Ludwig Boltzmann Institute for Experimental and Clinical Traumatology
in the Trauma Research Center of the AUVA, Vienna, Austria

Project supported by the Vienna Science and Technology Fund (WWTF) Grant
No. LS07-065.

Experimentally Approaching the Intensive Care Unit:
Monitoring Outcome-Based Responses in
the Two-Hit Mouse Model of Posttraumatic Sepsis

INAUGURAL DOCTORAL THESIS

for the academic degree of

DOCTOR MEDICINAE VETERINARIAE
at the University of Veterinary Medicine Vienna (VUW)

Submitted by
Mag. med. vet. Susanne Drechsler

Wien, September 2012
Acknowledgements

Dr. Soheyl Bahrami, Univ. Prof. DI.
Ludwig Boltzmann Institute for Experimental and Clinical Traumatology
at the AUVA Research Center, Vienna
Co-Director and Head of Intensive Care Medicine Research Group

Dr. Marcin F. Osuchowski, Ph.D., DVM
Ludwig Boltzmann Institute for Experimental and Clinical Traumatology
at the AUVA Research Center, Vienna
Head of the Sepsis Research Group

Dr. Heinz Redl, Univ.Prof.
Ludwig Boltzmann Institute for Experimental and Clinical Traumatology
at the AUVA Research Center, Vienna
Director

This study was published (in an abbreviated version) as an original paper in the Journal of Biomedicine and Biotechnology (IF 2.44).
1. Betreuer: Univ. Prof. DI. Dr. Soheyl Bahrami

2. Betreuer: Univ. Prof. Dr. med. vet. Rupert Palme

Special thanks to Marcin, Katrin, Pierre and Mohammad for teaching and supporting me during the past three years.
# Contents

1. Introduction ............................................................................................................................ 1
   1.1 The Epidemiology of Secondary Septic Complications in Trauma Patients .......... 1
   1.2 The Pathophysiology of Trauma/Hemorrhage and Sepsis ........................................ 2
      1.2.1 Immune Responses following Trauma and Hemorrhage (TH) ...................... 2
      1.2.2 Immune Responses in Sepsis ........................................................................ 4
   1.3 Sepsis Definitions ............................................................................................................. 6
      1.3.1 Systemic Inflammatory Response Syndrome (SIRS) ................................... 6
      1.3.2 Sepsis Syndrome ............................................................................................. 7
      1.3.3 Severe Sepsis .................................................................................................... 7
      1.3.4 Septic Shock ..................................................................................................... 7
   1.4 Rodent models of Trauma/Hemorrhage and Sepsis ............................................... 7
      1.4.1 Rodent Fracture Models ................................................................................ 8
      1.4.2 Hemorrhage Models ....................................................................................... 8
      1.4.3 Rodent Models of Trauma-Hemorrhage ....................................................... 10
   1.5 Sepsis Models ................................................................................................................. 11
      1.5.1. Exogenous Administration of a Toxin ............................................................ 11
      1.5.2 Exogenous Administration of a Viable Pathogen .......................................... 12
      1.5.3 Host-barrier Disruption .................................................................................. 13
   1.6 Rodent 2-hit Sepsis Models ......................................................................................... 15
      1.6.1 Hemorrhage and Endotoxin .......................................................................... 15
      1.6.2 Trauma and CLP ............................................................................................. 16
      1.6.3 Hemorrhage and CLP ..................................................................................... 16
      1.6.4 Trauma/Hemorrhage and Sepsis .................................................................. 17
   1.7 Blood Collection Methods in Mice ............................................................................ 18
      1.7.1 General Concerns ............................................................................................ 18
      1.7.2 Blood Collection without Anaesthesia ............................................................ 19
      1.7.3 Blood Collection under Anesthesia ................................................................. 21
   1.8. Aims .............................................................................................................................. 23

2. Animals and Methods ........................................................................................................... 24
   2.1 Animals ........................................................................................................................ 24
   2.2 Two-Hit Model............................................................................................................ 24
   2.3 Blood Sampling .......................................................................................................... 26
   2.4 Complete Blood Count ............................................................................................. 26
Figures

**Figure 1.** A Course of the Systemic Inflammatory and Immune Response in the 2-Hit Model (TH-CLP) ................................................................................................................................... 4

**Figure 2:** Retro-orbital Bleeding .............................................................................................................. 10

**Figure 3.** Schematic of the Cecal Ligation and Puncture Surgery. ......................................................... 14

**Figure 4.** Facial Vein Sampling .............................................................................................................. 19

**Figure 5.** Schematic of the two-hit model and repetitive blood sampling ........................................ 25

**Figure 6.** Unilateral femur fracture X-ray. ............................................................................................ 26

**Figure 7 A-C.** A 7-day survival curve in 3-month-old female mice subjected to the 2-Hit model (TH-CLP) ....................................................................................................................................... 29

**Figure 8 A-C.** Retrospective comparison of red blood cell, platelet count, and hemoglobin concentration in dying (DIE) vs. surviving (SUR) females in the post-TH phase (pre-CLP). 30

**Figure 9 A-B.** Retrospective comparison of neutrophil and lymphocyte count in dying (DIE) vs. surviving (SUR) females in the post-TH phase (pre-CLP) ............................................................ 31

**Figure 10 A-E.** Retrospective comparison of circulating glucose, AST, ALT, LDH, and urea in dying (DIE) vs. surviving (SUR) females in the post-TH phase (pre-CLP) .................................................. 33

**Figure 11 A-C.** Retrospective comparison of red blood cells, platelet count and hemoglobin concentration in dying (DIE) vs. surviving (SUR) females in the post-CLP phase ........................................... 34

**Figure 12 A-B.** Retrospective comparison of neutrophil and lymphocyte count in dying (DIE) vs. surviving (SUR) females in the post-CLP phase ................................................................. 36

**Figure 13. A-E.** Retrospective comparison of circulating glucose, AST, ALT, LDH and urea in dying (DIE) vs. surviving (SUR) females in the post-CLP phase ............................................. 37
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>Alanine Transaminase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>aPC</td>
<td>Activated Protein C</td>
</tr>
<tr>
<td>AST</td>
<td>Asparate Transaminase</td>
</tr>
<tr>
<td>CARS</td>
<td>Compensatory Anti-inflammatory Response Syndrome</td>
</tr>
<tr>
<td>CASP</td>
<td>Colon Ascendens Stent Peritonitis</td>
</tr>
<tr>
<td>CD14</td>
<td>Cluster of Differentiation 14</td>
</tr>
<tr>
<td>CLP</td>
<td>Cecal Ligation and Puncture</td>
</tr>
<tr>
<td>DIE</td>
<td>Died</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>G</td>
<td>Gauge</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High Mobility Group Box 1</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive Care Unit</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Interleukin 1 Receptor Antagonist</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LYM</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>MARS</td>
<td>Mixed Inflammatory Response Syndrome</td>
</tr>
<tr>
<td>MOF</td>
<td>Multi Organ Failure</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium-Chloride</td>
</tr>
<tr>
<td>NEU</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>NT-proCNP</td>
<td>N-Terminal fragment of C-Type Natriuretic Peptide</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PLT</td>
<td>Platelets</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear Leukocytes</td>
</tr>
</tbody>
</table>
PBS       Phosphate Buffered Saline
RBC       Red Blood Cells
sCD14     Soluble Cluster of Differentiation 14
SUR       Survived
SIRS      Systemic Inflammatory Response Syndrome
TF        Tissue Factor
TH        Trauma Hemorrhage
TBV       Total Blood Volume
TNF       Tumor Necrosis Factor
TLR       Toll-like Receptor
US        United States of America
WHO       World Health Organisation
1. Introduction

The multi-origin epidemiology of post-traumatic sepsis and the dynamic alterations of its immune and organ function responses emphasize that successful treatment of sepsis should rely on highly individualized therapeutic interventions (KOX et al., 2000). This requires pre-clinical modelling that sufficiently reproduces both multi-tier pathophysiology of a given type of septic complication(s) as well as the standard ICU monitoring protocol. Therefore, we combined the murine 2-hit model of trauma/hemorrhage and subsequent sepsis with daily monitoring via facial vein blood sampling.

1.1 The Epidemiology of Secondary Septic Complications in Trauma Patients

Only limited data is available concerning the epidemiology of sepsis as secondary complication in trauma patients. According to the World Health Organisation (WHO), traumatic injuries related to road traffic accidents are the worldwide leading cause of death across all age groups up to 60 years (PEDEN, 2005).

A South-African study conducted by MUCKART et al. (1997) reported a high incidence of secondary sepsis (14.4%) in a population of 450 trauma patients. The mortality rate was 10.1% in patients with sepsis, 13.1% in patients with severe sepsis and 63.7% in patients with septic shock.

A study by ERTEL et al. (1998) et al. investigating 1278 injured patients submitted to the Division of Trauma Surgery at the University Hospital Zurich detected the systemic inflammatory response syndrome (SIRS) in 57.6% of all patients. Septic complications developed in 13.8% of all patients and from those 10% developed sepsis, 2.7% severe sepsis and 1.2% septic shock. Interestingly, the incidence of septic complications correlated with the severity of trauma.

Another European study investigating 339 patients with mechanical trauma, who were admitted to the Division of Trauma Surgery at University Hospital Zurich, diagnosed severe
SIRS in 7.4% and an incidence of sepsis related to pneumonia in 13.3% of all cases (WANNER et al., 2000). Furthermore, 56% of those patients developed subsequent failure of one or more organs.

A large study conducted in the United States of America (US) by OSBORN et al. (2004) that included 30303 trauma patients, detected sepsis only in 2% of all patients but its occurrence was associated with a high mortality of 23%.

In their pan-European, multicenter, observational sepsis study, VINCENT et al. (2006) detected sepsis in 25% of all patients who were subsequently admitted to the intensive care unit after arrival at the emergency room (ER) and/or ambulance. Furthermore, the ICU mortality was significantly greater in patients with sepsis compared to their non-septic counterparts.

1.2 The Pathophysiology of Trauma/Hemorrhage and Sepsis

During the last decades, a number of experimental and clinical studies have underlined the influence of traumatic injury on the specific and non-specific immune responses. A traumatic-hemorrhagic insult can trigger a defence reaction regulated by immune cells, which may potentially progress into a state of either predominant hyperinflammation or immunosuppression, or both (Fig.1). This hyperactivation of the inflammatory system increases the risk for secondary septic complications (TSCHOEKE et al., 2007) and attempts are made to find biomarkers that could aid in identification of injured individuals who are prone to become septic. N-terminal fragment of the C-type natriuretic peptide precursor, NT-proCNP, is the most recent example of such a biomarker: it has of late shown a high accuracy for predicting the development of sepsis in multiple-traumatized patients (BAHRAMI et al., 2010).

1.2.1 Immune Responses following Trauma and Hemorrhage (TH)

The initial immune response after trauma and blood loss is directed towards hemostasis and protection of vital organ function. Therefore, the hemodynamic response to severe blood loss
induces a phase of tachycardia and an increase of vascular resistance with a maintained arterial pressure. This is then followed by a phase of decompensation with hypotension and a decrease in cardiac perfusion, which, if not counteracted, may result in hypovolemic shock. When suffering from a state of shock, the physiologic vasomotoric compensatory reflexes are diminished due to the hypoperfusion of the medullary vasomotor center (FOEX, 1999).

Simultaneously, at the site of trauma, local mediators like histamine, kinins and arachidonic acid metabolites act in a paracrine way at the site of injury to support hemostasis and to initiate tissue repair and wound healing. These bioactive substances cause increased capillary permeability, tissue edema and infiltration of immune cells. Furthermore, the early innate immune response to trauma is supported by complement components, which not only recruit additional immune cells to the site of tissue trauma but also facilitate phagocytosis via opsonisation (LENZ et al., 2007).

If the tissue damage is too severe, additional endogenous triggers for immune activation, referred to as alarmins, such as high mobility group box 1 (HMGB1), heat shock proteins, and defensins are released into the systemic circulation. They can either be produced by immune cells or released after non-programmed cell death and act as chemoattractants and activators for antigen presenting cells (APCs). Therefore, alarmins induce systemic release of both pro- and anti-inflammatory cytokines (OPPENHEIM et al., 2005; BIANCHI, 2007). Consequently, these inflammatory mediators strongly modulate immune system functions and induce further chemotaxis of immune cells to the site of damage. This complex systemic immune response can lead to a dysregulation of the immune system and therefore result in an overwhelming systemic inflammation followed by subsequent immunosuppression (Fig.1), which might predispose patients for sepsis and development of subsequent multiple organ failure (MOF) (LENZ et al., 2007).
1.2.2 Immune Responses in Sepsis

Sepsis is described as the body’s immune response against a known or suspected bacterial and/or fungal infection. Systemic spread of pathogens can originate from numerous organs/systems of the body – the lungs representing the most frequent site of infection (68%), followed by the abdomen (22%), blood (20%) and urinary tract (7%; VINCENT et al., 2006).

In gram-negative bacteria, the main structural component recognized by immune cells is lipopolysaccharide (LPS, endotoxin), which is located in the outer cell wall of the gram-negative microorganism (COHEN, 2002). Such recognition occurs via so-called pathogen-associated molecular patterns (PAMP). The initial interactions between immune cells and LPS are depending on LPS-binding protein, a soluble protein that interacts with LPS and enables binding to the opsonic receptor CD14, which is located on the surface of monocytes.

Figure 1. The Course of the Systemic Inflammatory and Immune Response in the 2-Hit Model. Trauma and sepsis induce a simultaneous release of pro-inflammatory and anti-inflammatory mediators termed the mixed anti-inflammatory response syndrome (MARS), which may lead to a subsequent depression of immune system functions. Within this phase of immuno-suppression patients may show an increased susceptibility for secondary septic complications.
and macrophages (DZIARSKI et al., 1998). In contrast, gram-positive bacteria do not contain LPS in the cell walls, but peptidoglycans and lipoteichoic acid, which are responsible for their biological activity via interaction with the CD14 receptor. It has been shown that stimulation of the human whole blood with gram-positive bacterial cell wall components induces release of the inflammatory mediators such as TNFα, IL-6 and IL-10 (WANG et al., 2000). In its soluble form, sCD14 interacts with various cells that are constitutively CD14 negative (i.e. dendritic cells, fibroblasts, vascular endothelium) to enable recognition of LPS by immune cells (PUGIN et al., 1994).

After initial contact, further activation of signalling pathways is mediated by Toll-like receptors (TLRs) (YANG et al., 1998). Once activated, mononuclear cells play a key role in the initially localized inflammatory response by rapidly releasing a palette of pro- and anti-inflammatory cytokines, chemokines, lipid mediators (i.e. platelet-activating factor, prostaglandins, leukotrienes) and oxygen radicals. When such a localized response is further propagated and spreads across the entire body (primarily via systemic circulation), it will produce so called systemic inflammatory response syndrome (SIRS) (PUGIN et al., 1994).

While the initial presumption stated that SIRS is characterized by production/release of pro-inflammatory cytokines, subsequently followed by a counter-reaction during which anti-inflammatory mediators are secreted (defined as a compensatory anti-inflammatory response syndrome or CARS), recent studies have disproved it. Experimental (OSUCHOWSKI et al., 2006; OSUCHOWSKI et al., 2010) studies supported by clinical (GROENEVELD et al., 2003; HOTCHKISS et al., 2009; NOVOTNY et al., 2012; TAMAYO et al., 2011) evidence demonstrated that sepsis triggers an immediate and simultaneous release of pro-and anti-inflammatory mediators into the blood, featuring a mixed inflammatory response syndrome or MARS-like response (OSUCHOWSKI et al., 2006). A patient that survives the initial hyperinflammatory phase of sepsis, enters a stage of immunosuppression (Fig.1), typically characterized by a failure to clear the primary infection and increased susceptibility to new, mostly nosocomial, infections (MONNERET et al., 2008). The weakened immunocompetence can be nevertheless accompanied by presence of circulating inflammatory mediators (both pro-and inflammatory), albeit at much lower concentrations compared to...
acute sepsis (OSUCHOWSKI et al. – in press; XIAO et al., 2006; OSUCHOWSKI et al., 2007)

Pro-inflammatory cytokines (e.g. IL-1, IL-6, TNF-α) released from activated leukocytes strongly influence coagulation by stimulating endothelial cells and mononuclear immune cells to continue the release of cytokines and tissue factor (TF) – main triggers of the inflammation-induced thrombin generation. The above process results in a procoagulant state characterized by an inadequate tissue perfusion and vascular instability, which in turn contribute to the development of microvascular fibrin depositions and subsequent organ failure. Simultaneously, anticoagulant pathways are inhibited: circulating antithrombin, a main inhibitor of thrombin formation and coagulation factor Xa, is reduced due to its overwhelming consumption. Under physiologic conditions, activated protein C (aPC) decelerates the coagulation pathway by inactivating several coagulation factors. During sepsis, expression of thrombomodulin (needed for activation of aPC) is severely down-regulated (LEVI et al., 2003). Additionally, the physiologic mechanisms responsible for fibrinolysis are impaired during sepsis, because high levels of circulating plasminogen-activator type-1 (PAI-1) inhibit the production of plasmin from its precursor plasminogen. The latter action provokes further deposition of fibrin clots in small vessels thereby decreasing tissue micro-perfusion and oxygenation (PUGIN et al., 1994; COHEN, 2002).

1.3 Sepsis Definitions

1.3.1 Systemic Inflammatory Response Syndrome (SIRS)

The International Sepsis Definitions Conference in 2001 defined SIRS as a systemic inflammatory response syndrome triggered by a variety of infectious and non-infectious (e.g. burns, trauma, pancreatitis, etc.) conditions (LEVY et al., 2003).
1.3.2 Sepsis Syndrome

Sepsis is defined as the presence of SIRS and a known or strongly suspected infection. Furthermore, the infection itself was defined as an invasion of normally sterile tissue or fluid or body cavity by pathogenic or potentially pathogenic microorganisms (LEVY et al., 2003).

1.3.3 Severe Sepsis

Severe sepsis is considered as sepsis complicated by organ dysfunction, hypoperfusion or hypotension. Hypoperfusion and perfusion abnormalities may include lactic acidosis, oliguria or an acute alteration in mental status (BONE et al., 1992).

1.3.4 Septic Shock

Septic shock is defined as acute circulatory failure with persistent arterial hypotension unexplained by other causes, which can not be counteracted by adequate volume resuscitation (LEVY et al., 2003).

1.4 Rodent models of Trauma/Hemorrhage and Sepsis

Animal models are the launch platform for virtually all clinical investigations since they feature a maximally controlled environment and optimal experimental reproducibility. The choice of an appropriate animal model should be based on the objectives of a given study, as well as on the clinical scenario aimed to be mimicked by the experiment. Different models have been developed to study the pathophysiology of trauma/hemorrhage and/or sepsis but primarily as “single hits”. For example PELINKA et al. (2004) studied neuron-specific enolase by subjecting rats to either hemorrhagic shock or bilateral femur fracture. Clinical relevant combinations of these insults (i.e. trauma/hemorrhage followed by sepsis) remain strongly underrepresented in the existing literature.
1.4.1 Rodent Fracture Models

In 1996, NAPOLITANO et al. investigated alterations of gut barrier integrity and immune system functions after closed and open femur fracture. To realise a femur fracture, two hemostats were applied adjacent to each other with opposing force under anaesthesia. Closed femur fracture was associated with significant immunosuppression and altered gastrointestinal permeability, while open fracture did not influence immune response or gastrointestinal permeability.

KOBBE et al. (2008a) performed bilateral femur fracture in mice by using a guillotine-like device. They demonstrated the role of fracture-associated soft tissue injury in combination with bilateral femur fracture on the induction of inflammatory response and remote organ dysfunction. The combination of both soft tissue injury and femur fracture induced further increased release of inflammatory mediators and induced a more severe organ damage (compared to sham) than each injury alone.

In their second study, KOBBE et al. (2008b) aimed to investigate the pattern of cytokine release and its association with the evolution of organ dysfunction following bilateral femur fracture. An increase in hepatic and pulmonary infiltration with polymorphonuclear cells (PMN) in the absence of elevated serum cytokine levels was observed.

A more recent model to simulate femur fracture was established by MENZEL et al. (2011), who successfully reproduced immune and organ function responses to bone fracture by exposing a previously injured muscle to intra muscular injection of crushed-bone components. This approach although successful in reproducing early post-traumatic inflammatory sequelae, fails in a longer-term setup due to resorption/inactivation of the stimulus.

1.4.2 Hemorrhage Models

In general, a massive blood loss occurs not as a single event but is typically combined with traumatic injury of a different magnitude. Since both the speed of hemorrhage and amount of shed blood influences the evolution of hypovolemic shock, different approaches have been made to mimic the clinical scenario of trauma-hemorrhage (TH).
1.4.2.1 Fixed-Pressure Hemorrhage Model

In the fixed-pressure model, anesthetized animals are catheterized via the jugular vein, the carotid artery and/or femoral artery and heparinized to prevent coagulation. Next, a variable amount of blood is removed from the animal’s circulation until the desired mean arterial blood pressure (MAP) is reached. This predetermined intensity of hypovolemic shock (defined over MAP) is then maintained for a fix time span. Afterwards, the catheter can serve to return shed blood or fluid replacement during resuscitation. Given that the speed of blood loss, as well as severity and duration of hypovolemic shock can be influenced by the investigator, this model is the least comparable to clinical situation. (LOMAS-NIERA et al., 2005).

1.4.2.2 Fixed-Volume Hemorrhage Model

In this model (VAN GRIENSVEN et al., 2002), the amount of shed blood is predefined and can count for up to 60% of total blood volume (TBV). Fixed-volume hemorrhage can be performed either with or without preceding catheterization of the femoral artery to monitor blood pressure. In either case, mice must remain under anaesthesia for this procedure. Without using a catheter, bleeding is induced via retro-orbital puncture, tail clip or heart puncture (Fig.2). An existing catheter can be used to return shed blood or fluid replacement during resuscitation. The rate of blood removal, which affects compensation and decompensation during hypovolemia, cannot be controlled by the investigator. Therefore, the fixed-pressure model more suitably mimics an acute hemorrhage scenario (LOMAS-NIERA et al., 2005).
1.4.2.3 Models of Uncontrolled Hemorrhage

Models of uncontrolled hemorrhage are defined as blood loss from/of varying source and volume until bleeding ceases spontaneously. Hemorrhage can be induced through massive splenic injury, laceration of the aorta or surgical instrumentation. These models are primarily suited to investigate fluid resuscitation strategies (HIRSHBERG et al., 2007). Although they reflect the clinical scenario better than fixed-pressure and fixed-volume hemorrhage models, there are disadvantages concerning the standardization and reproducibility of these techniques (LOMAS-NIERA et al., 2005; NAPOLITANO et al., 1995).

1.4.3 Rodent Models of Trauma-Hemorrhage

In their experimental setup in rats, AYALA et al. (1991) defined trauma as a midline laparotomy followed by fixed-pressure haemorrhage. They demonstrated that in contrast to TNFα, IL-6 was significantly increased after soft tissue trauma and before the blood loss.

WICHMANN et al. (1996b) demonstrated that combination of closed bone fracture of the right lower leg with hemorrhagic shock compromised the immune system even more severely.
than hemorrhage alone. Fracture was realized by 3-point bending applied with a custom-made fracture apparatus before fixed pressure hemorrhage.

Two years later, the same group showed in mice that soft-tissue trauma with unilateral bone fracture of the right lower leg prior to hemorrhagic shock produced an even more compromised immune response than either soft-tissue trauma or closed-bone fracture with fixed pressure hemorrhage alone (WICHMANN et al., 1998).

SZALAY et al. (2003) established a combined model of midline laparotomy (30min) and fixed pressure hemorrhagic shock of varying intensity (moderate to severe) defined by resuscitation strategy. The authors investigated the plasma kinetics of D-lactate (produced by bacteria in the intestinal tract) and concluded that its presence in the circulation is indicative of bacterial (and/or microbial products) leakage from the gut mucosa.

Furthermore, MOMMSEN et al. (2011) investigated the effect of unilateral femur fracture (realized by a guillotine device), moderate soft-tissue injury and volume based hemorrhagic shock on the immune function of alveolar macrophages and remote lung injury in wild-type compared to IL-6 knockout mice. They detected that even an isolated femur fracture had an impact on the response of alveolar macrophages.

PROBST et al. (2012) further expanded the above approach by adding a closed traumatic brain injury to femoral fracture and hemorrhagic shock. Their study demonstrated that combination of those three insults resulted in an increased release of inflammatory cytokines compared to either femur fracture and shock or weight drop brain injury itself.

1.5 Sepsis Models

1.5.1. Exogenous Administration of a Toxin

Over the past decades the most widely used approach to induce sepsis has typically employed a bolus administration of LPS (gram-negative bacteria) or lipoteichoic acid and peptidoglycans (gram-positive bacteria). Endotoxin challenge causes a massive release of pro-inflammatory mediators followed by a peak of anti-inflammatory cytokines. Initially, this
release pattern has been associated with the findings in human patients with sepsis-induced SIRS, and it was supported by the notion that it is the host response to bacteria, and not the pathogen itself, that leads to development of sepsis and organ dysfunction (DEITCH, 2005).

One of the most important mediators increased after LPS infusion was TNFα, followed by a delayed peak of IL-1β, both known to be detrimental to their host (REMICK et al., 1989; CHENSUE et al., 1991). After several clinical trials that implemented monoclonal antibodies against TNFα, and IL-1 receptor antagonist (IL-1ra) had failed to demonstrate any improvement of survival in septic patients, the endotoxin models were questioned and underwent diligent re-evaluation (FISHER, JR. et al., 1994; OPAL et al., 1997; ABRAHAM et al., 1998; ABRAHAM et al., 2001).

It has been established that one of the main reasons responsible for the lack of therapeutic success was due to the fact that the cytokine release induced by endotoxin does not reproduce the true profile of the inflammatory response generated in septic patients. In humans, the immune response is triggered by live bacteria and is much more protracted, delayed and remains at much lower levels (REMICK et al., 2005).

Furthermore, bolus injection of LPS also fails to reproduce the hemodynamic changes observed in human sepsis. While the administration of endotoxin results in a hypodynamic state with severe depression of cardiac output and blood pressure, human sepsis patients usually display a high cardiac output (FINK et al., 1990; DYSON et al., 2009).

1.5.2 Exogenous Administration of a Viable Pathogen

Infection with viable bacteria has been a useful tool to study the pathophysiology of sepsis. Both bacterial load and route of infection are important factors which influence the course of immune responses. To date, several application approaches have been introduced in sepsis modelling.

Intravenous infusion and intraperitoneal injection are the most common application routes to maximally mimic an exogenous bacterial sepsis. Bacteria have also been administered within fecal pellets covered by a gelatine capsule or as part of a fibrin clot, which lead to a prolonged
development of an intra-abdominal abscess. Unfortunately, the use of fecal pellets for induction of peritonitis has serious drawbacks as it is unfeasible in experimental conditions to control the number and/or species of the administered microorganisms (DEITCH, 2005).

Given that the lungs are the most frequent source of secondary septic complications in humans, preclinical models based on nasal/tracheal instillation of viable bacteria were recently developed (MUENZER et al., 2006; BURAS et al., 2005).

Despite a logical emulation of the administration route, a common disadvantage of this model is that, even high doses of exogenous bacteria do not typically colonize and replicate within the host due to lysis induced by the complement system (CROSS et al., 1993).

Additional aspect is that the intensity of hemodynamic changes after administration of viable bacteria is highly dose-dependent: an increase of the bacterial load leads to cardiovascular changes comparable to those seen in LPS, while the application of low doses is comparable to human sepsis patients.

1.5.3 Host-barrier Disruption

Host barrier disruption models affect the natural barriers in the body that normally protect sterile compartments, leading to a systemic spread of pathogens (BURAS et al., 2005). The two most commonly used models are the cecal ligation and puncture (CLP) and the colon ascendens stent peritonitis (CASP).

1.5.3.1 Cecal Ligation and Puncture (CLP)

The CLP model represents the human diseases of ruptured appendicitis and perforated diverticulitis. CLP surgery is performed by midline laparotomy, exterioration of the cecum, followed by ligation of the cecum distal to the ileocecal valve and puncture of the ligated part with a needle (Fig.3). In the next step, cecum is reposed and abdominal cavity and skin are closed using single button sutures. This surgery creates a continuous leakage of fecal content into the peritoneal cavity and therefore activates the inflammatory response due to spreading of mixed bacteria and the presence of necrotic tissue (i.e. ligated cecum). Furthermore, CLP
induced inflammatory response shows a high degree of similarity to the protracted course of immune response, hemodynamic and metabolic changes seen human sepsis (WICHTERMAN et al., 1980; REMICK et al., 2000).

A considerable advantage of the CLP model is that its severity can be easily adjusted by either altering the size of the ligated part of the cecum, changing needle size and/or the amount of punctures. Nevertheless, its precise standardization is difficult and is typically performed independently in each laboratory independently. The same experimental setup may lead to different results due to the lack of influence on the amount of leaking fecal material and other factors including age, sex and mouse strains (BURAS et al., 2005; DEJAGER et al., 2011).

Figure 3. Schematic of the Cecal Ligation and Puncture Surgery. The cecum is ligated below the ileocecal valve and punctured (typically twice) with a needle to create a polymicrobial abdominal sepsis focus.

1.5.3.2 Colon Ascendens Stent Peritonitis (CASP)

The CASP model also aims at creating polymicrobial abdominal sepsis. Following laparotomy, a stent of a defined diameter is implanted into the colon ascendens of mice. This induces a constant leakage of fecal content into the peritoneal cavity and therefore induces a polymicrobial infection with intestinal bacteria (BURAS et al., 2005; ZANTL et al., 1998).
The systemic cytokine response and bacterial counts are typically higher in CASP compared to CLP (MAIER et al., 2004) and the peak of lethality occurs 1-2 days after stent implantation. Similar to CLP, severity can be influenced by using stents with different diameters. Furthermore, removing the stent after a predefined time period, which has been defined as a maximum time span of 3 hours, improves survival of mice (ZANTL et al., 1998).

Although it is known that CASP may induce multiple organ failure (MOF) including the lung, liver and kidneys, the hemodynamic changes induced by CASP are yet not well characterized (FETEROWSKI et al., 2004). A possible disadvantage of CASP lays in the challenging surgical procedure of exactly placing the stent without causing abscess formation in the abdomen. Furthermore, in contrast to CLP-induced sepsis, CASP does not produce necrotic tissue, which is believed to be responsible for the more protracted immune response in CLP.

1.6 Two-hit Sepsis Models in Rodents

1.6.1 Hemorrhage and Endotoxin

ZERVOS et al. (1997) established a 2-hit model combining sublethal hemorrhage (by cardiac puncture) with LPS challenge (by intraperitoneal injection) in rats. They found out that preceding hemorrhage attenuated the IL-1 response to LPS challenge and significantly improved survival.

Another model in the rat combining fixed-pressure hemorrhagic shock with intravenous injection of LPS was established by ZHOU et al. (2002) to investigate the influence of apocynin on MAP, hemorrhagic shock, survival rate and lung injury. In their study, apocynin ameliorated the severity of the two injuries.

Upon realization that immune responses induced by endotoxin do not appropriately reflect immuno-inflammatory alterations occurring in septic patients, the attention has been re-focused on more complex models such as CLP and CASP. The current consensus is that CLP, the most frequently-used model of sepsis, constitutes the most accurate recapitulation of
human polymicrobial abdominal peritonitis gone systemic (DEJAGER et al., 2011; REMICK et al., 2000).

1.6.2 Trauma and CLP

In 1995, NAPOLITANO et al. investigated whether femur fracture (Fx) alone or in combination with CLP (induced four days later) influences the release of IL-10 and other immune functions. Furthermore, they tested if pre-treatment with ethanol could induce alterations in splenocyte cytokine profile. Unilateral femur fracture was realised by using a mosquito clamp and animals were sacrificed 14 days after CLP. Ethanol did not induce significant alterations and the authors detected a decrease in cellular immune response in both (CLP and Fx-CLP) groups independent of ethanol.

1.6.3 Hemorrhage and CLP

An early attempt to establish a 2-hit model combining fixed-pressure hemorrhage with subsequent sepsis was made by STEPHAN et al. (1987), who demonstrated a significant depression of cellular immunity following hemorrhage and increased mortality in subsequent sepsis.

In 1992, a study demonstrated that prostaglandins restored immunosuppression after hemorrhage and therefore increased the overall survival rates after sepsis in a model of fixed-pressure hemorrhage followed by CLP 72h later. Afterwards, a number of studies used the same approach to investigate the influence of different parameters on the hemorrhage induced immuno-suppression (ERTEL et al., 1992; KAHLKE et al., 2002; DIODATO et al., 2001; WICHMANN et al., 1996a).

ROQUILLY et al. (2010) combined fixed-volume hemorrhage via cardiac puncture with transtracheal insertion of bacterial inocula to create pneumonia in order to investigate the treatment effect of the TLR9 agonist CpG-ODN and TLR4 agonist monophosphoryl lipid A. Hemorrhage decreased the survival rate after subsequent pneumonia and increased the severity of sepsis induced lung lesions and alterations of dendritic cell subsets.
1.6.4 Trauma/Hemorrhage and Sepsis

In 1993, WANG et al. combined midline laparotomy (trauma) and fixed-pressure hemorrhage in rats as first hit with CLP-induced sepsis to investigate the influence of pentoxifylline on cardiac output and susceptibility to secondary sepsis. Intravenous infusion of pentoxifylline improved both survival and tissue perfusion.

To investigate how post-traumatic sepsis induces influences organ function, VAN GRIENSVEN et al. (2002) established a 2-hit model containing unilateral femur fracture, realized (by using a blunt guillotine) accompanied by volume-based haemorrhage (60% of total blood volume) followed by CLP 48h later. At 96h after induction of sepsis, histologic examination of organ samples revealed morphologic changes in the lungs and liver, with increased numbers of polymorphonuclear neutrophils, while the kidneys did not display any pathologic changes.

1.6.5 CLP and Pneumonia

Using another 2-hit approach, a model encompassing CLP-induced sepsis, followed by intratracheal administration of Pseudomonas aeruginosa (P.aeruginosa) was developed by STEINHAUSER et al. (1999). The authors showed that CLP had a strong effect on the innate immune system of the lung and thereby reducing the ability of alveolar macrophages to ingest and eliminate P. aeruginosa ex vivo.

A similar setup was used by MURPHEY et al. (2004) who combined CLP with intravenous injection of P. aeruginosa through the dorsal penis vein. Immune response of mice previously challenged with CLP resulted in the subsequent spread and proliferation of endogenous bacteria as well as P.aeruginosa, while other groups without CLP were able to destroy the infectious agent.

MUENZER et al. (2006) subjected mice to CLP as the first hit and used either intranasal application of Staphylococcus pneumoniae or P.aeruginosa as the second insult.
1.7 Blood Collection Methods in Mice

The relatively small total blood volume in the mouse (defined as 6% of total body weight (6-8ml)) has been a limiting factor in emulating the clinical ICU scenario (repeated blood sampling) in this species. To gain an appropriate plasma sample size for analysis of a larger number of parameters, mice have to be typically sacrificed at pre-defined time points. This dramatically increases the number of animals needed for each study and partially violates the 3R tenet. More importantly, pre-defined sacrifice time points provide only a snap-shot assessment and preclude a protracted follow up of a natural evolution of any investigated disease. Therefore, techniques that allow repeated blood sample measurements in mice without their periodical scarify are of great importance.

1.7.1 General Concerns

The choice of the appropriate sampling method should be defined by the needed frequency of sampling, the volume of blood required and whether the animal’s survival is an endpoint (DONOVAN et al., 2005). Restraining procedures and blood collection itself induce stress reactions in mice, which may consequently influence the experimental results. Therefore, mice need to be accustomed to fixation before the onset of each experiment. At the same time, diligent training of all operators involved in the experiment(s), is required to guarantee reliable results.

Depending on the sampling localization, repeated blood collections may be either possible (tail vein, saphenous vein, jugular veins) or not recommended (i.e. retro-orbital sampling). If the blood is drawn from retro-orbital sinus or jugular vein, anaesthesia is necessary to avoid unessential stress and pain, as well as to ensure a steady positioning of the animal.

For repeated blood samples, the blood collection site should be chosen thoughtfully (e.g. facial vein or tail vein), and should not be changed during the study as the values of many blood-based parameters (e.g. leukocyte counts) vary considerably between sampling sites (NEMZEK et al., 2004). The maximum amount of blood that can be removed daily is defined
as 2% of total blood volume given that adequate fluid resuscitation is provided intravenously, intraperitoneally or subcutaneously (HOFF, 2000).

1.7.2 Blood Collection without Anaesthesia

1.7.2.1 Blood Collection from the Saphenous Vein and Dorsal Pedal Blood Vessel

The saphenous vein is a common site for repeated blood sampling in mice. The amount of blood that can be drawn from this site is defined as 5% of circulating blood volume. It is located at the caudal surface of the thigh while the medial dorsal pedal vein can be found at the dorsal top of the foot. Before sampling, mice should be warmed to stimulate the blood flow. After placing mice into a restraining tube that leaves one leg accessible, hair is plucked or shaved and petroleum jelly can be applied in the sampling area. Afterwards, the respective vein is punctured with a 25 gauge (G) needle. Bleeding can be stopped by applying slight pressure on the sample location. (HEM et al., 1998; DIEHL et al., 2001).

1.7.2.2 Blood collection from the submandibular vein

For facial vein sampling, mice are restraint with one hand, especially focusing on the tension of the skin on the cheek. In this position, a small vascular bundle is located at the spot where the orbital veins, the submandibular veins and other vessels, join into the jugular vein and cautiously punctured with either a lancet or a needle (23gauge (G)) or lancet (GOLDE et al., 2005). The emerging blood drop is promptly gathered with a pipette (rinsed with EDTA) if small amounts are desired or collected within a blood sampling tube (Fig. 4). Once the required blood volume is drawn, a gaze swab is immediately pressed on the puncturing spot to stop further bleeding and avoid formation of haematomas. To further minimize the risk of hematomas, only well trained operator(s) may perform facial vein sampling. Additionally, cheeks should be alternated daily to provide healing time (WEIXELBAUMER et al., 2010). The method can be used to obtain both large and very small volumes.
1.7.2.3 Blood collection from the tail

The most commonly used methods are the lateral tail incision, amputation of the tip of the tail (tail clip), and tail tip puncture. For all three techniques mice have to be positioned in a restrainer although a gentle tail clip can be also performed in an unrestrained mouse by an experienced operator. Slight massage of the tail may be necessary to keep the blood flowing but the tail should not be rubbed too hard to avoid leukocytosis and leakage of the interstitial fluid. The site of lateral incision of the tail is located 2cm beneath the tail base and the cut realized by using a scalpel. The forming blood drop is then collected.

The amputation of the tip of the tail is commonly performed with scissors and should not exceed 1mm (max. 5mm) to avoid damage of proximate cartilages. Blood is then collected with a glass capillary. The obtained blood volume is usually 0.1 – 0.2ml. Diehl et al. recommended the use of generalized anesthesia for amputation technique (DIEHL et al., 2001). In contrast to amputation, the tip of the tail can be punctured with a lancet to obtain a small blood sample. For repeated sampling from the same site the fresh blood clot can be removed to reopen the wound (CHRISTENSEN et al., 2009).

Figure 4. Facial Vein Sampling. The vena submandibularis is located and punctured with a 23G needle. Identification of the correct spot is possible by locating the fur freckle on either of the cheeks.

Vena submandibularis
Location of facial vein puncture
In addition to the above mentioned methods, the lateral tail vein can also be punctured with a syringe or a needle. To facilitate the sampling process, vasodilatation can be induced by exposing the animals to higher temperatures or by applying local warming of the tail (i.e. warm water) Experimenters should be careful to avoid unnecessary stress caused by too high temperatures (CHRISTENSEN et al., 2009; DIEHL et al., 2001).

1.7.3 Blood Collection under Anesthesia

1.7.3.1 Blood Collection from the Retroorbital Sinus

To collect blood from the retroorbital sinus, mice have to be restraint with one hand. A small microhematocrit tube is carefully inserted underneath the eyeball at the medial canthus of the orbit (Fig. 2). With a rotating movement under slight pressure, the insertion of the capillary is facilitated. As soon as blood appears, the pressure is released and blood is collected into a suitable tube. After the capillary is removed, it may be required to apply soft pressure to the eye to cease the bleeding. Furthermore, a period of a two week recovery should be observed between two samplings to enable proper wound healing. Retroorbital blood collection requires a skilled operator to avoid lesions of the optical nerve and/or other intraorbital structures that may lead to various complications (i.e. blindness, ocular ulcerations, keratitis,…) (CHRISTENSEN et al., 2009; DIEHL et al., 2001).

1.7.3.2 Blood Collection from the Jugular Vein

To obtain a blood sample from the jugular vein, mice have to be restraint in a hyperextended position. The fur can be either shaved or moistened with alcohol. After locating of the jugular veins (lateral of the sternoclavicular junction), they can be punctured with a 1ml syringe armed with a 25G needle. To avoid unwanted collapse of the vessel, blood needs to be drawn very slowly (HOFF, 2000).
1.7.4 Terminal Blood Collection

All methods for terminal blood collections are performed under deep anesthesia. In general, these techniques allow the operator to collect larger blood volumes compared to the afore described methods. Animals must be euthanized immediately after blood collection prior to recovery from anesthesia.

1.7.4.1 Blood Collection by Cardiac Puncture

Mice can be held in one hand with their head up, placed on their back or on their side. Depending on the chosen position, a 1ml syringe armed with a 23-25G needle is then inserted into the chest either next to the thorax center at a 25-30 degrees angle (if mouse is restrained in one hand), parallel to the body beneath the sternum (back position) or a 90 degree angle through the rib cage (side). Blood should be removed slowly to avoid collapse of the heart (DOEING et al., 2003). The justification to use cardiac puncture only for terminal bleeding is based on the fact that this technique may induce complications as pericardial bleeding or cardiac tamponade (DIEHL et al., 2001).

1.7.4.2 Blood Collection from the Vena Cava

To access the vena cava posterior the abdominal cavity is opened. Next, the intestines are moved to the left side to reach the widest part of the vessel, which is located between the kidneys. A 1ml syringe armed with 23-25G needle is then used to slowly draw blood. If the vein collapses, a short break enables a refilling with blood without removing the needle (HOFF, 2000).

1.7.4.3 Blood Collection from the Axillary Vessels

Mice are placed dorsally and one forelimb is stretched out. A deep skin incision is performed in the armpit. Next, the underlying vessels are cut with a scalpel and blood is collected. Experimenters have to be aware that the gained sample may be contaminated with cells from the surrounding tissue.
1.8. Aims

The first aim of our study was to develop a murine two-hit model (TH-CLP) mimicking the clinical ICU-scenario of a patient who suffers from trauma and hypovolemic shock, and subsequently develops a severe intra-abdominal sepsis as secondary complication.

The second aim was to characterize the TH-CLP Model by protracted monitoring via the repeated blood sampling method (WEIXELBAUMER et al., 2010).
2. Animals and Methods

All animal procedures were approved by the Viennese (Austria) legislative committee (Animal Use Proposal Permission no: MA 000794/2009/13) and conducted according to National Institute of Health guidelines.

2.1 Animals

3 month old, female CD-1 mice (total n=120) with an average weight of 30g were used for all experiments. Mice were purchased from Harlan Laboratories (Udine, Italy) and were kept in groups of five per cage on a 12h light – dark cycle. Temperature was maintained between 22 – 24°C and standard rodent diet and water were provided ad libitum to all mice throughout the experiments. Cages were enriched with houses, wood wool for nesting as well as wooden boards, tunnels and small blocks for gnawing (Abedd Lab & Vet Service, Vienna, Austria) to facilitate natural behaviour prior to and throughout the experimentation.

2.2 Two-Hit Model

We modified the original two-hit trauma/hemorrhage protocol developed by (VAN GRIENSVEN et al., 2002) (Fig. 5). In brief, the first hit consisted of a non-comminuted, unilateral, midshaft femur fracture followed by a hemorrhage via retro-orbital puncture (Fig. 2). Femur fracture was produced by custom-designed blunt pliers and confirmed by X-ray (Fig.6). For hemorrhage, total blood volume (TBV) was calculated as 6% of total body weight and both 50 and 40% of TBV were tested to achieve survival of at least 95% at the end (0h) of the post-trauma/hemorrhage (TH) phase. Post-TH, mice were resuscitated with 0.9% sodium chloride subcutaneously, (NaCl) with four times the volume of shed blood: 1ml was administered immediately after hemorrhage, while the remaining volume 1h later.
To induce the second hit, mice were subjected to CLP 48h post-TH (Fig. 1). We followed the original CLP protocol by (WICHTERMAN et al., 1980) with a number of modifications specified elsewhere (Fig.5) (TURNBULL et al., 2004; ZANOTTI-CAVAZZONI et al., 2009). In order to receive an approximate mortality of 50% at day 5 post-CLP (day 7 post-TH), different grade of CLP severity was induced by increasing needle sizes (i.e. 20, 19, 18 or 17G). Starting with TH, mice were resuscitated twice daily (excluding the immediate post-hemorrhage resuscitation) with 1ml of 0.9% sodium chloride (NaCl) including analgesia (0.05mg/kg buprenorphine, Temgesic®). From 2h post-CLP onward, every mouse was resuscitated twice daily for 5 days with 1ml Ringer solution containing analgesia (as above) and wide-range antibiotic (25mg/kg imipenem, Zienam®) to emulate the "Surviving Sepsis Campaign Guidelines for Management of Severe Sepsis and Septic Shock" (DELLINGER et al., 2008). For all surgical procedures mice were anesthesized with isoflurane (Forane®) and survival was monitored for seven days post-TH. Sham surgeries were not performed to reduce the total number of mice required for the study.

Figure 5. Schematic of the two-hit model and repetitive blood sampling. 3-month-old female mice were subjected to trauma and hemorrhage (TH) followed by polymicrobial CLP-induced sepsis. Time-span of model was divided into a pre-and post-CLP phase. Starting at TH, a 20µl blood sample was collected daily (until day 7 post-TH/day 5 post-CLP) from each animal, including one extra sample at 6h post-CLP.
2.3 Blood Sampling

Beginning immediately prior to hemorrhage (~48h time-point), 20µl of blood was collected via facial vein puncture from each animal every 24h (including an additional sample at 6h post-CLP) until day 5 post-CLP as previously described by WEIXELBAUMER et al. (2010). All samples were immediately diluted 1:10 in PBS with EDTA (diluted 1:50). After centrifugation (1g, 5min, 22°C), 180µl of plasma was removed and stored at -80°C for further analysis.

2.4 Complete Blood Count

After removing plasma, the remaining blood pellet was resuspended with 180µl Cell-Dyn buffer with EDTA prior to complete blood count (CBC) analysis with a Cell Dyn 3700 counter (Abbott Laboratories, Illinois, USA).

2.5 Metabolic and Organ Function Parameters

Urea nitrogen (urea), glucose, lactate dehydrogenase (LDH), alanin transaminase (ALT) and aspartate transaminase (AST) were analysed in plasma samples with a Cobas c111 reader (Roche, Switzerland).

Figure 6. Unilateral femur fracture X-ray. Non comminuted fracture of the right femur was realized with a custom-built, forcipate device.
2.6 Statistical Analysis

Survival curves were plotted using Kaplan-Meier method. Data were tested for normality and for all further analyses mice were retrospectively divided into either surviving (SUR, alive by day 5 post-CLP) or dying (DIE, died between day 1-5 post-CLP). Due to the high variability and low n=3 at 96h (day 5 post-CLP), we excluded this time-point from all graphs. Regardless whether pre-CLP (Figs. 8-10) or post-CLP (Figs.11-13), SUR vs. DIE differences (each time-point separately) were evaluated by either Student’s t test (normally distributed data) or Mann-Whitney test (abnormally distributed data). One-way ANOVA followed by Dunn’s test was used to assess longitudinal fluctuations of all parameters in the pre-CLP phase. All pre-CLP data (Figs. 3-5) are presented as Tukey box-plot diagram, while post-CLP data are plotted as mean ± SEM (Fig.6-8). Statistical tests were carried out using Prism 5 (GraphPad Software Inc., San Diego, USA) and the level of significance was defined at p<0.05.
3. Results

3.1 Survival

The initial goal was to reach an overall mortality of approximately 50% at the end of the acute phase of sepsis (day 5 post-CLP, day 7 post-TH). All mice were subjected to trauma (unilateral femur fracture) and hemorrhage (50% of TBV) followed by CLP of varying severity 48h later (Fig. 7A). Hemorrhage of 50% of TBV resulted in an average mortality of 27% at day 2 post-TH. CLP surgery with 17G needle produced additional mortality of 34% leading to the overall mortality of approx. 60% at day 5 post-CLP (day 7 post-TH). CLP with remaining needles was either mildly lethal (10% by 19G) or non-lethal (20 and 18G) (Fig. 4A).

To eliminate the excessive loss of mice induced by TH, we reduced the hemorrhage volume to 40% of TBV. This significantly lowered the post-TH mortality from 27 to 4% (Fig. 7B). All post-TH deaths occurred within 1h of the hit.

Next, we tested whether the change in the amount of shed blood (50% vs. 40%) affected the post-CLP outcome. Mice were subjected to TH with either 50 or 40% of TBV followed by CLP with 17G needle (Fig. 7C). The day-5 (post-CLP) mortality was virtually identical for both groups (56% vs. 54%) (Fig. 7C).
Figure 7 A-C. A 7-day survival curve in 3-month-old female mice subjected to the 2-Hit model of trauma/hemorrhage (TH) followed by a polymicrobial CLP-induced sepsis. To achieve approximately 50% mortality at day 7 post-TH (50% hemorrhage of total blood volume, TBV), the severity of CLP was adjusted by different gauge needles (20G n=10, 19G n=10, 18G n=10, 17G n=22). To reach sublethality after hemorrhage, 40% blood loss (of TBV) was tested with a 17gauge needle (40%+17G n=65; 50%+17G n=16).

3.2. Pre-CLP (post-TH) phase:

3.2.1 Red blood cells, hemoglobin and platelets

In all subsequent experiments, all mice were consistently subjected to TH with 40% of TBV and CLP with 17G needle and retrospectively divided into either SUR or DIE for further comparisons (see statistical analysis).

TH hit caused a virtually identical reduction of red blood cell (RBC) counts (by approx. 32%) and hemoglobin (Hb) concentration (by approx. 31%) in both SUR and DIE animals at –24h (Fig. 8A and B) and the magnitude of this decrease remained virtually unchanged until CLP
(0h time-point). Prior to TH (-48h time-point), platelets (PLT) were lower in SUR by approximately 11% (p<0.05) compared to DIE mice (Fig. 8C). At -24h, PLT insignificantly decreased in SUR (by 13%) and in DIE (by 20%) animals (compared to the respective counts at -48h). At 0h, PLT recovered to their initial pre-TH values (both groups) with the SUR PLT count approx. 10% lower (p<0.05) than in DIE mice.

Figure 8 A-C. Retrospective comparison of red blood cells, platelet count, and hemoglobin concentration in dying (DIE) vs. surviving (SUR) females in the post-TH phase (pre-CLP). Displayed values were compared between DIE and SUR at-48h (DIE at least n=17, SUR at least n=18), -24h (DIE at least n=28, SUR n=32), 0h pre-CLP (DIE at least n=28, SUR n=32 for all parameters) and between time points. §p<0.05 between time points and *p<0.05 between DIE and SUR.
3.2.2 White blood cells

We investigated the effect of trauma and hemorrhage on neutrophil (NEU) and lymphocyte (LYM) population. TH hit did not affect circulating leukocytes (data not shown). Interestingly, while TH did not have an immediate effect upon circulating NEU (Fig. 9A), its delayed surge (identical in both groups) was evident immediately prior to CLP (0h vs. -48h and -24h, p<0.05). Neither longitudinal nor inter-group differences were observed in circulating LYM counts post-TH (Fig. 9B).

![Figure 9 A-B](image)

**Figure 9 A-B. Retrospective comparison of neutrophil and lymphocyte count in dying (DIE) vs. surviving (SUR) females in the post-TH phase (pre-CLP).** Displayed values were compared between DIE and SUR at-48h (DIE n=17, SUR n=18), -24h (DIE n=29, SUR n=32) 0h pre-CLP (DIE n=31, SUR n=34) and between time points. §p<0.05 between time points and *p<0.05 between DIE and SUR.

3.2.3 Organ function parameters

TH hit caused a virtually identical hypoglycemia in both SUR and DIE animals, as evidenced by the approx. 40% drop of circulating glucose at -24h (Fig.10A). Glucose remained low (approx. 70% of the -48h value) in both (SUR and DIE) groups until CLP (0h).

There was a strong AST spike in all mice at -24h (Fig. 10B) and this increase was 3-fold higher in DIE compared to SUR mice (192.7 vs. 578.7 U/l, p<0.05). However, there was no significant inter-group difference at 0h, as AST nearly recovered both in SUR (122U/l) and
DIE (172U/l) mice (Fig. 10B). In ALT, there was also a significant post-TH increase at -24h but in contrast to AST, its magnitude (average 48%) was similar in both SUR and DIE mice (Fig. 10C).

The same was true for LDH: an initial 64% post-TH surge at –24h (identical in SUR and DIE) was followed by its marked recovery at 0h (Fig. 10D). Levels of urea differed slightly (12%, p<0.05) between DIE and SUR prior to TH (-48h, Fig. 10E) but this dissimilarity disappeared at later (post-TH) time-points (-24h and 0h).
Figure 10 A-E. Retrospective comparison of circulating glucose, AST, ALT, LDH, and urea in dying (DIE) vs. surviving (SUR) females in the post-TH phase (pre-CLP). Displayed values were compared between DIE and SUR at -48h (DIE n=16, SUR n=18; AST: DIE n=6, SUR n=12), -24h (DIE n=23, SUR n=32; AST: SUR n=21), 0h pre-CLP (DIE n=23, SUR n=32; AST: DIE n=8, SUR n=21) and between time points. §p<0.05 between time points and *p<0.05 between DIE and SUR.
Figure 11 A-C. Retrospective comparison of red blood cells, platelet count and hemoglobin concentration in dying (DIE) vs. surviving (SUR) females in the post-CLP phase. Displayed values were compared between DIE and SUR group 6h (DIE at least n=30, SUR at least n=32), 24h (DIE at least n=28, SUR at least n=31), 48h (DIE at least n=16, SUR at least n=31), 72h (DIE at least n=11, SUR at least n=32) and 96h (SUR at least n=26 for all parameters) post-CLP. Dotted lines represent normal values. *p<0.05.
3.3 Post-CLP phase:

3.3.1 Red Blood Cells, hemoglobin, platelets

Regardless of outcome, RBC counts and Hb concentration remained lower throughout the entire post-CLP period. In DIE mice, RBC and Hb were slightly (but consistently) elevated (e.g. 15% and 13% at 6h) compared to SUR (Fig. 11A+B). Sepsis caused an almost identical PLT drop in both SUR and DIE groups until 48h post-CLP. Thereafter, PLTs began to recover in SUR mice (further decline in DIE), reaching approx 80% of baseline at 96h. A 2-fold separation between SUR and DIE mice was recorded at 72h (Fig. 11C).

3.3.2 White blood cells

Within the first 48h, CLP resulted in a drop of NEU in all mice, with NEU counts typically lower (e.g. by 31% at 24h and 21% at 72h) in DIE animals compared to SUR (Fig. 12A). From 48h onward, there was a gradual recovery of NEU, which at 72h either reached (in DIE) or exceeded (in SUR) baseline values.

Similar to NEU, there was a rapid LYM decrease in all mice within the first 48h of CLP, with initial LYM counts significantly higher (by 17% at 6h and 51% at 24h) in DIE compared to SUR (Fig. 12B). This was followed by a prominent shift between the SUR vs. DIE groups: while the 72h LYM count remained depressed in DIE mice, there was a robust LYM recovery in SUR from 48h onward. At 72h, the SUR LYM count was higher by 40% (p<0.05) (compared to the LYM count in DIE) and continued to increase toward baseline (approx. 84% of the lower baseline range at 96h) (Fig. 10B).
3.3.3 Organ function parameters

Regardless of the group, pre-existing (post-TH) hypoglycemia worsened after the CLP-induced sepsis. Compared to DIE, a slight glucose recovery in SUR mice was noted from 48h onward (by approx. 20% at 48h and 23% at 72h, p<0.05) (Fig. 13A). In both DIE and SUR groups, there was a notable post-CLP increase in AST. The peak inter-group difference (DIE 2-fold higher than SUR) was recorded at 48h (p<0.05) but disappeared at 72h (Fig. 13B). Such an inter-group separation was even more pronounced in ALT: while its activity continuously increased in DIE from 6h onward, it was gradually declining (transient peak at 24h) in SUR reaching baseline by 96h (Fig. 13C). The maximal, 6-fold difference in circulating ALT between SUR and DIE mice was recorded at 72h (0.3-fold at 24h and 4-fold at 48h).

Similar post-CLP response was observed for LDH and urea. Both parameters were elevated in DIE mice: urea by 0.3-fold at 6h and by 3.6-fold between 24-72h, while LDH by approximately 2-fold at 24h, 5-fold at 48h and 3.5-fold at 72h time-point (p<0.05) compared to SUR. In SUR mice, both urea and LDH values approached baseline from 48h onward (Fig. 13 D+E).
Figure 13. A-E. Retrospective comparison of circulating glucose, AST, ALT, LDH and urea in dying (DIE) vs. surviving (SUR) females in the post-CLP phase. Displayed values were compared between DIE and SUR group 6h (DIE n=23, SUR n=32; AST: DIE n=8, SUR n=20), 24h (DIE n=28, SUR n=34; AST: DIE n=14, SUR n=23), 48h (DIE n=19, SUR n=34; AST: DIE n=9, SUR n=23), 72h (DIE n=12, SUR n=34; AST: DIE n=3, SUR n=23) and 96h (SUR at least n=33; AST: SUR n=23) post-CLP. Dotted lines represent normal values. *p<0.05.
4. Discussion

Existing hurdles in modeling of human sepsis in laboratory animals are multi-tier (DYSON et al., 2009):

1. An inadequate recognition that any narrowly-defined scenario of experimental sepsis aims to represent exclusively the cohort of septic patients with the respective pathophysiological fingerprints (and should not be over-extended to other patients’ subpopulations that may feature different disease characteristics).

2. The extreme difficulty in simulating adequate scenarios of various septic events without full comprehension of their underlying pathophysiology.

3. Numerous technical challenges in reproducing the advanced ICU treatment and monitoring procedures in models already recognized as clinically relevant.

The growing awareness to the above-mentioned shortcomings has provoked attempts to develop more representative (two-hit) animal models aimed at investigating the pathogenesis of sepsis as the secondary insult. The primary goal of our study was to reproduce a scenario, in which an initial traumatic/hypovolemic event survived by an ICU-monitored patient is complicated by a severe intra-abdominal sepsis.

4.1 The 2-hit Model

4.1.1 Trauma-Hemorrhage

Given that an extensive blood loss typically coincides with injury and/or trauma, combining these two elements into an initial challenge is more clinically relevant than referring to established first-hit models consisting solely of hemorrhage (FOEX, 1999; WICHMANN et al., 1996b; WICHMANN et al., 1998). Studies have shown that in an affected subject, both (trauma/hemorrhage) are responsible for initiation of a robust immuno-inflammatory response that in later phases typically transitions into a suppression of innate and adaptive immunity (FOEX, 1999; ZELLWEGGER et al., 1995).
Both hemorrhage and trauma elements of the first hit were modified from the original protocol (VAN GRIENSVEN et al., 2002): guillotine was replaced with custom-built blunt pliers, while the hemorrhage step was initiated immediately after femur fracture (versus a 2h delay in the previous setup) and the hemorrhage volume was decreased by 20% (to 40% of TBV). The hemorrhage modifications were dictated by both the desire to reproduce a more clinically relevant timing and to produce survivable hypovolemic shock.

The femur was chosen as fracture localisation because of its surrounding soft-tissue. Furthermore, the decision to break the femur with a forcipate, soft-tissue injury creating device was based on the findings of two other studies which had previously demonstrated that soft-tissue trauma further compromises immune functions after bone fracture and/or hemorrhage (KOBBE et al., 2008a; WICHMANN et al., 1998). Given that in trauma patients fractures naturally diverge in terms of location, type and severity of concurrent soft tissue injury and extensive hemorrhage is frequently lethal, a relatively rigorous standardization of these elements can be viewed as the model’s shortcoming rather than advantage. Yet, in this particular two-hit setup, a relatively similar magnitude of the TH hit severity and maximal elimination of lethality is desired, as we aimed to verify our model by studying the outcome-depended responses triggered by the CLP hit (and not by the TH). It is also suggestive that discovery value of potential responses indicative of the post-CLP outcome would be more scientifically attractive, if such diverse reactions were produced by a conservatively standardized (hence generating more uniform responses) TH hit.

Also, the adherence to the Russell-Burch "3R" tenet (replace, refine, reduce) should not be overlooked (HOBSON-WEST, 2009). Although it was obviously impossible to replace animals by alternative methods, our experimental setup was “refined” by using adequate number of animals and the combination of insults (fracture, soft-tissue injury and hemorrhage) to further improve the clinical relevance. Furthermore, variation of results was controlled by using animals of same age, weight and gender, and by the relatively similar impact magnitude of the first hit (TH). All experiments were carried out in the morning to exclude variations due to the circadian rhythms. Moreover, by conducting comparisons between animals that died and those that survived, we were able to abandon a sham animal group and therefore to additionally “reduce” the total number of animals used in this study.
4.1.2 Sepsis

Since the systemic inflammatory response induced by colon ascendens stent peritonitis is comparable to immune responses to endotoxemia, we decided to subject mice to cecal ligation and puncture, which produces a more protracted immune response comparable to human sepsis (DEJAGER et al., 2011). Even though the cecal ligation and puncture model we used is by far the best choice for replication of human peritoneal sepsis, in the clinical setting nosocomial (ventilator-associated) pneumonia is a major cause of secondary septic complications in trauma patients with prolonged ICU stays (COOK et al., 2010; MAGRET et al., 2010; DYSON et al., 2009) although incidences of polymicrobial sepsis are also reported (CHERON et al., 2010).

An abdominal focus of infection may be viewed as relatively suboptimal given the prevalence of lung-associated secondary infections in polytrauma patients. In our model, however, the cecal ligation and puncture primarily served as a trigger to produce a severe polymicrobial challenge (as opposed to the typically monobacterial lung-associated sepsis) that spreads systemically in a protracted fashion, rather than a model of abdominal peritonitis per se.

Moreover, our model was deliberately deprived of relevant intangibles such as pre-existing comorbidities (e.g. diabetes, atherosclerosis) and operative interventions that are routinely observed/performed in patients and may significantly skew investigated parameters and outcome (XIAO et al., 2006; OSUCHOWSKI et al., 2010)We chose to use healthy mice since any confounding factor in addition to the two-hit challenge would likely generate too much variability and make data interpretation difficult. The CLP challenge itself provides an adequate variability regarding both the outcome and wide range of generated responses. The level of sepsis-induced mortality in our model (approx. 50% at day 5 post-CLP) was carefully set to closely reproduce clinical data (reporting between 20-60% incidence of deaths in the septic ICU patients) (POLI-DE-FIQUEIREDO et al., 2008). This is important since data generated in highly lethal CLP models might artificially enhance predictive value of some biomarkers (DELANO et al., 2009).
4.2. Monitoring of Immune Responses

Profound changes in the immuno-inflammatory status caused by trauma/hemorrhage, specifically those propagating development of progressive immunoparalysis, have been postulated to be responsible for the increased susceptibility to secondary sepsis (DELANO et al., 2009). Such immuno-inflammatory fluctuations frequently occur in patients over the period of several hours. Thus, a rigorous and continuous surveillance of the ICU patients’ immune status is fundamental in ensuring a most adequate therapeutic strategy and rapid adjustments of treatment to tailor it to the ongoing shifts of the immune system.

To enable such tactics in our TH-CLP model, we subjected all mice to daily low-volume blood sampling via facial vein puncture. This technique provides daily collection of blood (20µl/mouse) from critically ill mice with virtually no negative effects upon their outcome and/or health status (WEIXELBAUMER et al., 2010). Despite a limited sample volume available for analysis, advanced and highly sensitive techniques such as multiplex bead assays and various automated biochemistry analyzers allow accurate measurement of dozens of circulating biomarkers and/or cell subpopulations.

However, in the context of a joint critical disease/repetitive sampling protocol, its main advantage lies in an ability to investigate a natural evolution of a given disease: any relevant immuno-inflammatory, metabolic and/or organ function alterations can be followed over time in individual subjects to an undefined outcome. This is in stark contrast to studies in which animals are sacrificed at predefined time-points providing only a narrow snap-shot assessment of investigated endpoints/pathways without option to match recorded changes with outcome.

4.3 Relevance of the TH-CLP model

In our study, we employed the TH-CLP/monitoring combination to gain preliminary insight into outcome-related mechanisms developing during the acute phase of post-traumatic sepsis. In context of potential post-traumatic septic complications, the period between the first (trauma/hemorrhage) and the secondary (sepsis) challenge may be as decisive as the immediate time-span following the onset of sepsis.
If relevant risk-correlating marker(s) are identified in the post-traumatic but pre-septic phase, one could identify not only subjects with high risk of developing septic complications (post-trauma) but also those who display higher risk of death once sepsis sets in. While this model does not allow any insight into the first scenario (all mice undergo sepsis), it is well-matched to investigate the latter situation. We therefore performed outcome-based retrospective analysis of all analysed parameters to detect potential early (pre-CLP) and late (post-CLP) differences between surviving and dying individuals.

In the pre-CLP phase, there was a much stronger post-TH (24h pre-CLP) AST spike (indirectly indicative of hepatic injury) in mice that died within first few days after the onset of peritonitis (compared to survivors), whereas the same cohort (DIE) showed clear and profound metabolic/organ function disturbances in the post-CLP period. Further analysis could provide information about a possible development of a more intense dysfunction of selected organs prior to sepsis in post-TH mice (consequently predisposing them for unfavourable post-CLP outcome) and how exactly organ disturbances drive lethal septic responses both prior and after the onset of sepsis.

In any respect, such longitudinal assessments enabled by more ICU-like tailored models such as the one described above, generate valuable findings that may be then successfully extrapolated to respective clinical scenarios. This in turn may help in identifying ideal time-points and/or targets for successful therapeutic interventions in sepsis and other related critical diseases.

### 4.4 Conclusions

The modified and clinically relevant TH-CLP model allows a more adequate investigative insight into the course of septic complications following trauma. Additional integration of the daily blood sampling into the TH-CLP setup closely emulates one of the major components of the ICU protocol: repetitive monitoring of critically sick patients. The daily monitoring of TH-CLP mice was a basis for outcome-based (retrospective) stratification of responses they generated during the both two-hit phases. Such an approach enables in turn correlation of circulating cells/biomarkers to septic outcomes even prior to the development of sepsis. If
verified in a clinical setting, findings from such advanced models could not only help to
categorize patients into high-and low-risk of death after their traumatic insult gets
complicated by sepsis, but also to prevent the onset of septic sequelae altogether.
5. Abstract

To simulate and monitor the evolution of post-traumatic sepsis in mice, we combined a two-hit model of trauma/hemorrhage (TH, 1st hit) followed by abdominal sepsis (CLP, 2nd hit) with repetitive blood sampling. Anesthetized mice underwent femur fracture/sublethal hemorrhage and cecal ligation and puncture (CLP) 48h later. To monitor outcome-dependent changes in circulating cells/biomarkers, mice were sampled daily (facial vein) for 7 days and retrospectively divided into either dead (DIE) or surviving (SUR) by day 7 post-CLP. Prior to CLP, AST was 3-fold higher in DIE, while all other post-TH changes were similar between groups. There was a significant inter-group separation post-CLP. In SUR, RBC and Hb were lower, platelets and neutrophils higher, while lymphocytes mixed compared to DIE. In DIE, all organ function markers except glucose (decrease) were few-fold higher compared to SUR. In summary, the combination of daily monitoring with an adequate two-hit model simulates the ICU setting, allows insight into outcome-based responses and can identify biomarkers indicative of death in the acute post-traumatic sepsis in mice.
6. German Summary


Zusammenfassend lässt sich sagen, dass die Kombination von einem adequaten 2-Insulte Modell mit täglicher Überwachung die Situation auf der Intensivstation simuliert und somit ein klinisch relevantes Tiermodell darstellt. So wird ein Einblick in die Immunreaktionen der Tiere in Abhängigkeit von ihrem Überleben ermöglicht und es können mögliche Biomarker identifiziert werden, die bereits in der akuten post-traumatischen Phase die Gefahr anzeigen, später an septischen Komplikationen zu sterben.


9. Reference List


leukocyte antigen-DR expression is independently associated with the development of sepsis after major trauma. Crit Care, 14, R208.


Methodology Report

Experimentally Approaching the ICU: Monitoring Outcome-Based Responses in the Two-Hit Mouse Model of Posttraumatic Sepsis

Susanne Drechsler, Katrin M. Weixelbaumer, Heinz Redl, Martijn van Griensven, Soheyl Bahrami, and Marcin F. Osuchowski

Ludwig Boltzmann Institute for Experimental and Clinical Traumatology in the Trauma Research Center of AUVA, Donaueschingenstraße 13, 1200 Vienna, Austria

Correspondence should be addressed to Marcin F. Osuchowski, marcin.osuchowski@trauma.lbg.ac.at

Received 13 October 2010; Accepted 13 December 2010

Academic Editor: Monica Fedele

Copyright © 2011 Susanne Drechsler et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

To simulate and monitor the evolution of posttraumatic sepsis in mice, we combined a two-hit model of trauma/hemorrhage (TH) followed by polymicrobial sepsis with repetitive blood sampling. Anesthetized mice underwent femur fracture/sublethal hemorrhage and cecal ligation and puncture (CLP) 48 h later. To monitor outcome-dependent changes in circulating cells/biomarkers, mice were sampled daily (facial vein) for 7 days and retrospectively divided into either dead (DIE) or surviving (SUR) by post-CLP day 7. Prior to CLP, AST was 3-fold higher in DIE, while all other post-TH changes were similar between groups. There was a significant post-CLP intergroup separation. In SUR, RBC and Hb were lower, platelets and neutrophils higher, and lymphocytes mixed compared to DIE. In DIE, all organ function markers except glucose (decrease) were few folds higher compared to SUR. In summary, the combination of daily monitoring with an adequate two-hit model simulates the ICU setting, allows insight into outcome-based responses, and can identify biomarkers indicative of death in the acute posttraumatic sepsis in mice.

1. Introduction

With more than 751,000 cases per annum in USA [1] and an incidence ranging from 0.4 to 1 case/1000 people in Europe [2], sepsis is the leading cause of death in noncoronary intensive care units [3]. Sepsis is routinely described as the body's systemic response to an invading pathogen such as bacteria and/or fungus [4]. The current consensus implies that early (acute) septic deaths have been associated with an excessive exacerbation of the proinflammatory immune response (termed systemic inflammatory response syndrome or SIRS), which is then followed by the compensatory anti-inflammatory response syndrome (CARS), although biomarkers typical to either of those syndromes may be released simultaneously [5, 6]. Systemic spread of pathogens can originate from numerous organs/systems of the body—the lung representing the most frequent site of infection (68%), followed by the abdomen (22%), blood (20%), and urinary tract (14%) [7].

The pathophysiology of sepsis is further complicated by a variety of invading microorganisms, and that sepsis typically occurs as a secondary complication (second hit). Epidemiological data are inconsistent on the incidence of post-traumatic sepsis: existing studies demonstrated that post-injury sepsis/severe sepsis incidence ranges from 2% to over 30% [8–10]. Furthermore, it has been corroborated that trauma patients with sepsis have significantly higher ICU admission rates and mortality than their non-septic counterparts, mostly due to the development of multiple organ dysfunction syndrome (MODS) [10, 11].

Animal models are the launch platform to study the pathophysiology of sepsis and septic shock since they feature...
hemorrhage followed by CLP) with our recently validated daily blood-based monitoring method. Next, we tested this design by surveying the evolution of exemplary immunological, organ function, and metabolic alterations prior to and after intraabdominal sepsis. Specifically, differences between dying and surviving animals during both hit phases were investigated.

2. Materials and Methods

2.1. Animals. 3-month-old, female CD-1 mice (total \( n = 120 \)) with an average weight of 30 g were used for all experiments. Mice were purchased from Harlan Laboratories (Udine, Italy) and were kept in groups of five per cage on a 12 h light-dark cycle. Temperature was maintained between 22–24°C, and standard rodent diet and water were provided ad libitum to all mice throughout the experiments. All animal procedures were approved by the local legislative committee and conducted according to National Institute of Health guidelines.

2.2. Two-Hit Model. We modified the original two-hit trauma/hemorrhage protocol developed by van Griensven et al. [19]. In brief, the first hit consisted of a noncom-minated, unilateral, midshaft femur fracture followed by a hemorrhage via retro-orbital puncture (Figure 1). Femur fracture was produced by custom-designed blunt pliers and confirmed by X-ray. For hemorrhage, total blood volume (TBV) was calculated as 6% of total body weight and both 50% and 40% of TBV were tested to achieve survival of at least 95% at the end (0 h) of the posttrauma/hemorrhage (TH) phase. After-TH, mice were resuscitated with 0.9% sodium chloride subcutaneously, (NaCl) with four times the volume of shed blood: 1 mL containing analgesia (0.05 mg/kg buprenorphine, Temgesic) was administered immediately after hemorrhage (aimed to reproduce the phase of restricted resuscitation), while the remaining volume was given 1 h later (aimed to reproduce the phase of unrestricted resuscitation).

To induce the second hit, mice were subjected to CLP 48 h after-TH (Figure 1). We followed the original CLP protocol by Wichterman et al. [25] with a number of modifications specified elsewhere [26, 27]. In order to receive an approximate mortality of 50% at post-CLP day 5 (post-TH day 7), different grade of CLP severity was induced by increasing needle sizes (i.e., 20, 19, 18, or 17 gauge (G)). Starting with TH, mice were resuscitated twice daily (excluding the immediate posthemorrhage resuscitation) with 1 mL of 0.9% NaCl including analgesia (0.05 mg/kg buprenorphine, Temgesic). From 2 h after-CLP onward, every mouse was resuscitated twice daily for 5 days with 1 mL Ringer solution containing analgesia (as above) and wide-range antibiotic (25 mg/kg imipenem, Zienam) to emulate the “Surviving Sepsis Campaign Guidelines for Management of Severe Sepsis and Septic Shock” [28]. For all surgical procedures, mice were anesthetized with isoflurane (Forane), and survival was monitored for seven days after TH.
Given the aim of the experiment, sham surgeries were not performed to reduce the total number of mice in the study.

2.3. Blood Sampling. Beginning immediately prior to hemorrhage (~48 h time-point), 20 µL of blood was collected via facial vein puncture from each animal every 24 h (including an additional sample at 6 h post-CLP) until post-CLP day 5 as previously described by Weixelbaumer et al. [29] (Figure 1). All samples were immediately diluted 1:10 in PBS with EDTA (diluted 1:50). After centrifugation (1 g, 5 min, 22°C), 180 µL of plasma was removed and stored at −80°C for further analysis.

2.4. Complete Blood Count. After removing plasma, the remaining blood pellet was resuspended with 180 µL Cell-Dyn buffer with EDTA prior to complete blood count (CBC) analysis with a Cell Dyn 3700 counter (Abbott Laboratories, Ill, USA).

2.5. Metabolic and Organ Function Parameters. Urea nitrogen (urea), glucose, lactate dehydrogenase (LDH), alanine transaminase (ALT), and aspartate transaminase (AST) were analyzed in plasma samples with a Cobas c111 reader (Roche, Switzerland).

2.6. Statistical Analysis. Survival curves were plotted using Kaplan-Meier method. Data were tested for normality, and for all further analyses, mice were retrospectively divided into either surviving (SUR, alive by post-CLP day 5) or dying (DIE, died between post-CLP day 1 and 5). Due to the high variability and low n = 3 at 96 h (post-CLP day 5) in the DIE group, we excluded this time point from all graphs/analyses. Regardless of whether pre-CLP (Figures 3–5) or post-CLP
3. Results

3.1. Survival. The initial goal was to reach an overall mortality of approximately 50% at the end of the acute phase of sepsis (post-CLP day 5, post-TH day 7). All mice were subjected to trauma (unilateral femur fracture) and hemorrhage (50% of TBV) followed by CLP of varying severity 48 h later (Figure 2(a)). Hemorrhage of 50% of TBV resulted in an average mortality of 27% at post-TH day 2. CLP surgery with 17G needle produced additional mortality of 34% leading to the overall mortality of approx. 60% at post-CLP day 5 (post-TH day 7). CLP with remaining needles was either mildly lethal (10% by 19G) or nonlethal (20 and 18G) (Figure 2(a)). To eliminate the excessive loss of mice induced by TH, we reduced the hemorrhage volume to 40% of TBV. This significantly lowered the post-TH mortality from 27% to 4% (Figure 2(b)). All post-TH deaths occurred within 1 h of the hit.

Next, we tested whether the change in the amount of shed blood (50% versus 40%) affected the post-CLP outcome.
Mice were subjected to TH with either 50% or 40% of TBV followed by CLP with 17G needle (Figure 2(c)). The (post-CLP) day-5 mortality was virtually identical for both groups (56% versus 54%) (Figure 2(c)).

3.2. Pre-CLP (Post-TH) Phase

3.2.1. Red Blood Cells, Hemoglobin, and Platelets. In all subsequent experiments, all mice were consistently subjected to TH with 40% of TBV and CLP with 17G needle and retrospectively divided into either SUR or DIE for further comparisons (see Section 2.6).

TH hit caused a virtually identical reduction of red blood cell (RBC) counts (by approximately 32%) and hemoglobin (Hb) concentration (by approx. 31%) in both SUR and DIE animals at −24 h (Figures 3(a) and 3(b)), and the magnitude of this decrease remained virtually unchanged until CLP (0 h time point).

Prior to TH (−48 h time point), platelets (PLT) were lower in SUR by approximately 11% (P<.05) compared to DIE mice (Figure 3(c)). At −24 h, PLT insignificantly decreased in SUR (by 13%) and in DIE (by 20%) animals (compared to the respective counts at −48 h). At 0 h, PLT recovered to their initial pre-TH values (both groups) with the SUR PLT count approx. 10% lower (P<.05) than in DIE mice.

3.2.2. White Blood Cells. We investigated the effect of trauma and hemorrhage on neutrophil (NEU) and lymphocyte (LYM) population. TH hit did not affect circulating leukocytes (data not shown). Interestingly, while TH did not have an immediate effect upon circulating NEU, its delayed surge (identical in both groups) was evident immediately prior to CLP (0 h versus −48 h and −24 h, P < .05) (Figure 4(a)). Neither longitudinal nor intergroup differences were observed in post-TH circulating LYM counts (Figure 4(b)).

3.2.3. Organ Function Parameters. TH hit caused a virtually identical hypoglycemia in both SUR and DIE animals, as evidenced by the approx. 40% drop of circulating glucose at −24 h (Figure 5(a)). Glucose remained low (approx. 70% of the −48 h value) in both (SUR and DIE) groups until CLP (0 h).

There was a strong AST spike in all mice at −24 h (Figure 5(b)), and this increase was 3-fold higher in DIE compared to SUR mice (192.7 versus 578.7 U/L, P<.05). However, there was no significant intergroup difference at 0 h, as AST nearly recovered both in SUR (122 U/L) and DIE (172 U/L) mice (Figure 5(b)). In ALT, there was also a significant post-TH increase at −24 h, but in contrast to AST, its magnitude (average 48%) was similar in both SUR and DIE mice (Figure 5(c)).

The same was true for LDH: an initial 64% post-TH surge at −24 h (identical in SUR and DIE) was followed by its marked recovery at 0 h (Figure 5(d)). Levels of urea differed slightly (12%, P < .05) between DIE and SUR prior to TH (−48 h, Figure 5(e)), but this dissimilarity disappeared at later (post-TH) time points (−24 h and 0 h).

3.3. Post-CLP Phase

3.3.1. Red Blood Cells, Hemoglobin, and Platelets. Regardless of outcome, RBC counts and Hb concentration remained decreased throughout the entire post-CLP period. In DIE mice, RBC and Hb were slightly (but consistently) elevated (e.g., 15% and 13% at 6 h) compared to SUR. Sepsis caused an almost identical PLT drop in both SUR and DIE groups.
Figure 5: Retrospective comparison of circulating glucose, AST, ALT, LDH, and urea in dying (DIE) versus surviving (SUR) animals prior to CLP. 3-month-old female mice were subjected to trauma/hemorrhage prior to CLP. Displayed values were compared between DIE and SUR at −48 h (DIE n = 16, SUR n = 18; AST: DIE n = 6, SUR n = 12), −24 h (DIE n = 23, SUR n = 32; AST: SUR n = 21), 0 h before-CLP (DIE n = 23, SUR n = 32; AST: DIE n = 8, SUR n = 21) and between time points. §P < .05 between time points, and *P < .05 between DIE and SUR.
Figure 6: Retrospective comparison of red blood cell platelet count, and hemoglobin concentration in dying (DIE) versus surviving (SUR) animals after-CLP. 3-month-old female mice were subjected to trauma/hemorrhage followed by polymicrobial CLP-induced sepsis. Displayed values were compared between DIE and SUR group 6 h (DIE at least \( n = 30 \), SUR at least \( n = 32 \)), 24 h (DIE at least \( n = 28 \), SUR at least \( n = 31 \)), 48 h (DIE at least \( n = 16 \), SUR at least \( n = 31 \)), 72 h (DIE at least \( n = 11 \), SUR at least \( n = 32 \)), and 96 h (SUR at least \( n = 26 \) for all parameters) after-CLP. Dotted lines represent normal values. \( *P < .05 \).

3.3.2. White Blood Cells. Within the first 48 h, CLP resulted in a drop of NEU in all mice with NEU counts typically lower (e.g., by 31% at 24 h and 21% at 72 h) in DIE animals compared to SUR (Figure 7(a)). From 48 h onward, there was a gradual recovery of NEU, which at 72 h either reached (in DIE) or exceeded (in SUR) baseline values.

Similar to NEU, there was a rapid LYM decrease in all mice within the first 48 h of CLP, with initial LYM counts significantly higher (by 17% at 6 h and 51% at 24 h) in DIE compared to SUR (Figure 7(b)). This was followed by a prominent shift between the SUR versus DIE groups: while the 72 h LYM count remained depressed in DIE mice, there was a robust LYM recovery in SUR from 48 h onward. At 72 h, the SUR LYM count was higher by 40% (\( P < .05 \)) (compared to the LYM count in DIE) and continued to increase toward baseline (approx. 84% of the lower baseline range at 96 h) (Figure 7(b)).

3.3.3. Organ Function Parameters. Regardless of the group, preexisting (post-TH) hypoglycemia worsened after the CLP-induced sepsis. Compared to DIE, a slight glucose recovery in SUR mice was noted from 48 h onward (by approx. 20% at 48 h and 23% at 72 h, \( P < .05 \)) (Figure 8(a)). In both DIE and
SUR groups, there was a notable post-CLP increase in AST. The peak inter-group difference (DIE 2-fold higher than SUR) was recorded at 48 h (∗P < .05) but disappeared at 72 h (Figure 8(b)). Such an intergroup separation was even more pronounced in ALT; while its activity continuously increased in DIE from 6 h onward, it was gradually declining (transient peak at 24 h) in SUR reaching baseline by 96 h (Figure 8(c)). The maximal, 6-fold difference in circulating ALT between SUR and DIE mice was recorded at 72 h (0.3-fold at 24 h and 4-fold at 48 h).

Similar after-CLP response was observed for LDH and urea. Both parameters were elevated in DIE mice: urea by 0.3-fold at 6 h and by 3.6-fold between 24–72 h, while LDH by approximately 2-fold at 24 h, 5-fold at 48 h, and 3.5-fold at 72 h time point (∗P < .05) compared to SUR. In SUR mice, both urea and LDH values approached baseline from 48 h onward.

4. Discussion

The primary goal of our study was to reproduce a scenario, in which an initial traumatic/hypovolemic event survived by an ICU-monitored patient is complicated by severe polymicrobial sepsis. By combining the two-hit model with daily blood sampling, we were able to detect potential early and late differences between surviving and dying septic animals and follow the evolution of those changes prior and/or after each of the hits.

Given that an extensive blood loss typically coincides with injury and/or trauma, combining these two elements into an initial challenge is more clinically relevant than referring to established first-hit models consisting solely of hemorrhage [30]. Elimination of either trauma or hemorrhage component may significantly distort the ensuing pathophysiological immuno-inflammatory response, and in effect produce incomplete or erroneous conclusions. The growing awareness to the above-mentioned facts has provoked attempts to develop more representative (two-hit) animal models aimed at investigating the pathogenesis of sepsis as the secondary insult.

Nosocomial (ventilator-associated) pneumonia is a major cause of secondary septic complications in trauma patients with prolonged ICU stays [31, 32] although incidences of polymicrobial sepsis are also reported [33]. An abdominal focus of infection may be viewed as relatively suboptimal given the prevalence of lung-associated secondary infections in polytrauma patients. In our model, however, the cecal ligation and puncture primarily served as a trigger to produce a severe polymicrobial challenge (as opposed to the typically monobacterial lung-associated sepsis) that spreads systemically in a protracted fashion, rather than a model of abdominal peritonitis per se. Moreover, our model was deliberately deprived of relevant intangibles such as preexisting comorbidities (e.g., diabetes, atherosclerosis) and operative interventions that are routinely observed/performed in patients and may significantly skew investigated parameters and outcome [34, 35]. We chose to use healthy mice since any confounding factor in addition to the two-hit challenge would likely generate too much variability and make data interpretation difficult. The CLP challenge itself provides an adequate variability regarding both the outcome and wide range of generated responses. The level of sepsis-induced mortality in our model (approx. 50% at day 5 post-CLP) was carefully set to closely reproduce clinical data (reporting between 20% and 60% incidence of deaths in the septic ICU patients) [12]. This is important since data generated in highly lethal CLP models might artificially enhance predictive value of
Figure 8: Retrospective comparison of circulating glucose, AST, ALT, LDH, and urea in dying (DIE) versus surviving (SUR) animals after-CLP. 3-month-old female mice were subjected to trauma/hemorrhage followed by polymicrobial CLP-induced sepsis. Displayed values were compared between DIE and SUR groups 6 h (DIE n = 23, SUR n = 32; AST: DIE n = 8, SUR n = 20), 24 h (DIE n = 28, SUR n = 34; AST: DIE n = 14, SUR n = 23), 48 h (DIE n = 19, SUR n = 34; AST: DIE n = 9, SUR n = 23), 72 h (DIE n = 12, SUR n = 34; AST: DIE n = 3, SUR n = 23), and 96 h (SUR at least n = 33; AST: SUR n = 23) after-CLP. Dotted lines represent normal values. *P < .05.
some biomarkers [36]. For example, we noted a significant improvement in predictive accuracy for outcome of IL-6 in severe (LD₈₀) CLP model compared to a milder (LD₁₀) CLP injury (unpublished observation).

Both hemorrhage and trauma elements of the first hit were modified from our original protocol [19]: guillotine was replaced with custom-built blunt pliers while the hemorrhage step was initiated immediately after femur fracture (versus a 2 h delay in the previous setup), and the hemorrhage volume was decreased by 20% (to 40% of TBV). In our laboratory, the use of pliers better reproduced blunt soft tissue damage while still providing an excellent reproducibility of noncomminuted, midshaft femur fracture. The hemorrhage modifications were dictated by both the desire to reproduce a more clinically relevant timing and to produce a survivable hypovolemic shock. Given that in trauma patients fractures naturally diverge in terms of location, type and severity of concurrent soft tissue injury and extensive hemorrhage is frequently lethal, a relatively rigorous standardization of these elements can be viewed as the model’s shortcoming rather than advantage. Yet, in this particular two-hit setup, a relatively similar magnitude of the TH hit severity and maximal elimination of lethality are desired, as we aimed to verify our model by studying the outcome-dependent responses triggered by the CLP hit (and not by the TH). Additionally, extrapolability of potential responses indicative of the post-CLP outcome is more relevant if such diverse reactions were produced by a conservatively standardized (hence generating more uniform responses) TH hit. Finally, the adherence to the Russell-Burch “3R” tenet (replace, refine, reduce) should not be overlooked [37].

Profound changes in the immunoinflammatory status caused by trauma/hemorrhage, specifically those propagating development of progressive immunoparalysis, have been postulated to be responsible for the increased susceptibility to secondary sepsis [36]. Although, due to technical constraints, we were not able to determine the cytokine-based immunoinflammatory status of TH-CLP mice, this aspect requires careful evaluation in subsequent experiments. Given that immunoinflammatory fluctuations frequently occur in patients over the period of several hours, a rigorous and continuous surveillance of the ICU patients’ immune status is fundamental in ensuring the most adequate therapeutic strategy (e.g., rapid adjustments of treatment to tailor it to the ongoing shifts of the immune system). To enable such tactics in our TH-CLP model, we subjected all mice to daily low-volume blood sampling via facial vein puncture. This technique provides daily collection of blood (20 µL/mouse) from critically ill mice with virtually no negative effects upon their outcome and/or health status [29]. Despite a limited sample volume available for analysis, advanced and highly sensitive techniques such as multiplex bead assays and various automated biochemistry analyzers allow accurate measurement of dozens of circulating biomarkers and/or cell subpopulations. However, in the context of a joint critical disease/repetitive sampling protocol, its main advantage lies in an ability to investigate a natural evolution of a given disease: any relevant immuno-inflammatory, metabolic, and/or organ function alterations can be followed over time in individual subjects to an undefined outcome. This is in stark contrast to studies in which animals are sacrificed at predefined time points providing only a narrow snapshot assessment of investigated endpoints/pathways without option to match recorded changes with outcome.

In our study, we employed the TH-CLP/monitoring combination to gain preliminary insight into outcome-related mechanisms developing during the acute phase of post-traumatic sepsis. In context of potential posttraumatic septic complications, the period between the first (trauma/hemorrhage) and the secondary (sepsis) challenge may be as decisive as the immediate time span following the onset of sepsis. If relevant risk-correlating marker(s) are identified in the posttraumatic but preseptic phase, one could identify not only subjects with high risk of developing septic complications (posttrauma) but also those who display higher risk of death once sepsis sets in. While this model does not allow any insight into the first scenario (all mice undergo sepsis), it is well matched to investigate the latter situation. We therefore performed outcome-based retrospective analysis of all analysed parameters to detect potential early (pre-CLP) and late (post-CLP) differences between surviving and dying individuals. In the pre-CLP phase, there was a much stronger post-TH (24 h pre-CLP) AST spike (indirectly indicative of hepatic injury) in mice that died within first few days after the onset of peritonitis (compared to survivors), whereas the same cohort (DIE) showed clear and profound metabolic/organ function disturbances in the post-CLP period. Further detailed analysis is needed to establish whether some post-TH mice develop a more intense dysfunction of selected organs prior to sepsis (consequently predisposing them for unfavourable post-CLP outcome) and how exactly organ disturbances drive lethal septic responses both prior and after the onset of sepsis. In any respect, such longitudinal assessments, enabled by more ICU-like tailored models such as the one described above, generate valuable findings that may be then successfully extrapolated to respective clinical scenarios. This in turn may help in identifying ideal time points and/or targets for successful therapeutic interventions in sepsis and other related critical diseases.

5. Conclusion

The modified and clinically relevant TH-CLP model allows a more adequate investigative insight into the course of (polymicrobial) septic complications following trauma. Additional integration of the daily blood sampling into the TH-CLP setup closely emulates one of the major components of the ICU protocol: repetitive monitoring of critically sick patients. The daily monitoring of TH-CLP mice was a basis for outcome-based (retrospective) stratification of responses they generated during both two-hit phases. Such an approach enables in turn correlation of circulating cells/biomarkers to septic outcomes even prior to the development of sepsis. If verified in a clinical setting, findings from such advanced models not only could help
to categorize patients into high- and low-risk of death after their traumatic insult gets complicated by sepsis, but also to prevent the onset of septic sequelae altogether.

Acknowledgments

The study was supported by the Vienna Science and Technology Fund (WWTF Grant no. LS07-065). The authors gratefully acknowledge the expert help of Anna Khadem and Christine Kober with the technically challenging organ function assays.

References


