Aus dem Centrum für Muskuloskeletale Chirurgie
der Klinik für Unfall- und Wiederherstellungschirurgie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Immunomodulation of IL6 transsignaling in polytrauma – organ specific effects of in vivo simulation in a murine multiple trauma model

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Tom Eric Malysch

aus Potsdam

Datum der Promotion: 18.12.2020

To my family

Foreword

The successful implementation of the long-term murine multiple trauma model within this study has been published in the Journal of Orthopaedic Research in July 2015 with the title “Temporal profile of inflammatory response to fracture and haemorrhagic shock: Proposal of a novel long-term survival multiple trauma model”. [40]

Table of contents

List of Figures

List of Tables

List of Abbreviations

ADAM	A disintegrin and metalloproteinase
AIS	Abbreviated injury scale
AO	Arbeitsgemeinschaft für Ostheosynthesefragen
AProf	Assistant Professor
ARDS	Acute respiratory distress syndrome
ATN	Acute tubular necrosis
BSA	Bovine serum albumin
CARS	Compensatory anti-inflammatory response syndrome
CD	Cluster of differentiation
CO_2	Carbon dioxide
CRP	C-reactive protein
DAMP	Danger associated molecular pattern
DCR	Damage control resuscitation
DCS	Damage control surgery
ECG	Electrocardiogram
ECMO	Extra corporal membrane oxygenation
EDRF	Endothelium-derived relaxing factor
ELISA	Enzyme-linked immunosorbent assay
EMS	Emergency medical service
ETC	Early total care
FACS	Fluorescence-activated cell sorting
FFPE	Formalin fixation and paraffin embedding
Fx	Fracture
GCS	Glasgow coma scale
gp130	Glycoprotein 130
Hb	Haemoglobin
Hct	Haematocrit
HE	Haematoxylin eosin
I.D.	Inner diameter
ICU	Intensive care unit
IL1	Interleukin-1

IL6	Interleukin-6
IL6R	Interleukin-6 receptor
INR	International normalized ratio
JAK	Janus kinases
K wire	Kirschner wire
LAGeSo	Landesamt für Gesundheit und Soziales
Ly6G	Lymphocyte antigen 6 complex locus G6D
Mab	Monoclonal antibody
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MOF	Multiple organ failure
NO	Nitric oxide / nitrogen oxide
N_2O	Nitrous oxide
O.D.	Outer diameter
O_2	Oxygen
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCT	Procalcitonin
PE	Polyethylene
PERT	Pre-hospital and emergency department resuscitative thoracotomy
PFA	Paraformaldehyde
PGY1	Post graduate year 1
PhD	Doctor of Philosophy
PICS	Persistent inflammation, immunosuppression and catabolism syndrome
PT	Polytrauma
PTT	Partial thromboplastin time
Raf	Rapidly accelerated fibrosarcoma protein
Ras	Rat sarcoma G-protein
REBOA	Resuscitative balloon occlusion of the aorta
RT	Room temperature
sIL6R	Soluble interleukin-6 receptor

SIRS	Systemic inflammatory response syndrome
STAT	Signal transducer and activator of transcription proteins
TNFα	Tumor necrosis factor α
TRIS	Tris-aminomethane / trometamol
TSR	Transsignaling ratio

Abstract

Introduction

Polytrauma has a bimodal mortality peak. While the majority of mortality occurs prehospital, a significant number of patients die during the ICU phase of care because of a severe immune response, which is caused by the initial interaction of haemorrhagic shock and direct tissue trauma. The resulting ischemia and tissue destruction trigger the release of danger associated molecular patterns (DAMPs), leading to a systemic immune response syndrome (SIRS) resulting in organ failure. Interleukin-6 (IL6) plays a central role within that immune response, which signals through a membrane bound (IL6R) and a soluble IL6 receptor (sIL6R). IL6 and the sIL6R can form an agonistic IL6/sIL6R-complex, activating numerous cells that are usually not IL6 responsive, a process called transsignaling. IL6 transsignaling appears to be an important mechanism in trauma; while it is still poorly understood, it represents a promising target. We attempted to demonstrate that modulation of the IL6 transsignaling can attenuate the devastating immune response after polytrauma.

Methodology

Implementing a novel murine multiple trauma model, mice were assigned to one of three study arms, sham, fracture or polytrauma. The mice were given general anaesthesia and received an ipsilateral femur- and tibia fracture (fracture group), a haemorrhagic shock in addition to the fractures (polytrauma group), or no further intervention (sham group). Half of the animals received the intervention without immunomodulation, while the other half had application of a sIL6R antibody afterwards. After 6 h, 24 h, 48 h or 21 d the mice were euthanised, blood samples were analysed for IL6 and sIL6R serum levels, and organs were analysed for neutrophil infiltration and end organ damage.

Results

IL6 and sIL6R showed a rapid peak concentration at 6 hours after trauma. Both biomarkers increased moderately after fracture and much more markedly after polytrauma. Biomarkers were reduced significantly by blocking transsignaling via sIL6R Mab application. Murine shock organ analysis also illustrated significant neutrophil infiltration 6 hours following polytrauma, which was also abated via sIL6R Mab application. Furthermore, end organ damage was reduced by sIL6R Mab application in murine polytrauma.

Conclusion

The study results highlight the importance of IL6 transsignaling in polytrauma, with haemorrhagic shock being a major trigger of inflammatory response, and especially of sIL6R expression. Modulation of IL6 transsignaling shows promise in the prevention of adverse events like organ failure and should be targeted in future research.

Zusammenfassung (German)

Einleitung

Die Mortalität des Polytraumas stellt sich bimodal dar. Während ein Großteil der Todesopfer prähospital auftritt, verstirbt ein immer noch relevanter Anteil von Patienten während der Intensivtherapie aufgrund einer ausgeprägten Immunreaktion. Diese ist auf das initiale Zusammenwirken von hämorrhagischem Schock und direktem Gewebetrauma zurückzuführen, welches aufgrund von resultierender Ischämie und Gewebezerstörung die Freisetzung von „danger associated molecular patterns“ (DAMPs) bewirkt. Dies führt zu einer systemischen Immunantwort (SIRS), welches Multiorganversagen begünstigt. Hierbei spielt Interleukin-6 (IL6) eine zentrale Rolle, dessen Signalkaskade sowohl membrangebunden (IL6R), als auch mit löslichem Antikörper (sIL6R) möglich ist. IL6 und sIL6R können dabei einen agonistischen Komplex hervorbringen, welcher an zahlreichen, sonst nicht IL6 sensiblen Zellen wirken kann, ein Mechanismus der „Transsignaling“ genannt wird. IL6 Transsignaling scheint auch beim Trauma eine wichtige Rolle zu spielen, obwohl dies bisher noch wenig erforscht ist, weshalb in dieser Arbeit die Relevanz des Transsignaling dargestellt und die Möglichkeit der Immunmodulation zur Reduktion der massiven Immunantwort nach Polytrauma überprüft werden soll.

Methodik

Im Rahmen der Etablierung eines neuen murinen Polytraumamodells wurden Mäuse in die Studiengruppen Sham, Fraktur und Polytrauma aufgeteilt, erhielten eine Narkose und anschließend eine ipsilaterale Femur- und Tibiafraktur (Frakturgruppe), einen hämorrhagischen Schock zusätzlichen zu den Frakturen (Polytraumagruppe) oder keine weitere Intervention (Shamgruppe). Die Hälfte der Tiere bekam keine weitere Intervention, die andere Hälfte erhielt postinterventionell einen sIL6R Mab appliziert. Anschließend wurden die Mäuse nach 6 h, 24 h, 48 h oder 21 d euthanasiert, Blutproben zur Messung von IL6 und sIL6R gewonnen, sowie Organe entnommen zur Beurteilung von neutrophiler Infiltration und Endorganschaden.

Ergebnisse

IL6 und sIL6R zeigten einen rapiden Anstieg bereits 6 h nach Trauma. Beide Biomarker stiegen mäßig nach Fraktur an, zeigten jedoch einen massiven Anstieg nach Polytrauma, welches durch die Blockade des Transsignaling mittels sIL6R Mab wiederum signifikant reduziert werden konnte. Die Analyse der murinen Schockorgane bestätigte ebenfalls eine signifikante neutrophile Infiltration nach 6 h, welche ebenfalls durch die Applikation des sIL6R Mab reduziert werden konnte. Weiterhin viel ein verminderter Endorganschaden nach sIL6R Mab Gabe beim murinen Polytrauma auf.

Schlussfolgerung

Die Studienergebnisse stellen die zentrale Rolle des IL6 Transsignaling beim Polytrauma dar, wobei sich vor allem der hämorrhagische Schock als treibende Kraft der posttraumatischen Immunantwort zeigt, ebenso als Hauptauslöser für die Produktion von sIL6R. Die Immunmodulation des IL6 Transsignaling beschreibt eine vielversprechende Möglichkeit, um die Folgen der posttraumatischen Immunantwort und insbesondere den Endorganschaden zu minimieren und sollte somit weitergehend untersucht werden.

1. Introduction

1.1 Definition and epidemiology of polytrauma

Polytrauma is historically defined as a traumatic injury pattern that in itself, or in combination is potentially lethal [1]. In the clinical setting, diverse terminology is applied internationally, including “multiple trauma”, “major trauma” or “polytrauma”, to describe the subset of most severely injured patients after trauma. The 2014-proposed “Berlin definition of polytrauma” combines an anatomic approach to injuries using an Abbreviated Injury Scale (AIS) ≥ 3 for two or more different body regions, in conjunction with the physiologic response from one of the following: systolic blood pressure ≤90 mmHg, Glasgow coma scale (GCS) ≤8, metabolic acidosis with base excess ≤ -6, coagulopathy with INR ≥1.4 and PTT ≥40 sec, age ≥70 years [2]. Using this definition, polytrauma is the only term to uniformly and sufficiently describe the patient population with this significant injury pattern [3], which accounts for the most fatalities in young adults [4]. Furthermore, polytrauma is predicted to soon outpace infectious diseases worldwide in terms of productive life lost [5].

While the majority of traumatic death occurs in the pre-hospital phase, a second significant mortality peak develops during the intensive care unit (ICU) phase [6]. This means, a significant number of patients, having survived the initial traumatic event, still die after successful resuscitation. Early mortality is most commonly due to traumatic brain injury and/or refractory shock [7]. Therefore, the initial management within the ICU phase is based on restoring circulation and haemostasis, preventing hypoperfusion and hypoxia, as well as treating raised intracranial pressure [8]. Despite this, 50% of polytrauma patients still develop multiple organ failure (MOF) in their further course [9], which is largely as a result of the systemic immune response to severe trauma [10]. This interaction of haemorrhage and tissue trauma, consequently triggering that response, is fundamental in both the understanding and successful management of the polytrauma patient.

1.2 Haemorrhagic shock

Haemorrhagic shock is a form of hypovolemic shock in which severe blood loss leads to inadequate cellular oxygen delivery [11]. In addition to blood loss itself, shock-induced peripheral vasoconstriction contributes to hypoperfusion in organs that are usually well perfused, and these organs are increasingly bypassed from the normal blood supply (shock organs). Consequently, developing ischaemia leads to anaerobic metabolism and ultimately to cell destruction triggering an inflammatory response and end organ damage [12], dependent on the duration and severity of shock / ischemia [13].

Furthermore, oxygen debt and catecholamine surge cause endothelial dysfunction, characterised by a loss of ability to release endothelium-derived relaxing factor (EDRF), and thus a lack of nitrogen oxide (NO) [14]. The failure of endothelial-dependent functions results in reduced vasorelaxation, capillary leak, and thus worsening hypoperfusion. This further promotes inflammation as well as coagulopathy, playing a key role in the “lethal triad of trauma” - coagulopathy, acidosis (due to ischemia as described above) and hypothermia - setting up a vicious cycle leading to MOF [15].

The haemostatic response to trauma is another issue in the context of haemorrhagic shock. Trauma causes platelet activation and interaction of clotting factors [16, 17], which promotes proinflammatory mediators that excite the immune system [18]. Since, increased inflammation leads to further platelet activation [18], as well as the complement pathway (e.g. C5a) acting together with coagulation [19], a self-perpetuating cycle is generated. In severe trauma this leads to disseminated coagulation, and thus coagulopathy, as well as promoting a systemic immune response.

The initial trauma mortality due to exsanguination, the most common cause of preventable traumatic death [20], can be diminished by haemorrhage control (pelvic binder, tourniquets, etc.) and transfusion. However, the end organ damage from haemorrhage-induced hypoperfusion and endothelial dysfunction, resulting in MOF remains a significant challenge represented by the bimodal peak in trauma mortality [6].

1.3 Posttraumatic immune response

The immune system is composed of various components and pathways designed to protect the host from infection. Depending on the pathogen or substance, the immune system becomes activated by recognition of specific molecular structures, either expressed or secreted by the affected cell (MHC complexes, cytokines), or associated with the pathogen itself (pathogen-associated molecular patterns – PAMPs). Furthermore, molecular structures, such as heat shock proteins and mitochondrial DNA, once exposed to the immune system due to cell death, can also be detected by the innate immune system and provoke an immune response [21]. The release of molecules called “danger associated molecular patterns (DAMPs)” or “alarmins” can be caused by several non-infectious causes, such as burns, ischemia or trauma [22]. This initial reaction of an organism to immunological stress is referred to as “acute phase response”.

In polytrauma, severe ischemia due to haemorrhagic shock (as described above) induces a significant DAMP release and thus triggers an immediate and massive acute phase response, in particular within shock organs (e.g. liver, kidney, lungs) [23]. Since DAMP activation primarily refers to the innate immune system, cells like neutrophil granulocytes infiltrate shock organs based on cell priming [23, 24], depending on the level on inflammation [25]. These neutrophils deploy their mediators, such as proteases and reactive oxygen species, against healthy tissues causing localised organ damage, as well as releasing cytokines, all together promoting further inflammation [26].

In addition to ischemic release of DAMPs, direct tissue trauma causes cellular damage with comparable exposure of DAMPs. In that context, it is irrelevant whether tissue destruction is caused by the actual accident or by iatrogenic injury in the form of surgical procedures. Therefore, operative measures can set up a second immunological hit to the trauma patient and should be limited to physiological resuscitation rather than restoration of anatomy, as proposed within the principles of “Damage Control Surgery” (DCS) [27].

With an increasing amount of cellular death due to ischemia based on haemorrhage and direct tissue trauma, the level of inflammatory response increases exponentially, leading to a proinflammatory “cytokine storm” in polytrauma patients, which can result in “systemic inflammatory response syndrome” (SIRS) [28]. This, in conjunction with endothelial dysfunction, shunting and capillary leak reducing microcirculation,

contributes to the development of MOF [29], in addition to the non-inflammatory ischemic cascade inducing MOF (as described above, see 1.2).

Consequently, the initial “trauma load” of a patient directly correlates with the inflammatory response and thus the degree of organ failure later on. Additionally, the extent of medical management, particularly the choice of surgical approach (e.g. damage control surgery), influences the inflammatory response [30]. Thus, it is important to understand the immune response after trauma to predict and direct the further pathway of a polytrauma patient.

1.4 The Interleukin-6 cytokine system

When trauma-associated inflammation occurs due to local DAMP recognition by macrophages, mast cells, fibroblasts or endothelial cells (as described above) via pattern recognition receptors, chemokines like IL1 and/or TNFα are produced by those cells, attracting neutrophil granulocytes to the affected tissues [31]. As the main source of acute inflammation, neutrophil granulocytes release Interleukin-6 (IL6), as well as fibroblasts, macrophages and endothelial cells, which play a leading role in promoting further inflammation via an increased production of acute phase proteins within hepatocytes and augmentation of the cellular and humoral response of the innate immune system [31]. It also triggers the adaptive immune system, by attracting T- and B-cells as well as enhancing T-cell differentiation [32].

IL6 signals through an IL6 receptor consisting of two subunits, IL6 receptor alpha (CD126 or gp80) and the signal transducer protein gp130 (CD130 or IL6 receptor beta) [33]. When IL6 binds to its receptor (consisting of alpha and beta subunit), it initiates activation of the JAK-STAT pathway, as well as the Ras-Raf MAPK pathway, leading to gene transcription [34, 35].

Usually, the IL6 receptor is exclusively expressed on liver cells and leukocytes, but due to alternative splicing and shedding, a soluble IL6 receptor (sIL6R) is formed [33]. In alternative splicing, a common phenomenon described for many membrane-bound proteins, the alternatively spliced sIL6R-mRNA codes for a reading frame shift, without influence on the ligand binding of IL6 and accounts for approximately 10% of sIL6R in humans [36]. In mice, however, alternative splicing of the IL6 receptor is not described [36]. The enzymatic cleavage (shedding) of the IL6 receptor alpha subunit from cell surfaces occurs by the action of metalloproteinases ADAM10 and ADAM17 [37], which are commonly involved in regulating immune functions, proliferative pathways and cancer development [38]. While ADAM10-mediated release is characterised as a slow and constitutive process, ADAM17 is involved in more rapid release during inflammation, due to upregulation as part of the acute phase reaction [39]. Notably, this is not only based on endotoxemic inflammatory triggers, but “sterile triggers” such as haemorrhagic shock [40].

Notably, IL6 and the sIL6R can form an agonistic IL6/sIL6R-complex, activating cells that express gp130. Since gp130 is a widely spread transmembrane protein, IL6 extends its sphere across the organism, contributing to an overwhelming immune

response [41]. This mechanism is called transsignaling and is known to be factor in trauma, sepsis, cancer and autoimmune disease [30]. The literature shows that especially in areas with high volumes of neutrophil granulocytes, and thus acute inflammation, the amount of IL6 and sIL6R is elevated, leading to increased transsignaling.

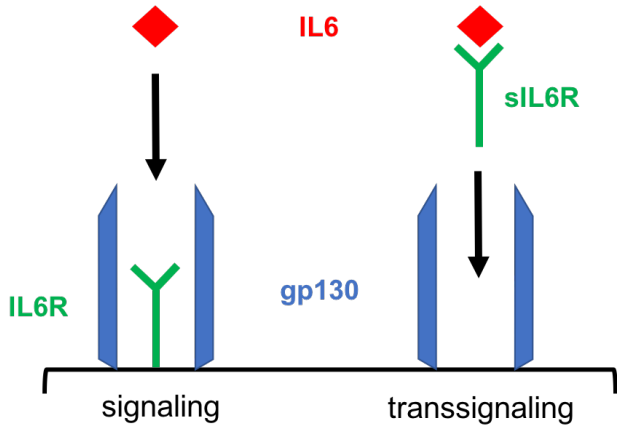

Figure 1 - IL6 signalling via membrane-bound IL6 receptor and transsignaling via soluble IL6 receptor.

Elevated IL6 serum levels are known to be associated with worse outcome in acute coronary syndrome [42], stroke [43] and cancer [44], as well as the severity of organ failure and mortality in critically ill patients [45, 46], by inducing further inflammatory pathways (as described above). Consequently, IL6 transsignaling, as one of the two IL6 signalling pathways, is involved in those inflammatory driven processes [47]. Furthermore, transsignaling leads to an increased signal strength compared to regular signalling via the membrane-bound IL6 receptor, because of reduced internalization of IL6 [48].

In Cardiovascular disease, IL6 transsignaling seems to be responsible for inflammation-related atherogenesis [49]. With 10-30% of cancer patients dying from cachexia [50], IL6 transsignaling has a possible role in cachexia development by inducing elevated macroautophagy, generating nutrients that benefit cancer cells to sustain tumor growth [51]. In sepsis IL6 levels correlate well with survival, although the membrane-bound IL6 signalling does not appear to affect mortality, but transsignaling was found to be of paramount importance in septic shock, due to the induction of vasodilation and capillary leak [52]. Lastly, IL6 transsignaling seems to have a major role in trauma [30], but is currently still poorly understood. Therefore, further evaluation of this mechanism is needed.

1.5 Transsignaling in polytrauma

Evidence on the role of transsignaling in polytrauma is still in its infancy. Most prior studies have focused on fracture healing in the context of transsignaling [53]. However, one preliminary clinical study demonstrated that 6-24 hours after trauma serum IL6 concentrations peaked, and were gradually elevated in comparison to less severely injured patients and non-injured patients [30]. IL6 increased faster than commonly used clinical inflammatory biomarkers such as CRP or PCT [54]. Further, sIL6R concentrations were shown to be lower in polytrauma patients, and are thus indicative of an increased transsignaling mechanism [30]. Supporting this hypothesis, the incidence of MOF and overall mortality is significantly increased in patients with a higher transsignaling ratio, a ratio implemented within that study to represent and estimate the transsignaling process [30]. This data is presented by one of the few papers suggesting the importance of transsignaling in polytrauma. Additionally, blocking the transsignaling process is known to improve fracture healing [53, 55], as well as improving outcome in severe inflammation [56]. Consequently, IL6 transsignaling represents an important mechanism to investigate trauma mortality and survival, as well as a promising target for immunomodulation in trauma.

1.6 Aim of dissertation

With IL6 transsignaling playing a key role in various critically ill patients (as described above), understanding this mechanism in trauma and employing immunomodulation in IL6 transsignaling may offer the potential to reduce MOF following polytrauma, and thus diminish trauma mortality, along with its associated global burden of major clinical and socioeconomic impact [57]. Therefore, the aim of this dissertation is to demonstrate that modulation of the IL6 transsignaling can reduce immune response following polytrauma.

We hypothesise that:

- IL6 serum levels rise after trauma and reflect the severity of trauma
- Applying an sIL6R antibody reduces IL6 serum levels
- sIL6R serum levels change after trauma, especially following haemorrhagic shock
- Applying an sILR6 antibody normalises sIL6 serum levels
- Transsignaling Ration (TSR) can be used to estimate the transsignaling process
- Applying an sILR6 antibody diminishes IL6/sIL6R transsignaling
- Polytrauma induces neutrophil infiltration in shock organs
- Applying an sILR6 antibody reduces the neutrophil infiltration in shock organs
- Polytrauma leads to tissue damage in shock organs
- Applying an sILR6 antibody decreases tissue damage in shock organs
- Polytrauma leads to end organ failure
- Applying an sILR6 antibody can reduce end organ failure from polytrauma

2. Methodology

2.1 Overall study design

12- to 14-week-old female C57BL/6N mice were housed at 22 ± 2 °C with a 12 h light/day cycle in macrolan-cages with conventional mouse pellet food and water *ad libitum*. One week after acclimatisation, animals were randomly assigned to three study groups and received the respective intervention. Following the intervention, the mice were kept in cages (see above) and received postoperative analgesia (first 3 days) with tramadol enriched drinking water (25 mg/L). At a pre-set time, blood samples were taken, the mice were euthanised and their organs were harvested for further histological analysis.

The experiments were performed according to international regulations
for animal experimentation and were approved by the local animal protection authorities (Landesamt für Gesundheit und Soziales, Government of Berlin –
LAGeSo, Nr: G 0274/10).

2.2 Study groups

The mice were assigned to three major study groups:

I: Sham (no operation; n = 30);

II: Fracture [Fx] (femur/tibia fracture; n = 48);

III: Polytrauma [PT] (haemorrhagic shock & fracture; n = 48)

Within groups II and III, half of the animals (n = 24) were operated on without further postoperative immunomodulation (“no modulation“ subgroups – groups IIa and IIIa); the other half received an sIL6-receptor antibody (“sIL6R Mab“ subgroups – groups IIb and IIIb) (100 µg, LEAF™ purified anti-mouse CD126 antibody IL6-Rα chain, BioLegend, San Diego, USA) following the operative procedure by intraperitoneal injection.

Within each subgroup, 6 mice were euthanised after 6 h, 24 h, 48 h and 21 d respectively. Within the sham group, only the antibody subgroup (group Ib) was subjected to the same protocol. The “no modulation“ subgroup (group Ia) was limited to 6 animals with all being euthanised at the same time point due to “reduction“ according to the 3Rs concept of experimental techniques [58].

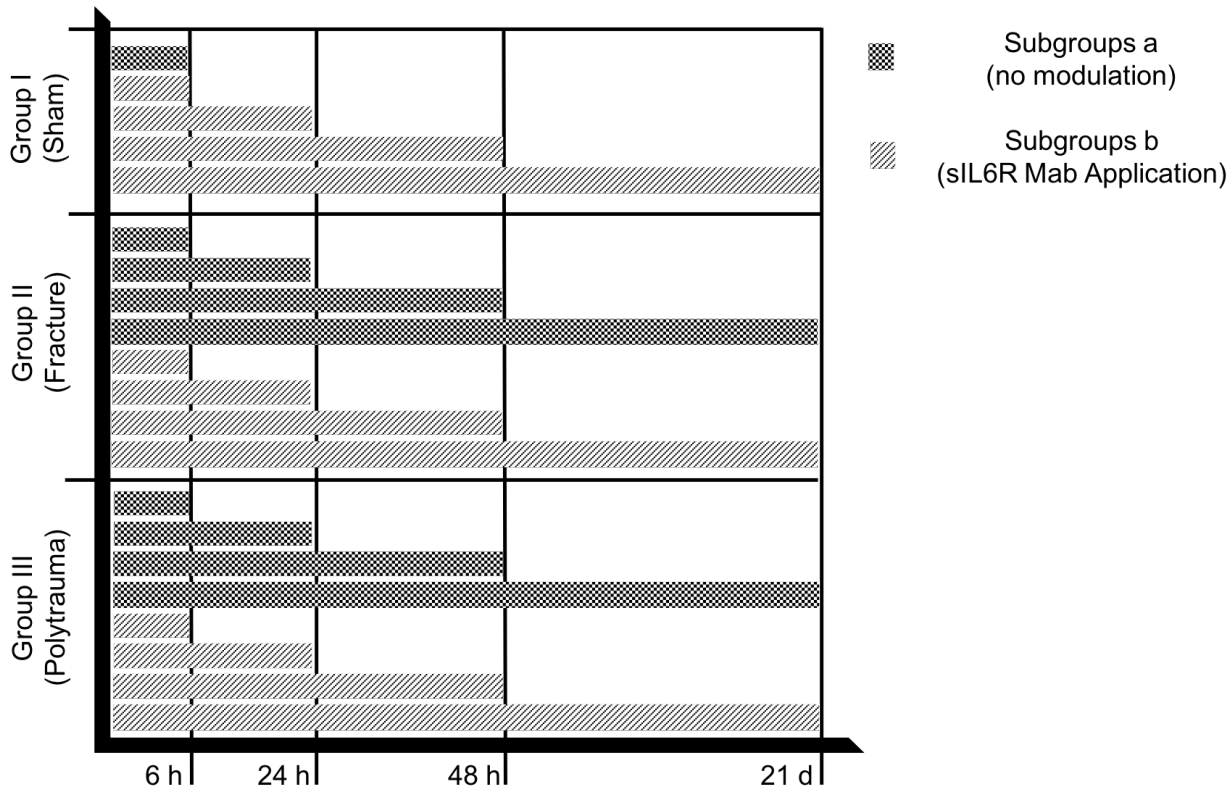

Figure 2 - Groups and subgroups of murine multiple trauma model. Each column represents 6 mice.

2.3 Murine multiple trauma model

2.3.1 General anaesthesia and monitoring

Within our newly proposed long-term survival murine multiple trauma model, it was possible to keep perioperative mortality for the polytrauma group (group III) below 17% by using sophisticated perioperative monitoring, without any death in the sham or fracture group (group I and II) [40]. In the protocol, induction was achieved in spontaneously breathing mice via exposure to volatile isoflurane anaesthesia (isoflurane 1.6 vol%; N_2O 0.3 L/min, O_2 1.8 L/min) using a custom-made mask (incl. exhaust system for backflow and expired gas) connected to a vaporizer. Additionally, mice were weighed and subcutaneous buprenorphine (0.03 mg/kg bodyweight) was administered. The correct depth of anaesthesia was monitored using respiratory rate (60 $\pm$ 10/min) and heart rate (350 $\pm$ 50/min). Perioperative antimicrobial prophylaxis was achieved by subcutaneous application of clindamycin (8 mg/kg bodyweight) as a standard mean within orthopaedic trauma surgery. The mice were positioned supine on a thermo-controlled heating plate (37 ± 0.5 °C, feedback-controlled by rectal temperature) for maintenance of body temperature. An electrocardiogram (ECG) was connected by needle electrodes (AD Instruments) to both fore and left hind leg. For monitoring and invasive blood pressure measurement, AD Instruments Power Lap 4/30, Animal Bio Amp ECG, Bridge Amp, Memscap SD844 physiological pressure transducer and LapChart 7 software (AD Instruments, Dunedin, New Zealand) were used.

2.3.2 Haemorrhagic shock

The induction of haemorrhagic shock was performed using a combination of the models proposed by Wichmann and Vollmar [59, 60]. Under general anaesthesia the mouse was placed in supine position for microsurgical cervical preparation. A Polyethylene PE10 tubing (I.D. 0.28 mm, O.D. 0.61 mm; Schubert Medizinprodukt) was inserted through the right common carotid artery (see Figure 3b) to measure arterial blood pressure (physiological pressure transducer, AD Instruments). Immediate blood backflow from the artery was used to obtain the first blood gas analysis (Na-heparin haematocrit-capillaries 60 µL; Hirschmann, Germany; ABL800 Flex, Radiometer, Germany). For induction of haemorrhagic shock, the animals were bled to a mean arterial blood pressure (MAP) of 35–40 mmHg. MAP was maintained at that level for 60 min, either by further blood

withdrawal or infusion of Ringer-solution (Braun Melsungen; Germany). pH, lactate, haemoglobin (Hb) and haematocrit (Hct) were routinely measured after induction of shock, as well as the partial pressures of CO_2 and O_2 to ensure sufficient oxygenation and ventilation (table 01). For resuscitation and thus shock termination, the animals received Ringer-solution at a volume equal to 1.5 times the shed blood volume within 20 min. After removal of the catheter, ligation of the carotid artery and suture of the skin using Prolene 5.0 (Ethicon), the mouse was transferred into a right lateral position for performance of femoral osteotomy and closed tibia fracture.

Table 1 - Blood gas analysis (median, IQR = interquartile range, SD = standard deviation) in polytrauma group (group III) after induction of haemorrhagic shock

	serum level	IQR	SD	unit
pH	7.19	0.14	0.07	
paCO2	38.9	21.0	16.5	mmHg
paO2	385.0	304.0	158	mmHg
HCO3	15.6	4.1	3.7	mmol/l
BE	-12.4	3.2	3.6	mmol/l
Hb	13.2	1.4	1.4	g/dl
Hct	40.5	4.3	4.2	%
lactate	26.0	15.0	8.2	mg/dl

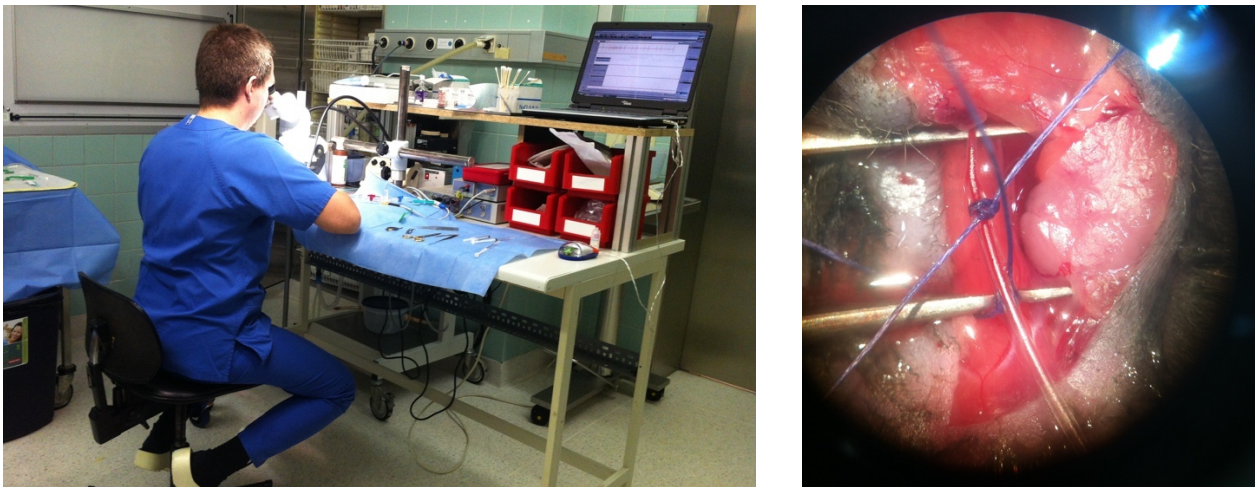

Figure 3a & b - Reinhold, JM. 2013. Murine trauma model working bench (3a, left) and microscopic (surgeon‘s) view of carotid artery with PE tube in place for invasive blood pressure monitoring (3b, right)

2.3.3 Fractures

A combination of ipsilateral femur and tibia fracture was used to increase the trauma load mimicking multiple skeletal trauma.

Simulation of a femur fracture was achieved via standardised midshaft osteotomy, after shaving and preparing with povidone iodine, opening the skin and fascia between the femoral flexor and extensor muscles, followed by blunt dissection along the intermuscular septum for exposure of the lateral aspect of the femoral cortex, and using a gigli-saw (Ø= 0.66 mm, AO Development Institute Davos, Swiss) for transverse femoral osteotomy. Prior to completion of femoral osteotomy, a mouse-specific external fixator (MouseExFix, RISystem, Davos, Switzerland) was installed with two pins each, proximal and distal to the intended osteotomy site and mounting of the stabiliser. Wound closure was achieved with Prolene 5.0 (Ethicon).

The standardised tibia fracture was achieved via a modified weight drop three-point bending technique, regularly resulting in a AO-type B tibia and fibula fracture with only moderate closed soft tissue damage (Grade II according to Tscherne) [61]. The tibia fracture was subsequently fixed using an antegrade intramedullary K-wire (BD Microlance 3, 27 G, 3/4´, 0.4 x 19 mm, Heidelberg, Germany), which was inserted medially to the patellar tendon. Again, wound closure was achieved with Prolene 5.0 (Ethicon).

Prior to termination of anaesthesia, both fractures (femur and tibia) and corresponding fixations were radiographically verified in two planes (28 kV, 8 sec, Faxitron MX20, Illinois, USA).

2.3.4 Euthanasia

According to prior randomisation, mice were euthanised at 6 h, 24 h, 48 h or 21 d after intervention (as described in 2.2). All animals were deeply narcotised via intraperitoneal application of ketamine (60 mg/kg bodyweight) and medetomidine (0.3 mg/kg bodyweight) prior to cardiac puncture with lethal blood aspiration, followed by hyperextension of the cervical spine. Afterwards, organs known to be affected by shock (liver, spleen, kidneys) were harvested through midline laparotomy.

2.4 Blood sampling and ELISA

Blood samples were drawn in terminally anaesthetised mice via cardiac puncture, prior to hyperextension of the cervical spine. After 30 – 60 min the samples were centrifuged, and the serum was pipetted into vials and stored at -80 °C until ELISA analysis was performed.

The IL6 serum concentrations were quantified in doublets by ELISA (dilution 1:2; Mouse IL-6 Quantikine Immunoassay M6000B; R&D Systems, Wiesbaden-Nordenstadt, Germany). The sIL6R serum levels were measured by ELISA in triplets (dilution 1:32; Mouse IL-6 R alpha DuoSet DY1830; R&D Systems). These samples (dilution 1:32) had previously been validated by spike and recovery test (100%, R&D Systems).

ELISA readout was performed within 30 min of finishing the assay using a microplate reader at 450 nm with correction at 570 nm, and evaluated with Microplate Manager Version 5.2 software (Bio-Rad Laboratories, Inc., Hercules, CA).

The Transsignaling ratio (TSR) was calculated by the quotient of IL6 (pg/ml) and sIL6R (ng/ml); therefore measured sIL6R serum levels were multiplied by a factor of 10^{-3}.

2.5 Histology of shock organs

2.5.1 Paraffin processing

Fixation and embedding

Shock organs were prepared for paraffin embedding utilising 4%-paraformaldehyde solution (PFA; Electron Microscopy Sciences, USA) for 48 h at room temperature (RT) immediately after harvesting, followed by a 45 min tap water flush, and lastly storage using 70% ethanol solution at 4 °C until further processing, which was performed using an automated tissue processor (Leica TP1020, Leica Biosystems, Germany).

Samples were put into a cassette system (Tissue Tek mega cassette system, Sakura Finetek, Japan), set into the tissue basket of the tissue processor, and processed as follows:

1. 70% ethanol for 60 min
2. 80% ethanol (#1) for 60 min
3. 80% ethanol (#2) for 120 min
4. 96% ethanol (#1) for 120 min
5. 96% ethanol (#2) for 120 min
6. 100% ethanol (#1) for 120 min
7. 100% ethanol (#2) for 120 min
8. 100% ethanol (#3) for 180 min
9. Xylene (#1) for 60 min
10. Xylene (#2) for 90 min
11. Paraplast wax (#1) for 120 min at 58 °C
12. Paraplast wax (#2) for 120 min at 58 °C

Afterwards, tissue samples were placed separately into a 58 °C melted paraffin bath for 15 min and then put onto a cooling plate for approximately 30 min to solidify.

Table 2 - Chemicals in paraffin embedding and fixation

Chemical	Company
Ethanol	Carl Roth, Germany
Xylene	J.T. Baker, USA
Paraplast wax	McCormick Scientific, USA

Sectioning

For sectioning, a microtome (RM2325, Leica Bioscience, Germany) was used, producing 5 µm sections, and these were transfered onto the surface of a 37 °C water bath (deionised water) and prepared onto slides (microscope slides, Marienfeld, Germany). Slides were put onto a warming block at 65 °C for 20 min and then stored at room temperature until staining.

Staining

Mayer's haematoxylin / eosin (HE) stain was used for paraffin-embedded lungs, kidneys, livers and spleens.

Table 3 - Mayer's HE staining protocol

Procedure	Company	Time	Temp.
4% formaldehyde	Electron Microscopy Sciences	15 min	RT
Distilled water		2x 5 min	RT
Haematoxylin	Merck Millipore	2 min	RT
Tap water		repetitive	RT
Tap water		5 min	RT
Eosin	Chroma Waldeck	1.5 min	RT
96% ethanol	Roth	2x 2 sec	RT
100% ethanol	Roth	2x 2 min	RT
Xylene	J.T. Baker	2x 2 min	RT
Vitrocloud	Langenbrinck		RT

Haematoxylin was used in a 1:2 dilution with distilled water. Following the haematoxylin stain, probes were flushed with tap water until the water had cleared and were left in tap water for 5 minutes afterwards.

2.5.2 Cryo processing

Fixation and embedding

Shock organs were prepared for cryo embedding (frozen section embedding) utilising 4%-paraformaldehyde solution (PFA; Electron Microscopy Sciences, USA) for 2 h at 4 °C immediately after harvesting, followed by a 10%-, 20%- and 30%-sugar solution for 24 h each, at 4 °C. Afterwards, organs were embedded into SCEM medium (SECTION-LAB Co. Ltd, Japan) and immersed into n-hexane, which itself was immersed into cold acetone, prepared with dry ice. Frozen samples needed to be stored immediately at −80 °C. This protocol is superior to traditional formalin fixation and paraffin embedding (FFPE) for immunohistochemistry, due to milder effects on the molecules' cross-linking [62].

Sectioning

For cryo sectioning, a refrigerated microtome (Cryostat, Leica CM3050 S, Leica Bioscience, Germany) was used at -19 °C, producing 5 µm sections onto charged adhesion slides (Superfrost OT, ThermoScientific). Slides with cryo sections were brought to room temperature and stored at -80 °C until staining.

Staining

Neutrophil leukocytes were stained using a Ly6G antibody (Ly6G/GR1 Mouse AM26331PU-N, Host: Rabbit, Acris Antibodies).

Table 4 - Ly6G staining protocol

Procedure	Company	Time	Temp.
Assemble Slides			RT
Acetone	SIGMA Aldrich	20 min	-20 °C
PBS 1:10	SIGMA Aldrich	2x 5 min	RT
Blocking	See below	30 min	RT
Primary antibody	See below	12 h	4 °C
PBS 1:10	See above	2x 5 min	RT
Secondary antibody	See below	30 min	RT
PBS 1:10	See above	2x 5 min	RT
AB Complex	BIOZOL Diagnostika	50 min	RT
PBS 1:10	See above	2x 5 min	RT
Chromogen buffer	See below	2x 5 min	RT

Substrate solution	BIOZOL Diagnostika	20 min	RT
PBS 1:10	See above	2x 5 min	RT
Distilled water		2x 5 min	RT
Methyl green	Merck Millipore	15 min	RT
96% ethanol	Roth	2 sec	RT
100 % ethanol	Roth	2x 2 min	RT
Xylene	J.T. Baker	2x 2 min	RT
Vitrocloud	Langenbrinck		RT

The primary antibody consisted of a Ly6G antibody (see above) in a 1:25 dilution with diluent (Dako, Agilent Pathology Solutions). Blocking was achieved with 5%-neutral rabbit serum (BIOZOL Diagnostika) in PBS-2%BSA solution, composed of PBS solution in a 1:10 dilution with distilled water and the addition of BSA (Sigma Aldrich) to constitute a 2% concentration.

The secondary antibody utilised 2% biotinylated, anti-rat, mouse adsorbed antibody (Vector Laboratories, USA) and 2% neutral-serum rabbit (BIOZOL) in a PBS-2%BSA solution. The chromogen buffer consisted of 3.96 g TRIS HCl (Sigma Aldrich), 0.54 g TRIS Base (Sigma Aldrich), 2.63 g NaCl (Merck Millipore) and 300 ml distilled water.

Mayer’s haematoxylin / eosin (HE) stain was used for additional analysis of kidneys. The treatment was to the same protocol as in 2.5.1 (paraffin processing).

2.5.3 Microscopy

Ly6G and HE stains were scanned with a Zeiss microscope (Axioskop 40) at 10 times magnification using the AxioCam MRc5 and AxioVision imaging software (Carl Zeiss Microscopy, release 4.8.2 SP3). Analysis was performed with ImageJ image processing software, version 1.51p (National Institute of Health, USA).

2.5.4 Analysis

Initially, paraffin-embedded sections of livers, kidneys, spleens and lungs of group IIa (fracture without immunomodulation) and group IIIa (polytrauma without immunomodulation) were analysed at 24 h with HE stain. Neutrophils were counted and subclassified into “banded” (young) and “segmented” (mature) using the following definition of the College of American Pathologists: “The nucleus is centrally or eccentrically placed and indented to more than half the distance from the farthest nuclear margin. The nucleus may appear in the shape of a band, sausage, letters C or U, or may be lobulated. If lobulated, the bridge or isthmus between the lobes must be wide enough to have two distinct parallel dark margins with light nuclear material in between” [63].

For further evaluation, cryo embedded sections of livers, kidneys and spleens of all study groups were analysed utilising Ly6G stain for neutrophil infiltration, using the imageJ manual counting tool (NIH, USA, version 1.51p). Counts were readjusted to the organ size (cells per cm²) for better comparability of the samples, and evaluated through the colour-, brightness-, and density-threshold settings of imageJ. The kidneys were re-analysed in HE stain, due to incompatibility of optic Ly6G stain and tubular background stain within kidneys.

During analysis, the observer was blinded for group and subgroup (including point of time) of analysed samples. Random samples were additionally and independently analysed by a second observer. These counts were included into the analysis by using the mean-value of both results.

2.6 Macroscopic lung analysis

To estimate extravascular lung water as a surrogate parameter for acute lung injury [64, 65], the right lung of each mouse was withheld from histological processing and put into a warming cupboard at 37 °C for three days. Lung weight was measured before and after the warming phase, to calculate the net difference (delta lung weight), representing the vaporised lung water.

2.7 Statistics

Statistical analysis was performed using IBM SPSS Statistics, Version 24 (IBM, USA). For statistical evaluation, the Mann-Whitney-U test (non-parametric, unpaired samples) was used. Data is displayed as mean values and standard deviations for parametric data and as median and interquartile range (IQR) for non-parametric data. Statistical significance was assumed with a p-value of 0.05 or less, but the data has explorative character and needs to be interpreted considering that limitation. Therefore, no adjustment for error-accumulation has been performed.

3. Results

3.1 ELISA

3.1.1 IL6

IL6 serum levels were significantly elevated at 6 h post trauma (group Ia vs. group IIa at 6 h $p < 0.01$; group Ia vs. group IIIa at 6 h $p = 0.01$) and returned to baseline from 24 h onwards, without relevant difference to group Ia. Within the polytrauma group the IL6 peak was significantly higher than in the fracture group (group IIa at 6 h vs. group IIIa at 6 h $p = 0.01$), without significant difference in the further course until 21 days.

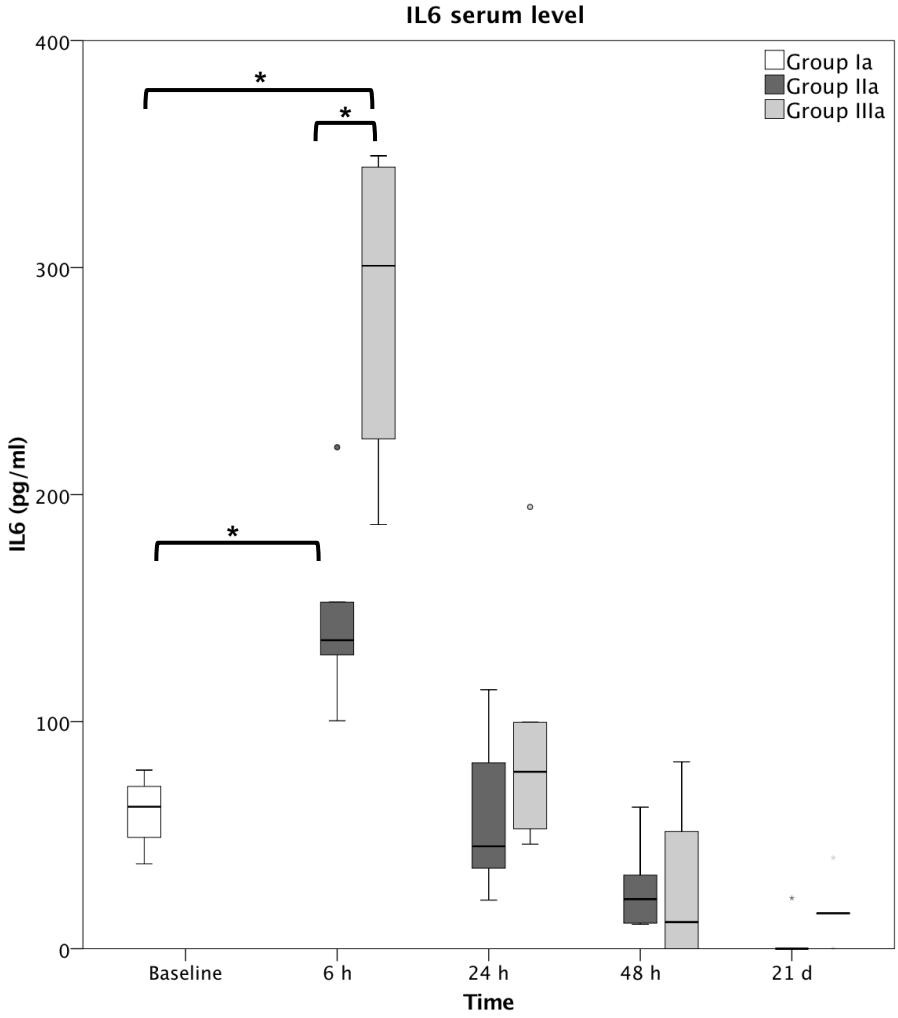

Figure 4 – IL6 serum levels following fracture without immunomodulation (group IIa) and polytrauma without immunomodulation (group IIIa). Group Ia represents sham mice. * represents $p \leq 0.05$.

When sIL6R Mab was applied to the polytrauma group (group IIIb), the IL6 serum levels were higher at 6 h than the IL6 serum levels of the polytrauma group that did not receive sIL6R Mab and sham (group IIIa at 6 h vs. group IIIb at 6 h p = 0.06, group Ia vs. group IIIb at 6 h p = 0.06), which was not significant. From 24 h until 21 d, IL6 serum levels of both polytrauma groups and sham approximate another, without significant difference between the groups.

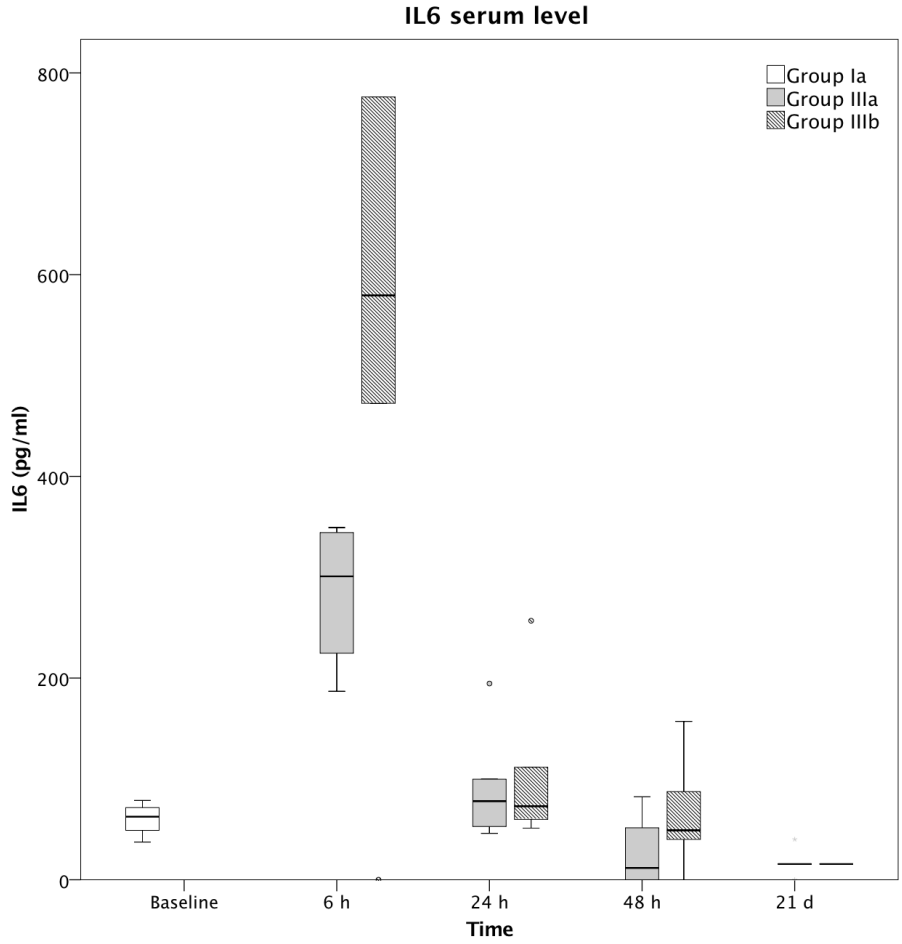

Figure 5 – IL6 serum levels following polytrauma with immunomodulation (group IIIb) and without immunomodulation (group IIIa). Group Ia represents sham mice. * represents p ≤ 0.05.

3.1.2 sIL6R

At 6 h and 24 h after fracture, sIL6R serum levels were significantly elevated from baseline (group Ia vs. group IIa at 6 h $p < 0.01$; group Ia vs. group IIa at 24 h $p < 0.01$), resetting around baseline at 48 h and 21 d. The same dynamics apply to the polytrauma group, but without significance (group Ia vs. group IIIa at 6 h $p = 0.06$, group Ia vs. group IIIa at 24 h $p = 0.18$). Among fracture and polytrauma groups (groups IIa and IIIa), no significant difference could be seen.

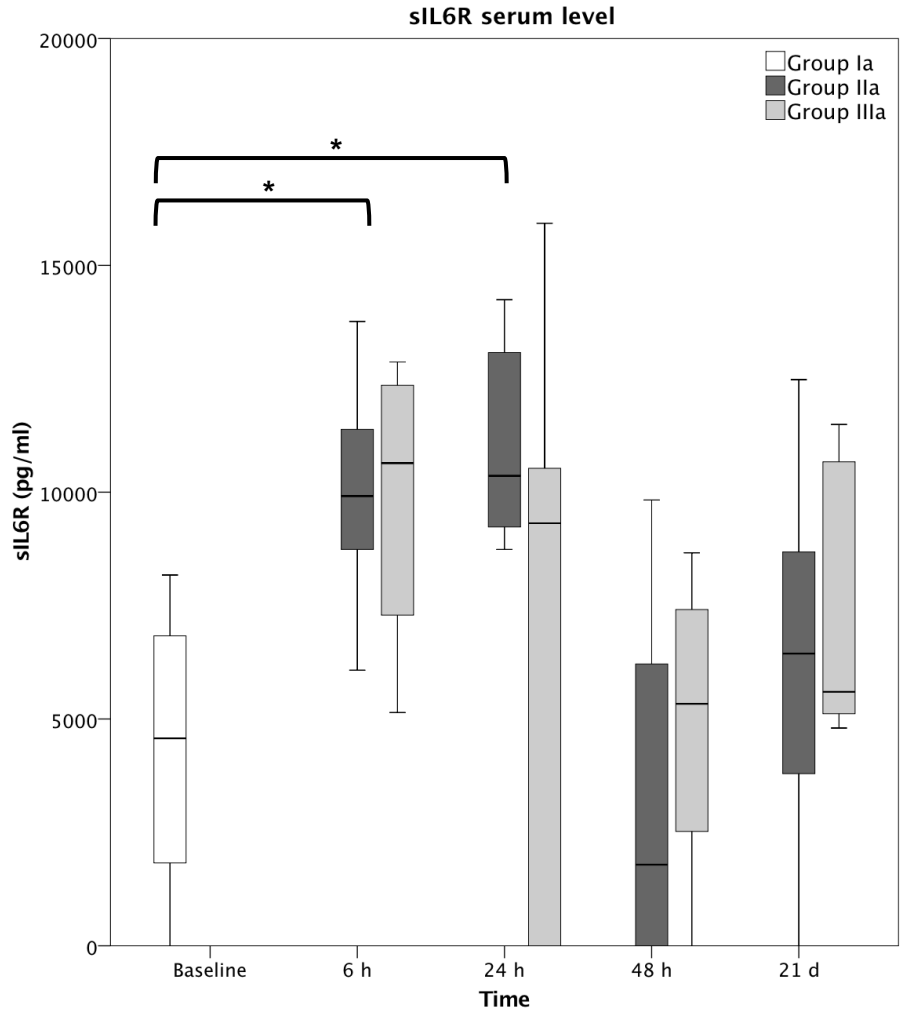

Figure 6 – sIL6R serum levels following fracture without immunomodulation (group IIa) and polytrauma without immunomodulation (group IIIa). Group Ia represents sham mice. * represents $p \leq 0.05$.

When sIL6R Mab was applied to the polytrauma group (group IIIb), sILR6 serum levels rose significantly compared to the previous levels of group IIIa at 6 h, 24 h and 48 h, all also being significant compared to baseline.

Group IIIa at 6 h vs. group IIIb at 6 h	p = 0.01
Group IIIa at 24 h vs. group IIIb at 24 h	p < 0.01
Group IIIa at 48 h vs. group IIIb at 48 h	p < 0.01
Group Ia vs. group IIIb at 6 h	p = 0.01
Group Ia vs. group IIIb at 24 h	p = 0.01
Group Ia vs. group IIIb at 48 h	p = 0.01

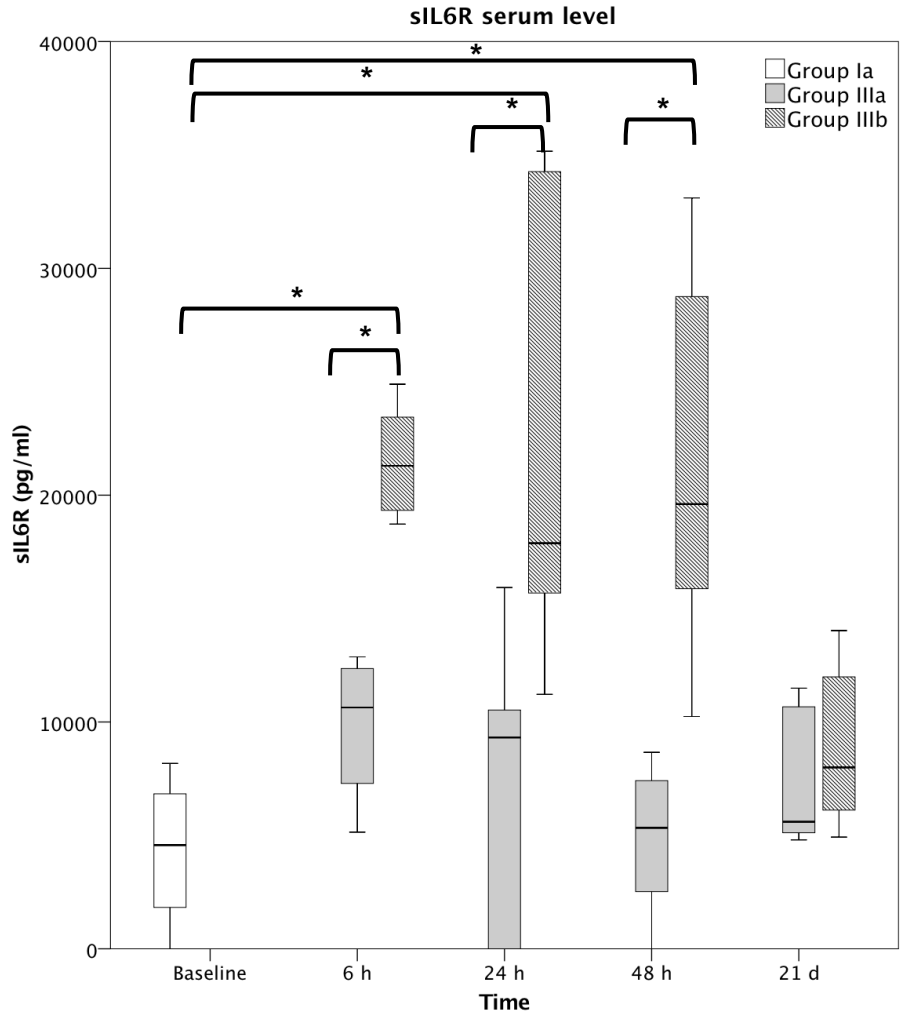

Figure 7 – sIL6R serum levels following polytrauma with immunomodulation (group IIIb) and without immunomodulation (group IIIa). Group Ia represents sham mice. * represents p ≤ 0.05.

3.1.3 TSR

The TSR at 6 h shows a significant change from baseline (group Ia vs. group IIa at 6 h $p < 0.01$; group Ia vs. group IIIa at 6 h $p = 0.03$), showing a significant difference between the fracture and polytrauma group at 6 h, 24 h and 21 d, but not at 48 h (group IIa at 6 h vs. group IIIa at 6 h $p = 0.02$; group IIa at 24 h vs. group IIIa at 24 h $p = 0.01$, group IIa at 21 d vs. group IIIa at 21 d $p < 0.01$).

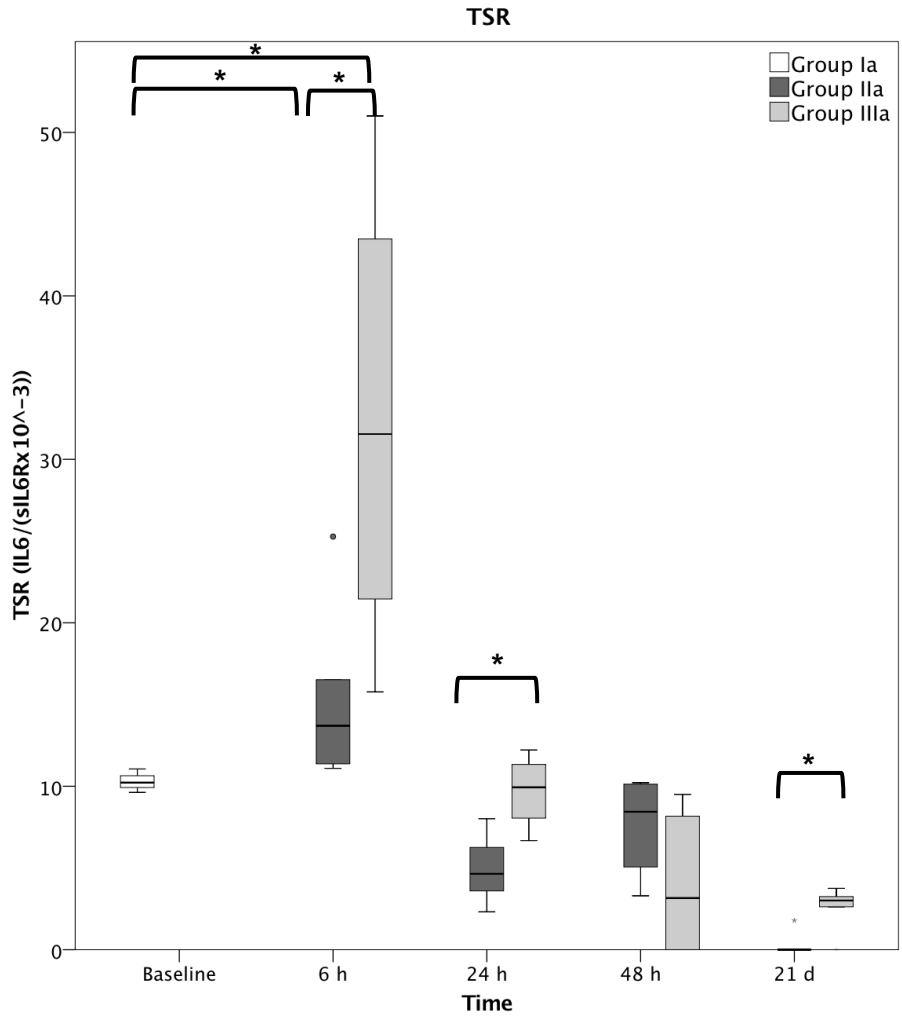

Figure 8 – Transsignaling ratio following fracture without immunomodulation (group IIa) and polytrauma without immunomodulation (group IIIa). Group Ia represents sham mice. * represents $p \leq 0.05$.

Application of sIL6R Mab in polytrauma (group IIIb) leads to a decrease in TSR at 6 h and significantly at 24 h (group IIIa at 6 h vs. group IIIb at 6 h p = 0.46, group IIIa at 24 h vs. group IIIb at 24 h p = 0.02). There was no significant difference at 48 h and 21 d from previous TSR levels.

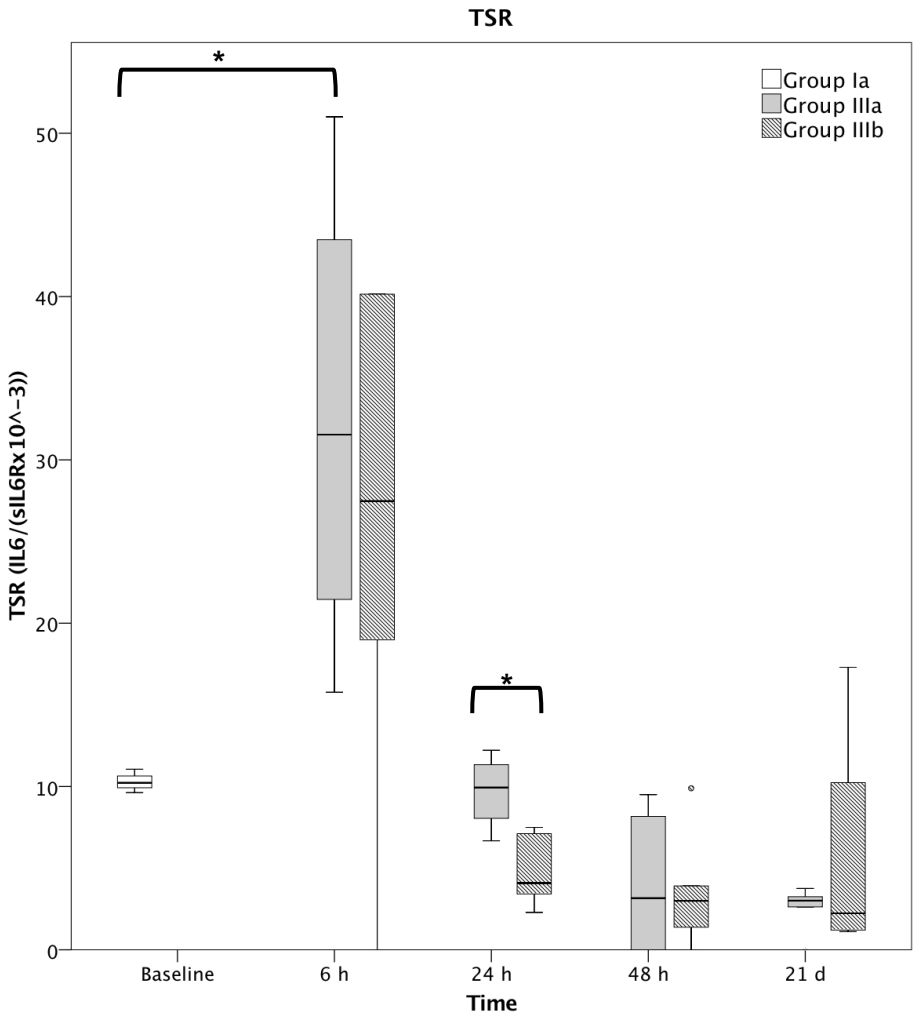

Figure 9 – Transsignaling ratio following polytrauma with immunomodulation (group IIIb) and without immunomodulation (group IIIa). Group Ia represents sham mice. * represents p ≤ 0.05.

3.2 Shock organs

3.2.1 Overall

In comparison to group IIa at 24 hours (fracture without immunomodulation), group IIIa at 24 hours (polytrauma without immunomodulation) had an increased accumulation of neutrophil granulocytes within all shock organs, being significant within lungs, kidneys and spleen.

Table 5 – Neutrophil granulocyte count following fracture and polytrauma at 24 hours without immunomodulation (groups IIa and IIIa at 24h).

organ	neutrophil granulocytes	group IIa - 24h	group IIIa - 24h	p-value
lung	banded	0	4.3 ± 2.3	0.005
	segmented	0.5 ± 0.8	25.7 ± 2.3	0.02
liver	banded	0.3 ± 0.5	2 ± 2.1	-
	segmented	12.7 ± 2.6	23 ± 7.7	-
kidney	banded	0.17 ± 0.4	1.2 ± 1.46	-
	segmented	2.5 ± 1.6	21.8 ± 5.2	0.006
spleen	banded	0.33 ± 0.52	2.5 ± 1.6	0.008
	segmented	4.67 ± 1.8	26.5 ± 2.3	0.004

3.2.2 Liver

The neutrophil count in the liver at 6 h after polytrauma was significantly higher than after fracture only (group IIa at 6 h vs. group IIIa at 6 h p= 0.05); however, the change from baseline was not significantly higher (group Ia vs. group IIIa at 6 h p = 0.1). From 24 h onwards neutrophil count returned to baseline, without any significant difference between the fracture and polytrauma groups (groups IIa and IIIa).

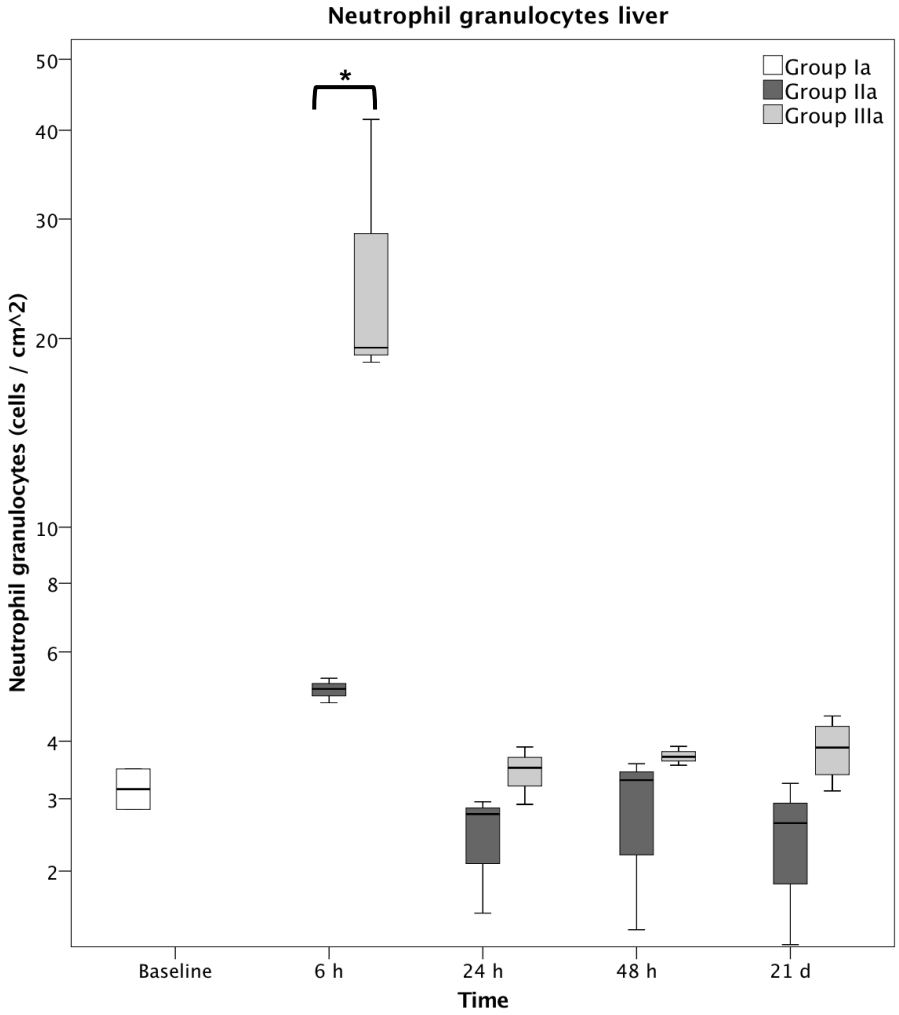

Figure 10 – Neutrophil granulocytes in liver following fracture without immunomodulation (group IIa) and polytrauma without immunomodulation (group IIIa). Group Ia represents sham mice. * represents p ≤ 0.05.

The elevated neutrophil count at 6 h after polytrauma was significantly reduced after application of sIL6R Mab (group IIIa at 6 h vs. group IIIb at 6 h p = 0.03). At 24 h and 48 h neutrophil counts did not show any significant difference from baseline or between groups. After 21 d neutrophils dropped below baseline within group IIIb, with no statistical significance between groups Ia or IIIa.

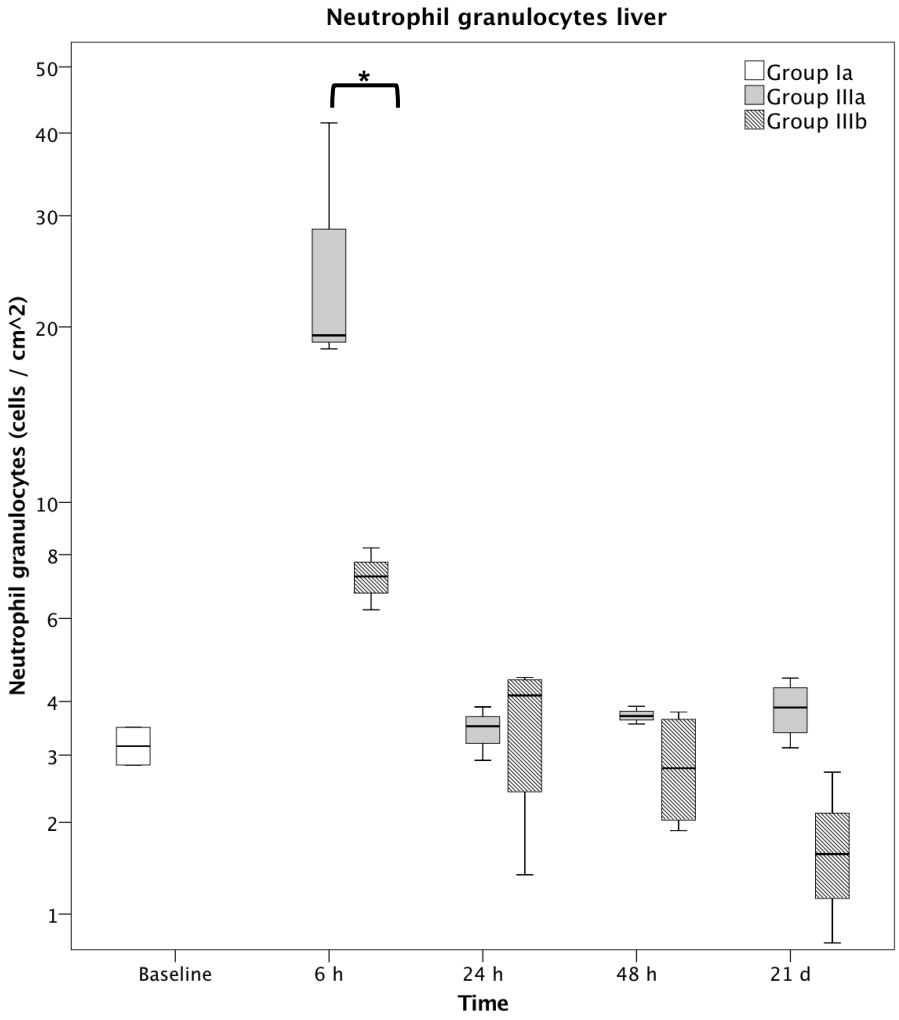

Figure 11 – Neutrophil granulocytes in liver following polytrauma with immunomodulation (group IIIb) and without immunomodulation (group IIIa). Group Ia represents sham mice. * represents p ≤ 0.05.

Liver tissue of polytrauma mouse without immunomodulation (group IIIa) with excessive neutrophil infiltration.

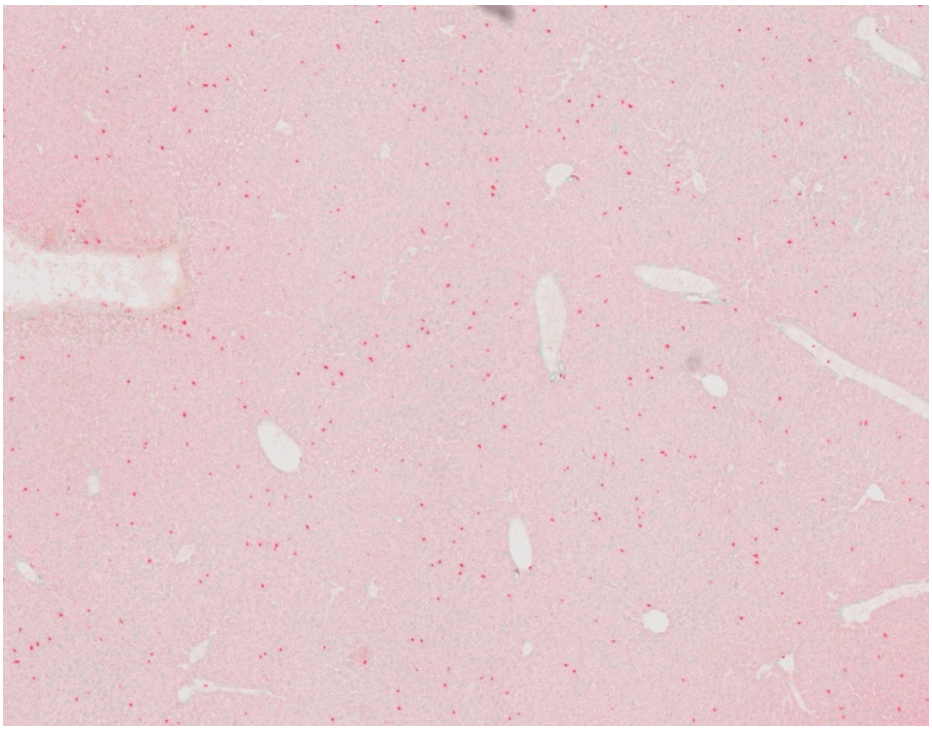

Figure 12 – Liver Ly6G stain of group IIIa at 6 h (polytrauma without immunomodulation)

Liver tissue of polytrauma mouse following sIL6R antibody application (group IIIb), demonstrating reduced neutrophil count as compared to group IIIa.

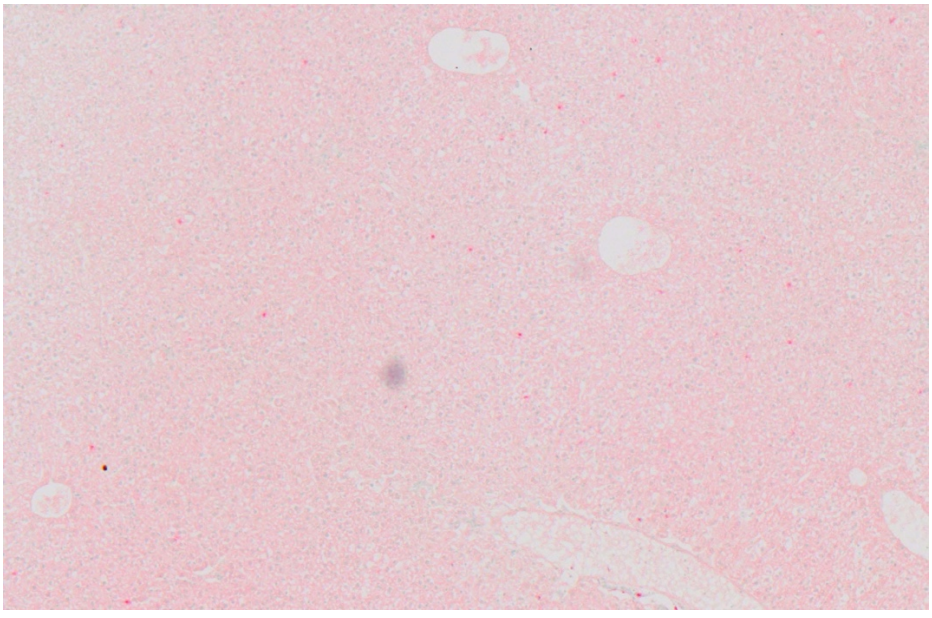

Figure 13 – Liver Ly6G stain of group IIIb at 6 h (polytrauma with immunomodulation)

3.2.3 Spleen

Pre-existent neutrophil granulocytes within the spleen led to excessive Ly6G stain in the sham group, which made any difference difficult to detect by optical means. Consequently, following treatment additional neutrophil infiltration was impossible to detect accurately. In macroscopic review, and also using imageJ software, there was no marked difference in staining area volume between the study groups.

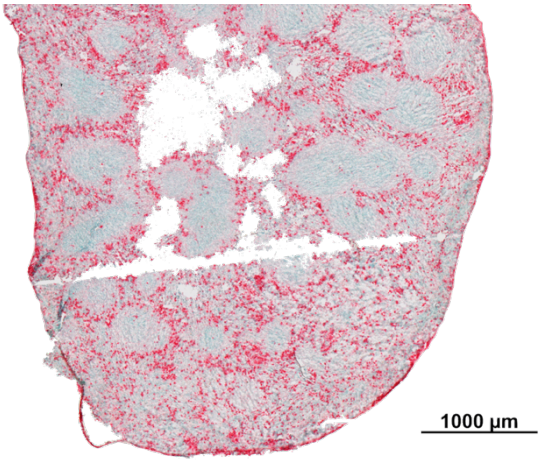

Figure 15 – Spleen Ly6G stain of group Ia mouse (sham).

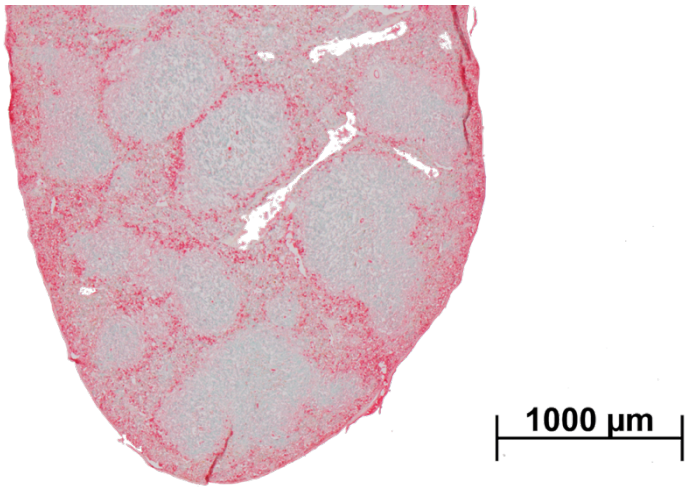

Figure 14 – Spleen Ly6G stain of group II mouse (fracture).

3.2.4 Kidney

Neutrophil granulocytes infiltrated into the tubular system. The nucleus of the neutrophil granulocytes cannot be sufficiently distinguished from the cells of the tubular system with Ly6G stain, and one therefore cannot distinguish between separate cells. Consequently, the kidneys were stained in HE to assess macroscopic incidence of kidney injury.

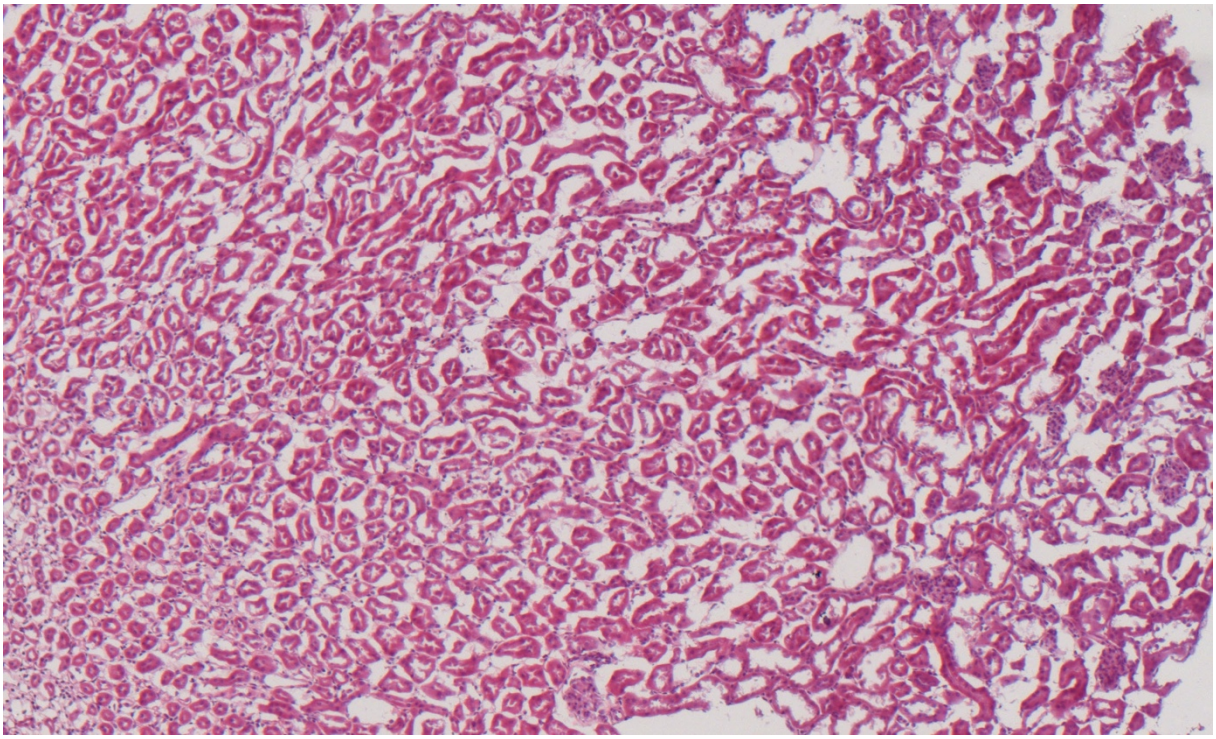

Figure 16 – Kidney HE stain of group Ia mouse (sham)

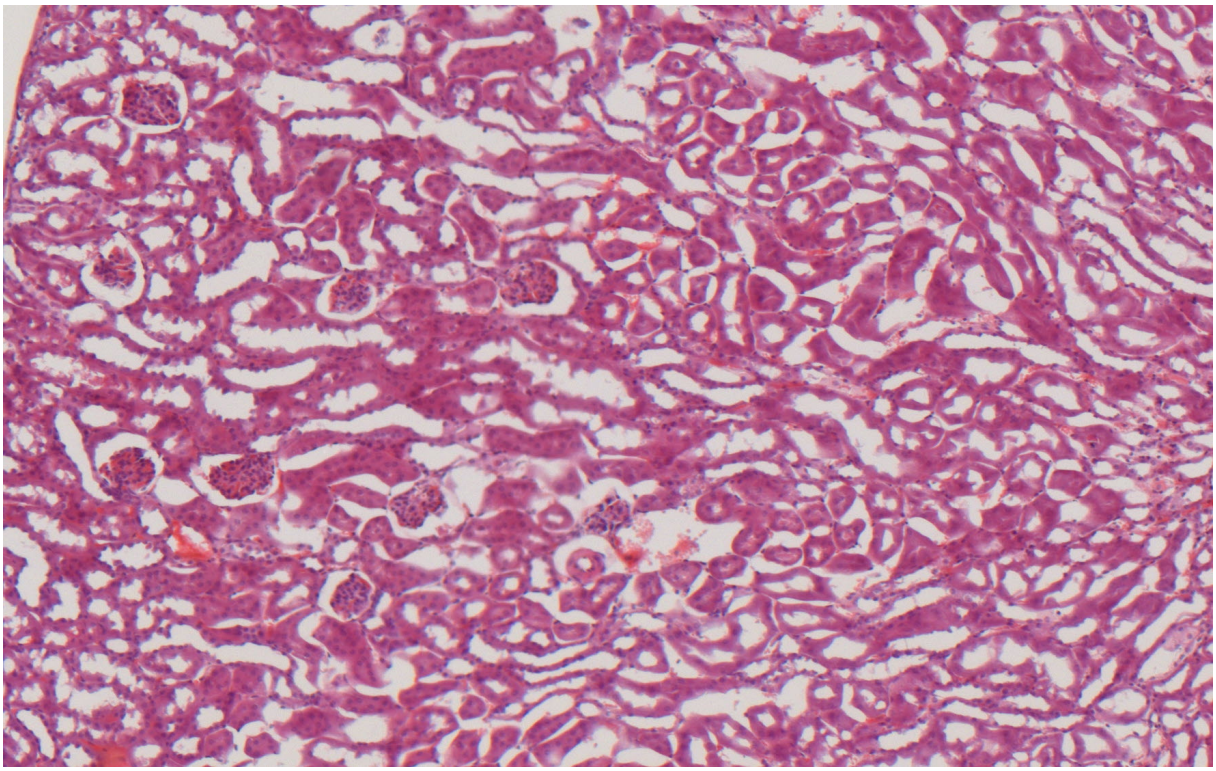

Figure 17 – Kidney HE stain of group IIIa mouse (polytrauma)

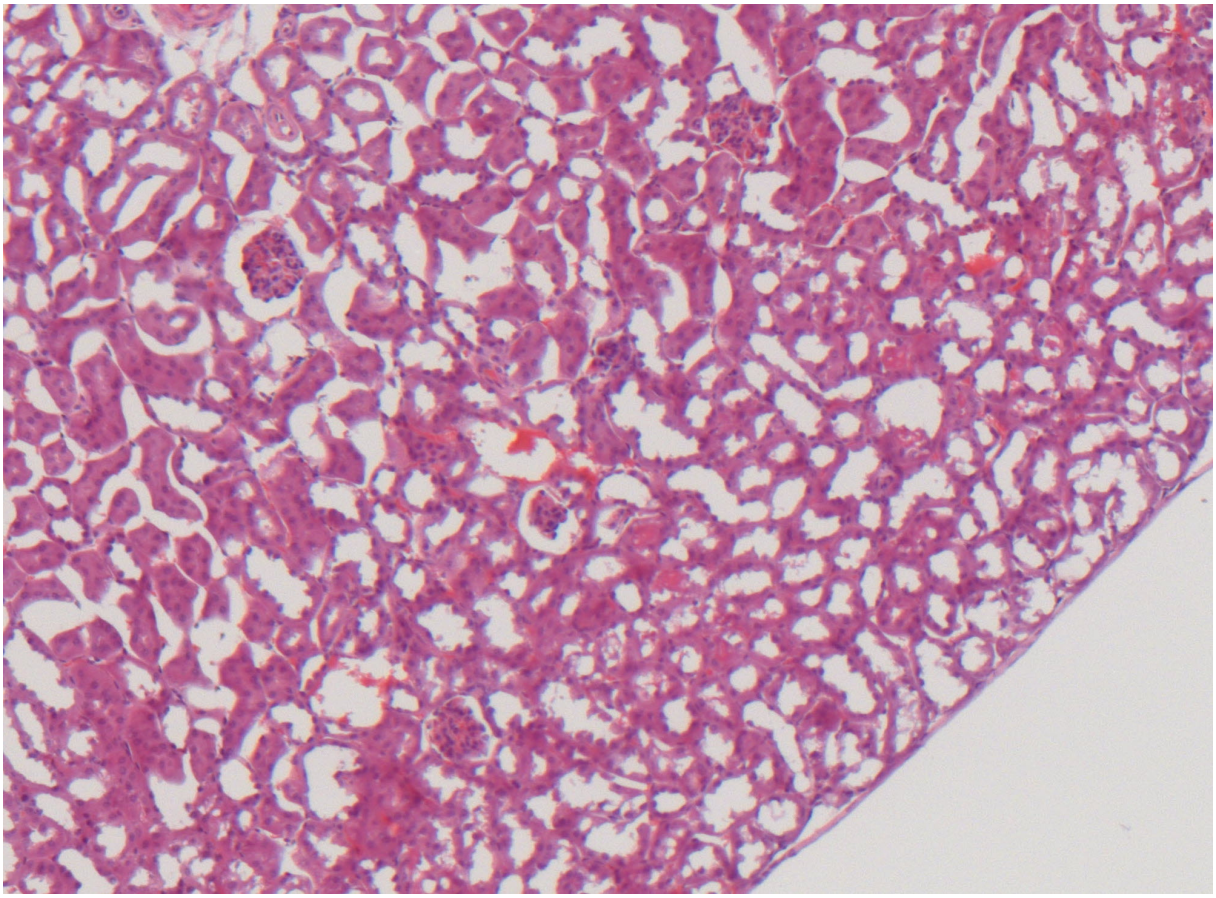

Figure 18 – Kidney HE stain of group IIIa mouse (polytrauma)

Reviewing the images, the tubular system within the polytrauma groups (groups IIIa and IIIb) appears blurry, unlike the fracture group (group IIa) and sham group (group Ia). Among group IIIa (polytrauma without immunomodulation) and group IIIb (polytrauma with immunomodulation) no difference could be seen.

3.2.5 Lung

The difference in lung weight at 6 h and 21 d was significantly higher in the polytrauma group than in the fracture group (group IIa at 6 h vs. group IIIa at 6 h p = 0.01; group IIa at 21 d vs. group IIIa at 21 d p = 0.01).

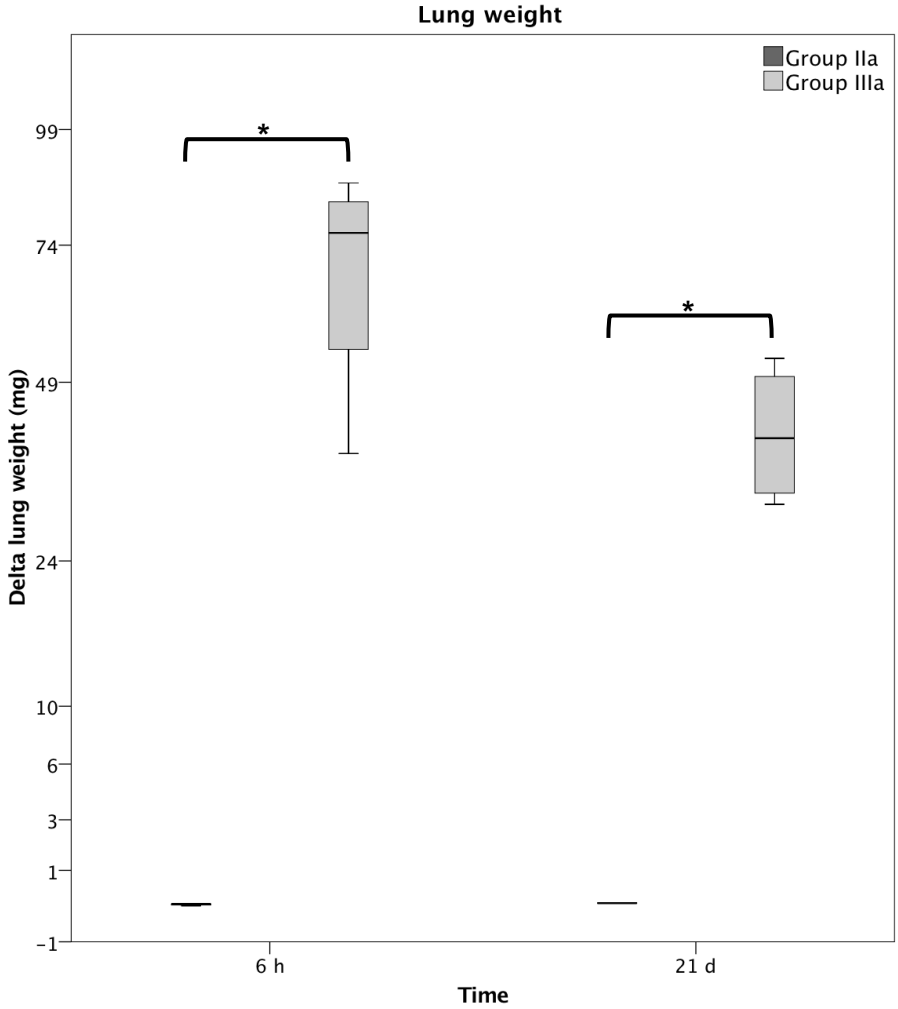

Figure 19 – Difference in lung weight following polytrauma with immunomodulation (group IIIb) and without immunomodulation (group IIIa). * represents p ≤ 0.05.

Following the application of sIL6R Mab, the difference in lung weight at 6 h within the polytrauma group decreased significantly (group IIIa at 6 h vs. group IIIb at 6 h $p = 0.04$). There was no significant change at 21 d.

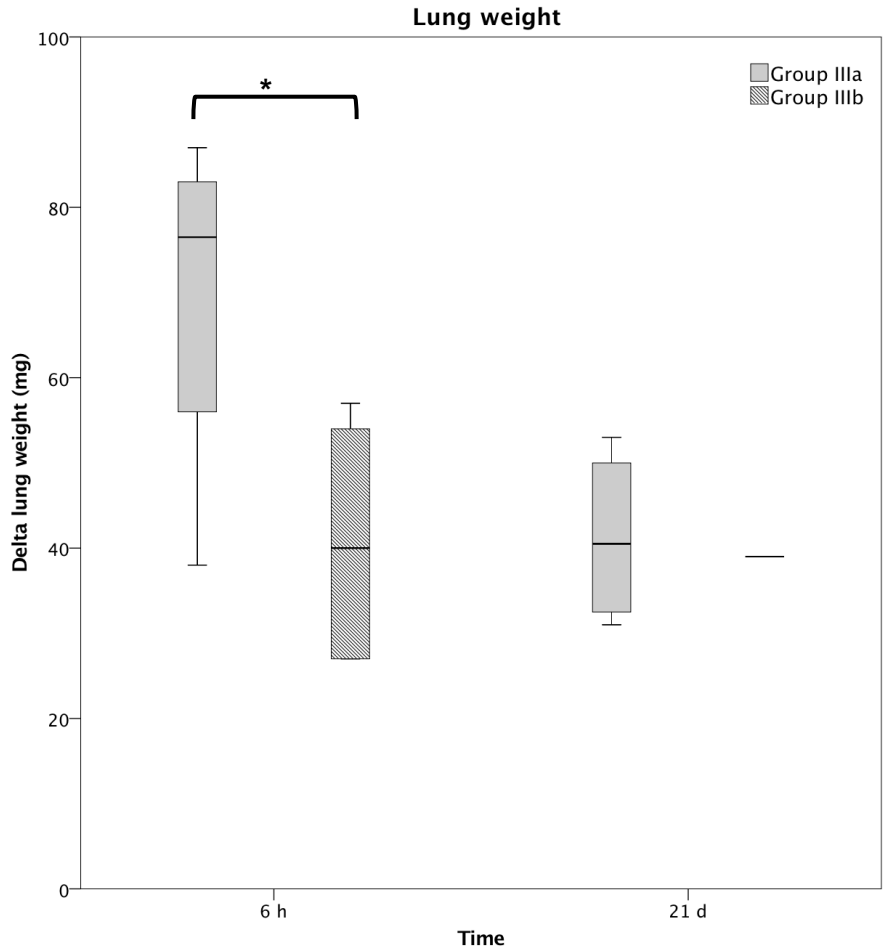

Figure 20 – Difference in lung weight following polytrauma with and without immunomodulation (groups IIIa and IIIb). * represents $p \leq 0.05$.

4. Discussion

4.1 ELISA

4.1.1 IL6

Our results show that trauma in general causes an increase in IL6 serum levels within 6 hours of trauma. Furthermore, the more severe the trauma, the more IL6 increased. These kinetics match data from human analysis [30], although IL6 stayed elevated at 24 h and 72 h in polytrauma patients. One has to bear in mind that the estimated immunological turnover of the mouse is approximately twice the rate of a human organism. The reported half-life of IL6 varies, but is generally reported as less than 24 hours [66]. Comparing human and murine data, the described murine multiple trauma model seems to accurately reflect the immunological response and modulation after polytrauma. Interestingly, these kinetics were observed even though the mice were housed in a hygienic laboratory environment, which potentially leads to an immature immune system, and therefore restricted immune response compared to mice that had received some “antigen exposure”, better reflecting normal human immunity [67].

Even though trauma in general led to a rise in IL6 serum levels, one needs to consider that the musculoskeletal trauma was the same between the fracture group and the polytrauma group. Therefore, it would appear to be the haemorrhagic shock primarily, in combination with the prolonged operative procedure, that is causative of the significant increase in IL6 in comparison to the “fracture only” animals. Therefore, haemorrhage would appear not only to be a key factor in prehospital trauma care but determine the further clinical course, even if the initial bleeding event has been controlled and resuscitation instituted.

The advantage of IL6 over more common clinical biomarkers used in inflammation such as CRP and / or PCT would appear to be with the early peak of IL6, whereas CRP and PCT need 48 to 72 h to peak [54]. IL6 is a standard test within laboratories and point-of-care tests are also available. IL6 could be of potential value in early estimation of the immunological hit of a trauma patient, and could help to identify patients who would benefit from initially prioritising physiological recovery over anatomical reconstruction, using the principles of damage control surgery and resuscitation [68]. Because operative and invasive procedures are known to initiate a second hit to the

patient [69, 70], IL6 could help to direct and time further treatment. In a future clinical setting, IL6 may represent an important marker to identify, risk stratify and direct further management for polytrauma patients and should be routinely used in assessment within the resuscitation room.

The administration of sIL6R antibody, and thus inhibition of the IL6 / sIL6R complex formation, causes a significant increase in IL6 serum levels, possibly by making more IL6 available for ELISA detection, as it is not bound with its receptor. Although the ELISA detection antibody is polyclonal and recognises multiple epitopes, it was raised against full length region antigens, and thus probably some epitopes that lie within the region of the IL6 / sIL6R complex formation. Therefore, we propose that the IL6 increase is not an expression of increased inflammation, but rather a technical phenomenon due to the prevention of normal complex formation. On the other hand, newly unbound IL6 could now signal through the membrane-bound IL6 receptor, shifting the momentum from transsignaling to increased regular IL6 signalling. If true, one could consider using an alternative blocking mechanism of transsignaling further downstream in the signalling pathway, e.g. the gp130 antibody. Further data will be available based on the histological evaluation of shock organs within this study.

4.1.2 sIL6R

sIL6R serum levels were increased at 6 h and 24 h after trauma, therefore trauma probably intensifies the shedding and splicing process that set up the soluble form of the IL6 receptor. This may induce metalloproteinases as part of the acute phase reaction. Using additional data from murine experiments, we were able to observe even higher serum levels induced by haemorrhagic shock [40].

In our human pilot study, we observed a decrease in sIL6R within the trauma patients compared to healthy individuals [30]. Human patients presumably had consumption of their sIL6R within the transsignaling process. Considering the massive shock that was induced within our murine trauma model (see Table 1), sIL6R formation might be induced more than it was consumed by transsignaling. In other words, the additional induction of sIL6R production needs a greater trauma load, in particular additional haemorrhagic shock, which would explain the different dynamics of both biomarkers after trauma, specifically low sIL6R serum levels after moderate trauma and high sIL6R serum levels following more severe trauma. Consequently, sIL6R might be of use to evaluate the degree of haemorrhagic shock in a trauma patient independently from other injuries that typically affect other biomarkers (IL6, lactate, etc.).

Blocking the IL6 / sIL6R complex formation by application of sIL6R Mab leads to a significant increase in sIL6R serum levels, demonstrating that more sIL6R was produced following trauma without immune modulation than expected from the data. Again, this might reflect the fact that sIL6R was bound within the IL6/sIL6R complex due to transsignaling and therefore could not be detected in total by the ELISA antibody. For IL6- and sIL6R detection we used ELISA Kits from the same company, with identical antibody development, and therefore superimposable features for epitope recognition.

4.1.3 TSR

The TSR, as the quotient of IL6 and sIL6R serum levels, was introduced by our study group to obtain a single value representing the transsignaling process [40]. At 6 h and 24 h after trauma, TSR showed a significant difference between polytrauma, fracture only and non-injured mice, matching our findings from human data, when TSR was able to distinguish between survivors and non-survivors at 6 h and 24 h after trauma [30]. Because of the more complex dynamics of sIL6R than was initially expected (see 4.1.2), it needs to be re-evaluated whether the quotient used to calculate TSR remains alone of sufficient value for trauma evaluation, or conversely, whether IL6 and sIL6R rather should be interpreted independently. This may enable separate evaluation of general tissue trauma using IL6 serum levels and of haemorrhagic shock by using sIL6R serum levels. Importantly, the biomarker serum levels (IL6, sIL6R) may help to identify severely injured patients that clinical examination and radiographic assessment may currently overlook or underestimate.

Application of sIL6R Mab, blocking transsignaling, reduced the TSR at 6 h and 24 h after trauma, which represent the time points when significant mortality differences occurred in the human data [30]. Following immune modulation, IL6 and sIL6R serum levels change significantly because of technical reasons, as described above, which makes it potentially more difficult to use them as predictive values following immunomodulation, unless another serum level range for immunomodulation can be established in the future. However, TSR initially seems to mathematically even out that effect, so that TSR values might be comparable again. Consequently, TSR could be included into the assessment panel in the future whenever immunomodulation is considered. Additionally, blood samples should be taken before a patient receives immunomodulating drugs, whenever possible, until this data has been further evaluated.

4.2 Shock organs

4.2.1 Overall

Comparing neutrophil granulocytes within shock organs after fracture and polytrauma revealed that adding haemorrhagic shock to a musculoskeletal trauma led to increased infiltration of those cells, consistent with the ELISA data of increased inflammation. Within the experimental phase, this was an important confirmation that the murine multiple trauma model was simulating immune response after trauma sufficiently, not just on the biomarker level, before subjecting the materials to more specific evaluations. The longer lasting surgical intervention of polytrauma mice (group III) in comparison to the fracture only mice (group II) needs to be considered, since extended surgical measures cause additional inflammation (as described in 1.3) [69, 71].

Furthermore, evaluating neutrophil infiltration is an important baseline parameter in the pathophysiology after trauma, along with the assessment of other parameters (biomarkers, cell infiltration patterns etc.), especially when using immunomodulation [72, 73].

Additionally, a rapid increase in neutrophils due to SIRS is associated with impaired bactericidal function, making patients more susceptible to infections [74], a recognised common complication of prolonged ICU stays in polytrauma patients [75, 76]. In combination with the phenomenon of anti-inflammatory response and immunosuppression, counteracting the systemic inflammatory response, termed CARS (compensatory anti-inflammatory response syndrome) [77], this can lead to a persistent inflammation, immunosuppression and catabolism syndrome (PICS) [78]. That “complicated clinical course” due to “immune paralysis” is also a target of immunomodulation and might be avoidable by preventing the neutrophil- and thus inflammatory peak.

Of course, the absolute count of neutrophils within the overall analysis does not represent the correct number of cells because of the nonspecific stain used and the missing adjustment for sample size, but it does show a tendency towards cell infiltration and thus inflammation. Therefore, the more specific Ly6G stain was used for further evaluation.

4.2.2 Liver

The specific analysis of neutrophil granulocytes using Ly6G stain confirmed our hypothesis that trauma does induce neutrophil infiltration, especially haemorrhagic shock, as the main difference found was between the fracture group (group II) and the polytrauma group (group III), again indicating a significant inflammatory boost. The peak of neutrophil infiltration matches the biomarker peak at 6 h after trauma and already returns towards baseline at 24 h. Therefore, we postulate that if a patient is kept from another immunological hit after trauma, such as extended surgical measures, the effects on the shock organs, represented by the degree of neutrophil infiltration, seems to decrease as quickly as the systemic immune response. This is illustrated by the reduction of IL6 serum levels from 6 h to 24 h after trauma. Since human data showed a prolonged IL6 peak for up to 72 hours as opposed to 6 h in the murine model, this effect may be similar for the neutrophil infiltration in humans and thus explain the period for which a trauma patient (namely, 72 hours) is extremely vulnerable for further complications [79-81].

The sIL6R Mab application led to a significant decrease of neutrophil infiltration within the expected peak at 6 h, as well as it decreasing IL6 serum levels and TSR at the same time point. Notably, TSR levels within our human data, especially at 6 h, were associated with the degree of organ failure and mortality, most likely associated with the degree of neutrophil infiltration as shown within the murine data, and thus reduction of the neutrophil granulocyte peak (and TSR) may potentially lead to less organ failure, as has previously been described for acute lung injury and liver injury [82, 83], and therefore potentially reduce mortality, which would be the ultimate goal of immunomodulation in trauma.

The effect of sIL6R Mab was seen for 6 to 24 hours within the ELISA data. The reduction of neutrophils within the liver only occurred at the 6-hour peak, whereas the number of neutrophils at 24 hours was not significantly affected compared to baseline. One drawback of using immunomodulation is completely inhibiting the immune response, but it would appear that targeting the sIL6R for immunomodulation merely prevents the immune system from “overreacting” without supressing it. This concept needs further evaluation, especially when varying dosage and dosing frequency. If confirmed, the application to the clinical setting may be beneficial for equilibrating the pro- and anti-inflammatory response after severe trauma at an early stage, without

influencing the immune response for common inflammatory complications during the ICU stay (e.g. pneumonia).

4.2.3 Spleen

Within the overall analysis of shock organs, using HE stain, the spleen had a significant increase in neutrophil infiltration after polytrauma (group III), compared to fracture only (group II). Certainly, the spleen appears important within the mechanisms following polytrauma, and especially with haemorrhagic shock. Aside from being a major organ system in serum immunology and the pooling of thrombocytes, the spleen has the capacity to provide extra erythrocytes to the circulation during hypoxia via contraction [84]. Additionally, there is evidence that hypovolemia triggers this response as well, providing extra oxygenation in response to physiological stress [85, 86], and therefore additional perfusion in haemorrhage. Currently, it remains unclear whether this effect is based upon a direct nerve response or humoral factors.

Furthermore, the splenic pool of neutrophil granulocytes, including aged ones that were previously removed from the blood stream, is mobilised in acute inflammatory responses and have been found to have an even higher activity than non-aged cells [87]. Consequently, it would be pertinent to see how immunomodulation would influence this migration, and what if any role these aged neutrophils play in the posttraumatic immune response.

Unfortunately, the pre-existing number of neutrophils within the spleen kept us from performing an adequate analysis using Ly6G stain in the context of bright-field microscopy, because Ly6G showed a more intense stain than HE stain, revealing far more neutrophil granulocytes, and subsequently making it impossible to adequately separate them by optic means only. For future experiments, Ly6G could be used in combination with a florescence, analysing it with spectrometer software or using flow cytometry (FACS), allowing a more detailed evaluation [88].

4.2.4 Kidney

Acute kidney injury is a common response to severe shock [89], due to a combination of pre- and intrarenal causes of renal failure, and is often one of the early organ failures on the way towards multi organ failure (MOF) [90]. Therefore, it is important to analyse the kidneys in the context of polytrauma and haemorrhagic shock.

Using bright field microscopy in combination with HE stain, we were able to see basic tissue changes that are described in acute kidney injury. Within the polytrauma group (group III), we saw blurry renal tubules at 6 h and 24 h, probably representing damage of the tubular cells, as opposed to unremarkable kidneys in the fracture group (group II). While there are no consistent histopathological findings within inflammatory processes [91], acute tubular necrosis (ATN) is well described for ischaemia-reperfusion experiments [92]. Thus, ischaemia due to haemorrhagic shock seems to be the key factor in triggering renal failure in polytrauma, more than the systemic immune response. However, due to ischaemia, transmigration of neutrophil granulocytes occurs and prevention of neutrophil accumulation has demonstrated a reduction in kidney injury [93]. As the application of sIL6R Mab reduced neutrophil infiltration within the liver, it could potentially reduce neutrophil granulocytes within the kidney, protecting against loss of kidney function. This might be an important factor in morbidity and mortality [90], since kidney injury in general, especially ATN, is associated with progression to chronic kidney failure [94, 95].

Analysing our histological samples, no marked difference between group IIIa (polytrauma without immunomodulation) and group IIIb (polytrauma with sIL6R Mab application) was seen. In general, tubular morphology seems to regenerate over 48 to 72 hours following ATN [92], but the lasting effects that we can influence via immunomodulation can be seen primarily via physiological parameters and should be targeted in further experiments

In the end, it was possible to see overall neutrophil infiltration using HE stain, but we were not able to accurately measure neutrophil infiltration in detail within the kidneys using Ly6G, due to overlap of the tubular staining with neutrophil staining. As described for the spleen, Ly6G could be used in combination with a florescence for future experiments [96].

4.2.5 Lung

Severe trauma can cause disruption of the lung endothelial and epithelial barriers, with consecutive loss of alveolar membrane integrity and therefore transepithelial neutrophil migration, which we were able to demonstrate within the overall shock organ evaluation (see 3.2.1). This neutrophil infiltration contributes to further membrane destruction and increased capillary permeability [97], permitting efflux of protein-rich fluid into the interstitial space and ultimately into the alveolar space. The resulting respiratory dysfunction with oedema-induced hypoxaemia based on oedema is called acute respiratory distress syndrome (ARDS).

In keeping with this pathophysiology, we saw an increased amount of lung water within our polytrauma mice, demonstrating measurable end organ damage. Therefore, it would appear that our results from serum level biomarker data and neutrophil infiltration counts seem to translate into objectifiable organ failure, as already hypothesised. Furthermore, it would appear to validate the accuracy of this murine multiple trauma model.

This organ damage, in terms of ARDS, can be categorised into mild, moderate and severe, based on the oxygenation index (Horovitz Index) according to the 2012 Berlin definition [98]. Based on our data, we were unable to distinguish between those categories, but considering the extensive shock that we were able to establish (see Table 1), this would at least cause a moderate form of ARDS within our polytrauma mice.

Using immunomodulation via sIL6R Mab application led to a decrease in lung water within the polytrauma mice (group IIIb), and thus suggests a reduction in ARDS. This effect only applies in the initial stages, whereas at 21 days no difference could be seen anymore, matching our previous data. However, the amount of lung water at 6 hours was reduced to approximately the same amount of lung water found in the mice that survived for 21 days, suggesting that there might be a potential survival benefit. IL6 in general has been proven to be a predictor of mortality in ARDS due to the extent of inflammatory response [99], and therefore modulation within the IL6 system could be promising for ARDS as well.

.

4.3 Conclusion

In polytrauma an understanding of the interaction of haemorrhage and tissue trauma in triggering an immune response would appear fundamental in improving mortality rates, which are the highest in young adults among all diseases. The central role of IL6 was used to evaluate and modulate that response and was therefore targeted in our novel long-term survival murine multiple trauma model. Consequently, biomarker levels, neutrophil infiltration and end organ damage were analysed following fracture and polytrauma with and without immunomodulation, and compared to sham mice, hypothesising that biomarkers, neutrophils and organ damage after trauma increase, but can be reduced by blocking IL6 transsignaling, a signalling process utilising the soluble IL6 receptor (sIL6R).

Our data revealed that IL6 as well as sIL6R showed a rapid peak within 6 h after trauma and are thus potentially of value before other serum biomarkers [54]. While both biomarkers in our murine model increased moderately after fracture, they increased significantly after polytrauma. Thus, the more severe the trauma, the greater the increase of IL6 and sIL6R, with haemorrhagic shock being the main difference in trauma load between our study groups. Calculating the transsignaling ratio (TSR), utilised to estimate the transsignaling process numerically, revealed an increase at 6 h and 24 h after trauma. Furthermore, TSR was reduced significantly by blocking transsignaling via sIL6R Mab application. Importantly, TSR is a parameter that was able to significantly predict mortality in human polytrauma patients [30].

Concurring with the ELISA data, murine shock organ analysis illustrated a significant neutrophil infiltration within 6 hours after polytrauma. The neutrophil peak returned to baseline at 24 h, potentially improving recovery from organ injury when another inflammatory stimulus (second hit) is prevented, altogether supporting the idea of damage control resuscitation / surgery (DCR / DCS). The initial neutrophil peak, considered responsible for organ failure and immune paralysis [26, 74], was prevented by blocking transsignaling via sIL6R application, demonstrated in the liver. That reduction of inflammation led to a notable reduction of end organ damage, as demonstrated within the lungs in terms of reduced lung water, representing the degree of acute lung injury.

Our results, therefore, underscore the important role of IL6 transsignaling in polytrauma, with haemorrhagic shock being a major trigger of the inflammatory response, and especially of sIL6R expression.

In vivo modulation of IL6 transsignaling via antibody application and blockade has the capability to prevent adverse events like organ failure, but future research needs to be targeted toward showing that neutrophil infiltration and end organ damage can be reduced by sIL6R Mab application, and especially demonstrating this in several separate organs. Moreover, it needs to be evaluated whether this ultimately leads to reduction in morbidity and mortality in the clinical setting. It needs to be noted that the effect of immunomodulation within our data is shown from 6 to 24 hours. Therefore, the dosage and frequency of antibody application needs to be further evaluated. It ist equally important that the changing dynamics of IL6 and sIL6R serum levels following immunomodulation should be investigated in more detail, to distinguish technical reasons, as proposed within our data, from as yet unidentified immunological phenomena.

Most traumatic death currently occurs prehospital due to haemorrhage. As more and more bleeding control measures, such as Tourniquets, haemostatic dressings and sophisticated techniques such as REBOA are available prehospital [100], allied to making blood units available in the field [101, 102], an increasing proportion of trauma patients, who previously would have died due to haemorrhage, may now reach the resuscitation areas of our hospitals. Considering that haemorrhagic shock caused the major difference in inflammatory response within our trauma model, controlling the massive immunological aftermath of these patients is likely to be a fruitful area of future innovation and research.

5. Bibliography

1. Tscherne, H.; Köle, W.; Heberer, G., *Chirurgie*. Springer: 1977.
2. Pape, H. C.; Lefering, R.; Butcher, N.; Peitzman, A.; Leenen, L.; Marzi, I.; Lichte, P.; Josten, C.; Bouillon, B.; Schmucker, U.; Stahel, P.; Giannoudis, P.; Balogh, Z., The definition of polytrauma revisited: An international consensus process and proposal of the new 'Berlin definition'. *J Trauma Acute Care Surg* 2014, *77* (5), 780-786.
3. Rau, C. S.; Wu, S. C.; Kuo, P. J.; Chen, Y. C.; Chien, P. C.; Hsieh, H. Y.; Hsieh, C. H., Polytrauma Defined by the New Berlin Definition: A Validation Test Based on Propensity-Score Matching Approach. *Int J Environ Res Public Health* 2017, *14* (9), 1045.
4. Buschmann, C. T.; Gahr, P.; Tsokos, M.; Ertel, W.; Fakler, J. K., Clinical diagnosis versus autopsy findings in polytrauma fatalities. *Scand J Trauma Resusc Emerg Med* 2010, *18*, 55.
5. Murray, C. J. L.; Lopez, A. D., *The Global burden of disease : a comprehensive assessment of mortality and disability from diseases, injuries, and risk factors in 1990 and projected to 2020*. World Health Organization , World Bank & Harvard School of Public Health: 1996.
6. Kleber, C.; Giesecke, M. T.; Tsokos, M.; Haas, N. P.; Schaser, K. D.; Stefan, P.; Buschmann, C. T., Overall distribution of trauma-related deaths in Berlin 2010: advancement or stagnation of German trauma management? *World J Surg* 2012, *36* (9), 2125-30.
7. Martin, N. D.; Kaplan, L. J., *Principles of Adult Surgical Critical Care*. Springer International Publishing: 2016.
8. Wilhelm, W., *Praxis der Intensivmedizin: konkret, kompakt, interdisziplinär*. Springer Berlin Heidelberg: 2013.
9. Frink, M.; van Griensven, M.; Kobbe, P.; Brin, T.; Zeckey, C.; Vaske, B.; Krettek, C.; Hildebrand, F., IL-6 predicts organ dysfunction and mortality in patients with multiple injuries. *Scand J Trauma Resusc Emerg Med* 2009, *17*, 49.
10. Lord, J. M.; Midwinter, M. J.; Chen, Y. F.; Belli, A.; Brohi, K.; Kovacs, E. J.; Koenderman, L.; Kubes, P.; Lilford, R. J., The systemic immune

response to trauma: an overview of pathophysiology and treatment. *Lancet* 2014, *384* (9952), 1455-65.

11. Gutierrez, G.; Reines, H. D.; Wulf-Gutierrez, M. E., Clinical review: hemorrhagic shock. *Crit Care* 2004, *8* (5), 373-81.
12. Brøchner, A. C.; Toft, P., Pathophysiology of the systemic inflammatory response after major accidental trauma. *Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine* 2009, *17* (1), 43.
13. Kalogeris, T.; Baines, C. P.; Krenz, M.; Korthuis, R. J., Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol* 2012, *298*, 229-317.
14. Lefer, A. M.; Ma, X., Endothelial Dysfunction as an Early Critical Event in Ischemia and Shock. In *Host Defense Dysfunction in Trauma, Shock and Sepsis.*, E., F.; J.L., M.; F.W., S., Eds. Springer: Berlin, Heidelberg, 1993; pp 107-112.
15. Moore, E. E.; Burch, J. M.; Franciose, R. J.; Offner, P. J.; Biffl, W. L., Staged physiologic restoration and damage control surgery. *World J Surg* 1998, *22* (12), 1184-90; discussion 1190-1.
16. Bennett, B.; Towler, H. M. A., Haemostatic Response To Trauma. *British Medical Bulletin* 1985, *41* (3), 274-280.
17. Hoffman, M.; Monroe, D. M., A cell-based model of hemostasis. *Thromb Haemost* 2001, *85* (6), 958-65.
18. Jenne, C. N.; Urrutia, R.; Kubes, P., Platelets: bridging hemostasis, inflammation, and immunity. *Int J Lab Hematol* 2013, *35* (3), 254-61.
19. Kanse, S. M.; Gallenmueller, A.; Zeerleder, S.; Stephan, F.; Rannou, O.; Denk, S.; Etscheid, M.; Lochnit, G.; Krueger, M.; Huber-Lang, M., Factor VII-activating protease is activated in multiple trauma patients and generates anaphylatoxin C5a. *J Immunol* 2012, *188* (6), 2858-65.
20. Kleber, C.; Giesecke, M. T.; Tsokos, M.; Haas, N. P.; Buschmann, C. T., Trauma-related preventable deaths in Berlin 2010: need to change prehospital management strategies and trauma management education. *World J Surg* 2013, *37* (5), 1154-61.
21. Maslanik, T.; Mahaffey, L.; Tannura, K.; Beninson, L.; Greenwood, B. N.; Fleshner, M., The inflammasome and danger associated molecular patterns (DAMPs) are implicated in cytokine and chemokine responses following stressor exposure. *Brain, Behavior, and Immunity* 2013, *28*, 54-62.

22. Land, W. G., The Role of Damage-Associated Molecular Patterns (DAMPs) in Human Diseases: Part II: DAMPs as diagnostics, prognostics and therapeutics in clinical medicine. *Sultan Qaboos Univ Med J* 2015, *15* (2), e157-70.
23. Reino, D. C.; Palange, D.; Feketeova, E.; Bonitz, R. P.; Xu, D. Z.; Lu, Q.; Sheth, S. U.; Peña, G.; Ulloa, L.; De Maio, A.; Feinman, R.; Deitch, E. A., Activation of toll-like receptor 4 is necessary for trauma hemorrhagic shock-induced gut injury and polymorphonuclear neutrophil priming. *Shock* 2012, *38* (1), 107-14.
24. Schlag, G.; Redl, H.; Hallström, S., The cell in shock: the origin of multiple organ failure. *Resuscitation* 1991, *21* (2-3), 137-80.
25. Tang, D.; Kang, R.; Coyne, C. B.; Zeh, H. J.; Lotze, M. T., PAMPs and DAMPs: signal 0s that spur autophagy and immunity. *Immunol Rev* 2012, *249* (1), 158-75.
26. Mittal, M.; Siddiqui, M. R.; Tran, K.; Reddy, S. P.; Malik, A. B., Reactive oxygen species in inflammation and tissue injury. *Antioxid Redox Signal* 2014, *20* (7), 1126-67.
27. Rotondo, M. F.; Schwab, C. W.; McGonigal, M. D.; Phillips, G. R.; Fruchterman, T. M.; Kauder, D. R.; Latenser, B. A.; Angood, P. A., 'Damage control': an approach for improved survival in exsanguinating penetrating abdominal injury. *J Trauma* 1993, *35* (3), 375-82; discussion 382-3.
28. Tsukamoto, T.; Chanthaphavong, R. S.; Pape, H. C., Current theories on the pathophysiology of multiple organ failure after trauma. *Injury* 2010, *41* (1), 21-6.
29. Stein, D. M.; Scalea, T. M., Capillary leak syndrome in trauma: what is it and what are the consequences? *Adv Surg* 2012, *46*, 237-53.
30. Kleber, C.; Becker, C. A.; Schmidt-Bleek, K.; Schaser, K. D.; Haas, N. P., Are pentraxin 3 and transsignaling early markers for immunologic injury severity in polytrauma? A pilot study. *Clin Orthop Relat Res* 2013, *471* (9), 2822-30.
31. Oberholzer, M. J., *Pathologie verstehen: molekulare Grundlagen der allgemeinen Pathologie*. Thieme: 2001.
32. Tanaka, T.; Narazaki, M.; Kishimoto, T., IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol* 2014, *6* (10), a016295.

33. Yawata, H.; Yasukawa, K.; Natsuka, S.; Murakami, M.; Yamasaki, K.; Hibi, M.; Taga, T.; Kishimoto, T., Structure-function analysis of human IL-6 receptor: dissociation of amino acid residues required for IL-6-binding and for IL-6 signal transduction through gp130. *EMBO J* 1993, *12* (4), 1705-12.
34. Rose-John, S.; Scheller, J.; Elson, G.; Jones, S. A., Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. *J Leukoc Biol* 2006, *80* (2), 227-36.
35. Heinrich, P. C.; Behrmann, I.; Haan, S.; Hermanns, H. M.; Müller-Newen, G.; Schaper, F., Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 2003, *374* (Pt 1), 1-20.
36. Chalaris, A.; Garbers, C.; Rabe, B.; Rose-John, S.; Scheller, J., The soluble Interleukin 6 receptor: generation and role in inflammation and cancer. *Eur J Cell Biol* 2011, *90* (6-7), 484-94.
37. Akeson, G.; Malemud, C., *A Role for Soluble IL-6 Receptor in Osteoarthritis*. 2017; Vol. 2, p 27.
38. Scheller, J.; Chalaris, A.; Garbers, C.; Rose-John, S., ADAM17: a molecular switch to control inflammation and tissue regeneration. *Trends Immunol* 2011, *32* (8), 380-7.
39. Yan, I.; Schwarz, J.; Lücke, K.; Schumacher, N.; Schumacher, V.; Schmidt, S.; Rabe, B.; Saftig, P.; Donners, M.; Rose-John, S.; Mittrücker, H. W.; Chalaris, A., ADAM17 controls IL-6 signaling by cleavage of the murine IL-6Rα from the cell surface of leukocytes during inflammatory responses. *J Leukoc Biol* 2016, *99* (5), 749-60.
40. Kleber, C.; Becker, C. A.; Malysch, T.; Reinhold, J. M.; Tsitsilonis, S.; Duda, G. N.; Schmidt-Bleek, K.; Schaser, K. D., Temporal profile of inflammatory response to fracture and hemorrhagic shock: Proposal of a novel long-term survival murine multiple trauma model. *J Orthop Res* 2015, *33* (7), 965-70.
41. Rose-John, S., IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6. *Int J Biol Sci* 2012, *8* (9), 1237-47.
42. Fanola, C. L.; Morrow, D. A.; Cannon, C. P.; Jarolim, P.; Lukas, M. A.; Bode, C.; Hochman, J. S.; Goodrich, E. L.; Braunwald, E.; O'Donoghue, M. L., Interleukin-6 and the Risk of Adverse Outcomes in Patients After an Acute Coronary Syndrome: Observations From the SOLID-TIMI 52 (Stabilization of

Plaque Using Darapladib-Thrombolysis in Myocardial Infarction 52) Trial. *J Am Heart Assoc* 2017, *6* (10).

43. Waje-Andreassen, U.; Kråkenes, J.; Ulvestad, E.; Thomassen, L.; Myhr, K. M.; Aarseth, J.; Vedeler, C. A., IL-6: an early marker for outcome in acute ischemic stroke. *Acta Neurol Scand* 2005, *111* (6), 360-5.
44. Wang, L.; Miyahira, A. K.; Simons, D. L.; Lu, X.; Chang, A. Y.; Wang, C.; Suni, M. A.; Maino, V. C.; Dirbas, F. M.; Yim, J.; Waisman, J.; Lee, P. P., IL6 Signaling in Peripheral Blood T Cells Predicts Clinical Outcome in Breast Cancer. *Cancer Research* 2017, *77* (5), 1119.
45. Watanabe, E.; Hirasawa, H.; Oda, S.; Matsuda, K.; Hatano, M.; Tokuhisa, T., Extremely high interleukin-6 blood levels and outcome in the critically ill are associated with tumor necrosis factor- and interleukin-1-related gene polymorphisms. *Crit Care Med* 2005, *33* (1), 89-97; discussion 242-3.
46. Srisangthong, P.; Wongsa, A.; Kittiworawitkul, P.; Wattanathum, A., Early IL-6 response in sepsis is correlated with mortality and severity score. *Critical Care* 2013, *17* (Suppl 2), P34-P34.
47. Scheller, J.; Garbers, C.; Rose-John, S., Interleukin-6: from basic biology to selective blockade of pro-inflammatory activities. *Semin Immunol* 2014, *26* (1), 2-12.
48. Peters, M.; Blinn, G.; Solem, F.; Fischer, M.; Meyer zum Büschenfelde, K. H.; Rose-John, S., In vivo and in vitro activities of the gp130-stimulating designer cytokine Hyper-IL-6. *J Immunol* 1998, *161* (7), 3575-81.
49. Hartman, J.; Frishman, W. H., Inflammation and atherosclerosis: a review of the role of interleukin-6 in the development of atherosclerosis and the potential for targeted drug therapy. *Cardiol Rev* 2014, *22* (3), 147-51.
50. von Haehling, S.; Anker, S. D., Cachexia as a major underestimated and unmet medical need: facts and numbers. *J Cachexia Sarcopenia Muscle* 2010, *1* (1), 1-5.
51. Pettersen, K.; Andersen, S.; Degen, S.; Tadini, V.; Grosjean, J.; Hatakeyama, S.; Tesfahun, A. N.; Moestue, S.; Kim, J.; Nonstad, U.; Romundstad, P. R.; Skorpen, F.; Sørhaug, S.; Amundsen, T.; Grønberg, B. H.; Strasser, F.; Stephens, N.; Hoem, D.; Molven, A.; Kaasa, S.; Fearon, K.; Jacobi, C.; Bjørkøy, G., Cancer cachexia associates with a

systemic autophagy-inducing activity mimicked by cancer cell-derived IL-6 trans-signaling. *Scientific Reports* 2017, *7* (1), 2046.

52. Krüttgen, A.; Rose-John, S., Interleukin-6 in sepsis and capillary leakage syndrome. *J Interferon Cytokine Res* 2012, *32* (2), 60-5.

53. Kaiser, K.; Prystaz, K.; Vikman, A.; Haffner-Luntzer, M.; Bergdolt, S.; Strauss, G.; Waetzig, G. H.; Rose-John, S.; Ignatius, A., Pharmacological inhibition of IL-6 trans-signaling improves compromised fracture healing after severe trauma. *Naunyn-Schmiedeberg's Archives of Pharmacology* 2018, *391* (5), 523-536.

54. Meisner, M.; Adina, H.; Schmidt, J., Correlation of procalcitonin and C-reactive protein to inflammation, complications, and outcome during the intensive care unit course of multiple-trauma patients. *Crit Care* 2006, *10* (1), R1.

55. Kleber, C., *Notfallmanagement des Polytraumas*. Charité - Universitätsmedizin Berlin: 2015.

56. Barkhausen, T.; Tschernig, T.; Rosenstiel, P.; van Griensven, M.; Vonberg, R. P.; Dorsch, M.; Mueller-Heine, A.; Chalaris, A.; Scheller, J.; Rose-John, S.; Seegert, D.; Krettek, C.; Waetzig, G. H., Selective blockade of interleukin-6 trans-signaling improves survival in a murine polymicrobial sepsis model. *Crit Care Med* 2011, *39* (6), 1407-13.

57. Lim, S. S.; Vos, T.; Flaxman, A. D.; Danaei, G.; Shibuya, K.; Adair-Rohani, H.; Amann, M.; Anderson, H. R.; Andrews, K. G.; Aryee, M.; Atkinson, C.; Bacchus, L. J.; Bahalim, A. N.; Balakrishnan, K.; Balmes, J.; Barker-Collo, S.; Baxter, A.; Bell, M. L.; Blore, J. D.; Blyth, F.; Bonner, C.; Borges, G.; Bourne, R.; Boussinesq, M.; Brauer, M.; Brooks, P.; Bruce, N. G.; Brunekreef, B.; Bryan-Hancock, C.; Bucello, C.; Buchbinder, R.; Bull, F.; Burnett, R. T.; Byers, T. E.; Calabria, B.; Carapetis, J.; Carnahan, E.; Chafe, Z.; Charlson, F.; Chen, H.; Chen, J. S.; Cheng, A. T.; Child, J. C.; Cohen, A.; Colson, K. E.; Cowie, B. C.; Darby, S.; Darling, S.; Davis, A.; Degenhardt, L.; Dentener, F.; Des Jarlais, D. C.; Devries, K.; Dherani, M.; Ding, E. L.; Dorsey, E. R.; Driscoll, T.; Edmond, K.; Ali, S. E.; Engell, R. E.; Erwin, P. J.; Fahimi, S.; Falder, G.; Farzadfar, F.; Ferrari, A.; Finucane, M. M.; Flaxman, S.; Fowkes, F. G.; Freedman, G.; Freeman, M. K.; Gakidou, E.; Ghosh, S.; Giovannucci, E.; Gmel, G.; Graham, K.;

Grainger, R.; Grant, B.; Gunnell, D.; Gutierrez, H. R.; Hall, W.; Hoek, H. W.; Hogan, A.; Hosgood, H. D.; Hoy, D.; Hu, H.; Hubbell, B. J.; Hutchings, S. J.; Ibeanusi, S. E.; Jacklyn, G. L.; Jasrasaria, R.; Jonas, J. B.; Kan, H.; Kanis, J. A.; Kassebaum, N.; Kawakami, N.; Khang, Y. H.; Khatibzadeh, S.; Khoo, J. P.; Kok, C.; Laden, F.; Lalloo, R.; Lan, Q.; Lathlean, T.; Leasher, J. L.; Leigh, J.; Li, Y.; Lin, J. K.; Lipshultz, S. E.; London, S.; Lozano, R.; Lu, Y.; Mak, J.; Malekzadeh, R.; Mallinger, L.; Marcenes, W.; March, L.; Marks, R.; Martin, R.; McGale, P.; McGrath, J.; Mehta, S.; Mensah, G. A.; Merriman, T. R.; Micha, R.; Michaud, C.; Mishra, V.; Mohd Hanafiah, K.; Mokdad, A. A.; Morawska, L.; Mozaffarian, D.; Murphy, T.; Naghavi, M.; Neal, B.; Nelson, P. K.; Nolla, J. M.; Norman, R.; Olives, C.; Omer, S. B.; Orchard, J.; Osborne, R.; Ostro, B.; Page, A.; Pandey, K. D.; Parry, C. D.; Passmore, E.; Patra, J.; Pearce, N.; Pelizzari, P. M.; Petzold, M.; Phillips, M. R.; Pope, D.; Pope, C. A.; Powles, J.; Rao, M.; Razavi, H.; Rehfuess, E. A.; Rehm, J. T.; Ritz, B.; Rivara, F. P.; Roberts, T.; Robinson, C.; Rodriguez-Portales, J. A.; Romieu, I.; Room, R.; Rosenfeld, L. C.; Roy, A.; Rushton, L.; Salomon, J. A.; Sampson, U.; Sanchez-Riera, L.; Sanman, E.; Sapkota, A.; Seedat, S.; Shi, P.; Shield, K.; Shivakoti, R.; Singh, G. M.; Sleet, D. A.; Smith, E.; Smith, K. R.; Stapelberg, N. J.; Steenland, K.; Stöckl, H.; Stovner, L. J.; Straif, K.; Straney, L.; Thurston, G. D.; Tran, J. H.; Van Dingenen, R.; van Donkelaar, A.; Veerman, J. L.; Vijayakumar, L.; Weintraub, R.; Weissman, M. M.; White, R. A.; Whiteford, H.; Wiersma, S. T.; Wilkinson, J. D.; Williams, H. C.; Williams, W.; Wilson, N.; Woolf, A. D.; Yip, P.; Zielinski, J. M.; Lopez, A. D.; Murray, C. J.; Ezzati, M.; AlMazroa, M. A.; Memish, Z. A., A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012, *380* (9859), 2224-60.

58. Russell, W. M. S.; Burch, R. L., *The principles of humane experimental technique*. Methuen: London,, 1959; p xiv, 238 p.

59. Vollmar, B.; Lang, G.; Menger, M. D.; Messmer, K., Hypertonic hydroxyethyl starch restores hepatic microvascular perfusion in hemorrhagic shock. *Am J Physiol* 1994, *266* (5 Pt 2), H1927-34.

60. Wichmann, M. W.; Ayala, A.; Chaudry, I. H., Severe depression of host immune functions following closed-bone fracture, soft-tissue trauma, and hemorrhagic shock. *Crit Care Med* 1998, *26* (8), 1372-8.
61. Hiltunen, A.; Vuorio, E.; Aro, H. T., A standardized experimental fracture in the mouse tibia. *J Orthop Res* 1993, *11* (2), 305-12.
62. Fischer, A. H.; Jacobson, K. A.; Rose, J.; Zeller, R., Cryosectioning tissues. *CSH Protoc* 2008, *2008*, pdb.prot4991.
63. Glassy, E. F.; Agosti, S. J.; College of American Pathologists. Atlas Subcommittee.; College of American Pathologists., *Color atlas of hematology : an illustrated field guide based on proficiency testing*. College of American Patholgists: Northfield, Ill., 1998; p ix, 370 p.
64. Kuzkov, V. V.; Kirov, M. Y.; Sovershaev, M. A.; Kuklin, V. N.; Suborov, E. V.; Waerhaug, K.; Bjertnaes, L. J., Extravascular lung water determined with single transpulmonary thermodilution correlates with the severity of sepsis-induced acute lung injury. *Crit Care Med* 2006, *34* (6), 1647-53.
65. Phillips, C. R.; Smith, S. M., Predicted body weight-indexed extravascular lung water is elevated in acute respiratory distress syndrome. *Crit Care Med* 2009, *37* (1), 377-378.
66. Oda, S.; Hirasawa, H.; Shiga, H.; Nakanishi, K.; Matsuda, K.; Nakamua, M., Sequential measurement of IL-6 blood levels in patients with systemic inflammatory response syndrome (SIRS)/sepsis. *Cytokine* 2005, *29* (4), 169-75.
67. Sbierski-Kind, J.; Kath, J.; Brachs, S.; Streitz, M.; von Herrath, M. G.; Kühl, A. A.; Schmidt-Bleek, K.; Mai, K.; Spranger, J.; Volk, H. D., Distinct Housing Conditions Reveal a Major Impact of Adaptive Immunity on the Course of Obesity-Induced Type 2 Diabetes. *Front Immunol* 2018, *9*, 1069.
68. Lamb, C. M.; MacGoey, P.; Navarro, A. P.; Brooks, A. J., Damage control surgery in the era of damage control resuscitation. *Br J Anaesth* 2014, *113* (2), 242-9.
69. Pape, H. C.; Schmidt, R. E.; Rice, J.; van Griensven, M.; das Gupta, R.; Krettek, C.; Tscherne, H., Biochemical changes after trauma and skeletal surgery of the lower extremity: quantification of the operative burden. *Crit Care Med* 2000, *28* (10), 3441-8.

70. Giannoudis, P. V.; Smith, R. M.; Bellamy, M. C.; Morrison, J. F.; Dickson, R. A.; Guillou, P. J., Stimulation of the inflammatory system by reamed and unreamed nailing of femoral fractures. An analysis of the second hit. *J Bone Joint Surg Br* 1999, *81* (2), 356-61.
71. Cruickshank, A. M.; Fraser, W. D.; Burns, H. J.; Van Damme, J.; Shenkin, A., Response of serum interleukin-6 in patients undergoing elective surgery of varying severity. *Clin Sci (Lond)* 1990, *79* (2), 161-5.
72. Hofer, H. P.; Egger, G.; Kukovetz, E. M.; Bratschitsch, G.; Steindorfer, P.; Schaur, R. J., The influence of trauma on changes in neutrophil granulocyte function assessed by an analysis of granulocyte migration. *Langenbecks Arch Chir* 1996, *381* (3), 148-54.
73. Faist, E.; Meakins, J. L.; Schildberg, F. W., *Host Defense Dysfunction in Trauma, Shock and Sepsis: Mechanisms and Therapeutic Approaches*. Springer Berlin Heidelberg: 2012.
74. Hietbrink, F.; Koenderman, L.; Althuizen, M.; Leenen, L. P., Modulation of the innate immune response after trauma visualised by a change in functional PMN phenotype. *Injury* 2009, *40* (8), 851-5.
75. Mondello, S.; Cantrell, A.; Italiano, D.; Fodale, V.; Mondello, P.; Ang, D., Complications of trauma patients admitted to the ICU in level I academic trauma centers in the United States. *Biomed Res Int* 2014, *2014*, 473419.
76. Pillai, J.; Yazicioglu, C.; Moeng, S.; Rangaka, T.; Monareng, T.; Jayakrishnan, R.; Veller, M.; Pinkus, D., Prevalence and patterns of infection in critically ill trauma patients admitted to the trauma ICU, South Africa. *J Infect Dev Ctries* 2015, *9* (7), 736-42.
77. Ward, N. S.; Casserly, B.; Ayala, A., The compensatory anti-inflammatory response syndrome (CARS) in critically ill patients. *Clin Chest Med* 2008, *29* (4), 617-25, viii.
78. Gentile, L. F.; Cuenca, A. G.; Efron, P. A.; Ang, D.; Bihorac, A.; McKinley, B. A.; Moldawer, L. L.; Moore, F. A., Persistent inflammation and immunosuppression: a common syndrome and new horizon for surgical intensive care. *J Trauma Acute Care Surg* 2012, *72* (6), 1491-501.
79. Howard, B. M.; Kornblith, L. Z.; Christie, S. A.; Conroy, A. S.; Nelson, M. F.; Campion, E. M.; Callcut, R. A.; Calfee, C. S.; Lamere, B. J.; Fadrosh, D. W.; Lynch, S.; Cohen, M. J., Characterizing the gut microbiome in trauma:

significant changes in microbial diversity occur early after severe injury. *Trauma Surgery & Acute Care Open* 2017, *2* (1).

80. Peitzman, A. B.; Schwab, C. W.; Yealy, D. M.; Rhodes, M.; Fabian, T. C., *The Trauma Manual: Trauma and Acute Care Surgery*. Wolters Kluwer Health: 2012.
81. Bentley, G., *European Instructional Lectures: Volume 11, 2011, 12th EFORT Congress, Copenhagen, Denmark*. Springer Berlin Heidelberg: 2011.
82. Abraham, E., Neutrophils and acute lung injury. *Crit Care Med* 2003, *31* (4 Suppl), S195-9.
83. Poggetti, R. S.; Moore, F. A.; Moore, E. E.; Bensard, D. D.; Anderson, B. O.; Banejee, A., Liver injury is a reversible neutrophil-mediated event following gut ischemia. *Archives of Surgery* 1992, *127* (2), 175-179.
84. Richardson, M. X.; Lodin, A.; Reimers, J.; Schagatay, E., Short-term effects of normobaric hypoxia on the human spleen. *Eur J Appl Physiol* 2008, *104* (2), 395-9.
85. Nordine, M.; Brauns, K.; Petricek, J.; Dosel, P.; Gunga, H.-C.; Stahn, A.; Opatz, O., The spleen: an endogenous reserve of red blood cell concentrate during acute physiological stress. 2014/09/16.
86. Yu, A. W.; Nawab, Z. M.; Barnes, W. E.; Lai, K. N.; Ing, T. S.; Daugirdas, J. T., Splanchnic erythrocyte content decreases during hemodialysis: A new compensatory mechanism for hypovolemia. 1997; Vol. 51, pp 1986-1990.
87. Uhl, B.; Vadlau, Y.; Zuchtriegel, G.; Nekolla, K.; Sharaf, K.; Gaertner, F.; Massberg, S.; Krombach, F.; Reichel, C. A., Aged neutrophils contribute to the first line of defense in the acute inflammatory response. *Blood* 2016, *128* (19), 2327-2337.
88. Rose, S.; Misharin, A.; Perlman, H., A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* 2012, *81* (4), 343-350.
89. Mayeur, N.; Minville, V.; Jaafar, A.; Allard, J.; Al Saati, T.; Guilbeau-Frugier, C.; Fourcade, O.; Girolami, J. P.; Schaak, S.; Tack, I., Morphologic and functional renal impact of acute kidney injury after prolonged hemorrhagic shock in mice. *Crit Care Med* 2011, *39* (9), 2131-8.

90. Faubel, S., Acute kidney injury and multiple organ dysfunction syndrome. *Minerva Urol Nefrol* 2009, *61* (3), 171-88.
91. Langenberg, C.; Bagshaw, S. M.; May, C. N.; Bellomo, R., The histopathology of septic acute kidney injury: a systematic review. *Crit Care* 2008, *12* (2), R38.
92. Ysebaert, D. K.; De Greef, K. E.; Vercauteren, S. R.; Ghielli, M.; Verpooten, G. A.; Eyskens, E. J.; De Broe, M. E., Identification and kinetics of leukocytes after severe ischaemia/reperfusion renal injury. *Nephrol Dial Transplant* 2000, *15* (10), 1562-74.
93. Bolisetty, S.; Agarwal, A., Neutrophils in acute kidney injury: not neutral any more. *Kidney Int* 2009, *75* (7), 674-6.
94. Amdur, R. L.; Chawla, L. S.; Amodeo, S.; Kimmel, P. L.; Palant, C. E., Outcomes following diagnosis of acute renal failure in U.S. veterans: focus on acute tubular necrosis. *Kidney Int* 2009, *76* (10), 1089-97.
95. Chawla, L. S.; Amdur, R. L.; Amodeo, S.; Kimmel, P. L.; Palant, C. E., The severity of acute kidney injury predicts progression to chronic kidney disease. *Kidney Int* 2011, *79* (12), 1361-9.
96. Williams, T. M.; Wise, A. F.; Alikhan, M. A.; Layton, D. S.; Ricardo, S. D., Establishing the flow cytometric assessment of myeloid cells in kidney ischemia/reperfusion injury. *Cytometry A* 2014, *85* (3), 256-67.
97. Zemans, R. L.; Colgan, S. P.; Downey, G. P., Transepithelial migration of neutrophils: mechanisms and implications for acute lung injury. *Am J Respir Cell Mol Biol* 2009, *40* (5), 519-35.
98. Ranieri, V. M.; Rubenfeld, G. D.; Thompson, B. T.; Ferguson, N. D.; Caldwell, E.; Fan, E.; Camporota, L.; Slutsky, A. S.; Force, A. D. T., Acute respiratory distress syndrome: the Berlin Definition. *JAMA* 2012, *307* (23), 2526-33.
99. Johnson, E. R.; Matthay, M. A., Acute lung injury: epidemiology, pathogenesis, and treatment. *J Aerosol Med Pulm Drug Deliv* 2010, *23* (4), 243-52.
100. Sadek, S.; Lockey, D. J.; Lendrum, R. A.; Perkins, Z.; Price, J.; Davies, G. E., Resuscitative endovascular balloon occlusion of the aorta (REBOA) in the pre-hospital setting: An additional resuscitation option for uncontrolled catastrophic haemorrhage. *Resuscitation* 2016, *107*, 135-8.

101. Lyon, R. M.; de Sausmarez, E.; McWhirter, E.; Wareham, G.; Nelson, M.; Matthies, A.; Hudson, A.; Curtis, L.; Russell, M. Q.; Kent, S. r. S. A. A. T., Pre-hospital transfusion of packed red blood cells in 147 patients from a UK helicopter emergency medical service. *Scand J Trauma Resusc Emerg Med* 2017, *25* (1), 12.

102. Rehn, M.; Weaver, A. E.; Eshelby, S.; Røislien, J.; Lockey, D. J., Pre-hospital transfusion of red blood cells in civilian trauma patients. *Transfus Med* 2017.

Affidavit

“I, Tom Eric Malysch certify under penalty of perjury by my own signature that I have submitted the thesis on the topic “Immunomodulation of IL6 transsignaling in polytrauma – organ specific effects of in vivo simulation in a murine multiple trauma model”. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (see above) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

Declaration of publications

Tom Eric Malysch had the following share in the following publications:

Publication 1:

Kleber C, Becker CA, **Malysch T**, Reinhold JM, Tsitsilonis S, Duda GN, Schmidt-Bleek K, Schaser KD. Temporal profile of inflammatory response to fracture and hemorrhagic shock: Proposal of a novel long-term survival murine multiple trauma model. *J Orthop Res.* 2015

Contribution in detail: operating on multiple trauma model, organ processing and histology, ELISA, data analysis and statistics

Signature, date and stamp of the supervising University teacher

_____

Signature of the doctoral candidate

_____

Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

List of publications

1. Kleber C, Becker CA, **Malysch T**, Reinhold JM, Tsitsilonis S, Duda GN, Schmidt-Bleek K, Schaser KD. Temporal profile of inflammatory response to fracture and hemorrhagic shock: Proposal of a novel long-term survival murine multiple trauma model. *J Orthop Res.* 2015

Acknowledgements

First of all, I want to thank Christian Kleber for introducing me to the world of research, the opportunity I was given by him with this topic and for guiding me to become a better researcher and clinician.

Additionally, I want to thank all my colleagues of the polytrauma working group at Charité Berlin for their support and their friendship over the last years. Specifically, I want to mention Jens Reinhold, who worked with me during sleepless nights and always provided me with moral support whenever I needed it. What I am most thankful for during the research period is the close bond of friendship that has built.

Furthermore, I am grateful to Katharina Schmidt-Bleek, Norma Schulz, Gabriela Korus and Claudia Schlundt from the Julius Wolff Research Institute in Berlin for their intense support, without whom this research work never would have been successful.

I also want to thank Jim Connolly and Andrew Coggins for their thorough advice.

Finally, I want to thank my family for everything they have done for me, in particular thank you to my wife Anja, who had to sacrifice a lot during the last years but always stood by my side and encouraged me. I love you.