Antibacterial Effect and Inflammatory Response in Relation to Antibiotic Treatment of Sepsis

PAUL SKORUP
Dissertation presented at Uppsala University to be publicly examined in Gunnesalen, Ingång 10, Akademiska sjukhuset, Uppsala, Friday, 8 February 2019 at 13:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish. Faculty examiner: Associate professor Kristoffer Strålin (Division of infection and dermatology; Department of medicine; Huddinge, Karolinska institutet).

Abstract

Sepsis defines as life-threatening organ dysfunction caused by a dysregulated host response to infection. The importance of early administration of antibiotics in septic shock is undisputed, but the optimal antibiotic choice remains uncertain. Some national guidelines advocate single β-lactam antibiotic treatment while others recommend a combination of β-lactam and aminoglycoside. This thesis aimed to investigate the anti-bacterial properties and antibiotic-induced inflammatory responses of β-lactam antibiotic compared with effects of the addition of an aminoglycoside in clinically relevant E. coli porcine intensive care sepsis/septic shock models. We also studied the host's antibacterial capacities in primary and secondary sepsis.

In Paper I the addition of an aminoglycoside, in comparison with single β-lactam antibiotic treatment, caused decreased bacterial growth in the liver and greater antibiotic-induced blood killing activity ex vivo. The results thereby constitute possible mechanisms to the previously reported improved survival in the most critically ill sepsis patients receiving the β-lactam/aminoglycoside combination. Also observed in this paper was that individual blood bactericidal capacity may have significant effects on antimicrobial outcome.

In Paper II we investigated endotoxin release in vivo after antibiotic treatment in comparison with no treatment. There were no differences, however, antibiotics did increase an inflammatory IL-6 response that was associated with leukocyte activation and pulmonary organ dysfunction. A secondary finding was that the addition of an aminoglycoside to a β-lactam induced trends towards less inflammation compared with β-lactam alone.

Paper III compared how challenge with different pre-killed E. coli activates the inflammatory response, resulting in higher cytokine responses, more leucocyte activation and inflammatory capillary leakage after single β-lactam compared with live or heat-killed bacteria. The addition of an aminoglycoside lowered the β-lactam-induced responses.

Paper IV demonstrated that animals with secondary sepsis exhibited an attenuated inflammatory response as expected; however, contrary to our hypothesis, the animals’ antibacterial capacities were intact and partly enhanced.

We conclude that there are likely several beneficial effects of the addition of an aminoglycoside to a β-lactam therapy regimen in septic shock. Because host antibacterial capacities in secondary sepsis are enhanced, the need for bactericidal antibiotic combinations is not greater in secondary than in primary sepsis.

Keywords: Sepsis, Septic shock, Antibiotics, Aminoglycoside, β-lactam, Bacteria, E. coli, Inflammation, Cytokines, Endotoxin, Porcine, ICU, Secondary sepsis, Endotoxin-tolerance.

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Till mina favoriter Elin, Gordon, 
Frank och Gloria
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*Photo of the author by Karl Sandström*

*Photo of the game “Pass the pigs” by Paul Skorup*
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


III Skorup, P., Maudsdotter, L., Lipcsey, M., Larsson, A., Sjölin, J. **Mode of Bacterial Killing Affects the Inflammatory Response and Associated Organ Dysfunctions in a Porcine E. coli Intensive Care Sepsis Model.** Submitted manuscript.


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## Abbreviations

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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>MODS</td>
<td>Multiple organ dysfunction syndrome</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>SOFA</td>
<td>Sequential organ failure assessment</td>
</tr>
<tr>
<td>FiO₂</td>
<td>Fraction of inspired oxygen</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>POx</td>
<td>Pulse oxymetry</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DOB</td>
<td>Dobutamine</td>
</tr>
<tr>
<td>EPI</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>K</td>
<td>Capsular antigen</td>
</tr>
<tr>
<td>O</td>
<td>Oligosaccharide-attached antigen</td>
</tr>
<tr>
<td>e.g.</td>
<td><em>Exempli gratia</em></td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>LSECs</td>
<td>Liver sinusoidal endothelial cells</td>
</tr>
<tr>
<td>i.e.</td>
<td><em>Id est</em></td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin-binding protein</td>
</tr>
<tr>
<td>CLP</td>
<td>Caecal ligation and puncture</td>
</tr>
<tr>
<td>iv</td>
<td>Intravenous</td>
</tr>
<tr>
<td>vs</td>
<td>Versus</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive end-expiratory pressure</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>Arterial carbon dioxide pressure</td>
</tr>
<tr>
<td>PRIM</td>
<td>Primary sepsis</td>
</tr>
<tr>
<td>SEC</td>
<td>Secondary sepsis</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming units</td>
</tr>
<tr>
<td>RR</td>
<td>Respiratory rate</td>
</tr>
<tr>
<td>CI</td>
<td>Cardiac index</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>MPAP</td>
<td>Mean pulmonary arterial pressure</td>
</tr>
<tr>
<td>I:E</td>
<td>Inspiratory:expiratory ratio</td>
</tr>
<tr>
<td>RA</td>
<td>Ringer acetate</td>
</tr>
<tr>
<td>CLED</td>
<td>Cysteine lactose electrolyte deficient</td>
</tr>
<tr>
<td>Log</td>
<td>Logarithmic</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
</tbody>
</table>
Introduction

Sepsis is derived from the Greek word σηψις (sipsi), an ancient word first mentioned in the literature more than 2700 years ago. The term refers to “decomposition of animal or vegetable organic matter in the presence of bacteria” [1]. The definitions and nomenclature for sepsis have thereafter shifted and since 1992, the words infection, inflammation and sepsis carry different meanings [2]. An infection occurs when an invading microorganism (e.g., bacteria or virus) enters a normally sterile body compartment while an inflammation is the host’s defence reactions to that intrusion. Sepsis is a result of the reactions between a dysregulated host defence and the invading organism. The 2016 Sepsis-3 definitions state that sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection [3]. Sepsis requires urgent treatment and care for the individual to survive. To improve sepsis care the World Health Organisation recently acknowledged sepsis as a "Global Health Priority" [4]. The understanding of sepsis inflammatory responses and its associations to antibacterial effects, antibiotics and morbidity/mortality are of increasing interest [5, 6]. The optimal empiric antibiotic treatment of sepsis/septic shock is, however, under debate and thus needs, together with sepsis pathophysiology, to be further investigated in clinically relevant sepsis models to amend current sepsis treatment and evolve future therapeutic strategies [7-11].
Background

Sepsis
Sepsis is a life-threatening disease that exists in all parts of care, with septic shock being the severest form of an infection. Sepsis mortality has decreased in the past 20 years and is about 20% in sepsis and approximately 40% in septic shock, but the incidence is increasing. Rapid management to determine the site of infection, the causative microorganism, provide empiric antibiotic treatment and initiating supportive care is lifesaving in these conditions [3, 12].

Clinical picture
In severe infections the clinical picture consists of pathogen-specific symptoms resulting from the invading microorganism and the site of its entry, as well as general symptoms due to inflammatory activation. The latter indicates the severity of the infection, while the former may give an idea of what specific agent is involved. General systemic inflammatory symptoms include fever, leucocytosis, tachycardia and respiratory depression. In the most severe cases a fall in blood pressure, systemic hypoperfusion, altered mental status, even worse impairments of pulmonary, renal and liver functions, as well as coagulopathy. Sepsis is demonstrated either by the fact that a patient with infectious symptoms also has evidence of organ dysfunction; or the reverse, that a patient with organ dysfunction appears to have a suspected infection [13]. If a sepsis presents with diffuse symptoms without an obvious local infection or pathogen-specific symptoms, it is associated with delayed time to antibiotics and higher in-hospital mortality [14].

Definitions

Sepsis
The former use of the word sepsis, meaning the presence of bacteria in the blood (i.e. bacteraemia) [1], was revised in the 1980s when it was discovered that not only trauma or an infection but also the host inflammatory response itself may be harmful and lead to septic shock, multiple organ dysfunction syndrome (MODS) and death [15, 16]. Whether bacteria are present in the
blood (bacteraemia) or not is of limited importance in the clinical situation in the emergency department when the physician is seeking to grade the severity of the infectious disease because bacteria may be present in the blood without severe disease; on the other hand, patients might die from infections without bacteraemia [17]. In addition, it takes time before blood cultures reveal the growth of bacteria in the blood.

In 1992, a group of key opinion leaders released the first consensus definition of sepsis (later termed sepsis-1), introducing the term systemic inflammatory response syndrome (SIRS) and redefined the term sepsis to a state in which SIRS is present together with a suspected or proven infection [2]. This terminology stated that sepsis could develop into severe sepsis and septic shock. A second consensus group in 2001 upgraded this terminology with the addition of threshold values for organ damage, later termed sepsis-2 [18-20]. However, complaints were proposed regarding the lack of sensitivity and specificity with the use of the SIRS criteria. For example, almost all intensive care unit (ICU) patients have SIRS [21].

In 2016, a consensus group updated the sepsis definitions and named them sepsis-3. Sepsis is now clinically an increase of ≥2 sequential organ failure assessment (SOFA) points in the setting of an infection, thereby putting more emphasis on scoring organ dysfunction [3]. The organs are represented by respiratory, cardiovascular, coagulatory, hepatic, central nervous system and renal functions (Figure 1).

<table>
<thead>
<tr>
<th>Cardiovascular. MAP: vasopressor/inotrope a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p: MAP &lt;70 mmHg</td>
</tr>
<tr>
<td>2p: DA &lt;5 or DOB</td>
</tr>
<tr>
<td>3p: DA 5-15 or EPI ≤0.1 or NE ≤0.1</td>
</tr>
<tr>
<td>4p: DA &gt;15 or EPI &gt;0.1 or NE &gt;0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CNS. Glasgow Coma Scale (score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p: 13-14</td>
</tr>
<tr>
<td>2p: 10-12</td>
</tr>
<tr>
<td>3p: 6-9</td>
</tr>
<tr>
<td>4p: &lt;6</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Respiration. PaO₂/FIO₂ (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p: &lt;53.3 (app. ≤ 96% POx at room air)</td>
</tr>
<tr>
<td>2p: &lt;40 (app. &lt; 92% POx at room air)</td>
</tr>
<tr>
<td>3p: &lt;26.7 with respiratory support</td>
</tr>
<tr>
<td>4p: &lt;13.3 with respiratory support</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Liver. Bilirubin (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p: 20-32</td>
</tr>
<tr>
<td>2p: 33-101</td>
</tr>
<tr>
<td>3p: 102-204</td>
</tr>
<tr>
<td>4p: &gt;204</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Renal. Creatinine (μmol/L)</th>
</tr>
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<tbody>
<tr>
<td>1p: 110-170</td>
</tr>
<tr>
<td>2p: 171-299</td>
</tr>
<tr>
<td>3p: 300-440</td>
</tr>
<tr>
<td>4p: &gt;440</td>
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</tbody>
</table>

<table>
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<tr>
<th>Urin output (ml/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3p: Urin output &lt;500</td>
</tr>
<tr>
<td>4p: Urin output &lt;200</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Coagulation. Platelets (x10³/μl)</th>
</tr>
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<tbody>
<tr>
<td>1p: &lt;150</td>
</tr>
<tr>
<td>2p: &lt;100</td>
</tr>
<tr>
<td>3p: &lt;50</td>
</tr>
<tr>
<td>4p: &lt;20</td>
</tr>
</tbody>
</table>

*Figure 1. SOFA scores. ≥2 points + infection = sepsis.*

*Abbreviations: POx, pulse oxymetry; DA: dopamine; DOB: dobutamine; EPI: epinephrine; NE: norepinephrine. a Catecholamine given μg/kg/min for at least 1 h.*
The terms SIRS and severe sepsis were eliminated and the concept sepsis in sepsis-3 may roughly be interpreted as a fusion between the former sepsis + severe sepsis terms. It may be speculated that the incidence in the latest sepsis definition probably will be somewhat higher than for severe sepsis, mainly because more patients meet the respiratory criterion. A Quick SOFA (qSOFA) was also introduced for bedside screening outside the ICU. The qSOFA uses three criteria, assigning one point for low blood pressure ($\leq 100$ mmHg), high respiratory rate ($\geq 22$ breaths per min), or altered mentation (Glasgow coma scale $< 15$). A presence of $\geq 2$ qSOFA points near the onset of infection is associated with a greater risk of death or prolonged intensive care unit stay [3, 22].

**Septic shock**

Septic shock is a medical condition that occurs when the patient is further impaired in the sepsis disease in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality. Definition: sepsis together with persisting hypotension requiring vasopressors to maintain mean artery pressure (MAP) $>65$ mmHg and having a serum lactate level $>2$ mmol/L despite adequate volume resuscitation [3].

**Secondary and tertiary sepsis**

Secondary sepsis is a condition in which patients have recently undergone a previous activation of the immune system (e.g., postoperatively or after a previous infection) before suffering a sepsis. This patient group is associated with weakened inflammatory responses as well as less organ dysfunction in experimental animal studies and in a clinical retrospective ICU study [23-25]. Long hospital care, particularly in an ICU due to sepsis, induces immunosuppression leading to increased susceptibility to nosocomial infections and secondary sepsis. A third patient group, tertiary sepsis, includes patients whose infectious condition meets the clinical criteria for sepsis. This group consists of intensive care patients who suffered multiple infectious hits and often suffered prolonged critical illness, possibly with the addition of surgery. The anti-inflammatory systems often dominate in tertiary sepsis so that organisms of low virulence (such as coagulase-negative staphylococci, enterococci or Candida) have the potential to invade [5, 25-28].

The burden and awareness of nosocomial infections (e.g., secondary sepsis) are increasing and the need for knowledge about their pathophysiology to optimise their antibacterial treatment is essential [29, 30]. Previous studies investigating whether the weakened inflammatory responses reported in secondary sepsis are associated with *in vivo* weakened bacteria killing efficacy report conflicting results and have not been investigated in large animal or human studies [24, 31, 32].
Incidence and prognosis

Sepsis and septic shock are the most frequent causes of mortality in critically ill patients worldwide, with at least 30 million cases annually. The incidence is reported to be increasing, probably because of the increasing age of the population, in addition to more frequent immunosuppressive medications, invasive surgery and growing antibiotic resistance. Sepsis mortality has decreased during the past 20 years, from a mortality of 35-40% to about 20%, probably because of the greater focus on early diagnosis, fluid resuscitation, rapid delivery of antibiotics and other improvements in supportive care. Mortality in septic shock is 40-50%. [33-40]. After the update to sepsis-3 definitions, the mortality in sepsis in future scientific reports may be higher because of higher morbidity included in the new defined term [22]. Recent studies report similarly increased levels of pro- and anti-inflammatory cytokines in sepsis, with an early predominance of pro-inflammation, rapidly followed by an anti-inflammatory counter-reaction. The majority of sepsis-related deaths do not occur during the early phase, but tend to take place during a prolonged immunosuppressive phase, probably due to failure to control the primary infection or in a secondary sepsis [5, 25, 41].

Bacterial activation of the immune system

Bacteria are the main cause of sepsis, but viruses, fungi and protozoans are possible pathogens as well. Different studies report conflicting results as to whether Gram-positive or Gram-negative bacteria are the most common causative pathogens in sepsis. Gram-negative bacteria dominate in sepsis in the ICU [35, 42, 43]. Bacteria activate the immune system powerfully with their cell wall components and extracellular products. In Gram-negative bacteria endotoxin, a constituent of the Gram-negative cell wall, has a dominant role in the immuno-activation of the host response. Gram-positive bacteria have no endotoxin but carry other pro-inflammatory cell wall structures, including peptidoglycan and lipoteichoic acid. Virulent exotoxins can be produced both by Gram-negative and Gram-positive bacteria [44, 45].

*Escherichia coli*

The paediatrician Theodor Escherich first isolated and described *Escherichia coli* (E. coli) in 1885 as *Bacterium coli commune* from the faeces of newborns. Later, its generic name was changed to *Escherichia*. It belongs to the family of Gram-negative bacteria and is one of the most intensively studied model organisms in biology. It constitutes a wide range of bacterial species, probably sharing only 20% of the genes among all the strains. Overlooking all blood stream infection, regardless of the severity of the infectious disease, *E.*
coli stands out as the most frequently found bacteria in community-onset bloodstream infections. Normally, *E. coli* live in the intestines of animals/humans where they thrive and constitute an important part of a healthy intestinal flora. In an optimal environment and without competitiveness the *E. coli* can grow rapidly with a generation time of 17 minutes (min); however, in the intestinal tract the generation time is estimated to be 12-24 hours (h). An important factor involved in the attacks of the pathogenic strains of *E. coli* is an ability to resist the bacterial defences in serum. Such strains are referred to as serum-resistant *E. coli*. The infectious disease caused by a certain strain of *E. coli* depends on their arsenal of virulence determinants, including capsules, toxins, adhesins and the ability to withstand different host defence mechanisms [46-49].

*E. coli* are traditionally named according to its antigens: the lipopolysaccharide O antigen, the capsular K antigen and the H antigen associated with the flagella. The presence of a K antigen on the *E. coli* is associated with serum resistance and more severe diseases (e.g., upper urinary tract infections and bloodstream infections). These K antigens can decrease the ability of complement and antibodies to bind to the bacterial surface and thereby avoid phagocytosis. K1 is the best-studied K antigen which, in addition to being antiphagocytic, also creates an antigenic disguise. One problem with *E. coli* experiments is that the most available strains are old laboratory strains (e.g., the O111B27, the K-12 and the O127). Unlike wild strains, these laboratory strains have lost their serum resistance and ability to thrive in the intestine and therefore probably are not suitable for *in vivo* experiments [50, 51].

Taking these virulence factors together, it seems obvious that preferable characteristics of an *E. coli* strain selected for an *in vivo* sepsis study should be i) serum resistant, ii) encapsulated and iii) a wild strain over an old laboratory strain.

Endotoxin

Considerable interest has been given to the release of bacterial products, especially regarding endotoxin. Endotoxin constitutes 75% of the Gram-negative bacterial cell-wall membrane. It consists of a lipophilic region, called lipid A, that is directed inwards into the bacteria, and an outward-directed hydrophilic polysaccharide portion, including the O antigen, giving the equivalent name lipopolysaccharide or LPS. Both during growth and death, Gram-negative bacteria release endotoxin [52-56].

Endotoxin is a very potent stimulator of the cells in the immune system. Indeed, it has been used extensively in the experimental sepsis field as an alternative to live bacteria because they mainly mediate the same inflammatory and physiologic responses as those seen in clinical sepsis. More than 70% of patients with sepsis and septic shock have elevated levels of plasma endotoxin.
In some studies higher plasma levels has been associated with increased mortality [57-59].

Endotoxin tolerance

Endotoxin tolerance, a term that has long been known, refers to reduction of the systemic inflammatory responsiveness in animals and humans to an endotoxin challenge following a first challenge with endotoxin. The multiple cellular mechanisms and signals are mainly unknown. Briefly, genetic reprogramming of leukocytes leads to shifting away from pro-inflammation, including cytokines, towards a response with anti-inflammatory features. Endotoxin tolerance is of interest in clinical situations when the inflammatory response has already been activated (e.g., postoperatively, posttraumatically or after a previous infection). Endotoxin tolerance is probably a protective mechanism suppressing immune activities to avoid an excessive inflammatory response and has in animal studies been shown to reduce mortality or organ dysfunctions. However, a presence of endotoxin tolerance genetically at initial sepsis presentation has been associated with organ dysfunction in patients [23, 60-62].

Not many studies has been done in large animals or human models on host antibacterial effects in secondary sepsis. Thus, the porcine 30-h intensive care model recently developed by our research group using endotoxin pre-treatment to create endotoxin tolerance was deemed suitable for this purpose [23].

Cytokines

Assaults to a host of microbes, bacterial cell components or even trauma activate a broad range of cells to release cytokines that are inflammatory mediators. A number of these small proteins are directly related to the development of the local and systemic inflammation. Some are more involved in pro-inflammation, whereas others are more involved in anti-inflammation. The best-known pro-inflammatory cytokines are interleukin-1 (IL-1) and TNF-α, whereas interleukin-10 (IL-10) acts mainly anti-inflammatorily [63]. Interleukin-6 (IL-6) is activated somewhat later than IL-1 and TNF-α and even if its activity per se is something in between pro- and anti-inflammation, its concentration reflects previous pro-inflammatory activity. Macrophages and endo/epithelial cells are the most potent and frequent producers of cytokines involved in the development of the inflammatory response. Every cytokine has its own matching cell-surface receptor, which induces signals intracellularly to alter the cell’s functional operation for optimal defence against invading pathogens. On the other hand, cytokines may dysregulate the immune response and promote tissue-damaging inflammation. In fact, some cytokines (e.g., TNF-α and IL-6) have been clearly associated with the pathophysiologic changes that ultimately lead to septic shock [64].
Because bacteria produce endotoxin, a range of extracellular products and other cell-wall constituents, which all might accelerate the inflammatory response, there is considerable interest on how antibiotic treatment may affect inflammatory responses during bacterial killing, especially in severe bacterial infections when many of the systems have already been activated.

Brief presentation of relevant antibacterial mechanisms
The antimicrobial defence consists of two major subdivisions: The innate immune system that is present in both vertebrates and invertebrates consisting of barriers but also of cellular defences; the other major subdivision is the adaptive immune system that is only present in vertebrates defending against immunologically memorised previously invading pathogens with antibody-mediated and cellular defences. The initial protection, the innate immune system, is believed to dominate situations with the development of sepsis [46, 65]. Some of its features will be briefly presented, focusing on i) leucocyte action, ii) systems contributing to the blood’s bactericidal capacity and iii) important immunoactive organs that remove circulating intruding bacteria.

Leucocyte action
Important in the innate immune system are leucocytes, of which monocytes, macrophages, neutrophils and dendritic cells are all involved in phagocytosis. After intruding microorganisms are detected, they are phagocytised and killed. However, the involved leukocytes also signal and recruit more phagocytising cells to the site of infection and those cells produce even more pro-inflammatory signals that are responsible for the initiation of other inflammatory cascades (e.g., coagulation, fibrinolysis, complement systems) [46, 66].

Neutrophil activation is central in sepsis. The series of activating inflammatory signals that intruders induce, recruit neutrophils and monocytes from the bone marrow and blood, leading initially to an increased amount of those cells in a blood count. But upon activation, they adhere to the local activated endothelial cells, roll along the luminal surface and then transmigrate through the dilated blood vessel wall in order to further migrate towards the bacteria, which during intense neutrophil-activations leads to depressed number of circulating neutrophils [67].

Blood-borne systems – bloods bactericidal capacity
The bloods bactericidal capacity consists of different systems that are important protector when intruders reach circulating blood, but if overstimulated they may enhance the sepsis reaction.
Circulating erythrocytes entrap and kill bacteria with oxidative stress, then release them into circulation, a mechanism that is most effective against non-capsulated bacteria [68, 69].

Neutrophil extracellular traps are a kind of “neutrophil beneficial suicide”. Within hours after the death of the neutrophil, subsequent to its successful microbe phagocytosis, it leaks threads of DNA impregnated with antimicrobial peptides on which new microbes are trapped and killed [70, 71].

Neutrophil cytotoxicity, reactive oxygen species that induce oxidative damage and granulae proteins may all exert their actions intravascularly [72].

Antimicrobial peptides are mostly associated with tissue-specific protection; however, some are also blood-borne. This diverse group of small defence proteins each has different mechanisms of action and one single target attacks either a virus, a bacteria or a parasite [73, 74].

The complement system constitutes small proteins in the blood activated via the classical pathway, the alternative pathway or the newly discovered lectin pathway. All pathways start a cascade reaction including different parts of nine complement components resulting in: 1) local pro-inflammation 2) chemotaxis, 3) opsonisation (marking intruders for phagocytes), and 4) lysis [75].

The coagulation system has antibacterial properties besides its clot sealing of extra-vasal bleedings. A clot induces different local antibacterial immune effector mechanisms and immunomodulatory functions but can also possess small peptide derivates with direct membrane lysis on bacteria [76].

When performing in vivo investigations to determine how bacteria survive in the body, it might be of interest to contemplate the contribution from these systems to the overall results.

Immunoactive organs in the innate immune system

Of major interest in the innate system and the initial response to microbial attacks is the liver and the spleen that collect, trap and kill bacteria from the blood circulation, both having large amounts of tissue macrophages [77-79]. Many macrophages reside in the lungs because of exceptional large contact with the outside environment but is not the focus of this paragraph because their macrophages are specialised in inhaled particles/toxins/air pathogens rather than active during bacteraemia.

The liver

This highly metabolic active organ is also immune-active in microbe phagocytosis, immune-regulation and induction of immune responses. Its huge number of macrophages, the Kupffer cells, line up the inside of the sinusoid vessel wall on the liver sinusoidal endothelial cells (LSECs) and are in contact with the blood circulation. The portal venous blood from the gut is drained within these sinusoids and the Kupffer cells effectively clear circulating blood from pathogens. These cells also digest old neutrophils and are immune-active in
cytokine signalling. The LSECs also serve as a platform for and interact with other immune cells such as lymphocytes, myeloid cells and specialised natural killer cells that lodge in the liver [78, 80].

The antibacterial mechanisms and effects in bacterial secondary sepsis are largely unknown and the few studies have only been performed in small animals [24, 31, 32, 70] and some using intracellular bacteria inducing other host mechanisms [81]. Anyhow, the liver seems antibacterially important. Further investigations in this field are needed to understand the pathophysiology and optimise the management of ICU infections and secondary sepsis.

**The spleen**
The spleen is profoundly involved in the innate immune system. It filters, collects and disposes non-functional erythrocytes and leukocytes but also bacteria if present. The smallest arteries in the spleen have strongly reduced blood flow and drains the blood and its content of cells and microbes freely into blood-filled open spaces known as the red pulp (Figure 2).

![Figure 2. The human spleen. Modified picture reused with permission from the U.S. National Cancer Institute.](image)

The erythrocytes outnumber leukocytes and other cells in these areas and thus colorizing it and naming that part. This area has reticular fibres forming red blood cell filters that retain defect cells and microbes in the red pulp. It also contains specialised red pulp macrophages that have an important role in the phagocytosis and killing of blood-borne bacteria. Surrounding the arteries are white pulp areas localised as islands involved in the adaptive immunity. Because of the liver's larger size, the liver often collects the majority of circulating bacteria but the spleen is equally active and can reach a higher bacterial concentration owing to its relatively smaller size [79, 82, 83].
If intruding bacteria are encapsulated, (e.g., pneumococci and *E. coli*), parts of the circulating second-line innate immune protection and early phagocytosis often have problems in detecting and binding to the bacterial surface, which implies a greater reliance on the ability of the spleen to remove this intruder from the circulation. In patients in whom the spleen is missing or non-functional, capsulated bacteria, especially the pneumococci, constitute a major threat [84].

Our knowledge of the spleen’s activity in bacterial secondary sepsis suffers the same limitations as the liver antibacterial effects [24, 31, 32, 70, 81], the spleen’s role though also needs to be further investigated.

**Treatment of sepsis and septic shock**

The more severely ill the sepsis patients get, which is due to the invading microbe and the pro- and anti-inflammatory responses, the more organs will fail. This failure leads to MODS (multiple organ failure) and increased mortality. Prompt management is necessary and consists of both antibiotic and non-antibiotic treatment options.

**Non-antibiotic treatment of sepsis**

Non-antibiotic treatment includes mainly support of vital function and failing organs. The patients may need supportive fluid, vasopressors, mechanical ventilation and other forms of supportive care. The continuously updated Surviving Sepsis Campaign provides evidence-based recommendations for clinicians in the management of sepsis in emergency departments and ICUs. Norepinephrine is the first line agent when vasopressors are indicated and treatment with corticosteroids is of value only if adequate fluid resuscitation and vasopressor therapy fail to restore hemodynamic stability [12, 85]. Passive immunotherapy with immunoglobulin in sepsis/septic shock is generally not recommended, but might be motivated in the most severe invasive manifestations caused by group A Streptococcus [12, 86].

During the past 20 years, many randomised controlled trials have sought to identify new ICU treatments based on the improved knowledge about the pathophysiology of sepsis to improve patient survival. Attempts such as antithrombin III, corticosteroid and activated protein C were initially promising. However, in later randomised clinical trials, they failed to reduce mortality and in some cases were even associated with increased risk of serious adverse events [87].
Antibiotic treatment of sepsis

Several studies have shown that mortality in sepsis is substantially increased if the patient does not receive an “adequate” antimicrobial agent, i.e. that the isolated pathogen found in the microbial investigations is in vitro susceptible to at least one of the antibiotics given [88, 89].

Timing of antibiotic

The timing of antibiotics is vital as demonstrated in the study by Kumar et al in which an increase of 7.6% in mortality was observed for each hourly delay of adequate antibiotics in patients with septic shock [90]. The more recent study by Ferrer et al [91] reports an increase in mortality of only 1-2% for each hourly delay of antibiotic treatment. However, confirming that even with the recent improvements in survival [40], the importance of prompt administration of antibiotics is still high and should be administered within 1 h after septic shock recognition [12]. A prompt administration (1-3 h) of antibiotics in sepsis without shock is probably also of great importance, though more debated [12, 92, 93].

Combination therapy

In the initial empirical treatment of these severe infections a combination of antibiotics is commonly used to ensure that the causative organism is covered by at least one active drug. However, there have also been discussions on whether there is an advantage of covering the probable pathogen with two active agents of different antimicrobial classes. The most common empirical treatment combination of the severest infections has been that between a β-lactam antibiotic and an aminoglycoside, although the β-lactam might also be combined with a fluoroquinolone or a macrolide [12, 94].

To date, no large randomised clinical trial has been done to evaluate the effect of the β-lactam combination with aminoglycoside or one of the other two antibiotics. Therefore, the advantages of combination therapy with two active antibiotics have been questioned. In fact, several meta-analyses have not been able to demonstrate beneficial effects of combination therapy in severe sepsis [7, 95]. However, a meta-regression study by Kumar et al [8] showed that combination therapy improves survival and clinical response but only in those with life-threatening infections, particularly septic shock. A following large multicentre cohort study found significant beneficial effects when using early combination therapy with at least two antibiotics with activity against the pathogen isolated in patients with septic shock, especially if an aminoglycoside, a fluoroquinolone or a macrolide was added to a β-lactam antibiotic [9]. In a retrospective study on Gram negative bacteraemia Martínez et al showed that the addition of an aminoglycoside to septic shock patients is an independent protective factor [11]. While fluoroquinolones and macrolides have been shown to have immunomodulatory effects [96], such an effect has...
not been shown for aminoglycosides, where fast bactericidal activity and synergism have been speculated to be more likely mechanisms.

Another later systematic review from Paul M et al [97], which is mainly an update of their previous reports [7, 95], once again argued that the β-lactam/aminoglycoside combination does not provides an advantage over β-lactam monotherapy in septic patients but increases nephrotoxicity. Similar results were recently reported in an observational study by Ong et al [10] who compared two Dutch hospitals with different traditions in adding gentamycin to β-lactam treatment of severe sepsis/septic shock (sepsis-2 definitions), although the morbidity/mortality interpretations are limited because of heterogeneity between the treatment groups, i.e. a need for vasopressors/inotropy support. A general drawback of the reports from both Paul et al and Ong et al is the lack of patient stratification according to the presence of septic shock and illness severity causing confounding by indication. Another limitation concerning toxicity is that the aminoglycosides are used for considerably longer periods in many of these cited studies. The toxicities associated with aminoglycosides are nephrotoxicity [98] and ototoxicity [99] and the risk of these side effects increases with the length of treatment, emphasising the usage of aminoglycosides in septic shock during only 1-3 days. A single dose of aminoglycoside has not been shown to be nephrotoxic [100, 101] and when an aminoglycoside is added to a β-lactam as an initial empirical sepsis treatment, it is generally only for 1 or 2 days until results of the cultures become available [102].

Whereas a synergistic effect of the β-lactam/aminoglycoside combination has been extensively demonstrated in vitro [103, 104], in vivo experimental data are more limited. Animal models have examined the addition of an aminoglycoside to a β-lactam for the treatment of Gram-negative bacterial infections in peritonitis, [105] pneumonia [106], pyelonephritis [107] and endocarditis [108]. With few exceptions, these studies indicate that the combination results in improvements of either microbiological results or in survival, but unfortunately, these studies have often been performed on local infections with difficult-to-treat organisms (such as *Pseudomonas* spp.) and duration of treatment has often been several days. Increased rate of bacterial clearance in the blood and reduced growth in the organs during the early phase of treatment are probably crucial factors in the treatment of life-threatening sepsis and septic shock.

Secondary sepsis is associated with weakened inflammatory responses and it would be of interest to study whether the host antibacterial capacity also might be weakened. If so, the recommended antibiotics in many nosocomial infections should be readdressed, wherein broader antibiotics and also an addition of a fast bactericidal antibiotic such as an aminoglycoside might be preferable.
Current guidelines for the addition of an aminoglycoside

The latest Surviving Sepsis Campaign guidelines suggest empiric combination therapy aimed at the most likely bacterial pathogen in septic shock [12]. National guidelines differ in their interpretation concerning the need for adding an aminoglycoside to a β-lactam antibiotic for empirical treatment in septic shock patients, advocated in several guidelines [109-111] but not in all [112-115]. The addition of an aminoglycoside in sepsis treatment when shock is not present, is regularly not recommended [12].

The need for further knowledge concerning pathophysiology, host antibacterial capacity and inflammatory responses in relation to antibiotic treatment of sepsis is presently of great concern. Considering the problems involved with randomising patients with sepsis/septic shock at the emergency department into clinical trials during hasty sepsis management, an experimental large animal model evaluating the β-lactam/aminoglycoside combination for the initial treatment of sepsis and septic shock would be suitable. To our knowledge, such a study has not yet been performed. Difficulties with clinical human sepsis trials will be discussed further in the paragraph dealing with ethical considerations.

β-lactam and aminoglycoside: mechanisms and endotoxin release

In a number of in vitro experiments endotoxin has been shown to be released during antibiotic-induced killing of bacteria [52, 54, 116]. In general, bactericidal antibiotics such as β-lactams and aminoglycosides, release initially more endotoxin than bacteriostatic antibiotics. Antibiotics that act on the cell wall (e.g., β-lactams) liberate more than antibiotics with other modes of action such as protein synthesis inhibitors (e.g., aminoglycosides). Within the β-lactam group there is also wide variation among antibiotics in the propensity to release endotoxin. This is mainly caused by the binding of the drug to the specific bacterial penicillin-binding protein (PBP), initiating interactions that lead to changes in bacterial cell morphology. Different β-lactam subtypes have distinct affinities for certain PBPs. Generally, PBP-1 inhibitors cause cell elongation and are potent triggers of lysis; PBP-2 inhibitors alter cell shape but do not cause lysis; and PBP-3 inhibitors influence cell division and can induce large filamentation before lysis. Especially if there is binding of a β-lactam to PBP-3 on Gram negative bacteria, the successive bacterial filamentation and lysis leads to the release of substantial amount of endotoxin [117].

The β-lactam antibiotics are commonly used for the treatment of sepsis/septic shock [12], of which piperacillin, cefotaxime and meropenem all are examples of β-lactam antibiotics that bind to PBP-3 [118, 119]. Treatment with PBP-3-acting antibiotics have been particularly associated with an increase in the inflammatory response [120, 121].

Aminoglycosides are protein synthesis inhibitors acting through binding to the ribosome causing mistranslation and misfolded membrane proteins as well
as changes in the bacterial surface. In contrast to β-lactam antibiotics, their killing of bacteria is associated with a low release of endotoxin [52, 116, 117]. When aminoglycosides are combined with β-lactam antibiotics, the β-lactam antibiotic-induced release of endotoxin is often markedly reduced [52, 116, 122].

Antibiotic-induced endotoxin release has been shown to be biologically active [123, 124]. Still, evidence that antibiotic-induced endotoxin release is of clinical relevance is restricted and conflicting [121, 125, 126]. It would be of interest to investigate the in vivo inflammatory responses after antibiotic treatment, and thereby possibly explanations to a phenomenon often observed in clinical practise and recently reported from an ICU study, namely, a clinical deterioration in many patients with sepsis after the start of effective antibiotic treatment [127]. Considering the serious problems involved with clinical trials in this patient group, models in large animals that investigate the release of endotoxin and effects on the cytokine and organ responses would be of great benefit. However, as far as we know, this has only been performed in mice [128] that have a substantially different susceptibility to endotoxin and immune system in comparison with humans [129, 130].

Experimental models of sepsis
Given the difficulties to perform experiments in humans, experimental sepsis models are important in the ever-growing need for knowledge about pathophysiology and the treatment of sepsis. Most experimental sepsis studies have been performed in rodents. However, whereas mice share only a 10% similarity to the human immune system, the pig is highly similar to humans with a similarity of 80% [130]. Furthermore, the similarities to humans with respect to circulation, heart, lungs, kidneys, spleen and central nervous system make the pig an excellent choice for experimental sepsis and multiple organ deteriorations [131]. The size of the pig enables monitoring, repeated blood sampling and intensive care with circulatory support and devices used for humans. These factors are practically suitable but also important in that these measures and care procedures affect the inflammatory response, adding clinical relevance to the pig model [132-135]. Large animals all suffer disadvantages. The use of dogs that is commonly considered “man’s best friend”, raises sensitive ethical issues. Rabbits are too small for adequate instrumentation and sheep differ significantly from humans in circulatory and respiratory physiology [129, 136].

The inflammatory response in sepsis can be mimicked with the use of endotoxin administered intravenously (iv) and the effect of endotoxin in pigs and the similarity to the response in humans have previously been described in
However, when focusing on antibiotic treatment effect and bacterial pathophysiology, live bacteria are necessary. For that, mainly four models can be used:

- local bacterial inoculation into the lung, brain or soft tissue
- caecal ligation and puncture (CLP)
- mixture of bacteria + a fibrin clot, installed intra-abdominally
- iv infusion

All these models have their pros and cons [138-140]. For the local bacterial inoculation, its time lag between inoculation of bacteria and start of sepsis symptoms makes it difficult to perform in a large animal study and introduces considerable variability as to the start and severity of sepsis [129]. The CLP model often induces a severe and fast septic shock, but unfortunately, this method does not offer control of species and the amount of bacteria and severity are often highly variable [141]. The bacteria + fibrin clot mixture offers a relevant model but there are risks of variability in the preparation and implantation of the clot and its subsequent bacteraemia. If it works smoothly the technique might be a clinically relevant model to induce bacteraemia [142]. To maximally standardise the challenge of bacteria within the bloodstream and the development of sepsis/septic shock, the iv-administration route is probably the sharpest. Criticism against this route would be that in human infections the bacterial focus may seed the body over a longer time, rather than one sudden iv administration that only occurs in conjunction with contaminated infusions [140]. Accordingly, when using the iv route, it is probably of benefit to infuse the bacteria over a period of hours instead of minutes.

Results from animal experiments must always be interpreted with caution. Besides species differences and that the experimental stimulus of the inflammatory response does not often reflect the human situation, it must be emphasised that experimental animals are regularly young and without comorbidities in contrast to the patients in the ICU. The advantages are clearly the controlled situation regarding exact stimulus, interventions, measurements and the availability of tissue samples at the end of the experiment [129].

**Ethical considerations**

Why do we perform animal experimentation to determine how to treat severe bacterial infections? The background is that studies of acute diseases with high mortality are difficult to study in humans. Performing potentially harmful experiments in humans with infusions of live bacteria or bacterial toxins and thereafter observing morbidity and taking organ samples are not possible without damaging the body. Even observational studies of humans entering the emergency department with septic shock are difficult because of the initial unknown specie and amount of attacking bacteria. Another problem is that the hastily life-saving management of these patients makes a lengthy inclusion in
a scientific study troublesome. If patients suffer an altered mental state, they are also not able to perform the obligatory informed consent. Animal models of severe bacterial infections are considered necessary to increase our knowledge and create ways to reduce human morbidity and mortality in these diseases.

Great care must be taken to reduce animal stress before and during the experiment. Moreover, adequate levels of sedation and pain relief must be provided at all time. We have chosen the pig (Figure 3) because of anatomic and physiologic reasons but also because its immune system resembles that of humans [130, 131, 136].

*Figure 3.* Experiments are ongoing in two animals. The author is measuring the cardiac output using the thermodilution method.
Aims and questions

- To investigate whether the addition of an aminoglycoside to a β-lactam antibiotic has an effect on the in vivo killing rate of *E. coli* during the early phase of treatment in a porcine intensive care model of severe sepsis/septic shock. A secondary aim was to analyse the effect of individual blood bactericidal capacity ex vivo at baseline on the subsequent bacterial growth in the blood and organs.

- To investigate the in vivo relevance of antibiotic-induced endotoxin liberation, inflammatory response and sepsis-induced organ dysfunction in a porcine intensive care model of *E. coli* sepsis/septic shock. Secondarily, in a pilot study, to explore whether the addition of an aminoglycoside to a β-lactam antibiotic reduces these responses in comparison with that caused by the β-lactam antibiotic alone.

- To investigate whether bacteria killed by a PBP-3 active antibiotic, such as cefuroxime, affects the early inflammatory response and organ dysfunction more than a corresponding amount of live or heat-killed bacteria in a porcine intensive care model of sepsis/septic shock. Secondarily, whether the addition of an aminoglycoside, such as tobramycin, is able to reduce the cefuroxime-induced response.

- To investigate the host bacterial killing in vivo in secondary sepsis compared with primary sepsis, using an intensive care *E. coli* sepsis model with endotoxin tolerant pigs. Secondarily, to compare blood bactericidal capacities ex vivo in primary versus (vs) secondary sepsis.
Materials and methods

"I accept chaos, I'm not sure whether it accepts me."
Bob Dylan

Animals and ethic approvals
The experiments discussed in this thesis includes healthy Norwegian landrace-breed piglets of both sexes weighing approximately 25 kg. All animals were anaesthetized upon arrival at our research facility and never woke up before euthanasia. They were handled according to the guidelines of the Swedish National Board for Laboratory Animals and the European Convention on Animal Care and the experiments have always been provided with approvals from the Animal Ethics Board (Uppsala djurförsöksetiska nämnd), the permits during these experiments have been: C225/8, C250/11 and C255/14.

In order not to misspend the lives of animals, some of the animals are included in more than one paper in these experiments.

Great care has been taken to reduce the animals stress before and during the experiment and to maintain adequate levels of sedation and pain relief at all time. At the end of the experiments the animals were euthanized with a high dose of iv potassium-chloride leading to cardiac arrest and thereafter the respirator was turned off.

Preparations
All preparations of the animals were done under aseptic conditions and prepared as described elsewhere [137]. In brief: Animals were sedated with an intramuscular injection of 6 mg x kg\(^{-1}\) tiletamine/zolazepam. An auricular vein was cannulated, thereafter a bolus of 20 mg morphine and 100 mg ketamine was injected before tracheal intubation (Paper I-III) or tracheotomy (Paper IV). Iv general anaesthesia was administrated containing sodium pentobarbital and morphine together with muscle-relaxant pancuronium (Paper I-III) or rocuronium bromide (Paper IV). An initial bolus of fluids was given 30-45 minutes (min) before experimental start, Paper I-III: succinylated gelofusin
solution, Paper IV: acetated Ringer’s solution. Continuous replacement of acetated Ringer’s solution was administered resulting in a total fluid administration rate of 10 mL x kg\(^{-1}\) x h\(^{-1}\) during the experiment.

During general anaesthesia the following blood vessels were catheterized: auricular peripheral veins, the superior caval vein, the pulmonary artery (Swan Ganz catheter) and a cervical artery. Using vesicostomy, a suprapubic urine catheter was inserted. After completed preparations a 30-40 min of stabilization-time was allowed before initiation of the experiment.

The animals were mechanically ventilated. Respiratory settings were as follows: volume-controlled mode, inspired oxygen fraction in air (FiO\(_2\)) 30%, respiratory rate 25 min\(^{-1}\) and positive end-expiratory pressure (PEEP) 5 cm H\(_2\)O. Tidal volume was adjusted during the upstart period to maintain an arterial carbon dioxide pressure (PaCO\(_2\)) of 5.0-5.5 kPa.

Every effort was made to minimize animal suffering and by signs from an animal that it had not been sedated deep enough during anaesthesia, an iv bolus of 100 mg ketamine was injected, and if a reaction to fore hoof pain stimuli was observed, 20 mg morphine iv was provided.

Experimental design

The experimental designs of Paper I-IV are depicted in Figures 4-7. Note that the fundamental design of Paper I and II are identical, but their samples and analyses differ.

![Experimental Design Diagram](chart.png)

*Figure 4. Experimental design in Paper I.*
Figure 5. Experimental design in Paper II.

Figure 6. Experimental design in Paper III.
Protocols

Paper I-II

All animals received an *E. coli* iv infusion initiated at 0 h and continued at a constant infusion rate during 3 h. 27 animals were randomised to three treatment groups: The cefuroxime group (n=9) received 750 mg cefuroxime. The combination group (n=9) received 750 mg cefuroxime + 7 mg x kg⁻¹ tobramycin simultaneously. The control group (n=9) received saline. Immediately before 0 h and repeatedly during the experiment, physiological data were recorded and blood and urine samples obtained for laboratory testing.

**Exclusively for Paper I:** Quantitative bacterial analysis and *Ex vivo* experiments were performed according to Figure 4. Antibiotic concentration tests were performed hourly when animals received antibiotics. Organ samples were obtained immediately post mortem and analysed for bacterial growth.

**Exclusively for Paper II:** The first 27 animals were recruited from Paper I. Aiming to compare antibiotic treated animals (n=18) with animals receiving no antibiotics, it was deemed motivated to add 9 consecutive control animals for this study, though reaching similar group sizes (both n=18). Endotoxin, IL-6 and TNF-α were recorded and individual differences to the values at baseline (i.e., at 2 h) were calculated (Figure 5). *In vitro* endotoxin experiments were performed.
Paper III
This study comprised 16 animals randomised into four treatment groups receiving an iv infusion of pre-killed or live *E. coli* initiated at 0 h (i.e., at baseline) and continued at a constant rate for 3 h (Figure 6). The groups were either live *E. coli* (n=4) or killed *E. coli* through pre-exposure to cefuroxime (n=4), heat (n=4) or the combination of cefuroxime + tobramycin (n=4). Immediately before baseline and repeated hourly during the experiment, physiological data were recorded and blood and urine samples were obtained for laboratory testing, such as bacteria, endotoxin and cytokines analysis. An extra dose of antibiotics was administered after 2 h in order to kill eventual remaining live circulating bacteria, the same antibiotics as utilized in the pre-killing, while the heat-group received single cefuroxime and the control group received saline.

Paper IV
The animals were randomised to either primary sepsis (PRIM, n=18) or secondary sepsis (SEC, n=18) investigation, upon arrival to the laboratory facility. All animals were prepared identically, but SEC animals were pre-exposed to endotoxin and intensive care during 24 h, before baseline (Figure 7). The *E. coli* 0111:B4 endotoxin was administered iv to the SEC animals with an initial gradual increase of the infusion rate in order to avoid pulmonary hypertension in the animals [143, 144]. At baseline all animals received live *E. coli* as a 3-hour iv infusion. Blood cultures *in vivo*, two *ex vivo* bacterial series and organ samples were analysed for bacterial growth (Figure 7). Organ parameters and laboratory tests were assessed for sepsis reactions and for maintenance of the intensive care. Baseline and peak cytokine levels in blood were obtained for evaluation of endotoxin tolerance. To prevent and treat eventual atelectasis during the 30-h experiments, the secondary sepsis animals’ body position were changed and alveolar recruitment manoeuvres were performed every 6 h.

Interventions to optimize intensive care
To resemble an intensive care setting and optimize intensive care, all animals were treated in accordance with a protocol to maintain vital parameters within pre-set limits (Table 1).
Table 1. Intensive care treatment protocol to maintain the vital variables within preset limits. Grey cursive markings: interventions only used in Paper IV. In Paper IV cardiac output was assessed instead of cardiac index, its threshold value <2 and interventions were unchanged.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Threshold values for intervention</th>
<th>Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO₂</td>
<td>&lt;10 kPa first time</td>
<td>Increase FiO₂ to 0.6</td>
</tr>
<tr>
<td></td>
<td>&lt;10 kPa thereafter</td>
<td>1. Increase FiO₂ to the next level 0.6 → 0.8 → 1.0 AND 2. Increase PEEP to the next level 5 → 8 → 10 → 14 cmH₂O AND 3. Alveolar recruitment*</td>
</tr>
<tr>
<td></td>
<td>&gt;20 kPa</td>
<td>Decrease FiO₂ to the next level 1.0 → 0.8 → 0.6 → 0.3</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>&gt;6.5 kPa</td>
<td>Increase tidal volume by 10% up to 15 ml x kg⁻¹</td>
</tr>
<tr>
<td></td>
<td>&lt;4.5 kPa</td>
<td>If RR ≤25, decrease tidal volume by 10% down to 4 ml x kg⁻¹. If RR &gt;25 Decrease RR by 10%</td>
</tr>
<tr>
<td>P pause</td>
<td>&gt;30 cmH₂O</td>
<td>Decrease tidal volume to 7 ml/kg and increase RR to the same minute volume.</td>
</tr>
<tr>
<td></td>
<td>&gt;30 cmH₂O persistently</td>
<td>I:E is set to 1:1</td>
</tr>
<tr>
<td>MAP</td>
<td>MAP=MPAP at &lt; 60 min after start of endotoxin or bacterial infusion. (&lt; 90 min in Paper IV)</td>
<td>Single dose of 40 μg of norepinephrine</td>
</tr>
<tr>
<td></td>
<td>MAP=MPAP at &gt; 60 min (&lt; 90 min in Paper IV)</td>
<td>1. Single dose of 20 μg of norepinephrine AND 2. Start norepinephrine infusion 0.07 μg x kg⁻¹ x min⁻¹. If ongoing, increase rate one step: 0.07 → 0.13 → 0.29 → 0.54 μg x kg⁻¹ x min⁻¹ AND 3. Single bolus dose of RA 15 ml x kg⁻¹</td>
</tr>
<tr>
<td></td>
<td>&lt;60 mmHg (&lt;50 is in Paper IV used as a threshold &lt; 90 min after start of endotoxin or bacterial infusion)</td>
<td>Start norepinephrine infusion 0.07 μg x kg⁻¹ x min⁻¹. If ongoing, increase rate one step: 0.07 → 0.13 → 0.29 → 0.54 μg x kg⁻¹ x min⁻¹ AND IF CO &lt;2.5 give RA bolus 15 ml x kg⁻¹</td>
</tr>
<tr>
<td></td>
<td>&gt;100 mmHg</td>
<td>Decrease norepinephrine rate one step 0.54 → 0.29 → 0.13 → 0.07 → 0 μg x kg⁻¹ x min⁻¹</td>
</tr>
<tr>
<td>CI</td>
<td>&lt;2</td>
<td>Start norepinephrine infusion 0.07 μg x kg⁻¹ x min⁻¹. If ongoing, increase rate one step: 0.07 → 0.13 → 0.29 → 0.54 μg x kg⁻¹ x min⁻¹</td>
</tr>
<tr>
<td>B-glucose</td>
<td>&lt;4.0 mmol/L</td>
<td>Single dose of 20 ml 30% glucose iv</td>
</tr>
</tbody>
</table>

PaO₂= arterial partial pressure of oxygen, FiO₂= inspired fraction of oxygen, PEEP= positive end expiratory pressure, RR= respiratory rate, MAP= mean arterial pressure, CI= cardiac index, MPAP= mean pulmonary arterial pressure, I:E= Inspiratory:Expiratory ratio, RA= Ringer acetate.

*PEEP was increased stepwise until a peak pressure of 35 cm H₂O was reached. At this point, an inspiratory hold was performed for 10 seconds. Thereafter, the PEEP was stepwise decreased to the PEEP defined by the protocol. If MAP decreased to the level of the MPAP, the recruitment maneuverer was aborted.
Organism

The chosen organism in these experiments was the *E. coli* strain B09–11822, a clinical isolate obtained from a patient with a bloodstream infection and septic shock. This strain is encapsulated and serum resistant (analysed to be serotype O-rough:K1:H7 at Statens Seruminstitut, Copenhagen, Denmark). In *in vitro* pilot studies prior to this study this strain demonstrated continuous growth in both serum and whole blood from pigs as well as from humans (data not shown). The bacteria were grown to logarithmic growth phase before the experiments. The number of bacteria of the infusate was controlled, in all experiments aiming for 5 x 10^8 colony-forming units (CFU).

Antibiotics

Cefuroxime was chosen as the β-lactam antibiotic and may be regarded as a representative for other antibiotics acting through PBP-3 (e.g., piperacillin, cefotaxime, meropenem) [118, 119] and was purchased as Zinacef. The aminoglycoside chosen was tobramycin, which was purchased as Nebcina. These antibiotics were preferred because their porcine pharmacokinetic profiles have been studied and shown to have similarities to those in humans [23]. The antibiotic doses selected are those commonly recommended as maximal doses for the treatment of human clinical septic shock. The minimal inhibitory concentrations for this *E. coli* strain, according to Etest, for cefuroxime and tobramycin were 4 mg x mL^{-1} and 0.5 mg x mL^{-1}, respectively.

Bacterial investigations

All quantifications of live bacteria were determined by plating on cysteine lactose electrolyte deficient (CLED) agar plates, then cultured 37°C overnight and CFU quantified with viable count technique (Figure 8).

*In vitro* bacterial killing (Paper I)

In order to overview the magnitude of the *in vivo* effects of the combination treatment, the *in vitro* effect of the addition of tobramycin to cefuroxime was analysed. Bacteria were suspended to 10^7 CFU x mL^{-1} in a Lysogeny broth medium containing cefuroxime or cefuroxime + tobramycin or no antibiotics. Bacterial survival was determined hourly 0-6 h with viable count technique.
Quantitative PCR (Paper I)

A PCR method was created for this specific \textit{E. coli} strain to be detected in porcine blood. DNA was extracted from whole blood and primers directed against the K1 capsule gene were used as previously described [145]. Hourly quantitative PCR analysis of the arterial \textit{in vivo} blood and also from the \textit{ex vivo} blood killing activity experiment were performed.

Bacterial growth \textit{in blood} (Paper I, II, III and IV)

Bacterial count in 0.1 mL arterial blood was quantified in triplicate by viable count technique. The detection limit was 10 CFU x mL$^{-1}$.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image8.png}
\caption{E. coli cultured overnight on CLED agar plates; ready for viable count.}
\end{figure}

Bacterial growth in organs (Paper I and IV)

Post-mortem biopsies were taken within 30 min post mortem from standardized locations in the left kidney, the liver and the spleen, thereafter placed in saline, homogenized and quantified with viable count technique.
Blood ex vivo investigations (Paper I and IV)

**Ex vivo bactericidal capacity at 0 h**
At baseline, before start of the *E. coli* infusion, blood was obtained from animals and inoculated ex vivo with $10^5$ CFU x mL$^{-1}$ *E. coli*. Quantifications was performed hourly 0-6 h with viable count technique. The bactericidal capacity at baseline was demonstrated as the growth after 6 h. The same investigation is named slightly different in the two papers where it was performed:
- Paper I: “Blood ex vivo bactericidal capacity of the animals at baseline”.
- Paper IV: “Ex vivo$_{PREBACT}“$.

**Ex vivo bactericidal capacity at 3:20 h**
Same preparations as “Ex vivo bactericidal capacity at 0 h” above, but performed in blood obtained at 3:20 h. Quantifications was performed hourly during 3 h, with viable count technique. The bactericidal capacity was demonstrated as the growth after 3 h. The same investigation is named slightly different:
- Paper I: “Blood ex vivo killing activity of the animals after 1 h of treatment”.
- Paper IV: “Ex vivo$_{POSTBACT}“$.

Killing of bacteria by in vitro pre-exposure (Paper III)
Paper III investigated animals receiving $5 \times 10^8$ CFU pre-killed *E. coli* compared with live. The in vitro pre-treatments were either: cefuroxime pre-exposure during 4 h, heat pre-exposure 95°C during 10 min, or pre-exposed during 1 h to the combination cefuroxime + tobramycin. Microscopic investigations of the final infused solutions containing live or pre-killed *E. coli* were performed.

Laboratory analyses
Haemoglobin and blood cell counts, i.e. leucocytes, troponin and platelets, were analysed on a CELL-DYN 4000™ haematology analyser. Blood gases were analysed on ABL™ 5 and Hemoximeter™ devises. Urine and plasma creatinine analyses as well as plasma tobramycin were performed on an Architect Ci8200 analyser. Plasma concentration of cefuroxime was analysed by reversed phase high-performance liquid chromatography and detected by mass spectrometry on an Agilent 1100 LC-MS. Commercial porcine-specific sandwich enzyme-linked immune-sorbent assay was used for the determination of TNF-α and IL-6 in plasma.
Analysis of endotoxin was performed with the limulus amebocyte lysate assay (Endochrome-K) [146]. Endotoxin-free equipment was used during all steps. In vitro and in vivo samples were immediately filtrated (0.45 µm) into vials, in order to remove any whole bacteria before storage and analysis.

Statistics

Statistica (Statsoft) was used in all statistical calculations. For analysis of normality Shapiro-Wilk’s test was employed. Normally distributed data are presented as mean ± standard deviation (SD), unless otherwise indicated. Data with a non-normal distribution are presented as median and inter-quartile range (IQR). In this model the logarithmic (log) growth of bacteria and also the log concentrations of cytokines and plasma endotoxin all approximate to normal distribution, with only occasional exceptions (Paper IV). A p-value of <0.05 was considered statistically significant, though in Paper II that p-value was considered significant only in the analyses of primary aims (endotoxin and IL-6) while for secondary analyses in Paper II p<0.01 was considered significant.

Paper I

The difference in bacterial growth in the organs between the combination and cefuroxime regimen alone was chosen as primary endpoint. Student’s t test was used in the comparison of bacterial growth. Correlation analysis combining all three groups were performed, with non-normally distributed organ bacterial counts due to antibiotics, between organ bacterial counts, in vivo bacterial count and baseline blood ex vivo investigation, using the Spearman rank correlation.

Paper II

Dynamics in concentrations of endotoxin and IL-6 in antibiotic treated animals and controls were the primary endpoints. Secondary endpoints were dynamics in TNF-α, and to compare differences between the combination treatment with cefuroxime alone. IL-6 was chosen as primary endpoint in the inflammatory response because TNF-α concentrations were expected to peak before initiation of antibiotic treatment. A coincidental difference between the groups at 2 h before division into different treatment groups were present in this study: The animals randomised into the group receiving single cefuroxime had fewer bacteria at 2 h. Therefore, all individual differences in endotoxin- and cytokine-concentrations and organ dysfunctions were calculated as comparisons to their values obtained at 2 h (i.e., the baseline). Concentration dynamics of endotoxin and cytokines were analysed by analysis of variance.
(ANOVA) for repeated measures using the group by time interaction and at specific time points by a one-way ANOVA. Correlation analyses of Pearson’s correlation coefficient was calculated.

Paper III
The primary endpoint was to study whether the dynamics in concentration of cytokines (TNF-α, IL-6 and IL-10) differed between animals receiving bacteria killed by cefuroxime and those receiving live or heat-killed bacteria. Further analyses on organ dysfunction and endotoxin were performed and secondary whether this inflammatory response was reduced after the addition of tobramycin. Differences between groups were analysed by ANOVA for repeated measures using the group by time interaction. If the interaction demonstrated significance when all four groups were included, additional ANOVAs were conducted to test differences between individual groups. Cytokine peak values were compared by one-way ANOVA. Non-normally distributed data were analysed by the Kruskal-Wallis test. In the correlation analyses between the inflammatory response and changes in cellular and organ dysfunction at 3 h Pearson’s correlation coefficient was calculated, except for creatinine, where the Spearman rank correlation was applied.

Paper IV
Bacterial growth in the organs at the end of the experiment constituted the primary endpoint and the student’s t test was used to compare the SEC with the PRIM group. Because the bacterial growth in the ex vivo series did not follow a normal distribution, differences between the groups at specific timepoints regarding bacterial growth in in vivo and in ex vivo were analysed by the Mann-Whitney U test and the paired analysis were calculated by the Wilcoxon matched-paired test.
Results

All animals in these experiments developed signs of sepsis/septic shock, these responses were seen despite the mitigating effect of intensive care measures [133-135]. No animals exhibited \textit{in vivo} growth of \textit{E. coli} or any contaminants before the bacterial infusion was initiated.

Results from Paper I

The sepsis-2 definitions were used and all animals developed signs of severe sepsis/septic shock. Antibiotic concentrations equalled those commonly recommended as maximal doses for the treatment of human clinical sepsis.

\textit{In vitro} investigation

The \textit{in vitro} investigation revealed that maximum bactericidal activity for single cefuroxime and the combination group was achieved after 5 and 1 h, respectively (Figure 9).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{\textit{In vitro} bacterial killing rate of the live \textit{E. coli} strain used in the experiment. The bacteria were exposed to cefuroxime alone, the combination of tobramycin and cefuroxime, or control medium.}
\end{figure}
Animal bacterial investigations

**In vivo blood bacteria quantification**

After completion of the bacterial infusion, there was a rapid bacterial clearance in all groups, with no live bacteria detectable at 15 min post-infusion or later, in both *in vivo* blood bacterial count and PCR (Figure 10) irrespective of treatment, revealing no differences between treatments in the *in vivo* bacterial killing rate.

![Graph](image)

*Figure 10. In vivo* bacterial DNA in the blood of treated animals.

**In vivo organ bacteria quantification**

Growth of bacteria in the spleen was reduced in the antibiotic groups compared with the controls; without difference between the two antibiotic groups (Figure 11). Bacterial growth in the liver was significantly lower in the combination group than in the cefuroxime group. Organ samples revealed no growth in the kidney.
Blood ex vivo bactericidal capacity at baseline

Blood bacterial capacity at baseline is presented in Table 2. The capacity was lower in the control group which probably was one component explaining the fewer bacteria in in vivo blood bacteria quantification at 2 h in the cefuroxime group. The blood bacterial capacity at baseline correlated negatively, when all groups were analysed together, to both in vivo bacterial count at 2 h ($r=-0.41$, $p<0.05$) and to spleen bacterial counts ($r=-0.42$, $p<0.05$).

Table 2. Dose of infused E. coli, bactericidal capacity of the blood at baseline and in vivo blood bacterial count before start of antibiotic treatment at 2 h.

<table>
<thead>
<tr>
<th></th>
<th>All animals</th>
<th>Cefuroxime</th>
<th>Cefuroxime + tobramycin</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose of infused bacteria, log$_{10}$ CFU</td>
<td>n=27</td>
<td>n=9</td>
<td>n=9</td>
<td>n=9</td>
</tr>
<tr>
<td>Blood bactericidal capacity at baseline</td>
<td>8.72±0.05</td>
<td>8.71±0.06</td>
<td>8.72±0.05</td>
<td>8.71±0.04</td>
</tr>
<tr>
<td>Bacterial count at 0 h, log$_{10}$ CFU x ml$^{-1}$</td>
<td>4.87±0.06</td>
<td>4.89±0.07</td>
<td>4.88±0.06$^a$</td>
<td>4.84±0.04</td>
</tr>
<tr>
<td>Bacterial count at 6 h, log$_{10}$ CFU x ml$^{-1}$</td>
<td>4.41±1.50</td>
<td>3.93±1.90</td>
<td>3.86±1.39$^{ab}$</td>
<td>5.26±1.03</td>
</tr>
<tr>
<td>Blood bacterial count in vivo at 2 h, log$_{10}$ CFU x ml$^{-1}$</td>
<td>2.94±0.29</td>
<td>2.70±0.28$^{b}$</td>
<td>2.99±0.15$^{ab}$</td>
<td>3.13±0.24</td>
</tr>
</tbody>
</table>

$a^{n}=8$, start inoculum in one animal outside the accepted limits. P-values were calculated using Student’s $t$ test: $^a$ significant vs control group, $p<0.05$. $^b$ significant vs the combination group, $p<0.05$.

Blood ex vivo killing activity of the animals after 1 h of treatment

In blood collected 1 h after administration of antibiotics (i.e. 3:20 h), the ex vivo blood killing activity in the animals treated with the combination of a β-
lactam antibiotic plus an aminoglycoside was markedly bactericidal, whereas blood after single cefuroxime demonstrated a more gradual killing (Figure 12). In contrast, no bacterial DNA reduction was detected in any of the groups, in the similar parallel *ex vivo* killing experiment using the PCR method (Figure 13).

*Figure 12. Ex vivo* killing activity in blood obtained from treated animals. P-values were calculated using Student’s *t* test.

*Figure 13. Bacterial DNA in the blood after ex vivo addition of live E. coli.*
Results from Paper II

*In vitro* endotoxin

*In vitro* endotoxin release after exposure to cefuroxime, the combination of cefuroxime + tobramycin or no antibiotics, is depicted in Figure 14.

![Endotoxin release in vitro](image)

*Figure 14.* Endotoxin release *in vitro* from the infused *E. coli* strain after exposure to the combination of cefuroxime + tobramycin or cefuroxime alone. The inserted figure: Bacterial killing from the same experiments, authorized reuse from PLoSOne.

In the control group endotoxin increased during growth. In spite of a significant killing after exposure to cefuroxime, the increase in endotoxin mimicked the control but the time concentration curve run a somewhat different course (p<0.05). For the cefuroxime + tobramycin combination, the concentration stabilized at a lower level vs cefuroxime alone and vs the control (p<0.001).

Animal investigations

**Endotoxin release in vivo**

At baseline (i.e., at 2 h) the endotoxin concentration was lower in the cefuroxim group, in comparison with the combination and control groups. After antibiotic treatment, there was a similar decline in endotoxin without any significant differences between the groups (Figure 15).
Figure 15. Endotoxin concentration in vivo. The arrow indicates the midpoint of the 20-min antibiotic administration.

**Cytokine release in vivo**

The IL-6 levels at baseline were low and increased markedly. The comparisons were performed to individual levels at baseline. Antibiotic-treated animals demonstrated a higher interleukin-6 response (p < 0.001) over time, than control animals (Figure 16). Cefuroxime treated animals had somewhat higher levels after treatment in comparison to those receiving the combination, but the difference was not significant.

The TNF-α levels in plasma reached peak concentration 1 h before the baseline. Beyond baseline the gradual and similar fall in TNF-α concentration did not differ between the groups (data not shown).
Figure 16. Change in interleukin IL-6 concentration after administration of antibiotics. The arrow indicates the midpoint of the 20-min antibiotic administration. The p-values over time are the results of the group-by-time antibiotic administration in the repeated-measures ANOVA, while p-values at timepoints are one-way ANOVA calculations.

**Inflammation and organ dysfunctions**

Leucocytes began to increase in controls, whereas in the antibiotic-treated animals, they followed a significantly different course with further leucocyte activation, shown as a decrease up to 5 h. This difference was mainly seen in the cefuroxime group (Table 3).

Organ dysfunctions: Static pulmonary compliance showed greater deterioration in the antibiotic-treated animals than in the controls, a difference that was noted in both cefuroxime-treated and combination-treated animals (Table 3). There were highly significant correlations between the dynamics, during 2h-6h, of IL-6 and leucocytes ($r = -0.56$, $p<0.001$) and between leucocytes and static pulmonary compliance ($r = -0.46$, $p<0.01$).
Table 3. Other parameters of inflammation, circulation and organ dysfunction. Group-by-time interaction in the repeated measures ANOVA was calculated, p<0.01 was considered significant.

<table>
<thead>
<tr>
<th>Parameter/Treatment</th>
<th>Before antibiotic treatment</th>
<th>After antibiotic treatment</th>
<th>Difference to the value before treatment at 2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>3 h</td>
<td>4 h</td>
</tr>
<tr>
<td><strong>INFLAMMATION</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucocyte count (10⁹ x L⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.4 ± 3.9</td>
<td>0.0 ± 2.2</td>
<td>0.4 ± 2.3</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>9.2 ± 4.0</td>
<td>0.1 ± 2.3</td>
<td>-1.3 ± 2.9</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>10.1 ± 4.8</td>
<td>0.3 ± 2.1</td>
<td>-1.9 ± 2.5</td>
</tr>
<tr>
<td>Cefuroxime+tobramycin</td>
<td>8.3 ± 2.9</td>
<td>0.0 ± 2.7</td>
<td>-0.7 ± 3.2</td>
</tr>
<tr>
<td><strong>Hemoglobin (g x L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>98 ± 11</td>
<td>4.6 ± 6.6</td>
<td>5.0 ± 8.7</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>87 ± 11</td>
<td>2.4 ± 4.1</td>
<td>5.6 ± 6.1</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>89 ± 13</td>
<td>1.0 ± 4.6</td>
<td>4.6 ± 7.4</td>
</tr>
<tr>
<td>Cefuroxime+tobramycin</td>
<td>85 ± 9</td>
<td>3.9 ± 3.2</td>
<td>6.6 ± 4.6</td>
</tr>
<tr>
<td><strong>CIRCULATION</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>89 ± 12</td>
<td>-3 ± 11</td>
<td>-3 ± 9</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>94 ± 16</td>
<td>-1 ± 16</td>
<td>4 ± 13</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>91 ± 16</td>
<td>-1 ± 19</td>
<td>7 ± 10</td>
</tr>
<tr>
<td>Cefuroxime+tobramycin</td>
<td>97 ± 16</td>
<td>-1 ± 14</td>
<td>2 ± 16</td>
</tr>
<tr>
<td>Cardiac index (L x min⁻¹ x m⁻²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.7 ± 0.6</td>
<td>-0.4 ± 0.6</td>
<td>-0.1 ± 0.7</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>3.7 ± 0.8</td>
<td>-0.7 ± 0.7</td>
<td>-0.7 ± 1.0</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>3.5 ± 0.7</td>
<td>-0.5 ± 0.8</td>
<td>-0.4 ± 1.1</td>
</tr>
<tr>
<td>Cefuroxime+tobramycin</td>
<td>4.0 ± 0.8</td>
<td>-0.9 ± 0.6</td>
<td>-0.9 ± 1.0</td>
</tr>
<tr>
<td><strong>Blood lactate (mmol x L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.4 ± 0.6</td>
<td>0.3 ± 0.8</td>
<td>0.0 ± 0.8</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>1.9 ± 1.2</td>
<td>0.1 ± 1.2</td>
<td>-0.2 ± 1.2</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>2.2 ± 1.5</td>
<td>0.0 ± 1.5</td>
<td>-0.5 ± 1.6</td>
</tr>
<tr>
<td>Cefuroxime+tobramycin</td>
<td>1.5 ± 0.5</td>
<td>0.3 ± 0.7</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td><strong>ORGAN DYSFUNCTION</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Static pulmonary compliance (ml x cmH₂O⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17.6 ± 3.8</td>
<td>-1.9 ± 2.1</td>
<td>-2.0 ± 2.6</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>20.6 ± 3.7</td>
<td>-2.9 ± 1.7</td>
<td>-3.0 ± 1.9</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>20.2 ± 3.6</td>
<td>-2.5 ± 1.7</td>
<td>-2.4 ± 1.2</td>
</tr>
<tr>
<td>Cefuroxime+tobramycin</td>
<td>21.0 ± 4.0</td>
<td>-3.2 ± 1.9</td>
<td>-3.6 ± 2.3</td>
</tr>
<tr>
<td>PaO₂/FiO₂ (kPa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>42 ± 11</td>
<td>-10 ± 10</td>
<td>-10 ± 8</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>56 ± 10</td>
<td>-12 ± 8</td>
<td>-14 ± 10</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>56 ± 9</td>
<td>-12 ± 11</td>
<td>-13 ± 11</td>
</tr>
<tr>
<td>Cefuroxime+tobramycin</td>
<td>56 ± 11</td>
<td>-12 ± 5</td>
<td>-15 ± 10</td>
</tr>
</tbody>
</table>

Group name abbreviations: Ab= antibiotic; contr= control; cefur= cefuroxime; comb= combination; NS= not significant.
Results from Paper III

*In vivo* bacterial growth

The groups receiving pre-killed *E. coli* had no live bacteria present *in vivo* blood bacterial counts, while the control animals receiving live *E. coli* exhibited growth during the bacteria infusion but negative thereafter.

**TNF-α, IL-6 and IL-10 dynamics**

Differences in dynamics of concentration over time between the four groups were found for the three cytokines (i.e. TNF-α, IL-6 and IL-10, all *p*<0.001), (Figure 17). Bacteria pre-exposed to cefuroxime resulted in higher values over time for all three cytokines than live bacteria (all *p*<0.05) or bacteria pre-exposed to heat (all *p*<0.01). The addition of tobramycin notably reduced the cefuroxime-induced cytokine response (all *p*<0.001).

*Figure 17. Dynamics in concentration of TNF-α, IL-6 and IL-10. The p-values are the results of the group-by-time interaction in the repeated-measures ANOVA after taking all time points into consideration. Inflammation, circulation and organ dysfunction*
Inflammatory and organ function parameters

Leucocytes: Decreases in leucocyte count, which reflect leucocyte activation differed significantly between the groups, with the most prominent changes in the cefuroxime group and the addition of tobramycin in the pre-exposure reduced these deteriorations (p<0.01).

Haemoglobin: Increases in haemoglobin, which reflect an increase in capillary permeability, differed between the groups, most deterioration observed in the cefuroxime group and the addition of tobramycin in the pre-exposure reduced that (p<0.01).

Norepinephrine: Also, the total dose of norepinephrine was higher in the cefuroxime group with median dose (range) of 2440 μg (60-4980), which was more than that administered to animals receiving live E.coli 0 μg (0-60) or bacteria killed by the combination that were not given any norepinephrine at all (both p<0.05). The norepinephrine treatment in the heat group were 30 μg (0-2540) and did not differ vs the single cefuroxime group.

There were more pronounced deteriorations in the group pre-exposed to cefuroxime but without significant differences in: plasma lactate, cardiovascular organ function represented by left ventricular stroke work index and MAP and renal organ function represented by creatinine and urine output (data not shown).

Morphologic visualisation of bacteria

Microscopic investigation of the final infused bacteria solutions demonstrated in solutions with bacteria killed by single cefuroxime elongated thread-like bacterial filaments without visible damage to the membranes. The other solutions containing either live E. coli, bacteria killed by heat or the combination of cefuroxime + tobramycin, visualised bacteria that could not be differentiated in quantity or morphology from each other.

Endotoxin levels

Endotoxin concentrations in the four groups revealed no differences in dynamics when all groups were analysed together, using the repeated-measures ANOVA (Table 4), therefore no further individual analyses were performed.

The peak endotoxin concentrations at 2 h correlated with peak TNF-α, IL-6, IL-10 values and leucocytes response at 3 h with r-values of 0.88, 0.85, 0.42 and -0.61, respectively (all p<0.05).
Table 4. Endotoxin levels (Log EU x L\(^{-1}\)) at different time points in the four treatment groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline, before challenge</th>
<th>0 h</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live bacteria</td>
<td>1.57 ± 0.14</td>
<td>2.67 ± 0.20</td>
<td>2.21 ± 0.15</td>
<td>1.82 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>1.53 ± 0.10</td>
<td>2.98 ± 0.36</td>
<td>2.27 ± 0.25</td>
<td>1.98 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>Heat</td>
<td>1.83 ± 0.16</td>
<td>2.76 ± 0.06</td>
<td>2.09 ± 0.13</td>
<td>1.77 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>Cefuroxime + tobramycin</td>
<td>1.50 ± 0.10</td>
<td>2.08 ± 0.10</td>
<td>1.52 ± 0.07</td>
<td>1.50 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>

Correlation tests

As shown in Table 5, the peak TNF-\(\alpha\) and IL-6 values correlated with changes at 3 h in lactate, platelets and PaO\(_2\)/FiO\(_2\) and the leucocyte response at 3 h to lactate and PaO\(_2\)/FiO\(_2\). For changes in creatinine, these correlations were stronger at 6 h: 0.86 for TNF-\(\alpha\), 0.81 for IL-6 and -0.64 for leucocyte peak responses.

Table 5. Correlations between the cytokine peak values and the leucocyte response at 3 h to each other and changes from baseline in blood haemoglobin, plasma lactate and circulatory, respiratory and renal organ function variables at the end of the bacterial infusion at 3 h.

<table>
<thead>
<tr>
<th></th>
<th>Log IL-6 (_{3h})</th>
<th>Log IL-10 (_{1h})</th>
<th>Leucocytes (_{3-0h})</th>
<th>Haemoglobin (_{3-0h})</th>
<th>LVSWI (_{3-0h})</th>
<th>Lactate (_{3-0h})</th>
<th>Platelets (_{3-0h})</th>
<th>Static pulm. compl. (_{3-0h})</th>
<th>PaO(_2)/FiO(<em>2) (</em>{3-0h})</th>
<th>Creatinine (_{3-0h})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log TNF-(\alpha) (_{1h})</td>
<td>0.87***</td>
<td>0.71**</td>
<td>-0.79***</td>
<td>0.32</td>
<td>-0.57*</td>
<td>0.88***</td>
<td>-0.63**</td>
<td>-0.19</td>
<td>-0.62*</td>
<td>0.44</td>
</tr>
<tr>
<td>Log IL-6 (_{3h})</td>
<td>-</td>
<td>0.47</td>
<td>-0.71**</td>
<td>0.34</td>
<td>-0.61*</td>
<td>0.77***</td>
<td>-0.65**</td>
<td>-0.29</td>
<td>-0.78***</td>
<td>0.39</td>
</tr>
<tr>
<td>Log IL-10 (_{1h})</td>
<td>-</td>
<td>-</td>
<td>-0.63**</td>
<td>0.14</td>
<td>-0.27</td>
<td>0.55*</td>
<td>-0.11</td>
<td>-0.24</td>
<td>-0.43</td>
<td>-0.05</td>
</tr>
<tr>
<td>Leucocytes (_{3-0h})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-0.40</td>
<td>0.34</td>
<td>-0.70**</td>
<td>0.38</td>
<td>0.49</td>
<td>0.77***</td>
<td>-0.07</td>
</tr>
</tbody>
</table>

LVSWI=Left ventricular stroke work index, Static pulm. compl.= static pulmonary compliance. P-values were calculated using Pearson’s correlation coefficient, except in the correlations to creatinine in which Spearman’s rank correlation test was calculated: *p<0.05; **p<0.01; ***p<0.001.

Results from paper IV

Animal experiment

SEC animals demonstrated an attenuated inflammatory response with lower peak TNF-\(\alpha\) and IL-6 values during bacteraemia, compared with PRIM animals (Table 6). The reduction in the inflammatory response in the SEC group was most evident in the levels of TNF-\(\alpha\).
Table 6. Baseline and peak plasma levels for TNF-α and IL-6 in animals with pri-
mary (PRIM) and secondary (SEC) sepsis.

<table>
<thead>
<tr>
<th></th>
<th>PRIM Log_{10} ng x L^{-1}</th>
<th>SEC Log_{10} ng x L^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=18)</td>
<td>(n=18)</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h = baseline</td>
<td>1.87 ± 0.04</td>
<td>1.80 ± 0.05</td>
</tr>
<tr>
<td>1 h = peak</td>
<td>4.70 ± 0.11</td>
<td>2.27 ± 0.11</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h = baseline</td>
<td>1.83 ± 0.10</td>
<td>2.09 ± 0.07</td>
</tr>
<tr>
<td>3 h = peak</td>
<td>3.40 ± 0.07</td>
<td>2.72 ± 0.07</td>
</tr>
</tbody>
</table>

Bacterial investigations

**Organ bacterial cultures**
The SEC group exhibited lower growth in the spleen compared with the PRIM group (p<0.05), (Figure 18). Bacterial growth in the liver was somewhat lower in the SEC, without reaching statistical significance.

**In vivo blood bacterial cultures**
The SEC animals showed fewer bacteria in cultures during the bacterial infu-
sions compared with the PRIM animals, significant 1 h after initiated infusion (p<0.05), (Table 7). After termination of the infusion, there was a rapid clearance of bacteria in all animals.
Table 7. Bacterial growth after bacterial challenge in blood cultures obtained \textit{in vivo} and \textit{ex vivo}.

![Table](https://example.com/table7.png)

P-values were calculated using The Mann-Whitney \textit{U}-test for differences between PRIM and SEC at each time point. The Wilcoxon matched-paired test compared differences between \textit{Ex vivo} PREBACT and \textit{Ex vivo} POSTBACT at 3 h. $^a = p<0.05$ PRIM vs SEC, $^b = p<0.01$ PRIM vs SEC, $^c = p<0.05$ \textit{Ex vivo} PREBACT vs \textit{Ex vivo} POSTBACT.

\textbf{Ex vivo} investigations for blood bactericidal capacity

\textit{Ex vivo} growth is demonstrated in Table 7. Blood bactericidal capacity was intact in the SEC animals compared with PRIM. A transient difference with increased killing in the SEC group was observed in the beginning of the \textit{Ex vivo} PREBACT serie. In both groups there was a reduction in \textit{Ex vivo} POSTBACT killing capacity compared with \textit{Ex vivo} PREBACT.
Discussion

Paper I

The main findings of this severe sepsis/septic shock study were that the addition of an aminoglycoside to a β-lactam antibiotic resulted in decreased bacterial growth in the liver (p<0.05) and greater antibiotic-induced blood killing activity \textit{ex vivo} (p<0.001) compared with single β-lactam treatment. Moreover, the knowledge that individual blood bactericidal capacity may have a significant effect on antimicrobial outcome was important.

Our model was designed to be a clinically relevant experimental intensive care sepsis study focusing on the bacteriological consequences of bacteria that have entered the bloodstream during the early phase of severe sepsis/septic shock. We found that even though this \textit{E. coli} is a serum-resistant and encapsulated wild type stem, characteristics which are emphasised in experimental models [50, 51], there was an extremely rapid \textit{in vivo} clearance of live bacteria from the blood with no relapse during the study period in any of the animals, including the controls. The rapid bacterial clearance is further highlighted by the bacterial DNA analyses demonstrating that bacterial DNA were present in a nearly constant concentration after antibiotic bacterial killing \textit{ex vivo}, whereas no DNA was detectable \textit{in vivo} once the bacterial infusion had been completed (Figures 10 and 13). The implication of this finding to clinical patients is unclear but with a healthy and effective innate immune system and during the initial phase of critical sepsis both live and killed bacteria were rapidly eliminated from the circulation, which has been proposed before in rat, rabbit and dog [77].

Our findings suggest that the majority of circulating bacteria are removed from the circulation and taken care of by the immunoactive organs: spleen and liver [78, 79]. Focusing on bacterial counts in control animals reveals that the liver contained approximately 10 times fewer bacteria than the spleen, but the pig’s liver out-sizes the spleen’s by approximately 10 times as previously reported [147]. Consequently, these organs may have contributed relatively equally in bacterial entrapment.

In the spleen cefuroxime alone and the combination of cefuroxime and aminoglycoside both resulted in a significant decrease in bacterial count of similar magnitude, whereas in the liver the addition of the aminoglycoside reduced bacterial count significantly compared with single cefuroxime treatment (Figure 11). Reasons for these organ differences probably relate to exposure time
to antibiotics because *ex vivo* results show that cefuroxime alone can reach a potential killing activity but have to be in contact with bacteria for more than 3 h to reach the same killing magnitude as the combination (Figure 12). Therefore, it may be speculated that the difference in exposure time to antibiotics and effects in these two organs might be explained by divergences in the organ setups: the spleens trapping mechanisms are in the red pulp [79] where bacteria are exposed during longer time to cefuroxime (exposure >3 h), thereby concealing the effects of the addition of aminoglycoside. The liver, in contrast, withdraws circulating bacteria mainly directly from the circulating blood during bacteraemia without specialised trapping mechanisms [78, 80], which disables cefuroxime’s time-consuming activity. Consequently, the superior bacterial killing activity of the combination that was proven *ex vivo* was seen only in the liver. Although there was rapid elimination of bacteria from the blood, our liver bacterial cultures indicate that antibiotic exposure was long enough for the combination to exert its antimicrobial effect before the bacteria were fully phagocytised and inaccessible for antibiotics.

Another explanation to the animals lower bacteria concentration in the liver could be if the liver is initially more active during bacteraemia in the withdrawal of bacteria from the circulation and has already eradicated liver-trapped bacteria at the time of organ sampling (i.e. 3 h after terminated bacterial infusion). However, earlier organ samples were not performed in this study in order to avoid misspending the lives of animals.

We found that stronger individual blood bactericidal capacity *ex vivo* at baseline showed negative correlation to *in vivo* bacterial cultures in blood and organ, thus significantly affected the antimicrobial outcome.

In the absence of prospective randomised human trials our findings are of clinical relevance and strongly supports the results of the studies by Kumar et al [8, 9] indicating that the addition of an aminoglycoside to a β-lactam antibiotic during the early phase of treatment results in beneficial antimicrobial effects in addition to merely broadening the antibacterial coverage. Furthermore, the individual blood bactericidal effect may confound the results in this type of experiment and hence should be considered when interpreting the findings.

**Paper II**

The main findings of Paper II were that treatment with antibiotics in sepsis/septic shock elicited an increased inflammatory IL-6 response that is associated with leucocyte activation and pulmonary organ dysfunction, without differences in plasma endotoxin concentrations. Considering the similarities between humans and pigs in endotoxin susceptibility, inflammatory response, physiology and pharmacokinetics of the two drugs used in the study [101, 130,
a comparable reaction will probably occur in patients as well. Consequently, this controlled study supports a recent investigation from an intensive care unit reporting deterioration in almost half of the patients with sepsis within 4 h after antibiotic treatment [127].

Employing a clinically relevant model, the present study tested whether the in vitro phenomenon of antibiotic-induced endotoxin liberation and subsequent inflammation existed in vivo in critical sepsis/septic shock. Cefuroxime acts on PBP-3 similar to most other β-lactam antibiotics (e.g., cefotaxime, ceftazidime, piperacillin and meropenem) used in the treatment of sepsis/septic shock [118, 119] and in the current study cefuroxime though is a representative for other PBP-3-acting antibiotics. The rapid elimination of live and dead bacteria from blood found in Paper I made it difficult to demonstrate any endotoxin release in plasma. However, the absence of an antibiotic-induced increase in plasma endotoxin concentration agrees with clinical observational findings [126, 127]. In contrast to plasma endotoxin levels, the study showed a significant difference in the inflammatory response with higher IL-6 concentrations as well as increased leucocyte activation and decreased pulmonary compliance in antibiotic-treated animals compared with controls. In the pilot study the addition of an aminoglycoside to the PBP-3-active cefuroxime was seen to lower these responses but did not reach statistical significance. Despite a lower bacterial count in the organs presented in Paper I, the antibiotic-treated animals showed an increased inflammatory response, probably caused by killed bacteria or the process of bacterial killing, such as the release of endotoxin. A direct antibiotic-induced inflammatory response [148] is less likely given that treatment with either a cephalosporin or an aminoglycoside has previously been shown to reduce IL-6 concentrations in a porcine endotoxin model [149].

The IL-6 response was associated with signs of leucocyte activation, which is an important component in the development of sepsis and organ dysfunction [150, 151]. Haemoglobin concentration, a surrogate marker for capillary permeability, showed similarities to the leucocyte response without reaching significance. Concerning organ dysfunction, the effects on circulation and oxygen delivery were offset by performed interventional and resuscitation according to the intensive care treatment protocol (Table 1), although there were no major differences in interventions between the groups. In contrast, there was significant deterioration in static pulmonary compliance that was associated with the IL-6 and leucocyte responses. Similar trends were seen in PaO2/FiO2, but this ratio is also affected by intra-pulmonary shunts and, to some extent, by therapeutical measures.

The increased leucocyte activation and decreased pulmonary compliance in correlations to IL-6 levels indicate that the differences in the inflammatory response are probably of clinical relevance in the most severe infections.

The focus of the present study was the initial hours and any effect on mortality is not known. However, in patients with sepsis initial IL-6 levels have
been associated with mortality in several studies [152-154]. Cefuroxime represents other PBP-3-active antibiotics [118, 119] and a mice sepsis study showed increased mortality when treated with the PBP-3-acting antibiotics ceftazidime or meropenem in comparison with the PBP-2-acting imipenem that releases only limited amounts of endotoxin [128], suggesting that differences in IL-6 and leucocyte activation observed in the present model might be harmful.

In the current study the differences in endotoxin liberation between the two antibiotic regimens demonstrated with this $E. coli$ strain in vitro were not reproduced in vivo. Nonetheless, differences between the treated and control animals in IL-6 and leucocyte activation were more evident in the animals treated with the PBP-3-active cefuroxime alone than with the combination, which were in focus as a pilot study. Similar trends were seen in haemoglobin, lactate and PaO2/FiO2 but not in pulmonary compliance. Thus, the advantage of adding an aminoglycoside to a $\beta$-lactam antibiotic, as previously shown by Kumar et al [9], might not exclusively depend on more effective killing demonstrated in Paper I, but also on a reduced antibiotic-induced inflammatory effect which, however, needs further investigation.

**Paper III**

In this experimental study the mode of bacteria pre-killing mechanism affects the host differently in the increase of cytokines, leukocyte activation, capillary leakage represented by haemoglobin and the need for supportive norepinephrine, with most deterioration seen in the group receiving bacteria pre-killed by the PBP-3-active cefuroxime. The addition of an aminoglycoside to cefuroxime was found to lower the cefuroxime-induced response. The inflammatory responses were different depending on which way the bacteria were pre-killed, indicating that the mode of killing caused the different host reactions.

The differences in the endotoxin concentration between animals given cefuroxime and those given live or heat-killed bacteria were limited, suggesting that other factors than released endotoxin are important in the activation of the innate inflammatory response.

The morphological appearance with elongation of bacteria and formation of filamentous forms associated with PBP-3 activity [155] were also seen in this experiment. Thus, these morphological features may be another factor in the activation of the innate inflammatory response. Still, the strong correlations between peak endotoxin levels and TNF-$\alpha$, IL-6 and leucocyte responses indicate that endotoxin may play a role. Because the supernatants after treatment with antibiotics or heat were removed before infusion into the animals, the endotoxin in this study was released in vivo from killed bacteria during the further process of fragmentation. This event also occurred in bacteria killed by heat. Live bacteria are quickly eliminated from the circulation and the main
process of bacterial killing probably takes place in the spleen and liver as reported in Paper I from where the systemic inflammatory response may be activated via endotoxic and non-endotoxic mechanisms, as has recently been shown, resulting in cytokine increase, leucocyte activation and organ dysfunction as reported in Paper II.

Differences in parameters reflecting cellular metabolism and organ function did not reach statistical significance. Yet, these parameters strongly correlated with cytokine responses, suggesting that differences in the inflammatory response are associated with changes in cell and organ dysfunction. In addition, effects on organ parameters concerning circulation and oxygen delivery may be neutralised by resuscitation measures. Nevertheless, there was a difference in the circulatory intervention between the groups with a significantly greater need for norepinephrine in the single PBP-3-active cefuroxime group and the addition of an aminoglycoside lowered that need.

IL-10, a marker of anti-inflammation, increased in parallel with TNF-α and IL-6, supporting initial activations of both pro- and anti-inflammatory responses in this model. This event is similar to early clinical responses shown in intensive care patients with sepsis [5, 156]. Consistently, IL-10 was found to mostly increase in the cefuroxime group as compared with the other groups.

The results of the present study support Mock et al’s findings that sepsis patients treated with PBP-3-active antibiotics showed higher mortality than patients treated with other antibiotics [120]. Randomised clinical studies comparing the effect of a PBP-3-active antibiotic with a non-PBP-3-active antibiotic have shown no or only trends towards differences in endotoxin levels, cytokines and outcome [121, 157-159]. However, considering the heterogeneity of sepsis patients, the chance of detecting a difference was minimal. A randomised clinical study designed for patients with septic shock would be of value but has not yet been performed, probably because of the inherent difficulties in conducting such a study. Nevertheless, the phenomena of an antibiotic-induced inflammatory response and deterioration is supported by Paper II and in agreement with Mignon et al’s study on ICU patients with sepsis in which deterioration occurred in almost half of the patients within 4 h after the start of antibiotic treatment [127].

As has previously been shown in vitro [123, 160], the addition of tobramycin reduced the cefuroxime-induced inflammatory response. This effect is probably mainly mediated by non-endotoxic effects, as discussed above; however, in agreement with several in vitro studies [52, 116, 122] the levels of endotoxin were lower in the combination group (Table 4) and peak endotoxin concentrations correlated with the inflammatory response, suggesting that differences in endotoxin levels might have contributed to the reduced inflammatory response in the combination group. Thus, in addition to its binding to the ribosome causing mistranslation and misfolded membrane proteins as well as changes in the bacterial surface, the aminoglycoside-induced inhibition of endotoxin synthesis may play a role [117, 161]. These results might offer an
explanation to Kumar et al’s clinical findings in which patients treated with
the combination of a β-lactam antibiotic and an aminoglycoside demonstrated
improved survival in comparison with propensity-matched control patients
[9].

Paper IV
In the presence of an expected reduced inflammatory response (lower TNF-α
and IL-6), the secondary sepsis animals exhibited - contrary to the hypothesis
- enhanced bacterial killing in the spleen than animals in the primary sepsis
group, with a similar trend in the liver. Furthermore, an intact and partly in-
creased bacteria clearance in secondary sepsis animals was also observed in
in vivo blood cultures and ex vivo investigations. Thus, the weakened inflam-
matory response in secondary sepsis was not associated with a reduced bacte-
ria killing capacity in the immune-active organs.

A second challenge of endotoxin has been performed extensively in ro-
dents, to some extent in rabbits and more seldom in large animals and humans.
This second challenge of endotoxin results in a suppressed inflammatory re-
sponse and a phenomenon referred to as endotoxin tolerance [23, 162-166].
Only a few studies have been performed in which live bacteria represent the
second challenge and where bacterial elimination has been reported. Results
in mice challenged with bacteria as a second challenge have demonstrated an
improved bacterial clearance [24, 32, 81]. However, because of difficulties in
extrapolating mice studies to humans (they only share 10% similarity in their
immune system) [130] and opposite results were observed in an endotoxin-
tolerant rabbit model challenged with E. coli [31], it was deemed worthy to
study this phenomenon. Concerning the difficulties in performing a study in
humans, the pigs' similarities in immune system and physiological responses
to humans as discussed above, we reason that the pig constitutes a rational
model to investigate bacterial elimination in secondary sepsis [130, 132]. The
intensive care treatment might additionally mitigate the inflammatory re-
sponse [133-135], which adds clinical relevance to this model.

Our results showed a reduced number of live bacteria in the spleen in the
SEC animals. This finding might be caused by reduced splenic uptake from
the blood or increased clearance of bacteria. Baseline blood bactericidal ca-
pacity before start of the bacterial infusion, expressed as the $E_x \text{vivo}_\text{PREBACT}$
growth after 6 h, may affect growth in the organs as reported in Paper I, but in
this respect, there were no differences between the PRIM and SEC groups,
which strengthens the present results. Thus, the increased bacterial clearance
in the SEC animals is likely caused by augmented splenic bacterial killing. A
lower bacterial count in vivo in the blood during bacteria infusion was seen,
suggesting an increased bactericidal capacity within the blood in the SEC an-
imals. Although there was no difference in growth in the end between the
groups, an initial increase in bacterial killing was noted in the independent Ex
vivo PREBACT-investigation in SEC animals, supporting the in vivo results. Thus,
increased killing within the blood of the SEC animals might have contributed
to some of the differences observed in the spleen.

Our results showing increased bacterial killing agree with those in mice
pre-treated with endotoxin [24, 32, 81]. Here, we speculate that the opposite
result in the rabbit study might have been caused by the short time interval (1
h) between endotoxin pre-exposure and secondary bacterial challenge in that
study [31]. The reduced ex vivo bactericidal capacity immediately after com-
pletion of the 3-h bacterial infusion (Ex vivo POSTBACT), in comparison with Ex
vivo PREBACT in our study, seems consistent with the finding in the rabbit model.

Based on the anticipation that the reduced inflammatory response might be
associated with reduced bacterial killing, it was hypothesised that there would
be an increased need of maximised bactericidal antibiotic therapy for the treat-
ment of secondary sepsis. Considering the present results, this matter no
longer seems urgent. However, even if endotoxin tolerance resembles the im-
mune depression that occurs in patients after a major infection or trauma [24],
our findings do not exclude a reduced killing capacity if the pro- and anti-
inflammatory responses are active for periods longer than 24 h.

This study has several limitations. The challenge of bacteria as an iv infu-
sion might clinically resemble the administration of contaminated infusions-
a rare event nowadays. The CLP technique better resembles common infec-
tions but has difficulties in bacteria standardisation. In our study only bacterial
findings from the spleen and liver were reported. These organs have been
shown to be the major sites that trap and kill bacteria from the blood circula-
tion [77-79], a finding also reported in primary sepsis in Paper I. Organ sam-
ps performed at different timepoints would have been interesting but could
not be performed in this investigation in order not to misspend the lives of
animals. In the first animals in this study cultures were taken from several
organs and only occasionally bacterial growth in low numbers was observed
in the lung.
Conclusions

I: Bacteria and gene fragments are rapidly eliminated from the circulation, regardless of the treatment. The beneficial pharmacodynamic effects of the combination of a β-lactam antibiotic and an aminoglycoside seen in vitro were also observed in the early phase of severe sepsis and septic shock, compared to single β-lactam antibiotic treatment. Furthermore, the individual blood bactericidal effect may confound the results in this type of experiment and hence should be considered when interpreting the findings.

II: Treatment with antibiotics elicited an increased inflammatory IL-6 response associated with leucocyte activation and pulmonary organ dysfunction, but no observable differences in plasma endotoxin concentrations. The reduction in cefuroxime-induced endotoxin release after the addition of an aminoglycoside in vitro could not be reproduced in vivo.

III: *E. coli* killed by a PBP-3-active antibiotic induces an increased inflammatory response when compared with live or heat-killed bacteria. This effect seems to be mediated by both endotoxic and non-endotoxic mechanisms. The increase in inflammation is associated with deteriorated organ and cellular function. The addition of an aminoglycoside reduces the cefuroxime-induced inflammatory response.

IV: Animals with secondary sepsis exhibit attenuated inflammatory response as expected, but contrary to the hypothesis, enhanced antibacterial capacities in the major immune-active organs compared with animals with primary sepsis. An intact and partly enhanced bacterial clearance in secondary sepsis were also observed in blood cultures and ex vivo investigations. These results indicate that the need for bactericidal antibiotic combinations is not greater in secondary than in primary sepsis.
Final summary and future research

Sepsis is the state in which the body’s response to infection causes life-threatening organ dysfunction. Septic shock is sepsis that results in tissue hypoperfusion, with vasopressor-requiring hypotension and elevated lactate levels [3]. Performing potentially harmful experiments in humans as regards these conditions, injecting live bacteria and taking organ samples are not possible without causing damage to the body. Even observational studies of humans with septic shock at the emergency department are difficult to conduct, with problems such as initially unknown microbe species and study enrolment during hastily performed sepsis management. The majority of sepsis studies using live bacteria have been performed in mice, but because of difficulties in extrapolating mice studies to humans (the immune system of mice has only 10% similarity to humans) [130], we focused on the pig whose immune system is 80% similar to humans. In addition, the pig demonstrates similar physiological responses as humans to sepsis [130, 132]. The pig’s physiognomy is suitable for intensive care treatment, including sedation, vasopressors and mechanically ventilation that additionally might mitigate the inflammatory response [133-135]. All this adds clinical relevance to the model.

The importance of early administration of antibiotics in septic shock is undisputed and should be administrated within 1 h after sepsis recognition [12], but the optimal antibiotic choice is debated [7-10, 97]. A β-lactam antibiotic with the addition of an aminoglycoside for the empirical treatment in critically ill sepsis patients is advocated in several guidelines [12, 109-111] but not in all [112-115]. Paper I investigated whether the addition of an aminoglycoside to a β-lactam antibiotic has an effect on the in vivo killing rate of E. coli during the early phase of treatment in a porcine intensive care model of severe sepsis/septic shock. A secondary aim was to study the effect of individual blood bactericidal capacity ex vivo at baseline on subsequent bacterial growth in vivo. Consistent with what has been found in vitro [103, 104], the results showed that the addition of an aminoglycoside resulted in both decreased bacterial growth in the liver and greater antibiotic-induced blood killing activity ex vivo compared with single β-lactam treatment. The results thereby constitute a possible mechanism to the findings in the meta-regression analyses by Kumar et al demonstrating an improved survival rate with two active antibiotics, such as the β-lactam/aminoglycoside combination, in the most critically
ill patients [8, 9]. Furthermore, the knowledge that individual blood bactericidal capacity may have a significant effect on antimicrobial outcome was also observed in this paper.

Endotoxin is a potent stimulator of the immune system that is found to be elevated in more than 70% of patients with sepsis/septic shock [57-59]. Gram-negative bacteria contain endotoxin that, if released, induce pro-inflammatory cytokines [56, 167] associated with morbidity and mortality in sepsis/septic shock [152, 153]. Clinical trials studying the concept of antibiotic-induced endotoxin liberation and inflammation are few and those that do exist are limited to milder sepsis, demonstrating no or only a trend towards increased inflammatory response [121, 157-159]. Several in vitro studies have demonstrated increased release of endotoxin when Gram-negative bacteria are exposed to different antibiotics, but decreased when aminoglycosides have been added to β-lactam antibiotics in vitro [52, 53, 116, 122-124, 160]. Paper II investigated the in vivo relevance of antibiotic-induced endotoxin liberation, and contrary to what has been shown in vitro, antibiotic treatment did not result in increased plasma endotoxin secretion in vivo. Nevertheless, treatment with antibiotics increased the inflammatory IL-6 response that was further associated with leucocyte activation and pulmonary organ dysfunction. Consequently, our study supports a phenomenon that others have observed in clinical practise and that has recently been reported from an intensive care unit, namely, a deterioration in almost half of the patients with sepsis within 4 h after antibiotic treatment [127]. Secondarily, we found that the in vivo addition of an aminoglycoside to a β-lactam antibiotic induced a trend towards less inflammation compared with the β-lactam treatment alone. This finding might offer an additional explanation to the increased bacterial killing observed in paper I to the improved survival of patients treated with the combination of a β-lactam antibiotic and an aminoglycoside [9].

The lack of changes in in vivo endotoxin levels after antibiotic treatment of Gram-negative sepsis in Paper II compared with no treatment, although simultaneously responding with increased inflammation and organ deterioration, led to the question of whether it is the process of in vivo antibiotic-induced bacterial killing or the existence of remaining antibiotic-killed bacteria that causes the increased inflammatory response after antibiotic treatment. β-lactam antibiotics with affinity to PBP-3 (such as piperacillin, cefotaxime and meropenem) are commonly used for the treatment of sepsis [12, 118, 119]. PBP-3-acting antibiotics result in considerable bacterial structural changes and have been particularly associated with an increase in the inflammatory response [120, 121], properties that aminoglycosides do not have. In paper III we aimed to investigate in vivo whether bacteria pre-killed in vitro by a PBP-3-active antibiotic have a greater effect on the early inflammatory response and organ dysfunction than corresponding amounts of live or heat-killed bacteria. We also studied whether the addition of an aminoglycoside could reduce the cefuroxime-induced response. We found that animals receiving bacteria
pre-killed by the PBP-3-active antibiotic cefuroxime demonstrated higher cytokine responses (TNF-α, IL-6, IL-10) over time than those receiving live or heat-killed bacteria and that this response was lowered by the addition of an aminoglycoside. Furthermore, the cefuroxime-killed group developed more leucocyte activation and inflammatory capillary leakage that were also reduced by the addition of tobramycin to cefuroxime in the pre-exposure.

Secondary sepsis, defined as a second infection after a primary activation of the systemic inflammatory response, is associated with attenuated inflammatory responses in experimental animal studies, as well as less organ dysfunction in a clinical retrospective ICU study [23-25]. However, previous studies investigating host bacterial killing capacity in secondary sepsis is conflicting [24, 31] and has not been performed in large animals or humans. We hypothesised that the attenuated inflammatory response might be associated with impaired bacterial clearance that should increase the need for maximally bactericidal antibiotic treatment of secondary sepsis. Paper IV investigated the host bacterial killing in vivo in secondary sepsis compared with primary sepsis, using an intensive care E. coli sepsis model with endotoxin tolerant pigs. As expected, animals with secondary sepsis demonstrated an attenuated inflammatory response (lower TNF-α and IL-6) than animals with primary sepsis. In contrast to the hypothesis, animals with secondary sepsis demonstrated fewer live bacteria in the spleen (statistically significant) and in the liver (statistically nonsignificant). An intact and partly enhanced bacterial clearance in animals with secondary sepsis was also observed in in vivo blood cultures and ex vivo investigations. Altogether, these results indicate that antibiotic treatment does not need to be more bactericidal in the treatment of secondary than of primary sepsis.

Our model was designed to be a clinically relevant experimental intensive care sepsis model focusing on the host defence, inflammatory responses and organ consequences of bacteria that have entered the bloodstream. We studied the effects after an intravenous E. coli challenge. We believe it would also be of interest to use this model to investigate other implanting routes to normally sterile compartments, such as installation of E. coli into the upper urinary tracts or peritoneal cavity, probably using a fibrin clot for the administration of bacteria [140]. The effect of other microbes such as Staphylococcus aureus or Streptococcus pyogenes would also be of pathophysiologic interest, subsequently investigating antibiotic treatment.

Organ dysfunction and eventual specific organ replacement therapies are of central concern during sepsis management [12]. Further studies in our model regarding the host’s different local organ inflammatory responses during sepsis might be of pathophysiologic relevance for future choice and timing of optimal organ-directed therapy. In addition, further knowledge is justified concerning in which part of the organs bacteria are trapped, killed or thrive
during sepsis, which might be open to investigation by repeated organ samplings in our primary and secondary sepsis models. Radioactive marking of bacteria is an interesting field that might help answer these questions [168].

The findings in Paper I-III on the probable beneficial effect of the addition of an aminoglycoside to a β-lactam antibiotic motivate randomised controlled clinical studies in patients with septic shock at the emergency department if ethical considerations and practical problems with regard to consent and randomization despite all obstacles, could be overcome. In such a study differences in mortality, development of organ dysfunction and toxicity between a single β-lactam antibiotic and the combination with an aminoglycoside could be analysed.

We found that the individual bactericidal capacity may have a significant effect on antimicrobial outcome, which could be further investigated in a patient trial. As a future therapeutic approach, the need for maximally bactericidal antibiotic treatment might be higher if the individual’s host bactericidal capacity is weak.

In the era of the emergence of multi-drug resistant microbial pathogens [169], it might be of worth to study other non-antibiotic treatments of sepsis/septic shock in this clinically relevant experimental intensive care sepsis model, both concerning our primary and secondary sepsis model. For example, prospective studies on hydrocortisone treatment or blood purification techniques would be beneficial, with perhaps looking at different effects on the inflammatory response and bacterial killing in secondary compared with primary septic shock.
Varje år drabbas cirka 7 av 1000 vuxna svenskar av sepsis, och dödligheten uppskattas till 20%. Denna avhandling visar sammanfattningsvis att kombinationsbehandling med två olika antibiotikasorter är bättre än en till de allra sjukaste sepsispatienterna, vi får rimliga förklaringar till det välkända fenomen som innebär att många sepsispatienter blir sjukare strax efter sin antibiotikabehandling samt att om en sepsis drabbar redan svårt sjuka patienter har dessa faktiskt förbättrad bakterieavdödning.

Vad är sepsis? När en kropp utsätts för en bakterieinfektion påbörjar immunförsvaret både en lokal bakterieavdödning och samtidigt inflammatoriska kedjereaktioner som engagerar fler försvarsmekanismer. Dessa försvårar är fullt nödvändiga för vår överlevnad, men i kraftigare infektioner kan immunförsvaret felregleras till överreaktion som ger värre sjuklighet och sviktande organfunktioner, vilket heter sepsis (blodförgiftning).

Att som experiment ge människor stora mängder bakterier för att studera sepsis och testa behandlingar är oetiskt, varför sepsisstudier anses berättigade på djur. All medicinsk forskning med djurmodeller regleras av lagar och skall godkännas av en etiknämnd. I de experiment som återges här föll valet på grisar, eftersom de på många sätt liknar människan i organens funktioner och immunförsvar. Under experimenten sövdes djuren och intensivvårdades under människoliknande förhållanden i respiratorer.


I en andra artikel utreds det varför sepsispatienter trots behandling med effektiv antibiotika ändå ofta fortsätter försämras i infektionen med tilltagande

I den **tredje artikeln** studerar vi i grissepsismodellen skillnader mellan djur som ges avdödade bakterier (d.v.s. bakterierna för-avdödats i provrörm) jämfört med djur som utsätts för levande bakterier. Hur farliga är egentligen döda bakterier att ha i kroppen, bakterier som t.ex. just avdödats av antibiotika? Det visade sig att redan avdödade bakterier starkare bidrog till immunförsvarets skadliga överreaktion än levande bakterier. Kombinationsbehandling med tilllagd aminoglykosid till β-laktamantibiotika, följdes även i detta arbete av en önskvärt mildare inflammatorisk försvarsreaktion.

Problemen och bördan av vårdrelaterade infektioner har uppmärksammats allt mer de senaste åren. När en ytterligare sepsis drabbar redan svårt sjuka patienter som sjukhusvårdas för annan allvarlig sjukdom, till exempel en kirurgisk operation eller en ”vanlig” sepsis, blir detta en ”andra smäll” för immunförsvaret. Detta kallas för **sekundär sepsis**. I vår **fjärde artikel** studerar vi sekundär sepsis i grissepsismodellen. Det är välkänt att en sekundär sepsisinfektion åtföljs av en försvagad inflammatorisk försvarsreaktion och mindre kroppslig sjuklighet hos patienter, än det stormigare sjukdomsförlopp som en ”vanlig” sepsis orsakar. Vi misstänkte att individer som utsätts för sekundär sepsis också kan ha svagare förmåga att avdöda bakterier. En sådan insikt skulle ändra valet av antibiotikabehandling av sekundär-sepsispatienter till att då vilja ge dem ännu starkare antibiotika. Vi fann dock, tvärt emot förväntat utfall, att djur med sekundär sepsis hade förbättrad bakterieavdödning jämfört med ”vanlig” sepsis. Sammanfattningsvis så ökar detta vår kunskap kring sekundär sepsis, men det ändrar inte synen på hur de bör antibiotikabehandlas.
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**A leucocyte at the liver is about to phagocytize a very large virus while a bacteria is trying to sneak off.** From the TV-series “**En cellsam historia**”, broadcasted 1987, fascinating pathophysiology for the author as a 10-year old. Authorized reuse: Creator Albert Barillés family.
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