentration implying initiation of the antiendotoxin strategy very early on in the septic course. In clinical practice, however, this will be difficult and, therefore, it may be of greater interest to focus on investigations evaluating the effect in major surgery, in which an antiendotoxin procedure can be initiated before the start of the operation.
The concept of endotoxin tolerance is very similar to the inflammatory situation seen in patients suffering from sepsis in the post-operative period or secondary to trauma. Several studies have reported beneficial effects of endotoxin tolerance. Many experimental in vivo models of endotoxin tolerance have used repeated single doses of endotoxin to induce tolerance, while in many in vitro models cells are incubated with endotoxin for a time of varying length. In our model we used an in vivo model where the animals were subjected to a continuous endotoxin load. The reason for choosing a continuous infusion in the present study was two-fold. First, it minimizes the risk of procoagulatory and proinflammatory components in the form of a generalized Shwartzman reaction, a phenomenon that has not been studied to any appreciable extent in porcine models. Second, and more importantly, the ambition was to create a model that more closely resembles Gram-negative bacteremia in patients with a sustained release of endotoxin from infecting Gram-negative bacteria or increased translocation from the gastrointestinal tract. To increase the clinical relevance of the model the animals were treated according to general intensive care principles with mechanical ventilation and interventions as depicted in Table 1.

Pronounced endotoxin tolerance has been demonstrated in humans after exposure to very low doses of endotoxin. Therefore, it was of interest to study whether there existed an on-off mechanism or a more gradual dose response in the development of endotoxin tolerance and in challenge doses in this in vivo large animal model. When the animals preexposed to endotoxin were challenged with a 4- to 64-fold increase in the endotoxin dose after baseline, there was, as expected, a significant attenuation in the TNF-α response compared with that in animals not pre-exposed to endotoxin. In this TNF-α response an obvious dose dependency in the endotoxin tolerance could be demonstrated, which is in agreement with results in an in vivo rabbit model.

In the present study some type of endotoxin tolerance was noted in all variables studied (Figure 9). The attenuation in hypoperfusion and organ dysfunction, measured at the time of the largest response, was uniformly present. Because the animals not pre-exposed to endotoxin improved towards the end of the experiment, the differences between these animals and the endotoxin-tolerant animals became smaller with time. In the animals given the high-dose endotoxin pre-exposure there was a trend toward a more pronounced endotoxin tolerance in the inflammatory variables, whereas such a trend was not as apparent for circulatory and organ dysfunction variables. In the endotoxin tolerant animals the inflammatory variables were also more pronounced in the high-challenge groups than in the low-challenge groups. In contrast to circulatory variables, hypoperfusion and kidney function, in which no dose dependency was seen, pulmonary function clearly differed in response to the high- and low-dose challenges in the animals pre-exposed to endotoxin (Figure 10). In the groups receiving the high-dose endotoxin
challenge respiratory dysfunction continued to deteriorate during the last three hours of the experiment. This development was not observed after the low-dose challenge or in the animals not pre-exposed to endotoxin. After twenty-four hours of mechanical ventilation and exposure to endotoxin, the $\text{PaO}_2/\text{FiO}_2$ and pulmonary compliance were lower at baseline in the thirty-hour groups than in the six-hour groups. This event, together with the effect of the high-dose challenge, resulted in an outcome in respiratory function that, at the end of the experiment, was even worse than in the animals not pre-exposed to endotoxin.

Some of the known risk factors in the development of ventilator-induced lung injury are high volume ventilation, high inflation pressure and low PEEP. In this experiment all animals were ventilated with a TV of $<10 \text{ mL x kg}^{-1}$. No animals had plateau airway pressures above 30 cm H$_2$O during the first day and all animals were treated with PEEP. In addition to this, a reasonably low-dose fluid regimen of 10 mL crystalloid fluid x kg$^{-1}$ x h$^{-1}$ was used during the entire experiment. Finally, alveolar recruitment maneuvers were performed every third hour to prevent the development of ventilator-induced lung injuries. The saline control group demonstrated only a moderate reduction in $\text{PaO}_2/\text{FiO}_2$ and pulmonary compliance after twenty-four hours of ventilation, indicating that the ventilator-associated injury was limited and in the magnitude of that often seen in patients treated with mechanical ventilation in the ICU.

The lungs reacted differently than the other organs, which cannot be explained by endotoxin pre-exposure, mechanical ventilation or fluid replacement because these factors were the same in the high- and low-dose challenge groups during the first twenty-four hours of the experiment. Recently, it was demonstrated that alveolar macrophages in the human lung were primed after instillation of endotoxin as reflected by an increased $\text{ex vivo}$ endotoxin-induced expression of IL-1$\beta$ and IL-6 genes six hours after first exposure. Furthermore, in mice it has been demonstrated that bronchoalveolar cells are less likely than splenocytes, peritoneal cells and bone marrow cells to develop endotoxin tolerance. However, reduced pulmonary neutrophil infiltration and pulmonary edema associated with tolerance have been demonstrated after a second hit of endotoxin in the rat. A similar result with less neutrophil infiltration in the lung was observed in a study in mice, but in this study the finding was associated with a profound deterioration of pulmonary function, pulmonary edema and increased mortality despite the reduction in systemic cytokine response. To some extent the threshold effect of the endotoxin challenge found in tolerant animals in the present study might explain some of the differences in the results from other studies. The dose dependent result of our study is in fact consistent with the clinical study by Buttenschoen et al., in which there was an association between endotoxin tolerance and the development of
ARDS, because in that study it was also found that the higher the endotoxin levels, the worse the severity of ARDS.

The survival value of this pulmonary sensitivity to endotoxin is uncertain but it may be speculated that there is an evolutionary advantage in the sense that the lungs, as opposed to other organs that are not in contact with the outside world, must maintain some degree of the inflammatory response for the defense against virulent agents also in a state of general endotoxin tolerance. However, while maintaining the sensitivity to endotoxin the risk of an exaggerated response remains as demonstrated in the present study. This finding seems to be in agreement with the results of a recent metaanalysis study in ventilator-associated pneumonia (VAP) in which it was demonstrated that prognosis is worse in VAP that is caused by Gram-negative, endotoxin-containing bacteria than in VAP that is caused by Gram-positive bacteria. Of special interest is that prognosis was shown to be more severe in cases in which VAP develops as a second hit, e.g., after an infection such as sepsis or community-acquired pneumonia.

One of the key events in the development of ARDS is the sequestration of white blood cells, mainly neutrophil granulocytes and macrophages, in the alveolar space. In the present study the high- and low-challenge endotoxin pre-exposure groups reacted differently in the leukocyte response, with decreasing leukocyte levels after baseline in the high-challenge groups but not in the low-challenge groups. Additional to activation via CD14-TLR4 activation, endotoxin may activate the neutrophil granulocytes via other mechanisms, including complement activation that leads to neutrophil adhesion to endothelial cells.

In paper II there was a small but beneficial effect of reduced plasma endotoxin concentration with improved gas exchange in the lungs as well as slightly improved pulmonary compliance. There was no effect whatsoever on the TNF-α response but leukocyte levels indicated reduced adhesion. It may be speculated that at low plasma levels of endotoxin the lungs are protected by the endotoxin tolerance; on the other hand, at high levels leukocyte activation pathways not involving the cytokine-releasing CD14-TLR4 pathway outplay this effect. Thus, the pulmonary pathology might be more dependent on these alternative pathways than other organ systems. However, underlying mechanisms as well as the effect of timing and dosing of the second hit need further investigation.

In paper III we induced tolerance with a continuous endotoxin infusion for twenty-four hours before challenging the animals with the second endotoxemic hit. We made the choice of the duration of endotoxin preexposure based on prior studies. Endotoxin tolerance, as indicated by a low TNF-α increase after ex vivo stimulation with endotoxin, was observed at different times in paper IV. In order to study the dose response in the development of endotoxin tolerance, two endotoxin infusion rates were employed. In spite of some differences in the initial physiological
responses, no significant dose dependency in the TNF-α response could be demonstrated; neither in vivo nor after endotoxin challenge ex vivo. This indicates that endotoxin tolerance may be marked already after low amounts of endotoxin, which is in agreement with previous human data. In contrast to an undetectable dose effect, there was a significant variation in the endotoxin tolerance over time. The time of the strongest suppression of TNF-α in ex vivo stimulated samples, and thus the highest level of endotoxin tolerance, was at six hours, i.e. quite early after the initial proinflammatory peak. Contrary to the hypothesis that a continuous endotoxin infusion would lead to a constant suppression of ΔTNF-α, the maximal suppression was followed by a variation of the level of endotoxin tolerance that was highly significant (Figure 11). This was mainly caused by the higher TNF-α values after ex vivo endotoxin stimulation at fifteen to twenty-one hours. This is also seen in Figure 12 in which ΔTNF-α at different time points was compared with that at time zero, i.e. before the start of the endotoxin infusions. At all times the level of ΔTNF-α was reduced in comparison with baseline, although significantly so only at six to twelve hours and, in addition at twenty-four hours. Whether this represents a true biphasic course due to an early anti-inflammatory response that is followed by a transient anti-anti-inflammatory response needs more investigation as does the mechanisms behind. However, our results indicate that the time of administration of a second hit is of more importance than the magnitude of the preexposure dose.

The individual ΔTNF-α values at baseline and at the end of the experiment correlated strongly to each other and to ΔTNF-α at the time of maximal endotoxin tolerance, i.e. six hours. However, only ΔTNF-α values at six hours and at the end of the experiment correlated to physiological parameters at the end of the experiment. Since ΔTNF-α is inversely proportional to endotoxin tolerance, any parameter with a positive correlation to ΔTNF-α correlates negatively to endotoxin tolerance, and vice versa. With the exception of platelet count and hemoglobin, only the circulatory parameters and oxygen consumption (VO₂) showed significant correlations to ΔTNF-α. A higher ΔTNF-α level at six hours correlated to a lower CI and SvO₂ in addition to higher MAP and SVRI and the conclusion must be that the opposite is true for the individual level of endotoxin tolerance. Thus, the higher the tolerance level, the higher CI and SvO₂ and the lower the MAP and SVRI. If one connects these associations, the highly interesting conclusion is that at the time of maximal tolerance, the more tolerance to endotoxin the animal displays, the more hyperdynamic the animal’s circulation will be eighteen hours later. DO₂ did not correlate significantly to ΔTNF-α. As this is a derived parameter dependent on hemoglobin, arterial saturation and PaO₂ apart from CI, the interpretation is that the low correlation coefficients of the non-circulatory parameters attenuate the total association to endotoxin tolerance. In contrast to the
delivery of oxygen, lower oxygen consumption correlated with a higher level of endotoxin tolerance.

Proinflammatory cytokines induce the hypothalamic-pituitary axis (HPA) leading to ACTH secretion and subsequent systemic glucocorticoid and vasopressin release. These effects are markedly suppressed following repeated endotoxin exposure. Furthermore, there is evidence of hypothalamic NO being involved in the suppressed HPA response to repeated endotoxin challenges. Several authors have reported on the roles of iNOS in modulating endotoxin tolerance. Dias et al showed that mice deficient of the iNOS gene were not rendered tolerant to endotoxin and that in wild-type endotoxin tolerant mice given the specific iNOS-antagonist aminoguanidine, the normal physiological response to endotoxin returned. Although not investigated in this experiment, one might hypothesize that animals with a higher level of tolerance have higher iNOS-activity and more suppression of the HPA-axis-controlled stress reaction. This would result in endotoxin tolerant animals resolving the initial endotoxin-induced vasoconstriction earlier and the NO-induced vasodilatation weighing over. An increased iNOS-activity resulting in a nitric oxide-induced mitochondrial dysfunction might explain the lower oxygen consumption. This mechanism may also be behind the association between thrombocytopenia and endotoxin tolerance since NO has been shown to be a potent activator of platelets.

In our study, the respiratory parameters did not correlate to the level of endotoxin tolerance. At a glance these results contradict the interesting study by Buttenshoen et al where patients with ARDS (PaO2/FiO2 ratio <200) had lower ΔTNF-α than in patients with ALI (PaO2/FiO2 ratio <300). The results of our present study show the importance of the temporal variation of endotoxin tolerance, which could be of importance when interpreting the results of the study of Buttenshoen et al, since most probably the patients with the more severe condition had been ill for a longer time and deteriorated from the ALI population to the ARDS population. More importantly, the threshold effect in the pulmonary response to endotoxin in endotoxin tolerant animals demonstrated in paper III together with the fact that the plasma levels of endotoxin in the study of Buttenshoen et al. correlated with the severity of the disease, might explain the discrepancy.

Paper IV has several limitations. First, we did only study the development of tolerance up until twenty-four hours after the start of the infusion. However, since we expected the ΔTNF-α response to be constantly suppressed during a continuous endotoxin infusion, we did not aim for a longer experimental period in this study. Second, even if the porcine model has the advantage of great resemblance to human sepsis and the possibility of treating the animals similarly to patients in the intensive care unit, there are at present only a limited number of porcine mediators that are possible to
analyze in order to achieve a more mechanistic approach. However, such analyses are under development and will be included in future studies.

A septic shock that is caused by virulent bacteria, such as *Streptococcus pneumoniae, Streptococcus pyogenes, Neisseria meningitides* or other Gram-negative bacteria, occurring in healthy individuals with a host response not previously activated has been called a primary septic shock 161. In accordance with this, septic shock that occurs secondary to trauma, surgery or an initial infection may be regarded as a secondary septic shock. In this type of shock the inflammatory response will probably to be modified and more influenced by anti-inflammatory responses that are due to endotoxin tolerance and other similar mechanisms. Thus, it is reasonable to speculate that effects of immunomodulating drugs and other therapeutic modalities may differ in primary and secondary sepsis. Whereas endotoxin injections into healthy animals may resemble the situation in primary septic shock, the model described in the present study might be considered as one of conceivable models of secondary septic shock.

In summary, the responses to the different modulating interventions of this thesis differed between the organ systems studied. As to the innate immune response, TNF-α was not at all influenced by reduced plasma endotoxin concentration while profoundly attenuated, dose-dependently, in endotoxin tolerant animals. The IL-6 response, as previously shown by Lipcsey et al. more dependent on endotoxin concentration than other parameters 86, showed a tendency towards lower concentrations following decreased plasma endotoxin concentration and was, similar to TNF-α, dose-dependently attenuated in endotoxin tolerant animals. The leukocyte response showed an improved recovery towards baseline values following the antiendotoxin measure and reacted different in tolerant animals as regards the challenge dose. The leukocyte response was similar to that of the pulmonary organ dysfunction parameters where improved blood gases and compliance were seen after the antiendotoxin measure and quite distinctive reactions as regards the high- or low-dose challenge in tolerant animals were noted. However, no correlation to the individual level of endotoxin tolerance was seen for the pulmonary organ dysfunction parameters. Hypoperfusion and cardiac organ dysfunction did not correlate to the individual level of endotoxin tolerance and similarly no effect of the antiendotoxin measure was seen. In endotoxin tolerant animals hypoperfusion and cardiac organ dysfunction was almost unaffected by the endotoxin challenge. The circulatory organ dysfunction was not at all influenced by reduced plasma endotoxin concentration and the reaction to the endotoxin challenge in the endotoxin tolerant animals approximated that seen for hypoperfusion and cardiac function. However, the circulatory parameters were associated with the level of endotoxin tolerance. As to the renal organ dysfunction parameters, they were not influenced by the addition of an aminoglycoside or by reduced plasma endotoxin concentration. A clear picture of endotoxin
tolerance was seen with less prominent development of renal dysfunction following the endotoxin challenge in tolerant animals, however not associated with the individual level of tolerance.

The picture with distinctive patterns in the responses of different organs to different interventions underlines the importance of using whole animal models when trying to decipher the complex relationship between systemic inflammation and the development of organ dysfunction. The purity of the systemic inflammatory reaction induced by endotoxin as opposed to the possible confounding effects of a disseminated infection with live bacteria multiplying in the organs, provides a good argument for the use of endotoxemic models in the study of inflammatory-induced organ dysfunction. Given the possibility to expand the model resembling different types of sepsis such as secondary sepsis, the endotoxemic large animal model used in this study will be even more useful in the future.
Conclusions

- No additional effect on the development of renal dysfunction by the aminoglycoside tobramycin was found, indicating that a single high dose of tobramycin does not further compromise renal function in systemic inflammatory-induced renal dysfunction.

- Antiendotoxin treatment had no measurable effect on TNF-α-mediated toxicity once the inflammatory cascade was activated. In contrast to the lack of effect on hemodynamics, hypoperfusion and circulatory and renal function, there was a small effect on the leukocyte response that was associated with slight improvements in respiratory function and microcirculation, making it impossible to rule out fully the beneficial effect of the antiendotoxin concept. However, the effects were limited in relation to the magnitude of the endotoxin concentration reduction and the very early application of the antiendotoxin measure.

- Endotoxin tolerance resulted in attenuated effects following a second endotoxemic hit on systemic inflammatory response-induced manifestations, such as disturbed circulation, hypoperfusion and organ dysfunction.

- The lungs stood out compared to the other organ systems as having a threshold endotoxin dose for the protective effect of endotoxin tolerance. As to the development of hypoperfusion and circulatory and renal dysfunction, tolerance to endotoxin was evident regardless of the endotoxin preexposure and challenge dose.

- There was a temporal variation of endotoxin tolerance that did not follow changes in plasma TNF-α concentrations and maximal tolerance was seen very early in the course. More pronounced endotoxin tolerance at the time of maximum tolerance was associated with a more marked hyperdynamic circulation, reduced oxygen consumption and thrombocytopenia eighteen hours later, whereas no such associations were present for cardiac, pulmonary and renal function.

- Since the biological response after a second challenge of endotoxin differs from that in unexposed animals, it might be of interest to use this experimental model of long-term endotoxemia followed by a second hit, which has been designed to resemble an intensive care setting, for the study of treatment effects of immunomodulating therapies in secondary sepsis.
Future perspectives

One of the scientific goals for the group’s work is to use experimental large animal models to answer clinical questions impossible or difficult to answer in clinical trials. To successfully do this, the model continuously needs to be developed. The interesting finding of this study that the pulmonary response to both reduced endotoxin concentrations as well as following a second hit to tolerant animals differed from the responses in other organ systems opens up for a more mechanistic approach to develop the model and the knowledge of the lung response.

A study is underway were healthy pigs ventilated with respiratory settings used in this study (TV 10 mL x kg\(^{-1}\), PEEP 5, RR 25) are compared with pigs ventilated with low tidal volumes (6 mL x kg\(^{-1}\)). After two hours of ventilation, the pigs are challenged with an endotoxin infusion and the extra-pulmonary organ responses are followed. In a planned continuation of the project a VAP-model will be developed using bronchial inoculation of live bacteria, with the aim to compare whether the two ventilation patterns above lead to any differences as to the development of VAP and subsequent extra-pulmonary organ injury. Furthermore we will use our model of long-term endotoxemia to induce a state of endotoxin tolerance followed by bronchial inoculation of live bacteria. The response will be compared to that of non-tolerant animals receiving intra-bronchial bacteria to test the hypothesis that the alveolar sequestration of neutrophils seen in ALI/ARDS as well as during endotoxemia can be attenuated by bacterial invasion leading to phagocytosis, and equally hypothetically earlier resolution of the pulmonary inflammatory process.

The mechanistic factors involved in the development of endotoxin tolerance need to be further investigated as previously discussed. A study comparing the effect of a single injection of endotoxin with that of a continuous infusion to induce a state of endotoxin tolerance is underway. In this study the impact of oxidative stress, NO and anti-inflammatory cytokines on the development of tolerance will be investigated.
Sepsis, i folkmun kallat blodförgiftning, är en av de vanligaste orsakerna till död på våra intensivvårdsavdelningar. Trots medicinsens framsteg i övrigt är risken för död vid sepsis fortfarande stor. Den största orsaken till sjuklighet och död i samband med sepsis är den generella inflammationsreaktion som startas av det naturliga immunförsvaret efter kontakt med invaderande mikroorganismer, vanligtvis bakterier. Då klinisk forskning på sepsispatienter är behäftad med stora metodologiska svårigheter och stora kunskapsluckor föreligger i framför allt orsakerna till multiorgansvikt och död vid sepsis, behövs experimentella sepsismodeller.


I delarbete I var frågeställningen om en enkel hög dos med aminoglykosidantibiotika ytterligare försämrar njurfunktionen hos individer med inflammationsorsakad njurskada. Försöksdjuren erhöll kontinuerligt intravenöst endotoxin som efter tre timmar orsakade betydande njurfunktionsnedsättning med stigande kreatininvärden, kraftigt nedsatt antibiotikaclearance och sjunkande urinproduktion hos alla djur. De djur som dessutom erhöll aminoglykosidbehandling i en kliniskt relevant dos upprisade inga tecken till ytterligare försämring av njurfunktionen, vare sig i kreatininkoncentration, elektrolythalt, antibiotikaclearance, urinproduktion eller bedömt med elektronmikroskopi. Sålunda sågs inga tecken till att en kliniskt relevant aminoglykosiddos ytterligare försämrar njurfunktionen vid akut inflammationsorsakad njurskada.

I delarbete II skapades en modell för endotoxinelimination; en behandlingsform baserad på blodaferes som genomgått kliniska prövningar på patienter med positiva resultat i singelcenterstudier men inte i större i multicenterstudier. Endotoxineliminationen efterliknades genom att en grupp
djur erhöll endotoxin under hela försökstiden (sex timmar) medan infusionen stängdes av efter en respektive två timmar i interventionsgrupperna. Trots en mycket snabb och kraftig sänkning av endotoxinnivån i plasma i interventionsgrupperna sågs ingen skillnad i inflammationssvår eller cirkulationssvår mellan grupperna. Sålunda blir slutsatsen att endotoxinelimination som behandlingsform med mycket liten sannolikhet kommer att ha effekt vid septisk chock.

I delarbete III och IV undersöcktes fenomenet endotoxintolerans vilket innebär ett minskat fysiologiskt svar på endotoxin efter att individen tidigare utsatts för substansen. Endotoxintolerans är ett fenomen med klinisk relevans, men ett komplext sådant då man hos exempelvis patienter med akut lungskada (ALI/ARDS) har visat att de sjukaste patienterna uppvisar mer endotoxintolerans än de mindre sjuka.


I delarbete IV tillsattes endotoxin till blodprov från djuren i delarbete III. På detta sätt kunde graden av endotoxintolerans bedömas utifrån blodets förmåga att svara med cytokinproduktion, där en lägre cytokinproduktion tolkades som högre grad av endotoxintolerans. Djuren uppvisade den kraftigaste graden av endotoxintolerans redan sex timmar efter försöksstart följt av en minskande tolerans de följande timmarna. Vidare sågs en koppling mellan graden av endotoxintolerans vid sex timmar och cirkulationsstatus och organpåverkan vid försöksens slut. Högre grad av endotoxintolerans tidigt i försöket korrelerade med en mer hyperdynamisk cirkulation (dvs. lägre blodtryck, högre hjärtminutvolum och lägre systemvaskulär resistans), lägre syrgaskonsumtion samt lägre trombocytinivåer vid försöksens slut.

Delarbete III och IV har utvecklat en modell för att studera hur individer med redan aktiverat inflammationssvår reagerar vid förnyad systeminflammation orsakad av sepsis. Detta kan ses som en modell för så kallad sekundär sepsis, dvs. sepsis som uppstår sekundärt till trauma, bränskador eller som sekundärinfektion. Sekundär sepsis är en vanlig form av sepsis på våra intensivvårdsavdelningar och skapandet av en experimentell modell ger möjligheten att pröva effekterna av t ex. immunmodulerande läkemedel vid detta tillstånd.
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References

13. Castellheim A, Brekke OL, Espevik T, Harboe M, Mollnes TE. Innate immune responses to danger signals in systemic


46. Galani V, Tatsaki E, Bai M, et al. The role of apoptosis in the pathophysiology of Acute Respiratory Distress Syndrome (ARDS):


102. Opal SM, Scannon PJ, Vincent JL, et al. Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein


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