The effect of C5a activity on adrenal functionality (following polytrauma) in male C57BL/6 mice.

Dissertation

submitted to the Faculty of Medicine of the Ulm University
in Fulfillment of the Requirements
for
Doktor der Medizin

Julia Franziska Kunze

born in Kempten

2019
current dean: Prof. Dr. Thomas Wirth
first commentator: Prof. Dr. Stefan Reber
second commentator: Prof. Dr. Markus Huber-Lang
day of promotion: July 3rd 2020
dedicated to my beloved parents
# Table of contents

ABBREVIATION INDEX VI

1 INTRODUCTION 1

1.1 EPIDEMIOLOGIC ASPECTS OF TRAUMA 1

1.2 CAUSES OF TRAUMA AND INJURY PATTERNS 2

1.3 EXPLANATION OF THE TERM POLYTRAUMA 3

1.4 PATHOPHYSIOLOGICAL ASPECTS OF POLYTRAUMA 4

1.4.1 INFLAMMATORY RESPONSES FOLLOWING POLYTRAUMA 6

1.4.2 ANTI-INFLAMMATORY RESPONSES FOLLOWING TRAUMA 12

1.4.3 THE INTERRELATION BETWEEN THE HPA-AXIS AND THE IMMUNE SYSTEM 17

1.5 AIMS OF THE STUDY 19

2 METHODS 22

2.1 MATERIAL 22

2.1.1 ANIMALS 25

2.2 EXPERIMENTAL PROCEDURES 25

2.2.1 EXPERIMENT 1 25

2.2.2 EXPERIMENT 2 27

2.2.3 EXPERIMENT 3 28

2.3 ELISA FOR CORT 28

2.4 ORGAN PREPARATION 29

2.5 OIL-RED-O STAINING 29

2.6 DIGITAL IMAGE ACQUISITION 30

2.7 IMAGE PROCESSING WITH REIMAGE.EXE 30

2.8 SOFTWARE VALIDATION WITH IMAGE J 31

2.9 STIMULATION OF ADRENAL EXPLANTS WITH ACTH IN VITRO 32

2.10 DETERMINATION OF RELATIVE ADRENAL CORT CONTENT 33

2.11 STIMULATION OF ADRENAL EXPLANTS WITH CSA, CSA-SPIEGELMER AND CSA+CSASPIEGELMER IN VITRO 33

2.12 STATISTICAL ANALYSIS 34

3 RESULTS 35

3.1 COMPARISON OF REIMAGE.EXE AND IMAGE J FOR QUANTITATIVE ANALYSIS OF LIPID DROPLET STAINING 35

3.2 EFFECTS OF CSA SPIEGELMER ON BODYWEIGHT AND HPA- PARAMETERS FOLLOWING POLYTRAUMA 35

3.2.1 EFFECTS OF CSA SPIEGELMER ON ADRENAL IN VITRO ACTH SENSITIVITY FOLLOWING POLYTRAUMA 35

3.2.2 EFFECTS OF CSA SPIEGELMER ON ADRENAL WEIGHT FOLLOWING POLYTRAUMA 37

3.2.3 EFFECTS OF CSA SPIEGELMER ON BODYWEIGHT FOLLOWING POLYTRAUMA 38

3.2.4 EFFECTS OF CSA SPIEGELMER ON ADRENAL CORTICAL LIPID DROPLET CONTENT FOLLOWING POLYTRAUMA 39

3.2.5 EFFECTS OF CSA SPIEGELMER ON ADRENAL CORT- CONTENT FOLLOWING POLYTRAUMA 40

3.2.6 CORRELATION ANALYSES 41

3.4 EFFECTS OF CSA, CSA-SPIEGELMER AND THE COMBINATION OF BOTH ON ACTH SENSITIVITY OF ADRENAL EXPLANTS IN VITRO 47

4 DISCUSSION 48

4.2 ADRENOCORTICAL CONTENT OF LIPID DROPLETS IS NOT AFFECTED BY POLYTRAUMA OR CSA SPIEGELMER 52

4.3 ADRENAL CORT CONTENT IS NOT AFFECTED BY POLYTRAUMA OR CSA SPIEGELMER 54

4.4 CORRELATION ANALYSES OF CORT CONTENT, LIPID DROPLETS AND ACTH SENSITIVITY FOLLOWING PT AND CSA SPIEGELMER TREATMENT 58
4.5. C5a and C5a SM do not affect in vitro CORT production of naïve mice 59
4.6. C5a enhances ACTH sensitivity of adrenal explants from naïve mice in vitro 62
4.7. Conclusion and outlook 64
5 Abstract 66
6 Zusammenfassung 68
7 References 70
Appendix 96
Acknowledgments 99
Curriculum Vitae 101
## Abbreviation Index

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACAT</td>
<td>acyl-coenzyme A cholesterol transferase</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Acyl CoA</td>
<td>acyl-coenzyme A</td>
</tr>
<tr>
<td>AI</td>
<td>adrenal insufficiency</td>
</tr>
<tr>
<td>AIS</td>
<td>Abbreviated Injury Score</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C5aR1</td>
<td>C5a-receptor-1</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CARS</td>
<td>compensatory anti-inflammatory response syndrome</td>
</tr>
<tr>
<td>CBG</td>
<td>corticosteroid-binding globulin</td>
</tr>
<tr>
<td>CE</td>
<td>cholesteryl ester</td>
</tr>
<tr>
<td>CH50</td>
<td>complement hemolytic activity-50</td>
</tr>
<tr>
<td>CORT</td>
<td>corticosterone</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotropin-releasing factor</td>
</tr>
<tr>
<td>CRF-R</td>
<td>corticotropin-releasing factor receptor</td>
</tr>
<tr>
<td>DALY</td>
<td>disability-adjusted life year</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger-associated molecular pattern</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>e.g.</td>
<td>for example</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GC</td>
<td>glucocorticoid</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HMG CoA</td>
<td>hydroxyl-methyl-glutaryl coenzyme A</td>
</tr>
<tr>
<td>HPA-axis</td>
<td>hypothalamic–pituitary–adrenocortical axis</td>
</tr>
</tbody>
</table>
HSL: hormone sensitive lipase
i.a.: inter alia
Ig: immune globuline
IL: Interleukin
ISS: Injury Severity Score
l: liter
LC: locus coeruleus
LD: lipid droplet
LDL: low-density lipoprotein
MAC: membrane attack complex
MASP: MBL-associated serine protease
MBL: mannose-binding lectin
MC2R: melanocortin-2 receptor
mg: milligram
min: minute
ml: milliliter
MODS: multiple organ dysfunction syndrome
MOF: multiple organ failure
MR: mineralocorticoid receptor
MRAP: melanocortin-2 receptor associated protein
MWU: Mann-Whitney-U test
ORO: Oil-Red-O
PAMP: pathogen-associated molecular pattern
PBS: phosphate buffered saline
PKA: protein kinase A
PN: paraventricular nucleus
POMC: proopiomelanocortin
PT: polytrauma
PTSD: post-traumatic stress disorder
QOL: quality of life
RKI: Robert-Koch institute
ROI: region of interest
RT: room temperature
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>scc</td>
<td>side-chain cleavage</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SIRS</td>
<td>systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SNS</td>
<td>sympathetic nervous system</td>
</tr>
<tr>
<td>SR-B1</td>
<td>scavenger receptor B1</td>
</tr>
<tr>
<td>StAR</td>
<td>steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like-receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>vs</td>
<td>versus</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
</tbody>
</table>
1 Introduction

1.1. Epidemiologic aspects of trauma

Barely any other stroke of fate might change a person’s and its family’s lives as dramatically and unexpectedly as the occurrence of a health delimitating event. Hereby, severe injuries belong to those health issues, that out of the sudden can compromise the well-being of a person completely. The extent to which injuries threaten people’s lives all over the world is clarified by the fact that injury-related morbidity as well as mortality make a large contribution to the overall global burden of disease[215,230,316]. According to the Robert-Koch-Institute (RKI), every tenth person in Germany gets involved in a traumatic event every year and a large proportion of those accidents ends up fatal with over 20.000 people dying due to injuries per year[258]. Indeed, trauma is the third-leading cause of death in industrialized countries throughout all ages following cardiovascular diseases and malignancies[6]. Making it even more captivating, the scale of that problem is expected to further enlarge in the future and trauma-related deaths are predicted to increase by nearly 30% until 2030 [192,319]. Although trauma affects people throughout their lives, the risk to sustain a serious accident is especially high amongst the younger population. Until the age of 45, trauma is the most frequent cause of death and every fourth adult dying at an age between 25 and 29 loses his or her life because of one or more harmful event(s)[118].

Nevertheless, deadly accidents represent only the tip of the iceberg, since the amount of not lethal traumata is not negligible[13,155,217,303]. For every person that dies from the consequences of trauma, there are even more becoming disabled persistently by the latter. In this context, the calculation of the so-called disability-adjusted life years (DALY) provides more comprehensive information about the epidemiology. The DALY-concept actually includes not only the life years that are lost due to a health issue but also years that are spent with adverse health outcomes. In 2015, the “Global Burden of Disease Study 2015” [216] revealed that almost 250 million DALYs could be traced back to injuries, which corresponded to a proportion of nearly 11% of all DALYs worldwide[303].
Besides direct physical restriction, also personal, social, and environmental perspectives might further aggravate the burden of trauma patients – an aspect that recently has been investigated in a study evaluating the quality of life (QOL) of injury victims[163]. Accordingly, not only the somatic consequences involving bodily pain but also psychological sequelae of trauma such as feelings of dependency, social impoverishment, impaired mental and emotional well-being contribute to the reduction of the QOL. That again can be regarded in the context of an enhanced risk for psychiatric complications like anxious or depressive symptoms[114,225,301], emotional maladjustments[121,122,123,197,225] and even increased incidence of posttraumatic stress disorder (PTSD)[40,101,118,123,144,174,195].

Taken together, trauma and its consequences undoubtedly represent a far-reaching problem in terms of mortality as well as considering physical or mental morbidity and restrictions concerning one’s social life.

1.2. Causes of trauma and injury patterns

There is a large variety of incidences that can result in the experience of a trauma. Those can range from willful violence or self-harm on the one hand to unintended traumata like home-, work- or sports-related accidents on the other[182]. For instance, falls have often been described to rank first regarding accidents occurring in Germany[159,258,]. When it comes to the most fatal injuries, however, most trauma-related deaths can be attributed to traffic accidents[93,182,318]. In fact, more than 1.2 million fatalities each year can be attributed to road crashes and by the year 2030, traffic accidents are assumed to belong to the four major causes of deaths in the world[192]. In the course of such traumata, a body might absorb blunt and sharp forces implicating injury mechanisms with an especially high probability to be life-threatening[161,244]. Regarding the most probable patterns of injuries, many studies quote high incidences for musculoskeletal and head-neck injuries followed by visceral traumata concerning the thorax and abdomen[3,35,50,253]. Moreover, vascular injuries followed by post-traumatic hemorrhage possibly resulting in ischemia is known to be a frequent trauma complication[59,158,159], as excessive bleeding has shown to be one of the most possibly avoidable reasons for injury-related death[59].
In summary, those trauma patterns have in common, that they per se pose a high
danger for injury victims. But, there is rising evidence that it rather is the concurrence
of multiple injuries that further aggravates the situation of trauma patients[1].
Revealingly, Gabbe et al. determined a correlation between sustaining more than one
injury and the degree of disability one year after trauma[104]. This is in line with
supporting studies showing that combined traumata extend the therapeutic complexity
of spinal cord injury[272] or paraplegia[246] and worsen the outcome after severe
head injury[309]. Moreover, Kleber et al. recently has described multiple traumatization
as being the main reason for injury-related deaths in Berlin[159] and has therefore
concluded that a combination of injuries imply an increased probability of becoming
life-endangering.

Knowing that the accumulation of injuries impairs the recovery and prognosis of injured
subjects, there is considerable interest in getting further insight into the pathogenetic
mechanisms of the multiply injured. In this context, the term polytrauma (PT) has
frequently been used in clinical and scientific practice.

1.3. Explanation of the term polytrauma

In the last decades, the concept of PT has frequently been applied to describe an
exceeding degree of severity of injury and is particularly common throughout the
European trauma literature[287]. In the American literature, however, PT is
synonymously referred to as major trauma or multiple trauma[44,257]. Although many
efforts have been made to characterize the term PT in more detail, the current
terminology still lacks clearness and a generally valid definition of PT is still
missing[31,44]. More than 40 years ago, a first formal definition was proposed by
Border and colleagues defining a PT patient by sustaining two or more “significant
injuries”[31]. Later, this concept was refined by Faist et al., who defined PT as the
concurrence of at least two injuries of which at minimum one injury or the sum of all
injuries is life-threatening[95]. This explanation still makes an impact on today’s
understanding of PT and suggests the amount as well as the extent of injuries to be
the major criteria. Indeed, classification systems like the “Injury Severity Score” (ISS)
[16] or the “Abbreviated Injury Score” (AIS)[251] have been introduced to determine
anatomical sites of traumata. But again, the usage and interpretation of these scores,
e.g. the ISS, is highly fluctuating[6,13,27,132,200,204,228]. What further complicates these discrepancies is the fact that the term PT recently has also been applied not only for physical but also for psychosocial traumata[19,120].

Especially important for the present thesis is that the organism’s response to trauma has more and more been included in the process of finding a proper description of PT. According to that, the general understanding of PT has progradiently get completed by the sequential systemic reactions implicating the dysregulation and -in the worst case- the break-down of vital organ functions[26,220]. Amongst a large variety of pathophysiological occurrences that have been discussed to be incorporated in a PT definition, the inflammatory response seems to play a key role in the pathogenesis of the latter[91]. For that reason, investigations further focused on the concept of the systemic inflammatory response syndrome (SIRS) to characterize PT and predict posttraumatic outcomes[91].

Summing it up, despite of a co-existence of distinct approaches to specify PT, pathophysiological processes, particularly post-traumatic immune responses, certainly are at least as indicative for PT as the bare concurrence of more than one injury. Together with the fact that trauma patients with an adverse outcome differ from low-risk trauma victims in terms of their response, in particular their inflammatory response, to injuries[62,322], this substantiates the need to obtain a more comprehensive knowledge of the pathogenesis of PT. This might help to develop novel approaches to early identify high-risk patients and pave the way to patient-specific treatment.

1.4. Pathophysiological aspects of polytrauma

Since PT constitutes a life- threatening situation for an organism, it is accompanied by a range of pathophysiological reactions within the host to respond towards damage and to restore physiological conditions.

The so called “two hit theory” [260,269] describes primary and secondary post-traumatic challenges that initiate a complex cascade of defense mechanisms. At first, direct forces of the traumatic event result in initial tissue injury involving the massive release of agents, so called “danger-associated molecular patterns” (DAMPs) [162,325] such as nucleic acids, cell organelles or components of the extracellular
matrix or cell membrane[186]. Trauma might further destroy physiological barriers like the skin or mucous membranes (e.g. in the gastrointestinal tract). The immune system is thereby confronted with exogenous pathogens. In detail, it has to respond to specific molecules associated with groups of pathogens, also known as “pathogen-associated molecular patterns” (PAMPs)[196]. Additionally, intrinsic complications involving respiratory and metabolic distress (e.g. hypoxia, hypothermia or hemodynamic instability) and extrinsic (iatrogenic) burden (e.g. surgery, anesthesia etc.) account for so called “second hits” [80,305] that, in addition to DAMPs and PAMPs, disturb an organism’s homeostatic balance.

Such “danger signals” are then detected by a machinery of interrelating defense systems that imply two substantial ways of facing the traumatic burden: i) a “systemic inflammatory response” (SIRS) and ii) counter-regulatory processes that in the sum have also been referred to as the “compensatory anti-inflammatory response syndrome” (CARS). For a long time, these two mechanisms were described to occur sequentially. Nowadays, though, there is a growing body of evidence that both rather happen simultaneously. Indeed, this concept has additionally been supported by cohort studies revealing an increase of pro-inflammatory but also of anti-inflammatory markers 2-24 h after trauma[143,146,266,321]. Both components of the posttraumatic immune response (SIRS and CARS) are each characterized by a variety of an organism’s mechanisms mediating pro- or anti-inflammatory effects, respectively.

The complement system is generally noted for being a representative mediator of posttraumatic inflammation. On the opposite, inflammation is importantly modulated by neuroendocrine responses. In detail, the hypothalamus-pituitary-adrenal (HPA)-axis is supposed to mediate a fine equilibrium between SIRS and CARS to eventually initiate recovery. A general overview of the main aspects of the SIRS, especially the complement system, and CARS including the modulation via the HPA-axis are addressed in Figure (Fig.) 1 and in more detail in the following chapters.
Figure 1: Pathophysiological aspects of the posttraumatic immune response. According to the “two-hit-theory”, first and second hits constitute a danger situation within the host organism. Danger-associated molecular patterns (DAMPs) as well as pathogen-associated molecular patterns (PAMPS) serve as mediators that induce (i) a “systemic inflammatory response syndrome” (SIRS) and simultaneously (ii) inflammation modulating reactions, characterized as a “compensatory anti-inflammatory response syndrome” (CARS). An essential mediator during SIRS is the complement system translating the danger situation into upregulation of immune competent cells and pro-inflammatory cytokines (tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β) and IL-6, -8, -12, -18). Concerning CARS, neuroendocrine modulation takes place in terms of activation of the hypothalamus-pituitary-adrenal (HPA) axis, that is capable to ameliorate immune cell activation and to induce the secretion of anti-inflammatory cytokines (IL-10, Transforming growth factor-β (TGF-β), soluble TNF-receptor (sTNFr) and IL-1 receptor antagonist (IL-1-ra)). Illustration by Julia Kunze.

1.4.1. Inflammatory responses following polytrauma

The posttraumatic state is characterized by a systemic inflammatory response that begins about half an hour after the traumatic incident. It is rather a response towards tissue damage (DAMPs) or blood loss than towards infection (PAMPS)[325]. Thus, a variety of cell types build up an interplay characterized by a massive release of pro-inflammatory cytokines, also referred to as a “cytokine storm”[302]. The latter contributes to the manifestation of a hyper-inflammatory condition[261]. Early after trauma (one to two hours) increased levels of tumor necrosis factor-α (TNF-α) and Interleukin-1β (IL-1β) can be observed, followed by a subacute rise in for instance IL-6, -8, -12 and -18[115]. This in turn leads to the recruitment and activation of immunocompetent cells such as neutrophils and triggers them to secrete other inflammatory substances[36,252]. Moreover, the release of free oxygen radicals
Introduction

(“oxidative stress”/ “respiratory burst”), enzymes and other cytokines results in an exacerbation of inflammation and holds up a self-perpetuating circuit of inflammation responses[219].

A systemic inflammatory response is inevitable to accomplish the process of recovery. If it is exaggerated in extent or duration, however, primarily beneficial inflammatory processes may convert into detrimental effects. Supporting this, a prolonged, overshooting SIRS condition also indicates an enhanced risk for irreversible organ damage up to multiple organ dysfunction syndrome (MODS) and multiple organ failure (MOF)[6,63,89,146]. An extended pro-inflammatory cytokine increase correlates with the severity level of PT[190,267,289] and predicts the onset of MODS or MOF after severe traumatic events like traumatic brain injury (TBI) [184] or PT[102]. By means of this, it also has been possible to early diagnose trauma victims that are at a particularly high risk for complications or even a lethal outcome[67,74,76,107,145].

1.4.1.1. The complement system

For the establishment of an effective post-traumatic inflammatory reaction, the translation of danger signals like DAMPs and PAMPs into cellular reactions is inevitable for the host’s defense against potentially life-threatening immune challenges.

The complement system is an adequate tool for this task as it provides a panel of plasmatic mediators instructing cells of the innate and the adaptive immune systems to induce elementary functions like chemotaxis, opsonizing, phagocytosis and cell killing.

Already by the end of the 19th century, Paul Ehrlich specified the complement system as the feature of the blood serum to literally complement the activity of antibodies[312]. Subsequently, in the following decades, the structure and functionality of the complement cascade has been a major subject of research.

Today, it is known, that the complement cascade is an essential part of the innate immune system consisting of about 30 glycoproteins mainly being synthesized in the liver. The complement components, numbered C1-C9, are constantly present in the plasma in their inactive form. Upon stimulation, mainly via proteolysis, complement factors are enabled to activate each other proteolytically thereby forming a chain-like cascade of enzymatic activity.

The critical step in the generation of activated complement products is the assembly of the C3 convertase that cleaves C3 into C3a and C3b. Basically, there are three main
activation pathways to accomplish this: the classical, the alternative and the lectin pathway[85,221]. The longest known pathway of complement activation is the **classical** way[113], which is triggered by the presence of antibodies in case a prior antigen recognition by immunoglobulins (Ig) of type M or G (IgM, IgG) has occurred. In the course of that, antibodies bind an antigen with their antigen binding fragment (Fab), whereas the constant part (Fc; fragment crystallizable) is able to interact with protein C1 of the complement system that in turn consists of the subunits C1q (the first and essential protein of the C1 cascade), C1r, and C1s. C1s then enables serine proteases to acquire their proteolytic activity and cleave C4 and C2 resulting in the formation of the C4b2a, also known as C3a convertase[12].

The second way is the **alternative** way of complement activation. In the proper sense, this pathway is rather defined as a failure to regulate the continuous generation of C3 convertase skipping C1, C2 and C4, than a true complement activating pathway. In fact, a smaller proportion of C3 constantly undergoes spontaneous hydrolysis that is physiologically kept in check in healthy cells. Thereby, a molecule resembling C3b is permanently generated. In case of tissue damage, bacterial endotoxins and other microbial components can be bound by this fraction of C3b and (via intermediate steps) evolves a C3 convertase independently from C1, C2, C4[239,308]. Later, it was revealed that, also the mannose-binding **lectin** (MBL)/ MBL-associated serine protease (MASP) is able to activate the complement cascade[105,228]. The proteins initiating this pathway are multimeric lectin complexes called collectins. In particular MBL and ficolin, which interact with mannose-containing carbohydrate pattern that are unusual for the host[194]. Interestingly, MBL shares structural similarities with C1q and the serine proteases MASP-1 and -2 are similar to C1r and C1s[300]. This might explain why lectin-mediated complement activation resembles the C1-activated classical activation pathway. Here again, cleavage of C3 is the critical event in which the lectin pathway results in[194].

Interestingly, apart from those mechanisms, accessory pathways have been identified. For instance, components of the coagulation cascade like thrombin[139] and others (e.g. Factor Xa, plasmin) [32] give rise to biologically active complement factors. This supports the idea that various plasmatic cascades, that **per se** are activated in the early phase after trauma, act in concert.
Every pathway converges in the formation of the C3 convertase, that successively evolves C3a and C3b[12]. By incorporating C3b into the C3 convertase, the generation of an enzyme that cleaves C5 is induced. The latter results in the production of C5a and C5b[166].

Subsequently, the cleavage products C3b and C5b facilitate phagocytosis and elimination of pathogens and/or circulating immune complexes by opsonization. Moreover C5b can additionally interacts with C6, C7, C8, and C9 in order to generate the membrane attack complex (C5b-9; MAC), which is used by the host to lyse gram-negative bacteria[164,209].

Regarding the constitution of an inflammatory state, however, the activity of the remaining complement components (C3a, C4a and C5a) is more significant. By targeting various immune cells[188,326], C3a, C4a and C5a act as so called anaphylatoxins, that attract inflammatory cell to the site of interest and thus initiate chemotaxis. Subsequent degranulation of mast cells, eosinophils and basophile granulocytes[141,142,271] is i.a. triggered, as well. Accordingly, vasoactive mediators, reactive oxygen species chemokines and cytokines increase vascular permeability and amplify the inflammatory response[77].

In this context, C5a has frequently been described to be the most powerful anaphylatoxin[121]. Since its receptors are widely expressed[108,140], it exerts a broad spectrum of effects to drive inflammation involving the release of cytokines, like TNF-α, IL-1β, and IL-6, IL-8[135,286], for instance. Together with its properties to i.a. induce phagocytosis[55], recruit neutrophils[280] and to enhance their adhesive properties to endothelial cells[66,216], to heighten lymphocyte reactivity[111] or to initiate a delay in apoptosis of immunocompetent cells[235], it is reasonable that C5a also represents an effective way to transmit humoral into cellular inflammatory mechanisms.

1.4.1.2. Complement activation and C5a following (poly)trauma

Having demonstrated the relevance of the complement cascade for the response toward danger signals, it is not surprising that the early phase after a severe event, such as a PT, is subsequently characterized by an excessive activation of complement proteins. Accordingly, it could be verified in both human and animal studies that the serum complement hemolytic activity (CH50)[41,84], a clinical method to monitor
complement activity, significantly decreases after both trauma and blood loss, meaning that those events reliably go along with a rise in complement activity. At this point, it is of special importance that an exaggerated activation of complement factors in turn leads to their consumption or even results in their depletion. Together with an altered expression of C5a receptors and complement regulators on circulating leukocytes this phenomenon has already been referred to as the so called “complementopathy” that then again includes an increased risk for complications and a worsening in terms of the chances for recovery[4]. High concentrations of anaphylatoxins clearly correlate with an adverse degree of severity and bad posttraumatic prognosis[127].

In this context C5a, again, takes a special position and has even been described to be occasionally “too much of a good thing” [109,138]: Exaggerated C5a elevation indeed is a central problem in the pathogenesis of sepsis (a lethal complication of PT), which implies C5a-mediated breakdown of several immune functions and the risk for organ failure[224].

Taking together, the extent of generalized inflammatory processes is decisive for the course after PT and the complement system, especially C5a, is a predominant driver for this. Owing to that, complement inhibitory or modulatory strategies may provide promising therapeutic strategies for balancing an overshooting immune response.

1.4.1.3. Complement activation following trauma and complement modulatory strategies

A considerable number of investigations have yet focused on the approach to inhibit complement activity at nearly all levels of the cascade using various animal models for injury manifestations like TBI[149,175], thoracic injury[97,211] musculoskeletal trauma and ischemia/ reperfusion injuries[46,54,157,172,315] or hemorrhagic shock[46,53,172,315].

Amongst many approaches, a larger number of studies focused on inhibiting C5a signaling by blocking its interaction with its receptors[157].

For instance, the C5a-receptor 1 (C5aR1) antagonist CCX168 (ChemoCentryx) has been under clinical investigation for the inflammatory diseases but so far has not been used for SIRS-related indications[137].
The first therapeutic compound applied in human SIRS or sepsis studies is the anti-C5a monoclonal antibody IFX-1 (InflaRx), which has been investigated in a clinical phase II trial (NCT02246595) to evaluate its potential in preventing early organ dysfunction in sepsis. Moreover, a phase II study focused on SIRS-induced organ dysfunction after complex cardiac surgery[137].

Corresponding data convincingly illustrate beneficial effects of antagonizing complement under inflammatory conditions[98], but, an optimal way of using complement factors as a pharmacological target remains unrevealed. Rodent studies e.g. have already demonstrated that a complete knock-out of C3 or C5 is rather incompatible with survival, probably because of a loss of vital complement functions like opsonization and the MAC[97].

Thus, in order to manage the detrimental inflammatory processes of exuberant complement activity but at the same time to preserve vital benefits of complement activation such as MAC-dependent defense (i.e. leaving the C5b-9 pathway open for killing of bacteria), it is particularly reasonable to interfere more downstream of the complement signaling (thus at the level of C5a).

A relatively novel and promising concept of neutralizing C5a function is the application of an L-ribonucleic acid aptamer, known under the trade name Spiegelmer (SM). The name Spiegelmer derives from the German term “Spiegel” meaning mirror and thus reflects that it is made up of nucleotides in the mirror-image (L-)configuration, while in nature all nucleic acids are exclusively in the D-configuration. Consequently, C5a Spiegelmer (C5a SM) consists of non-physiological nucleotides that are resistant towards plasma nucleases and build up a three-dimensional structure, an “L-aptamer”, that is capable of enveloping C5a and subsequently inhibit the C5a molecule from performing its biological effects[134,323] (Fig. 2).
Figure 2: Schematic illustration of the principle of the C5a Spiegelmer. Spiegelmers are composed of non-physiological nucleotides which make them resistant to plasma nucleases and therefore a promising tool for therapeutic use. C5a is a potent anaphylatoxin being produced during the complement cascade and might efficiently be targeted by Spiegelmer. The Spiegelmer’s structure shows a complex three-dimensional structure (L-aptamer) that arranges itself around C5a and thus envelopes it and inhibits the C5a-molecule to perform its biological effects. This Illustration contains elements of a figure originally published in Yatime L, Maasch C, Hoehlig K, Klussmann S, Andersen GR, Vater A. Structural basis for the targeting of complement anaphylatoxin C5a using a mixed L-RNA/L-DNA aptamer. Nat Commun. 2015;6:6481. Published 2015 Apr 22. doi:10.1038/ncomms7481 distributed under CC BY 4.0, https://creativecommons.org/licenses/by/4.0/legalcode.

1.4.2. Anti-inflammatory responses following trauma

As described above, rather simultaneously with pro-inflammatory forces, counter-regulatory mechanisms compromise an overshooting inflammation by a course of events, that can be summarized by the term CARS. In detail, this includes e.g. the production of anti-inflammatory cytokines (e.g. IL-10 or transforming growth factor-β (TGF-β))[92,291] and endogenous cytokine inhibitors (soluble TNF- receptors, IL-1 receptor antagonists)[130,156,321] as well as an enhanced apoptosis of immune effector cells[136]. Interestingly, those processes seem to be interrelated since the elimination of e.g. apoptotic cells by macrophages and dendritic cells further drives immune suppression by inducing the release of anti-inflammatory mediators[130,136,303].

To sum up, CARS can be characterized as the development of an “immune-paralysis” Similarly to its counterpart (SIRS), CARS also potentially endangers the host in case it appears overstatedly or inadequately timed. Such circumstances can result in an increased susceptibility for infections, which might imply deleterious consequences like sepsis and MOF[136,267]. Fig. 3 summarized the interplay of SIRS and CARS in respect to the course of trauma.
Introduction

Figure 3: Schematic relationship between pro- and anti-inflammatory reactions of the posttraumatic immune response. The posttraumatic immune response is characterized by a systemic inflammatory syndrome (SIRS) that is counterbalanced by a compensatory anti-inflammatory syndrome (CARS). Overshooting SIRS (a) but also exaggerated CARS (c) results in complications, up to multi-organ dysfunction syndrome (MODS) and multi-organ failure (MOF). Establishing an equilibrium between SIRS and CARS is critical for recovery and survival (b). Important to mention is that this figure is simplified and that pro-and anti-inflammatory processes happen rather simultaneously than sequentially. Illustration by Julia Kunze.

1.4.2.1. Neuroendocrine response

After portraying that the post-traumatic immune status is shaped by the synergy of both pro-inflammatory (SIRS) and anti-inflammatory (CARS) resonances, it is now relevant to emphasize that neuroendocrine responses play a key role in balancing the post-traumatic immune condition. As already indicated above and especially in Fig.1, the immune and the neuroendocrine system are strongly interconnected and share a constant reciprocal communication. Hereby, a major position is undertaken by the HPA-axis.

1.4.2.2. The HPA-axis

To highlight the fundamental role of the HPA axis in the regulation of immune challenges, it is reasonable to sum up the general anatomical and physiological aspects of it. The HPA axis represents a vital neuroendocrine system that is in large parts responsible for regulating an organism’s response to stressors (i.e. stimuli that dys-balance the body’s homeostatic equilibrium). The HPA axis' properties are
achieved by the interaction between its three major endocrine organs, the hypothalamus, the pituitary gland and the adrenal glands. HPA axis activation starts with the secretion of corticotropin releasing hormone (CRH), which i.a. is concentrated in neurons that originate in the parvocellular subdivision of the paraventricular nucleus of the hypothalamus and terminate in the external layer of the median eminence where CRH is released into the hypophyseal portal circulation[257,297]. Although the physiological effects of the CRH peptide are communicated by two different receptor subtypes (CRH-receptor (CRH-R) 1 and CRH-R2), the neuroendocrine properties of CRH are mediated via CRH-R1 in the anterior pituitary[64,237]. In corticotropic cells of the anterior pituitary gland (also called neurohypophysis) CRH binds to CRH-R1, induces a rapid release of adrenocorticotropic hormone (ACTH) from cellular stores into the systemic circulation and initiates the delayed synthesis of the ACTH precursor proopiomelanocortin (POMC) to regenerate intracellular reservoirs for ACTH[28,52,173,250]. Once ACTH reaches the adrenal gland, it acts on its specific receptor, the melanocortin type 2 receptor (MC2R). This primarily happens in the zona fasciculata of the adrenal cortex. Subsequently, MC2R leads to an activation of multiple signal transduction cascades. The cyclic adenosine mono-phosphate (cAMP) dependent protein kinase A (PKA) pathway, however, is central for hormonally driven activation of adrenal synthesis of steroids such as glucocorticoids (GC). Fig. 4 outlines a short overview the HPA axis.
Figure 4: Schematic representation of the hypothalamus-pituitary-adrenal axis. Hypophysiotropic neurons located in the paraventricular nucleus (PVN) of the hypothalamus, i.a. release corticotropin releasing hormone (CRH) and secrete into portal vessels of the pituitary. CRH then binds to its receptor (not shown) and induces the secretion of adrenocorticotropic hormone (ACTH) into systemic circulation, which targets its receptor melanocortin type 2 receptor (MC2R) in adrenal cortical cells to stimulate glucocorticoid, especially corticosterone (CORT) synthesis via intracellular signaling depending on cyclic adenosine monophosphate (cAMP), which then activates protein kinase A (PKA). GC further inhibit HPA activation via intracellular receptors that are widely expressed throughout the brain and peripheral tissue. Illustration by Julia Kunze.

Due to the lipophilic properties of steroids and in contrast to cells that synthesize polypeptide hormones, steroidogenic cells produce newly synthesized steroids upon hormonal stimulation instead of building up intracellular storages[106]. For that reason, various mechanisms within adrenal cortical cells guarantee the sufficient supply for cholesterol as the essential substrate for steroidogenesis[124]. There are four main mechanisms how a steroidogenic cell can ensure steroid supply. First, adrenal glands are capable of absorbing cholesteryl esters (CE) in low-density lipoproteins (LDL) by receptor-mediated endocytosis and directing it to endosomes, where CEs are hydrolyzed into un-esterified (free) cholesterol and exported in the cytoplasm[25,38]. Secondly, the scavenger receptor B Type 1 (SR-B1)-mediated uptake of LDL as well as of high-density lipoprotein (HDL)-CE provides a “selective” way of cholesterol delivery independently from lysosomal hydrolyzation[14,311]. In this context, it could be proven, that rodent adrenals derive most of their cholesterol for steroidogenesis from SRB-1-delivered CE[15,311], whereas this pathway evidently appears to play a minor role in human steroid synthesis[49,180].
In addition to plasma-derived cholesterol sources, cholesterol can also be synthesized *de novo* from acetate and acyl-coenzyme A (Acyl CoA) via the rate limiting enzyme hydroxyl-methyl-glutaryl coenzyme A (HMG CoA)-reductase in the endoplasmatic reticulum of the cell (third way). Forth, free cholesterol can be esterified by the Acyl CoA-cholesterol-acyltransferase (ACAT) and preserved in intracellular dynamic organelles, so called lipid droplets (LDs). LDs can be found in a variety of eukaryotic cells and contain a neutral lipid core being surrounded by a surface phospholipid monolayer with proteins embedded in or bound to the phospholipid layer[25]. In particular, in steroidogenic tissue LDs establish an intracellular reservoir for esterified cholesterol[241], which in turn can be accessed and mobilized by the neutral CE-hydrolase called hormone sensitive lipase (HSL). Observations could confirm, that HSL-induced hydrolysis of CE in LD provides the preferred source for gathering cholesterol for adrenal GC synthesis and consistently, HSL deficiency actually is associated with a marked decrease in GC response upon ACTH stimulation[167-169,276,]. Furthermore, and supporting the central role of the HSL for adrenal cholesterol utilization especially upon hormonal stimulation, there is evidence that HSL activity is also highly involved in the cholesterol delivery via the relevant SR-B1 pathway[167].

Via its protein–protein interaction with the steroidogenic acute regulatory protein (STAR), HSL moreover facilitates the transport of cholesterol to mitochondria[277,284], where it then is metabolized to pregnenolone (a precursor of most steroids) by the cholesterol side-chain cleavage (scc) enzyme P450scc and other enzymes in the steroidogenic pathway i.a. resulting in the end product CORT. The pathways described in this chapter are furthermore illustrated in detail in Fig. 5.
Figure 5: Schematic illustration of cholesterol utilization in an adrenal cortical cell. The illustration shows a simplified schema of a steroidogenic cell within the adrenal cortex. Adrenal cortical cells take up low-density lipoprotein either via receptor-mediated endocytosis via the LDL-receptor (LDL-R) or via the selective uptake via scavenger receptor B1 (SR-B1), which also binds high-density lipoprotein (HDL). Cholesterol might additionally be produced de novo from acyl-coenzyme A (Acyl CoA) by the enzyme hydroxy-3-methylglutaryl (HMG) CoA reductase. Independent from its source cholesterol can be esterified by Acyl-CoA: cholesterol transferase (ACAT) and then stored in lipid droplets (stainable by Oil-Red-O). In turn, free cholesterol can be regained by the activity of the enzyme hormone-sensitive lipase (HSL). After having been transported to the mitochondria by vesicular or non-vesicular traffic (not shown), the steroidogenic acute regulatory protein (STAR) moves cholesterol to the inner mitochondrial membrane where it can be converted to induce further steps of steroid synthesis. Illustration by Julia Kunze.

1.4.3. The interrelation between the HPA-axis and the immune system

Importantly, HPA signaling cannot be fully understood without being aware that there is an intensively geared and bidirectional linkage between the immune and the endocrine, especially the HPA-, system. It is well established that immune and hormonal processes operate in a rather coordinated fashion. Humoral and cellular immune responses can be modulated by the neuroendocrine environment in that they occur and vice versa. This manifests a neuroendocrine-immune feedback loop[20,23,208]. On one side, immune activation triggers HPA signaling: Inflammatory mediators deriving from the systemic bloodstream can reach the portal circulation via the anterior hypophyseal arteries and are transported into brain structures either through areas
lacking a blood brain barrier (BBB) or else via specific transporters[199]. In addition, systemic inflammation may additionally cause a breakdown of the BBB affording traffic of blood-borne cytokines to deep brain structures[274]. Interestingly, complement components, and in particular C5a, are expressed both by astrocytes and endothelial cells and may play a key role amongst many factors that contribute to the collapse of the BBB or the destruction of tight junctions[107].

Then, by targeting organs of the HPA axis, cytokines provoke the release of the HPA-hormones CRH in the hypothalamus and ACTH in the pituitary, respectively[148,176,191,292,304]. At the level of the adrenal glands, studies could show that a large spectrum of cytokines enhance adrenal steroidogenesis in vivo and in vitro[200,205,290]. Together with evidence that inflammatory mediators, e.g. IL-6 and its receptors, both are expressed in the adrenal cortex[148,24,39,229] this supports the idea that also the adrenal gland additionally participates in the neuro-immune crosstalk.

Regarding the opposite direction, HPA signaling in turn has an impact on immune functions. Although there are direct immunomodulatory effects of CRH and ACTH[205,248], the most conspicuous hormonal influences on immune function are achieved by adrenocortical steroids. Already more than 30 years ago, Muck et al. claimed that “the physiological function of (…) increases in glucocorticoid levels is to protect not against the source of stress itself, but against the normal defense reactions (e.g. immune response/ inflammation) (…). Glucocorticoids accomplish this by dampening off such defense reactions and thus preventing them from overshooting and in turn themselves threatening homeostasis”[212, page 28]. Supporting that, immune cells are equipped with receptors for neurotransmitters and hormones, e.g. CORT, by activation of which GC are capable of affecting immune processes[73,206]. Recent research attends that immunological cells (e.g., lymphocytes) alter their responsiveness towards neurotransmitters and hormones during stress[259]. This involves that GCs might either prevent exuberant pro-inflammatory responses or increase local immunity if necessary[88,100,255,259].

On one side, GC limit inflammatory processes by ameliorating the proliferation of immune cells[195,263] or by inducing apoptosis of lymphocytes[240]. An increase of GC levels actually has been associated with a decline of pro-inflammatory cytokines and chemokines[71,89,320,279]. Nonetheless, many immunosuppressive properties
of corticosteroids were observed after pharmacological rather than physiological doses of the hormone[88,178,255]. In fact, HPA axis activity has additionally been associated with enhancing immune activation, i.a. by transiently increasing pro-inflammatory cytokines like IL-6[71,326], TNF-α[71,283] or IL-β[71]. Fittingly, an appropriate adrenal functioning has recently been proved to be vital for the proper activity of immune cells, e.g. natural killer cells[18].

Putting it together, the HPA axis bares an outstanding role in modulating immune activity – a fact that is further confirmed by studies in adrenalectomized animals that are characterized by increased mortality after injection of pro-inflammatory cytokines, whereas administration of GC improves survival in those animals[22,96,247]. Moreover, and referring to trauma again, adrenal insufficiency is a feared complication after (poly-)trauma[54,56,125,190,218,223]. That clearly emphasizes the significance of an adequate HPA-axis functionality for the survival severely injured. In clinical practice the administration of glucocorticoids is a common tool to reduce inflammatory responses[79,129]. However, clinical or scientific experience yields rather contradictory conclusions whether GC treatment would result in significant improvement and a decreased mortality following trauma and sepsis[7] or worsens the course of such diseases and prime deleterious inflammatory cascades resulting in death, though[61,222,324].

In addition, data investigating HPA axis functionality in the early period after severe multiple injuries, especially at the level of the adrenal glands (thus GC release) is limited. That further motivates to highlight HPA-functionality in trauma- related, particularly C5a- mediated, inflammation.

1.5. Aims of the study

Given that 1) PT, is characterized by an inflammatory response that is i.a. effectively induced by C5a, 2) the immune system and the HPA axis share a bidirectional communication, i.e. inflammation pathophysiologically is accompanied by neuroendocrine modulation in terms of an activation of the HPA- axis and its end-organ the adrenal gland, and 3) less is known about adrenal reactivity following trauma and potential influence of C5a on the latter, the main aims of this study are i) to investigate if treatment of polytraumatized mice with a C5a SM, which is supposed to reduce C5a-
mediated immune activation also affects the functionality of the adrenals glands in PT vs. SHAM-treated animals (experiment 1), ii) to elucidate if C5a and/or its SM has/have potential direct topical effects on the basal in vitro CORT synthesis of the adrenal glands of untreated mice (experiment 2), and if C5a and/or its SM affect(s) adrenal in vitro CORT production in response ACTH (experiment 3).

To achieve aim i), the in vitro ACTH responsiveness, the amount of steroid precursor molecules (CEs in LDs) as well as the adrenal CORT content via ELISA was determined in the adrenals of mice that were exposed to either PT or SHAM and subsequently injected with either C5a SM or vehicle (VEH).

For aim ii) two further experiments were conducted. In experiment 2 adrenal glands of a set of untreated (neither PT nor SHAM-exposed) mice were stimulated in vitro with either C5a, C5a SM or the combination C5a+C5a SM. In experiment 3, adrenal glands of another set of untreated mice were pre-incubated with C5a, C5a SM or the combination C5a+C5a SM before being stimulated with ACTH in vitro.

Additionally, a smaller, third aim of this study was to validate the software Reimage.exe © PD Dr.med. Michael Noll-Hussong (abbreviated in the following by Reimage.exe), a program that has been used for image processing and e.g. quantifying Oil-Red-O (ORO) staining, in the Reber Lab for the first time. To achieve this aim, digital images of adrenal cortices from experiment one were by comparison analyzed with the commercial software program Image J and Reimage.exe, respectively for quantification of LDs. For an overview of the major aims of the present thesis see Fig.6.
Aims of the study

aim i)

Figure 6: The major aims of this thesis. This thesis (i) investigates HPA-functionality following polytrauma-induced, especially C5a-mediated, inflammation by using a C5a-Spiegelmer in a murine model for polytrauma. Secondly (ii), direct effects of C5a, C5a-Spiegelmer and the combination of both on adrenal corticosterone (CORT) production are supposed to be investigated first without and then with an additional in vitro stimulation with adrenocorticotropic hormone (ACTH). As a novel image processing software *Reimage.exe* is ought to be compared to the commercial software *Image J* for quantitative analysis of lipid droplet (LD) staining by Oil-Red-O (ORO) (aim iii)). Illustration by Julia Kunze.
## Material and Methods

### 2.1. Material

#### Chemicals

<table>
<thead>
<tr>
<th>Description</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH Fragment 1-24</td>
<td>Sigma-Aldrich, Steinheim, Germany</td>
</tr>
<tr>
<td>Albumin bovine (BSA) Fraction 5</td>
<td>BIOMOL GmbH, Hamburg, Germany</td>
</tr>
<tr>
<td>C5a</td>
<td>Hycult Biotech, Uden, The Netherlands</td>
</tr>
<tr>
<td>C5a Spiegelmer</td>
<td>Noxxon Pharma AG, Berlin, Germany</td>
</tr>
<tr>
<td>Di-sodium hydrogen phosphate</td>
<td>ROTH, Karlsruhe, Germany</td>
</tr>
<tr>
<td>DMEM/F-12</td>
<td>Life Technologies, Paisley, UK</td>
</tr>
<tr>
<td>Ethanol 99.8 %</td>
<td>SIGMA - ALDRICH, Steinheim, Germany</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>Sanofi, Frankfurt am Main, Germany</td>
</tr>
<tr>
<td>Oil -Red-O</td>
<td>WALDECK GmbH &amp; Co. KG, Muenster, Germany</td>
</tr>
<tr>
<td>Paraformaldehyde 3.5-3.7 %</td>
<td>Otto Fischar GmbH &amp; Co. KG, Saarbruecken, Germany</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>ROTH, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>ROTH, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Propan-2-ol</td>
<td>VWR, Darmstadt, Germany</td>
</tr>
<tr>
<td>Sevoflurane</td>
<td>Sevorane Abbott, Wiesbaden, Germany</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>SIGMA - ALDRICH, Steinheim, Germany</td>
</tr>
<tr>
<td>Tissue-Tek®</td>
<td>Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands</td>
</tr>
</tbody>
</table>
### Commercial kits and enzymes

<table>
<thead>
<tr>
<th>Enzyme/Kit</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA for CORT</td>
<td>IBL International GmbH, Hamburg, Germany</td>
</tr>
<tr>
<td>Roti®-Mount Aqua</td>
<td>ROTH, Karlsruhe, Germany</td>
</tr>
</tbody>
</table>

### Solutions and buffers

<table>
<thead>
<tr>
<th>Solution/Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffered saline (PBS), 10x</td>
<td>8 g Sodium chloride</td>
</tr>
<tr>
<td></td>
<td>1.16 g di-sodium hydrogen phosphate</td>
</tr>
<tr>
<td></td>
<td>0.2 g Potassium dihydrogen phosphate</td>
</tr>
<tr>
<td></td>
<td>0.2 g Potassium chloride</td>
</tr>
<tr>
<td></td>
<td>ad 1 l dH₂O</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS), 1x</td>
<td>100 ml 10x PBS</td>
</tr>
<tr>
<td></td>
<td>900 ml dH₂O</td>
</tr>
</tbody>
</table>

### Technical equipment

<table>
<thead>
<tr>
<th>Device designation</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical balance CPA 100001</td>
<td>Sartorius Weighing Technology GmbH, Goettingen, Germany</td>
</tr>
<tr>
<td>Analytical balance MSE225P</td>
<td>Sartorius Weighing Technology GmbH, Goettingen, Germany</td>
</tr>
<tr>
<td>Blood pressure analyzer</td>
<td>DSI, St. Paul, Minnesota, USA</td>
</tr>
<tr>
<td>Easypet 3</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Intravenous catheter</td>
<td>Föhr Medical Instruments, Seeheim/Ober-Beerbach, Germany</td>
</tr>
<tr>
<td>Leica CM1950 Cryostat</td>
<td>Leica Biosystems, Nussloch, Germany</td>
</tr>
<tr>
<td>Leica DMI6000B</td>
<td>Leica Biosystems, Nussloch, Germany</td>
</tr>
<tr>
<td>MICRO STAR 17®</td>
<td>VWR, Darmstadt, Germany</td>
</tr>
<tr>
<td>Multipipette® Stream</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Olympus DP73</td>
<td>Olympus GmbH, Hamburg, Germany</td>
</tr>
</tbody>
</table>
### Material and Methods

**Research Plus Pipettes**
Eppendorf, Hamburg, Germany

**Venti-line ®**
VWR, Darmstadt, Germany

**VORTEX Genie 2**
Bohemia, NY, United States

### General consumable material

<table>
<thead>
<tr>
<th>Description</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well plate,</td>
<td>Sarstedt, Nuembrecht, Germany</td>
</tr>
<tr>
<td>Combitips advanced®</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Falcon 15 ml, 50 ml</td>
<td>CORNING, Tewksbury, MA, USA</td>
</tr>
<tr>
<td>Folded qualitative filter paper, 303</td>
<td>VWR, Darmstadt, Germany</td>
</tr>
<tr>
<td>Microscope cover glasses</td>
<td>ROTH, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Pipette tips</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>R35 microtome blades</td>
<td>Feather Safety Razor Co., Osaka, Japan</td>
</tr>
<tr>
<td>RotilaboR-embedding cassettes, POM</td>
<td>ROTH, Karlsruhe, Germany</td>
</tr>
<tr>
<td>S35 microtome blades</td>
<td>Feather Safety Razor Co., Osaka, Japan</td>
</tr>
<tr>
<td>SuperFrost® Plus (microscope slides)</td>
<td>VWR, Darmstadt, Germany</td>
</tr>
<tr>
<td>SuperFrost® Plus (microscope slides)</td>
<td>ROTH, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Surgical Disposable Scalpels</td>
<td>BRAUN, Tuttlingen, Germany</td>
</tr>
<tr>
<td>Tissue-Tek® Cryomold ®</td>
<td>Weckert, Kitzingen, Germany</td>
</tr>
<tr>
<td>Eppis 1.5 ml, 2 ml</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
</tbody>
</table>
2.1. **Animals**

Experiment 1 and 2 were performed with Male C57BL/6 mice aged 8 to 12 weeks with a mean body weight of 25g (± 2.5g) (Jackson Laboratories, Bar Harbour, Maine). For experiment 3 male C57BL/6N mice (Charles River, Sulzfeld, Germany) weighing 19-22 g were used. Experimental mice were kept under standard laboratory conditions (12 h light/ 12 h dark cycle, lights on at 0600 AM, 22 °C, 60 % humidity) and had free access to tap water and standard mouse diet. All experimental protocols were approved by the Committee on Animal Health and Care of the local government, and conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

2.2. **Experimental procedures**

2.2.1. Experiment 1

The animals were randomized into four experimental groups. Two groups were subjected to PT and received either the C5a SM (10 mg/ kg bodyweight) or VEH (i.e. an inactive version of the C5a SM) via a catheter inserted into the right jugular vein of the animals. The other two groups of animals served as SHAM (i.e. not traumatized) controls and were also randomly assigned to the injection with either C5a SM or VEH, respectively.

Concerning the PT protocol, mice were anesthetized with 2.5% sevoflurane (Sevorane Abbott, Wiesbaden, Germany)/97.5% oxygen, which was continued during the entire procedure and observation period. For analgesia, 0.03 mg/kg buprenorphine was administrated by subcutaneous injection. Then, mice were exposed to a defined combination of the following trauma types (for an overview of this procedure see also figure 7): a blunt bilateral chest trauma (TXT), a traumatic brain injury (TBI), and a closed transverse femoral fracture (inclusive soft tissue injury) as previously described[70,306]. After the induction of PT, the left hind limb of the anaesthetized mice was shaved and cleaned before the left femoral artery was microsurgically catheterized (Föhr Medical Instruments, Seeheim/Ober-Beerbach, Germany) for blood pressure monitoring (blood pressure analyzer, DSI, St. Paul, Minnesota, USA) and a controlled blood loss (hemorrhagic shock (HS)). Another incision was created along the ventral cervical skin and a further catheter was inserted into the right jugular vein allowing the resuscitation procedure, the controlled infusion of catecholamines and the
C5a SM (Noxxon Pharma AG, Berlin, Germany). For the induction of HS, mice were bled for 5 to 10 min to reach a mean blood pressure of 30 mmHg (±5 mmHg) which was kept stable for 60 min. Subsequently, mice were re-perfused via the jugular vein with the 4-fold volume of the drawn blood with balanced electrolyte solution (ionosterile) over 30 min. During the observation period (lasting 2h) after the HS, animals were subjected to a preset protocol, adjusting anesthesia and norepinephrine support (0.01–0.12mg/kg/min) (Sanofi, Frankfurt am Main, Germany) in a standardized manner to maintain a mean arterial blood pressure (MAP) of 50 mmHg. Four hours after PT, animals were sacrificed by cardiac puncture.

SHAM animals underwent an analogous procedure compared to PT animals concerning the duration and anesthesia, but no trauma was applied.

Table 1 demonstrates the four animal groups used in experiment 1 and figure 7 clarifies the timeframe as well as the process of the PT protocol that has been applied in the first experiment in more detail.

At this point, it is important to emphasize that the experiments that are described and discussed in this present thesis (i.e. the analysis of adrenal parameters concerning the adrenal gland) only represent a part of the body of investigation that is depicted here. Since the present study is a collaboration with the research group of Prof. Dr. Huber-Lang (Institute for Clinical and Experimental Trauma Immunology; University Clinic Ulm), scientists belonging to the latter group performed several further investigations that will not be addressed in the following.

Table 1: Experimental groups of the polytrauma experiment (experiment 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Injection</th>
<th>C5a-activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM VEH</td>
<td>SHAM</td>
<td>Vehicle (VEH)</td>
<td>normal</td>
</tr>
<tr>
<td>SHAM C5a SM</td>
<td>SHAM</td>
<td>C5a-neutralizing Spiegelmer</td>
<td>blocked</td>
</tr>
<tr>
<td>PT VEH</td>
<td>polytraumatization</td>
<td>vehicle (VEH)</td>
<td>normal</td>
</tr>
<tr>
<td>PT C5a SM</td>
<td>polytraumatization</td>
<td>C5a-neutralizing Spiegelmer</td>
<td>blocked</td>
</tr>
</tbody>
</table>

Table by Julia Kunze.
Methods

2.2.2. Experiment 2

For the second experiment, adrenal glands of untreated mice (n=9) were prepared (see chapter 2.4) and the halves were randomly assigned to four experimental groups for the in vitro stimulation with VEH, C5a (C5a (item number: HC1101, Hycult biotech, Uden, The Netherlands), C5a SM or the combination C5a+C5a SM (n=9 adrenal halves per group). For details of the experimental procedure see Fig. 8 and of details of the in vitro stimulation see chapter 2.11.
2.2.3. Experiment 3

For the third experiment, adrenal glands of untreated mice (n=12) were prepared (see chapter 2.4) and the halves were randomly assigned to four experimental groups for the *in vitro* stimulation with C5a, C5a SM or the combination C5a+C5a SM; incubation with VEH (i.e. the medium C5a SM is dissolved in) was performed as a control (for details see chapter 2.11) (n=6 adrenal halves per group). The incubation with C5a, C5a SM, or C5a+C5a SM was performed after all adrenal glands were halved, weighed and medium was replaced by new one. After ca. 4h (corresponding to the duration of the treatment phase of the PT procedure of experiment one) the medium containing VEH, C5a, C5a SM, or C5a+C5a SM was replaced by fresh medium. After another pre-incubation time of 1.5h, adrenal halves were stimulated *in vitro* with ACTH or saline (n=6 adrenal halves per group) for 1h. For details of the experimental procedure see Fig.9 and of the *in vitro* stimulation with ACTH see chapter 2.9.

Figure 9: Illustration of the experimental procedure of experiment 3. Following sacrifice, adrenal glands of untreated mice were removed, pruned from fat, weighed, bisected and again weighed. After adrenal halves were incubated with either C5a, C5a- Spiegelmer (C5a SM) or the combination for ca. 240 min, which corresponds with the duration of the treatment phase of the PT procedure (see also figure 6). As controls, one group of adrenal halves was incubated with incubation medium (vehicle (VEH)). Subsequently organ halves were pre-incubated with incubation medium for 90 min before being stimulated *in vitro* with adrenocorticotropic hormone (ACTH) or saline (for control) for 60 min at the beginning of the dark phase. After 60 min, supernatants were removed and stored at -20°C until quantification of corticosterone (CORT). Illustration by Julia Kunze.

2.3. ELISA for CORT

To determine the CORT concentration, a commercially available ELISA for corticosterone (analytic sensitivity <1,631nmol/l, intra-assay ≤ 4,08 %and inter-assay coefficients of variations ≤ 6.35 %, IBL International, Hamburg, Germany) was performed according to the manufacturer’s manual.
2.4. **Organ preparation**

After euthanizing the animals, left and right adrenal glands were removed and stored in ice-cold Dulbecco’s Modified Eagle Medium (DMEM/F12, Life Technologies, Paisley, UK) containing 0.1% bovine serum albumin (BSA) (Albumin bovine Fraction 5; BIOMOL GmbH, Hamburg, Germany) before being pruned from fat and connective tissue. After weighing the organs using microscales (MSE225P; Sartorius Weighing Technology GmbH, Goettingen, Germany), the right adrenal was embedded in protective freezing medium (Tissue-Tek®; Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands) and stored at −80 °C for cryo-cutting and CORT extraction. The left adrenal glands, were used to conduct an explant stimulation with ACTH *in vitro*.

2.5. **Oil-Red-O Staining**

For each animal, a series of 5 μm cryo-sections from the mid-part of the adrenal glands, containing both a cortical and medullary part, was produced using a cryostat (Leica CM1950 Cryostat; Leica Biosystems, Nussloch, Germany). Therefore tissue sections were thaw-mounted onto microscope slides (SuperFrost®Plus; VWR, Darmstadt, Germany), dehumidified at room temperature (RT) for ten minutes and stored at -20°C. Adrenal cuts were then fixed in paraformaldehyde (3.5 -3.7%; Otto Fischar GmbH & Co. KG, Saarbruecken, Germany) for 72h at RT. 24 hours before the staining procedure, a 0.5% Oil-Red-O (ORO; WALDECK GmbH & Co. KG, Muenster, Germany) dissolved in absolute isopropyl alcohol (propanol-2; VWR, Darmstadt, Germany) stock solution was prepared, which was then thinned down to a 60% solution with distilled water. Slides with fixed cryo-cuts were shortly washed with distilled water, rinsed in 60% isopropyl alcohol for 5 minutes and stained with freshly filtered 60% ORO-solution for 15 minutes before briefly being differentiated in 60% isopropyl alcohol and replaced into distilled water until preserving them with Roti® Mount Aqua (ROTH, Karlsruhe, Germany) and microscope cover glasses (ROTH, Karlsruhe, Germany).
2.6. Digital image acquisition

Per adrenal, three sections were used for further analysis. For quantification of the amount of lipid droplets in the adrenal cortex, each adrenal cut was imaginarily divided into four quarters containing both a medullary and cortical part of the organ. From each area, a microscopic image at 20x magnification was collected by a light microscope (Leica DMI6000B; Leica Biosystems, Nussloch, Germany) plus camera (Olympus DP73; Olympus GmbH, Hamburg, Germany).

2.7. Image processing with Reimage.exe

The software Reimage (© Dr. Michael Noll-Hussong) was used for image analysis. Within the cortical zone of each section, a region of interest (ROI; 300x 300=70624 pixels) was defined for further image evaluation. The amount of ORO positive pixels and unstained pixels were determined by color thresholding and a ratio between those two areas was calculated. The adrenal medulla and the outermost layer of the adrenal gland (zona glomerulosa), both easily distinguishable by eye, were not taken into account in this evaluation as the main site for corticosterone synthesis are the zona fasciculata and reticularis of the adrenal cortex. The average, calculated from the 12 measures per animal, was used for statistical analysis. The precise process of image analysis employing Reimage.exe is demonstrated in Fig.10.
Methods

Figure 10: Image processing with Reimage.exe. A) Digital image of lipid droplet staining of the adrenal gland. B) Region of interest (ROI) within the cortical zone. C) Color thresholding for quantitative evaluation of the proportion of stained area in relation to ROI. D) ROI after applying the color threshold. Illustration by Julia Kunze.

2.8. Software validation with Image J

Quantitative data generated using the first-time application of the image editing program Reimage.exe were compared to data acquired with the freely available software “Image J” to prove the validity of Reimage.exe. Therefore, in addition to the analysis with Reimage.exe, one image per animal of the SHAM A group was
subsequently processed and evaluated by *Image J*. Fig. 11 illustrates the procedure of quantitative analysis of lipid-droplet staining in the adrenal gland using *Image J*.

![Figure 11: Image processing with *Image J*. A) Digital image of lipid droplet staining of the adrenal gland. B) Region of interest (ROI) within the cortical zone. C) Color thresholding for quantitative evaluation of the proportion of stained area in relation to the ROI. D) ROI after applying the color threshold. Illustration by Julia Kunze.](image)

2.9. **Stimulation of adrenal explants with ACTH *in vitro***

ACTH stimulation was performed as described previously[256] except for differences in durations and pre-incubations (see below). After organ preparation including pruning
them from fat and connective tissue left adrenals were bisected, whereby both halves consisted of both a medullary and a cortical part. After the weight of the organ halves was taken and pre-incubated in 200µl DMEM/F-12+0,1% BSA (37°C, 95% O₂, 5% CO₂). Immediately before the stimulation with ACTH, medium was then replaced by 100µl fresh DMEM/F-12+0,1% BSA before adding either 25µl of isotonic saline solution (Braun Melsungen AG, Melsungen, Germany) for the basal condition or 25µl of a 500nm ACTH (ACTH Fragment 1-24, Sigma-Aldrich, Steinheim, Germany) and incubating the adrenal halves with a final concentration of 100nm ACTH (representing a pharmacological dose of ACTH) for 1 h at 37 °C (95% O₂, 5% CO₂) at the beginning of the dark phase. As controls the second adrenal halves were incubated with saline instead of ACTH under equal conditions, the latter is the dissolvent of ACTH. After stimulation of the explants, supernatants were removed and stored at -20 °C until CORT concentration were analyzed via a commercially available ELISA for CORT (IBL International, Hamburg, Germany). Subsequently, CORT concentrations were calculated in relation to the weight of the respective adrenal explants.

2.10. Determination of relative adrenal CORT content

After completing cryo-sectioning of the right adrenal glands, the embedded organs were thawed in ice-cold 1x phosphate buffered saline (PBS) until the freezing medium was dissolved completely and the adrenal tissue could be dried and weighed. To extract CORT out of the adrenal tissue, the samples were then homogenized with 50µl of each (Ethanol 99,8%, Sigma-Aldrich, Steinheim, Germany) in 1 x PBS on ice and centrifuged at 4 °C (4000rpm, 5 min). Supernatants were collected and stored at -20°C until CORT concentration were analyzed via a commercially available ELISA for CORT (IBL International, Hamburg, Germany). CORT-content was calculated in relation to the weight of the respective tissue (relative adrenal CORT content).

2.11. Stimulation of adrenal explants with C5a, C5a-Spiegelmer and C5a+C5aSpiegelmer in vitro

For the stimulation of adrenal explants with C5a, C5a SM and C5a+C5a SM in vitro, both left and right adrenals were prepared, halved and weighed as described in section 2.10. The four organ halves per animal were randomly assigned to four conditions (C5a, C5a SM and C5a+ C5a SM). After pre-incubation at 37°C, 5% CO₂ and 95% O₂ in 200µl culture medium (DMEM/F-12+0,1% BSA), medium was substituted by 100µl
DMEM/F-12+0,1% BSA for the basal, C5a and C5a-Spiegelmer condition or 75µl for the stimulation with the combination C5a+C5a-Spiegelmer. Depending on the four options for the explant stimulation either 25µl of DMEM/F-12+0,1% BSA, 25µl of C5a (500ng/ml), 25µl of C5a SM (500µg/ml, i.e. the end-concentration of C5a SM in the incubation well was 100ng/ml), or 25µl of C5a and 25 µl of C5a SM were added, explants were incubated for 1h (37°C, 5% CO₂ and 95% O₂) at the beginning of the dark phase and supernatants were removed carefully before being stored at -20°C. CORT concentration were measured by an ELISA kit and consequently CORT concentrations were adjusted to the respective adrenal mass.

2.12. Statistical analysis

For statistical comparisons, the software package IBM SPSS statistics (version 23.0) was used. To test the acquired data sets for normal distribution, Kolmogorow-Smirnov test using Lilliefors’ significance was employed. Normally distributed were proved for significant outliers were using Grubb’s test. Consequently, normally distributed data sets were analyzed using parametric statistics, i.e. parametric Student’s t-test (one factor, two independent samples) or two-way analysis of variance ANOVA (two factors, two or more independent samples). Non -normally distributed data sets were analyzed using non-parametric statistics, i.e. Man-Whitney U (MWU) test (two factors, two independent samples) Kruskal- Wallis-H test (more than two independent samples). All tests comparing more than two samples were conducted when a significant main effect was found, by post-hoc analysis using Bonferroni pairwise comparison. Normally distributed data are presented as bars (mean ± SEM). Non-normally distributed data are presented as box-plots (median; mean;10th, 25th, 75th and 90th percentile; outliers). The level of significance was set at \( P<0.05 \). Considering experiment one, linear correlations between variables were assessed applying Pearson's correlation analysis. All tests were two-sided and the level of significance was 0.05.
3 Results

3.1. Comparison of Reimage.exe and Image J for quantitative analysis of lipid droplet staining

The statistical analysis using Student’s t-test showed that there was no significant difference in the staining of adrenocortical LDs between Reimage.exe and Image J (Fig.12).

![Figure 12: Comparison of Reimage.exe and Image J for quantitative analysis of lipid droplet staining. Content of Oil-Red-O stained area was quantified using the software systems Reimage.exe or Image J, respectively. Reimage (n=9), Image J (n=9). Data represent mean +SEM. Graph by Julia Kunze.](image)

3.2. Effects of C5a Spiegelmer on bodyweight and HPA-parameters following polytrauma

3.2.1. Effects of C5a Spiegelmer on adrenal in vitro ACTH sensitivity following polytrauma

In vitro stimulation (Fig. 13) of adrenal explants with ACTH resulted in a significant increase of adrenal CORT production compared to basal values in all experimental groups (MWU Test; SHAM VEH: basal vs. ACTH: p=0,019; SHAM C5a SM: basal vs. ACTH: p=0,001; PT VEH: basal vs. ACTH: p=0,016; PT C5a SM: basal vs. ACTH: p=0,015). Statistical analysis also revealed significant differences within the ACTH stimulated groups. In VEH-treated groups MWU Test revealed an increased CORT
Results

The production of the PT Group compared to the SHAM group (p = 0.05). ACTH-stimulated adrenals of PT mice secrete significantly less CORT (p=0.05) when treated with active C5a SM compared to VEH. Moreover, comparing ACTH-stimulated SM-treated groups, MWU revealed that the PT group by trend (p=0.07) showed lower CORT levels than the SHAM group.

Figure 13: Effects of C5a Spiegelmer on in vitro ACTH sensitivity following polytrauma. Following euthanization 2h after polytrauma or respective SHAM treatment, both left and right adrenal glands were removed, prepared, bisected and the halves were incubated either with saline (basal) or 100nm adrenocorticotropic hormone (ACTH). Corticosterone (CORT) concentration was measured in the supernatants after stimulation (1h). SHAM vehicle (VEH) (n=9), SHAM C5a Spiegelmer (C5a SM) (n=9), polytrauma (PT) VEH (n=10), PT C5a SM (n=11). Solid line represents median, dashed line represents the mean for each data set. Lower boxes indicate 25th, upper boxes indicate 75th percentile. Also shown are the 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles). #: p ≤ 0.05; ##: p ≤ 0.01: ACTH compared to basal values. * p≤ 0.05 vs. respective indicated group. Graph by Julia Kunze.
3.2.2. Effects of C5a Spiegelmer on adrenal weight following polytrauma

Two-way ANOVA analysis revealed that neither the factor PT nor the factor C5a SM influenced the overall adrenal weight of the animals (Fig. 14).

Figure 14: Effects of C5a Spiegelmer on adrenal weight following polytrauma. After euthanization, adrenals were removed and weighed separately. Shown is the absolute adrenal weight of both adrenals (left and right adrenal weights of each mouse were summed up). SHAM VEH (n=9), SHAM C5a SM (n=9), PT VEH (n=10), PT C5a SM (n=11). Data represent mean +SEM. Graph by Julia Kunze.
3.2.3. Effects of C5a Spiegelmer on bodyweight following polytrauma

Statistical analysis via MWU Test revealed, that there was no effect of the factor PT or C5a SM on the overall bodyweight of the mice between all respective groups. This was indicated by no significant differences between SHAM VEH vs. PT VEH, between SHAM C5a SM vs. PT C5a SM, between SHAM VEH vs. SHAM C5a SM and between PT VEH vs. PT C5a SM (Fig. 15).

Figure 15: Effects of C5a activity on bodyweight following polytrauma. Following euthanization bodyweight was taken. SHAM vehicle (VEH) (n=9), SHAM C5a Spiegelmer (C5a SM) (n=9), polytrauma (PT) VEH (n=10), PT C5a SM (n=11). Solid line represents median, dashed line represents the mean for each data set. Lower boxes indicate 25th, upper boxes indicate 75th percentile. Also shown are the 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles). Graph by Julia Kunze.
3.2.4. Effects of C5a Spiegelmer on adrenal cortical lipid droplet content following polytrauma

Two-way ANOVA analysis revealed no statistical effect of the factor PT or the factor C5a SM on the relative ORO stained (LD) area of the adrenal cortices (Fig. 16 A & B).

Figure 16: Effects of C5a Spiegelmer on lipid droplets in the adrenal cortex following polytrauma. A) Relative area of lipid droplets to cortex area in the adrenal glands after cryosectioning and Oil-Red-O (ORO) staining. SHAM vehicle (VEH) (n=9), SHAM C5a Spiegelmer (C5a SM) (n=9), polytrauma (PT) VEH (n=10), PT C5a SM (n=11). Data represent mean +SEM. B) Representative images of ORO stained adrenal cortex of the distinct groups acquired at 20 x magnification. The scale bar within the images represents 200µm. Graph by Julia Kunze.
3.2.5. Effects of C5a Spiegelmer on adrenal CORT- content following polytrauma

Statistical analysis via MWU revealed no statistical effect of the factor PT and no statistical effect of the factor C5a SM on the CORT- content of the adrenal glands. This was indicated by no significant differences between SHAM VEH vs. PT VEH, between SHAM C5a SM vs. PT C5a SM, between SHAM VEH vs. SHAM C5a SM and between PT VEH vs. PT C5a SM (Fig. 17), analyzed by MWU, respectively.

Figure 17: Effects of C5a Spiegelmer on adrenal CORT-content following polytrauma. Following euthanization 2h post-traumatization, right adrenals glands were removed, pruned from fat, weighed and used for cryo-sectioning. Thereafter, adrenal glands were homogenized in 20% ethanol and adrenal corticosterone (CORT) content [ng/ml/mg tissue] was determined via ELISA in the supernatants. SHAM vehicle (VEH) (n=9), SHAM C5a Spiegelmer (C5a SM) (n=9), polytrauma (PT) VEH (n=10), PT C5a SM (n=11). Solid line represents median, dashed line represents the mean for each data set. Lower boxes indicate 25th, upper boxes indicate 75th percentile. Also shown are the 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles). Graph by Julia Kunze.
3.2.6. Correlation analyses

3.2.6.1. Correlation between *in vitro* CORT values of basal and ACTH stimulated adrenal glands and adrenal CORT content in polytraumatized mice

A positive correlation between *in vitro* CORT production (basal (rho=0,925; p<0,001) and CORT following stimulation with ACTH (rho=0,524; p=0,01)) and adrenal CORT content in all polytraumatized mice of experiment 1 (Fig. 18 A & B)) could be revealed.

![Graph](image)

**Figure 18:** Correlation between *in vitro* CORT values of basal and ACTH stimulated adrenal glands and adrenal CORT content in polytraumatized mice. Depicted are significant correlations between corticosterone (CORT) values of the *in vitro* stimulation (basal and following stimulation with adrenocorticotropic hormone (ACTH)) and adrenal CORT content in all polytraumatized mice of experiment one (n =21). Both, basal CORT concentrations (A) and CORT concentrations after ACTH (B) stimulation positively correlated with CORT content of adrenal glands. Graph by Julia Kunze.
3.2.6.2. Correlation between *in vitro* CORT values of the basal and ACTH stimulated adrenal glands and adrenal CORT content in C5a Spiegelmer-injected polytraumatized mice

There was a positive correlation between CORT values of the *in vitro* stimulation (basal (rho=0.963; p<0.001) and following stimulation with ACTH (rho=0.683; p=0.021)) and adrenal CORT content in polytraumatized mice that were injected with C5a SM (Fig. 19 A & B)).

*Figure 19: Correlation between *in vitro* CORT values of basal and ACTH stimulated adrenal glands and adrenal CORT content in C5a Spiegelmer-injected polytraumatized mice.* Depicted are significant correlations between adrenal corticosterone (CORT) content and CORT values of the *in vitro* stimulation (basal and following stimulation with adrenocorticotropic hormone (ACTH)) and in all polytraumatized mice of experiment o1 that were injected with C5a Spiegelmer (n=11). Both basal CORT concentrations (A) and CORT concentrations after ACTH (B) stimulation positively correlated with CORT content of adrenal glands. Graph by Julia Kunze.
3.2.6.3. **Correlation between cortical area containing lipid droplets and adrenal CORT content in polytraumatized mice**

Correlation of the data of experiment 1 revealed a negative correlation between the area of lipid droplets and adrenal CORT content in all polytraumatized mice of experiment one (\(\rho = -0.509; p = 0.022\)) (Fig. 20).

*Figure 20: Correlation between the cortical area containing lipid droplets and the adrenal CORT content in polytraumatized mice. Illustrated is the significant correlation between the area containing lipid droplets (LD) (following staining with Oil-Red-O) and adrenal corticosterone (CORT) content in all polytraumatized mice (n=21). The relation between the area of LDs and the whole adrenal cortical area negatively correlated with the CORT content. Graph by Julia Kunze.*
3.2.6.4. Correlation between the cortical area containing lipid droplets and adrenal CORT content in C5a Spiegelmer-injected polytraumatized mice

Correlative investigation revealed a negative correlation (rho=-0.775; p=0.005) between the area of lipid droplets (in relation to the entire area of the adrenal cortex) and adrenal CORT content in polytraumatized mice that were injected with C5a SM (Fig.21).

Figure 21: Correlation between the cortical area containing lipid droplets and adrenal CORT content in C5a Spiegelmer-injected polytraumatized mice. Illustrated is the significant correlation between the area containing lipid droplets (LD) (following staining with Oil-Red-O) and adrenal CORT content in all polytraumatized mice of experiment one (n=21). The relation between the area of LDs and the whole adrenal cortical area negatively correlated with the CORT content. Graph by Julia Kunze.
3.2.6.5. **Correlation between in vitro CORT values of the basal and ACTH-stimulated adrenal glands and the amount of cortical lipid droplets in C5a Spiegelmer-injected polytraumatized mice**

Detailed analysis of the data of experiment 1 revealed a negative correlation between basal CORT values of the *in vitro* stimulation (\(\rho=-0.615; p=0.044\)) as well as CORT concentrations following stimulation with ACTH (\(\rho=-0.617; p=0.045\)) and the area of lipid droplets in polytraumatized mice of experiment one that were injected with C5a SM. (Fig. 22 A) & B).

![Figure 22: Correlation between in vitro CORT values of the basal and ACTH-stimulated adrenal glands and the amount of cortical lipid droplets in C5a Spiegelmer-injected polytraumatized mice. Depicted are significant correlations between adrenal corticosterone (CORT) values of the *in vitro* stimulation (basal and following stimulation with adrenocorticotropic hormone (ACTH) and the area containing lipid droplets (LD) in relation to the whole adrenal cortical area (following staining with Oil-Red-O) in polytraumatized mice of experiment one that were injected with C5a Spiegelmer (n =11). Both basal CORT concentrations (A) and CORT concentrations after ACTH (B) stimulation negatively correlated with the quotient area of LDs/ area cortex of adrenal glands. Graph by Julia Kunze.](image)

3.2.2.6 **Correlation between adrenal parameters in SHAM animals**

Correlative calculations of the data from SHAM-treated animals of experiment one revealed no significant correlations between any of the investigated parameters.
3.3 **Effects of C5a, C5a-Spiegelmer and the combination of both on basal adrenal CORT production of adrenal explants *in vitro***

The Kruskal-Wallis-$H$-Test showed no statistical differences of *in vitro* CORT secretion between stimulation with C5a, C5a SM, C5a+C5a SM and VEH (Fig. 23).

**Figure 23: Effects of C5a, C5a-Spiegelmer and the combination of both on basal adrenal CORT production of adrenal explants *in vitro*.** Following euthanization, both left and right adrenal glands were removed, weighed, bisected and the halves were incubated either with C5a (500ng/ml), C5a-Spiegelmer (C5a SM) (500µl/ml), the combination C5a+C5a SM or incubation medium (vehicle (VEH)). Subsequently, corticosterone (CORT) concentration was determined in the supernatants. VEH (n=9), C5a (n=9), C5a SM (n=9), PT C5a SM (n=9). Solid line represents median, dashed line represents the mean for each data set. Lower boxes indicate 25th, upper boxes indicate 75th percentile. Also shown are the 10th (lower error bar) and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles). Graph by Julia Kunze.
3.4 Effects of C5a, C5a-Spiegelmer and the combination of both on ACTH sensitivity of adrenal explants in vitro

*In vitro* adrenal CORT production was significantly increased in ACTH stimulated vs. basal values in all groups. (Two-way ANOVA; VEH: basal vs. ACTH: p=0.003; C5a: basal vs. ACTH: p <0.000; C5a SM: basal vs. ACTH: p <0.000; C5a+C5a SM: p <0.000). Statistical analysis further revealed that there were no differences in CORT production within the basal and the ACTH-stimulated groups, respectively. Performing a two-way ANOVA between VEH vs. C5a stimulated groups alone revealed that C5a-preincubated adrenal halves had a significant higher CORT production following ACTH stimulation *in vitro* compared to adrenal glands that have been pre-incubated with VEH (p=0.005). (Fig. 24 A &B)).

![Graph](image)

**Figure 24 Effects of C5a, C5a-Spiegelmer and the combination of both on adrenal ACTH sensitivity of adrenal explants in vitro.** Following euthanization, both left and right adrenal glands were removed, weight, bisected and the halves were incubated either with vehicle (VEH), C5a, C5a Spiegelmer (C5a SM) or C5a+C5a SM for 4 hours. Afterwards, adrenals were incubated for 1,5 hours in fresh medium, before being stimulated with saline (basal) or 100nM adrenocorticotropic hormone (ACTH) for 1 hour. CORT concentration was measured in the supernatants. A) VEH (n=6), C5a (n=6), C5a SM (n=6), C5a+C5a SM (n=6); Data represent mean +SEM; ###: p ≤ 0.001: ACTH compared to basal values. B) VEH (n=3) and C5a (n=3); #: p ≤0.01 ####: p ≤0.001: ACTH compared to basal values;**: p ≤ 0.01 : between indicated groups. Graph by Julia Kunze.
4 Discussion

The present study comprises rather novel approaches to investigate the interplay between adrenal functionality and C5a activity (in the context of the exposure to a PT). For a brief synopsis of the major findings, firstly, it is accenting that exposing mice to multiple traumatization increased the responsiveness of adrenal glands towards ACTH. Secondly, in turn, the application of a C5a SM, which blocks the functionality of the anaphylatoxin C5a, ameliorated the PT-induced increase of adrenal ACTH sensitivity. Further analysis, however, did not reveal any effect of the factor PT or the treatment with C5a SM on adrenal weight, the amount of adrenocortical ORO-stained LDs (containing CEs) or adrenal CORT content.

Subsequent in vitro studies with adrenals from not polytraumatized untreated (naïve) mice unveiled that neither C5a, nor C5a SM or C5a+C5a SM affects adrenal CORT production per se.

Finally, the combination of the experimental setup of the second experiment (i.e. the pre-incubation of adrenal explants from naïve mice with C5a, C5a SM or C5a+C5a SM in vitro) with a subsequent stimulation with ACTH in vitro made obvious that C5a significantly increases CORT secretion in response to ACTH.

4.1. Polytrauma enhances adrenal in vitro ACTH sensitivity whereas the application of C5a Spiegelmer ameliorates it

Trauma, in particular PT, is a critical illness establishing a condition of both severe, sustained (physical) stress and immune-activation /inflammation[306]. PT-related or induced boost of C5a are supposed to play a key role in these processes[41,67,307]. At the same time, an adequate “stress response” is inevitable to provide energy, to modulate the immune response and to ensure hemodynamic homeostasis[273]. Thus, a pronounced activation of the HPA axis is generally known to be pivotal to survive adverse conditions like PT[7,125,222,324]. Despite rising experimental and clinical evidence highlighting the critical role of the immune–neuroendocrine interactions in the context of trauma[265], the present study is a rather novel approach for unravelling mechanisms of post-traumatic adrenal functioning and the potential impact of C5a in this context. To pursue this purpose of examining a potential effect of post-traumatic C5a signaling on HPA-axis activation, adrenal glands of mice that previously have
been exposed to PT procedure have been investigated. This rodent model for multiple injury has been combined with the injection of C5a-SM (or VEH, respectively), which is supposed to block biological activity of the anaphylatoxin C5a and thereby ought to alter the pathophysiological severity of the outcome of PT. A major readout of this experimental design was the sensitivity of the adrenal glands (that have been removed after the termination of the PT procedure and the euthanizing of the mice). Concerning that, an established protocol to stimulate the adrenal glands with ACTH has been applied[256]. As a result of that first experiment, interestingly, it became apparent that adrenal glands from VEH-treated PT mice react with an enhanced CORT production in vitro in response to ACTH compared to mice that solely experienced SHAM treatment. That is a result which confirms the hypothesis that harmful events (such as PT) are accompanied by an upregulation of HPA reactivity[11,187,265].

But how could this effect be mediated though?

First, one has to keep in mind that the PT procedure conducted in this study (see also chapter 2.2.1.) includes various insults like thoracic trauma, femur fracturing and TBI but also “iatrogenic effects” like instrumentation, anesthesia and analgesia. All of the latter have in common that they per se might act as potent “stressors” threatening an organism’s homeostasis that result in an activation of the hormonal stress system – e.g. the HPA axis. Thus, trauma is regarded as a severe threat that, especially in its early phase, evokes stress owing to pain or a lack of oxygen within tissues - a matter that has already been approved before[143,171,177,265]. For instance, it has been shown in mice that adrenal responsiveness to plasma ACTH is increased under hypoxic conditions[249].

As outlined in the introduction (chapter 1.4.), a further potential factor that can influence PT-induced ACTH responsiveness is the activity of the immune system, especially C5a. However, although at this point it is not possible to surely attribute the enhancement of ACTH sensitivity after PT to C5a-activity (post-trauma), it additionally could be demonstrated that adrenals from PT-treated animals injected with C5a SM had an ameliorated in vitro adrenal CORT responsiveness towards ACTH when compared to PT-mice that did not receive treatment with C5a SM. So, together with that second result of the ACTH in vitro stimulation, this justifies to speculate that C5a might be a probable mediator for increased adrenal reactivity following PT.
In line, these results are supported by a study investigating HPA response after surgery: the thereby observed HPA axis activation occurred after rather than during surgical intervention, which suggests that HPA response might happen rather “secondarily” (e.g. subsequently to the establishment of an inflammatory condition) than directly due to tissue damage[116]. Furthermore, that time course is fitting well with the described increase in circulating pro-inflammatory markers (e.g. TNF-α, IL-1 and IL-6) remaining raised for about 24 hours before returning to baseline[65].

For comparing this first result with findings in the literature, first, it is critical to notice that evidence about adrenal response towards ACTH in the context of multiple physical injury is rather scarce. More studies, however, aimed at highlighting the status of the HPA axis in the context of critical illness, such as sepsis or septic shock which, however, are acknowledged complications in the course of PT[145,179,267]. In addition to that, opinions about adrenal functioning in the (clinical) setting of critical illness appear to be highly controversial, as well. To cite examples, some publications hold the view that disrupted adrenal function or thus a “collapse” of adrenal capacities, contribute to poor outcome, especially in patients with septic shock[8,57,58,68,189]. In detail, during the past decades, terms such as critical illness–related corticosteroid insufficiency and relative adrenal insufficiency (AI) were being used to describe a failure of such an adequate GC response in proportion to the degree of severity of the subject’s illness. Presently, this issue constitutes one of the most controversial problems in critical care[78,87,298,328] and has been discussed by experts and consensus conferences in order to elaborate guidelines to diagnostic and therapeutic options[87,298,328]. In this context, the standard corticotropin test represents a clinical diagnostic tool that has been introduced for the assessment of adrenocortical functioning. It includes the measurement of total plasma CORT concentrations at distinct points of time after intravenous administration of 250µg ACTH[189,314]. Although the test remains highly debated as to what it reveals about adrenal functional reserve[69,189,298] and its suitability in the setting of critical illness is not fully definite, it at least represents a method to measure adrenal sensitivity towards ACTH in a way that is similar to the method used in the present study. Recent reports even downgrade the recommendation for performing the test[268]. Current available evidence does not support the use of the corticotrophin test in the setting of acute critical illness to assess
adrenocortical function nor should it be used to guide steroid therapy in this setting[69,298].

Apart from human (clinical) studies also animal models for TBI, for instance, picture a positive correlation between severity of brain injury and HPA dysregulation[262] up to the development of critical illness–related corticosteroid insufficiency[54]. Other reports, on the other hand, provide evidence that the response of the HPA axis towards traumatic events rather involves a sort of “hyper-reactivity” of adrenals that might be more detrimental due to immunosuppressive properties of an “overdose” of GC[37,193,285,291,293].

To make it short, opinions are divided on the question whether adrenal glands from traumatized subjects react with an increased or rather an ameliorated GC secretion, instead. Current literature[37,54,193,262,285,291,293], however, yields the idea that adrenal response following PT might perchance be dependent on the duration of the traumatic event and, thus, it might be a matter of the duration that an organisms is exposed to inflammatory signals such as C5a. A rather acute influence of a trauma (i.e. also C5a) might trigger an enhanced adrenal GC secretion, whereas rather long-term or chronic effects of trauma-induced inflammation might involve the development of an adrenal insufficiency resulting in a decrease of GC.

Taken together, PT so far has implicated an increase in vitro ACTH-sensitivity, while the injection with C5a SM following PT could prevent the latter.

But, given that (i) the ACTH stimulation test in vitro comprises a pharmacological dose of ACTH being significantly higher than physiological ACTH concentration, (ii) previous evidence doubts the significance of ACTH stimulated GC levels in the context of critical illness and (iii), above all, the ACTH in vitro stimulation cannot fully predict potential underlying mechanisms how C5a SM might affect posttraumatic adrenal functioning, a further question was, if (and if yes, how ) PT and/ or the treatment with C5a SM affects steroidogenic activity of adrenal glands independently from an additional stimulation with a “supra-physiological” concentration of ACTH in vitro.

In this concern, one of the next approaches was the quantification of LDs - the intracellular stores for CORT precursor molecules in the adrenal cortex.
4.2. **Adrenocortical content of lipid droplets is not affected by polytrauma or C5a Spiegelmer**

As the next attempt to identify potential effects of PT and the presence of C5a signaling on adrenal steroidogenesis, the amount of LDs within the adrenal cortex was quantified by ORO staining followed by digital evaluation.

Before discussing the result of this histological analysis, it briefly should be approached that this study additionally included the purpose to validate the establishment of a novel software program for digital image processing and analysis: Reimage.exe. To approach this, images of adrenal ORO staining were analyzed by Reimage.exe and by applying Image J (a commercially available software). This assessment resulted in comparable accounts for ORO-positive area within adrenal cortices indicating that Reimage.exe can be used as an equivalent to the commercially available software system Image J to analyze LD content, for example.

As already described in the introduction, LDs represent intracellular reservoirs for the substrate cholesterol within the adrenal cortex for further steps of the steroidogenic pathway. Indeed, such droplets are well described to be short term stores for cholesterol since they are rapidly mobilized in response to an ACTH stimulus\[201,210,234,241,242\]. On ground of this knowledge, the initial hypothesis was that due to physical stress together with the establishment of an inflammatory state, PT might imply the initiation of steroidogenic pathways including the mobilization of CEs in LDs for CORT synthesis (and, consequently, the application of C5a SM in the context of a PT might prevent the latter).

There is sophisticated evidence that lipid depletion in the zona fasciculata of both human and rodent adrenals is characteristic for various immune challenges, e.g. in the course of septic shock, experimental endotoxemia and also fatal non-septic illness- a phenomenon that positively correlated with inflammation, necrosis and hemorrhage within the adrenal glands\[265\]. In agreement, adrenal glands from long-term patients in intensive care units contain nearly 80% less CEs than those from controls\[29\]. In the present study, however, adrenals from PT or SHAM animals, respectively, show comparable densities of cortical LDs. However, although this is not in line with the
expectation that CEs in adrenals from PT mice would be diminished, it recently has been reported that, in contrast to enduringly critically ill subjects, adrenal glands from short-term intensive care patients are not characterized by significant CE-depletion[30]. This suggests that it might rather be a matter of duration of a critical illness like PT that profoundly affects adrenocortical structure, especially the status of adrenal LDs. Thus, depletion of CEs may contribute to release enough GCs rather during an extended course of disease instead of during the acute phase of or after a traumatic event. Fittingly, the latter might be the case in the present study, since adrenal glands have been harvested about 4 hours after the PT procedure.

Not to forget, the mobilization of LDs represents only one out of various possibilities to cover the demand of cholesterol for steroidogenesis (for more details see chapter 1.4.2.2). Adrenocortical cells are endued with various options for gathering free cholesterol via bypassing the mobilization of the CE-reservoir in LDs. In turn, this implicates that despite of an unchanged amount of LDs, it is possible that the effects that could have been seen in the in vitro ACTH stimulation of left adrenal gland explants (experiment 1) can be accounted for by alterations concerning distinct steps of the steroidogenic pathway rather than by the depletion of LDs, though.

In this concern, receptors on the cell surface such as the LDL-receptor and SR-B1 are key players to regulate cholesterol uptake and supply of steroidogenic cells such as in the adrenal cortex. In particular, SR-B1, for instance, majorly contributes in providing lipoprotein-derived cholesterol in rodent adrenals[103] and, strikingly, SR-B1 expression is upregulated following stress exposure[103]. Vice versa, the lack or absence of SR-B1 has recently been proved to impair adrenal GC response and results in uncontrolled inflammation and high mortality rates in SR-B1 deficient mice[47,117]. This is a further hint that SR-B1 is a vital element for an effective HPA response. Therefore, SR-B1 might be an essential mediator for covering the enhanced GC demand that arises i.a. due to stress[103] and/or immune challenges[46,103,117].

Besides the latter, steroidogenic enzymes such as the HMG CoA- reductase, which is the rate limiting enzyme for cholesterol de novo synthesis in the endoplasmatic reticulum of the cell or mitochondrial steroidogenic enzymes like CYP11A1[21] might be probable candidates for altered GC production in the course of PT. In the literature,
the expression of steroidogenic in the context of immune activation or inflammation has been described neither explicitly nor consistently, yet. For instance, there is one study attesting no change of HMG CoA-reductase in animals facing an immune challenge compared to those that did not[47]. However, in contrast, another recent rodent study could approve that the initial elevation of GC after an inflammatory stimulus is associated with *de novo* synthesis together with an observable increase of STAR[252]. As a reminder, STAR accomplishes the transport of cholesterol from the cytoplasm to the inner membrane of the mitochondria and therefore fulfills a major task for enabling steroid synthesis in the mitochondria of the cell[233]. The authors of this study[233] additionally succeeded in demonstrating that the upregulation of STAR is associated with an increase of the MCR2-accessory protein (MRAP) – an important regulator for the surface expression of the MCR2 receptor and binding affinity of ACTH towards the latter[203]. This additionally justifies why an alteration of STAR expression might be an effective mechanism to manage an increase of CORT (in response to ACTH) despite of unchanged amounts of LDs.

Given that those parameters have not been assessed in this study, they represent ideal targets for future studies to further elucidate the exact mechanisms underlying the adrenal alterations following of PT and the influence of C5a on those. Nevertheless, all of those distinct pathways of providing cholesterol have in common that they- in the end- enable the synthesis of CORT.

That is the reason why, as a next investigation, content of CORT within adrenal glands has been determined.

### 4.3. Adrenal CORT content is not affected by polytrauma or C5a Spiegelmer

Concerning the ambition to unveil adrenal activity in the context of PT and C5a (SM), it has been appealing to detect not only the intracellular reservoir for GC, but moreover systemically available GC within the animals in experiment 1. In this concern, blood samples of those mice of the first experiment were not available for the determination of plasma CORT concentration since they were needed for further measurements. But, relevantly, there is literature clearly confirming that the CORT content within adrenal glands is indicative for CORT levels in the systemic circulation and indirectly provides information about the systemically available CORT within the organism[296]. For that reason, it has been decided to perform the extraction of CORT out of tissue of the right
adrenals (including its quantification via ELISA afterwards) and use that procedure as a suitable method for drawing a conclusion on systemic CORT levels within the respective animals.

Against the hypothesis that PT ought to result in an increased systemic need for GCs and thus in an enhanced adrenal CORT content, quantification did not indicate any differences of adrenal CORT content in SHAM vs. PT-rodents.

Nevertheless, it is conspicuous that the overall amount of CORT within adrenal tissue considering all animals, independently of whether they were faced by a PT or not, seemed to be relatively high when compared to adrenal CORT content concentrations of comparable mice in the literature[296]. In consideration of that finding, a supplemental investigation has been done: CORT content of adrenal glands of untreated even-aged mice of similar bodyweight was measured. Those animals so to speak represent controls for the animals in experiment 1. On average, CORT content values of the control animals did not deviate from those in experiment 1 (own unpublished data; CORT content of untreated mice).

Conclusively, the relatively high CORT content values of experiment 1 would suggest that the experimental setting per se might imply a rather stressful situation for the animals- not only for those that were exposed to PT procedure but also for those that solely experienced SHAM. But, by means of the additional finding using control animals, it can be excluded that SHAM treatment might be as “stressful” as the PT procedure. Subsequently, you can conclude that neither SHAM or PT nor C5a SM injection affect the adrenal CORT content, i.e. the amount of systemically available CORT.

When you compare this finding to previous studies about HPA- and GC- response in the context of traumatic incidents, the state of knowledge again is not consistent. Interestingly, most hypothalamus-pituitary-peripheral-hormonal axes that collaborate in achieving metabolic and immunological regulation the course of critical illness typically follow a biphasic response pattern. While ACTH rises solely very transiently and then drops off again (maybe even lower than normal plasma ACTH) from quite early after admitting the concerned patients to intensive care treatment, levels of plasma cortisol observed to be consistently increased[30]. Indeed, many studies described elevated GC levels to be characteristic for the acute as well as the prolonged
phase in patients suffering from severe trauma, for instance[30,202,232,310]. The degree of illness-severity, and thus the risk for a fatal outcome, has been positively correlated with the proportion of plasma GC elevation[310]. On the other side, recent research unraveled that, if at all, GC secretion is only slightly increased in the context of risky illnesses or injuries. Instead, the frequently cited hypercortisolemia in the course of critical illness might majorly account for a reduced expression of GC-metabolizing enzymes in the liver or kidney rather than a more of GC production due to HPA axis upregulation[29,231]. This concept is strengthened by recent experimental research in mice being deficient in a gene encoding for 5 alpha–reductase type 1 (a crucial enzyme for GC metabolism). The latter attests that peripheral CORT breakdown implicates a higher increase of CORT and, in the long term, adrenocortical insufficiency[181]. Reminiscing that in the present study CORT was measured following an extraction of it out of adrenal tissue, metabolic activity in the liver and kidneys ought to have no influence on CORT content. This might provide an explanation for unchanged CORT content between PT and SHAM animals in the first experiment.

In addition, GCs, once secreted into the bloodstream, are transported predominantly being bound to corticosteroid-binding globulin (CBG, i.e. transcortin[81,214]) and to a lesser extent to other proteins (e.g. albumin[81]). Importantly, solely free (unbound) GC exert biological activity. Thus, low CBG levels are accompanied by enhanced cortisol availability at the tissue level[236]. In subjects after multiple trauma, plasma CBG concentrations have shown to be promptly decreased which goes along with elevated free cortisol levels. In literature, this is generally discussed for explaining a several-fold increase in plasma free cortisol concentrations. Here again, this has to be taken into account considering the present study. Since the measurement of CORT content within adrenal tissue has been performed as an equivalent for plasma CORT concentrations (for more details see earlier in this chapter), the effect of plasma protein binding on adrenal CORT content levels is assumed to be rather negligible. That might interpret unaltered adrenal CORT content here, which deviate from heightened GC levels in previous studies.

Interestingly, the result of unaltered adrenal CORT content levels in experiment 1 are consistent with the finding of the in vitro stimulation showing unchanged CORT values under basal conditions (i.e. not having been stimulated with ACTH) between all groups
assessed. So, it is striking that PT and/ or C5a SM seem to affect adrenal functionality only in the presence of ACTH.

This brings about the idea that adrenal functionality might be affected by a potential interrelation between ACTH and C5a-signalling. In this regard, one possibility could be a resemblance of the receptors for ACTH and C5a expressed in the adrenal cortex. Indeed, the receptor for ACTH (MC2R) and that for the anaphylatoxin C5a are similarly structured. C5a exerts its biological activity via receptors belonging to the large family of G-protein coupled receptors (GPCR) representing a main group of transmembrane receptors in mammals[213]. Of note, C5a exerts its functions via two different receptors: C5a receptor (C5aR) 1[111] and C5aR2[48], which are (amongst various locations) also expressed in the adrenal gland[17,97,98]. The main pro-inflammatory and regulatory activities of C5a are hereby mediated via C5aR1[60]. Given that GPCRs share the signal transduction cascade via cAMP and PKA (for details see chapter 1.4.2.1), it might be possible that C5a, after binding its GPCR and initiating a rise in PKA, might additionally contribute in launching cAMP-dependent steroidogenesis (for illustration see supplementary figure 1 shown in the appendix). However, such an interplay between ACTH- and C5a-signaling is not proven and further investigations are required for unrevealing why PT and C5a SM seem to exert an effect on adrenal ACTH responsiveness in vitro but not on parameters representing adrenal steroidogenesis independently from an additional stimulation with its secretagogue ACTH.

Additionally, a potential plasticity of the receptor(s) for C5a in the adrenal cortex might provide an explanation for the contrary findings concerning post-traumatic adrenal activity having been mentioned in the chapter 4.1. Since it has been discussed, that a rather chronic (in contrast to an acute) exposure towards C5a might result in an amelioration instead of an increase of the adrenal CORT response, the potential internalization or a reduction of the expression of the receptors for C5a (e.g. C5aR1) might be possible mechanisms to substantiate that.
4.4. Correlation analyses of CORT content, lipid droplets and ACTH sensitivity following PT and C5a Spiegelmer treatment

Despite the findings that neither PT nor antagonizing of C5a by C5a SM affects the content of LDs or the content of CORT in adrenal glands, there can be seen an interesting interrelation between those parameters.

One substantial result from the correlation calculations was that significant correlations could only be observed after analyzing data from mice that experienced PT. For SHAM animals, however, none of the investigated parameters of adrenal functioning correlated significantly with each other.

So, firstly, correlative analysis in PT mice revealed that CORT content negatively correlated with the density of LDs within the adrenal cortex. This supports the idea that LDs indeed serve as a reservoir for steroidogenesis in these mice. The more completely synthesized GC an adrenocortical cell holds available, the less is its intracellular storage for esterified cholesterol in LDs. Since in contrast to the PT group, this correlation could not be approved for SHAM animals, this mechanism seems to be pronounced in mice that have sustained a PT. This in turn might indicate that under stressful conditions adrenocortical cells make recourse to LDs as a preferential source of cholesterol, whereas the mobilization and hydrolyzation of CEs out of LDs might take more minuscule positions for cholesterol supply under less stressful circumstances. At this point, it would be particularly interesting to quantify the HSL- the major cholesteryl esterase of the adrenal cortex[167,168,275]. Reflecting this idea, there is evidence that acute stress goes along with an increase gene expression of the HSL[126] and that phosphorylation (i.e. activation) of the HSL is a crucial step for rapidly increasing free cholesterol within adrenal cells for the further process of steroidogenesis[282].

Moreover, it could be elaborated that, again within the PT group, those mice that additionally were injected with C5a SM, the amount of LDs not only correlates negatively with the CORT content, but also with the CORT values of the in vitro stimulation (both basal and ACTH-stimulated CORT). In other words, although adrenals from VEH-injected animals, within which C5a activity was not blocked, react with an increased CORT secretion in vitro towards an ACTH stimulus compared to C5a SM-injected mice, this suggests that the injection with C5a SM provokes that adrenocortical cells extendedly resort to LDs as a source of cholesterol to make it
available for CORT synthesis and secretion upon the *in vitro* stimulation with ACTH. This mechanism appears to be less prevailing in adrenals from mice that had not been treated with C5a SM. In quest of an explanation for that you can speculate that the C5a SM-treatment might interfere with the mobilization of CEs out of LDs making those droplets an ascendant option to cover CORT demand after an ACTH stimulation. If a C5a SM injection does not take place, other sources of cholesterol (that have been described earlier in more detail) might be more predominant.

4.5. **C5a and C5a SM do not affect in vitro CORT production of adrenal glands of naïve mice**

In a synopsis of the three major readouts of experiment 1, i) adrenal ACTH sensitivity *in vitro*, ii) adrenal LD content and iii) adrenal CORT content, it can mainly be resumed that, except for adrenal CORT production following ACTH stimulation *in vitro*, those parameters remain rather unaffected by the PT procedure and/or C5a SM treatment. Provided that PT involves an increase of C5a, this proposes that C5a might elicit an enhanced adrenal responsiveness inasmuch as a pronounced stimulation by ACTH takes place. The finding that antagonizing C5a via C5a SM leads to an attenuation of PT-induced increase of ACTH-responsiveness supports the hypothesis that C5a activity and adrenal functioning are interconnected. Despite that it remains unsure whether this effect can be affiliated to a direct effect of C5a in the adrenal gland, it is tempting to discuss some possibilities via which C5a might affect adrenal activity in the context of trauma (rather indirectly):

For this, one should reminisce that C5a is the major anaphylatoxic agent of the complement cascade, thus initiates a bunch of humoral and cellular inflammation-activating effects (for details see chapter 1.4.1.1) that in turn might vice versa mediate adrenal activation and might act as regulators contributing to increase the availability of GC during critical illness such as PT.

In this concern, there is sophisticated evidence that the functioning of the adrenal cortex is highly dependent on its (cellular) environment and, in particular, immune and also endothelial cells are critically involved in immune–adrenocortical communication for modulating systemic GC levels[150,151,198]. Thus, immune cells take a critical position for homeostatic functions not only in the non-stressed state but as much more
under “stress conditions”, such as inflammation by detecting pathogens, eliminating apoptotic cells and inducing tissue regeneration via secretion of growth factors[150,153]. During systemic inflammation adrenal immune cell populations alter dynamically to establish close and both-sided cell–cell contacts i.a. with surrounding cells[152,153,313]. In addition to that, adrenal hormonal production has proved to be controlled in a paracrine way by the secretion of cytokines, such as IL-1 and 6[24] implying that generally, cytokines play an important role for steroidogenesis[33,153,185] since those are capable of inducing GC production within the adrenal cortex per se[5,24,33,153,185,264].

A further aspect for gaining a comprehensive understanding of the adrenocortical function is the impact of the adrenal medulla to affect the adrenal cortex. Adrenal medullary (chromaffin) cells are vital for adrenocortical steroidogeneses as evidenced by results from co-culture systems affirming that the addition of chromaffin cells to adrenocortical cells enhanced the secretion of GC by up to ten times[34,90]. In turn, adrenal GC initiate the synthesis and trigger the release of catecholamines from chromaffin cells[83]. Knowing that the adrenal medulla receives innervation by splanchnic nerve fibers deriving from the sympathetic nervous system and that the sympathetic nervous system, if confronted with a threatening stimulus (e.g. PT) gets activated synergistically to the HPA axis[119], it is possible that some of the increased adrenal sensitivity observed in PT mice derives from increased splanchnic nerve firing[82,90,294].

Furthermore, it is known that multiple injury brings along an enhanced risk of infections due to tissue damage involving the disruption of physiological barriers (e.g. skin and mucous membranes) and the invasion of pathogens. The pathogen–host interactions amongst others are executed by pattern recognition receptors, such as toll-like receptors (TLR). Interestingly, human and mouse adrenocortical cells express TLRs[153] suggesting TLR signaling in adrenocortical cells or in other cells of the adrenal surrounding, including recruited leukocytes, to participate in the immune–adrenal interaction and steroid synthesis during systemic inflammation[150,153]. For instance, mice in which TLR signaling was inactivated in immune or in adrenocortical cells are characterized by an abolition of the HPA-response after an immune
challenge. That is a finding that confirms the critical role for an intact TLR signaling in HPA axis functioning[152].

Considering more possibilities for the (dys-) regulation of steroidogenesis in the course of injury, it is critical that the adrenal gland is a highly vascularized organ captivating way more blood flow than its organ volume might justify[268]. Given that, its vulnerability for endothelial dysfunction and hemorrhage should be discussed, as well. In fact, there is a clear interrelation between adrenal vasculature and steroidogenesis[9,147]. Within the adrenal cortex, every steroidogenic cell is in close vicinity with at least one sinusoid. So, it is plausible that, in addition to their actions on the adrenal vasculature, endothelial secretory products are capable of regulating and/or increasing steroidogenesis[10,133,151].

To sum it all up, the matters that

i) dysfunction of the endothelial system is a hallmark of systemic severe trauma/ inflammation[2] (and its complications like sepsis or MOF) and

ii) the PT procedure applied in this study also involved the exposure towards an hemorrhagic shock

make it likely that the observed increase in CORT production following in vitro stimulation with ACTH may come from changes in adrenal perfusion. And, here again, you can ascertain a link to C5a activity: Indeed, C5a is reported to cause the endothelium layer to get “leaky” [183] and thus might interfere in vasculature-mediated processes affecting steroidogenesis not only by releasing endothelium-derived mediators[207,245] but also by initiating immune cell invasion from the circulation into the adrenal gland via vascular adhesion molecules[165,281].

To put it all together, those effects of affecting adrenal functioning that have been discussed in the previous paragraphs and are additionally illustrated in supplementary figure 2 in the appendix are cited to be driven by or at least to be under the influence of C5a.

But, limitingly, one so far cannot exclude whether the observed enhancement of adrenal sensitivity towards ACTH in vitro (in experiment 1) can be dedicated to C5a
per se or is rather driven by further immune-activating processes in the context of PT that, if at all, might be mediated by C5a indirectly.

Basing on this complex concept of adrenocortical functioning in the context of immunooactivation, the second experiment of this thesis aimed at investigating the topical influence of C5a and its SM on the adrenal gland from naïve animals in vitro and independently from multiple trauma. In other words, the idea was to substitute the PT procedure by the stimulation of adrenals with C5a intending to reveal a possible potential of C5a on adrenal functioning per se in the context of PT and to exclude confounding factors owing to the PT procedure itself, that might have an impact on adrenal activity, as well.

As a result and against the hypothesis that C5a ought to trigger adrenal GC production, CORT secretion was not changed following in vitro stimulation of adrenal explants with C5a or C5a SM, respectively. One possible explanation therefore could be that, firstly, C5a alone might not be sufficiently capable to induce enhanced CORT response of adrenals in vitro itself. Perhaps, it is rather the combined effect of C5a together with other stress- and inflammation-provoking mechanisms that occur following PT (as shown in Fig. 26 and in chapter 4.1). Those factors are rather negligible for experiment 2, since in vitro stimulation with C5a and C5a SM was intentionally carried out in mice that did not experience any trauma before. Secondly, it is important that the in vitro stimulation discussed here did not involve a stimulation with ACTH. Hereby, the result of unchanged CORT values following stimulation with C5a and C5a SM fits rather well to the main results of experiment 1 using PT mice. Specifically, if ACTH is not additionally present, C5a or C5a SM in vitro (experiment 2), in correspondence with PT and C5a SM in vitro (experiment 1) seem not to have any effect on adrenal in vitro CORT production.

Taken together, the fact that in vitro incubation with C5a and its SM does not elicit an effect on adrenal CORT secretion might either derive from missing PT or rather the lack of additional stimulation with ACTH.

4.6. C5a enhances ACTH sensitivity of adrenal explants from naïve mice in vitro
Strikingly, the finding that neither C5a nor C5a SM (and nor the combination of both) alters adrenal CORT production in vitro (experiment 2) together with unchanged basal (i.e. not ACTH-stimulated) CORT values following PT and/or C5a SM injection (experiment 1), prompts one to suppose that the presence of ACTH possibly might undertake a decisive role in mediating the effect of C5a on adrenal functioning.

On ground of that, a third experiment was performed to clarify if, and if yes to what extent, C5a is involved in influencing adrenal activity in combination with an in vitro stimulation with ACTH. The strategy was to pre-incubate adrenal glands of untreated (not injured) mice with C5a, which is the agent that is one of the strongest candidates to be responsible for the posttraumatic immune response and, via neuro-immune interactions, also for activating the adrenal gland. This approach then was combined with a subsequent in vitro stimulation with ACTH.

So to speak, the intention was to mimic the in vivo condition of experiment 1 by “replacing” the PT procedure including C5a SM injection by an in vitro incubation of adrenal explants of naïve mice with C5a or C5a SM, respectively. Following the pre-incubation, adrenal explants from untreated mice were stimulated in vitro with ACTH (analogously to the in vitro ACTH sensitivity assay that had already been performed in the first experiment).

By means of that final experimental approach, it became apparent that pre-incubation with C5a is capable of enhancing ACTH responsiveness of adrenal explants in vitro in mice that have not experienced a prior PT.

So, this result is in line to what was expected due to the findings of experiment 1 and might affirm the capability of C5a to heighten adrenal response towards ACTH per se. The effect of decreasing ACTH-dependent CORT secretion by injection with C5a SM in experiment 1 could not be reproduced in in vitro conditions in experiment 3, though, since in vitro C5a SM could not significantly lower the adrenal CORT secretion towards ACTH compared to the group that has been pre-incubated with C5a.

Anyways, despite of not being significant, the data give the impression that ACTH-stimulated CORT values from C5a SM-incubated adrenal explants are slightly lower than those from the C5a- incubated ones. This notice might be an appropriate reason to perform further investigations including a higher n-number, to elucidate whether C5a
SM indeed might be capable to ameliorate adrenal ACTH sensitivity also in naïve (not polytraumatized) rodents.

To this concern, one can suppose that C5a SM might admittedly be able to alleviate C5a-mediated immune-enhancing and thus activating effects on the adrenal gland. But, here that appears to be relevant only in a milieu of pronounced C5a activity due to an immune challenge (such as PT in experiment 1).

Since in the setting of the third experiment animals have not experienced any injury before, accordingly, there might be no occasion for a triggered C5a response including a lack of an inflammatory reaction. Hereof, C5a SM might be lacking of appropriate targets (i.e. C5a molecules) to elicit its effects. This guess would be in favor of C5a SM as a pharmacological compound since this would imply that C5a SM might be “medically active” in the presence of an inflammatory state whereas it might remain inoperative under non-inflammatory (“healthy”) circumstances.

4.7. Conclusion and outlook

Pursuing the approach to get more insight into steroidogenic functioning of the adrenal gland in the context of PT and supposing a PT-induced rise in C5a, the present study succeeded in demonstrating that the exposure to PT is accompanied by an increase of adrenal responsiveness towards a supra-physiological dose of ACTH in vitro, whereas basal CORT production (in the absence of ACTH) remained unaltered. The intravenous application of C5a SM in the context of PT implied an amelioration of adrenal ACTH sensitivity in vitro. But, the attempt to further comprehend the direct effect of C5a signaling on adrenal steroidogenesis lead to the finding that neither C5a nor C5a SM does elicit any effect on adrenal glands under basal conditions, since incubation with C5a of adrenals from not-injured mice did not alter CORT secretion in vitro. Conducting an additional stimulation with ACTH in vitro, however, could enhance adrenal CORT response.

Admittedly, this study bears some limitations that have partly been addressed earlier. Most importantly, future studies are inevitable to explain the conspicuous finding, why PT or C5a SM seem to affect adrenal activity in a way that seems to be dependent on the stimulation with ACTH. As well, the present findings necessarily have to be discussed in view of the fact that the experimental procedures have been conducted.
Discussion

in this manner for the first time. Given the establishment of methods (e.g. the stimulation of adrenal explants with C5a and ACTH), experimental procedures might have to be optimized in the future in terms of adjusting them to the precise timing of the PT schedule, for instance. Also, especially concerning the third experiment, the n-number of adrenal explants should be increased to affirm or further investigate the relation between C5a and ACTH-responsiveness and, suspiciously, whether C5a SM in vitro might also be enabled to decrease CORT production of adrenals after ACTH stimulation- an effect that, although not being significant, the third experiment hints at.

Since it has been discussed that the impact of C5a activity on adrenocortical functioning might be in dependence of the duration of the antecedent trauma (and the subsequent inflammatory/complement response), it definitely is worthwhile to consider not only the acute (short-term) effect of C5a on the adrenal gland but also to look at adrenal response in the context of a rather chronic (long-term or recurrent) influence of C5a in up-coming experiments, though.

Given that previous studies showed that the inflammatory response decisively predicts the outcome after PT, whereby C5a is known to take a dominant position, the present data suggest that the investigation of adrenal functioning in the course of trauma might be an indirect, but promising, approach to evaluate the extent of the potentially detrimental immune response within polytraumatized subjects and thus, ideally, their prognosis.

Nonetheless, the novel strategy to make use of the potential of a C5a SM to antagonize C5a signaling and, consequently, to regulate posttraumatic (hyper-) inflammation including the accompanying adrenal response provides an encouraging strategy for future studies. Concerning the long-term perspective, that might bring out a valuable therapeutic opportunity for diseases that are characterized by a “too much” of an overwhelming and potentially detrimental inflammatory response such as in the course of multiple injuries.
Abstract

Polytrauma (PT), defined as a combination of severe injuries, results in massive systemic immune activation, frequently cumulating in lethal multiple organ failure (MOF). The complement system, especially its anaphylatoxin C5a, thereby plays an important role. Given the function of the hypothalamus-pituitary-adrenal (HPA) axis, especially its effector hormone cortisol (humans) /corticosterone (CORT, rodents), to regulate immune responses and the vast interaction between the stress and the immune system, it is not surprising that many components of the immune system can vice versa activate the HPA axis, including the adrenal glands- the organs that synthesize cortisol/CORT. Thus, quantification of HPA axis activity, especially at the level of the adrenal glands, could potentially provide an indirect but meaningful approach to assess the extent of PT-induced, and C5a-mediated inflammatory processes.

To strengthen this approach, the general purpose of this thesis is to investigate the influence of C5a on adrenal functionality in both polytraumatized but also in naïve mice. In detail, a first experiment aimed for whether treatment of polytraumatized mice with a C5a Spiegelmer (C5a SM), which is supposed to antagonize C5a, affects the functionality of the adrenal glands in PT vs. SHAM-treated animals. Moreover, another aim of this thesis was to investigate the topical influence of C5a or C5a SM on in vitro CORT production of naïve adrenal glands under basal conditions (experiment 2) as well as in response to ACTH stimulation (experiment 3).

To achieve those aims, one set of mice (aim 1; experiment 1) was exposed to a 20-minute procedure of multiple traumatization (PT) or SHAM before being injected with either C5a SM or vehicle (VEH). Four hours after PT, animals were sacrificed and adrenals were removed. Right adrenals were used to determine the amount of steroid precursor molecules (cholesteryl esters) within the adrenal cortex employing Oil-Red-O (ORO) staining as well as to measure the adrenal CORT content via enzyme-linked immunosorbent assay (ELISA). In vitro adrenocorticotropic hormone (ACTH) responsiveness was assessed in left adrenals. Further on, adrenals of another set of untreated mice (aim 2) were incubated in vitro with C5a, C5a SM or the combination thereof to examine the topical influence of C5a or C5a SM on adrenal CORT production (experiment 2). Additionally, in the third experiment, adrenal glands of a third set of
animals were stimulated in vitro with ACTH after being incubated with C5a, C5a SM or the combination of C5a and C5a SM. The results of experiment 1 revealed that PT increases ACTH responsiveness, an effect that was blocked by the systemic blockade of C5a via C5a SM. However, the assessment of adrenal weight, CE-stores and CORT content showed no significant difference between the groups. Although neither C5a nor C5a SM nor C5a+C5a SM changed basal adrenal CORT in vitro compared to VEH values in experiment 2, pre-incubation with C5a in vitro of adrenal explants significantly enhanced adrenal ACTH sensitivity in experiment 3. This finding, however, could not be seen in ACTH-stimulated adrenals having been pre-incubated with C5a SM or C5a+C5a SM.

Although immune parameters were not directly determined in the present study and provided that PT goes along with a marked rise in C5a, the results that (i) C5a SM downregulated adrenal ACTH responsiveness in vitro in response to PT and (ii) C5a per se enhanced adrenal ACTH responsiveness in vitro in naïve mice, quantifying HPA axis activity might indeed serve as an indirect but reliable measure for immune system activation.

The observation that C5a and C5a SM, respectively, do not elicit any effect on in vitro CORT synthesis in not-PT (naïve) mice, indicates that there might be a bunch of other pathophysiological factors after PT that, except from C5a, affect adrenal functioning. The last experiment demonstrated that an additional in vitro stimulation with ACTH in not-PT animals results in an increase in CORT of C5a-preincubated adrenal explants, though. Conclusively, one can justify to assume a potential interrelation between adrenal C5a and ACTH signaling- a matter that certainly calls for further investigation in future studies.
6 Zusammenfassung


Ein zweites Experiment verfolgte das Ziel, den direkten bzw. topischen Einfluss von C5a bzw. C5a SM auf die Nebennierenaktivität unabhängig von dem Einwirken eines
Zusammenfassung

PTs aufzuklären. Hierfür wurden explantierte Nebennieren nicht polytraumatisierter Tiere in vitro mit C5a, C5a SM oder der Kombination C5a +C5a SM inkubiert. Dieser Versuchsansatz wurde im dritten Experiment abschließend noch durch ein zusätzliche in vitro-Stimulation mit ACTH erweitert, um die adrenale ACTH-Sensitivität nach Inkubation mit C5a bzw. C5a SM zu untersuchen bzw. die in vivo Situation aus Experiment 1 in vitro und unabhängig von einem PT nachzustellen.

Die Ergebnisse des ersten Experiments zeigten, dass PT (im Vergleich zu SHAM) zu einem höheren Ansprechen der Nebennieren auf den Stimulus ACTH in vitro führt, was durch die Injektion des C5a SM abgeschwächt werden konnte. Die Parameter Nebennierengewicht, Cholesterylesterdichte im adrenalen Cortex sowie der adrenale CORT-Gehalt zeigten sich unverändert.

Weiterhin offenbarte das zweite Experiment, dass weder C5a noch C5a SM oder C5a+C5a SM die CORT Produktion in vitro signifikant beeinflussen.

Eine Prä-inkubation mit C5a im dritten Experiment brachte jedoch einen Anstieg der ACTH-Sensitivität in vitro hervor.

Obwohl die vorliegende Arbeit Immunparameter nicht direkt bestimmt und die Annahme voraussetzt, dass ein PT eine deutliche Komplementantwort, ergo einen Anstieg von C5a mit sich führt, sprechen die Erkenntnisse, dass i) ein PT mit einem erhöhten Ansprechen der Nebenniere auf ACTH einhergeht und ii) C5a SM diesen Effekt abschwächt, dafür, dass die Untersuchung der Nebennierenaktivität einen indirekten Ansatz zur Bestimmung der Entzündungsantwort nach PT bieten könnte.

Die Beobachtung, dass C5a bzw. C5a SM unabhängig von dem Einwirken eines PT keinen Einfluss auf die adrenale in vitro CORT Synthese nehmen, könnte darauf hinweisen, dass neben der Einwirkung von C5a auf die Nebenniere auch weitere systemische Faktoren im Zuge eines PT Einfluss auf die adrenale Aktivität ausüben.

7 References


References


94. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM: Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production


103. Füchsl AM, Uschold-Schmidt N, Reber SO: Chronic psychosocial stress in male mice causes an up-regulation of scavenger receptor class B type 1 protein in the adrenal glands. Stress.,16:461-468(2013)


References


143. Increased levels of interleukin-6, -8 and -10 are associated with fatal outcome following severe traumatic brain injury. Brain Inj., 28:1311-1316(2014)


156. Keel M, Bonaccio M, Steckholzer U, Ungethüm U, Gallati H, Trentz O, Ertel W: Increased plasma level of Type I (p55) and Type II (p75) TNF-receptors following trauma. Swiss Surg.,5:241-244(1995)


164. Koski CL, Ramm LE, Hammer CH, Mayer MM, Shin ML: Cytolysis of nucleated cells


214. Murphy BE: Some studies of the protein-binding of steroids and their application to the routine micro and ultramicro measurement of various steroids in body fluids by competitive protein-binding radioassay. J Clin Endocrinol Metab. 27:973-990(1967)


References


References


267. Sapan HB, Paturusi I, Jusuf I, Patellongi I, Massi MN, Pusponegoro AD, Arief SK, Labeda I, Islam AA, Rendy L, Hatta M: Pattern of cytokine (IL-6 and IL-10) level as inflammation and anti-inflammation mediator of multiple organ dysfunction syndrome


References


# Appendix

**Table of figures and tables**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Pathophysiological aspects of the posttraumatic immune responses</td>
<td>6</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Schematic illustration of the principle of the C5a Spiegelmer.</td>
<td>12</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Schematic relation between pro- and anti-inflammatory reactions of the posttraumatic immune response.</td>
<td>13</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Schematic representation of the hypothalamus-pituitary-adrenal axis.</td>
<td>15</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Schematic illustration of cholesterol utilization in the adrenal cortical cell.</td>
<td>17</td>
</tr>
<tr>
<td>Figure 6</td>
<td>The major aims of this thesis.</td>
<td>21</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Illustration of the polytrauma procedure.</td>
<td>27</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Illustration of the experimental procedure of experiment 2.</td>
<td>27</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Illustration of the experimental procedure of experiment 3.</td>
<td>28</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Image processing with Reimage.exe.</td>
<td>31</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Image processing with Image J.</td>
<td>32</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Comparison of Reimage.exe and Image J for quantitative analysis of lipid droplet staining.</td>
<td>35</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Effects of C5a Spiegelmer in vitro ACTH sensitivity following polytrauma.</td>
<td>36</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Effects of C5a Spiegelmer on adrenal weight following polytrauma.</td>
<td>37</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Effects of C5a activity on bodyweight following polytrauma.</td>
<td>38</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Effects of C5a Spiegelmer on lipid droplets in the adrenal cortex following polytrauma.</td>
<td>39</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Effects of C5a Spiegelmer on adrenal CORT-content following polytrauma.</td>
<td>40</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Correlation between in vitro CORT values of basal and ACTH stimulated adrenal glands and adrenal CORT content in polytraumatized mice</td>
<td>41</td>
</tr>
<tr>
<td>Figure 19</td>
<td>Correlation between in vitro CORT values of basal and ACTH stimulated adrenal glands and adrenal CORT content in C5a Spiegelmer-injected polytraumatized mice</td>
<td>42</td>
</tr>
<tr>
<td>Figure 20</td>
<td>Correlation between the cortical area containing lipid droplets and the adrenal CORT content in polytraumatized mice.</td>
<td>43</td>
</tr>
</tbody>
</table>
Adrenocorticotropic hormone (ACTH) binds to its receptor, the melanocortin 2 receptor (MC2R), on the membrane of the adrenocortical cells, which increases cyclic adenosine mono phosphate (cAMP). So do the receptors for C5a (here for simplification: C5a receptor (C5aR)). cAMP stimulates protein kinase A (PKA), that leads to the release of cholesterol from cholesteryl esters (CE) in lipid droplets (LD) into the cytoplasm (enzyme: hormone sensitive lipase (HSL)) and de novo-production from acyl coenzyme A (Acyl CoA) via the enzyme hydroxy-3-methylglutaryl(HMG)-CoA reductase in the endoplasmatic reticulum. PKA increases the expression of the steroidogenic acute regulatory protein (STAR) to transport cholesterol (the substrate for steroid hormones) from the cytoplasm to the inner membrane of the mitochondria where steroidogenesis proceeds. A long-term effect of PKA involves the upregulation of cholesterol uptake (scavenger-receptor class B, member 1 (SR-B1), LDL receptor (LDL-R) and cholesterol synthesis. Blue lines indicate PKA-mediated effects. Illustration by Julia Kunze.
Supplementary figure 2: Discussion of potential concepts of the adrenal stress response following polytrauma and the possible link to C5a activity. Functioning of the adrenal gland highly depends on its (micro-) environment. In the context of an immune challenge, immune cells get activated, proliferate and subsequently accumulate in and around the adrenal gland. Either by direct action on the cells of the adrenal cortex or rather due to the secretion of cytokines, e.g. interleukin (IL) 1 or 6 deriving from, among others, immunocompetent cells, adrenal corticosterone (CORT) (rodents) or cortisol (humans) synthesis in the context of critical illness like polytrauma (PT) might be differentially regulated. Especially under non-sterile or septic conditions accompanying or following PT, bacterial and other (e.g. viral) toxins occur and can modify adrenal steroidogenesis by targeting toll-like receptors. Hypothalamic-pituitary-adrenal axis function is further influenced by adrenal vasculature involving blood flow factors deriving from endothelial cells. In addition, (sympathetic) innervation involving splanchnic nerve firing targeting the adrenal gland, and particularly adrenal medullary chromaffin cells might have an impact on adrenal functioning post-trauma. In support, chromaffin cells of the adrenal medulla are capable of eliciting effects on adrenocortical cellular activity due to a bidirectional interrelation between those two cell types. As indicated by red dashed lines, C5a is hypothetically capable of influencing the illustrated mechanisms of adrenal control in the context of a critical illness. But, in addition, PT is accompanied by way more pathophysiological processes that might affect adrenal functioning, which is why it remains speculative to attribute the illustrated mechanisms of influencing the adrenal gland to the activity of C5a. Illustration by Julia Kunze.
Acknowledgments

Looking back at my years as a medical student, I am now strongly convinced that the time I could spend in and with the “Reber Lab” yielded an opportunity to gain experiences, that for me belong to the most enriching during my studies in Ulm! I am sure that my time in the Laboratory for Molecular Psychosomatics considerably contributed not only to my medical or academic education but also - at least as much- to my further personal development. For that reasons, it is my main concern to heartily say thank you to all those people that afforded me to accomplish this.

First of all, I want to thank Prof. Dr. med. Harald Gündel for giving me the chance to write my medical dissertation in the Clinic for Psychosomatics and Psychotherapy in the University Clinic Ulm. I am honestly thankful for the International Graduate School in Molecular Medicine Ulm (IGradU) for supporting me and my work on this experimental MD thesis with a scholarship. Not less, I want to give thanks to Mrs Mihr’s effort from the promotion office.

My outstanding thanks go to my doctoral supervisor Prof. Dr. rer. nat. Stefan Reber. Since my first day in your lab, Stefan, you constantly and exhaustlessly placed your trust in me and my work. You reliable supported and encouraged me whenever there was any kind of doubt or worries. I did and do appreciate not only your scientific proficiency and what you have achieved so far. At least as much, I do cherish your fair, up-lifting, optimistic but at the same time also promoting way of how you deal with the people that work for and with you. You have succeeded to create an unique “team spirit” in your lab that makes it so enjoyable to work there. That and you motivated and inspired me to always give my very best. I am so glad that you have given me this special chance to learn from you. I am sure that I am a “lucky one” that could get to know you and your great team!

Furthermore, this thesis could not be possible without Prof. Dr. med. Markus Huber-Lang and his lab. Thank you very much for entrusting me to work on your research topic and for the cooperation with your team as well as with the Noxxon Pharma AG. Especially, I want to give thanks to Dr. Axel Vater for diligently proofreading this thesis. I am especially thankful for the great support and help from Dr. Annette Palmer and Sonja Braumüller.
Moreover, I want to thank Dr. Michael Noll-Hussong, who significantly contributed to this thesis by developing the software program Reimage.exe and helping me with the first steps of the digital analysis of my histological images. 

Furtheron, it is a special matter for me to dearly say thank you to the entire staff of the research group around Prof. Reber. I am so grateful that you, Dr. rer. nat. Dominik Langgartner have taught, supervised and advanced me in a way that I have appreciated from the very beginning! Not solely your expertise and your way of working so deliberately made it so easy for me not to give up (even after the 16th Western Blot) and to always carry on. Not less, I want to thank Sandra Förtsch for also supporting and encouraging me with your broad knowledge and your technical competence in the lab and in the animal facility. But at least as important: Sandra, I hope you know that I became not just a fan of your professional know-how but also of you and your personal attitude! Without you both I would not have learned so much in the lab and I would miss two great people, that became true friends for me! 

Thank you, Petra Hornischer and Ulrike Binder for not just helping be out whenever there was any technical question. You also spread so much encouragement and patience and always had an open ear for me! In addition, there are so many people that have got me to carry on with energy and optimism and have made me feel to be “at the right time at the right place”.

With you, Maike Erber and Anna Weiss, I got to know two precious people and I can’t express how thankful and happy I am that our paths crossed in the “AG Reber”. You and of course so many people more have given me the feeling to never walk alone! 

Last but not least, I want to thank my family, that I do love so much: Mama und Papa, ich hoffe ihr wisst, wie sehr ich euch schätze und liebe- dafür, dass ihr mich schon immer so bedingungslos geliebt habt und mir auf unermüdliche Art und Weise Kraft gebt, bei allem was ich tue! Ich hoffe ich kann euch in Zukunft zumindest einen Teil davon zurückgeben, was Ihr für mich und auch für Linda geleistet habt!
Curriculum vitae

This part has been removed due to protection of data privacy.
This part has been removed due to protection of data privacy.
This part has been removed due to protection of data privacy.
This part has been removed due to protection of data privacy.