Therapeutic Inhibition of Complement Component C5a in a Mouse Model of Post-traumatic Sepsis

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Submitted by:
Tanja Spenlingwimmer

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External Supervisor:
Dr.med.vet. Susanne Drechsler

Internal Supervisor:
Ass.-Prof. Mag.rer.nat. Dr.rer.nat. Priv.-Doz. Teresa Valencak

Consultant:
Univ.-Prof. Dr.rer.nat. Armin Saalmüller
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1. Question and Introduction

The aim of this bachelor thesis was to investigate whether the immunomodulating function of PMX-53, an antagonist of complement component C5a, improves survival of mice in post-traumatic sepsis.

To fully acknowledge the importance of sepsis research, it is essential to understand the underlying mechanisms of the disease. The following chapter begins with epidemiologic data on sepsis followed by introduction to the complex pathophysiology of post-traumatic sepsis and septic shock. It ends with an attempt to define the role of complement component C5a in sepsis.

Trauma and sepsis are among the leading causes of death worldwide and are closely related. For example, trauma patients do not typically die from the initial insult but rather from secondary complications such as sepsis (Mayr F. et al. 2014). In uncomplicated patients, each injury promotes a systemic inflammatory response, which is normally counterbalanced by an anti-inflammatory response. In patients with severe trauma (e.g. ischemia/reperfusion injuries, blood loss), the trauma itself and/or together with damage-control surgery can result in an exaggerated pro-inflammatory response. This can in turn cause a dysfunction and exhaustion of the immune response, which predisposes those patients for secondary infections and sepsis (Cirello V. et al. 2013, Angus D.C. and Van der Poll T. 2013, Brøchner A.C. and Toft P. 2009).

In the United States, the incidence of severe sepsis is estimated to be 300 cases per 100,000 people per year, the mortality of septic shock approached 50 % (Mayr F. et al. 2014). A study identified an increase of sepsis incidence in the United States over a 22 year period, from 82,7 cases per 100,000 population in 1979 to 240,4 cases per 100,000 in 2000 (Vincent J. et al. 2006). Incidences and mortality rate of sepsis continue to increase annually (Rhodes et al. 2016). This is most likely due to aging of the population, growing burden of chronic health conditions and increased use of immunosuppressive therapies, transplantations, chemotherapies and invasive procedures (Mayr F. et al. 2014).

People at high-risk for sepsis are typically infants, elderly (i.e. older than 65 years) and individuals who are immunosuppressed or suffer from chronic health conditions (e.g. diabetes) (Lindenauer P.K. et al. 2012). The survival rate rapidly decreases with increasing patient’s age: a 65-year-old septic patient had a survival rate of about 80 %, while the average survival rate of an 80-year-old individual was approximately 35 % (Turnbull I.R. et al. 2003). Although confirmed in numerous preclinical rodent studies (Drechsler S. et al. 2012, Diodato M.D. et al. 2001, Zellweger R. et al. 1997), the existence of a gender related outcome benefit for septic women is widely discussed given that data from clinical studies do not offer a clear consensus on that point (Hubacek J. et al. 2001, Combes A. et al. 2009, Vincent J. et al. 2006). The mortality of trauma patients with mild injury
who developed sepsis was greater than the mortality of those with moderate or severe trauma and sepsis (Osborn T.M. et al. 2004). With a mortality rate of about 20%, the mortality of post-traumatic septic patients remains relatively high (Wafaisade A. 2011).

There is no specific therapy for human septic patients; there are only detailed recommendations that depend on the patient’s status. This treatment approach is merely a supportive one, consisting of source control, resuscitation to improve tissue/organ perfusion, and administration of broad-spectrum antibiotics as the central anti-microbial intervention. The Surviving Sepsis Guidelines (Rhodes A. et al. 2016) recommend obtaining microbiologic cultures primarily from the blood before starting the antibiotic treatment whenever sepsis is suspected. In some patients, the use of vaspressors like norepinephrine, vaspressin and dopamine combined with resuscitation is required to restore adequate hemodynamics and therefore to improve survival. In cases of severe anemia, blood transfusion has to be performed. Occasionally, only platelets are transfused to restore blood coagulation (Rhodes A. et al. 2016).

There were phase III clinical trials in which IL-1 (Abraham E. et al. 1997, Abraham E. et al. 2001, Fisher C.J. et al. 1994) and TNF-α (Fisher C.J. et al. 1994, Fisher C.J. et al. 1996) were blocked to improve survival of septic patients. Yet, these therapeutic approaches failed to deliver the expected results. This was mostly due to improper animal models based on LPS administration that produced symptoms similar to sepsis/septic shock but failed to recapitulate the true immune-inflammatory dynamics of sepsis. Statistical analyses of preclinical studies (Eichacker P.Q et al. 2002) and human clinical trials (Knaus W.A. et al. 1996) indicate that anti-inflammatory treatments in patients that show an increased risk of mortality may indeed be beneficial. The pre-requisite for such a successful intervention must be, however, based on proper identification and stratification of septic patients who would likely benefit from such an anti-inflammatory therapy.

**Immune response to infection/trauma:** Both trauma and infection lead to the release of either damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) which are both recognized by pattern recognition receptors (PPRs) such as toll-like-receptors (TLRs). This interaction activates the innate immunity, which is on one hand essential for survival of infection and injury but on the other hand plays a pathologic role in tissue and organ injury associated with sepsis and trauma (Zhang Q. et al. 2010, Murphy K. and Weaver C. 2017). Interactions of pathogen and damage signals lead to the formation of protein complexes in the cytosol (called inflammasomes), which are essential for promotion and amplification of the inflammatory response (Petrilli V. et al. 2007).

Inflammation itself is a biological response initiated by harmful stimuli such as infection, physical trauma or a local immune response. In general, inflammatory processes are a protective attempt of an organism to remove injurious stimuli and to initiate the healing process. Infection (bacterial, viral,
fungal, parasitic) is among the most common causes of inflammation. In case of sepsis, the resulting inflammatory response in immuno-competent hosts leads to severe systemic reactions that cause extensive organ and/or tissue injury. The outcomes of reactions to infection are largely determined by the type of pathogen and to a large extent by characteristics of the host. Those include age, gender, acute and chronic health comorbidities (e.g. diabetes or HIV) – any factors that can influence the status of the innate and the adaptive immunity of the patient (Murphy K. and Weaver C. 2017, Springer Nature. 2017, Lindenauer P.K. et al. 2012).

In many cases, predominantly in immuno-compromised individuals, even localized infections can have broad systemic effects. Those effects are triggered (and directly contributed) by cytokine-induced systemic reactions which are collectively called the acute-phase response. The most important cytokines in this phase are TNF-α, IL-1 and IL-6, together with various types of interferons and chemokines responsible for chemotaxis of immuno-competent cells. The acute-phase response is accompanied by the following clinical symptoms and pathophysiological alterations: a) the first (most apparent) sign is fever, caused by so-called pyrogens, which induce the release of prostaglandins from vascular and perivascular cells of the hypothalamus. Exogenous pyrogens (e.g. lipopolysaccharides) induce the secretion of cytokines such as IL-1 and TNF-α, which then act as endogenous pyrogens by increasing the activity of cyclooxygenase (COX) that converts amino-acids to prostaglandins. b) The second characteristic is the release of acute-phase-proteins (e.g. C-reactive protein, serum amyloid A, fibrinogen) that are synthesized in the liver. During the acute-phase response plasma concentration of acute-phase-proteins can increase by several hundred-folds. The most essential function of acute-phase-proteins is the opsonization of pathogens and necrotic cells to facilitate their subsequent elimination by phagocytes. c) The third symptom is leukocytosis that can be rapidly detected by the basic blood count analysis. IL-1 and TNF-α cause an accelerated release of cells from the bone marrows post-mitotic reserve pool, resulting in an increased number of immature circulating neutrophils. Other non-specific symptoms that develop during the acute-phase response are increased blood pressure and pulse, rigors, chills, anorexia and somnolence (Kumar V. et al. 2016, Murphy K. and Weaver C. 2017).

Based on the newest Sepsis-3 guidelines, sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer M. et al. 2016). In other words, the aberrant host response and the presence of organ dysfunction are criteria that allow differentiation between infection and sepsis. Bacteria, viruses and fungi are the most frequent causes of sepsis. In the majority of cases, the primarily affected organs are the lungs, followed by gut and urinary tract infections. Dysfunction of two or more organ systems is termed multiple organ dysfunction syndrome (MODS) and strongly affects the cardiovascular system resulting in a reduction of peripheral vascular tone (vasodilatation because of nitric oxide), increase in capillary permeability
(capillary leak, causing edema), alterations of the regular blood flow to specific organs, microvascular occlusions and myocardial depression (Holzheimer R.G. et al. 2001). It has been shown that failure of three organs increases the mortality rate to 70 % (Martin G.S. et al. 2003, Vincent J.L. et al. 2006). If the underlying abnormalities in circulatory and cellular metabolism (e.g. *disseminated intravascular coagulation, hypotensive shock* and *metabolic disturbances* such as insulin resistance and hyperglycemia) become profound enough to substantially increase mortality, septic patients are diagnosed with septic shock (Singer M. et al. 2016, Kumar V. et al. 2016).

The **innate immune system** plays an essential role during infection, because its components (natural killer cells, dendritic cells, granulocytes, mast cells, macrophages and complement system) are the first responders to pathogens. Their other important function is elimination of damaged cells and foreign bodies (Kumar et al. 2016). Hence, restoration of hemostasis by a suitable modulation of the dysregulated immune response is a promising therapeutic target in animal models of sepsis or septic shock.

The **complement system** is a part of the innate immunity and consists of specific plasma proteins (complement components C1 to C9) that act together as a defense against pathogens in the extracellular space. It can be activated via different pathways and typically occurs as a response to infection. The complement system is able to kill pathogens directly via the membrane attacking complex (MAC), which creates a pore in the cell membrane of the pathogen that leads to its lysis, or via complement proteins that coat pathogens (i.e. opsonization) followed by their removal by phagocytes (Murphy K. and Weaver C. 2017).

**C5a** is a pro-inflammatory complement component which originates from the cleavage of C5 through a C5-convertase during complement activation. In contrast to the simultaneously released C5b (which is important for opsonization of pathogens and MAC formation) C5a is not involved in the generation of the MAC but rather functions as an anaphylatoxin. It plays an essential role for chemotaxis and has strong pro-inflammatory local and systemic effects like contraction of smooth muscle cells, increase of the vascular permeability, activation of the expression of adhesion molecules on endothelial cells to allow increased migration of leukocytes, activation of neutrophils and macrophages and release of histamine and TNF-α from mast cells. It also acts directly on neutrophils and monocytes to increase their adherence to endothelial cells, their migration towards the sites of antigen deposition and their phagocytic capacity (Murphy K. and Weaver C. 2017). C5a also plays a role in the formation of neutrophil extracellular traps and the activation of the inflammasome (Kalbitz M. et al. 2016, McDonald et al. 2016). Although complement component C3b adheres to the surface of pathogens, the activation of macrophages via C5a is essential to initiate phagocytosis. When produced in large amounts the release of C5a and C3a could cause a shock-alike syndrome similar to the effect of massive IgE release, termed anaphylactic shock.
(Murphy K. and Weaver C. 2017).

**Figure I: Interaction of C5a with C5aR during sepsis, resulting in i.e. compromised function of innate immunity, increased expression of pro-inflammatory mediators and coagulopathy (Ward P.A. and Gao H. 2009).**

**Preclinical evidence** showed that different complement components are involved in sepsis pathophysiology. In a rodent sepsis model example, the interaction of C5a with polymorphonuclear leukocytes (PMN) lead to an intense suppression of phagocytosis, chemotaxis and the respiratory burst. The same study demonstrated that macrophages simultaneously started to produce and release high amounts of cytokines (Huber-Lang M.S. et al. 2002). As shown by the Ward group, the upregulation of the C5a-receptor in different tissues and its massive interaction with C5a results in apoptosis via the intrinsic pathway (Ward P.A. et al. 2010).

During sepsis, C5-deficient mice showed a decreased ability to fight off invading microorganisms with a 400 fold increase of systemic bacteria compared to wildtype mice, because C5 is required for the assembly of the MAC. Those mice had significantly decreased levels of pro-inflammatory mediators, IL-1 receptor antagonist and IL-10 and showed severe neutropenia after CLP. Phagocytosis and the formation of oxidative bursts were also inhibited. This deficiency also caused a general loss of hemolytic complement activity under control and septic conditions (Flierl M.A. et al. 2008).

Therefore, the Flierl laboratory postulated already in 2008 that instead of blocking C5, the blockade of C5a and/or its receptors during sepsis constitutes a promising strategy against this disease. The expected benefit was that a blockade of C5a still allows MAC assembly, while the unfavorable and strong pro-inflammatory effects of C5a are prevented. They showed that an inhibition of C5a with a specific antibody during experimental sepsis in rats prevented the breakdown of the blood-brain
barrier and pituitary dysfunction (Flierl M.A. et al. 2009).

Cecal ligation and puncture (CLP)-induced experimental sepsis in rats led to a significantly elevated concentration of C5a already at 24h and its maximum release at 48h post-CLP (Flierl M.A. 2008). C5a antagonist treatment of rats challenged with CLP prevented organ damage assessed by specific multi-organ-failure parameters (Huber-Lang M. et al. 2001). The same treatment also showed in a CLP rat model that due to the administered therapy the activation of coagulation was nearly recovered (Laudes I. et al. 2002). Treatment with a C5a-receptor-antagonist reversed the lack of production of hydrogen oxide of neutrophils due to sepsis and improved the survival of mice (Huber-Lang M. et al. 2002). Overall, inhibition of C5a had protective effects in preclinical studies using a mouse and a rat model of polymicrobial sepsis (Hoehlig K. et al. 2013, Flierl M.A. et al. 2009, Ward P.A. et al. 2003).

Taken together, sepsis is a very complex reaction of an organism to an infection which involves overwhelming reactions of the immune system, ending up in severe systemic reactions causing extensive tissue injury. In the setting of post-traumatic sepsis, the immune system of the host is exposed to three activation challenges: a) traumatic insult, b) initial invasion of pathogens and c) dysregulation during developing sepsis. All three elements make the immune system an attractive target to modifications by experimental therapies. In this study, we aimed at modulating the effects of C5a by using a C5a antagonist (PMX-53) in a two-hit mouse model of trauma and sepsis. The main objective of using the PMX-53 antagonist was to prevent the strong pro-inflammatory effects while simultaneously retaining the positive effects of C5a concerning the defense mechanism against pathogens.
2. Animals and Methods
In this study a well-established two-hit-model of post-traumatic sepsis was used. It consisted out of traumatic hemorrhagic shock (TH) as first hit and cecal ligation and puncture (CLP) inducing polymicrobial sepsis as second hit.

Figure II shows the experimental setup of the performed two-hit model, consisting of traumatic hemorrhagic shock at -48 h followed by cecal ligation and puncture, 48h later. Blood sampling intervals are included indicated by the presence of a red blood drop at the time of sampling. CLP was considered as 0 h, because it was the time point of the polymicrobial sepsis onset. Generally blood samples were taken every 24 h, including one extra sample at 6 h post-CLP for the IL-6 measurement. IL-6 stratification has been validated via several studies in human patients (Groeneveld A.B. et al. 2003, Hack C.E. et al. 1989) and mice (Osuchowski M.F. et al. 2006, Remick D.G. et al. 2002). The specific time point of IL-6 measurement at 6 h post-CLP predicted the risk of death within the next 24 h very precisely (Remick D.G. et al. 2002). IL-6 deficient mice showed increased sepsis severity and more rapid mortality compared to wildtype mice, whilst nearly identical mortality rate, which suggests that IL-6 serves as a marker of disease severity and modulates physiologic responses (Remick D.G. et al. 2005).

2.1 Mice
Three-month-old female Balb/c mice purchased from Charles River (Germany) with an average weight of 20 g were used (total n=61) for all experiments. Mice were kept five per cage, the light-dark cycle was 12:12 and the temperature within the room was maintained between 22 °C and 24 °C. Standard rodent diet and water were provided ad libitum throughout the experiments. Cages were enriched with wood wool, tissues, wooden boards and small wooden blocks for gnawing to enable natural behavior.

2.2 First hit – Trauma-Hemorrhage (TH)
The first step was to administer 3-3.5 % Isoflurane (Forane®, Abbott, Germany) anesthesia via inhalation. Trauma was induced by a sinistral midshaft femur fracture performed with custom designed blunt pliers. This procedure was followed by hemorrhage (loss of 40% of total blood volume, defined as 6 % of total body weight) via punctuation of the retro bulbar vein plexus.
performed under the use of local analgesia (0.4 % Oxybuprocainhydrochlorid, Novain®, Agepha, Austria). This 40 % mark has been previously determined to ensure a survival of 95 % at the time of CLP (0 h) (Drechsler et al. 2010).

Post-TH, mice were resuscitated with 0,9 % ringer solution s.c. using three times the volume of the shed blood, first third ringer solution containing buprenorphine (0,05 mg/kg, s.c., Bupaq®, Richter Pharma, Austria) was administered immediately after TH, the remaining volume was given 1 h after the first administration.

The buprenorphine treatment (0,05 mg/kg in 0,5 ml Ringer solution, s.c., Bupaq®, Richter Pharma, Austria) was administered twice a day until CLP.

### 2.3 Second hit – Cecal ligation and puncture (CLP)

CLP was performed 48 h after TH using a 22 G needle to achieve a clinically relevant mortality of 50 % within the acute phase of sepsis (first five days after CLP). Buprenorphine (0,05 mg/kg in 0,5 ml Ringer solution, s.c., Bupaq®, Richter Pharma, Austria) was administered prior to anesthesia with 3,5 % Isoflurane (Forane®, Abbott, Germany) via inhalation. For CLP surgery, at first the abdomen was shaved and disinfected with Betaisodona solution (Betaisodona solution®, Multipharma, Austria).

Median laparotomy was performed via the linea alba to gain access to the abdominal cavity. The cecum got carefully pulled out through the incision with an anatomical forceps and the ligation was made with silk (Silkam® 4.0, B. Braun, Austria) underneath the ostium cecolicum (connection between cecum and colon) and the ostium ileale (connection between ileum and cecum), leaving the ileocecal valve unblocked. Gastrointestinal passage has to be preserved, to avoid causing an ileus instead of polymicrobial sepsis alone. After the ligation, cecum was punctured twice with a 22 G needle, one puncture situated at the apex caeci, the other one at the basis caeci and some of the intestinal content was pressed out of the cecum through the two holes.

The last step was to put the cecum back into the abdominal cavity and close the abdominal wall using two single-button sutures with Silkam 4.0 (B. Braun, Austria) and the skin with tissue glue (Histoacryl®, B. Braun, Austria). Two hours after CLP, antibiotic treatment with Imipenem and Cilastatin (25mg/kg, in 1ml Ringer solution s.c., Zienam®, MSD, Austria) was started and continued combined with the analgesic treatment (0,05 mg/kg, s.c., Bupaq®, Richter Pharma, Austria) twice a day until day 5 post-CLP. Survival was monitored for 28 days. Between day 5 and 28, buprenorphine treatment was resumed whenever an animal showed signs of deterioration or pain.
2.4 Blood sampling
Periodical sampling was performed at several time points throughout the experiment (as shown in Figure II, red blood drops indicate blood sampling) via punctation of the \textit{V. facialis} with a 23 G needle. Before analysis the blood samples had to be diluted due to the limited amounts of blood that are allowed to be withdrawn from mice daily (GV-SOLAS. 2017). Therefore 180 µl PBS with EDTA (1:50) were filled in 1,5 ml Eppendorf tubes before sampling. For sampling a small amount concentrated EDTA was collected within the pipette tip to avoid blood clots. 20 µl blood was collected from facial vein and immediately diluted in the previously prepared tube. All samples were centrifuged for 5 minutes at 5,0 rpm and 4 °C. After centrifugation 180 µl of the supernatant (diluted plasma) was carefully collected and stored at -80 °C for further analysis. The remaining pellet was resuspended with 180 µl of Cell-Dyn buffer (Diluent ST1600/2000, Abbott Laboratories, United States of America) containing EDTA (1:50). This 1:10 diluted sample was used for a complete blood cell count performed with a CELL-DYN3700SL high-resolution flow cytometry system (Abbott Laboratories, United States of America).

2.5 Blood cell count
The CELL-DYN3700SL system (Abbott Laboratories, United States of America) is a multi-parameter, automated hematology analyzer developed for diagnostic use. The EDTA-anticoagulated blood was analyzed for white blood cells, neutrophils, lymphocytes, red blood cells, platelets and hemoglobin. This system works via performance of simultaneous laser and electronic impedance measurements on blood cells, more specific this technology is called multi-dimensional cell classification or multi-angle polarized scatter separation. Four distinct light-scattering measurements are made on each individual white blood cell (0 °, 10 °, 90 ° and 90 ° depolarized) to differentiate them without the use of fixatives or cyto-stains. This technique allowed the identification blood cells very precisely even if they show abnormal pathology (Abbott Laboratories, 2013).

2.6 IL-6: cut-off predicting post-CLP outcome
Mice were stratified for outcome based on an IL-6 cut-off of 20 ng/ml. If the value of IL-6 at 6h post-CLP was higher than 20 ng/ml, the mouse was predicted to die and assigned to the P-DIE cohort. Mice with a value lower than 20 ng/ml were predicted to survive and assigned to the P-SUR cohort. This cut-off was calculated by statistical analysis of a pilot study (Fig. III). The stratification approach used in the study allowed identification/selection of mice with two contrasting disease phenotypes: a) high and b) low risk of death.
2.7 Cytokine ELISA

For IL-6 measurement the Bio-Plex® Suspension Array System and Multiplexing Technology was used (Bio-Plex 200 system, Bio-Rad Laboratories Inc., United States of America). The results were viewed with system specific software (Bio-Plex Manager Software 6.1, Bio-Rad Laboratories Inc., United States of America). Two different kits were necessary for performing this assay, the basic (ProcartaPlex® Simplex Bead Mouse Basic Kit, eBioscience, Austria) and the IL-6 specific kit (ProcartaPlex® Mouse IL-6 Simplex, eBioscience, Austria).

This immunoassay was based on magnetic microsphere technology that enabled the detection and quantitation of multiple protein targets. In this study only IL-6 specific beads were used.

The reagents were prepared according to the user manual from ProcartaPlex Mouse IL-6 Simplex. The first step was to prepare the antigen standard, afterwards the simplex beads had to be diluted, vortexed and 50 µl of the beads were pipetted into each well. 25 µl of the universal assay buffer and 25 µl of standards, controls or samples were added in each well. Next followed an incubation step for 120 minutes at room temperature on an ELISA-plate shaker. The next step was to wash with the provided washing buffer (repeated 3 times), followed by addition of 25 µl of the prepared detection antibody. After another 30 minutes of incubation at room temperature on the plate shaker, the washing step was repeated and 50 µl of Streptavidin-PE were added before another 30 minutes of incubation. After incubation the washing step had to be repeated again and then the beads had to be resuspended in 120 µl reading buffer and incubated for 5 minutes at room temperature placed on the plate shaker before the measurement with the Bio-Plex 200 multiplex suspension array system (Bio-Rad Laboratories Inc., United States of America) was started.

2.8 C5a-Antagonist and Vehicle

The used antagonist binds the human C5aR-C-peptide region, but it is able to inhibit the same region in mice or rats to a large extent. The antagonist (PMX-53) was spun down before opening to avoid any loss. The antagonist obtained from Prof. Lambris (University of Pennsylvania, School of Medicine) was soluble in PBS at 0.5 mg/ml, 0.5 mg were dissolved in 1 ml DPBS. This step took a considerable amount of time due to the poor solubility of the antagonist; to prevent heating it was important to put it on ice after two minutes of gentle stirring. The dosage for mice was 1 µg/g bodyweight. The antagonist was administered about 12-13 h post-CLP. Saline (0,9 %) was used as vehicle. Double randomization was performed. I.e. first, the P-DIE and P-SUR mice were randomly assigned to the treatment or control group, then the second randomization took place at the step of treatment administration, i.e. the person performing the tail vein injection did not know whether a given animal received treatment or vehicle.
2.9 Statistical analysis
The statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, USA). 28-day survival curves were plotted using Kaplan-Meier method. Data was tested for normality using the D’Agostino, Pearson and Sharpiro-Wilk test. Non-Gaussian data were log-transformed prior to further analyses. Differences between the groups were evaluated by Student’s t-test with usage of Welch-correction if needed or with 1-Way ANOVA with Turkey test for post-hoc for comparisons between different groups/time points. IL-6 levels were visualized via Box and Whiskers plots. Level of significance was set at p<0.05.

It was essential for this study to define an IL-6 cut-off for stratification of the mice into P-SUR and P-DIE cohort. Data from a pilot study was used to define the IL-6 cut-off at 6 h post-CLP, it was set at 20 ng/ml to provide a high enough sensitivity and specificity and therefore to minimize the incidents of false positives.

![Figure III: IL-6 cut-off 6 h post-CLP – data from pilot study](image-url)
3. Ethics statement

All animal procedures were approved by the Viennese (Austria) legislative committee (Animal Use Proposal Permission No. GZ: 343130/2013/14) and conducted according to the National Institutes of Health guidelines.

Due to the severity of the study maximally diligent observation of all mice was essential throughout the duration of the study to minimize suffering within the frames of the experimental design. The institute’s small animal facility allowed frequent and optimal monitoring therefore the overall health status was checked by trained professionals (i.e. DVMs and/or MDs) at least three times per day. Given that this study was designed as a survival study, post traumatic septic death was a critical endpoint. To avoid unnecessary suffering, a modified general condition score as recommended by Nemzek et al. in 2004 was used. Animals were scored at least once daily starting 24 h post-CLP until the end of acute sepsis (day 5 post-CLP). Our score included assessment of fur (not altered/dirty/blunt), posture (not altered/hunched/flat of tremors), mobility (not altered/upon disturbance/no movement) and alertness (not altered/decreased/no reaction). In addition body weight (loss of 0.6 g or more/no loss to 0.5 g loss/any gain), body temperature (>34 °C/ >28 and <34 °C/ <28 °C) and startle reflex were monitored. Mice were scored based on increasing severity, resulting in evaluation of a) 0 for no deterioration, b) 1 for medium severity changes and c) 2 for severe changes. A score of 8, body temperature below 28 °C or a missing startle reflex were criteria for immediate euthanasia. Animals were killed using deep inhalation anesthesia (isoflurane, Forane®, Abbott, Germany) followed by cervical dislocation. The body temperature cut-off of <28 °C has been previously established and identified dying septic mice with 94 % specificity, while a body temperature ≥35 °C identified surviving mice with 100 % specificity (Drechsler et al. 2015).

In order to respect the 3R (replace, reduce, refine) guidelines this study was planned and executed to minimize the amount of animals. Due to the intensive study design and severity of the study only 15 to 20 mice were operated at once and only two rounds were performed to guarantee best care and optimal monitoring.
4. Results

4.1 PMX-53 did not improve survival in secondary sepsis

A total of 61 mice were used for this study, based on IL-6 stratification 18 were assigned to the P-DIE cohort, 43 to the P-SUR cohort. The exact distributions are shown in Table I.

<table>
<thead>
<tr>
<th>Group</th>
<th>n(mice)</th>
<th>n(deaths)</th>
<th>n(survivors)</th>
<th>%-deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-DIE treatment</td>
<td>9</td>
<td>7</td>
<td>2</td>
<td>78 %</td>
</tr>
<tr>
<td>P-DIE vehicle</td>
<td>9</td>
<td>8</td>
<td>1</td>
<td>89 %</td>
</tr>
<tr>
<td>P-SUR treatment</td>
<td>21</td>
<td>7</td>
<td>14</td>
<td>33 %</td>
</tr>
<tr>
<td>P-SUR vehicle</td>
<td>22</td>
<td>5</td>
<td>17</td>
<td>9 %</td>
</tr>
</tbody>
</table>

Table I: Distribution of P-DIE and P-SUR subgroups and results of 28-day survival monitoring

As expected P-SUR mice survived better than P-DIE mice (p<0.05) and this effect was independent of the treatment group. Most deaths occurred within the first 5 days after CLP (Fig. VI). Treatment with PMX-53 did not improve survival of the P-DIE mice. Similarly, it did not negatively affect the outcome of P-SUR mice when compared to vehicle (Fig. VI). Occasional deaths of mice that were predicted to survive were related to the IL-6 stratification cut-off, which was chosen very conservatively to ensure maximal sensitivity (i.e. to minimize the incidents of false positives).

4.2 IL-6 based cut-off ensured adequate stratification of outcome

Evaluation of the IL-6 values at 6 h post-CLP showed that those P-SUR mice that died had IL-6 levels close to or higher than 16 ng/ml compared to other P-SUR mice (please refer to 9. Appendix, Tab. II). The P-SUR died cohort suggests that the IL-6 cut-off of 20 ng/ml provides an adequate sensitivity for identification of majority of mice with imminent death. Also, P-SUR stratification is short-term (for approximately 48 h); mice identified as P-SUR may deteriorate in the later stage of sepsis and eventually die. Thus, deaths after 48 h of P-SUR prediction do not invalidate the stratification approach.
Figure IV: Comparison of IL-6 level in each subgroup. P-SUR that survived (n=31), P-SUR that died (n=12), P-DIE that survived (n=3), P-DIE that died (n=15). Dotted line represents the chosen cut-off (20 ng/ml).

Figure V shows the IL-6 concentration of all mice treated with PMX-53 compared to those administered the vehicle. Data demonstrate that the IL-6 concentration was similar in both groups prior to the treatment indicating that, the study findings are not biased by the dissimilar disease severity at entry.

Figure V: Comparison of IL-6 level between treatment (n=30) and control (n=31) group mice.

Figure VI: 28-day survival of all subgroups divided based on the outcome-prediction and treatment.
4.3 PMX-53 treatment did not affect complete blood cell count

Complete blood cell count analyses were performed to check the influence of the treatment onto the number of certain blood parameters and cell types. Leukocytes (WBC), hemoglobin (Hb), neutrophils (NEU), lymphocytes (LYM), erythrocytes (RBC) and platelets (PLT) were screened. We did not find any statistically significant differences between treatment and control subgroups. In general, TH induced erythropenia (approximately 30 % decrease within 48 h, p<0.05), while at the same time leukocytosis (2-fold) with neutrophilia (3-fold) and lymphocytosis (2-fold) occurred when compared to the baseline (p<0.05). Despite the blood loss during TH, PLT decreased but returned to baseline values 24 h post-TH. After CLP, they reached their minimum (40 % drop compared to baseline) at 24 h and returned to baseline values within 96 h. After induction of sepsis, WBC, LYM and NEU decreased (by 42 %, 24 % and 59 %, respectively) within 6 h (p<0.05). Those parameters started to recover after reaching their minimum, typically between 24 and 48 h post-CLP. RBC and Hb decreased by 30 % after TH and remained relatively constantly low until the 96 h time point.
Figure VII: Cell counts and hemoglobin concentration of all mice in the first week of the experiment. red=P-DIE control, blue=P-DIE treatment, yellow=P-SUR control, green=P-SUR treatment; The number of samples per group was n= at least 7 from TH to 24 h post-CLP, and n= 2 in P-DIE control group at 72 h and 96 h. P-DIE treatment group had at least n= 6 from TH to 24 h post-CLP and n= at least 4 samples at 72 h and 96 h. In P-SUR groups for all time points n= at least 9.
5. Discussion

This is the first study testing the effect of PMX-53 (C5a receptor antagonist) on survival and hematologic parameters in a two-hit mouse model of post-traumatic sepsis. To further improve the value of the study, we used a treatment stratification approach to divide mice into high and low risk of death subgroups based on their circulating IL-6 concentration measured at 6 h post-CLP. This stratification facilitates better homogeneity within the stratified subgroups and is consistent with the recommendation from the clinical sepsis field that advises treatment of patients with more defined/characterized sepsis phenotypes thereby increasing the probability for a beneficial response to a given therapy.

Overall, the personalized treatment with a single dose of PMX-53 did not improve 28-day outcome in secondary post-traumatic sepsis in any of the subgroups.

There might be several reasons for this lack of protective effect. First, none of the previous in vivo studies which have demonstrated great potential of C5a inhibition in experimental sepsis were done in a two-hit setup with trauma as the first hit (Hoehlig K. et al. 2013, Flierl M.A. et al. 2006, Flierl M.A. et al. 2009, Huber-Lang M. et al. 2001, Huber-Lang M. et al. 2002). Severe trauma initiates an inflammatory response and subsequent immune dysfunction that can influence immune responses in secondary sepsis (Gentile L.F. et al. 2013). Evidence for an involvement of the complement system in the post-traumatic immune response has been recently provided. Activation of the complement system happens immediately after a traumatic injury as a response to PAMPs and DAMPs. This activation has various effects e.g., on the coagulation cascade, the cytokine/chemokine network and fracture healing. Complement induced hyper-activation or dysfunction of various systems promotes/leads to complications such as sepsis and MODS. Trauma results in generation of C3a and C5a, and it has been demonstrated in rodent studies that those complement factors compromise fracture healing. Inhibition of C5a after trauma showed to normalize fracture healing processes (Huber-Lang M. et al. 2013, Huber-Lang M. et al. 2015).

Second, we chose to administer PMX-53 at a later time point compared to previous studies. Huber-Lang M. et al. described the life-saving effect of PMX-53 in a CLP model when PMX-53 was administered immediately after CLP (Huber-Lang M. et al. 2002).

It is known that C5a can be detected about 2 h after the initiation of experimental sepsis and it stimulates the production of further pro-inflammatory mediators (Flierl M.A. et al. 2006). Its receptor (C5aR) has been found to be expressed on both inflammatory (Chenoweth D.E. and Hugli T.E. 1978) and non-inflammatory cells (Osaka H. et al. 1999, Fayyazi A. et al. 2000). C5aR on neutrophils is downregulated when C5a is extensively expressed due to internalization of C5aR, which results in innate immunity dysfunction and impaired ability of neutrophils to produce IL-8 (Xu R. et al. 2016) and to release an oxidative burst (Huber-Lang M. et al. 2002). Increased
expression of C5a and C5aR on polymorphonuclear cells and endothelial cells causes increased inflammatory injury of organs in sepsis (Guo R.F. and Ward P.A. 2006). Due to the upregulation of C5a within 2 h of the initiation of experimental sepsis and the consequential simultaneous regulation of C5a receptors on different cell types and organs, the harmful effects of C5a might have already taken place before the delayed PMX-53 administration in the present study.

Although the earlier treatment time points proved the protective effects of PMX-53 on the immune system, they are not translatable into clinical scenario given that sepsis is diagnosed within a few hours after its onset at the earliest and typically later, especially in outpatients. Our choice to provide therapy at 12 h post-CLP was therefore not only based on the fact that IL-6 as an outcome predictor in mice is reliable at 6h post-CLP (Remick D.G. et al. 2002). We also wanted to test whether the administration of the antagonist after onset of the first symptoms was still powerful enough to prevent adverse outcomes in mice with a high risk of death. However, a possible limitation of this study is the duration of the IL-6 Multiplex assay. Stratification of mice in P-DIE and P-SUR cohort before administration of any treatment took place at approximately 12 h post-CLP, with a delay due to blood sampling at 6 h after CLP and another 6 h delay due to subsequent IL-Multiplex analysis. This means that when treatment was administered, the first symptoms of sepsis were already apparent. On one hand, this was intended given that it recapitulated the situation in human septic patients who receive treatments relatively late (with ostensible sepsis symptoms). On the other hand, this could have meant that the therapeutic window had already closed and it was too late for PMX-53 to effectively save mice predicted to die.

In addition, although we did not yet investigate any parameters indicative for organ injury, the ~78 % mortality rate of the PMX-53 treated P-DIE mice in our study suggests that organ injuries caused by the strong pro-inflammatory host response had already progressed to a life-threatening level before PMX-53 was administered. Hence, it is possible that the antagonist slowed down the progression of organ injury for a short time but due to the one-time administration and the short half-life of PMX-53, it did not have the desired long-lasting effect. To improve survival, it would therefore be a good approach to administer the antagonist several times during the acute phase to overcome the problem of the short half-life of PMX-53. The early, rapid-clearance phase (≤ 3 h after administration) of PMX-53 is characterized by a mean half-life of ~4h. In the late phase (24-72 h) PMX-53 is characterized by a half-life of ~12h (Huber-Lang M. et al. 2002). Thus, repeated administration would prolong the time in which the pro-inflammatory effects of C5a are inhibited and might offer a chance for physical recovery from the initial organ injuries.

In accordance with the lack of effect on survival, single treatment with PMX-53 in post-traumatic sepsis did not affect the dynamics of the circulating blood cells, the readouts remained comparable and congruent between all groups until 24 h post-CLP. From this time point onwards the number of
individuals per time point decreased especially in the P-DIE groups. Changes between the groups after 24h must therefore be viewed with caution, given that e.g. only 2 P-DIE control samples were available at 72 h and 96 h post-CLP. No statistically significant differences could be shown at those time points.

The IL-6 cut-off was chosen based on preliminary data for female Balb/c mice. During our experiments several mice of the P-SUR cohort died. This might have been due to the conservative cut-off set at 20 ng/ml. While the original cut-off would have been 16 ng/ml, it was increased to 20 ng/ml in order to minimize the probability of false positives (i.e. increase specificity), while simultaneously the probability of false negatives increases. This justification is supported by the noticeable tendency that those P-SUR mice that died had rather high IL-6 levels close to 16 ng/ml or higher 6h post-CLP (please refer to 9. Appendix, Tab. II).

After CLP, encapsulation of the ligated cecum results in the formation of an abscess due to progressing infection and necrosis (Kumar V. et al. 2016). Formation of an abscess occurs towards the end of the acute phase of sepsis. It is possible that the abscess effuses spontaneously into the peritoneum, which is a possible reason for the sudden deaths of P-SUR mice during the 28-day monitoring period.

Mice that did not recover from CLP surgery and died early within 12 h post-CLP were excluded from the study because those deaths could not reliably be accounted for by sepsis and could have biased the results. Technical shortcomings could have been possible reasons; e.g. too narrow ligation of the cecum, which constipates the ileo-cecal valve, resulting in an ileus. Mice that died within the acute phase of sepsis tended to have very high IL-6 levels (please refer to 9. Appendix, Table II).

Recapitulating, the best possibility to improve the outcome in this particular experimental setup is to minimize the delay between 6 h sampling and administration of the treatment (challenging due to the duration of the IL-6 ELISA). In addition, the current design is closer to the clinical setting, because the administration of the treatment takes place after diagnosis of the first septic symptoms. Increasing the administered PMX-53 dosage is possible, but it has been shown that 1 µg/g bodyweight is the ideal dosage for mice, and even a 3-fold dose increase failed to improve survival (Huber-Lang M. et al. 2002). Another viable alternative is a repeated application of PMX-53 as it could help to prolong its effects by overcoming the short half-life time of the antagonist.
6. Summary

6.1 Abstract
The aim of this study was to test whether the therapeutic inhibition of complement component C5a has a positive effect on survival in a mouse model of post-traumatic sepsis.

To induce post-traumatic sepsis, a well-established two-hit model was performed. It consisted of trauma and hemorrhage as the first hit, which was defined as unilateral midshaft femur fracture and blood loss of 40% of the total blood volume (calculated as 6% of the body weight). The second hit was performed 48 h later and consisted of the ligation and puncture of the cecum (CLP), which is a standard model to for induction of polymicrobial sepsis from the abdominal source.

For stratification of the mice into predicted to survive (P-SUR) and predicted to die (P-DIE) cohorts, a blood sample was taken 6 h post-CLP via puncture of the facial vein to measure the IL-6 concentration via ELISA. For stratification, a cut-off was set at 20 ng/ml based on data from previous studies. Individuals with an IL-6 concentration below this cut-off were assigned to the P-SUR, while all mice with an IL-6 concentration above 20 ng/ml were assigned to the P-DIE cohort. After stratification and randomization, the C5a-antagonist (PMX-53) or the control treatment were administered to the half of each cohort. The general condition of the mice was assessed over 96 h, antibiotics and painkillers were administered twice a day during this period. General condition and survival of the mice was monitored for 28 days. In our study, we were not able to find any survival advantage of P-DIE mice due to PMX-53 treatment. To increase probability of treatment benefits, we suggest changing the study design to prolong the time of C5a inhibition with repeated injections in the post-CLP period.
6.2 Zusammenfassung

In dieser Bachelorarbeit wurde mittels einer in vivo Maus-Studie ermittelt, ob die therapeutische Inhibition des Komplementfaktors C5a mittels eines Antagonisten einen positiven Effekt auf das Überleben einer posttraumatischen Sepsis bewirkt.


Zur Stratifizierung in jene Tiere, die mit hoher Wahrscheinlichkeit überleben (P-SUR) und jene, die mit hoher Wahrscheinlichkeit sterben (P-DIE), wurde eine Blutprobe 6 h nach der CLP durch eine Punktion der Vena facialis entnommen und anschließend die IL-6 Konzentration mittels ELISA ermittelt. Um eine Stratifizierung zu ermöglichen, wurde der Schwellenwert auf 20 ng/ml festgelegt, hierfür wurden Daten aus einer vorangegangenen Studie herangezogen. Alle Tiere mit einer IL-6 Konzentration geringer als 20 ng/ml wurden zur P-SUR Kohorte zugeordnet, bei einer Konzentration größer 20 ng/ml erfolgte die Zuordnung zur P-DIE Kohorte. Nach dieser Einteilung erfolgte bei je 50% jeder Kohorte die randomisierte Verabreichung des C5a-Antagonisten (PMX-53) oder der Kontrollsubstanz. Schmerzmittel und Antibiotikum wurden während der ersten 96 h nach der CLP zwei Mal täglich verabreicht, außerdem wurde der Allgemeinzustand der Tiere bewertet und festgehalten. Der Allgemeinzustand und das Überleben der Tiere wurden anschließend für die folgenden 28 Tage beobachtet. In dieser Studie konnte kein Überlebensvorteil jener P-DIE Tiere festgestellt werden, die mit PMX-53 behandelt wurden. Um die Wahrscheinlichkeit für positive Effekte auf Grund der Behandlung mit PMX-53 zu erhöhen, empfehlen wir eine Veränderung des Studiendesigns zu Gunsten der Verlängerung der C5a-Inhibition durch mehrfache Verabreichung in der akuten Phase nach der CLP.
7. List of references


54. Turnbull I.R., Wizorek J.J., Osborne D., Hotchkiss R.S., Coopersmith C.M., Buchman T.G.


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Table II: IL-6 concentration 6 h post-CLP, stratification via P-SUR/P-DIE and survival, treatment group and survival period of all individuals