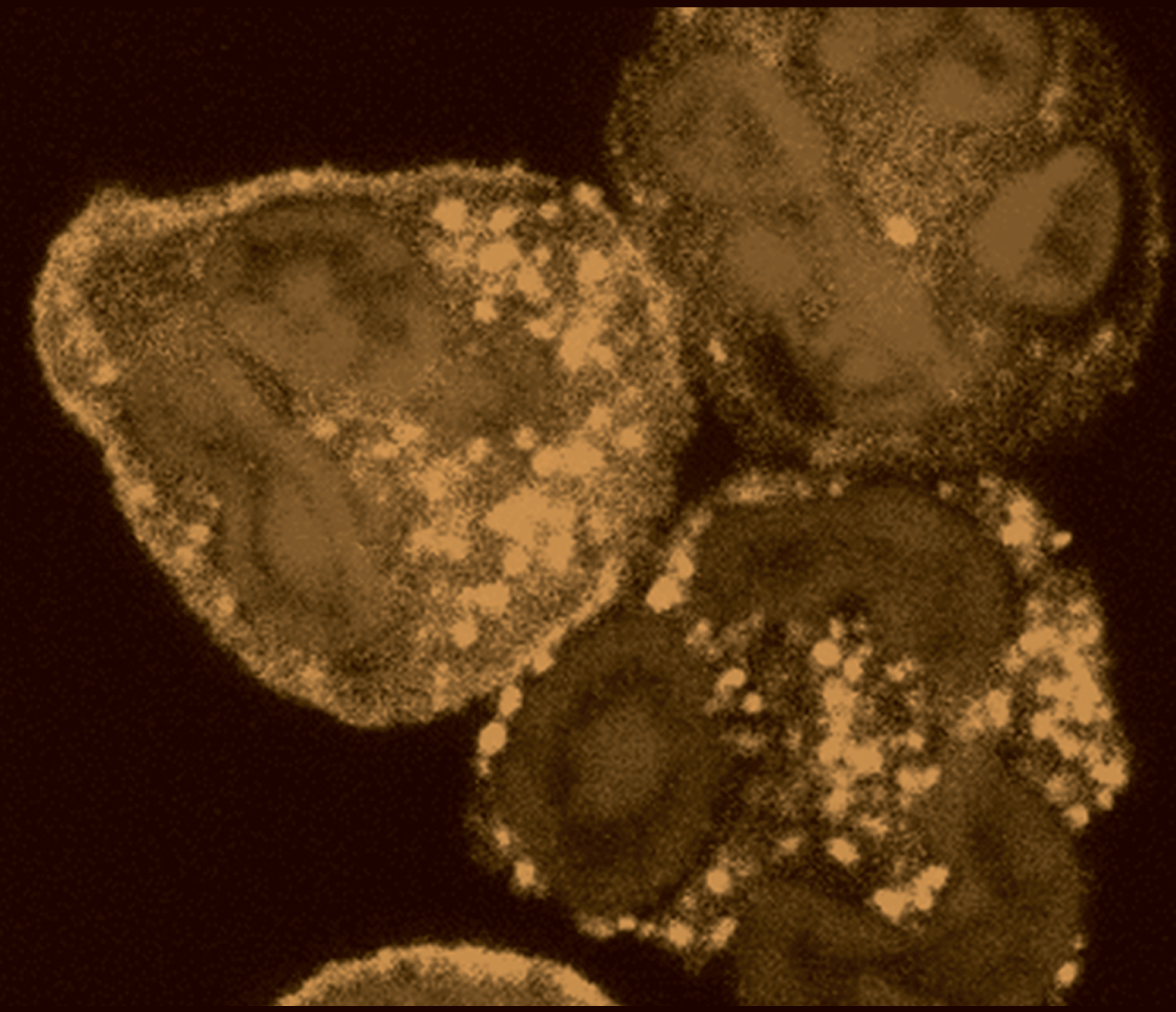
Mediators of Inflammation

Posttraumatic Immune Response and Its Modulation

Guest Editors: Frank Hildebrand, Sascha Flohe, Loek Leenen, Martijn van Griensven, and Michael Frink



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Editorial Board

Contents

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Editorial

Posttraumatic Immune Response and Its Modulation

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Following accidental trauma, an inflammatory response is induced involving hormonal, metabolic, and immunological mediators. This posttraumatic immune response is a physiological process required for tissue repair and regulation of the healing process. An overwhelming response after major trauma causes a disbalance between pro- and anti-inflammatory mediators resulting in complications like an increased susceptibility to infection, sepsis, and the Multiorgan-Dysfunction-Syndrome (MODS) [1]. Therefore, profound knowledge of this inflammatory response is a major interest in order to anticipate and control these potential posttraumatic complications.

Following major trauma, a local release of mediators such as cytokines, acid metabolites, and histamine increases the capillary permeability resulting in tissue edema and local infiltration of immunocompetent cells. Intrinsic leukocytes and affected endothelial cells produce and release pro- and anti-inflammatory cytokines acting locally as well as on remote cells in different organs like the lung [1, 2]. In this issue P. Mommsen et al. investigated the role of IL-6 on cytokine production capacity of splenocytes after femoral shaft fracture, isolated or in combination with hemorrhage in male mice. The authors showed that an isolated femoral fracture resulted in a suppression of *in vitro* cytokine synthesis of splenocytes, which was further enhanced in case of an additional hemorrhagic shock. In IL-6 knockout mice, the splenic immunodepression after femoral fracture and hemorrhagic shock attenuated. These results give evidence that the posttraumatic modulation of IL-6 synthesis might be a potential target for therapeutic interventions. As contradictory findings in animal models of trauma and sepsis impede a rationale for therapies directed against IL-6 in general, selective inhibition of IL-6 transsignaling using soluble IL-6R seems to be a potential treatment strategy [3].

P. Kobbe et al. (in this issue) described the effects of inhalative IL-10 administration on the pulmonary and systemic inflammatory response after hemorrhage in male mice. The authors found that inhalative IL-10 application resulted in a significant decrease of the inflammatory reaction in the lung without affecting the systemic immune response after hemorrhage. From the clinical point of view, generally the lung is the first organ to fail after injury. Furthermore, respiratory failure is most frequent in patients developing MODS, and these patients have the highest mortality rate [4]. Therefore, the results of P. Kobbe et al. have the potential to build the basis for the development of another treatment option after severe trauma.

Priming and activation of leukocytes blood trigger the systemic immune response eventually leading to inflammatory complications. On the other hand, anergy of leukocytes may lead to infectious complications. Neutrophils are the main types of effector cells in the innate immune system [5]. Their activation causes the release of neutrophil extracellular traps (NETs) which exert strong antimicrobial and immunomodulating properties. However, high local NETs concentrations might also result in severe tissue damage with subsequent organ dysfunction. In a study of W. Meng et al. (in this issue) the authors observed the significance of NETs and the natural counter regulator deoxyribonuclease (DNase) for the development of sepsis in multiple trauma patients.

They found that levels of NETs and DNase were significantly increased in the very early phase of sepsis or even before clinical manifestation. Therapeutic strategies that limit NETs activity might therefore prevent neutrophil-derived pathological effects possibly resulting in posttraumatic organ failure. Diverse neutrophil CD molecules have been shown to represent reliable marker for the incidence of complications after multiple trauma [2]. However, these parameters have not been implemented in clinical practice due to complex flow cytometric analysis. In this issue M. Groeneveld et al. demonstrated that analysis of CD molecules on a routine haematology analyser is reliable and fast so that these parameters might now be more suitable for implementation in the clinical routine. M. Kolackova et al. (in this issue) found a postoperative increase of systemic IL-10 concentrations which was dependent on the invasiveness of the surgical procedure. Furthermore, they described a correlation between increased IL-10 levels and a higher percentage of neutrophils after surgery. This suggests a functional relationship between both parameters and underlines the complexity of the posttraumatic immune response.

Beside neutrophil function, this special issue focuses on other aspects of the posttraumatic immune response. B. Auner et al. investigated Leukotriene B4, a proinflammatory lipid mediator, as a predictor for pulmonary complications in a population of 100 patients with severe injuries. They demonstrated increased plasma levels of Leukotriene B4 on admission as compared to a healthy control group. Moreover, increased plasma levels of Leukotriene B4 were associated with pulmonary complications but not with the severity of suffered chest injuries. Thus, Leukotriene B4 seems to be a candidate for an early identification of patients on risk for pulmonary complications following major trauma which may have an impact on further therapeutic strategies.

Matrixmetalloproteinases (MMPs) represent another family of inflammatory mediators playing a central role in tissue remodeling of extracellular matrix during the early posttraumatic period. In a clinical study of M. Brumann et al. (in this issue) including 60 polytrauma patients, MMP-9 significantly decreased over a 72 h period while tissue inhibitor of matrixmetalloproteinases- (TIMP-) 1 increased. Furthermore, this effect was dependent on injury severity. Therefore, MMP-9 and TIMP-1 may serve as an indicator for the posttraumatic immune system disbalance.

Proteasomes seem to have a biological role in the extracellular alveolar space, while their role during inflammatory processes remains to be elucidated. In this issue S. U. Sixt et al. found that the total proteasome concentration was increased in the bronchoalveolar lavage (BAL) in patients with ARDS as compared to healthy patients. The immunoproteasome proteins LMP2 and LMP7 were only identified in patients suffering from ARDS. Moreover, the proteasomal enzyme activity pattern was different between patients with ARDS and healthy subjects. Quantitative immunoproteasome measurements in BAL may help to discriminate disease activity and efficacy of therapy.

In the current special issue two new experimental trauma models are introduced. C. Probst et al. presented first results of a murine multiple trauma model including traumatic brain injury, hemorrhage combined with a femoral fracture. The model was characterized by plasma cytokine pattern and lymphocyte subtype populations. In another polytrauma model in rats, the group of M. Huber-Lang et al. combined a closed head injury, chest trauma, and lower leg fracture with soft tissue trauma. The trauma was characterized by systemic and BAL cytokine concentrations and histological analyses as well as changes of the complement system activity.

The current issue provides a broad overview regarding the posttraumatic immune response. Focusing not only on characterization of neutrophils and the impact of cytokines but also on less established parameters like matrixmetalloproteinases and proteasomes, the special issue presents new insights into immunological processes following trauma. The results may serve as an impulse for further research in this field. Molecular mechanisms of the posttraumatic immune response are essential for improvement of treatment strategies in patients suffering from major trauma.

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Clinical Study

Concentration Kinetics of Serum MMP-9 and TIMP-1 after Blunt Multiple Injuries in the Early Posttraumatic Period

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Metalloproteinases are secreted in response to a variety of inflammatory mediators and inhibited by tissue inhibitors of matrixmetalloproteinases (TIMPs). Two members of these families, MMP-9 and TIMP-1, were differentially expressed depending on clinical parameters in a previous genomewide mRNA analysis. The aim of this paper was now to evaluate the posttraumatic serum levels and the time course of both proteins depending on distinct clinical parameters. 60 multiple traumatized patients (ISS > 16) were included. Blood samples were drawn on admission and 6 h, 12 h, 24 h, 48 h, and 72 h after trauma. Serum levels were quantified by ELISA. MMP-9 levels significantly decreased in the early posttraumatic period ($P < 0.05$) whereas TIMP-1 levels significantly increased in all patients ($P < 0.05$). MMP-9 and TIMP-1 serum concentration kinetics became manifest in an inversely proportional balance. Furthermore, MMP-9 presented a stronger decrease in patients with severe trauma and nonsurvivors in contrast to minor traumatized patients (ISS ≤ 33) and survivors, initially after trauma.

1. Introduction

Posttraumatic immune activation and dysfunction in form of systemic inflammation response syndrome (SIRS), subsequent multiple organ dysfunction syndrome (MODS), and multiple-organ failure (MOF) still remains a leading cause of late posttraumatic morbidity and mortality [1]. MOF exerts a profound influence on patient outcome, as it occurs in one-fourth of all patients suffering from blunt multiple injuries and accounts for 27.5% of death among trauma patients [2].

Previous genomewide studies have linked specific mRNA expression patterns in monocytes with adverse outcome. Among these differentially expressed genes that were significantly connected to distinct clinical parameters like injury severity or outcome, matrix metalloproteinase-9 (MMP-9) and its specific tissue inhibitor-1 (TIMP-1) could be identified to play an important role in trauma patients in the early posttraumatic period [3].

MMP-9 is part of the matrix metalloproteinase (MMPs) family presenting a group of genetically distinct, but structurally related zinc-containing proteolytic enzymes. Collectively, MMPs play a central role in tissue remodeling of extracellular matrix (ECM) being capable of degrading all kinds of matrix components. Participating in ECM degradation they are involved in many biological processes such as embryogenesis, angiogenesis, and wound healing [4]. They are secreted as nonactive proenzymes in response to a variety of inflammatory mediators and they are inhibited in vivo by TIMPs [5]. Under physiological conditions MMP activities are precisely regulated at several levels like transcription or precursor zymogen activation and inhibition by their specific endogenous inhibitors.

MMP-9 (92 kDa gelatinase) is a type IV collagenase implicated in various aspects of inflammation including accumulation of inflammatory cells, healing of tissue injury, and remodeling processes. Specifically, it has been shown

to mediate vascular leakage and to initiate the migration of inflammatory cells inducing wound repair.

Furthermore, it is stored in the tertiary granules of polymorphonuclear leucocytes, which are key effectors in acute inflammatory diseases. Various cell lines such as keratinocytes, eosinophils, neutrophils, and macrophages can also express MMP-9 [6].

TIMP-1 works as a natural inhibitor of MMP-9 and is found in most tissues and body fluids. By inhibiting MMPs activities, TIMPs are involved in tissue remodeling and regulation of ECM metabolism. The TIMP family consists of four members sharing important structural features as well as the ability of MMP inhibition. Under normal physiological conditions, TIMPs bind MMPs in a 1 : 1 stoichiometry [4].

Consequently, a loss of activity control may result in a variety of diseases such as arthritis, cancer, arteriosclerosis, and fibrosis. Thus, the balance of MMP and TIMP activities plays the pivotal role in both physiological and pathological events [4].

Neither MMPs nor TIMPs have been described in patients suffering from multiple major trauma and subsequent posttraumatic immune system alterations.

In dependence on the persuasive results of a serial screening analysis of monocyte mRNA expression patterns after blunt multiple injuries , main intention of this investigation was the try to reproduce the MMP-9 and TIMP-1 transcriptional profiles of an immune cell fraction (monocytes) in serum samples of major trauma patients and their potential relationship to distinct clinical parameters.

2. Patients and Methods

In this study, performed at a Level I trauma center according to Good Clinical Practice (GCP), 60 adult patients (age > 18 years) presenting with multiple injuries and Injury Severity Score (ISS) of greater than 16 points were included. Patients were enrolled if the emergency department was reached within 90 minutes after the traumatic event.

Signed informed consent was obtained from each patient or their legal representatives over the course of time. Ethical Committee Permission was obtained from the Ludwig-Maximilians University, Munich, Germany (reference number: 012/00). Patients who did not survive the first 24 hours after trauma were excluded.

After initial resuscitation and/or primary surgical intervention according to standard of care, patients were admitted to intensive care unit.

Retrospectively, patients were distributed to different groups in regard to the two following clinical parameters: injury severity assessed by ISS according to AIS98 and outcome expressed by the survival of 90 days after trauma.

Serum samples were collected on admission (0 h), 6 h, 12 h, 24 h, 48 h, and 72 h after trauma. Afterwards they were stored at −80°C.

Protein concentrations were quantified by enzyme linked immunosorbent assay (ELISA; Human-MMP-9 and Human-TIMP-1 ELISA, Bender MedSystems GmbH).

For the analysis of MMP-9 and TIMP-1 protein levels in serum, sample dilutions of approximately 1 : 100 to 1 : 500 for MMP-9 and 1 : 1000 to 1 : 5000 for TIMP-1 were required to minimize the numbers of non evaluable results.

Resulting data was statistically analyzed using Mann-Whitney Rank Sum Test (SigmaStat/SigmaPlot). A P value of $P < 0.05$ was considered significant.

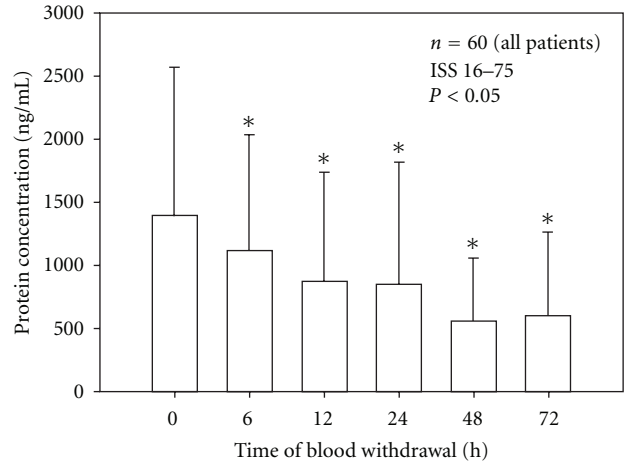

Figure 1: Significant decrease of mean MMP-9 serum levels in the early posttraumatic period in comparison to initial mean MMP-9 serum concentrations at 0 h after the traumatic event. Data were analyzed by Mann-Whitney Rank Sum test and presented by mean ± standard error of the mean (*$P < 0.05$).

3. Results

3.1. Patient Collective and Clinical Data. Altogether 60 patients fulfilled the entry criteria and were included into the study. Patients who did not reach the emergency department within 90 minutes after trauma, patients who were younger than 18 years, and patients who were pregnant or under guardianship were excluded.

There were 42 male and 18 female major trauma patients (mean age: 45 years; range: 18 to 93 years).

Patients were separated into different groups according to injury severity and outcome. Injury severity was coded according to ISS. We separated the patient collective into two groups regarding the severity of trauma; that is, we used a cut-off value of 33 points (group 1a: ISS ≤ 33, $n = 33$; group 1b: ISS > 33, $n = 27$). Median ISS in all patients was 33 (SD 11; range: 17 to 66). Median ISS of group 1a was 27 points (SD 5); median ISS of group 1b was 50 points (SD 22). Outcome was defined as a survival of 90 days after trauma. Six patients deceased during this time frame (group 2a: survivors, $n = 54$; group 2b: non-survivors, $n = 6$).

Six patients did not survive the traumatic event (mean age of non-survivors: 54 years; range: 37–93 years).

3.2. Serum Protein Concentrations

3.2.1. Time Course. Mean serum levels of MMP-9 and TIMP-1 were detected by immunosorbent assay in a multiple-trauma patient collective in the early posttraumatic period. Figure 1 shows MMP-9 concentration in ng/mL during

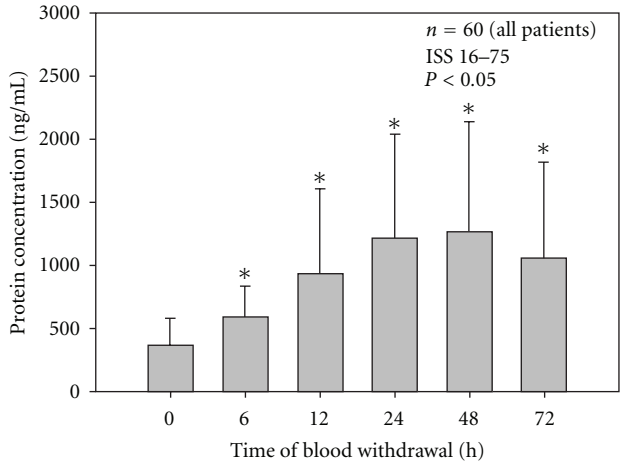

Figure 2: Significant increase of mean TIMP-1 serum concentration levels 6 h–72 h after the traumatic event compared to initial mean TIMP-1 serum level at 0 h after trauma. Data were analyzed by Mann-Whitney Rank Sum test and presented by mean ± standard error of the mean (*$P < 0.05$).

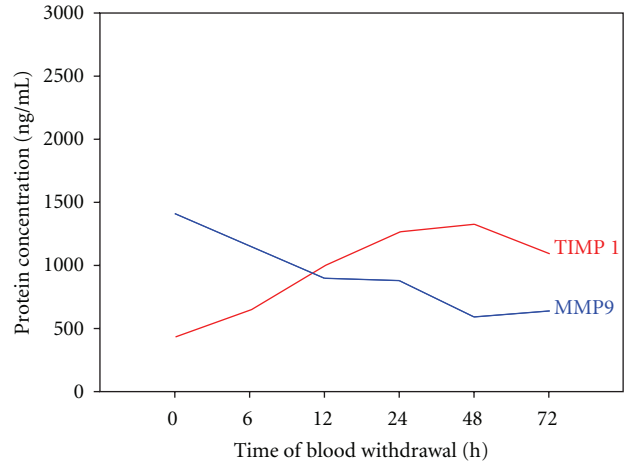

Figure 3: Significant inversely proportional concentration kinetics of MMP-9 and its specific inhibitor TIMP-1 during the observed posttraumatic time period of 72 h. The blue line indicates the connection of all measured time points concerning the significant decrease of MMP-9 serum concentration whereas the red one indicates the connection of all measured time points regarding the significant increase of TIMP-1 serum concentration.

the time after trauma; Figure 2 demonstrates TIMP-1 concentration in ng/mL over the time.

MMP-9 reached a high concentration level in all investigated patients initially after trauma at the time point of 0 h. Afterwards, mean serum levels of MMP-9 decreased significantly over the posttraumatic time period (6 h–72 h) compared to serum concentration detected directly after trauma (0 h).

In contrast to that, TIMP-1 started with low serum concentration, which was continuously increasing during this observation period (Figure 2). Mean serum levels of TIMP-1 significantly increased during the observed time period compared to the initial serum level (0 h).

Regarding the characteristics of MMP-9 and TIMP-1 serum levels, Figure 3 combined time course of MMP-9 and its specific inhibitor TIMP-1 during the observed time period of 72 h after trauma. Both proteins demonstrated a significant universal concentration pattern in all investigated patients after severe injury, as MMP-9 showed a significant downregulation whereas its physiological inhibitor TIMP-1 presented a significant upregulation in the posttraumatic period. This study's results show a specific posttraumatic serum concentration kinetics expressed by an inversely proportional balance of MMP-9 and TIMP-1 in the early time frame after multiple injuries.

3.2.2. ISS. Patients of group 1b presenting with an ISS greater than 33 points showed a remarkable lower mean MMP-9 serum concentration level initially (0 h) and in the following 12 h after major injury than patients of group 1a representing a collective of patients suffering from moderate injury with an ISS between 16 and 33 points (Figure 4). Concerning the following time points, mean MMP-9 serum concentrations of both groups decreased continuously without any remarkable significant difference.

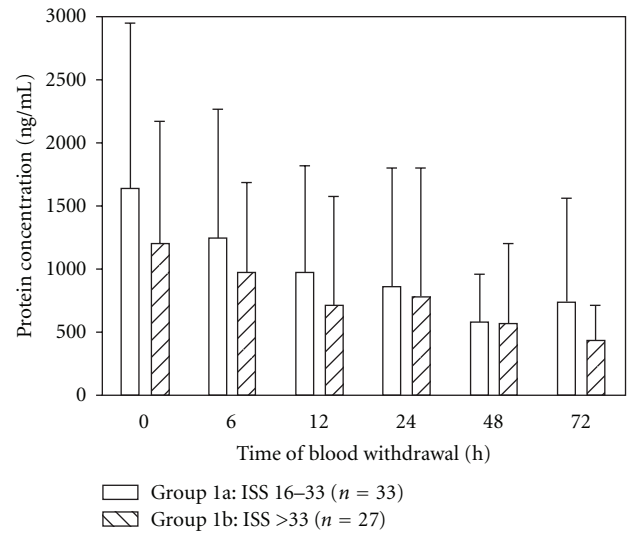

Figure 4: There is a lower serum MMP-9 activity in patients presenting with an ISS > 33 points in contrast to those with an ISS ≤ 33 regarding the first 12 h after trauma. Concerning the other points of time, there is no further remarkable significant difference between group 1a and 1b. Data were analyzed by Mann-Whitney Rank Sum test and presented by mean ± standard error of the mean.

Mean TIMP-1 serum concentration levels of both groups did not distinguish significantly (Figure 5). TIMP-1 concentration levels increased in the observed time period of 72 h after trauma in group 1a and 1b without notable difference.

3.2.3. Outcome. Mean MMP-9 serum levels of patients who did not survive the traumatic event (group 2b) during an observation period of 90 days were compared to those who survived (group 2a) in Figure 6. Remarkably,

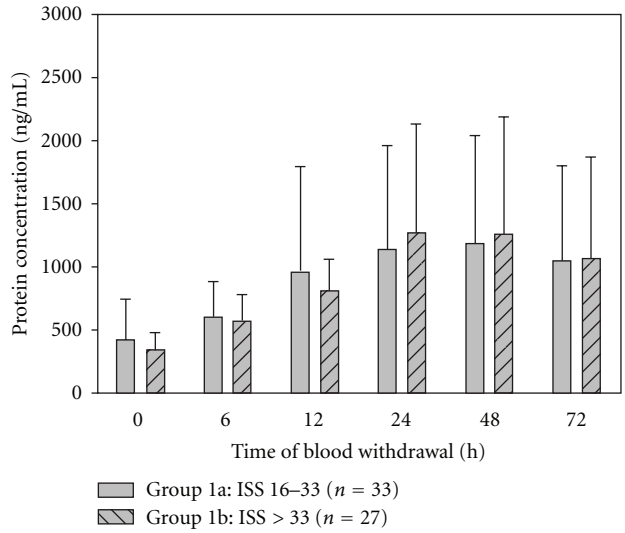

Figure 5: Patients after major trauma (ISS > 33) and patients after moderate injury (ISS ≤ 33) showed nearly equal mean serum levels of TIMP-1 in the early posttraumatic period over 72 hours. There were no significant differences found between these two groups. Data were analyzed by Mann-Whitney Rank Sum test and presented by mean ± standard error of the mean.

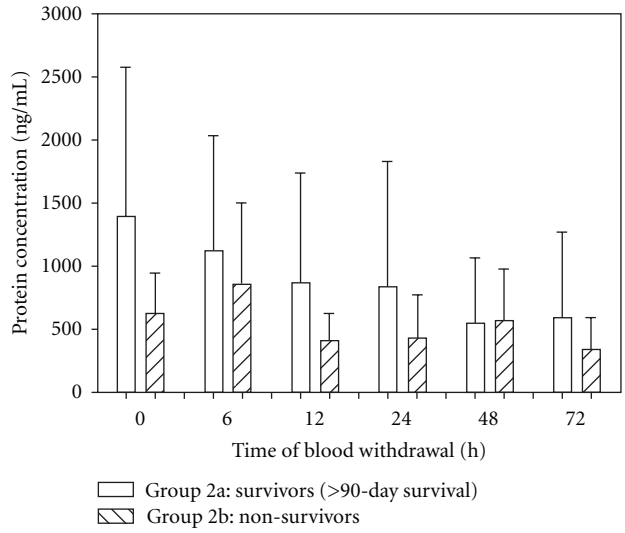

Figure 6: Mean MMP-9 serum levels were remarkably lower in non-survivors (group 2b) than those in survivors, especially within the 24 h after the traumatic event. Data were analyzed by Mann-Whitney Rank Sum test and presented by mean ± standard error of the mean.

MMP-9 revealed considerable lower mean serum levels in non-survivors (group 2b) than in survivors (group 2a) immediately after multiple major injuries within the first 24 h after the traumatic event (Figure 6). In the subsequent time interval, there were no significant differences between the two groups. All in all, mean MMP-9 serum levels of patients who did not survive the posttraumatic 90-day time frame revealed lower serum protein concentrations than those who survived the traumatic event.

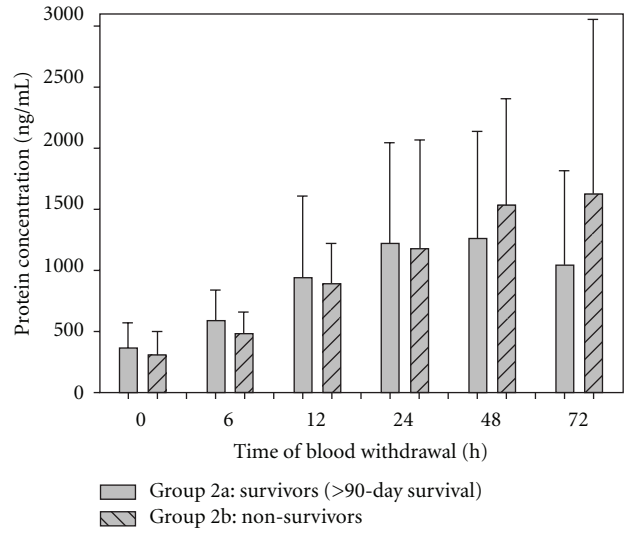

Figure 7: Mean TIMP-1 serum levels were lower in non-survivors (group 2b) than those in survivors (group 2a) within the first 24 h after multiple major injuries. 48 h and 72 h after trauma, mean TIMP-1 serum levels of non-survivors were higher than mean protein levels of patients who survived 90-days after the traumatic event. Data were analyzed by Mann-Whitney Rank Sum test and presented by mean ± standard error of the mean.

In Figure 7, mean TIMP-1 serum levels of survivors (group 2a) and non-survivors (group 2b) are illustrated. Observing the first 24 hours (0 h–24 h), mean TIMP-1 serum levels of non-survivors are lowered in comparison to mean serum TIMP-1 levels of survivors. Regarding TIMP-1 serum levels at the moment of 48 h and 72 h, patients who did not survive after trauma expressed definite higher mean TIMP-1 serum levels.

4. Discussion

A precedent serial, sequential oligonucleotide microarray analysis of initial posttraumatic monocyte messenger RNA could evaluate differential expression profiles in patients suffering from multiple injuries. Thereby, specific gene expression profiles have been identified to be significantly associated with important clinical parameters like injury severity and outcome [3].

Two of those identified eligible genes that were differentially expressed according to clinical parameters code for the two proteins were investigated in this study: MMP-9 and its specific endogenous inhibitor TIMP-1.

Furthermore, matrix metalloproteinases are regarded to be key role molecules in inflammation [7], as they are involved in pathophysiological remodeling of the vascular wall [8]. The aforementioned results of a previous genomewide mRNA analysis on the one hand and the gained prominence of MMPs as key role effectors in tissue turnover, inflammation, and several diseases on the other hand generated the intention to examine serum MMP-9 and TIMP-1 kinetics, to evaluate their balance and to identify

their outcome clarifying potential in patients after multiple major injuries in the early posttraumatic period.

Consequently, the primary aim of this study was to evaluate the balance of these proteins and to reproduce the outcome clarifying monocyte mRNA signature in serum samples of multiple injured patients.

In this context, serum level alterations of MMP-9 and TIMP-1 depending on clinical outcome parameters could have been the answer to early identify patients who are at risk to develop severe posttraumatic complications in response to their posttraumatic immune dysfunction leading to an unfavorable outcome.

Especially, since a genomewide mRNA analysis is particularly expensive, time-consuming, and restricted in terms of a widely spread availability, altered outcome predicting serum levels in multiple trauma patients could offer an instant and easily performable chance to receive an individual view on the immune status of each single patient and thereby evaluate the individual risk of posttraumatic morbidity and mortality.

4.1. Protein Serum Levels

4.1.1. Time Course and Balance. The main and most important result of this study is the identification of the convincing universal concentration pattern of MMP-9 and TIMP-1 in all investigated patients in the early posttraumatic period. For the first time, this study's results can state a significant decrease of mean MMP-9 serum levels in the first 72 h after trauma and simultaneously demonstrate a significant increase of its specific inhibitor TIMP-1 in serum of multiple traumatized patients immediately after the traumatic event. Both proteins state an inversely proportional balance after major injury, as it has not been evaluated in any other study so far.

Neither MMP-9 nor TIMP-1 levels have yet been examined in patients suffering from blunt multiple injuries. However, both proteins have built the basic of a multiplicity of studies but have never been described in context of multiple traumas. MMP-9 serum concentration levels have been described in the context of several diseases such as multiple sclerosis [9], coronary artery disease [10], multiple myeloma [11, 12], and chronic lymphocytic leukemia [13, 14]. TIMP-1 serum concentration levels have been examined in patients suffering from several types of cancer such as colorectal cancer [15] and lung cancer [16], and also in terms of acute disseminated encephalitis [17] or liver fibrosis [18].

Regarding the results of this current study, MMP-9 and TIMP-1 present a characteristic concentration pattern and contemporaneously a functional imbalance in patients after multiple injuries, since the concentration kinetics became manifest in an inversely proportional posttraumatic time course. In regard to ISS and MOF, our results demonstrate strong changes of MMP-9 concentration levels, especially in severely injured patients (ISS > 33) and non-survivors initially after trauma within the first 24 h after trauma. This initial downregulation of MMP-9 in these two groups may be understood as an answer to the severity of trauma, due to the fact that it implicates nearly a loss of MMP-9 serum concentration and in this context a loss of remodeling potential.

There might be different conceivable explanations for these phenomena. In healthy subjects, MMP- 9 is cosecreted with TIMP-1 in 1 : 1 stoichiometry. TIMPs are specific inhibitors of MMPs whereupon TIMPs 1–4 can inhibit all MMPs. TIMPs inhibit all MMPs tested so far, except that TIMP-1 fails to inhibit MT1-MMP [19].

Under pathological conditions, which are associated with an imbalance of MMP activities, changes of TIMP levels seem to play an important role as they directly influence the level of MMP activity [4].

Thus, in patients with multiple major injuries, there is a significant overproduction of TIMP-1 over MMP-9 as investigated in this study. The functional consequence of the TIMP-1/MMP-9 imbalance in multiple traumatized patients is impressive and may in this context be understood as a generalized downregulation of MMP-9 activity and consequently its remodeling potential induced by the overexpression of TIMP-1 in the very early posttraumatic period.

Remodeling is defined as to "model again or reconstruct". As already mentioned, the main role of MMPs remains ECM and basement membrane breakdown in the context of tissue remodeling and angiogenesis, whereas the key role of TIMPs is the maintenance of the essential balance between deposition and degradation of ECM by their function as endogenous inhibitors of MMP activities. The major intention of wound healing is the restoration of a functional connective tissue. Additionally, this regenerative process depends upon the accumulation and deposition of ECM molecules and besides the remodeling of ECM by MMPs [19].

In accordance with these physiological mechanisms, this study's result may be construed in the following way. The significant decrease of MMP-9 in the early posttraumatic period induced by a coincidental, significant overproduction of its physiological inhibitor in all patients after severe injury might reflect the organism's reaction on the distinct tissue injury and immune reaction caused by the traumatic event. MMP-9 activity—if correlated with the "remodeling potential"—has to be reduced significantly to cope with the posttraumatic damage. In consequence, the severely injured organism restricts the regenerative process depending upon MMP activity. This can be understood as a "rescue" response to the impact of trauma and the entailed overwhelming posttraumatic immune alteration.

Nevertheless, these protein serum results depending on injury severity and outcome could only demonstrate tendencies and could not reproduce the highly significant changes in monocyte mRNA expression patterns initially after trauma in a genomewide analysis so far.

There are several suggestions in the literature about monocytes playing a pivotal role in posttraumatic immune alterations, as they are an important part of innate immune system [20]. Hypothetically, the very early monocyte mRNA expression changes cannot be found in serum, as MMP-9 concentration changes once initiated in the intracellular space have not been secreted into serum so far.

On the other hand, monocytes only represent a small cell fraction of the cellular blood ingredients (2–8% of all

leucocytes), in comparison to the multiple ways of secretion, activation, and inhibition seen in patients' serum. This may explain why the distinct significance in the monocyte signature cannot be clearly reproduced in serum.

4.1.2. Blood Sampling. The determination of matrix metalloproteinases in peripheral blood as a noninvasive and easily performable possibility of diagnosis and monitoring of several diseases has already been recommended, since MMP activities on cellular level are reliably reflected in body fluids such as serum and plasma [21]. Peripheral blood contains several forms of MMPs. It can be found in soluble constitutive form in plasma and in form of intracellular zymogenes in platelets and leukocytes, which is followed by a release of proteinase into the circulation particularly in terms of a disease [22]. Thus, changes of MMP activity in serum measurements have been detected in several diseases and can therefore act as an indicator for pertinent intracellular protease activity [23].

However, discrepancies in MMP concentration levels depending on blood sampling procedure have been described in several studies [24]. Possible preanalytical errors of TIMP and MMP measurement in peripheral blood have been a matter of debate. In this context, elevated serum MMP levels have been described as serum levels of matrix metalloproteinases are likely influenced by MMP release following degranulation of leukocytes and platelets during the ex vivo blood clotting [25].

Nevertheless, most studies work with serum as medium to evaluate MMP concentration levels, because serum represents a likely affordable and reliable blood sampling procedure to easily investigate MMP and TIMP concentration levels in patients suffering from several diseases. Serum concentrations have been detected in terms of neurodegenerative diseases, cardiovascular diseases, or malignant proliferation, for example [23].

4.1.3. Patient Collective and Clinical Data. In reference to mean age (45 years) and distribution between the sexes of nearly 2 : 1 (men : women), this study's patient collective is well comparable to patient collectives described in assimilable European and American studies of multiple injured patients [26].

In accordance with the aforementioned entry criteria, only patients who reached the emergency department within 90 minutes after trauma were included in this study. In regard to other research groups' investigations on circulating neutrophils, the chosen blood-sampling time points in this study were set in the following way: on admission, which means within 90 minutes after trauma, and again 6 h, 12 h, 24, 48 h, and 72 h after the traumatic event [27].

It is well known that particularly the very early posttraumatic period sets the course for the appearance of a later multiple-organ failure [28]. Furthermore, trauma-induced alterations in the innate immune response lead to a subsequent immune cell-regulated defense reaction ending in hyperinflammation, immunoparalysis, or a combination of both, particularly in the early posttraumatic period [29]; therefore, a serial analysis of inflammation parameters including this vulnerable posttraumatic time frame after multiple major injuries is indispensable.

The definition of injury severity and the development of multiple-organ failure as well as setting the respective cut-off points in order to build dichotomous clinical groups requires the use of scoring systems as it was done in our study using ISS.

For this reason, serum samples were only collected if patients suffered from blunt multiple injuries with an injury severity greater than 16 points, as assessed by the ISS-Scoring System, the standard method of rating severity of injury [30]. In regard to injury severity, a cut-off level had to be defined to differentiate between minor and major traumatized patients. That is why we depended on building two opposite groups for our analysis, as the number of our investigated patient collective was too few to calculate a reliable multiple regressive analyses respecting the particular ISS count in every single patient. For these biostatistical reasons, we switched to the analysis of two dichotomous groups. Concerning injury severity, a cut-off of 33 points was appointed in this study to distinguish between major and minor traumatized patients, for an ISS of 33 points was the median ISS in our patient collective. This might be a limiting factor of this study. However, there is no definitive ISS cut-off level differentiating between minor and major traumatized patients published in literature so far.

In contrast to ISS mentioned previously, outcome is a simple and straightforward clinical criterion as it is just defined as a survival of more than 90 days after trauma. The evident advantage of this clinical criterion is the clear definition whereas its main limitation might be the inhomogeneity exhibited in both groups (survivors, non-survivors) as neither the cause of death nor the variety of posttraumatic individual complications after surviving the initial trauma is attended.

5. Conclusions

Regarding the time course of significantly decreasing MMP-9 and significantly increasing TIMP-1 serum levels, we can state a universal concentration pattern after blunt multiple injuries in all investigated patients. Both proteins show a typical inversely proportional serum concentration kinetics in the first 72 h after trauma.

Furthermore, the very early posttraumatic period is characterized by a remarkable decrease of MMP-9 serum concentration initially after trauma in patients with a major injury (ISS > 33) and those who did not survive the traumatic event in contrast to patients with moderate injury and those who survived. This might be due to the fact that the remodeling potential of MMP-9 is strongly downregulated in patients suffering from severe traumatic injury as well as in non-survivors.

The inversely proportional balance between MMP-9 and its endogenous inhibitor TIMP-1 and the decrease of MMP activity in severely injured (ISS > 33) and non-survivors suggest a potentially new mechanism of posttraumatic

immune system dysbalance and SIRS/MOF precondition following multiple injuries.

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Review Article

Facts and Fiction: The Impact of Hypothermia on Molecular Mechanisms following Major Challenge

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Numerous multiple trauma and surgical patients suffer from accidental hypothermia. While induced hypothermia is commonly used in elective cardiac surgery due to its protective effects, accidental hypothermia is associated with increased posttraumatic complications and even mortality in severely injured patients. This paper focuses on protective molecular mechanisms of hypothermia on apoptosis and the posttraumatic immune response. Although information regarding severe trauma is limited, there is evidence that induced hypothermia may have beneficial effects on the posttraumatic immune response as well as apoptosis in animal studies and certain clinical situations. However, more profound knowledge of mechanisms is necessary before randomized clinical trials in trauma patients can be initiated.

1. Introduction

A great number of patients with major injuries [1–3] suffer from accidental hypothermia ranging from 12 to 66% [4, 5]. In the current literature, multiple classifications using different definitions for hypothermia are described. In most classifications, hypothermia is defined as a core temperature below 35°C. Since an increase of mortality has been demonstrated below a core temperature of 34°C in patients with multiple injuries, this temperature threshold seems to be critical in trauma patients and therefore a modified definition is reasonable [6]. Thereby, the extent of hypothermia correlates with the overall injury severity and is increased by pelvic or abdominal surgery [7]; furthermore, hypothermic polytrauma patients suffer from an increased incidence of posttraumatic complications [2, 4, 8–10] as well as a an increased mortality [2, 8, 11].

Depending on its origin, three entities of hypothermia are known: endogenous, induced, and accidental hypothermia.

Endogenous hypothermia results from a metabolic dysfunction with a decreased heat production (e.g., hypothyreodism, hypoglycaemia, hypoadrenalism) or a disturbed thermoregulation (e.g., intracranial tumor, degenerative neurologic disorders).

Accidental hypothermia is characterized by an unintentional decrease of the core temperature due to exposure to a cold environment without a thermoregulative dysfunction [12]. This can be aggravated by therapeutic interventions. The infusion of cold fluids as, for example, 2 l of crystalloids (18°) decreases core temperatures about 0.6°C [13]. In addition, a reduced oxygen supply with an anaerobic state decreases the amount of adenosine trisphosphate (ATP) and subsequent heat production. Furthermore, the application of anaesthetics and skeletal muscle relaxants prevents shivering and vasoconstriction and therefore advances heat loss.

While the first two entities do not play a major role after trauma, accidental hypothermia is common in trauma patients as well as patients undergoing major surgery [4, 5]. In contrast to accidental hypothermia that needs to be addressed in the treatment of severely injured patients due to its detrimental effects, induced hypothermia is commonly used, that is, in elective cardiac surgery [14]. In addition there exists a strong recommendation for the induction of hypothermia after cardiopulmonary reanimation, and

there exists a growing body of evidence that suggests the application of hypothermia after blunt brain injury. Some articles were published regarding the influence of hypothermia in context of elective surgery but also on the posttraumatic immune response in animal models. However, an overview about the influence of hypothermia on the humeral and cellular immune response with special focus on apoptosis is missing. This paper outlines the molecular mechanisms by which hypothermia influences apoptosis as well as the immune response following severe trauma and major surgery.

2. Apoptosis

In general, cell death following hemorrhage and ischemia occurs either as necrosis of affected cells or as a complex process of programmed cell death called apoptosis. In contrast, necrosis represents the premature death of cells caused by external factors, that is, trauma, infections, or exposure to toxins.

Apoptosis is associated with a cascade of enzymatic reactions in which proteolytic caspase enzymes play a major role. Apoptosis could be initiated either in an extrinsic or intrinsic way [60]. The extrinsic way is characterized by the interaction of ligands (e.g., TNF-α) with "death receptors" on the cell surface (e.g., CD95) activating the enzymatic cascade by caspase-8 [61]. The intrinsic activation of apoptosis is triggered by tumor suppressing factors (e.g., p53) resulting in an increased expression of proapoptotic factors of the bcl-2 family (e.g., bax, bad) and an increased mitochondrial release of cytochrome-c [62]. Binding Apaf-1 (apoptotic protease activating factor) cytochrome-c activates the apoptotic cascade via caspase-9 [63, 64]. Finally, programmed cell death is mediated by "effector caspases," especially caspase-3, in both ways of apoptotic activation. Besides the proteins of the bcl-2 family (antiapoptotic bcl-2; proapoptotic bax and bad), the apoptotic process is regulated by Mitogen-activated protein kinases (MAP-kinases) like extracellular-signal-regulated kinase 1/2 (ERK 1/2), cJun N-terminal protein kinase 1/2 (JNK 1/2), and p38 MAP-kinase. Additionally, other pathways involving Phosphatidylinositol-3 kinases (PI3K) generating phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P(3)) and other phospholipids interacting with Akt are known to play an important role in regulating cell survival [65]. Furthermore, besides kinases the transcription factor NF-kappaB regulates cellular apoptotic programs [66].

In various studies, hypothermia was shown to prevent additional tissue injury by interrupting both, the intrinsic, and extrinsic apoptotic pathway [15, 67–70]. Interestingly, hypothermia seems to affect the very early steps in the apoptotic process including inhibition of activation of caspase enzymes, preserving mitochondrial function and decreased overload of excitatory transmitters. In contrast, apoptosis occurs relatively late following tissue challenge but it was shown that the process continues for up to 3 days [70–72]. Due to the delay of the apoptotic process, modulation of the apoptotic cascade could serve as a therapeutic target in early stages of polytrauma management after the initial resuscitation process in which the patient is stabilized with the aim to prevent additional damage. In this context, it is of special importance that the rate of apoptosis in neutrophils is dramatically decreased in multiple trauma patients [73].

2.1. In Vitro Studies. Cultured hepatocytes showed suppressed FAS-mediated apoptosis detected by a decreased mitochondrial damage following moderate hypothermia. Besides an attenuated cytochrome-c release, hypothermia suppressed the activation of caspase-7 and -9 [74]. This data suggests potential organ protective effects of hypothermia regarding apoptosis, which were confirmed in various animal models. On the other hand, murine neutrophils revealed a reduced spontaneous and TNFα-induced apoptosis under mild hypothermia of 35°C [75]. This fact could result in a prolonged exposure to activated neutrophils after trauma resulting in secondary organ damage.

2.2. Experimental Animal and Clinical Studies. Profound hypothermia was not only shown to preserve Akt in cardiomyocytes and inhibit caspase-3 activation but also activate antiapoptotic proteins such as bcl-2 in an experimental model of hemorrhagic shock [19] and ischemic insult [20]. In ischemia/reperfusion injury, hypothermia reversed activation of apoptosis stimulating fragment (FAS)/caspase-8, the increase of bax (an proapoptotic protein), and decrease of bcl-2 in endothelial cells [16]. This was accompanied by an inhibition of JNK 1/2 activation via MKP-1 induction [16]. Following ischemia and reperfusion, isolated cardiomyocytes showed increased Phospho-Akt levels associated with attenuation of reactive oxygen species production, which was blocked by Akt but not cGMP inhibition [76]. Additionally, hypothermia was associated with downregulation of the TNF receptor (TNFR)1 and its proapoptotic ligand FAS in rat cerebral cortices following a moderate fluid percussion model of traumatic brain injury [77].

Most of the clinical studies regarding the influence of hypothermia on apoptosis are limited to ischemic injuries following cardiac arrest or brain ischemia [78, 79].

In summary, there is clear evidence that hypothermia reduces ischemic neuronal apoptosis in global cerebral ischemia as a result of attenuated p53 expression and increased bcl-2 release [78]. Information regarding multiple injuries is not available to date. The effects of hypothermia on apoptosis are summarized in Table 1.

3. Immune System

3.1. Humoral Inflammatory Response. Immune response following major surgery or trauma consists of a complex set of pro- and anti-inflammatory reactions in order to restore homeostasis. The balance or imbalance of the pro- and anti-inflammatory immune response in part influences the clinical course. Whereas predominance of the pro-inflammatory response leads to the Systemic Inflammatory Response Syndrome (SIRS), the anti-inflammatory reaction also named as compensatory anti-inflammatory response syndrome (CARS) might result in immune suppression with an enhanced risk of infectious complications. SIRS as well as

Table 1: Effects of hypothermia on apoptosis.

Authors	Study design	Insult	Degree of hypothermia	Effects
Xu et al. [15]	In vitro study (mouse neurons)	Apoptosis using serum deprivation	33°C	caspase-3/8/9 ↓, cytochrome-c ↓, JNK ↓ (no effect on bcl-2, bax)
Yang et al. [16]	In vitro study (HUVEC*)	Ischemia using oxygen-glucose deprivation	33°C	caspase-3/8 ↓, bcl-2 ↑, bax ↓, JNK ↓
Pastuszko et al. [17]	Experimental study (piglet neurons)	Ischemia	33°C	caspase-3 ↓, bax ↓ (no effect on bcl-2)
Sahin et al. [18]	Experimental study (rat neurons)	Ischemia	34°C	caspase-3/9 ↓, bcl-2 ↑, bax ↓
Shuja et al. [19]	Experimental study (rat cardiac tissue)	Hemorrhage	15°C	bcl-2 ↑
Xiong et al. [20]	Experimental study (rat neurons)	Ischemia	32-33°C	caspase-3 ↓, bcl-2 ↑

* Human umbilical vein endothelial cells.

Table 2: Inflammatory cytokines.

	Induction	Synthesis	Effects
TNF-α	hemorrhage, hypoxia, ischemia, endotoxine	macrophages, monocytes, T-lymphocytes	primary, proinflammatory cytokine IL-1β and IL-6 expression activation of coagulation prostaglandine E2, steroid release
IL-1β	hemorrhage, hypoxia, ischemia, endotoxine C5a, TNF-α	macrophages, monocytes, endothelial cells	primary, pro-inflammatory cytokine Synergistic effects with TNF-α
IL-6	LPS, IL-1β, TNF-α	T-/B-lymphocytes, monocytes, endothelial cells	Secondary, pro-inflammatory cytokine induction of acute phase proteins (e.g., CRP, PCT) differentiation of lymphocytes activation of von NK-cells and neutrophils anti-inflammatory effect (IL-1β ↓, TNF-α ↓)
IL-8	IL-1β, TNF-α, bacteria, LPS, hypoxia	T-lymphocytes, monocytes, neutrophils, endothelial cells	Secondary, pro-inflammatory cytokine chemotactic effect on leukocytes
IL-10	TNF-α, IL-1β, endotoxine, LPS, prostaglandine E2	T-/B-lymphocytes, monocytes, macrophages	anti-inflammatory cytokine

immune suppression plays a decisive role in the development of sepsis and the Multiple Organ Dysfunction Syndrome (MODS) after trauma. Cytokines, released from various cell types including immunocompetent and intrinsic cells, regulate the specific and unspecific immune response. These mediators are detectable in the peripheral blood and several compartments like the lung and the liver. They serve not only as a marker of injury severity or outcome predictors but also as a tool for decision-making regarding timing of elective surgery during the clinical course [80, 81]. The most important cytokines in this regard include TNF-α, IL-1β, IL-6, IL-8, and IL-10 (Table 2). As another essential step of the systemic immune response, chemotactic cytokines, so-called chemokines (IL-8, MCP-1, MIP-1α, or MIP-1β) mediate neutrophil infiltration into the affected tissue [82]. Thereby, extravasation of neutrophils is mediated by different adhesion molecules [83]. The initial neutrophil-endothelial interaction, so-called rolling, is mediated by members of the selectin family of adhesion molecules. Integrins (CD11/CD18) and immunoglobulin superfamily receptors (ICAM-1, VCAM-1, ELAM-1) are important for the following firm adhesion and cell diapedesis [84, 85]. In various experimental as well as clinical studies, an effect of hypothermia on the inflammatory response by altering the expression of pro- and anti-inflammatory cytokines, chemokines and adhesion molecules has been shown [38, 86–88].

In brain injury, cytokines can have neuroprotective as well as neurotoxic properties. There is profound evidence that an inadequate or disproportionate posttraumatic immune response not only increases the risk for brain cell injury but also the extent of damage [89–95]. In this process, the IL-1 family plays a pivotal role. Elevated levels of IL-1 as well as an increased expression of IL-1 mRNA were detected following experimental brain injury in rodents, respectively [96–100]. While IL-1 does not cause brain damage itself, injection of IL-1 increased cell death following various brain

Table 3: Effects of hypothermia on inflammatory cytokines.

Authors	Study design	Insult	Degree of hypothermia	Effects
Zheng et al. [21]	Experimental study (piglets)	Ischemia	18°C	TNF-α ↓, IL-6 ↓
Sipos et al. [22]	Experimental study (pigs)	Ischemia	19/24/30°C	No effect TNF-α, IL-6, IL-10
Qing et al. [23]	Experimental study (pigs)	Ischemia	20/28°C	TNF-α ↓, IL-10 ↑
Meybohm et al. [24]	Experimental study (pigs)	Ischemia	33°C	TNF-α ↓, IL-1β ↓, IL-6 ↓, IL-10 ↓
Su and Li [25]	Experimental study (pigs)	Ischemia	Not defined	TNF-α ↓, IL-6 ↓
Lim et al. [26]	Experimental study (rats)	Inflammation	27°C	IL-1 β ↓, IL-10 ↑
Fujimoto et al. [27]	Experimental study (rats)	Inflammation	32°C	IL-6 ↓, IL-10 ↑
Stewart et al. [28]	Experimental study (mice)	Inflammation	32°C	IL-6 ↑, IL-10 ↑; no effect on TNF-α, IL-1β
Kim et al. [29]	Experimental study (rats)	Hemorrhage	27–30°C	IL-6 ↓, IL-10 ↑
Gundersen et al. [30]	Experimental study (rats)	Hemorrhage	32.5–33°C	IL-6 ↓, no effect on TNF-α, IL-10
Beiser et al. [31]	Experimental study (mice)	Hemorrhage	33°C	IL-6 ↓
Wagner et al. [32]	Experimental study (mice)	Septic shock	27°C	IL-6 ↓
Vitarbo et al. [33]	Experimental study (rats)	Trauma (TBI)	33°C	TNF-α ↓
Morita et al. [34]	Experimental study (rats)	Trauma (Lung)	34°C	TNF-α ↓, IL-6 ↓, IL-10 ↑
Lee et al. [35]	Experimental study (rats)	Hypothermia (isolated)	30°C	IL-2 ↓, IL-10 ↑
Qayumi et al. [36]	Experimental study (pigs)	Lung transplantation	ex vivo preservation at 4°C	No effect on TNF-α, IL-2, IL-4, IL-10, Thromboxan
Shiozaki et al. [37]	Clinical study	Trauma (TBI)	34°C	No effect onTNF-α, IL-6, IL-10
Aibiki et al. [38]	Clinical study	Trauma (TBI)	32-33°C	IL-6 ↓

damage models [101–103]. The hypothesis that IL-1 increases brain damage is supported by experiments in which an IL-1 antagonist prevented cell death in experimental brain injury [102–105]. Similar results were observed following treatment with an IL-1 converting enzyme (ICE) inhibitor in cerebral ischemia [106]. Thus, modulation of cytokine release by hypothermia may serve as a therapeutic approach following major injury.

3.1.1. In Vitro Studies. Peripheral blood mononuclear cells stimulated with lipopolysaccharide (LPS) from healthy volunteers showed decreased TNF-α release, while release of IL-1 and IL-6 was delayed when incubated at 33°C as compared to incubation at 37°C [86]. In a similar study with human macrophages, early secretion of TNF-α and IL-6 was blunted and in human monocytes early IL-6 and IL-1β secretion was decreased [107]. Additionally, a shift towards anti-inflammatory cytokines was detected in microglia cells following LPS treatment [108]. In contrast, hypothermia of 33°C raised the levels of IL-1β, IL-6, and TNF-α produced by monocytes from healthy volunteers stimulated with LPS [109]. This controversial findings suggest diverse effects of hypothermia on different cell types.

3.1.2. Experimental Animal Models. In a rat model of acute hemorrhage, Gundersen et al. evaluated the effect of hypothermia on immune response and corresponding organ damage. Moderate hypothermia had an organ protective effect in liver and kidney, which was associated with a decreased release of IL-6 as well as a reduction of reactive oxygen species [30]. In contrast, mild hypothermia did not affect systemic levels of IL-1, IL-6, and IL-10, while serum TNF-α levels were even increased following hemorrhagic shock suggesting different responses of cytokines or their respective sources [110].

In a study using a swine model of uncontrolled lethal hemorrhage, the authors were able to detect a decreased proinflammatory (IL-6) and an increased anti-inflammatory (IL-10) immune response following profound hypothermia. Furthermore, the potentially protective chaperone heat shock protein-70 (HSP 70) was preserved. The authors, therefore, concluded a beneficial modulation of the immune system due to hypothermia in this hemorrhage model [111].

Only a few publications investigated the anti-inflammatory effects of hypothermia in a combined trauma-hemorrhage setting. In a two-hit model consisting of a femoral fracture and hemorrhage, systemic IL-10 levels were elevated following mild hypothermia [44] confirming results from other experimental studies [35, 112, 113]. The increased anti-inflammatory response induced by hypothermia was also associated with a conversion from Th-1 to Th-2 cytokine pattern [35].

In a nonbacterial-driven sepsis model using intraperitoneal lipopolysaccharide injection, hypothermia also induced elevated plasma IL-10 levels [114].

The effects of hypothermia on the release of inflammatory cytokines are presented in Table 3.

3.1.3. Clinical Studies. Experimental studies of traumatic brain injury, in which hypothermia decreased systemic

Table 4: Effects of hypothermia on chemokines.

Authors	Study design	Insult	Degree of hypothermia	Effects
Dalen et al. [39]	In vitro study (human neurons)	Ischemia using oxygen-glucose deprivation	33°C	No effect on IL-8, MCP-1
Diestel et al. [40]	In vitro study (HUVEC*)	Inflammation	17/32°C	IL-8 ↓, MCP-1 ↓
Sutcliffe et al. [41]	In vitro study (HCEC**)	Inflammation	32°C	IL-8 ↓
Zheng et al. [21]	Experimental study (piglets)	Ischemia	18°C	IL-8 ↓
Li et al. [42]	Experimental study (rats)	Ischemia	32-33°C	MCP-1 ↓
Callaway et al. [43]	Experimental study (rats)	Ischemia	33°C	No effect on MCP-1 and MIP-1α
Hildebrand et al. [44]	Experimental study (mice)	Hemorrhage	27–30/30–33/33–35°C	MCP-1 ↓
Eggum et al. [45]	Clinical study	Surgery	25/32°C	RANTES and MCP-1↓
Menasche et al. [46]	Clinical study	Surgery	~26°C	No effect on IL-8

*human umbilical vein endothelial cells.
**human cerebral endothelial cells.

Table 5: Effects of hypothermia on adhesion molecules.

Authors	Study design	Insult	Degree of hypothermia	Effects
Haddix et al. [47]	In vitro study (HUVEC*)	Inflammation	25°C	E-Selectin ↓
Sutcliffe et al. [41]	In vitro study (HCEC**)	Inflammation	32°C	No effect on ICAM-1, CD 18 (Integrin)
Frohlich et al. [48]	In vitro study (human leukocytes)	Inflammation	33/35°C	CD 11b (Mac-1) ↑, no effect on CD62L (L-Selectin)
Johnson et al. [49]	In vitro study (HUVEC*)	Hypothermia (isolated)	25°C	E-Selectin ↓
Meybohm et al. [24]	Experimental study (pigs)	Ischemia	33°C	ICAM-1 ↓
Callaway et al. [43]	Experimental study (rats)	Ischemia	33°C	No effect on ICAM-1
Wang et al. [50]	Experimental (rats)	Ischemia	33°C	ICAM-1 ↓
Deng et al. [51]	Experimental study (rats)	Ischemia/Inflammation	33°C	ICAM-1 ↓
Hanusch et al. [52]	Experimental study (rats)	Hypothermia (isolated)	Ex vivo storage after flushing lungs with cold fluid (4°C)	ICAM-1 ↓, VCAM-1 ↓, ELAM-1 ↓
Choi et al. [53]	Experimental study (rats)	Ischemia	33°C	ICAM-1 ↓
Menasche et al. [46]	Clinical study	Surgery	~26°C	No effect on P- und E-Selectin
Boldt et al. [54]	Clinical study	Surgery	27-28°C	No effect on ICAM-1, VCAM-1, ELAM-1

*human umbilical vein endothelial cells.
**human cerebral endothelial cells.

cytokine levels, were confirmed by a clinical study showing a suppression in cytokine release associated with an improved outcome [38, 88]. In cerebral trauma patients, hypothermia of 32-33°C suppressed systemic IL-6 levels, which was associated with an increased Glasgow outcome scale 6 months postinjury as compared to patients treated under normothermic conditions [38].

3.2. Adhesion Molecules and Chemokines. The effects of hypothermia on chemokine levels and the expression of adhesion molecules were investigated in experimental as well as clinical studies (Tables 4 and 5).

3.2.1. In Vitro Studies. In vitro studies of human umbilical vein endothelial cells showed decreased MCP-1 as well as IL-8 levels under hypothermic conditions [40]. In contrast, no effect of hypothermia on ICAM-1 expression in human cerebral endothelial cells was shown under stress conditions [41]. In cultured human umbilical vein cells, hypothermia inhibited the transcription and expression but not the induction of E-selectin [49].

3.2.2. Experimental Animal Models and Clinical Studies. In a model of cerebral ischemia and reperfusion, mild hypothermia reduced local expression of MCP-1 [42]. The same finding could be confirmed in a murine multiple trauma model [44]. In another cerebral ischemia model, intraischemic as well as delayed hypothermia decreased ICAM-1 expression as well as intracerebral neutrophil infiltration [51].

In pediatric cardiopulmonary bypass (CPB) surgery, hypothermia reduced systemic levels of the chemokine regulated on activation normal T cell expressed and secreted (RANTES) and MCP-1 [45]. In contrast, no effect of hypothermia on MCP-1 levels was detected in a model of cardiac arrest in rats [43].

In transient focal cerebral ischemia, mild hypothermia reduced ICAM-1 expression, which was associated with reduced neutrophil and monocyte infiltration [50].

In contrast to experimental studies, circulating adhesion molecules were not altered by hypothermia in aortocoronary artery bypass grafting [54]. The effects of hypothermia on adhesion molecules are presented in Table 5.

In summary, there is evidence that induced hypothermia decreases pro-inflammatory cytokines as well as chemokines and adhesion molecules. Besides this, an increased anti-inflammatory cytokine response is observed in various trauma models. However, information of human studies is spare.

3.3. Cellular Immune Response

3.3.1. Experimental Studies. Hypothermia influences the cellular immune response, which was especially studied following brain injury. In various animal models, neutrophil and macrophage function was attenuated leading to a decreased extent of secondary brain injury and infarction size [115]. Furthermore, posttraumatic hypothermia decreased early and prolonged accumulation of neutrophils and myeloperoxidase activity suggesting hypothermia as a potential mechanism to modulate outcome [116]. These findings confirmed an earlier study of Toyoda et al. showing a decreased neutrophil infiltration following intraischemic hypothermia in a focal ischemia reperfusion injury [55], which was also shown after delayed hypothermia in another cerebral ischemia model [51].

Additionally, posttraumatic hypothermia reduced neutrophil accumulation on the injury site at 24 h in a model of spinal cord injury [57].

Although most studies were conducted in traumatic brain injury, similar findings were shown in other organs. Already in the 1980s, it was shown that hypothermia reduced local neutrophil infiltration in an experimental pleuritis model, while the number of circulating neutrophils was not affected [117]. Other studies suggest a hypothermia-induced decrease of circulating neutrophils after soft-tissue injury in piglets [118], supporting an older study with prolonged hypothermia of 29°C in pigs [119]. The reduced infiltration can be explained by a decrease of adhesion molecules due to hypothermia. However, phagocytosis of opsonised bacteria is even increased at a lower temperature suggesting a temperature-dependent activation of neutrophils [48].

Following major injuries, infiltrated neutrophils release proteolytic enzymes as well as free radicals causing tissue damage which may subsequently lead to organ dysfunction and failure. Hypothermia was able to reduce the amount of free radicals in ischemic brain injury. In contrast, hypothermia did not affect the formation of free radicals in a rat model of hemorrhagic shock [120–122]. The reduced number of free radicals is of great benefit since the capacity of antioxidative mechanisms is limited. Interestingly, the prevention of free radical production is linearly linked to the temperature [123]. Most of free radicals following brain injury are synthesized by nitric oxide synthase and by deregulated mitochondrial electron transporters [124–127]. Thus, it was speculated that the prevention of release or synthesis of free radicals may be induced by preserved mitochondrial function [128]. Interestingly, mitochondrial function plays also a pivotal role in the development of apoptosis through inhibition of the caspase cascade activation. The essential role of the nitric oxide synthase was supported by another experiment, in which attenuation of acute lung injury by induced hypothermia following hemorrhagic shock was associated with less myeloperoxidase activity and decreased gene expression of iNOS. Furthermore, gene expression of heat shock protein (HSP-72), a molecular chaperone known to exert protective effects in ischemia-reperfusion injury, was detected in hypothermic but not in normothermic rats [29].

In contrast to these results, another study using a model of pressure-controlled hemorrhagic shock revealed no differences in serum-free 8-isoprostane (a marker of lipid peroxidation by free radicals) between the two groups at either baseline or resuscitation time 1 hr [120]. In a forebrain ischemia and recirculation model, hypothermia prevented production of hydroxyl radicals following hyperthermia [129]. In another study, postischemic leukotriene production a well as edema development was reduced 2 h but not 10 min following reperfusion [130]. The effects of hypothermia on the respiratory burst are summarized in Table 6.

3.3.2. Clinical Studies. Controlled mild hypothermia had no effect on the number of circulating T lymphocytes in patients with severe brain injury [131]. A clinical study including infants and children with severe traumatic brain injury showed a preserved antioxidant reserve in cerebrospinal fluid, suggesting an attenuation of oxidative stress following hypothermia in severe brain injury [128].

4. Functional Parameters

4.1. Experimental Studies. Less neuron damage was detected at a temperature of 34°C following brain ischemia [132] confirming another study of thoracic aortic ischemia-reperfusion injury in which hypothermia prevented and delayed paralysis by preserving cells of the central nervous system [133]. In another study, a beneficial long-term effect of mild (35°C) and moderate (32°C) hypothermia was detected after spinal cord ischemia and reperfusion until 28 days following the injury [134].

In a rat model of ischemia/reperfusion of the lower extremity, local hypothermia protected skeletal muscle from capillary leakage, which was prevented after treatment with heme oxygenase and nitric oxide synthase inhibitors [135].

Hypothermia not only prevents damage on site of injury but also distant organ damage. In mesenteric ischemia-reperfusion injury, remote lung injury was prevented as measured by leukocyte trafficking, alveolar hemorrhage, and

Table 6: Effects of hypothermia on proteins/molecules associated with the production of reactive oxygen species.

Authors	Study design	Insult	Degree of hypothermia	Effects
Toyoda et al. [55]	Experimental study (rats)	Ischemia	30°C	MPO ↓
Lim et al. [26]	Experimental study (rats)	Inflammation	27°C	MPO ↓
Kim et al. [29]	Experimental study (rats)	Hemorrhage	27–30°C	Malondialdehyde (TBARS)↓, MPO ↓, iNOS ↓, NO ↓
Duz et al. [56]	Experimental study (rats)	Trauma (spinal cord)	27–29°C	Malondialdehyde (TBARS) ↓
Chatzipanteli et al. [57]	Experimental study (rats)	Trauma (spinal cord)	32°C	MPO ↓
Grezzana Filho et al. [58]	Experimental study (rats)	Ischemia	26°C	Malondialdehyde (TBARS)↓,
Qayumi et al. [36]	Experimental study (pigs)	Lung transplantation	ex vivo preservation at 4°C hypothermia)	No effect on Superoxide-Dismutase, GR*, GP**
Hsu et al. [59]	Experimental study (rats)	Heatstroke	Perfusion with cold fluid (4°C)	Malondialdehyde (TBARS) ↓ Glutathione ↑, GP** ↑, GR* ↑, Superoxid-Dismutase ↑
Eggum et al. [45]	Clinical study	Surgery	25/32°C	No effect on MPO

*GR (Glutathione-Reduktase).
**GP (Glutathione-Peroxidase).
***GST (Glutathione-S-Transferase).

increased BAL protein and wet/dry ratios [136]. These results confirmed another study using a hepatic model of ischemia/reperfusion injury, in which hypothermia reduced the associated lung injury [137].

In a rat hemorrhagic shock model, mild hypothermia (34°C) improved survival associated with modulation of the immune response [110].

4.2. Clinical Studies. Clinical studies are mainly focused on patients with traumatic brain injury. In general, there is a gap between experimental studies and clinical experience. In a multicenter study of 392 patients with severe head injuries, hypothermia of 33°C did not influence outcome. In 5 meta-analyses, conflicting results regarding outcome were shown [138–142]. Only 2 of them [141, 142] described a marginal benefit regarding mortality and neurological outcome. Patients with elevated intracranial pressure seem to be the cohort with the most benefit of hypothermia [141]. Regarding these controversial results, in current guidelines hypothermia is recommended as a level III treatment option [143].

5. Cooling Rewarming Procedure

5.1. Experimental Studies. Only few experimental studies investigated the effect of different cooling and rewarming strategies.

Hypothermia at time of occlusion decreased infarct size in a myocardial ischemia-reperfusion injury supporting the results showing an increased cellular tolerance to ischemia under hypothermic conditions [144]. These results suggest starting hypothermia as soon as the hemodynamic and hemostaseological parameters were stabilized. In contrast, there is consistent evidence that rapid rewarming reverses the beneficial effects of induced hypothermia in traumatic brain injury [145]. Furthermore, gradual rewarming improved survival and attenuated remote acute lung injury after intestinal ischemia and reperfusion injury as compared to speed rewarming [146]. The mechanism behind this phenomenon is only partly understood. Considering the current literature, it seems likely that abrogation of the beneficial effects is associated with ATP depletion, energy failure, and consecutive mitochondrial dysfunction [145]. To date, detailed knowledge about exact mechanisms in different rewarming strategies is lacking.

5.2. Clinical Studies. The European Resuscitation Council recommends early hypothermia (32–34°C) for 12–24 hours following cardiac arrest and a slow rewarming procedure with 0.25–0.5°C per hour avoiding hyperthermia [147]. In a clinical study in 57 hypothermic patients, continuous arteriovenous rewarming resulted in less fluid requirement and less mortality as compared to standard rewarming procedures [11].

In summary, there is only minor understanding of the rewarming process but slow rewarming is recommended based on mostly experimental studies.

6. Perspective

Before induced hypothermia can be introduced in the clinical management of patients several limitations of the presented studies need to be considered. A potential limitation of all studies in which hypothermia is induced by a cardiopulmonary bypass machine is the fact that increase of cytokines may

be due to the bypass procedure itself [111]. While numerous experimental as well as clinical studies regarding cardiac surgery, brain injury, or cardiac arrest are available information regarding hypothermia following major injuries is spare. Since there are still divergent results in experimental studies that are mostly limited to traumatic brain injury, the mechanisms by which hypothermia influences the posttraumatic immune response after multiple trauma need to be elucidated.

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Clinical Study

Circulating Leukotriene B4 Identifies Respiratory Complications after Trauma

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Background. Leukotriene B4 (LTB4), a proinflammatory lipid mediator correlates well with the acute phase of Acute Respiratory Distress Syndrome (ARDS). Therefore, LTB4-levels were investigated to determine whether they might be a useful clinical marker in predicting pulmonary complications (PC) in multiply traumatized patients. Methods. Plasma levels of LTB4 were determined in 100 patients on admission (ED) and for five consecutive days (daily). Twenty healthy volunteers served as control. LTB4-levels were measured by ELISA. Thirty patients developed PC (pneumonia, respiratory failure, acute lung injury (ALI), ARDS, pulmonary embolism) and 70 had no PC (ØPC). *Results*. LTB4-levels in the PC-group [127.8 pg/mL, IQR: 104–200pg/ml] were significantly higher compared to the ØPC-group on admission [95.6 pg/mL, IQR: 55–143 pg/mL] or control-group [58.4 pg/mL, IQR: 36–108 pg/mL]. LTB4 continuously declined to basal levels from day 1 to 5 without differences between the groups. The cutoff to predict PC was calculated at 109.6 pg/mL (72% specificity, 67% sensitivity). LTB4 was not influenced by overall or chest injury severity, age, gender or massive transfusion. Patients with PC received mechanical ventilation for a significantly longer period of time, and had prolonged intensive care unit and overall hospital stay. *Conclusion*. High LTB4-levels indicate risk for PC development in multiply traumatized patients.

1. Introduction

Trauma patients are at high risk of developing respiratory complications such as pneumonia, respiratory failure, Acute Lung Injury (ALI), Acute Respiratory Distress Syndrome (ARDS), and pulmonary embolism. Following multiple organ failure (MOF) and sepsis, respiratory complications are among the most common causes of morbidity and mortality for trauma patients surviving the initial postinjury phase [1–5]. The overall mortality from ARDS is still up to 50% [6–8]. Multiply traumatized patients have shown 10% mortality following ALI [9]. Approximately 20% of major trauma admissions develop ARDS or ARDS like pulmonary dysfunction. This represents one of the most frequent complications in these patients and is the major contributor to morbidity and mortality in trauma patients [3, 10, 11].

Several airway diseases including ALI/ARDS are closely associated with neutrophil infiltration of the airway wall [12]. Neutrophils release a variety of oxidants, as well as degradative and proteolytic enzymes, which induce lung inflammation with subsequent airway remodelling, microvascular damage, and lung tissue injury [12–14]. Persistence of neutrophils in the lungs is an important contributing factor to poor survival [15, 16].

Leukotriene (LT) B4 is a proinflammatory lipid mediator derived from the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism [17–20]. LTB4 is a potent chemoattractant which also exerts leukocyte activating abilities and plays a crucial role in neutrophil migration [21–24]. LTB4 induces neutrophil adherence to endothelial cells, promotes chemotaxis, stimulates the generation and release of oxidants, and increases 5-LO activation in neutrophils, resulting in enhanced LTB4 synthesis [17, 18, 25]. Patients with pulmonary disease have elevated levels of LTB4 indicating its proinflammatory role [25–27]. LTB4 concentrations are enhanced in bronchoalveolar lage (BAL) fluid of ALI/ARDS and chronic obstructive pulmonary disease (COPD) patients [27, 28]. Recently, it has been reported that LTB4 and its metabolites, due to a "priming" effect on neutrophils, plays an important role in the development

of polymorphonuclear-neutrophils-(PMN-) induced lung injury [29]. The priming effect of sequestered neutrophils in the lungs leads to their "hyperfunction." This results in an exaggerated inflammatory cell response to a secondary stimulus potentially inducing lung complications [30–32]. Early identification of high-risk patients for respiratory complications after trauma is important in determining subsequent treatment. The potential prognostic role of LTB4 in major trauma patients, suffering lung complications in a later postinjury phase, remains unclarified.

We hypothesize that high levels of LTB4 in the plasma of multiply traumatized patients indicate not only a strong proinflammatory response, but may also serve to identify patients at risk for imminent lung complications.

2. Methods

2.1. Ethics. This study was performed in the Goethe University Hospital with ethical approval (167/05, in accordance with the Declaration of Helsinki and following STROBE-guidelines) [33]. All patients signed the informed consent forms themselves or informed consent was obtained from relatives in accordance with ethical standards.

2.2. Patients. Inclusion criteria consisted of a history of acute blunt or penetrating trauma with an Injury Severity Score (ISS) $\geq$ 16 in any patient between 18 and 80 years of age. Burns, concomitant acute myocardial infarction, and/or lethal injury were exclusion criteria.

Blood samples were obtained from 100 multiply traumatized patients on admittance to the emergency department (ED), and daily for 5 days following the trauma. Upon arrival at the ED, vital signs were documented. Trauma severity was scored using the Abbreviated Injury Scale (AIS) [34–36]. In addition, ISS was calculated [37]. Patients with an ISS from 16–24 were classified as substantially injured patients, patients with an ISS from 25–39 were substantially/severely injured patients and patients with an ISS $\geq$ 40 were considered severely injured patients.

Pulmonary complications were defined as nosocomial pneumonia, ALI/ARDS, pulmonary embolism, and/or respiratory failure as described below. Pneumonia was defined by radiologic, clinical, and bacteriologic findings with the presence of new pulmonary infiltrates and at least one of the following criteria: positive blood culture, BAL, and/or sputum culture [38]. Lung injury was assessed using the American-European Consensus Conference criteria for ARDS [39]. Pulmonary embolism was diagnosed by computed tomography (CT), and pulmonary edema was diagnosed either by CT scan or chest X-ray. Respiratory failure was defined as the need for prolonged weaning or reintubation.

The control group included 20 healthy nonsmoking volunteers with un known chronic disease and no history of abdominal trauma or abdominal surgery within the past 24 months.

2.3. Blood Processing and Analysis. Blood samples were collected as early as possible after injury in prechilled ethylenediaminetetraacetic acid (EDTA) vacuum tubes (BD vacutainer, Becton Dickinson Diagnostics, Aalst, Belgium) and kept on ice. Blood was centrifuged at 2000 $\times$g for 15 minutes at 4°C. The supernatant was stored at -80°C until batch sample analysis.

The mean time between the injury and first blood sample taken directly upon admittance to the ED was 83 $\pm$ 7 min. Specimens were used for duplicate measurement of LTB4 levels. LTB4 was determined using a highly specific commercially available ELISA (LTB4 Parameter Assay Kit, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. The detection limit was 27.6 pg/mL for LTB4.

2.4. Statistics. Kolmogoroff-Smirnoff-Lillieford's test showed that the plasma concentration of LTB4 was not Gaussian-distributed. Median LTB4 levels for each of the 3 groups were compared using the Kruskal-Wallis test and the post hoc analysis was performed with Dunn's multiple comparison test. Data are presented as the median (interquartile range, IQR) or mean $\pm$ sem unless otherwise stated. A P value < 0.05 was considered statistically significant. Receiver-operator curves were generated to analyze the optimal cutoff levels. GraphPad Prism 5.0 software (GraphPad Software Inc. San Diego, CA) was used to perform the statistical analysis and computations.

3. Results

The total group consisted of 100 patients (24 female, 76 male), 98% suffering from blunt and 2% from penetrating trauma. All patients were substantially injured (ISS: 34.0 $\pm$ 1.7). Of these, 30 patients with an ISS of 33.7 $\pm$ 1.6 developed secondary pulmonary complications. Seventy patients with an ISS of 34.1 $\pm$ 1.3 had no pulmonary complications. The AIS_{chest} was comparable in both groups (3.1 $\pm$ 0.2 in the ØPC group and 3.5 $\pm$ 0.2 in the PC group). Time on mechanical ventilation, length of stay in the ICU and hospital were significantly prolonged in the PC group. Additionally, more patients developed sepsis ($P < 0.05$), organ failure, and MOF in the PC group. In-hospital mortality was also increased. Table 1 summarizes general patient characteristics and physiologic parameters in the study population. Table 2 depicts the type, severity, and cause of injury.

Figure 1 shows the distribution of plasma LTB4 values in the first sample obtained in the ED and subsequent daily measurements for five consecutive days. Median concentrations (and IQR) of LTB4 in trauma patients on admission were significantly increased compared to healthy controls (106.1 (62–159) pg/mL versus 58.4 (36–108) pg/mL, $P < 0.05$, Figure 1). The LTB4 levels on admission were also significantly elevated compared with levels at day 1 until day 5.

To investigate the relation between the injury severity and LTB4 concentrations determined in the ED, the study population was subdivided into three groups: seriously injured patients (ISS: 16–24, $n = 17$), seriously/severely injured patients (ISS: 25–39, $n = 54$), and severely injured patients (ISS: $\geq$40, $n = 29$). Plasma LTB4 concentrations in each group were markedly enhanced (112.8 (68–167) pg/mL, 107.6 (48–164) pg/mL, and 105.3 (62–148) pg/mL, resp.) compared with healthy volunteers 58.4 (36–108) pg/mL, but this tendency was not significant (Figure 2(a)).

Table 1: Summary of patient characteristics and physiologic parameters in the investigated groups (ØPC: no pulmonary complications, PC: pulmonary complications and ctrl, data are presented as mean ± SEM unless otherwise stated).

Patient characteristics	All patients (n = 100)	PC (n = 30)	ØPC (n = 70)	ctrl (n = 20)	P value(PC versus ØPC)
Age (years)	39.2 ± 1.7	42.8 ± 3.4	37.9 ± 1.9	32.3 ± 2.8	0.2241
Sex (male)	76	20	56	7	—
Injury severity score	34.0 ± 1.0	33.7 ± 1.6	34.1 ± 1.3	—	1.0000
Surgery after admission	69.0%	76.7%	70.8%	—	0.6218
Thoracic drainage	34.0%	53.3%	25.7%	—	0.0191
Packed red blood cells (pRBC)/250 mL (24 h)	7.4 ± 1.2	8.6 ± 1.9	7.2 ± 1.5	—	0.2739
Massive transfusion (≥10 units pRBC in 24)	20.0%	26.7%	17.4%	—	0.6007
Haemoglobin (g/dL)	11.5 ± 0.3	9.9 ± 0.7	12.6 ± 0.3	—	0.0044
Intubation	73.0%	96.7%	81.4%	—	0.0762
Intubation duration (d)	9.9 ± 1.1	18.4 ± 2.7	6.4 ± 0.8	—	<0.0001
Rotational bed therapy	62.0%	86.7%	51.4%	—	0.0020
Rotational bed Therapy (d)	3.9 ± 0.5	5.3 ± 0.7	3.4 ± 0.6	—	0.0070
Intensive care period (d)	12.8 ± 1.3	22.4 ± 3.1	8.9 ± 0.9	—	<0.0001
Hospital stay	24.1 ± 2.0	32.5 ± 5.2	20.5 ± 1.7	—	<0.0001
Sepsis (n/%)	16/16.0%	11/36.7%	5/7.1%	—	0.0162
Organ failure (n/%)	8/8.0%	5/16.7%	3/4.3%	—	0.3137
MOF (n/%)	4/4.0%	3/10.0%	1/1.4%	—	0.4889
Hospital mortality (n/%)	6/6.0%	3/10.0%	3/4.3%	—	0.6390
LTB4 (pg/mL, interquartile range)	105.9 (96.7)	127.8 (96.5)	95.6 (88)	58.4 (72.1)	0.0140

Table 2: Overview of type and mechanisms of injuries within the two investigated groups groups (ØPC: no pulmonary complications, PC: pulmonary complications and ctrl, data are presented as mean ± SEM).

Group	All patients (n = 100)	PC (n = 30)	ØPC (n = 70)	P value (PC versus ØPC)
Abbreviated injury severity-scale (AIS, mean ± sem)				
AIS head	2.5 ± 0.2	2.3 ± 0.3	2.6 ± 0.2	0.4332
AIS chest	3.2 ± 0.1	3.5 ± 0.2	3.1 ± 0.2	0.1008
AIS abdomen	1.9 ± 0.1	1.9 ± 0.2	2.0 ± 0.2	0.8208
AIS extremities & external	2.2 ± 0.1	2.4 ± 0.2	2.2 ± 0.2	0.3601
Mechanism of Injury (n)				
Blunt	98	29	69	—
Penetrating	2	1	1	—
Accident mechanism (n/%)				
Road accident	69/69.0%	21/70.0%	48/68.6%	
Motorcycle	30/30.0%	4/13.3	26/37.1%	
Car	28/28.0%	12/40.0%	16/22.9%	
Pedestrian	6/6.0%	4/13.3%	2/2.9%	
Bicyclist	5/5.0%	1/3.3%	4/5.7%	
Fall	24/24.0%	6/20.0%	18/25.7%	
>3 m	20/20.0%	6/20.0%	14/20.0%	
<3 m	4/4.0%	—	4/5.7%	
Other	7/7.0%	3/10.0%	4/5.7%	

The severity of chest trauma was assessed using the AIS chest scores. Patients without a relevant chest injury were graded as $AIS_{chest} \leq 2$ (n = 21). Patients with serious and serious/severe chest injury (AIS_{chest} = 3 or 4) occurred most frequently (n = 23, and n = 49, resp.), whereas patients with an AIS_{chest} = 5 occurred less often (n = 7). Taken together, LTB4 levels were increased in all four trauma patient groups ($AIS_{chest} \leq 2$: 113.9 (61–162), AIS_{chest} = 3: 105.8 (47–200), AIS_{chest} = 4: 107.7 (64–156), and AIS_{chest} = 5: 94.9 (65–133) pg/mL) compared with healthy volunteers, but this difference was not significant (Figure 2(b)).

However, comparing LTB4 levels taken in the ED of those patients who developed pulmonary complications (n = 30) following injury with those patients who did not develop pulmonary complications (n = 70) and healthy volunteers revealed a significant difference (127.8 (104–200) versus

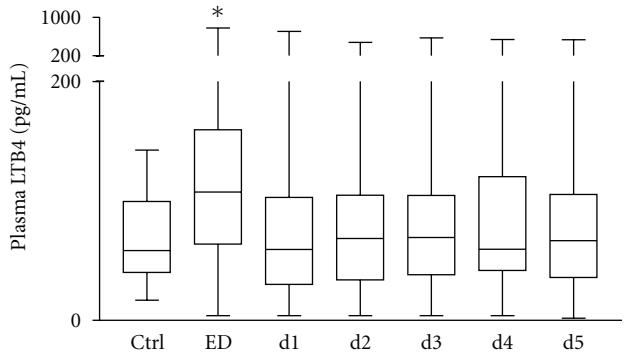

Figure 1: Summary of median (interquartile range) LTB4 (pg/mL) in multiply traumatized patients over a 5-day time course after admission ($n = 100$), and ctrl ($n = 20$). $^*P < 0.05$ versus other groups.

95.6 (55–143) and 58.4 (36–108) pg/mL, resp., $P < 0.05$, Figures 3(a) and 3(b)). Follow-up LTB4 levels (day 1–day 5) showed that increased LTB4 levels in the ED in patients with pulmonary complications diminished in a time-dependent manner (Figure 3(a)). This effect was irrespective of the patients ISS or AIS_{chest} since subgroup analysis according to ISS or AIS_{chest} revealed no differences in LTB4 levels.

Receiver operating characteristics (ROCs) analysis for LTB4 shows an optimal cutoff of 109.6 pg/mL with 72% specificity (95% CI: 0.61–0.81) and 67% sensitivity (95% CI: 0.49–0.84), for predicting pulmonary complications in a later posttraumatic course (Figure 4). The area under the ROC curve is 0.73.

Multiply traumatized patients with pulmonary complications needed significantly prolonged mechanical ventilation compared with patients without secondary pulmonary complications (18.4 ± 2.7 days versus 6.4 ± 0.8 days, resp., $P < 0.05$, Table 1). The mean ICU stay of all patients was 12.8 ± 1.3 days. Patients with pulmonary complications had a mean ICU stay of 22.4 ± 3.1 days versus 8.9 ± 0.9 days in patients without pulmonary complications ($P < 0.05$, Table 1). Patients with pulmonary complications also had significantly prolonged hospital stay compared with patients without pulmonary complications (32.5 ± 5.2 days versus 20.5 ± 1.7 days, resp., $P < 0.05$, Table 1). Sixteen patients developed sepsis, of those 69% had pulmonary complications (Table 1). Eight patients suffered from organ failure, and 4 patients had MOF, of those 63% and 75%, respectively, had pulmonary complications (Table 1). The mortality rate was enhanced for patients with pulmonary complications (10%) compared with patients without pulmonary complications (4%, Table 1).

4. Discussion

Respiratory complications, such as pneumonia, respiratory failure, ALI/ARDS, and pulmonary embolism are, next to MOF and sepsis, among the most common causes of late morbidity and mortality after trauma [1–5, 40]. An increased rate of pulmonary complication in severely injured trauma patients is closely associated with an excessive systemic and local inflammatory response including neutrophil influx [12, 40–42]. LTB4 represents a potent neutrophil chemoattractant and enhanced LTB4 levels are associated with pulmonary disease [25–28]. Despite the close association with airway disease, it remains unclear whether LTB4 is a reliable parameter for early identification of high-risk patients for pulmonary complications after multiple trauma.

This study shows that multiply traumatized patients with high LTB4 levels (cutoff at 109.6 pg/mL) in the initial phase are at high risk to develop posttraumatic pulmonary complications. The association of increased LTB4 in BAL fluid of patients with ALI/ARDS and COPD has previously been reported [27, 28]. The majority of clinical studies focused on the proinflammatory role of LTB4 in neutrophil infiltration and the subsequently induced lung injury in ICU patients [27–32]. The decisive role of neutrophils in several airway diseases, including ALI/ARDS, has been described [12–14]. Their persistence in the lungs is closely associated with poor survival [15, 16].

LTB4 is biosynthesized from arachidonic acid by the action of cytosolic phospholipase A_2, 5-LO together with 5-LO-activating protein (FLAP) and leukotriene A4 hydrolase [43]. 5-LO activity is considered a key factor in LTB4 biosynthesis. LTB4 levels have been shown to correlate with tumor necrosis factor alpha levels and the number of neutrophils recovered from the BAL fluid of patients with ARDS [44]. Furthermore, LTB4 and its metabolites have been shown to cause increased neutrophil adherence to the pulmonary endothelial cell surface, reflecting neutrophil sequestration in the lung and the capillary bed and increasing vascular permeability [17, 29, 45, 46]. Inhibiting the 5-LO rate-limiting enzyme in LTB4 biosynthesis by intratracheal application of IL-8 has been shown to prevent lung injury and perfusate LTB4 increase in the lungs. Neutrophil chemotaxis *in vitro* was also inhibited [47]. Therefore, it might be concluded that the impact of enhanced systemic LTB4 concentrations in trauma patients presenting at the ED may reflect an ongoing systemic inflammation. This, in turn, may lead to the development of pulmonary complications. Therefore, the predictive relevance of LTB4 should be considered in this highly heterogeneous group of patients. Employing timely appropriate treatment (e.g., kinetic therapy, operative and ventilatory strategies) could thereby improve patient outcome.

Other factors have been reported to be associated with the development of posttraumatic pulmonary complications. The extent of chest trauma has been shown to increase respiratory complications, such as ALI/ARDS [48, 49]. Interestingly, we found no significant correlation between the degree of chest injury assessed by the AIS and the rate of posttraumatic pulmonary complications. This may be due to the study having been conducted at a single clinic with a limited number of patients ($n = 100$). In a multivariate statistical analysis, we found that the effect of trauma severity on LTB4 levels as well as the development of pulmonary complications is considered not significant (data not shown).

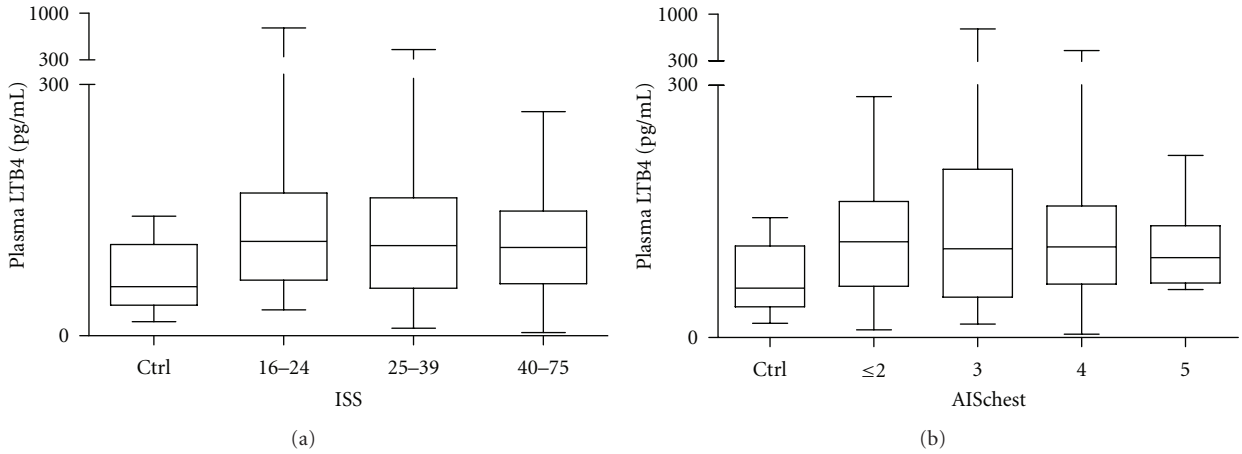

Figure 2: Summary of median (interquartile range) LTB4 (pg/mL) in ED samples in different groups of patients based on the overall injury severity (a) and the severity of chest injury (b). (a) ISS: 16–24, $n = 17$, ISS: 25–39, $n = 54$, ISS: ≥40, $n = 29$ and ctrl group, $n = 10$. (b) $AIS_{chest} \leq 2$, $n = 21$, $AIS_{chest} = 3$, $n = 23$, $AIS_{chest} = 4$, $n = 49$, $AIS_{chest} = 5$, $n = 7$ and ctrl, $n = 20$.

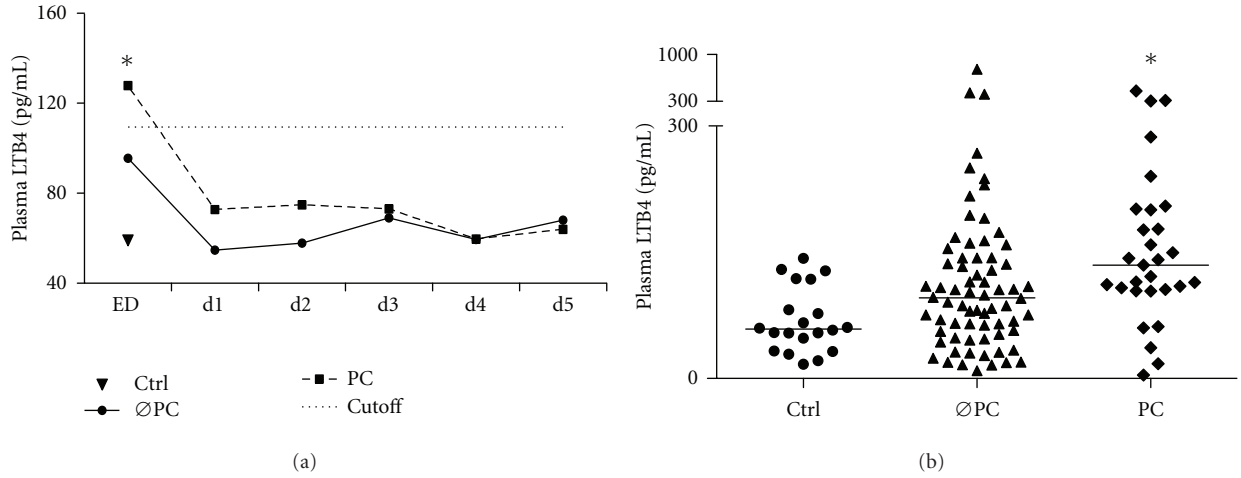

Figure 3: Summary of median LTB4 (pg/mL) in two patient groups based on the development of pulmonary complications (ØPC: no pulmonary complications, $n = 70$, and PC: pulmonary complications, $n = 30$) and ctrl, $n = 20$, $^*P < 0.05$ versus other groups: (a) time course in both groups, (b) LTB4 levels at the ED.

Evaluation of data from patients with hypoxemic respiratory failure ($n = 8$) has shown that LTB4 levels (at ED) were significantly enhanced in this group compared to healthy volunteers, but did not differ markedly from 22 patients with nonhypoxemic respiratory complication (data not shown). In the present study, the mortality was 6% in a cohort of trauma patients with considerable injury (mean ISS > 33). Blunt trauma as the major type of injury in over 90% of patients was in accordance with other European studies [50, 51]. Dysregulated immune response after trauma has been suggested to contribute to complications, such as sepsis and MOF. The incidence of sepsis and MOF vary strongly in the literature [2, 52–56]. In the present study, sepsis occurred in 16% and MOF in only 4% of patients. In line with the literature, trauma patients with pulmonary complications constitute the majority of patients who develop sepsis and/or MOF [52]. Interestingly, in the present study, patients developing pulmonary complications were not more severely injured than patients without pulmonary complications, as had been expected from previous reports [52]. The clinical course was strongly affected by the presence of respiratory complication with a prolonged ICU stay, days on mechanical ventilation, and longer hospital stay.

Pulmonary complications after severe trauma markedly affect the clinical course. The predisposing factors for these patients at risk are not fully understood and their identification before clinical manifestation of complications remains a challenge. Most clinical scoring systems have been developed to compare populations, while their predictive power is limited. The lung organ failure scoring (LOFS) method has been developed to estimate the risk for pulmonary complications in trauma patients [57]. Its effectiveness still needs to be assessed in prospective clinical studies.

In conclusion, to stratify the risk for later pulmonary complications the results presented here encourage LTB4 assessment early after trauma. In the present study, the LTB4

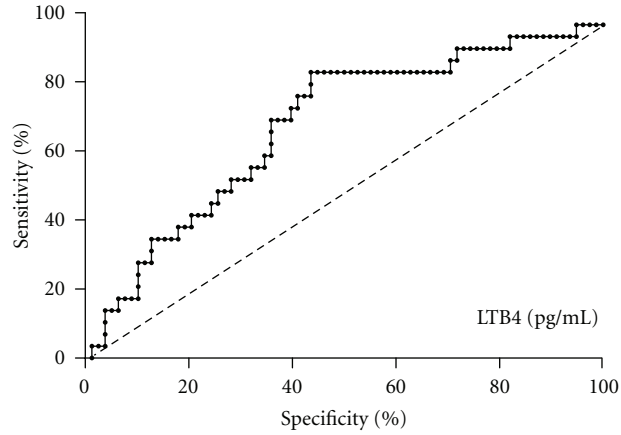

Figure 4: Receiver operating curve showing the optimal cutoff for LTB4 levels (109.6 pg/mL in predicting the presence or absence of postinjury pulmonary complications with 72% specificity and 67% sensitivity).

AUC is quite small. Enhanced patient numbers, especially in the group of patients with pulmonary complications, could strengthen the hypothesis that LTB4 may be of predictive value. While the pathophysiological sequelae of increased LTB4 release is principally understood, identification of relevant effects such as neutrophil adherence or edema formation need demonstration in the clinical setting. Therefore, clinical studies with larger numbers of patients are required to clarify the role of LTB4 in pulmonary complications and resolve its predictive efficacy.

Authors' Contributions

B. Auner and E. V. Geiger contributed equally in this research.

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Research Article

Systemic Inflammatory Effects of Traumatic Brain Injury, Femur Fracture, and Shock: An Experimental Murine Polytrauma Model

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Objective. Despite broad research in neurotrauma and shock, little is known on systemic inflammatory effects of the clinically most relevant combined polytrauma. Experimental investigation in an animal model may provide relevant insight for therapeutic strategies. We describe the effects of a combined injury with respect to lymphocyte population and cytokine activation. *Methods*. 45 male C57BL/6J mice (mean weight 27 g) were anesthetized with ketamine/xylazine. Animals were subjected to a weight drop closed traumatic brain injury (WD-TBI), a femoral fracture and hemorrhagic shock (FX-SH). Animals were subdivided into WD-TBI, FX-SH and combined trauma (CO-TX) groups. Subjects were sacrificed at 96 h. Blood was analysed for cytokines and by flow cytometry for lymphocyte populations. *Results*. Mortality was 8%, 13% and 47% for FX-SH, WD-TBI and CO-TX groups ($P < 0.05$). TNFα (11/13/139 for FX-SH/WD-TBI/CO-TX; $P < 0.05$), CCL2 (78/96/227; $P < 0.05$) and IL-6 (16/48/281; $P = 0.05$) showed significant increases in the CO-TX group. Lymphocyte populations results for FX-SH, WD-TBI and CO-TX were: CD-4 (31/21/22; P = n.s.), CD-8 (7/28/34, $P < 0.05$), CD-4-CD-8 (11/12/18; P = n.s.), CD-56 (36/7/8; $P < 0.05$). *Conclusion*. This study shows that a combination of closed TBI and femur-fracture/ shock results in an increase of the humoral inflammation. More attention to combined injury models in inflammation research is indicated.

1. Introduction

Trauma and especially multiple trauma including traumatic brain injury (TBI) are the most common killers in children and adults before age 50 around the world [1–3]. Mortality of multiple trauma including TBI following traffic accidents ranges between 18% and 25% [1, 4]. But not only the first hours and days are critical; for years, more and more multiple trauma patients have been surviving the initial accident and acute care period but only to develop systemic inflammatory response syndrome (SIRS), sepsis, or multiple organ dysfunction syndrome (MODS) in the course of the few weeks of critical care [2, 5, 6].

For 5000 multiple trauma patients in Germany, the German Trauma Registry showed an in-hospital mortality of 12% in 2010. In the same report, critical care complications such as SIRS, sepsis, and MODS are reported for 15% of patients. Other groups report similar rates of SIRS, sepsis, and MODS [2, 6, 7]. It is generally known that these complications again deteriorate the prognosis and outcome of the initially surviving trauma patient and increase late mortality to up to 50% if manifest MODS is developed [2, 7, 8].

To understand pathophysiological processes leading to multiple organ failure, SIRS, and sepsis and taking place during this devastating illness, profound pathophysiological

knowledge on the effects of the initial trauma ("first hit") is essential to minimize additional harm by the following surgical or critical care treatment (potential "second hit") [9–11].

Therefore, numerous neurotrauma as well as shock research studies with many different animal and clinical models showed inflammatory changes after trauma [12–14]. Only recently, Semple et al. reported on the important role of CCL2 in C57Bl/J6 mice, peaking at 4 to 12 h following closed head injury and leading to improved functional outcome and lesion size reduction as well as less secondary brain damage [15]. Another group showed that the substance Minozac may attenuate the effect of proinflammatory cytokines after closed TBI in mice. In the same study, the authors discussed if Minozac may also increase long-term neurofunction after TBI [16]. On the other hand, femur fracture and shock in mice are known to trigger an inflammatory response for years, and recently several groups discussed therapeutic strategies to modulate this inflammatory response [12, 14].

However, little attention has been paid to the immune reaction following combined trauma and the interaction of the brain and the other organs of the body. This is even more important, since the injury pattern of closed TBI, lower extremity fracture, and shock is very common in multiply injured patients treated in trauma centers around the world. Therefore, we investigated the humoral and cellular inflammatory changes of the combined TBI, shock, and femur fracture in a polytrauma model of the mouse.

2. Material and Methods

2.1. Animal Care. The study was approved by the Animal Research Committee of the Hannover Medical School (Medizinische Hochschule Hannover, MHH) and the county government of Lower Saxony (Bezirksregierung Niedersachsen), no. 03/672.

45 male C57BL/J6 mice aged 8–10 weeks with a mean weight of 27.4 g (±3.7 g) were used for the study. The animals were bred and raised in the central animal facility of our institution. All animals were handled at room temperature for 7 days before treatment. Throughout the study period, pelleted mouse feed (Altromin 1324) and water were available ad libitum. The lighting was maintained on a 12-hour cycle. Room temperature was maintained at a constant 20° ± 2°C.

2.2. Anesthesia. Analgetic therapy during the whole time of the study was provided by Metamizol 25 mg/1000 mL (Novaminsulfon ratiopharm, Ratiopharm GmbH, Ulm) in the drinking water of the animals ad libitum.

All surgical and trauma procedures were performed at a level of deep surgical anesthesia during maintained spontaneous breathing. For anesthesia, subcutaneous injection of Ketamine (Ketanest, Parke-Davis GmbH, Karlsruhe, Germany) with a dose of 100 mg/kg body weight and 2% Xylazine (Rompun, Bayer Vital GmbH, Leverkusen, Germany) with a dose of 16 mg/kg body weight were used. The eyes of the animals were covered by Dexpanthenol (Bepanthen, Hoffmann-La Roche AG, Grenzach-Wyhlen, Switzerland). The trauma and perioperative care was performed under an infrared heating lamp. Thereby body temperature of 36°C could be maintained.

2.3. Weight Drop Traumatic Brain Injury (WD-TBI). 30 of the animals underwent a standard WD-TBI following a model published previously by Shapira et al. [17] with modification according to Chen et al. [18]. Under a surgical level of anesthesia, the animal was positioned in a stereotaxic frame, the head was shaved, and the exposed scalp disinfected by Softasept N (Braun, Melsungen, Germany). A midline incision was performed, and the skull was exposed. Next, the 3 mm diameter probe was positioned on the planned impact point at the left of the sagittal suture and caudad to the coronal suture. The trauma was now performed with a mean velocity of 3 m/s by a software-guided process ("Labview," National Instruments, Austin, TX, USA).

Animals were taken out from the stereotaxic frame while still anesthetized. The wound was irrigated and closed by 4.0 prolene sutures. (Ethicon, Norderstedt, Germany). Afterwards, the animals remained under continuous observation and warming until full recovery was reached.

2.4. Femur Fracture. Femur fracture was applied by a blunt guillotine device according to Van Griensven et al. [19], We used a weight of 400 g falling out of a height of 160 mm on the fixed femur. Success of the fracture mechanism was controlled clinically (crepitation and instability), while the animal was still in anesthesia. If both of the clinical signs were absent, the trauma was repeated. The repeated procedure was required in one animal only. The guillotine procedure resulted in an A-type femur fracture combined with a moderate soft tissue injury. After completion of the injury, we performed a closed reduction and splinting of the fracture by a piece of wood in proper length that was tied to the fractured extremity proximally and distally of the fracture.

2.5. Shock. After both traumata were set, we draw 60% of the blood volume of the respective mouse via an orbital puncture with a heparinized capillary. Volume was restored after 1 hour of shock with Ringer's solution four times the extracted blood volume into the tail vein. This resulted in an individual resuscitation procedure. Only after this resuscitation was completed and the animal showed 36°C of body temperature, anesthesia was terminated and the animal awoke [19].

2.6. Analysis of the Cytokines and Lymphocytes. Subjects were sacrificed 96 hours after trauma by exsanguination in deep surgical anaesthesia, while blood samples for cytokines were extracted. As well the spleen was harvested, and a suspension was produced. This suspension was used for lymphocyte analysis as described earlier [19]. Lymphocytes were analyzed using standard flow cytometry (FACS analysis) for CD-4, CD-8, CD-4-CD-8, and CD-56 cells (FACS Calibur, BD

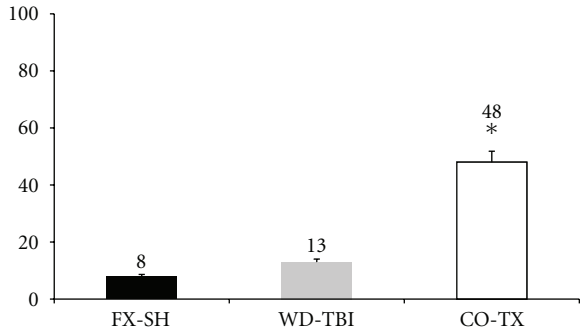

Figure 1: Mortality rates (%) of the 3 trauma subgroups. FX-SH: femur fracture and shock; WD-TBI: weight drop traumatic brain injury; CO-TX: combined injuries. $(*)P = 0.0263$. The CO-TX group showed a significantly higher mortality.

Biosciences, Heidelberg, Germany). The procedures have been described earlier [19].

Serum cytokines (tumor necrosis factor α, TNFα; che mokine CC2 ligand, CCL2; interleukins 6 and 12, IL-6 and IL-12) were analyzed by cytometric bead array technique (BDTM Cytometric Bead Array Mouse Inflammation Kit, BD Biosciences, Pharmingen, Germany) and FACS as described earlier [19].

2.7. Statistics. Analysis of continuous parameters was by ANOVA and a post hoc Tukey test. Mortality ratios were analyzed by chi-squared test. Results are reported as mean $\pm$ standard error of the mean (SEM). For statistical analysis, SPSS 14.0 software was used. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Mortality. The polytraumatized animals showed a significantly higher mortality than the animals of both singular trauma groups (Figure 1). There was no immediate death. Deaths occurred between 24 and 72 hours after trauma.

3.2. Humoral Inflammatory Markers. TNFα, CCL2, and IL-6 showed significantly higher concentrations at 96 h after trauma in the CO-TX group, while the single-trauma groups seem to raise their cytokine levels clearly less and without significant differences between groups WD-TBI and FX-SH (Figure 2). IL-12 showed no difference in plasma concentration between the groups at 96 h after trauma.

3.3. Lymphocyte Populations. The CD-8 ratio was significantly lower, and the CD-56 ratio was significantly higher in the FX-SH mice than in each of the other groups. CD-4 and CD-4-CD-8 ratios showed no difference between groups (Figure 3).

4. Discussion

While the intracranial effects of traumatic brain injury have been a subject of multiple studies, the aim of this study was to investigate the interaction of the brain injury and systemic shock after lower extremity trauma in regard to changes in the inflammatory response.

Our main results were:

(1) increased levels of TNFα, CCL2, and IL-6 in the CO-TX group,

(2) lower rates of CD-8 and higher rates of CD-56 cells in the FX-SH group,

(3) an increased mortality in the combined trauma animal group.

However, some weaknesses of our study have to be kept in mind. As always in animal studies, the findings cannot completely be extrapolated to human pathophysiology and clinical treatment strategies. Nonetheless, mouse models are very common due to their availability, ease of handling, and practicability. Anyhow, a correlation with similar large animal models and additional studies in humans are required to complete our findings and value the clinical relevance. Furthermore, the injury pattern may seem artificial in its constellation since one huge challenge of human polytrauma studies is the diversity of injury patters requiring complex inclusion and exclusion procedures to gain comparable populations in terms of injury severity and injury pattern. However, in our point of view, this strengthens our study: the injury pattern and severity of our individuals is highly standardized, and the model and both its components are robust and reproducible. So comparability of our injuries and populations is granted, even more so with regard to the complex hypothesis of a combined trauma being more than only the sum of its parts. This holds even true for standard parameters such as weight, size, sex, and age, that usually also affect human studies of polytrauma patients.

Our first finding supports any injury severity-related changes in inflammatory response reported from humans [2, 20–22]. Our CO-TX group simply suffered more severe injuries than the FX-SH and WD-TBI groups. Looking at each monotrauma, our plasma cytokine levels are comparable to those reported earlier in literature [12, 14]. However, the levels of the proinflammatory cytokines are elevated not only by factor 2 but by factors 3 to 10, respectively, even as late as 96 hours after trauma. Other groups found similar results: TNFα was associated with injury severity earlier. Likewise, in a murine trauma-haemorrhage model, other authors reported TNFα elevations [23, 24]. The same holds true for TBI in humans [25, 26] and clinically relevant models of TBI [27]. While these groups reported elevated TNFα levels 1 to 4 h after trauma, later publications also found TNFα effects on neuronal recovery and prevention of secondary brain damage [28]. One group reported elevated plasma TNFα levels after TBI. So, while the importance of TNFα after peripheral or brain injury remains rather clear and reciprocal effects were described to some extent, we found no earlier report on the extent of TNFα plasma level rise after combined TBI and trauma haemorrhage. Concerning IL-6, Maegele et al. reported a 3-4 fold increase in circulating plasma levels after combined fracture and fluid percussion TBI in rats [29]. In humans, IL-6 was classified

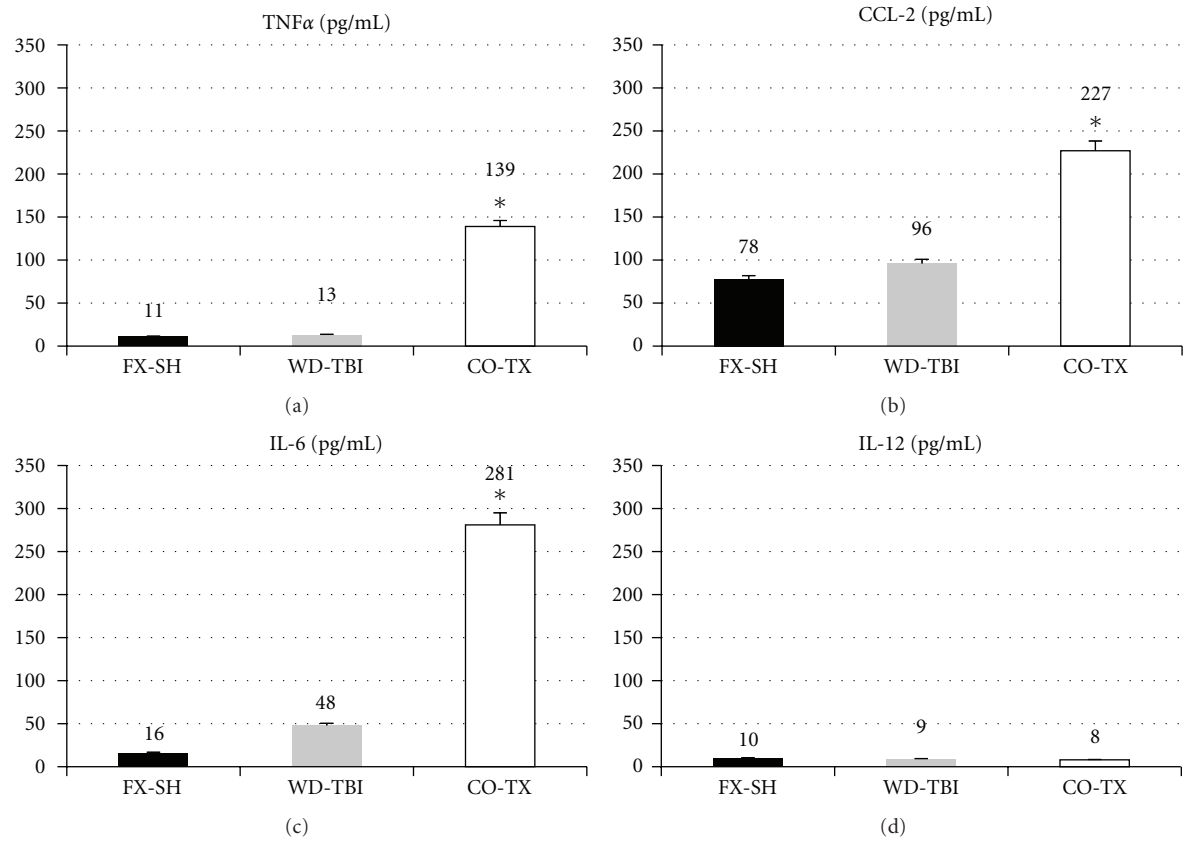

Figure 2: Cytokine levels (pg/mL) of the three subgroups. FX-SH: femur fracture and shock; WD-TBI: weight drop traumatic brain injury; CO-TX: combined injuries. $(*)P < 0.05$.

as the most important secondary cytokine in trauma [30], not only because of its multitude of mainly proinflammatory effects and its association to the severity of trauma, but also because of its prognostic relevance differing survivors and nonsurvivors. So, highest IL-6 levels in the most severe trauma group and the highest mortality are in line with earlier results in literature. However, the magnitude of the increase was rarely described. Similarly, CCL-2 is increased in the brain after TBI and leads to increased migration of intravascular leukocytes across a damaged blood-brain barrier [31, 32]. As well CCL-2 was found to play a key role in the development of organ dysfunction after trauma by activating monocytes/macrophages to migrate from the intravascular space into peripheral tissues. While Thibeault et al. showed no association between IL-6 and CCL-2 after trauma, they could show such an association between TNFα and CCL-2 [33]. So, while being in line with recent literature, our findings also remain conclusive within themselves with respect to the inflammatory cascades described earlier. With respect to proinflammatory IL-12, our nonsignificantly low serum levels are backed up by recent literature. In TBI, one author described differences in IL-12 after 24 h but not later [30]. In human multiple trauma patients suffering from chest trauma, IL-12 was reported to inversely correlate with mortality [30]. Furthermore, IL-12 seems to exert some major effects via CD56+-(NK-) cells [30]. However, depressed IL-12 production in trauma patients was associated with a shift towards a CD4+(TH2) type pattern of adverse clinical outcome [30].

While numerous reports focus on intravascular lymphocyte populations and function after trauma, the populations crucial for MODS to develop are those in peripheral tissues [30]. The significant decrease of the CD-8+ ratio in the splenic suspension of our FX-SH group is supported by the earlier finding of a decreased ratio of CD-8+ lymphocytes in peripheral tissues after trauma hemorrhage compared to controls [34]. However, we found no report on lymphocyte populations in peripheral tissues after traumatic brain injury. Yet, the WD-TBI group might be comparable to controls for peripheral trauma and hemorrhage (our FX-SH group) and therefore shows higher CD-8+ ratios. Still, this hypothesis does not explain the CD-8+ ratio of the CO-TX group. Restoration of the low CD-8+ ratio after FX-SH by WD-TBI might be speculated on but so far no theory on responsible mechanisms is known. Since this result was rather surprising for our group, detailed evaluation of key processes for lymphocyte populations after combined neuro- and peripheral trauma and hemorrhage must be left for future experiments. CD-56-(NK-) cells were found in a significantly higher concentration in the splenic suspension of our FX-SH group compared to our WD-TBI and CO-TX groups. This also is supported by earlier results as in

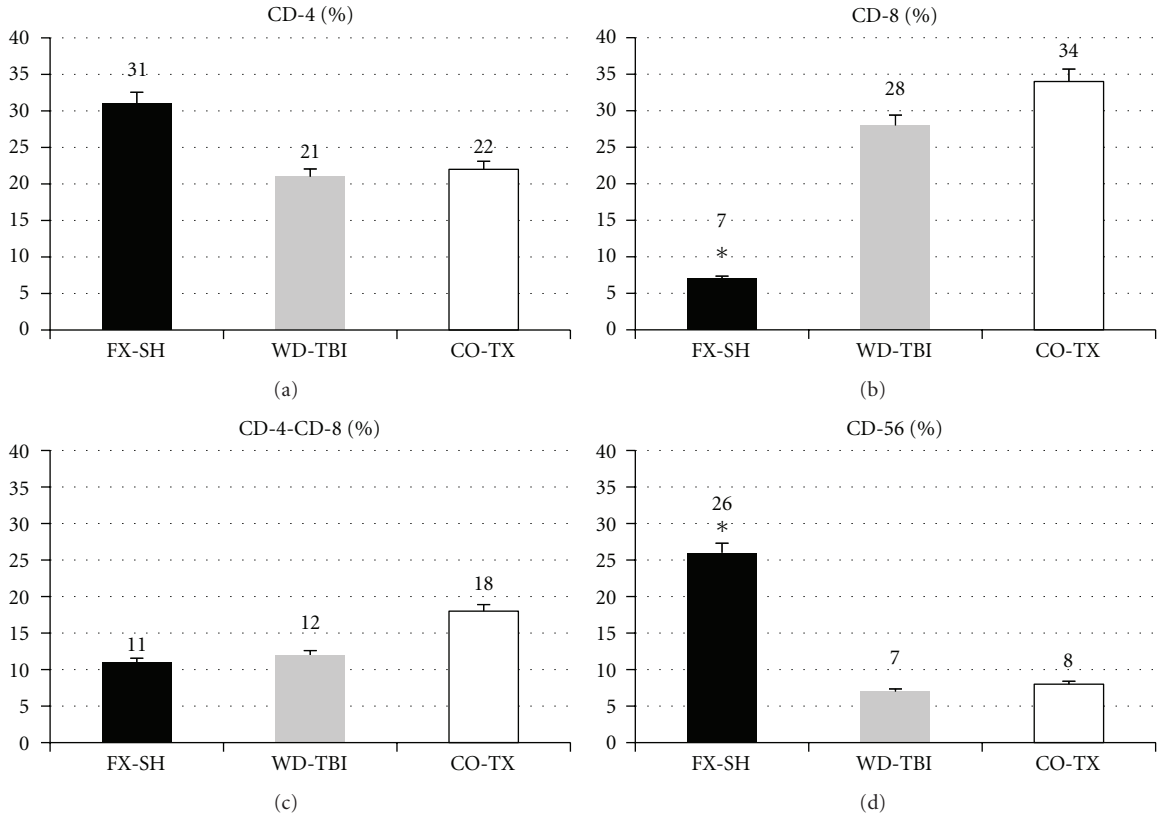

Figure 3: Lymphocyte subpopulations (%). FX-SH: femur fracture and shock; WD-TBI: weight drop traumatic brain injury; CO-TX: combined injuries.

CD-8+ cells [34]. While the same reasons as above for the physiologic CD-56 ratio in our WD-TBI group may be discussed, in the CO-TX group one more aspect needs consideration: raised TNFα levels in a murine sepsis model were associated with an increased ratio of NK cells [35, 36]. However, for CD-56+ cells as well as for CD-8+ cells, the detailed mechanisms and reasons underlying the differences found in our study cannot readily be explained. Further experiments should focus on lymphocyte populations in peripheral tissues after TBI and combined TBI and trauma hemorrhage.

Mortality may just reflect the inflammatory changes and/or trauma load [1, 12]. In humans, injury severity clearly is associated with mortality. Likewise, injury severity was associated with an increased inflammatory response as measured by increased IL-6 levels. So was mortality, an increased IL-6 level in multiply injured patients was associated with increased mortality. IL-6 even was suggested to be used for prognosis in severe trauma [2, 30]. In murine models, other groups support the findings in humans and of our study [12, 14, 37].

5. Conclusion

Our animal model of combined extremity fracture, shock, and closed traumatic brain injury clearly showed that the inflammatory response of the combined trauma is severalfold elevated as compared to the respective monotrauma in terms of humoral parameters. The almost physiological findings for peripheral tissue lymphocyte populations in the isolated TBI and in the combined trauma group as compared to the significantly elevated ratios of the trauma-hemorrhage group are not readily explained and need further evaluation. Mortality in our combined trauma model is clearly associated with trauma load and inflammation and such parallels the findings in multiple trauma patients. This shows the value of our model for future investigations.

Authors' Contribution

C. Probst and M. J. Mirzayan contributed equally to this study.

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Research Article

A New Experimental Polytrauma Model in Rats: Molecular Characterization of the Early Inflammatory Response

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Background. The molecular mechanisms of the immune response after polytrauma are highly complex and far from fully understood. In this paper, we characterize a new standardized polytrauma model in rats based on the early molecular inflammatory and apoptotic response. *Methods*. Male Wistar rats (250 g, 6–10/group) were anesthetized and exposed to chest trauma (ChT), closed head injury (CHI), or Tib/Fib fracture including a soft tissue trauma (Fx + STT) or to the following combination of injuries: (1) ChT; (2) ChT + Fx + STT; (3) ChT + CHI; (4) CHI; (5) polytrauma (PT = ChT + CHI + Fx + STT). Sham-operated rats served as negative controls. The inflammatory response was quantified at 2 hours and 4 hours after trauma by analysis of "key" inflammatory mediators, including selected cytokines and complement components, in serum and bronchoalveolar (BAL) fluid samples. *Results*. Polytraumatized (PT) rats showed a significant systemic and intrapulmonary release of cytokines, chemokines, and complement anaphylatoxins, compared to rats with isolated injuries or selected combinations of injuries. *Conclusion*. This new rat model appears to closely mimic the early immunological response of polytrauma observed in humans and may provide a valid basis for evaluation of the complex pathophysiology and future therapeutic immune modulatory approaches in experimental polytrauma.

1. Introduction

Trauma is still one of the leading causes of death among people aged 45 and younger. The annual economic burden of direct and indirect costs in Germany alone is estimated to be around 40 billion Euros annually [1]. Due to the heterogeneity of trauma, complex injury patterns, and broad variability of therapeutic options, it is an enormous challenge to collect valid data in prospective or retrospective studies concerning posttraumatic pathophysiological changes and possible treatment options.

Of all polytraumatized patients 86% sustain an injury to the extremities, 69% to the head, and 62% to the chest [2]. In this regard, the effects of a combined injury on the patient are not comparable to that after isolated trauma. Trupka et al. indicated that mortality rises from 7% to 18% when an additional trauma to the chest is present. Musculoskeletal trauma induces the systemic release of diverse "danger molecules" DAMPs (danger-associated molecular patterns) [3] which lead to a pronounced early immunological and inflammatory response [4]. The release of these DAMPs is a tremendous challenge for the immune system in

severe trauma as explained by the "danger model" of the immune system (danger sensing, transmission, response, elimination) [3, 5]. As a consequence of the injury severity and the immune status of the patients, the posttraumatic inflammatory response often results in an overindulging and uncontrolled activation of the complement system with an increased release of proinflammatory mediators [6] often resulting in multiple organ dysfunction and death. In this regard, IL-6 has been shown to be elevated at the scene of injury and a positive correlation between these IL-6 levels, the severity of the injury and the increased rate of complications including enhanced mortality has been demonstrated [7, 8]. In addition, increased systemic levels of the anaphylatoxins C3a and C5a in severe polytraumatized patients seem to be correlated with a higher risk of sepsis and a poor clinical outcome [6, 9]. However, up to date, little is still known about the immune response after severe polytrauma on a cellular and molecular basis. In particular, it is still unclear whether the early immune response is qualitatively or quantitatively different after severe polytrauma compared to an isolated injury. Furthermore, there is a lack of pathophysiology-based therapeutic remedies for polytrauma patients to prevent posttraumatic immunosuppression. Therefore, highly standardized experimental polytrauma models are required to define the early complex pathophysiology of severe combined trauma and to examine novel surgical or immunomodulatory treatment options.

In this study, we describe a new polytrauma model in rats and characterize the early systemic and local inflammatory response accompanied by a rapid activation of the complement system similar to that seen in humans.

2. Materials and Methods

2.1. Animals and Anesthesia. The study protocol was approved by the University Animal Care Committee and the federal authorities for animal research, Tuebingen, Germany. The experiments were performed in adherence to the National Institutes of Health Guidelines for the use of laboratory animals including a total of 352 male Wistar rats (250 g, 10–12 weeks, Jackson Laboratories, Bar Harbour). Anaesthesia was applied i.p. using 75 mg/kg Ketamin (Ketavet, Pfizer Pharma, Karlsruhe, Germany) and 0.4 mg/kg KG Medetomidine i.p. (Dormitor, Pfizer Pharma, Karlsruhe, Germany). Animals which underwent blunt chest trauma were anesthetized with a mixture of 4% sevoflurane (Sevorane Abbott, Wiesbaden, Germany) and 96% oxygen under a continuous flow of 2 L/min and received the aforementioned i.p anesthesia after the chest trauma.

2.2. Individual Trauma Models. The rats were randomly assigned to the different trauma groups (each $n = 6$–10). Narcotized rats underwent either a Sham operation (Sham), a blunt bilateral chest trauma (ChT), a blunt bilateral chest trauma and a lower leg (tibia/fibula) fracture with a contralateral soft tissue trauma (ChT + Fx + STT), a closed head injury (CHI), a bilateral blunt chest trauma and a head injury (ChT+CHI), or a combination of chest trauma, head trauma, fracture, and soft tissue trauma referred to as polytrauma (PT). Sham animals were anesthetized rats with an incision and surgical closure of the aponeurotic galea. All animals were placed on a heating pad after undergoing the surgical procedures. Continuous reflex status and vital signs of all animals were checked.

Animals subjected to the blunt bilateral chest trauma were narcotized and fixed in a supine position. The trauma was induced by a single blast wave as previously described [10–12]. The traumatic brain injury was induced by a weight drop device inducing a focused blunt injury over an intact skull after 1.5 cm incision of the aponeurotic galea as described in detail elsewhere [13, 14]. After trauma, the incision was closed by a monofil suture. For the right side tibia/fibula fracture a weight drop device (650 g, 13 cm) induced a reproducible closed transverse fracture of the lower leg, as reported in the past [15]. On the contralateral gastrocnemic region a soft tissue injury was applied using a weight drop device (170 g, 180 cm) as previously described [16]. Vital signs were documented during the whole experiment and blood gas analysis was performed. Waking the animals after trauma was strictly avoided and anaesthesia was deepened with repeated i.p. injections as necessary. After 2 hrs or 4 hrs, respectively, all rats were sacrificed and the blood and organs harvested.

2.3. Blood-, Lung-, and BAL-Fluid Preparation. Whole blood was spun at 500 ×g at 4°C for 10 min, the serum stored at −80°C until final analysis. After death the trachea was dissected and cannulated, the left lung clamped, and the right lung flushed 3 times with 5 mL PBS including 1 : 1000 broad spectrum protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA). The BAL fluids were then centrifuged 450 ×g at (4°C) for 10 min and stored at (−80°C) until analysis. Left lung tissue was filled with formalin after BAL was taken and immediately stored in 10% formalin until evaluation.

2.4. Cytokine ELISA. Interleukin (IL)-6, tumor necrosis factor (TNF)-α, complement anaphylatoxin 3a (C3a), monocyte chemoattractant protein (MCP)-1 (all BD OptEIA ELISA SET, BD Pharmingen, San Diego, CA), and cytokine-induced neutrophil chemoattractant (CINC) (R&D, Minneapolis, MN, USA) concentrations of BAL-fluids were determined by sandwich-enzyme-linked immunosorbent assay technique (ELISA) according to the manufacturer's recommendation. In serum, IL-6, TNF-α, and CINC were detected.

2.5. Serum Complement Hemolytic Activity (CH 50). The activity of the complement system was assessed by CH50 measurements. A dilution series of the samples was made (1/20–1/480) using Tris-buffered saline (TBS) and the diluted samples were incubated for 60 min at 37°C with sheep erythrocytes (Colorado Serum Company, Denver, CO, USA). The hemolytic complement system reaction was stopped using ice-cold TBS and centrifugation at 700 ×g for 5 min. The absorption of the supernatant was measured at 541 nm by spectrophotometry.

2.6. Flow Cytometry Analysis. EDTA whole blood (100 μL) was incubated with indicated FITC- or PE-labelled fluorochrome-conjugated monoclonal antibodies at room temperature for 20 min in the dark. Immediately after the incubation period, 2 mL of FACS lysing solution (BD Biosciences) was added to each tube, followed by incubation for 10 min at room temperature in the dark. The tubes were then centrifuged for 5 min at 340 ×g. After centrifugation, the supernatant was removed, and 2 mL of Dulbecco phosphate-buffered saline (DPBS) per tube was added, followed by an additional centrifugation step. After a second washing step, the cell pellet was resuspended in 100 μL of CellFIX Solution (BD Biosciences) for final flow cytometric analysis. Leukocyte populations (neutrophils, monocytes, and lymphocytes) were discriminated by forward/sideward scatter and additional CD45 staining. For each measurement, a minimum of 10,000 events was analyzed. For quantification of C3aR and CRegs (CD35, CD55, and CD59) expression, mean fluorescence intensity (MFI) emitted by the FITC- or PE-conjugated antibodies was calculated by subtracting the corresponding isotype control.

2.7. Histological Evaluation. Formalin-fixed lung tissue was dehydrated using ethanol, dissolved with Xylol and embedded in paraffin. Sections of 1 μm were prepared followed by H&E staining. Following that a semiquantitative analysis of the sections including a cell count was performed.

2.8. Preparation of PMNs. Whole blood of rats was drawn from the portal vein. Blood was transferred into syringes containing EDTA. Polymorphonuclear neutrophils (PMNs) were isolated using Ficoll-Paque (Pharmacia Biotech, Stockholm, Sweden) gradient centrifugation (340 ×g, 30 min, room temperature). The use of this technique may be responsible for some artificial activation of cells in vivo [17]. For neutrophil isolation, red blood cells (RBCs) were sedimented with dextran and residual RBCs were removed by hypotonic lysis. Neutrophils were resuspended in DPBS and finally diluted at 2 Mio cells per mL. 1 mL aliquots of PMNs were centrifuged at 800 g for 5 min, supernatants were removed, and cells were resuspended in 100 μL of Pharmigen Cell Lysis Buffer (BD Biosciences). After 30 min of incubation on ice, samples were pelleted (16000 ×g, 5 min, 4°C) and supernatants were frozen at −80°C.

2.9. Statistics. Results are presented as mean ± SD. A one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test as a post hoc test for multiple comparisons was performed to determine significant differences between experimental means. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Hematologic Findings. The blood gas analysis (2 and 4 hrs after injury) was indicative of stable Hemoglobin (Hb) and Hematocrit (Hct) values in this polytrauma model. The Hb values did not significantly differ in Sham and PT animals (Sham 2 h 16.5 ± 0.4 g/dL versus PT 2 h 16.2 ± 0.4 g/dL Sham 4 h 16.5 ± 0.32 g/dL versus PT 4 h 15.4 ± 0.4 g/dL; data not shown). The pO_2 levels stated no hypoxic state in the anaesthetized animals (Sham 4 h 87.7 ± 2.0 mmHg versus PT 4 h 90.0 ± 3.6 mmHg, data not shown).

3.2. Systemic Inflammatory Response following Trauma. As early as 2 hrs after injury, systemic IL-6 levels were not significantly increased in PT animals compared to the sham group. However, 4 hrs after polytrauma there was a significant increase in the IL-6 concentrations (Figure 1(a)). Similarly, CINC serum concentrations were significantly enhanced in ChT + Fx + STT, ChT + CHI, and PT groups compared to the Sham group 4 hrs after injury (Figure 1(b)). Systemic TNF-α levels were rather unchanged in all groups up to 4 hr after injury (Figure 1(c)).

3.3. Local Inflammatory Response following Trauma. In addition to the systemic inflammatory response, the local inflammatory changes were investigated with focus on the lungs. The local concentrations of IL-6 in the BAL fluids were increased in PT, ChT + CHI and ChT rats 4 hrs after injury compared to Sham-treated rats (Figure 2(a)). Four hours after trauma, the local TNF-α levels were increased in all trauma groups except CHI alone (Figure 2(b)). CINC and MCP-1 levels in BAL-fluids were significantly increased in all groups compared to Sham and CHI animals 4 hrs after injury (Figures 2(c) and 2(d)).

3.4. Histological Changes in Lung Tissues after Trauma. Early morphological changes were assessed on H&E stained lung tissue sections analyzed with 20x magnification. Lung sections of the control animals showed physiological lung parenchyma identical to the morphology seen in the traumatic brain injury (CHI) group. After bilateral blunt chest trauma plentiful erythrocytes could be found intra-alveolar, intra-bronchial and sub-pleural. In addition an increased number of alveolar macrophages, some damage to the alveolar wall and tissue edema were found. Similar qualitative morphological changes could be detected in PT-animals but all in a markedly aggravated intensity (Figure 3).

3.5. Complement Response after Trauma. The trauma-induced changes of the complement system were first screened by CH50 testing (Figure 4(a)). After 2 hrs there was a minimum decrease in CH50 values in the PT-group compared to the Sham group, but after 4 hrs this difference reached statistically significance, indicating systemic complement activation. Focusing on the central complement activation product C3a, BAL-fluid levels of C3a were significantly increased in PT animals compared to Sham animals 4 hrs after trauma (Figure 4(b)). The related C3aR revealed a significant loss of surface expression on neutrophils isolated 4 hrs after polytrauma, whereas the C3aR profile remained rather unchanged on monocytes and lymphocytes (Figure 4(c)). Protectin, also known as CD59, exhibited no difference in the expression on neutrophils after trauma (Figure 5). In contrast, complement receptor 1 (CD35) was

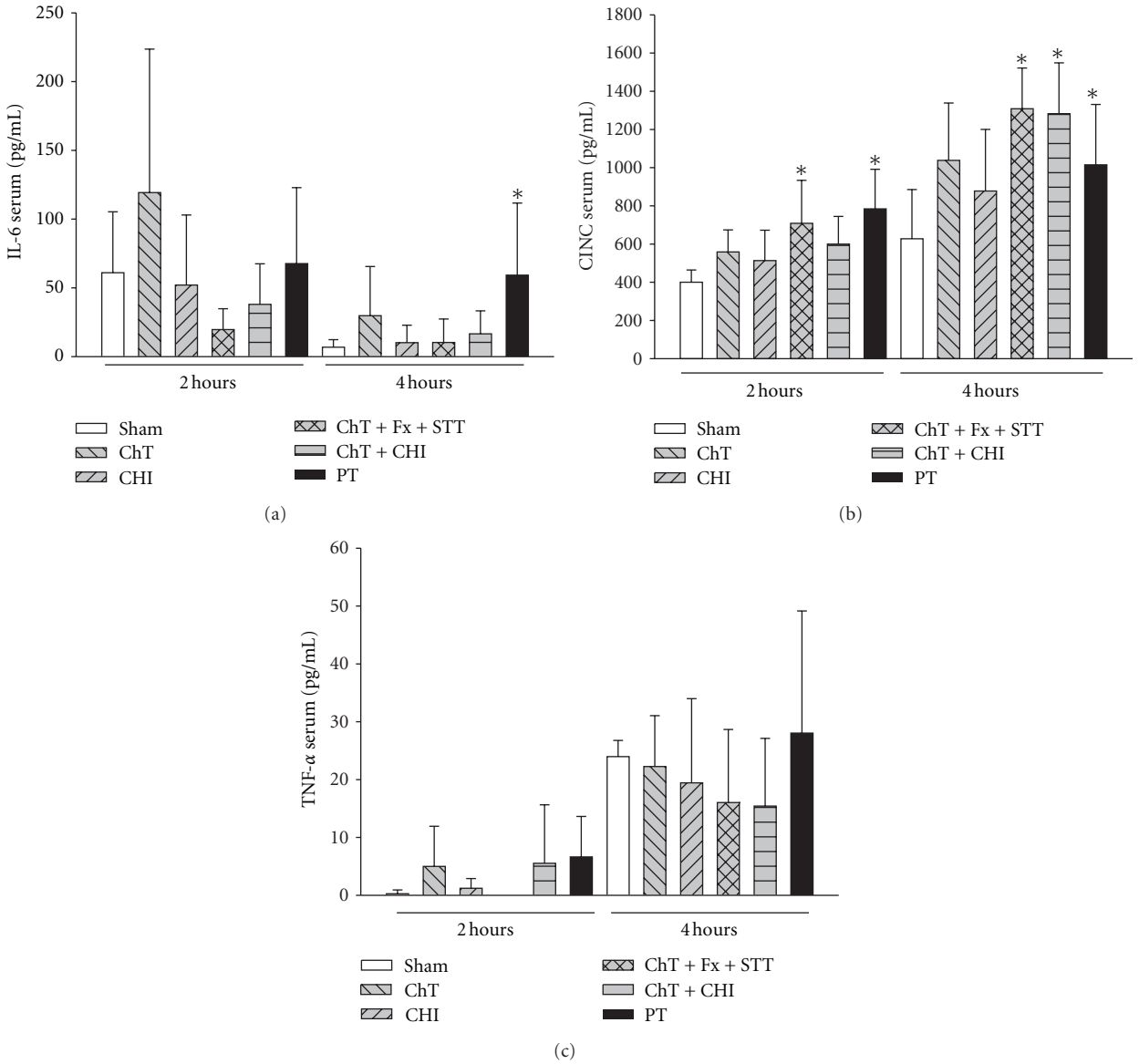

Figure 1: *Systemic inflammatory response following trauma*, (a) shows the systemic IL-6 (pg/mL) serum levels in Sham versus PT animals 2 and 4 hrs after trauma, (b) the CINC (pg/mL) serum concentrations in Sham versus PT animals 2 and 4 hrs after trauma and (c) the TNF-α (pg/mL) serum levels in Sham versus PT animals 2 and 4 hrs after trauma. All data are presented as mean ± SD. $^*P < 0.05$. $n = 6$–10 rats/group.

significantly decreased in PT animals versus Sham rats (Figure 5). A slight but insignificant decrease in expression of CD55 could be detected on neutrophils in PT-rats (Figure 5).

4. Discussion

The clinical and molecular danger management after polytrauma is rather complex and often associated with fatal complications. However, the resulting immune and inflammatory response is still in the dark. The aim of this study was to establish a highly standardized and reproducible rodent polytrauma model capable of closely mimicking the posttraumatic inflammatory response found in humans.

Patients at the scene are dying mostly based on severe brain injury or massive blood loss following an injury to a large blood vessel. In contrast, mortality of patients in the hospital several days after major trauma is often associated with a severe systemic inflammatory response (SIRS) and resulting systemic changes such as coagulopathy and complementopathy, breakdown of physiological barriers, impairment of the immune defense, and finally organ dysfunction and failure [6]. The proinflammatory cytokines (e.g., IL-6, IL-8, etc.) and complement anaphylatoxins (e.g., C3a, C5a) were experimentally and clinically proposed to be crucially involved in the early damage response after severe trauma and somehow indicative of the degree of tissue damage and clinical outcome [6, 9]. In the cecal

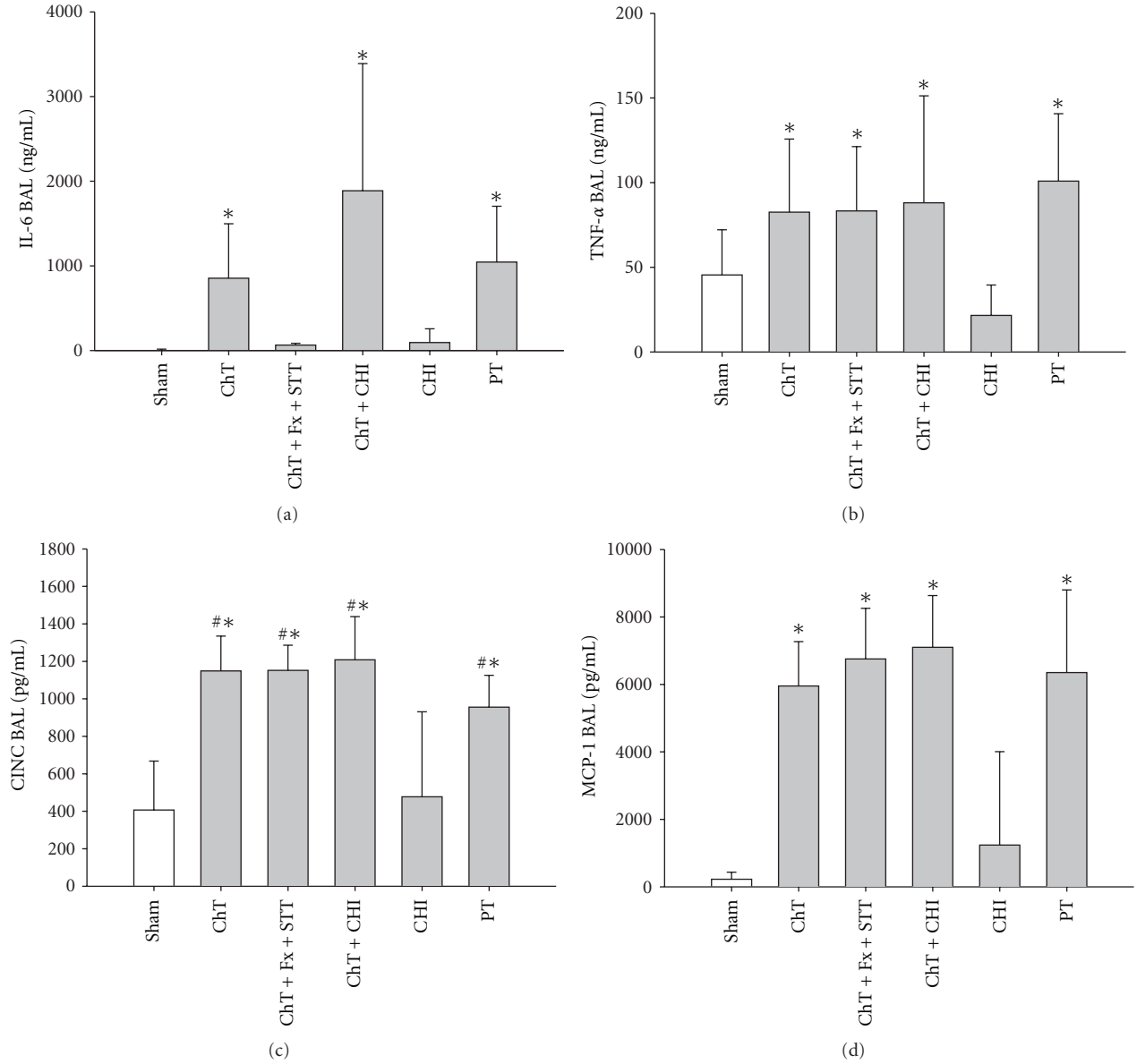

Figure 2: *Local inflammatory response following trauma,* (a) shows IL-6 (ng/mL) BAL-Fluid levels in Sham-, ChT-, ChT + Fx + STT-, ChT + CHI-, CHI- and PT-rats 4 hrs after trauma, (b) TNF-α (ng/mL) BAL-Fluid levels in Sham-, ChT-, ChT + Fx + STT-, ChT + CHI-, CHI- and PT-rats 4 hrs post trauma, (c) CINC (pg/mL) BAL-Fluid concentrations in Sham-, ChT-, ChT + Fx + STT-, ChT + CHI-, CHI- and PT-rats 4 hrs after trauma and (d) MCP-1 BAL-Fluid levels in Sham-, ChT-, ChT + Fx + STT-, ChT + CHI-, CHI- and PT-rats 4 hrs after trauma. All data are presented as mean ± SD. $^{*}P < 0.05$ to Sham; $^{\#}P < 0.05$ significant to CHT. $n = 6$–10 rats/group.

ligation and puncture (CLP) sepsis model in rodents, the complement activation product C5a has been shown to contribute significantly to the development and progression of the systemic inflammatory response. Furthermore, blockade of the C5a-C5aR interaction was associated with improved function on a molecular, cellular, and organ-level and finally resulted in a beneficial outcome [18–20]. However, it is rather unclear if a similar immune modulation would provide some advantageous effects in the setting of multiple injuries. This needs to be investigated further using this new polytrauma model. In the context of the complexity of the polytrauma danger response and the lack of effective therapeutics in the early posttraumatic phase, it is somehow surprising, that despite highly effective anaesthesia protocols there is a worldwide lack of valid rodent models closely simulating multiple injuries of humans. The present blunt trauma model was designed to combine clinically important injury patterns. The single traumata used has been well established and are highly standardized and reproducible in rodents [11–13, 15, 21, 22]. Upon adjustment to the combined injury application, this model provides the unique opportunity to study the impact of an individual trauma and different trauma combinations on the following immune response. A lethality of up to 20% could be detected in

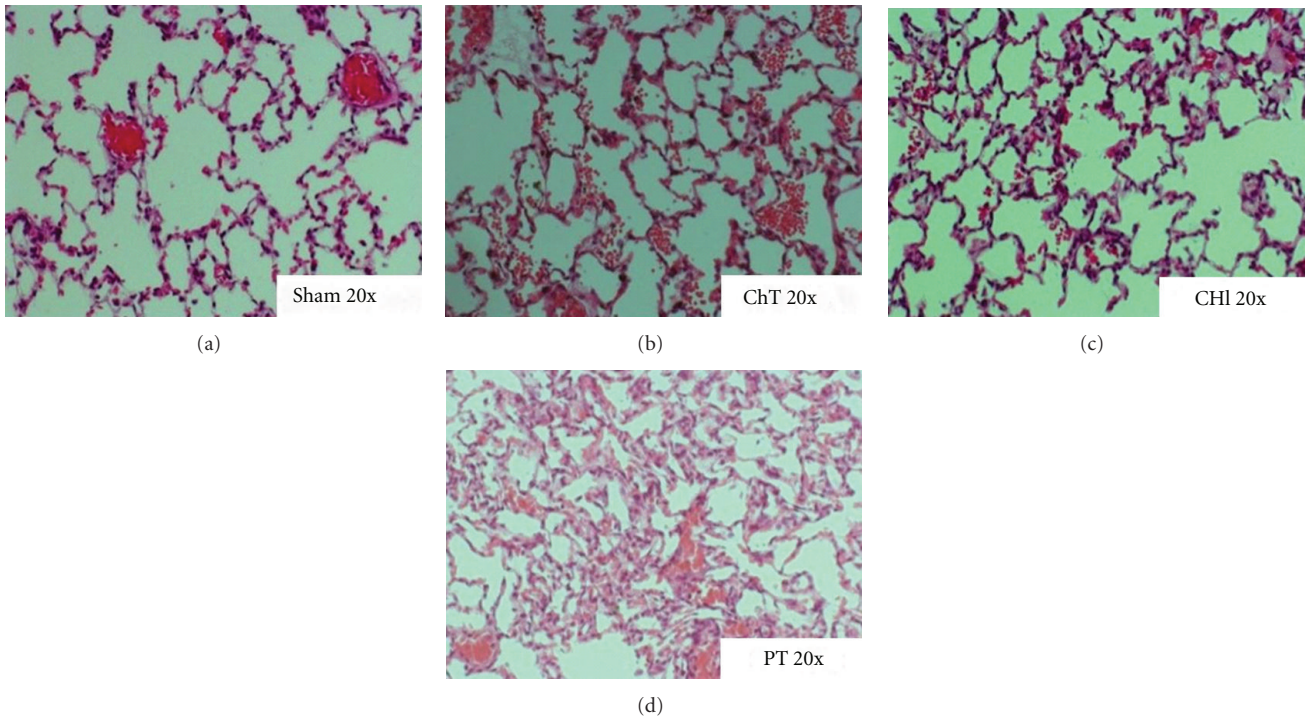

Figure 3: *Histological changes in lung tissues after trauma,* H&E stained lung tissue sections of Sham-, ChT-, CHI and PT-rats analysed by light microscopy with 20x amplification.

this model which reflects the lethality of polytraumatized humans (annual report of the German trauma register 2009). Care has been taken to first design a hemodynamically stable polytrauma model to rule out hemorrhagic shock effects on the inflammatory response. According to Sauaia et al. hemorrhage following traumatic injury accounts for 30–40% of deaths [23]. This could be excluded by BGA measurements showing stable haemoglobin and hematocrit values. However, hemorrhage/shock as well as abdominal trauma certainly represents certainly a major trigger of the inflammatory response [24]. Thus, pressure-controlled hemorrhage or CLP may be included in this model in future studies to discriminate their contribution to the inflammatory response as well.

The early systemic inflammatory response in the present multiple injury model was reflected by enhanced serum levels of IL-6 and CINC which are also enhanced in polytrauma patients [25, 26] and somehow associated with the injury severity and predictive of the clinical outcome. In comparison to Sham-treated animals, the systemic TNF-α levels of PT littermates were rather unchanged in the early posttraumatic phase. In contrast, TNF-α levels in multiply injured patients were found to be increased early after trauma [27] but rather irrelevant for the prediction of injury severity or outcome. In ex vivo experiments, even a decrease in TNF-α levels in stimulated whole-blood samples from polytraumatized patients 2–4 hrs after trauma was reported in relation to healthy donor samples [28].

The local inflammatory polytrauma response with focus on the lungs as "the engine of multi-organ dysfunction" [2] was reflected by increased concentrations in BAL fluids of cytokines and chemokines early after trauma. Similar chemokine profiles have been reported for blunt chest trauma alone by our group [29]. In the clinical setting of blunt chest trauma, there is a lack of data in BAL-fluids [30] but a distinct increase of these cytokines in whole blood with an overall correlation with lung injury severity [8, 26]. Present histological analysis of polytraumatized rats revealed severe intra-alveolar, intrabronchial, and subpleural hemorrhage as well as the presence of interstitial oedema, atelectasis, and an increased amount of alveolar macrophages. These changes have been described in experimental blunt chest trauma alone [22], but in PT animals an aggravation of the histological alterations is evident. The pathophysiological reasons for this enhanced lung injury are rather speculative and might be due to the accompanied brain injury with subsequent disturbances of the neurohormonal-stress-inflammation axis or due to a specific mediator release by the fracture. Fremont et al. described that polytraumatized patients with systemically increased plasma levels of TNF-α, IL-8, or IL-10 are more likely to develop ARDS [31].

The complement response globally screened by CH50 changes was unaltered early after polytrauma. However, 4 hrs after multiple injuries, a decent but significant decrease in CH50 was observed. It is noteworthy that already a reduction in CH50 by 10% can be considered as evidence of significant systemic complement activation. These findings are supported by recent reports showing complement activation after major trauma in humans [32].

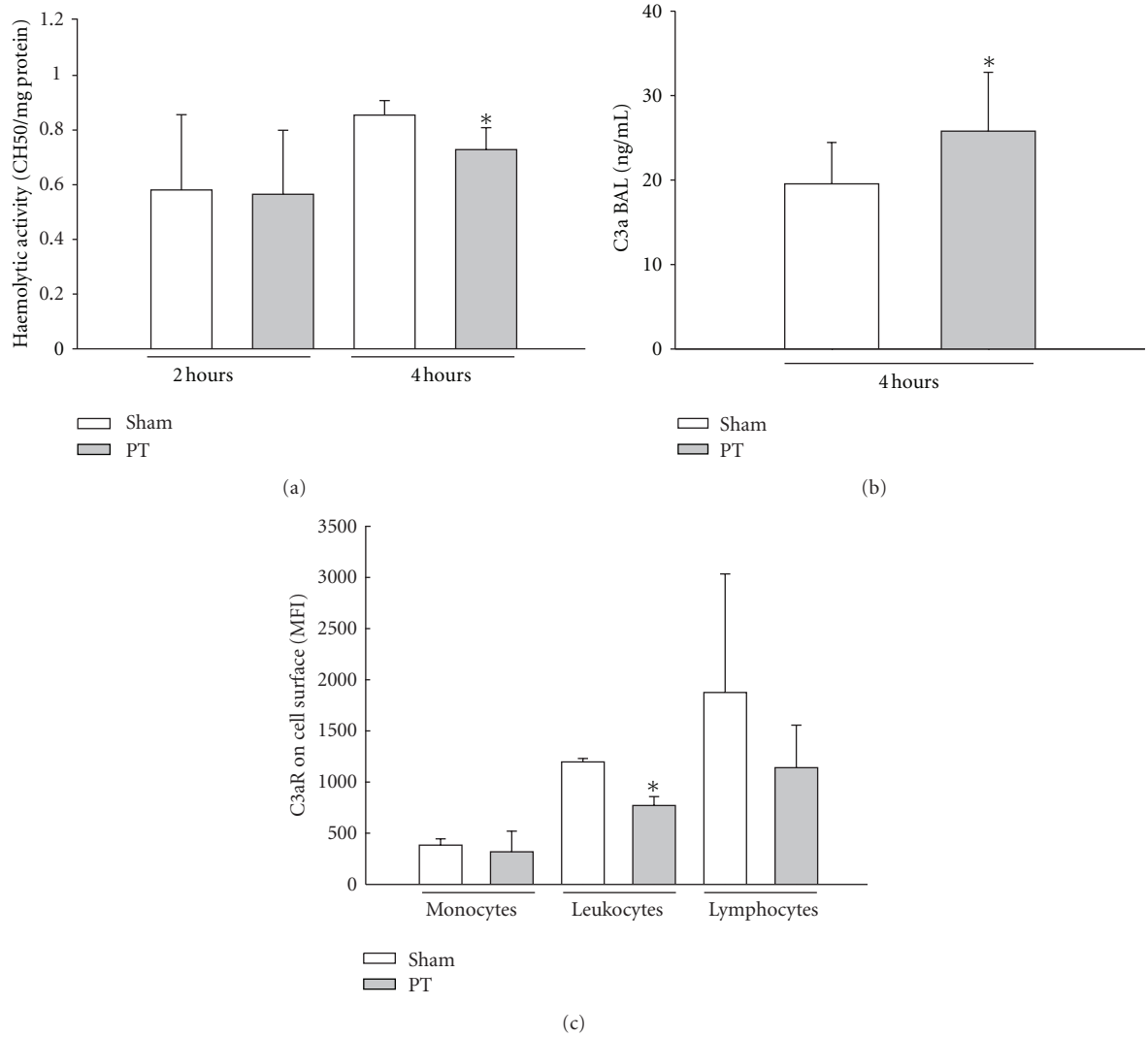

Figure 4: *Systemic complement response after trauma,* (a) presents the hemolytic activity (CH50/mg) on Sham- and PT-rats 2 and 4 hrs after trauma, (b) the C3a (ng/mL) BAL-Fluid levels in Sham- versus PT-rats 2 and 4 hrs post trauma and (c) the C3aR Expression (MFI) on Monocytes, Neutrophils and Lymphocytes in Sham- and PT-rats 4 hrs after trauma. All data are presented as mean ± SD. $^*P < 0.05$. $n = 6$–10 rats/group.

Complement regulation after severe tissue trauma has been recently addressed by our group, describing a specific leukocyte expression profile of the complement regulatory proteins (CRegs) CD55, CD59, and CD35 early after polytrauma. Whereas CD59 and CD55 were rather unchanged, CD35 expression on neutrophils in the PT versus Sham-group was significantly reduced. The dynamic of CReg expression beyond the 4 hr observational period remains to be seen. However, based on the extremely heterogenous patient groups and the complexity of the pathophysiological reaction, the apoptotic response has been reported rather controversially in polytrauma patients [33, 34]. Guo et al. described during sepsis induced by CLP a C5a-induced decrease of the neutrophil apoptosis rate along with increased levels of Bcl-XL and decreased levels of Bim [35]. Addressing the posttraumatic regulation of the reduced neutrophil apoptosis rate further mechanistic studies need to be performed in the present novel polytrauma model.

The present study has some limitations. During the short observation period, 4 hrs maximum according to the Animal Care Committee protocol, after injury the organisms may be incapable to building up the full inflammatory response especially in the absence of shock symptoms. In addition, the applied traumata and early posttrauma phase occurred in deep anaesthesia, which certainly exclude an additional stress-load seen in reality. On a technical level, although performed within 7 min, the traumata could not be applied simultaneously as it usually occurs in reality.

The role of each single trauma on the pathophysiology of polytrauma leading to SIRS, Sepsis, and MOF is still unknown. However, each isolated injury is survivable, the combination may become lethal. The patient's prognosis

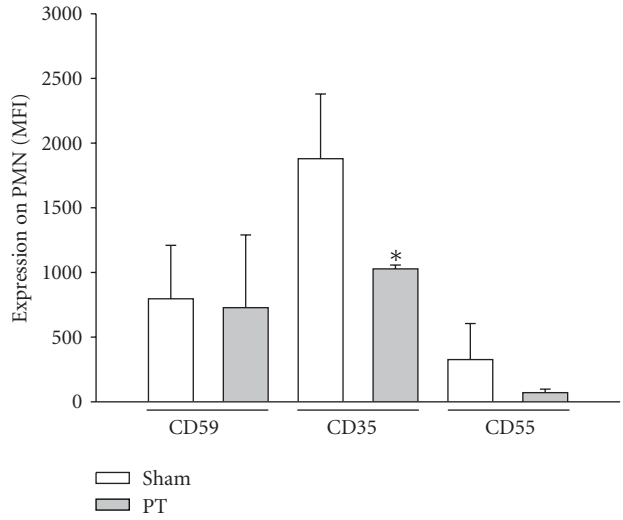

Figure 5: *Complement regulatory response after trauma,* CReg Expression (MFI) CD59, CD35 and CD55 on neutrophils (PMNs) in Sham- and PT rats 4 hrs after trauma. All data are presented as mean ± SD. $^*P < 0.05$. $n = 6$–10 rats/group.

and the outcome mainly depends on the presence of the traumatic brain injury and the associated secondary brain damage [36].

In summary, this study is—to our knowledge—the first to demonstrate very early inflammatory changes in a highly standardized, reproducible, hemodynamically stable polytrauma rodent model, mimicking most important injuries of polytraumatized patients excluding hemorrhagic shock. The present polytrauma model may therefore represent the basis for further investigation of the pathophysiology of polytrauma and the evaluation of early therapeutic interventions.

Conflict of Interests

The authors declare that they have no competing interests as defined by *Journal Mediators of Inflammation* or other interests that might be perceived to influence the results and discussion presented in this paper.

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Research Article

Immunophenotyping of Posttraumatic Neutrophils on a Routine Haematology Analyser

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Introduction. Flow cytometry markers have been proposed as useful predictors for the occurrence of posttraumatic inflammatory complications. However, currently the need for a dedicated laboratory and the labour-intensive analytical procedures make these markers less suitable for clinical practice. We tested an approach to overcome these limitations. *Material and Methods.* Neutrophils of healthy donors were incubated with antibodies commonly used in trauma research: CD11b (MAC-1), L-selectin (CD62L), FcγRIII (CD16), and FcγRII (CD32) in active form (MoPhab A27). Flow cytometric analysis was performed both on a FACSCalibur, a standard flow cytometer, and on a Cell-Dyn Sapphire, a routine haematology analyser. *Results.* There was a high level of agreement between the two types of analysers, with 41% for FcγRIII, 80% for L-selectin, 98% for CD11b, and even a 100% agreement for active FcγRII. Moreover, analysis on the routine haematology analyser was possible in less than a quarter of the time in comparison to the flow cytometer. *Conclusion.* Analysis of neutrophil phenotype on the Cell-Dyn Sapphire leads to the same conclusion compared to a standard flow cytometer. The markedly reduced time necessary for analysis and reduced labour intensity constitutes a step forward in implementation of this type of analysis in clinical diagnostics in trauma research.

1. Introduction

Trauma is a major cause of morbidity and mortality in people under the age of 50 in the western world [1]. This can be a direct result of the trauma and injury itself, or the post-injury immunological complications [2]. In response to tissue damage, due to trauma, an excessive immune reaction occurs. Dysfunctional polymorphonuclear (PMN) leukocytes play a clear role in the excessive immune response. This overwhelming immune response is considered to be a major risk factor in the development of posttraumatic organ failure [3, 4]. However, there remains a lack in the full understanding of the critical mechanisms and the identification of patients at risk for the development of systemic complication after trauma [5].

Inflammatory markers have been proposed as useful predictors for the occurrence of acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS) [6]. The development of immune status monitoring of trauma patients will not only help in the selection of patients at risk for posttraumatic complication but also may help in the choice of the most effective treatment protocol [7–9].

There are several markers indicated as possible indicators to predict the clinical course and clinical outcome of the trauma patient [10]. Our laboratory previously has shown that fMLF-induced active FcγRII (MhoPhab A27) can aid in early prediction which trauma patients are prone to develop inflammatory complications [10, 11]. This and other research on posttrauma immune responses depend on inflammatory markers measured by fluorescent monoclonal antibodies in a flow cytometer. However, experimental studies analysing inflammatory markers with flow cytometry need a dedicated laboratory and intensive laboratory work which is

required for these analysis. This makes these inflammatory markers less attractive in clinical practice [5, 12]. Therefore, there is an indisputable need for a quick and easy-to-use, reliable, and cost-effective diagnostic analyser. So more research on the mediator cascade an diagnostic determination of trauma patients at risk can take place.

In most clinical laboratories of many hospitals worldwide, the routine analysis of blood is performed with an automated haematology analyser designed for counting peripheral blood cells in whole blood samples. One of these machines, the CELL-DYN Sapphire (CD-Sapphire), also offers fluorescent flow cytometry capacities. Compared to a conventional flow cytometer, the CD-Sapphire could provide a greater degree of automation and could thus be operated with relatively little training [13].

We, therefore, evaluated the performance of the CD-Sapphire in comparison to our routine flow cytometer for analysis of inflammatory markers.

2. Material and Methods

2.1. Reference Flow Cytometer. The reference flow cytometer used during this study was the FACSCalibur (Becton Dickinson, Mountain View, CA, USA). The FACSCalibur uses an argon gas laser at a fixed emission of 488 nm. The instrument is capable of detecting six parameters: forward scatter (FSC), side scatter (SSC), and three fluorescent emissions (FL-1: 530 ± 30 nm, FL-2: 585 ± 42 nm, and FL-3: >670 nm) utilizing the first laser. A smaller diode laser emitting red light at 635 nm is used to detect within the fourth fluorescent detector (FL-4: 661 ± 16 nm). Cells can be identified according to their specific forward- and side-scatter signals [14].

2.2. Cell-Dyn Sapphire. The CD-Sapphire (Abbott Diagnostics, Santa Clara, CA, USA) is a routine haematology analyser which uses spectrophotometry, electrical impedance, laser light scattering (multi angle polarized scatter separation, (MAPPS)), and 3 color fluorescent technologies to classify blood cells. There are detectors that are used for optical scatter (Axial Light Loss (ALL), 0°, cell size) and Intermediate Angle Scatter (IAS), 7°, cell complexity. Moreover, the analyser is equipped with an integrated fluorescence (488 blue diode) laser and three fluorescent detectors (FL-1: 530 ± 30 nm, FL-2: 580 ± 30 nm, and FL-3: 630 ± 30 nm). Fluorescence channels FL-1 and FL-2 are not routinely utilized. Therefore, they could be used for immunophenotyping [15]. The FL-3 fluorescence channel is used to quantify nucleated red blood cells and leukocyte viability with a propidium iodide staining. However, in a fresh sample, the large majority of cells is viable, and, therefore, also FL-3 could be used for the analysis of other fluorochromes.

The CD-Sapphire has a fully automated modus (CD3/4/8 modus) designed for sample preparation and determination of fluorescent characteristics of T-cell subsets, namely, $CD3^+CD4^+$ T-Helper and $CD3^+CD8^+$ T-suppressor cells [16]. The CD3/4/8 procedure uses three consecutive rack positions, where the first position is occupied by the patient sample and the second and third position are, respectively, occupied by the directly labelled CD3/CD4 and CD3/CD8 antibody mixtures. The analyser takes fixed volumes of blood from the first (patient) sample tube and injects this into the two reagent tubes. After mixing and a timed incubation period, aliquots of the blood antibody mixtures are aspirated and diluted prior to passage through the optical flow cell where measurements of the optical scatter and fluorescence are made. No sample washing is required, and red cell lysis is incorporated by the analyser in the automated procedure. The total time required for analysis and data acquisition is eight minutes.

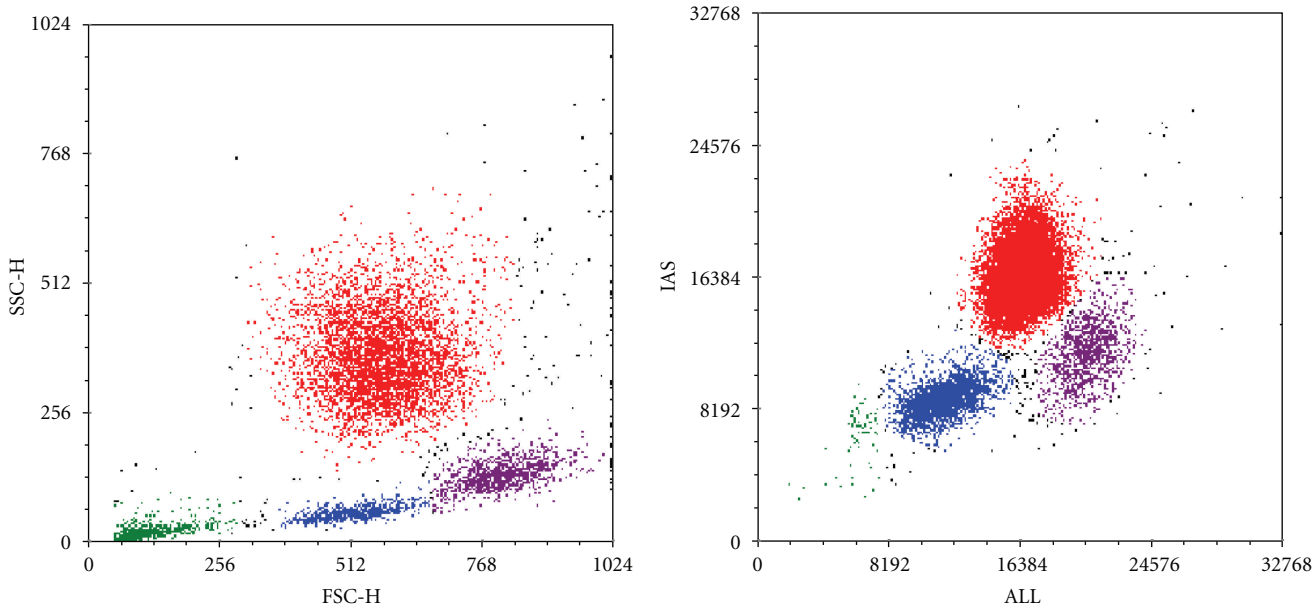
The hardware and software configurations of the CD-Sapphire can be adapted to enable using this modus for alternative fluorescent reagents [15].

2.3. Sampling and Analysis. Blood (9 mL) of ten healthy adult donors was collected in sodium-heparine coated sterile tubes (Vacuette). Within one hour of sampling, blood samples were analysed for neutrophil phenotype with the use of flow cytometry. The analysis of neutrophil receptor expression profiles was described previously [11]. In short, the samples were cooled on melting ice and kept on ice during the whole procedure. Directly labeled monoclonal antibodies were added to whole blood according the recommendations of the manufacturer and incubated for 60 minutes on ice. We chose four antibodies that are known for their significant importance in research in trauma patients: RPE-labeled CD11b (clone 2LPM19c) from Dako, Glostrup, Denmark, Alexa Fluor 647-labeled CD16 (clone 3G8), from Scientific Group, BD Pharmingen, Milnerton for the experiment on the CD-Sapphire and ECD-labeled CD16 (clone 3G8) for the experiment on the FACSCalibur, from Beckman Coulter, Marseille, France, FITC-labeled CD62L (clone Dreg56) from BD Pharmingen, USA, and FITC-labeled A27, a monoclonal phage antibody, which recognizes active FcγRII (active CD32), was manufactured in the Department of Respiratory Medicine at the University Medical Centre Utrecht (clone MoPhab A27) [17].

After incubation, the red cells were lysed with ice-cold isotonic NH_4Cl. After a final wash with PBS2+ phosphate-buffered saline with added sodium citrate (0.38% wt/vol) and pasteurized plasma proteins (10% vol/vol), each of the samples were both analysed on the reference flow cytometer and on the CD-Sapphire.

To test the applicability of the fully automated CD3/4/8 modus, whole blood samples of ten healthy volunteers were stimulated at 37°C for 5 minutes with N-formyl-methionyl-leucyl-phenylaline (fMLF 10^{-6} mol/L). After stimulation, the samples were kept on room temperature until analysis with the CD3/4/8 modus. As described previously [15, 18], the two reaction tubes normally containing CD3/CD4 antibodies were replaced by two barcoded nonanticoagulate Vacutainer tubes, containing FITC-labeled A27, the antibodies directed against active FcγRII.

2.4. Data Processing. On completion of analysis on the CD-Sapphire and FACSCalibur, raw data files were downloaded and transferred to a PC for manual software analysis (FCS



(a) FACSCalibur (b) Cell-Dyn Sapphire

Figure 1: Morphological display of leukocytes. Representative example of the morphological plots of a blood sample from a healthy volunteer on (a) a FACSCalibur and (b) a Cell-Dyn Sapphire. The cells in red are the neutrophils, the purple population are the monocytes, the blue cells are lymphocytes, and the puple in green is debris. (a) Forward Scatter (FSC)—Side Scatter (SSC) display from a FACSCalibur. FSC represents the size/cell volume of cells, SSC represents the inner complexity of the particle. (b) Multi Angle Polarised Scatter Separation (MAPSS) leukocyte differential plot from a Cell-Dyn Sapphire. Axial Light Loss (ALL) is an indicator of cell size, Intermediate Angle Scatter (IAS) represents the cell complexity.

Express Version 3; De Novo Software, Thornhill, Ontario, Canada) of fluorescence.

2.5. Statistical Analysis. Data from individual experiments are depicted as median fluorescence intensity (MFI) in arbitrary units (AUs) and standard deviation of at least 5.000 events. Results in figures are presented as means ± standard error of means (SEMs), unless otherwise specified. To test the association between results from the two flow cytometers, a Spearman correlation coefficient was defined. Statistical analysis of paired measurements was performed with the nonparametric Wilcoxon signed rang test. Statistical significance was defined as $P < 0.05$. All data was analysed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

Representative images of the gating strategies for individual leukocyte populations can be seen in Figure 1. These data show that the scatter plots for size (ALL/FSC) and complexity (IAS/SSC) produced by both analysers are very similar. This allows a comparable gating strategy in both analysers.

The agreement between the measurements on the routine flow cytometer and the CD-Sapphire was good for all markers (Figure 2). For MAC-1, the Spearman correlation coefficient was 0.976 ($P < 0.001$), L-selectin had a coefficient of 0.799 ($P = 0.0072$), FcγRIII of 0.407 (P = ns), and for active FcγRII the association was 1.000 ($P < 0.0001$).

The average time for preparation and analysis of one sample on the routine flow cytometer was approximately 125 minutes, whereas sample preparation and analysis on the CD-Sapphire took approximately 130 minutes. Calculations of absolute fluorescence/cell values took about 10 minutes in both machines.

In the second set of experiments, we used a fully automated staining procedure by the CD-Sapphire, including pipetting, lysing of erythrocytes and measuring fluorescence (Figure 3). This approach resulted in the identification of significant differences in expression of active FcγRII, between samples without preincubation with fMLF (mean 148.1 ± 53.0), and samples with fMLF preincubation (mean 753.9 ± 168.5, $P < 0.001$). The average time for the whole procedure, including sample preparation and analysis of one sample on the CD-Sapphire, took approximately 18 minutes. Manual off-line calculations of absolute fluorescence/cell values took about 10 minutes.

4. Discussion

This study evaluated the implementation of monoclonal antibodies for the determination of the posttraumatic systemic immune responses on a routine haematology analyser. The CD-Sapphire is such an analyser with a capability for analysis of fluorescent cells, by applying methods that are

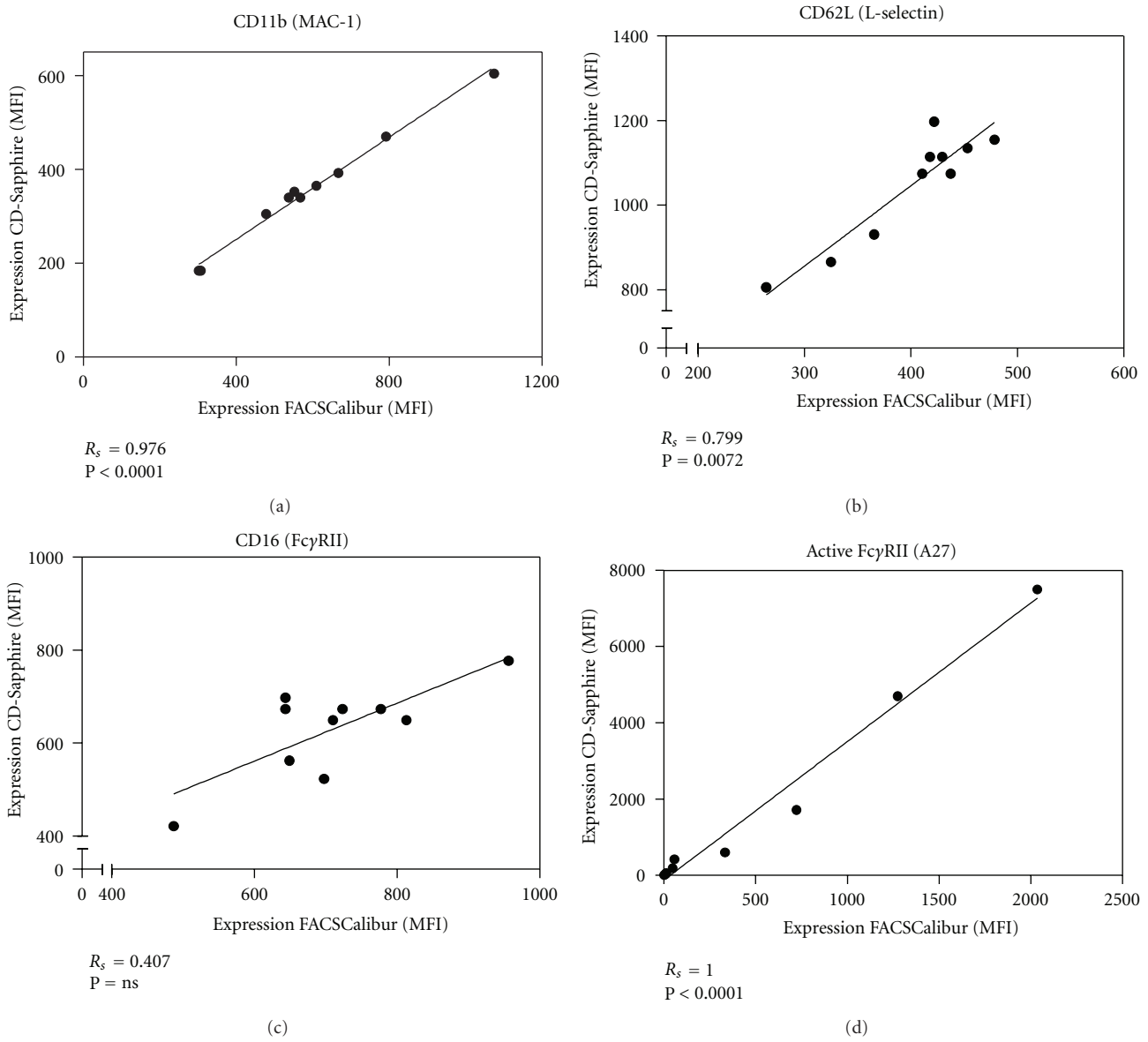


Figure 2: Agreement between two flow cytometers. Samples of whole blood of ten healthy volunteers were measured on both a FACSCalibur and a Cell-Dyn Sapphire to demonstrate the agreement between median fluorescent intensity (MFI) of monoclonal antibodies on neutrophils. Whole blood was incubated with (a) RPE-labeled CD11b (MAC-1); (b) FITC-labeled CD62L (L-selectin); (c) Alexa Fluor 647-labeled CD16 (FcγRIII) for the experiment on the CD-Sapphire and ECD-labeled CD16 (FcγRIII) for the experiment on the FACSCalibur; (d) FITC-labeled MhoPhab A27, a monoclonal phage antibody, which recognizes active FcγRII (CD32). The solid lines are regression lines. R_s = Spearmans rank correlation coefficient. ns = Significant.

analogous to conventional flow cytometry. This study demonstrates the feasibility of analysis of the activation phenotype of neutrophils by using the extended immuno-fluorescent modus of the CD-Sapphire analyser. This was shown for inflammatory markers that are commonly used in trauma research.

The results from the CD-Sapphire showed a high agreement to those of a routine flow cytometer, when paired samples were analysed. These results are in line with studies from the field of clinical chemistry, showing that results from the CD-Sapphire were comparable to their conventional flow cytometry in diagnostic measurements [13, 15, 18]. In addition, the machine has a fully automated modus for staining and incubating whole blood samples with antibodies, lysis of erythrocytes, and subsequent data acquisition [14]. Using this modus, we were able to show that the haematology analyser could clearly distinguish neutrophils that were activated with fMLF compared to control cells. This automated analysis only required a fraction of the time needed for regular flow cytometric analyses. In addition, this analysis did not require sample preparation by technicians. Implementation of such screening on activation markers on neutrophils of

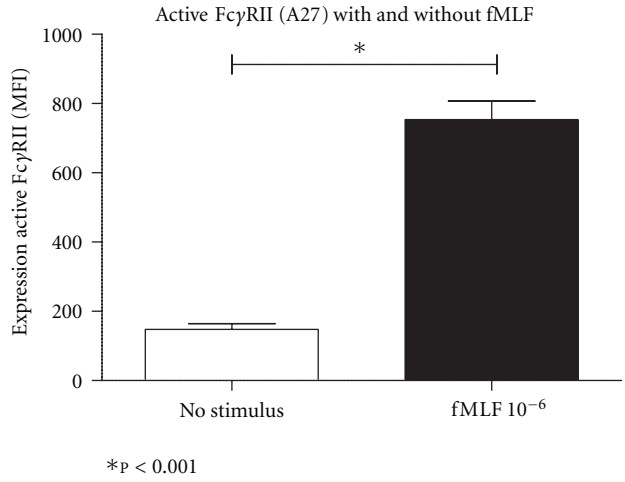


Figure 3: Incubation of monoclonal antibodies by the Cell-Dyn Sapphire. In ten healthy volunteers, two blood samples were taken. Half of the whole blood samples ($n = 10$) were preincubated for 5 minutes with fMLF 10^{-6}, and half of samples ($n = 10$) were not preincubated. The samples were then put in a Cell-Dyn Sapphire, and this analyser fully automatic performed flow cytometry analysis with the monoclonal phage antibody A27 that recognizes the active FcγRII (represented as expression of active FcγRII). MFI = median fluorescence intensity.

trauma patients with the CD-Sapphire greatly facilitates research on the posttraumatic immune response and its modulation. Furthermore, the rapid and fully automated modus does not need specialized staff trained in flow cytometry and is likely cost effective.

There are some limitations to these experiments. Firstly, the FACSCalibur and the CD-Sapphire have slightly different fluorescent detectors. We, therefore, could not use the same fluorochrome in our CD16 antibody (clone 3G8) preparations.

Secondly, we measured the expression of FcγRIII in the FL-3 channel, normally used to test the viability of cells with Propidium Iodine. We used fresh blood samples (<1 hour of sampling), assuming that the large majority of cells had a full viability. Müller et al. and others previously showed that proportions of nonviable leukocytes (especially neutrophils) progressively increase between twelve and 72 hours of storage at room temperature [19]. In comparison, Hedberg and Lehto found that white blood cell differential parameters are stable for up to 48–72 hours, when stored at >4°C [20]. We, therefore, advise that analysis of additional fluorescent markers in FL-3 of the CD-Sapphire should be performed without delay after sampling of the whole blood.

Thirdly, CD-Sapphire software includes fully automated data analysis of fluorescent measurements in the CD3/4/8 modus for lymphocytes. However, to obtain absolute fluorescent information of other cell populations, currently measured raw file information must be extracted from the machine and subjected to manual data analysis in off-line flow analysis software, such as FCS Express. These manual interventions could be overcome by the development of automated software, such as the Leuko64 QuantiCALC software that already exists for the analysis the LeukoCD64 Assay kits [21].

5. Conclusion

In conclusion, this study shows that the CD-Sapphire haematology analyser can be used for analysis of inflammatory antibodies to determine the posttraumatic immune response in trauma patients. It is, not only, comparable with conventional flow cytometry, but it is also more suitable for personnel without specific flow cytometry training. The reduced time necessary for analysis and potentially reduced labour cost constitutes a step forward in further research possibilities and for implementation in clinical diagnostics.

Authors' Contribution

K. M. Groeneveld and M. Heeres contributed equally to the paper.

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Clinical Study

The Effect of Conventional and Mini-Invasive Cardiopulmonary Bypass on Neutrophil Activation in Patients Undergoing Coronary Artery Bypass Grafting

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Interleukin-10 (IL-10) is considered to be a cytokine with potent anti-inflammatory properties, which have been previously linked to increased incidence of sepsis. The level of IL-10 is elevated by cardiac surgery when cardiopulmonary bypass (CPB) and methylprednisolone are used. In our study, we compare the level of IL-10, IL-10 Receptor (IL-10R), and percentage of neutrophils between two groups of cardiac surgical patients undergoing Coronary Artery Bypass Grafting, both of which were not given methylprednisolone. The first group was operated with conventional CPB, while the second group was operated with minimally invasive CPB (mini-CPB). We detected enhanced level of IL-10 during surgery and at the end of surgery in both groups of patients. While no correlation between IL-10 and IL10R was found, IL-10 was positively correlated with increased percentage of neutrophils at the time points when the level of IL-10 peaked.

1. Introduction

Cardiac surgery is connected to profound inflammatory response, characterized by increased level of both proinflammatory and anti-inflammatory mediators. Cardiopulmonary bypass (CPB) is considered to be a potential trigger of cytokine release, therefore, the impact of cardiac surgery on morbidity and mortality is often correlated with the use of CPB [1, 2]. Surgery conducted with the use of CPB is accompanied by ischemia-reperfusion injury, and above that, CPB also represents the environment where blood cells become activated by contact with artificial surfaces, direct air-contact, and also nonturbulent flow. The effort to remove the harmful effect of conventional CPB has led to a development of new surgical devices and techniques. Mini-CPB, that has recently been introduced and successfully used, is designed to reduce blood cell activation: it provides decreased area of extracorporeal circuit, reduces priming, and also lessens air-blood interface. Another favorable feature of mini-CPB is a biocompatible coating that induces higher tolerance by blood cells. The impact of mini-CPB on suppressing inflammatory response is already described and published [3, 4].

As the blood reaches all the body compartments, inflammatory response to surgical injury exceeds local reaction and becomes systemic. Although the increased production of cytokines is essential for protection against infection and also healing the wounds, unregulated inflammatory response is likely to have a harmful effect. Regulating mechanisms consist of anti-inflammatory molecules such as cytokines, cell surface enzymes, receptor antagonists, and soluble receptors [5]. The net impact of the produced cytokines, whether with proinflammatory or anti-inflammatory properties, is determined by the ability of the immune system to balance the inflammatory response properly [6].

IL-10 is a regulatory and immunosuppressive cytokine that is produced by a variety of cells [7]. Upon binding to its receptor (IL-10R), IL-10RB subunit transmits a signal into the cell which activates STAT3- and STAT3-resposive

Table 1: Demographic and preoperative data.

	Conventional CPB		Mini-CPB		*P* value
Patients (no.)	22		22		
Women/men (no.)	4/18		2/20		0.66
Age (years)	68	(63–71)	69	(66–74)	0.57
Body mass index	28.5	(25.3–32)	26.5	(25–31.3)	0.38
Acetylsalicylic acid (no.)	21		21		
Beta blockers (no.)	21		20		
ACE inhibitors (no.)	16		18		0.72
Statins (no.)	21		20		
Diabetes mellitus (no.)	4		6		0.72
COPD (no.)	3		4		
Prior myocardial infarction (no.)	12		9		0.37
Leukocytes (cells $\times 10^9$/L)	7.5	(6.8–8.5)	7.1	(6.3–8.8)	0.8
Ejection fraction (%)	64.5	(51.3–69.8)	60	(48.5–69.5)	0.35

Parameters marked as "no." display the number of positive cases in a group of patients or number of patients treated with a given medication. Other parameters are characterized by median value and interquartile range in brackets. If both groups contain the same number of cases or if they unequal by a case, then *P*-value is above 0.99 and is not displayed. ACE: angiotensin-converting enzyme; COPD: chronic obstructive pulmonary disease.

genes [8]. As a result, the production of proinflammatory cytokines, such as IL-1 and TNF-α, is inhibited [9].

The changes in production of IL-10, IL-1, and TNF-α have already been extensively evaluated in patients undergoing CABG surgery with conventional CPB [10]. However, the information about kinetics of IL-10 in mini-CPB patients is sparse. We compared the changes of IL-10 serum level, the expression of IL-10 membrane receptor, and the percentage of neutrophils in conventional and mini-CPB patients, as well as between these groups of patients. We also examined the incidence of sepsis and acute renal dysfunction in both groups.

2. Material and Methods

2.1. Patients. Forty-four patients, undergoing elective coronary artery bypass grafting (CABG) surgery on an arrested heart using CPB, were enrolled into our study. All patients were well informed about the purpose of the study and they confirmed their unconstrained participation by a written consent. The study project was approved by the Ethics Committee of the University Hospital in Hradec Kralove, Czech Republic. Patients, included in the study group in the period from December 2006 to December 2007, were randomly assigned to surgery either with the use of conventional CPB ($n = 22$) or mini-CPB ($n = 22$). Exclusion criteria consisted of acute inflammation, urgent operation, reoperation, combined operations, operative risk more than 5% (according to logistic Euroscore), preoperative level of serum creatinine above 130 μmol/L, hepatic disease, and malignancies. The demographic and preoperative data of our patients are shown in Table 1.

2.2. Conventional Cardiopulmonary Bypass (CPB). CPB was established using a two-stage venous drainage and ascending aortic return. A roller pump (Stöckert Instrumente GmBH, München, Germany), a membrane oxygenator (Dideco SrL, Mirandola, Italy) in a closed modification with a collapsible reservoir, a cardiotomy suction device, and a 40 μm arterial line filter (Dideco SrL, Mirandola, Italy) were integrated into the extracorporeal circuit. The system surface was not treated with any hemocompatible substance. The priming solution consisted of 500 mL of Ringer's lactate, 500 mL of Rheodextran (Rheomacrodex), 5,000 IU heparin, 80 mL of Natrium Bicarbonate ($NaHCO_3$ 8,4%), 20 mL of 10% Magnesium Sulphate, and Mannitol (at 1 g/kg body weight). The priming volume was calculated so that hematocrit level reached above 0.22. Heparin was administered intravenously at 300 IU/kg body weight to maintain an activated clotting time (ACT) above 480 s during bypass procedure. Patients received neither aprotinin nor corticosteroids intravenously. Pump flow rates averaged 2.4 L/min/m^2 of body surface area with pressure maintained at 50–60 mmHg. The patients were kept normothermic. Cardioplegic arrest was induced with a cold blood cardioplegic solution, which consisted of blood mixed with St. Thomas solution (Ardeapharma, Sevetin, Czech Republic) in 4 : 1 ratio. It was administered antegradely into the aortic root with doses added every 20 min or as needed. All patients received an internal artery mammary graft to the left anterior descending coronary artery. The central aortovenous anastomoses were performed during the reperfusion phase of cardiopulmonary bypass with the heart beating. After the termination of CPB, heparin anticoagulation was antagonized using Protamine Sulphate at a dose of 1 : 1.

2.3. Minimally Invasive Cardiopulmonary Bypass (Mini-CPB). Mini-CPB was established using a small 22F two-stage venous drainage and ascending aortic return. Minisystem Synergy (Sorin Group SrL, Mirandola, Italy) consisted of a centrifugal pump, membrane oxygenator, 40 μm arterial line filter, and a venous bubble trap. Cardiotomy suction was not used. The whole system, consisting of a closed loop with the surface treated with PH.I.S.I.O phosphorylcholine

coating (Sorin Group SrL, Mirandola, Italy) and very short tubing, was placed close to the operating field. The priming solution, heparinization, pump flow, temperature, and surgery technique were identical with the conventional CPB procedure described above. Cardioplegic arrest, induced according to the Calafiore warm blood-cardioplegia protocol, was administered antegradely into the aortic root. At the beginning of CPB, crystalloid priming solution was flushed retrogradely together with the blood coming from the arterial line to minimize the hemodilution of the patient.

2.4. Anesthesiological Management. All patients were anesthetized according to the current protocol of our department. Anesthesia was induced using Thiopental and Midazolam. Muscular relaxation was achieved with Cisatracurium. Anesthesia was maintained with Isoflurane and intermittent use of Sufentanyl. Continuous infusion of Propofol was used as a supplement if needed. Volume-controlled ventilation with FiO_2 0.5 was employed. Mean arterial pressure was kept above 50 mmHg, with norepinephrine administered as required.

2.5. Sample Collection and Data Acquisition. Blood samples were withdrawn from subclavian vein before and during surgery and from antebrachial vein in postsurgery period. The samples were collected into anticoagulant-untreated Vacutainer tubes as well as heparinized Vacutainer tubes (Becton Dickinson, UK) at the following time points: before surgery (introduction of anesthesia), at the beginning of CPB, at termination of CPB, at the end of surgery, and on the 1st, 3rd, and 7th postoperative day.

Serum was separated from the blood cells by centrifugation at 1000 g for 15 min. Samples were stored at -80°C prior to analysis. IL-10 concentration was determined by enzyme-linked immunosorbent assay (ELISA), with a sensitivity of 1 pg/mL. ELISA kit was purchased from Bender MedSystems (Austria). IL-10 concentrations were measured using spectrophotometer (Labsystems Multiskan RC, USA) with Genesis software.

Heparinized blood samples were processed immediately after collection. 50 μL of blood was incubated with titrated monoclonal antibodies anti-CD3 FITC/IL-10R PE/CD14 PerCP/CD16 APC. Antibodies, except for anti-IL-10R, were purchased from BD Biosciences (USA), while anti-IL-10R was purchased from BioLegend, USA. Following the 20 min incubation, red blood cells were lysed by hypotonic lysis and then the samples were washed. The data acquisition was performed with FACS Calibur flow cytometer using CellQuest software (BD Biosciences, USA), and the analysis was done using FlowJo software (Tree Star, USA). Expression of IL-10R on cells was displayed as median fluorescence intensity (MFI). Expression of IL-10R during surgery was not assessed.

2.6. Statistical Analysis. Values of IL-10, IL-10R, and percentage of neutrophils during and after surgery were compared to preoperative values. Normal distribution of the data was determined by Shapiro-Wilk test. Changes within a group were evaluated using ANOVA for repeated measures and Dunnett's test or Friedman ANOVA and Wilcoxon paired test. To determine the differences between both groups of patients, values of IL-10, IL-10R, and percentage of neutrophils were compared at matching time points using two-way ANOVA for repeated measures and Fisher's LSD test or Mann-Whitney U test. The relationship between IL-10 and percentage of neutrophils was assessed by Spearman correlation coefficient. Demographic and clinical data were analyzed by Fisher exact test, Mann-Whitney U test, and Student's t-test. All tests were performed at a significance level of 0.05. Bonferroni correction was used in case of multiple comparisons. Results of statistical analysis were expressed as medians unless stated otherwise.

3. Results

3.1. Differences in IL-10 in Serum within the Groups. Changes in IL-10 serum level were similar in both groups of patients, with respect to the preoperative serum level of 1 pg/mL in conventional CPB group and 1.45 pg/mL in mini-CPB group. In the conventional CPB group, serum level of IL-10 significantly increased at the termination of CPB (52.7 pg/mL, $P < 0.001$), at the end of surgery (50.5 pg/mL, $P < 0.001$), and on the 1st postoperative day (7.3 pg/mL, $P < 0.01$). Similarly, the mini-CPB group experienced an increase in serum level at the termination of CPB (from 1.5 pg/mL to 10.6 pg/mL, $P < 0.01$), at the end of surgery (21.2 pg/mL, $P < 0.001$), and on the 1st postoperative day (5.8 pg/mL, $P < 0.05$). Postsurgery monitoring revealed that the level of IL-10 was gradually decreasing in both groups and did not significantly differ from preoperative levels (Figure 1).

3.2. Differences in IL-10 in Serum between the Groups. There was significant serum-level difference ($P < 0.01$) between both groups at the termination of CPB: the conventional CPB group topped the mini-CPB group by reaching the value of 52.7 pg/mL. At this time point, IL-10 in mini-CPB group was enhanced, but only reached 10.6 pg/mL. In mini-CPB group, the level of IL-10 was highest at the end of surgery (21.2 pg/mL). In postsurgery period, the level of IL-10 decreased and did not differ between both groups (Figure 1).

3.3. Percentage of Neutrophils. Percentage of neutrophils was significantly increased ($P < 0.001$) in both groups of patients at the end of CPB, at the end of surgery, and also on the 1st and 3rd postoperative day. The baseline was 55% of neutrophils in conventional CPB group and 53% in mini-CPB group. The highest percentage of neutrophils was measured on the 1st postoperative day in both groups of patients (80% in conventional CPB group and 81% in mini-CPB group). There was statistically significant difference in percentage of neutrophils between both groups at the end of surgery ($P < 0.05$) when conventional CPB group reached 79% and mini-CPB group reached 75% of neutrophils (Figure 2).

The percentage of neutrophils correlated with the maximum level of IL-10 at the termination of CPB ($r_s = 0.73$, $P < 0.001$), at the end of surgery in conventional CPB group

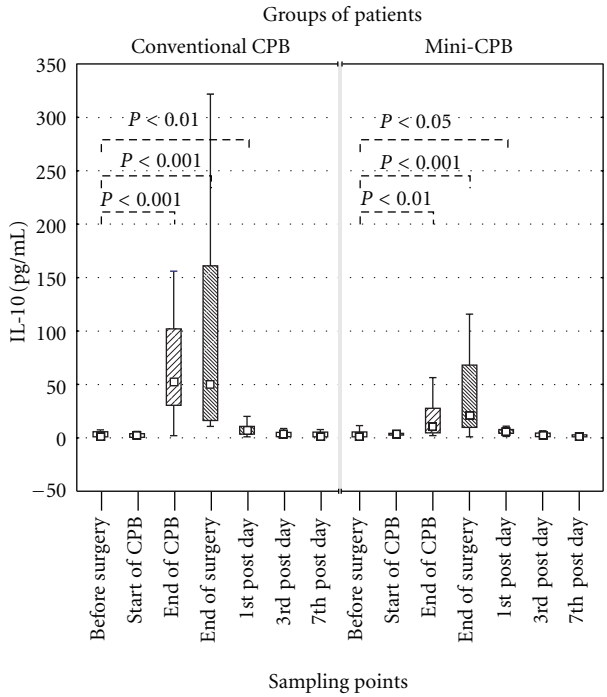


Figure 1: IL-10 level in serum of patients operated either with conventional CPB or mini-CPB. The IL-10 level was detected by ELISA. The level of IL-10 differed between conventional CPB and mini-CPB group after termination of CPB ($P < 0.01$). Even though the difference at the end of surgery was not statistically significant, IL-10 greatly varied when comparing both groups. Squares display median, boxes are quartiles, and whiskers display the range of nonoutlier values.

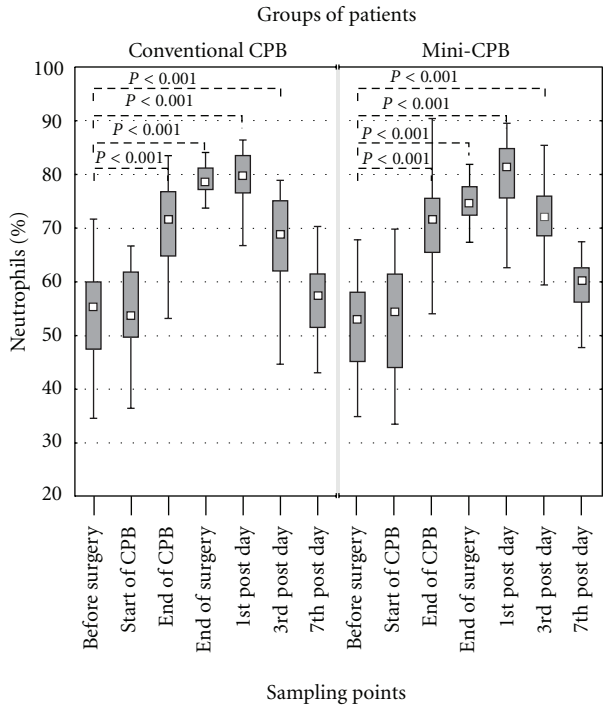


Figure 2: Percentage of neutrophils in peripheral blood samples of patients with conventional CPB or mini-CPB. The percentage of neutrophils was evaluated by flow cytometry. The percentage of neutrophils significantly differed between both groups of patients at the end of surgery (79% of neutrophils in conventional CPB group versus 75% of neutrophils in mini-CPB group, $P < 0.05$). Squares display, median, boxes are quartiles, and whiskers display the range of nonoutlier values.

($r_s = 0.54$, $P < 0.01$), and at the end of surgery in mini-CPB group ($r_s = 0.54$, $P < 0.01$) (Figures 3(a)–3(c)).

3.4. Expression of IL-10 Receptor (IL-10R). Since IL-10R is expressed on T lymphocytes, neutrophils, and monocytes, the expression was statistically evaluated in all of these populations. In lymphocytes and neutrophils, no differences were found at any time point when comparing MFI values within a group of patients or between both groups of patients.

The expression of IL-10R on monocytes significantly decreased at the end of surgery in both groups: the preoperative expression of IL-10R dropped from MFI of 10.2 to 7.6 ($P < 0.01$) in conventional CPB group, as well as in mini-CPB, where the preoperative level dropped from MFI of 9.8 to 7.6 ($P < 0.05$). On the 3rd postoperative day, the expression of IL-10R on monocytes was significantly enhanced (Figure 4), reaching MFI of 10.6 in conventional CPB ($P < 0.05$) and 11.3 in mini-CPB ($P < 0.05$). After that, the IL-10R expression decreased again and there was no significant difference between the preoperative value and the value on the 7th day after surgery in both groups (Figure 5).

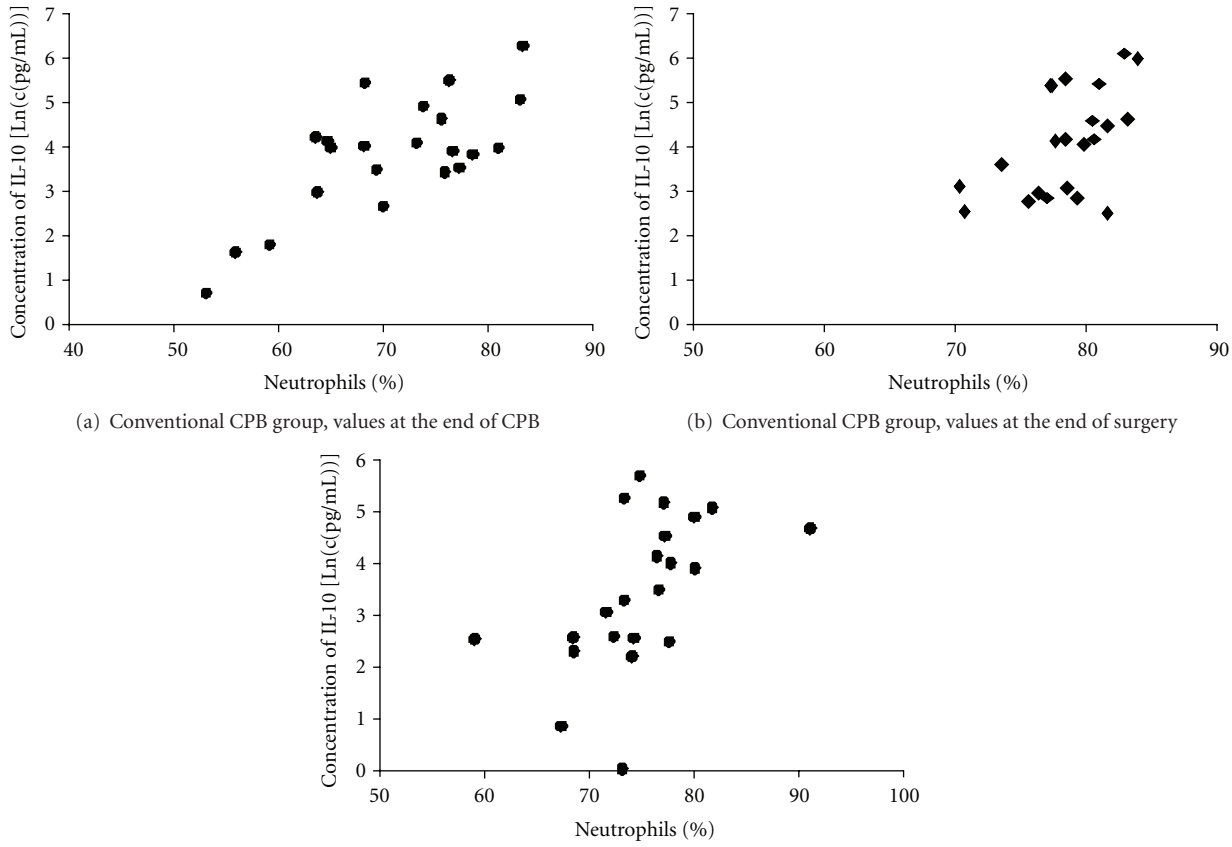
There was no correlation between serum level of IL-10 and expression of IL-10R on monocytes.

No difference in IL-10R expression on monocytes was observed when comparing both groups of patients (Figure 5).

We observed single- as well as multiple-organ dysfunction or failure in both groups of patients. IL-10 level in each group is listed in Table 2. Only two patients were considered suffering from sepsis. Intraoperative and postoperative data of patients are displayed in Table 3.

4. Discussion

IL-10 is an important cytokine, maintaining the proinflammatory response balanced. Although IL-10 can keep cells unresponsive, receptors on the cells affected by IL-10 are still capable of binding and internalizing proinflammatory cytokines. As a result, the cytokines are removed from blood circulation without subsequent activation of the cells [11, 12]. This kind of decoy activity is thought to be of favorable contribution of IL-10 in ischemic heart after myocardial reperfusion, and it is likely to prevent against heart failure [13]. However, high level of IL-10 can also have an adverse effect by increasing the likelihood of sepsis. Nonetheless, a significant association between sepsis and higher level of IL-10 determined by—1082 base pair single polymorphism in promoter region of IL-10 gene is equivocal [14, 15]. It has been



(a) Conventional CPB group, values at the end of CPB

(b) Conventional CPB group, values at the end of surgery

(c) Mini-CPB group, values at the end of surgery

Figure 3: Relationship between percentage of neutrophils and serum level of IL-10. (a) Correlation, $r_s = 0.73$, in conventional CPB group at the termination of CPB ($P < 0.001$). (b) Correlation, $r_s = 0.54$, in conventional CPB group at the end of surgery ($P < 0.01$). (c) Correlation, $r_s = 0.54$, in mini-CPB group at the end of surgery ($P < 0.01$)

Table 2: IL-10 level in groups of patients without and with organ dysfunction or failure.

	No organ affected ($n = 15$)	One and more organs affected ($n = 7$)	One organ affected ($n = 5$)	More organs affected ($n = 2$)
Conventional CPB ($n = 22$)	89 (41–451)	203 (148–731)	181 (148–385)	467 (203–731)
	No organ affected ($n = 11$)	One and more organs affected ($n = 11$)	One organ affected ($n = 6$)	More organs affected ($n = 5$)
MINI-CPB ($n = 22$)	35 (13–219)	75 (23–192)	105 (23–183)	55 (49–192)

Groups of patients were divided by number of organs suffering from dysfunction or failure. Upper value displays median of IL-10 (pg/mL), values in the brackets describe the range from minimum to maximum characterizing a group.

reported that CPB induces the release of IL-10, which can be further enhanced by the use of corticosteroids in patients undergoing CABG [16]. In our study, corticosteroids were not applied, and yet we observed that CPB induced a profound release of IL-10. We found significant difference in IL-10 production between the two different types of CPB used. Mini-CPB, which is considered to be a less harmful approach of cardiac surgery, elicited a lower release of IL-10. In spite of this fact, the increase of IL-10 does not seem to impact clinical outcome. Although the level of IL-10 was lower in mini-CPB group, the number of patients suffering from organ dysfunction or failure was nonsignificantly higher in mini-CPB group than in conventional CPB group ($P < 0.22$) (Table 2). While we would be able to predict organ dysfunction or failure according to IL-10 level in conventional CPB group, the similar relation was unclear in mini-CPB group. We also looked at the IL-10 value in patients with sepsis and patients without sepsis. Microbiologically confirmed, there were only two septic patients, one in each group, both reaching values of IL-10 in serum that fell within quartiles of IL-10 calculated for each sampling time point in any given group. Therefore, in our study groups, even though the IL-10 level was very high in some patients, IL-10 could not be used as a predictive marker related to sepsis. Increased level of IL-10 along with the medical treatment (Ampicillin-sulbactam) of our patients might have prevented the onset of

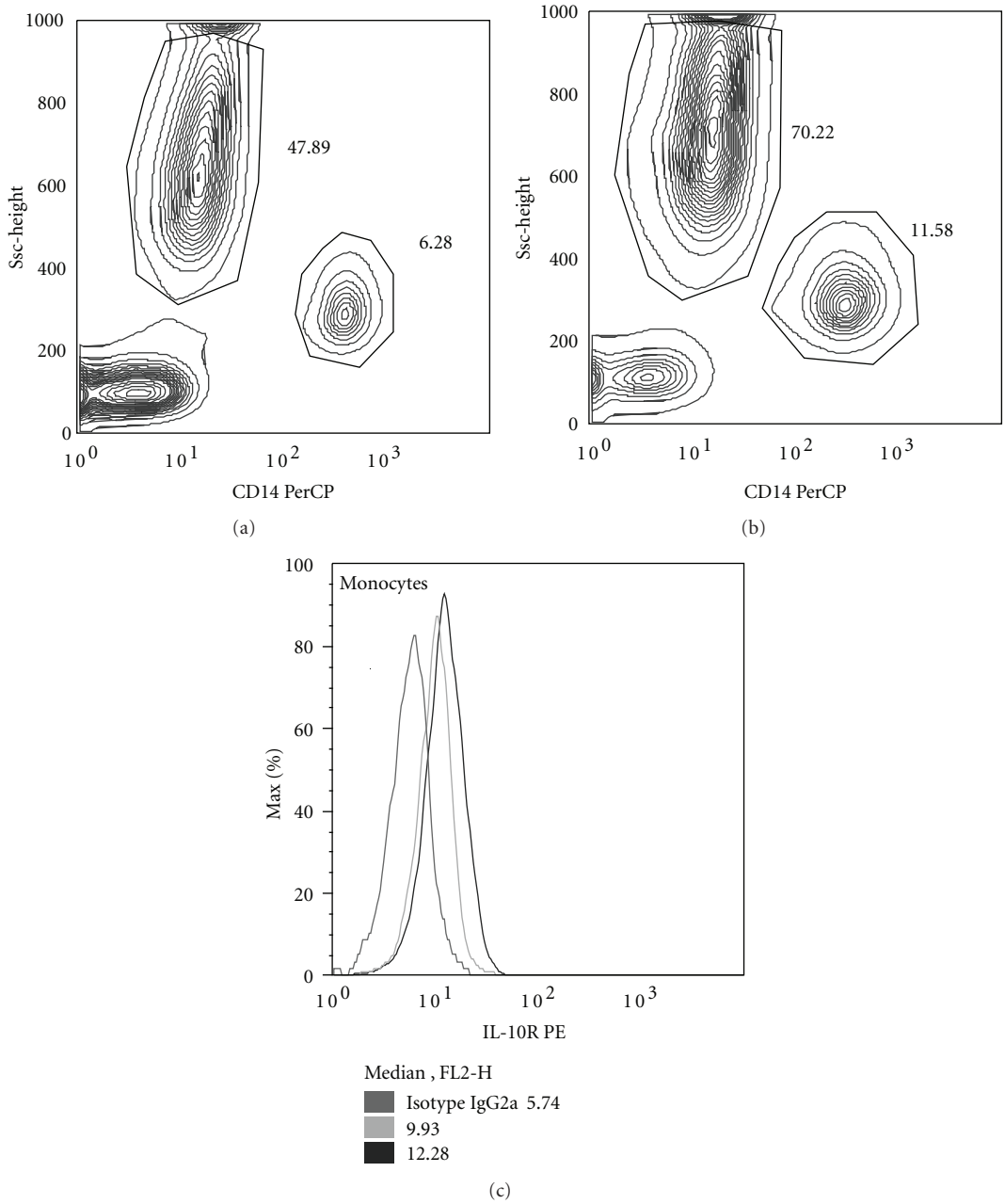


Figure 4: Illustration of changes in the expression of IL-10R on monocytes of conventional CPB patient. Monocytes are discriminated from other populations of cells by high expression of CD14 (x-axis). (a) Leukocytes before surgery. (b) Leukocytes on the 3rd postoperative day. (c) Overlaid histograms of monocytes from (a) and (b). Although the expression of IL-10R on monocytes enhances on the 3rd day after surgery (black line), it is relatively weak considering the isotype control (dark grey line). Intracellular staining did not reveal higher expression of IL-10R in cells (data is not shown).

sepsis. Such a beneficial role of IL-10 is in concordance with experimental studies [17, 18].

In previous works, IL-10 level was also found to be significantly increased in patients who suffered from postoperative renal dysfunction [19], which was characterized by creatinine level raised above 176 μmol/L [20]. The mini-CPB group had two patients that were suffering from acute renal dysfunction, while there was only one patient in conventional CPB group. The patient from conventional CPB group reached 522.5 pg/mL at the termination of CPB. This patient had the highest value of IL-10 in serum out of both groups. However, the other two patients did not exceed quartiles of IL-10 calculated for each sampling time point. According to this result, there is no simple relation between enhanced level of IL-10 and increased probability of acute renal dysfunction.

We compared the percentage of neutrophils to IL-10 level when IL-10 in serum was at its highest level. We observed significant correlation between IL-10 and percentage of

Table 3: Intraoperative and postoperative data of patients.

	Conventional CPB		Mini-CPB		P value
Duration of surgery (min)	210	(161–250)	165	(155–203)	0.15
Duration of CPB (min)	70	(56–111)	62	(55–76)	>0.1
Priming solution (mL)	1600	(1425–1800)	1100	(1000–1300)	<0.001
Anastomoses (no.)	2	(2-3)	2	(2-3)	0.7
Intraoperative blood loss (mL)	1000	(725–1000)	700	(500–950)	0.14
Postoperative blood loss in 24 h (mL)	650	(450–1075)	600	(500–950)	>0.1
Acute renal dysfunction and failure (no.)	1		2		
Respiratory dysfunction and failure (no.)	4		9		0.19
Postsurgery myocardial dysfunction and AMI (no.)	5		6		
Sepsis (no.)	1		1		

Parameters marked as "no." display the number of positive cases in a group of patients with the exception for number of anastomoses which denotes median value. All other parameters are characterized by median value and interquartile range in brackets. If both groups contain the same number of cases or if they unequal by a case, then P-value is above 0.99 and is not displayed. AMI: acute myocardial infarction.

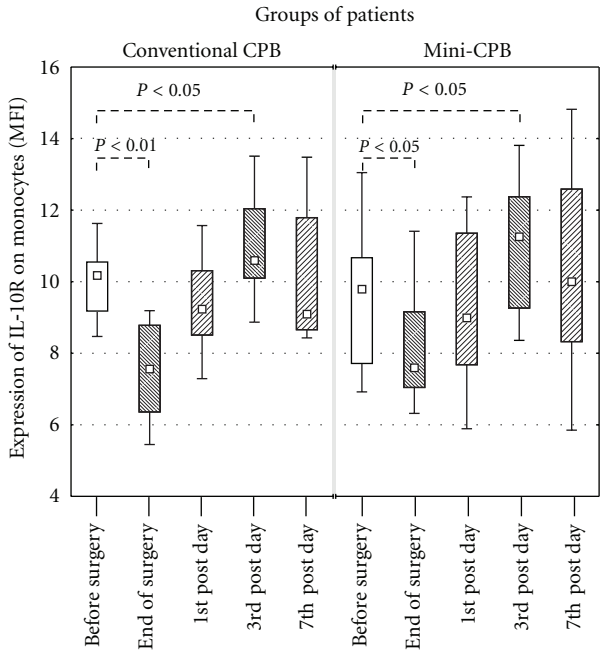


Figure 5: Expression of IL-10R on monocytes in peripheral blood samples of conventional CPB or mini-CPB group as a result of flow cytometry data analysis. The expression of IL-10R did not significantly differ between both groups of patients. Squares display median, boxes are quartiles, and whiskers display the range of nonoutlier values.

neutrophils at the termination of CPB in conventional CPB group ($r_s = 0.73$, $P < 0.001$) and at the end of surgery in mini-CPB group ($r_s = 0.54$, $P < 0.01$). We also found lower—but still significant—correlation ($r_s = 0.54$, $P < 0.01$) between IL-10 and percentage of neutrophils at the second highest level of IL-10 (at the end of surgery) in conventional CPB group. Our data suggests that neutrophils were the main producers of IL-10 in most of our cardiac surgical patients. However, the lower correlation coefficient at the end of surgery indicates other cells might also have participated in IL-10 production. The observation is in agreement with recently published findings that show that neutrophils are an important source of IL-10 [21] and that mini-CPB attenuates neutrophil activation and cytokine release after coronary bypass surgery [3].

IL-10 exerts its function through the binding to IL-10R [22]. It seems that higher level of IL-10 in conventional CPB group is exclusively linked to the surgery technique and devices used. Since we found no significant difference in expression of IL-10R between both groups of patients, we can hypothesize that the expression of IL-10R on hematopoietic cells exceeds the maximum level of its ligand, IL-10. It has been discovered that other cells, such as fibroblasts and epithelial cells [23, 24], also express IL-10RA after induction; thus IL-10 may have a much broader effect on tissues and organs, an effect which cannot be explained by the possible correlation of serum IL-10 and the expression of IL-10R on monocytes.

5. Conclusion

IL-10 level and percentage of neutrophils are significantly affected by the type of cardiac surgery employed. Although IL-10 level may have statistical relation to sepsis or renal dysfunction, the generally accepted critical level that would enable to unambiguously distinguish between patients with worse or good prognosis does not exist. However, in certain conditions, like using conventional CPB, IL-10 may represent a supporting tool, that, along with other parameters, would help rank the patients in likelihood of organ dysfunction or failure. The observed correlation between increased level of IL-10 and higher percentage of neutrophils in both groups of patients suggests functional relationship between both parameters.

Acknowledgments

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Research Article

Effects of Trauma-Hemorrhage and IL-6 Deficiency on Splenic Immune Function in a Murine Trauma Model

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Splenic immune function is known to be depressed following hemorrhage. The present study investigates the effects of femoral shaft fracture, isolated or in combination with hemorrhage, on early stage cytokine production capacity of splenocytes and observes the role of IL-6 under these conditions. Male IL-6 knockout (IL-$6^{-/-}$) and wild-type mice (WT) were randomly divided into three groups: sham (S), isolated femoral fracture (Fx), and femoral fracture + volume controlled hemorrhage (TH-Fx) ($n = 6$ per group). Animals were sacrificed four hours after induction of hemorrhage and fracture. Cytokine release (TNF-α, IL-6, and IL-10) of isolated and LPS-stimulated splenocytes was determined by cytometric bead array. Femoral fracture with or without hemorrhage caused a suppression of in vitro cytokine production capacity of splenocytes at an early posttraumatic stage in WT and IL-$6^{-/-}$. In the absence of IL-6, the profile of splenic cytokine secretion is significantly altered, identifying this cytokine as a potential therapeutic target to modulate the posttraumatic immune response.

1. Introduction

The posttraumatic immune response is characterized by a complex set of pro- and anti-inflammatory reactions in order to restore homeostasis [1] and was shown to be organ and cell specific [2, 3]. Splenic immune response and cellular immunity are known to be depressed after trauma-hemorrhage leading to an increased susceptibility to infectious complications [4, 5]. Several studies have demonstrated that splenocyte functions, such as proliferative and cytokine production capacity as well as macrophage antigen presentation function are suppressed following trauma hemorrhage [6–9]. In regards to T-cell function, a posttraumatic shift from splenic Th1 cytokine (IL-2, IFN-γ, and TNF-α) to Th2 cytokine (IL-4, IL-5, IL-10) production has been reported [10–15]. These splenic immune alterations are well described in rodent trauma models consisting of laparotomy and hemorrhagic shock [3, 16–19] and appear already 2 hours after insult induction [12, 15, 20–23]. Simple hemorrhage and laparotomy alone were demonstrated to result in a marked depression of splenic immune function, and no differences were seen in the extent of depression in the early stage, if these two insults were combined [24]. However, a prolonged splenic immunodepression of 7 days was seen after combined trauma hemorrhage [25], whereas splenic immune suppression only persists up to 3 days following isolated hemorrhage or laparotomy [26]. With a later onset at day 7, splenic immune alterations following femoral fracture and hemorrhage seem to differ from the aforementioned trauma models [27]. However, results are inconsistent due to different study designs (e.g., animal gender, genetic strain, and protocol of fracture induction). Data regarding the role of isolated femur fracture in early stage splenic immune alterations are lacking, but are of special clinical interest as femoral fracture itself as well as the stabilization by femoral nailing seems to have a significant impact on the inflammatory response and the susceptibility to infectious complications [28]. Furthermore, it is still controversially discussed whether Interleukin (IL)-6, produced by many different cell types (e.g., macrophages, endothelial, and T cells), exerts pro- or anti-inflammatory effects in the posttraumatic immune response. IL-6 seems to have both, pro- and inflammatory effects, depending on whether it is acting in a paracrine or endocrine manner [29]. Additionally, the effects of IL-6 seem to be influenced by the stimulus and the model of inflammation used in experimental studies [30]. The aim of the present pilot study

was to investigate whether a femoral fracture with or without hemorrhagic shock has an impact on the splenic cytokine production capacity at an early posttraumatic stage (4 hours) and to observe the effects of IL-6 under these conditions.

2. Materials and Methods

2.1. Animal Care. Prior to initiation, the study was approved by the animal welfare committee of the state of Lower Saxony. The experiments were performed in 18 male C57Bl/6 IL-6 knockout (IL-$6^{-/-}$) mice aged 8–10 weeks and weighing 22.0 ± 3.0 g. As a control 18 male C57Bl/6 mice of similar weight (wild type; WT) were used. The animals were bred and raised under specific pathogen-free conditions in the central animal facility of our institution. Throughout the study period, pelleted mouse feed (Altromin 1324) and water were available ad libitum. Lighting was maintained on a 12-hour cycle and temperature at 20°C ± 2°C. WT and IL-$6^{-/-}$ animals were randomly assigned into sham groups (S) (each of 6 animals, only anaesthesia) and 2 experimental groups, in which either an isolated femur fracture or a combination of hemorrhage and femoral fracture were induced.

2.2. Induction of Haemorrhage and Femur Fracture. All procedures were performed after deeply anesthetizing the animals with 2.2 ± 0.3 mg of ketamine (Ketanest 100 mg/kgBW) and 0.33 ± 0.045 mg of xylazine (Rompun 15 mg/kgBW). Anesthesia was maintained during the entire study period.

A standardized femur fracture was induced in experimental groups using a blunt guillotine device with a weight of 500 g as previously described [31]. This resulted in an A-type femoral fracture combined with a moderate soft-tissue injury. A hemorrhagic shock was induced by withdrawing 60% of the total blood volume (body weight (in g) x 0.04 mL) via puncturing the orbital plexus. Resuscitation using sterile ringer's lactate was performed with four times the shed blood volume in the tail vein after 1 hour. After resuscitation, splint fixation of the femoral fracture was performed.

Sham animals were only anaesthetized without performing any surgical procedure.

2.3. Harvesting Procedure and Preparation of Splenocyte Cultures. Experimental animals were sacrificed four hours after induction of hemorrhage and fracture, whereas sham mice were four hours after the first anaesthesia. The spleen was removed aseptically and processed as follows.

Splenocytes were isolated as previously described [32]. In brief, spleens were gently ground between frosted microscope slides to produce a single cell suspension. This suspension was centrifuged at 300 × g for 10 min at 4°C. The erythrocytes were lysed with lysis buffer and the remaining cells were washed with PBS by centrifugation (300 × g, 15 min, 4°C). After centrifugation, cells were resuspended in RPMI 1640 (Gibco) containing 10% heat inactivated FBS and antibiotics (50 U/mL penicillin, 50 μg/mL streptomycin and 5 μg/mL gentamycin, all from Gibco) to get a final concentration of 1×10^6 cells/mL. The splenocytes were then cultured in the presence of 10 μg/mL LPS (LPS from *E.coli* 0111:B4, Sigma-Aldrich Inc., Steinheim, Germany) at 37°C, 95% humidity, and 5% CO_2 for 24 hours. After incubation, the cell free suspension was collected and stored at −80°C until further analysis.

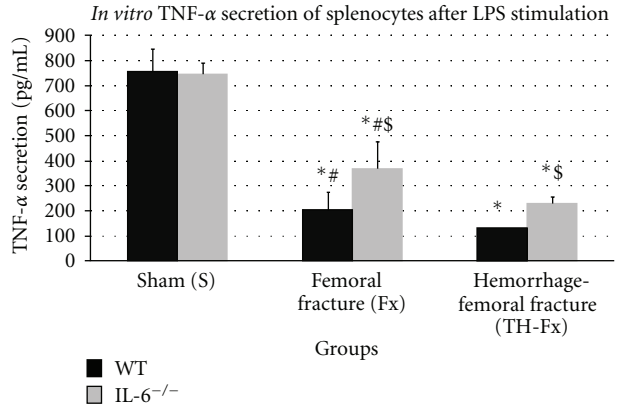

Figure 1: TNF-α productive capacity of splenocytes in response to their stimulation with LPS in wild-type (WT) and IL-6 knockout (IL-$6^{-/-}$) mice in sham animals (group S), after an isolated femoral fracture (group Fx) and after the combination of a femoral fracture and hemorrhage (group TH-Fx). *stat. significance ($P < 0.05$) S vs. Fx and TH-Fx, #stat. significance ($P < 0.05$) Fx versus. TH-Fx, $stat. significance ($P < 0.05$) WT vs. IL-$6^{-/-}$.

2.4. Flow Cytometry. Cytokine concentrations in cell-free supernatants were determined with cytokine Bead Array inflammatory kits using flow cytometry according to the manufacturer's instructions (Bender MedSystems, Vienna, Austria).

2.5. Statistics. Statistical analysis was performed using SPSS computer software (SPSS 11.5, Chicago, IL). Statistical significance was assumed where probability values of less than 0.05 were obtained. Comparisons between groups were performed using one-way analysis of variances (ANOVA), Student's t-test or the rank-sum test (Mann-Whitney U-test). Results are expressed as mean ± standard error of the mean (SEM).

3. Results

The *TNF-α* secretion of stimulated splenocytes was significantly reduced following isolated femoral fracture in WT and in IL-$6^{-/-}$ mice compared to sham animals (Figure 1). In IL-$6^{-/-}$ mice, the decrease of the TNF-α release was significantly attenuated in comparison to WT animals ($P < 0.05$). In sham groups, no significant differences concerning the TNF-α secretion were observed between WT and IL-$6^{-/-}$ mice ($P > 0.05$). Combined hemorrhage and femoral fracture resulted in a further significant decrease of the TNF-α release in WT as well as in IL-$6^{-/-}$ mice compared to animals with an isolated femoral fracture ($P < 0.05$). Again, a significantly attenuated depression of the TNF-α production

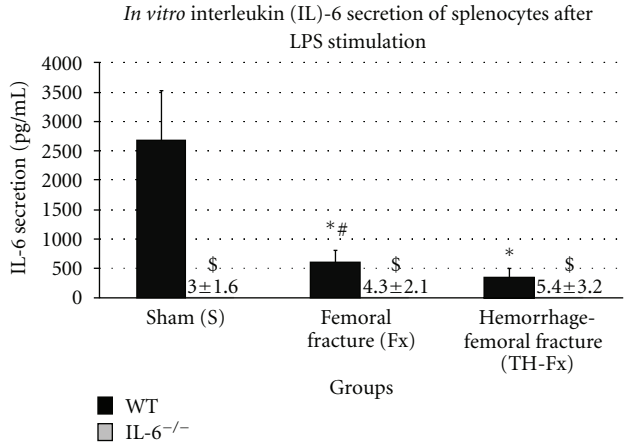

Figure 2: IL-6 productive capacity of splenocytes in response to their stimulation with LPS in wild-type (WT) and IL-6 knockout (IL-$6^{-/-}$) mice in sham animals (group S), after an isolated femoral fracture (group Fx) and after the combination of a femoral fracture and hemorrhage (group TH-Fx). *stat. significance ($P < 0.05$) S vs. Fx and TH-Fx, #stat. significance ($P < 0.05$) Fx versus. TH-Fx, $stat. significance ($P < 0.05$) WT versus. IL-$6^{-/-}$.

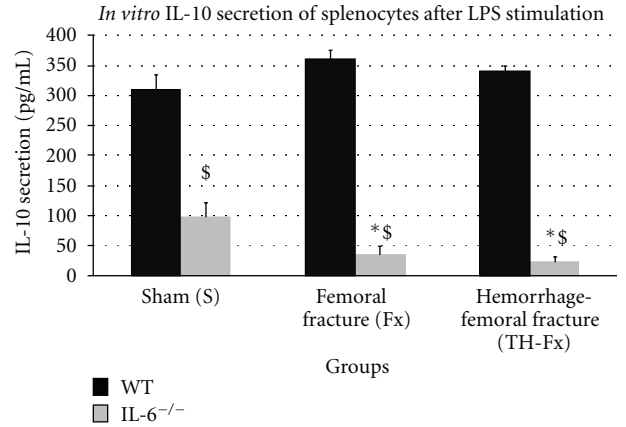

Figure 3: IL-10 productive capacity of splenocytes in response to their stimulation with LPS in wild-type (WT) and IL-6 knockout (IL-$6^{-/-}$) mice in sham animals (group S), after an isolated femoral fracture (group Fx) and after the combination of a femoral fracture and hemorrhage (group TH-Fx). *stat. significance ($P < 0.05$) S versus. Fx and TH-Fx, $stat. significance ($P < 0.05$) WT versus. IL-$6^{-/-}$.

was demonstrated in IL-$6^{-/-}$ mice compared to WT animals ($P < 0.05$) as shown in Figure 1.

The *IL-6* productive capacity of stimulated splenocytes was significantly decreased after femoral fracture compared to sham animals ($P < 0.05$). The combination of hemorrhagic insult and femoral fracture resulted in an additional significant suppression of the IL-6 release ($P < 0.05$) as shown in Figure 2. In IL-$6^{-/-}$ mice, the mean IL-6 secretion was <6 pg/mL in all study groups due to the lacking IL-6 gene. Therefore, no significant differences in IL-6 release between different study groups were observed in IL-$6^{-/-}$ mice ($P > 0.05$).

In WT mice, femoral fracture with or without hemorrhagic shock did not result in a significant increase of the IL-10 production of splenocytes compared to sham animals ($P > 0.05$). In IL-$6^{-/-}$ mice, the IL-10 release of stimulated splenocytes was significantly decreased in all study groups compared to WT animals (Figure 3). Thereby, the IL-10 production was significantly decreased following femoral fracture with or without hemorrhagic shock compared to the sham group ($P < 0.05$). No significant differences concerning the IL-10 release were demonstrated between IL-$6^{-/-}$ mice with an isolated femoral fracture and animals with an additional hemorrhagic shock ($P > 0.05$).

4. Discussion

Trauma results in a significant impairment of splenic immune functions which has been supposed to increase the susceptibility to septic complications as a result of depressed cellular immunity [33–37]. Operative stabilization of major fractures (e.g., femoral fractures) is supposed to additively enhance the trauma-induced inflammatory changes [38, 39], which has resulted in an ongoing discussion about the optimal timing of definitive fracture stabilization.

Despite the important role of the spleen in the immune response after trauma, the effects of a femoral fracture as an isolated injury or in combination with hemorrhagic shock on the early-stage splenic cytokine production has yet not been investigated. Furthermore, the role of IL-6 within these splenic immune alterations has not been elucidated. In this context, it is well described that systemic cytokines are mainly released by Kupffer cells as the largest population of tissue macrophages after trauma hemorrhage and infectious stimulus [2, 40]. As the pathophysiological role of splenic IL-6 has been discussed controversially, we focused on the importance of splenocytes and locally generated IL-6 in the posttraumatic inflammatory response.

The main findings of the present study may be summarized as follows.

(1) An isolated femoral fracture resulted in a significantly depressed cytokine production capacity of splenocytes at an early stage of the posttraumatic course. The depression of splenic cytokine release was enhanced in case of an additional hemorrhagic shock. These alterations were observed in WT and IL-$6^{-/-}$ mice.

(2) The splenic immunodepression in terms of reduced TNF-α secretion of splenocytes was significantly attenuated in the absence of IL-6.

(3) The production capacity of splenocytes for IL-10 was suppressed in the absence of IL-6 in all study groups. In contrast to WT animals, splenic IL-10 release was significantly decreased following femoral fracture with and without hemorrhagic shock.

In the present study, we were able to show that femoral fracture with and without hemorrhage results in a marked alteration of splenic cytokine production capacity at an early posttraumatic stage (4 h). In accordance, several studies reported a splenic immunodepression 2 h and 4 h following laparotomy and hemorrhagic shock [12, 15, 20–23, 41, 42]. In contrast, splenic immune depression after femoral fracture and hemorrhage seems to appear later in the posttraumatic course. Mack et al. described that the typical splenic Th1/Th2 shift was evident at day 7, but not at day 1 after trauma hemorrhage [27]. Further studies reported comparable results with marked alterations of splenic cytokine release in response to femoral fracture and hemorrhage at day 7 after insult induction [43–46]. On the other hand, Monroy et al. [47] as well as Stapleton et al. [38] found no significant alterations in splenic cytokine secretion seven days after combined femoral fracture and hemorrhage. These inconsistent results might be explained by differences in the study design. Several studies used female animals in different estrus cycle status, which probably have a marked impact as splenic immune response exhibits a gender dimorphic pattern [10]. Additionally, we induced a closed femoral shaft fracture, which was shown have an greater impact in terms of splenic immune suppression compared to open femoral fracture used in other studies [48]. As previous studies described an association between the extent of splenic immune depression and the posttraumatic susceptibility to infectious complications [12, 13, 15, 49, 50], the enhanced depression of splenic cytokine production capacity following combined femoral fracture and hemorrhagic shock might have clinical implications in the setting of multiple trauma in terms of priority and timing of definite fracture stabilization. However, the transfer of our results to the clinical setting is limited as measuring the cytokine production capacity of splenocytes only reflects a part of the T cell (namely, CD4+ T-cells) and splenic immune function. Based on our data, we could not make any statement concerning CD8+ T-cell function and proliferation capacity of splenocytes. Furthermore, it is not possible to differentiate whether depressed cytokine release is caused by a decreased production capacity of CD4+ T-cells, splenic macrophages, and/or dendritic cells as we measured cytokine secretion in the overall splenocyte culture. As the splenic immune response after trauma hemorrhage has been reported to result in a shift from Th1 to Th2 cytokines, we would have expected an increased production capacity of splenocytes for IL-10. In contrast, we only observed a nonsignificant increase of splenic IL-10 release following femoral fracture and hemorrhage in WT animals. This might be explained by the fact that we used C57BL6 mice, which were shown to be hyporesponsive to trauma hemorrhage compared to animals of the C3H/HeN strain used in other studies [19]. Moreover, it might be argued that femoral fracture and hemorrhage does not result in a Th1/Th2 shift because of the missing effect on the Th2 cytokine release in our study. In this context, it might be suggested that the depression of splenic TNF-α and IL-6 release results from a decreased production capacity of splenic macrophages rather than CD4+ T cells. In order to clarify this point separate isolation of splenic macrophages and/or assessment of more CD4+ T-cell-specific mediators (IL-2, INF-γ, IL-4, and IL-5) would have been helpful. Furthermore, significant differences in the splenic IL-10 release may not become apparent already 4 hours after trauma hemorrhage. Also for answering the question when the splenic immune function is restored after femoral fracture and hemorrhage, further studies with additional time points (e.g., 24 hours, day, 3 days, and 7 days) are needed.

Based on our results, it could be assumed that IL-6 might exert anti-inflammatory effects on the splenic immune function after trauma-hemorrhage. In the present study, we observed the principle depressive effect of femoral fracture and hemorrhagic shock on the splenic TNF-α release in WT and IL-$6^{-/-}$animals. However, the depression of cytokine release was significantly attenuated in the absence of IL-6. The increased TNF-α levels might be explained by a potentially anti-inflammatory role of IL-6 in order to restore TNF-α production (negative feedback mechanism), which itself has been described as a potent inductor of IL-6 synthesis [51, 52]. The significance of this observation on immune competent cells in other tissue compartments as well as on the incidence of infectious complications and outcome after trauma has to be clarified in further studies. In the present study, the secretion of IL-10 was significantly lower in IL-$6^{-/-}$ mice in all study groups, again pointing towards an anti-inflammatory effect of IL-6 on splenocytes, possibly by inducing IL-10 synthesis. Similar results were reported by Yang et al. for mucosal cells, where only a little or no increase of IL-10 mRNA was observed in IL-$6^{-/-}$ mice after hemorrhage, whereas a marked increase in WT mice was found [29]. In the same study, systemic IL-10 levels were found to be comparable between WT and IL-$6^{-/-}$ mice [29]. In contrast, after a septic insult significantly increased IL-10 plasma levels were observed in IL-$6^{-/-}$ mice compared to WT animals [53]. Due to the results of the present studies and the observations made in the literature it might be assumed that the kind of the initiating inflammatory stimulus as well as the observed body compartment might be essential for the net effect of IL-6 [29]. It has been postulated that the net effect of IL-6 on the host inflammatory response reflects a balance of two opposing effects, a paracrine effect of IL-6 that promotes inflammation, and an endocrine effect of IL-6 that diminishes inflammation [54]. As IL-$6^{-/-}$ mice were used in the present study, the results have to be interpreted carefully. In IL-$6^{-/-}$ mice, there might be a functional redundancy in the regulation of the immune response following a traumatic insult [1, 55, 56]. In IL-6-deficient mice, other proinflammatory mediators may take over effects of IL-6 resulting in an only partially impaired inflammatory response compared to WT animals [55]. Furthermore, the present study is limited by the fact that FACS analyses of cell free supernatants were done only once. This may lead to a significant variation of data as shown by the standard deviations in the present study.

5. Conclusions

The depression of splenic cytokine production capacity after femoral fracture with and without hemorrhagic shock at

an early posttraumatic stage underlines the importance of adequate treatment strategies and identification of high risk patients in order to prevent posttraumatic complications. The absence of IL-6 results in significant changes of posttraumatic splenic cytokine release indicating that the posttraumatic modulation of IL-6 synthesis might be a potential target for therapeutic interventions. However, further studies especially with an additional infectious stimulus are needed to identify the relevance of such therapies.

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Research Article

Distinct Proteasome Subpopulations in the Alveolar Space of Patients with the Acute Respiratory Distress Syndrome

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There is increasing evidence that proteasomes have a biological role in the extracellular alveolar space, but inflammation could change their composition. We tested whether immunoproteasome protein-containing subpopulations are present in the alveolar space of patients with lung inflammation evoking the acute respiratory distress syndrome (ARDS). Bronchoalveolar lavage (BAL) supernatants and cell pellet lysate from ARDS patients ($n = 28$) and healthy subjects ($n = 10$) were analyzed for the presence of immunoproteasome proteins (LMP2 and LMP7) and proteasome subtypes by western blot, chromatographic purification, and 2D-dimensional gelelectrophoresis. In all ARDS patients but not in healthy subjects LMP7 and LMP2 were observed in BAL supernatants. Proteasomes purified from pooled ARDS BAL supernatant showed an altered enzyme activity ratio. Chromatography revealed a distinct pattern with 7 proteasome subtype peaks in BAL supernatant of ARDS patients that differed from healthy subjects. Total proteasome concentration in BAL supernatant was increased in ARDS (971 ng/mL ± 1116 versus 59 ± 25; $P < 0.001$), and all fluorogenic substrates were hydrolyzed, albeit to a lesser extent, with inhibition by epoxomicin ($P = 0.0001$). Thus, we identified for the first time immunoproteasome proteins and a distinct proteasomal subtype pattern in the alveolar space of ARDS patients, presumably in response to inflammation.

1. Introduction

The proteasome is a multicatalytic enzyme complex responsible for the degradation of the vast majority of intracellular proteins [1]. Proteasomes are involved in many basic cellular processes including the cell cycle, apoptosis, the stress response, and also in the regulation of immune and inflammatory responses [2–5]. The 26S proteasome consists of a catalytic 20S proteasome core and two 19S (cap) regulatory complexes.

The 20S proteasome itself is a 660–700 kDa [2, 6] multicatalytic proteinase complex with a cylinder-shaped structure arranged as four axially stacked heptametrical rings composed of seven α subunits (outer rings) and seven β subunits (inner rings), respectively [7]. The α type subunits have highly conserved N-terminal extensions which were proposed to have regulatory and targeting function [38]. The proteolytic activities of the 20S proteasome are described as trypsin, chymotrypsin, and peptidyl-glutamyl peptide hydrolyzing activity and are exclusively associated with the proteasome subunits β_1, β_2, and β_5 [8, 9]. Five of the seven β type subunits are synthesized as precursor proteins with N-terminal propeptides that are cleaved off during 20S proteasome biogenesis [13–15] that is mediated by accessory proteins like the proteasome maturation protein (POMP) [10].

In cells exposed to IFN-γ or TNF-α, however, the standard β subunits can be replaced by so-called immuno-subunits β_{1i} (LMP2), β_{2i} (MECL-1), and β_{5i} (LMP7) that are incorporated cooperatively into newly synthesized proteasomes named "immunoproteasome". In case that only partial replacement takes place "intermediate-type proteasomes" are formed [11].

Table 1: Clinical characteristics of ARDS patients.

PaO_2/FiO_2 ratio [mmHg]	82 ± 30
Positive end-expiratory pressure (PEEP) [mbar]	16 ± 4
Venous admixture [%]	45 ± 11
Compliance [mL/mbar]	26 ± 15
Lung injury score (LIS)	3.4 ± 0.4
ECMO therapy [%]	50
In-hospital mortality [%]	53.6
Simplified acute physiology score (SAPS)	63.5 ± 13.6
Sepsis-related organ failure assessment (SOFA)	15.1 ± 3.2

Means ± SD from 28 patients with ARDS. Data were obtained within 24 hours of admission.

The immunoproteasome is more likely to generate peptides with hydrophobic and basic C-terminal residues and less likely to generate peptides with acidic C-terminal residues [12–14]. These short peptides (8–10 amino acids) are subsequently translocated by the transporter associated with antigen processing (TAP) to the endoplasmic reticulum (ER), where a small part of them are loaded on major histocompatibility complex class-I molecules (MHC-I) and presented to cytotoxic T lymphocyte [15] on the cell membrane. Concomitant with immunoproteasome synthesis induced by IFN-γ, other components of the antigen presentation machinery, like TAP [16] or the proteasome activator 28 (PA28), are also upregulated, and a decreased concentration of standard intracellular 26S proteasome is observed [17].

While a prior paradigm was that the proteasome is located only intracellularly, it is now accepted that proteasomes can also be present extracellularly [10]. Recently, we have reported the presence of biologically active 20S proteasome in the extracellular alveolar space in healthy subjects [18] and in patients with the acute respiratory distress syndrome (ARDS) [19]. Since ARDS goes along with pulmonary inflammation [20], proinflammatory mediators [21, 22] like IFN-γ and TNF-α are produced, and the alveolar proteasomal system could be altered. Accordingly, we investigated whether alveolar proteasomal populations are changed in lung inflammation and whether immunoproteasomes are present in the alveolar space of ARDS patients.

2. Material and Methods

2.1. Patients and Clinical Procedures. Twenty-eight adult patients with severe ARDS (13 men, 15 women, mean age: 41 years ± 16 SD) were studied prospectively after approval of the Ethics Committee of the University of Essen Medical School. Characteristics of ARDS patients are depicted in Table 1. To assess disease severity, lung injury score [23], simplified acute physiology score (SAPS) [24], and sepsis-related organ failure assessment (SOFA) [25] were measured. Twenty-two patients (79%) had an ARDS of pulmonary origin, 50% underwent therapy with extracorporeal membrane oxygenation (ECMO), and overall in-hospital mortality was 53.6%.

Patients were considered to suffer from ARDS and eligible for BAL and blood sampling if they met the criteria proposed by Bernard [20]: PaO_2/fraction of inspired oxygen (F_IO_2) ratio of ≤200 mmHg while on a positive end-expiratory pressure (PEEP) ≥10 cm H_2O, bilateral radiographic pulmonary infiltrates, and no clinical evidence of left atrial hypertension or a pulmonary artery occlusion pressure of 18 mmHg or less. The bronchoalveolar lavage (BAL) was performed during sedation/anesthesia in the lung segment showing radiological consolidation and infiltration.

Ten adult subjects without lung disease (7 men, 3 women, mean age: 30 years ± 5) served as controls. They were free of lung, cardiac, infectious, and allergic disease, had no history of chemotherapy or radiation therapy, and they were nonsmokers. In these individuals, BAL and blood sampling were performed during local anesthesia.

2.2. Bronchoalveolar Lavage (BAL). Within 24 h of admission, ARDS patients underwent BAL [26, 27] for routine workup of bacterial and viral infections. Four aliquots of warm (37°C) sterile isotonic saline (40 mL) were instilled via a bronchoscope wedged into a segmental bronchus and gently withdrawn. The BAL of healthy controls BAL was performed by instilling saline into the right middle or left lingular lob. A volume of greater than 50% was recovered, filtered through cotton gauze [28], and centrifuged (500 g, 10 min, 5°C). The BAL supernatant was immediately frozen using liquid nitrogen, stored at −80°C, and served as a sample of the extracellular alveolar fluid.

In the pellet, cell counts were assessed by counting an aliquot in a Neubauer chamber [28]. For cell differentiation, smears were air-dried and stained according to May-Grünwald-Giemsa [27]. The remaining cell pellet was immediately frozen in liquid nitrogen and stored at −80°C. After cell lysis, the cell pellet was ultracentrifugated (30000 g, 30 min, Beckman, München), and the upper portion of this centrifugation step was used for further analysis.

2.3. Blood Samples. To detect immunoproteasome proteins, if present, EDTA blood samples were drawn from all ARDS patients and healthy controls. Blood was centrifugated (500 g, 10 min, 5°C) to separate the supernatant (plasma) from cell pellet.

2.4. Measurements

2.4.1. SDS-PAGE Gelelectrophoresis. SDS-PAGE was performed with Mini-Protean 3 Electrophoresis (Bio-Rad) with 15% gels according to [18]. 50 μg protein per lane were applied. The molecular weight standard was SeeBlue Pre-Stained Standard obtained from Invitrogen.

2.4.2. Detection of Immunoproteasome Proteins by Western Blots. To detect the presence of proteasomal proteins samples (50 μg per lane) from 28 ARDS patients and from 10 healthy subjects the samples were subjected to SDS/PAGE and transferred to PVDF (BioRad) under semidry conditions with the use of a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (BioRad). After blocking the PVDF membranes by incubation with TBS-Tween buffer (5% Tween 20, 150 mM NaCl, 20 mM Tris/HCl, pH 7.6) and StartingBlock Blocking

Buffer (Pierce, Rockford) for 24 hours at 4°C, the membranes were incubated with rabbit polyclonal antibody to 20S proteasome subunit β_{1i} (LMP2) (Biomol International LP; PW 8840) (dilution 1 : 1000, 2 h, room temperature), rabbit polyclonal antibody to 20S proteasome subunit β_{5i} (LMP7) (dilution 1 : 2500, 2 h, room temperature), and with *rabbit* polyclonal antibody to proteasome activator 28 (PA28) (dilution 1 : 1000, 2 h, room temperature), as described elsewhere [29].

After washing with TBS-Tween buffer (5% Tween 20, 150 mM NaCl, 20 mM Tris/HCl, pH 7.6), the membranes were incubated (1 : 10000, 1 h, room temperature) with peroxidase-conjugated affinity-isolated goat anti-rabbit IgG (Sigma Aldrich). After washing, the chemoluminescence method was employed to detect the peroxidase activity using an ECL kit (SuperSignal West Pico Chemiluminescence Substrate, Pierce).

2.4.3. Determination of Total Proteasome Concentration in BAL Supernatant. Proteasome concentration was measured [30] by ELISA in BAL supernatants of all ARDS and of all healthy subjects. Microtitration plates were coated overnight with mouse monoclonal antibody to 20S proteasome subunit α6 (HC2) (Biomol International L.P., Exeter, UK) 1 : 4500 in PBS (Invitrogen GmbH, Karlsruhe, FRG), pH 7.4. The BAL supernatants were diluted with an equal volume PBST-BSA (PBS, Tween 20, 0.1%, and 1% bovine serum albumin) and applied to each well for 3 hours at room temperature. All measurements were covered by the linear portion of the respective ELISA standard curve.

Standard curves were established for every microtitration plate using 20S proteasome protein standards (Biomol International L.P., Exeter, UK) of concentration ranging from 19.5 ng mL^{-1} to 2500 ng mL^{-1} (8 linear dilution steps). The 20S proteasome was diluted in PBS-T (PBS and Tween 20, 0.1%). The plates were washed once, and a rabbit polyclonal antibody (Biomol International L.P., Exeter, UK) to 20S proteasome (dilution 1 : 4000) was added for 2 hours at room temperature. Following another four washing steps peroxidase-conjugated mouse anti-rabbit IgG (Sigma-Aldrich, Saint Louis, USA) was used for antigen detection (incubation period: 1 h at room temperature). The bound antibodies were detected using tetramethylbenzidine (Sigma-Aldrich, Saint Louis, USA) as substrate. The reaction was stopped with sulphuric acid, and OD-values were determined at 450 nm. To exclude nonspecific binding, wells were filled with bovine serum albumin (Sigma-Aldrich, Saint Louis, USA), PBS, or PBS-T instead of BAL supernatant and incubated with the antibody. No reaction was observed under these control conditions.

2.4.4. Purification of Proteasomes from BAL Supernatant. 20S proteasomes from 5 patients with ARDS and from 5 healthy subjects were purified as described elsewhere [31]. All purification steps were performed at 4°C. To the pooled BAL supernatant from 5 ARDS patients the same volume of TEAD buffer (20 mM Tris/HCl, 1 mM EDTA, 1 mM NaN_3, 1 mM DTT, pH 7.5) was added, and the mixture was homogenized by use of a Dounce homogenizer (20 strokes) under ice cooling. Undissolved material was separated by centrifugation (50 min at 20000 g). The supernatant was then subjected to a column (1 × 8 cm) of DEAE-Toyopearl 650S (TOSOH Biosep GmbH, Stuttgart, Germany) equilibrated with TEAD buffer. After washing the column with 50 mM NaCl/TEAD buffer, proteins bound to the resin were eluted with a linear gradient of 50–500 mM NaCl dissolved in TEAD buffer. Fractions of 1 mL were collected and tested for their proteasome activity with the fluorogenic substrate Suc-LLVY-AMC. Proteasome-containing fractions were then pooled, and 20S proteasomes were purified by successive chromatographies on Superose 6 (Pharmacia HR 10 × 30), Mono Q (HR 5/5) and Phenyl-Superose (HR 5/5) in conjunction with the FPLC system. All chromatographies were run in TEAD buffer. For elution of the enzyme from MonoQ a gradient of 0–500 mM NaCl and from Phenyl-Superose a gradient of 1.2–0 M $(NH_4)_2SO_4$ were used, respectively. The purified enzyme was finally dialyzed against TEAD buffer.

2.4.5. Purification of Proteasomes from Human Spleen, Cells, and Plasma. Purification of proteasomes from human erythrocytes and plasma was performed exactly as described by Zoeger et al. [32]. Briefly, "fraction II" was prepared from cell extract by use of DEAE-Sephacel, which was then used to obtain by ammonium sulphate (30–80% saturated with $(NH_4)_2SO_4$) precipitation a proteasome-containing fraction. The enzyme was then purified by successive chromatography on DEAE-Toyopearl 650S, preparative Superose 6, and MonoQ. For all chromatographic TEAD buffer was used. Finally, the enzyme was subjected to affinity chromatography with an antibody to subunit α3 as ligand, as described elsewhere [32], and was then dialysed against TEAD buffer.

Normal human spleen tissue purchased from Enzo Life sciences Ltd.

2.4.6. Two-Dimensional Polyacrylamidegel Electrophoresis (2D-PAGE). Preparation and performing 2D-PAGE with purified proteasomes from BAL supernatant of ARDS patients in 8 × 10 cm gels were exactly done as described by Schmidt et al. [33]. Designation of proteasome subunits corresponded to that used by Schmidt et al. [33] and by Froment et al. [34] without applying the nomenclature of the minor subforms of the α- and β-subunits. Proteasome concentration of healthy subjects after purification was too low to allow additional 2-D PAGE electrophoresis.

2.4.7. Proteasomal Activity. The proteasomal activity was measured fluorometrically in BAL supernatant in all ARDS patients and in all healthy controls using specific fluorogenic substrates and techniques previously described (19). We tested for peptidyl-glutamyl peptide-hydrolysing activity (PGPH) with 200 μM benzoyloxycarbonyl-LLE-7-amido-4-methylcoumarin (Z-LLE-MCA), for trypsin-like activity (Try) with 200 μM benzoyl-VGR-MCA (Bz-VGR-MCA), and for chymotrypsin-like activity (Chtr) with 100 μM succinyl-LLVY-MCA (Suc-LLVY-MCA) as substrates (46, 47). All measurements were performed in duplicate and averaged for each subject. To describe the specific enzyme activity of

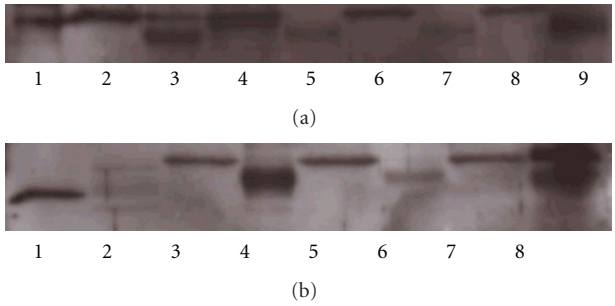

Figure 1: Western blots with a polyclonal antibody directed against LMP2 or LMP7 subunits of the immunoproteasome of samples of BAL supernatant and BAL cell pellet lysate obtained from ARDS patients. (a) LMP2 immunoproteasome protein was detected in both BAL supernatant and cell pellet in all ARDS patients. Lanes are identified as follows: Lane 1: 1 μg immunoproteasome (human spleen); Lane 2: cell pellet ARDS patients 1; Lane 3: BAL supernatant ARDS patients 1; Lane 4: cell pellet ARDS patients 2; Lane 5: BAL supernatant ARDS patients 2; Lane 6: cell pellet ARDS patients 3; Lane 7: BAL supernatant ARDS patients 3; Lane 8: cell pellet ARDS patients 4; Lane 9: BAL supernatant ARDS patients 4. (b) LMP7 immunoproteasome protein was detected in both BAL supernatant and cell pellet in all ARDS patients. Lanes are identified as follows: Lane 1: 1 μg immunoproteasome (human spleen); Lane 2: 1 μg 20S standard proteasome (human erythrocyte); Lane 3: cell pellet ARDS patients 1; Lane 4: BAL supernatant ARDS patients 1; Lane 5: cell pellet ARDS patients 2; Lane 6: BAL supernatant ARDS patients 2; Lane 7: cell pellet ARDS patients 3; Lane 8: BAL supernatant ARDS patients 3.

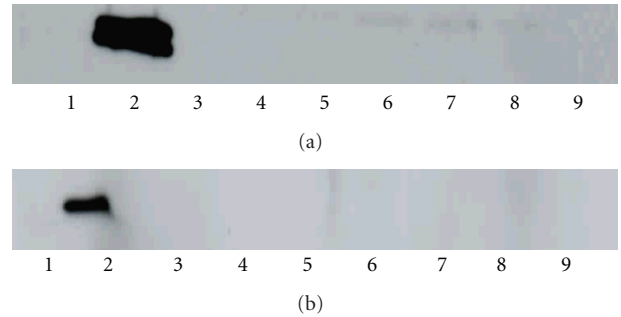

Figure 2: Representative western blots with a polyclonal antibody directed against LMP2 or LMP7 subunits of the immunoproteasome of samples of BAL supernatant obtained from healthy subjects. (a) LMP2 immunoproteasome protein could not be detected in BAL supernatant of any healthy subject. Lanes are identified as follows: Lane 1: 1 μg 20S standard proteasome (human erythrocyte); Lane 2: 1 μg immunoproteasome (human spleen); Lane 3: BAL supernatant healthy subject 1; Lane 4: BAL supernatant healthy subject 2; Lane 5: BAL supernatant healthy subject 3; Lane 6: BAL supernatant healthy subject 4; Lane 7: BAL supernatant healthy subject 5; Lane 8: BAL supernatant healthy subject 6; Lane 9: BAL supernatant healthy subject 7. (b) LMP7 immunoproteasome protein could not be detected in BAL supernatant of any healthy subject. Lanes are identified as follows. Lane 1: 1 μg 20S standard proteasome (human erythrocyte); Lane 2: 1 μg immunoproteasome (human spleen); Lane 3: BAL supernatant healthy subject 1; Lane 4: BAL supernatant healthy subject 2; Lane 5: BAL supernatant healthy subject 3; Lane 6: BAL supernatant healthy subject 4; Lane 7: BAL supernatant healthy subject 5; Lane 8: BAL supernatant healthy subject 6; Lane 9: BAL supernatant healthy subject 7.

extracellular proteasomes we used fluorogenic substrate cleavage (pmol/min $\times\mu$g).

2.4.8. Analysis of Proteasome Subtypes. Purified 20S proteasomes from 5 pooled BAL supernatants of ARDS patients were separated by high-resolution anion exchange chromatography (in conjunction with a SMART-Chromatography System; Amersham Biosciences) on Mini Q equilibrated with TEAD-buffer exactly as described elsewhere [35]. Purification of 20S proteasome from pooled BAL of 5 healthy subjects turned out to be impossible due to the low 20S proteasome concentration in BAL supernatant.

2.4.9. Lactate Dehydrogenase Activity in BAL Supernatant. Total (LDH_1–LDH_5) lactate dehydrogenase (LDH) activity was measured by a kinetic uv-test (Diaglobal GmbH, Berlin, FRG) using an optimized standard method (IFCC).

2.4.10. Total Protein Concentrations in BAL Supernatant. Total protein concentration was determined after trichloroacetic acid (TCA) precipitation (5%), washing, and resolubilization according to Lowry using an autoanalyzer (Technicon) employing bovine serum albumin (BSA) as a standard.

2.5. Chemicals. All chemicals were of highest available or analytical grade. Water was deionized, distilled, and passed through a Milli-Q-System (Millipore, Witten) before use.

2.6. Statistical Analysis. Analyses were performed with SPSS, version 9 (SPSS, Inc., Chicago, USA). Continuous variables are presented as means ± standard deviation (SD). Nonparametric variables were compared by using the Mann-Whitney *U*-test, as indicated. Data are presented as median and range and were not normally distributed. Comparison of values of variables between groups (ARDS versus healthy subjects) was performed using the Mann-Whitney *U* test. Differences were regarded as statistically significant with an a priori alpha-error *P* of less than 0.05.

3. Results

Most important, all ARDS patients showed both LMP2 and LMP7 immunoproteasome proteins in the BAL supernatant and also in their cell pellet lysate (Figures 1(a) and 1(b)). In contrast, LMP7 and LMP2 were not detected in the BAL supernatant (Figures 2(a) and 2(b)) of any healthy subject. LMP2 was only detected in the cell pellet of healthy controls whereas LMP7 was not.

The molecular weight of the immunoproteasome positive protein bands in the western blots of the BAL cell pellet lysate from ARDS patients was greater than that in their BAL supernatants, suggesting that extracellular immunoproteasome protein-containing proteasomes are assembled from larger intracellular pro-proteins.

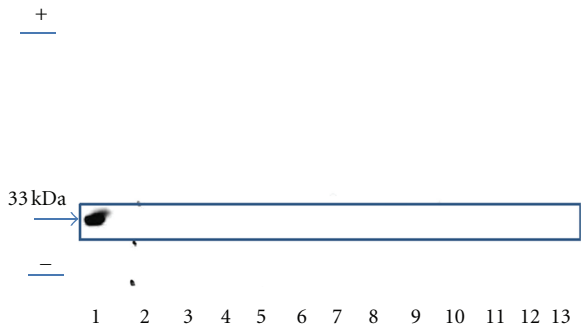

Figure 3: Representative Western blot with a polyclonal antibody directed against PA28 in BAL supernatant of twelve patients with ARDS. PA28 protein could not be detected in the BAL supernatant of ARDS patients. Start and front of the gel were marked as + and −. Lanes are identified as follows: Lane 1: 1 μg PA28 (standard); Lane 2–13 BAL supernatant of twelve ARDS patients.

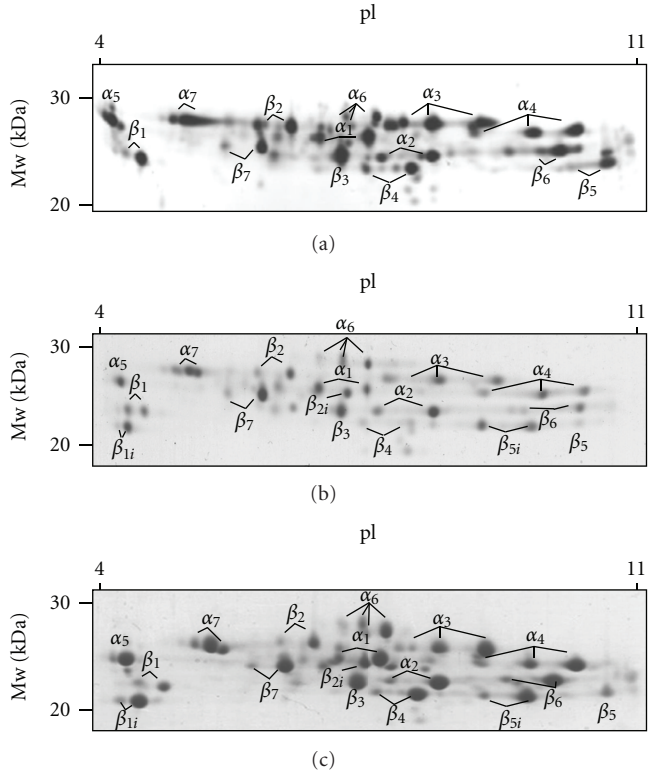

Figure 4: 2D-PAGE of purified 20S proteasomes from (a) red cells (5 μg), (b) BAL-supernatant (20 μg) from ARDS patients, and (c) spleen (30 μg). Detection of protein spots was performed by silver staining and Coomassie BB G250, respectively. Standard 20S proteasome was exclusively detected in red cells (a). Samples of human spleen and of the BAL supernatants from ARDS patients showed both standard and immunoproteasome proteins (panels (b) and (c)).

PA28 could neither be detected in BAL supernatants of all patients with ARDS nor in healthy controls. Figure 3 shows a western blot with an antibody directed against the PA28 activator.

Purification and 2-D gelelectrophoresis of the BAL supernatant from ARDS patients showed 20S proteasomal core proteins (Figure 4(a)). Immunoproteasome subunits β_{1i} (LMP2), β_{2i} (MECL-1), and β_{5i} (LMP7) were detected in the two-dimensional polyacrylamide gelelectrophoresis (Figure 4) confirming the data derived from the western blots. Like BAL supernatant from ARDS patients samples of splenic tissue, but not human red cells, revealed immunoproteasome subunits.

Comparison of the specific activities of purified proteasome (Table 2) from pooled BAL supernatant of healthy controls and of ARDS patients showed a lower proteasomal activity in ARDS patients but also a different ratio of the individual proteasomal enzyme activities (Table 2) suggesting a change of proteasomal subunit composition. With a ratio of peptidyl-glutamyl peptide-hydrolysing activity (PGPH) to trypsin-like activity (Try) of 11.2 versus 14.6, a ratio of chymotrypsin-like activity (Chtr) to trypsin-like activity of 33 versus 14.5, and a ratio of the chymotrypsin-like activity to the peptidyl-glutamyl peptide-hydrolysing activity (Chtr/PGPH: 2.95 versus 0.99) these activity ratios were different in ARDS patients when compared to healthy controls.

Chromatography (Figure 5) of a pooled sample of BAL supernatants from 5 ARDS patients revealed a new proteasomal subtype pattern with distinct numbers and proportions of seven peaks (I–VII) unlike that of human circulating plasma proteasome. In fact, since the alveolar subtype pattern seen in ARDS patients was not even similar to the subtype patterns found in erythrocytes, platelets, monocytes, and T lymphocytes (32), respectively, the extracellular alveolar proteasome found in ARDS patients is unlikely to derive from the blood stream.

In contrast to the BAL supernatant of healthy individuals, the plasma and the BAL cell pellet of all healthy subjects and of all ARDS patients showed both LMP2 and LMP7 proteins in the western blots (data not shown).

Total proteasome concentration in BAL supernatants of ARDS patients was higher (971 ± 1116 ng/mL) compared to healthy subjects (59 ± 25; $P < 0.001$) (Table 3), and all fluorogenic substrates were hydrolyzed by BAL supernatants of ARDS patients (Suc-LLVY-AMC: 3.1 ± 6.2 pkat/mg; Bz-VGR-AMC: 1.8 ± 2.5; Z-LLE-AMC: 0.8 ± 1.1) and of healthy subjects (Suc-LLVY-AMC: 7.3 ± 3.7 pkat/mg; Bz-VGR-AMC: 5.6 ± 3.2; Z-LLE-AMC: 2 ± 1.2), with inhibition by epoxomicin ($P = 0.0001$).

There was no significant correlation ($P = 0.16$) in ARDS patients between proteasome concentration in BAL supernatant and in their plasma. In addition, there was no correlation between LDH activity and proteasome concentration in BAL supernatant ($P = 0.21$), or between BAL cell count and proteasome concentration in BAL supernatant ($P = 0.26$), ruling out cell lysis as a major source of proteasome in the extracellular alveolar space.

Our patients by any criteria had severe ARDS (Table 1) and also showed marked physiological derangements, as indicated by a high simplified acute physiology score and sepsis-related organ failure assessment.

Table 2: Specific activities of proteasomes isolated from healthy controls and ARDS patients.

	Chtr (pmol/min μg)	Try (pmol/min μg)	PGPH (pmol/min μg)	Chtr/PGPH	PGPH/Try	Chtr/Try
Healthy controls	24.31	0.73	8.22	2.95	11.2	33.3
ARDS patients	9.87	0.68	9.93	0.99	14.6	14.5

Proteolytic activities of purified 20S proteasome from BAL supernatant of healthy controls and of ARDS patients, as measured with specific proteasomal fluorogenic substrates. BAL supernatants were pooled from 5 healthy subjects and from 5 ARDS patients, respectively. The ratio of enzyme activities differs between ARDS patients and healthy subjects, suggesting a rearrangement of proteasomal subunit composition.
PGPH: peptidyl-glutamyl peptide-hydrolysing activity; Try: trypsin-like activity; Chtr: chymotrypsin-like activity.

Table 3: Characteristics of BAL in ARDS and healthy subjects.

	ARDS patients ($n = 28$)	Healthy subjects ($n = 10$)	P value
Proteasome concentration in BAL supernatant [ng/mL]	971 ± 1116	59 ± 25	<0.001
Proteasome concentration in plasma [ng/mL]	2855 ± 2422	348 ± 126	<0.001
Suc-LLVY-AMC proteasome activity in BAL supernatant [pkat/mg]	3.1 ± 6.2	7.3 ± 3.7	<0.001
BZ-VGR-AMC proteasome activity in BAL supernatant [pkat/mg]	1.8 ± 2.5	5.7 ± 3.2	<0.001
Suc-LLE-AMC proteasome activity in BAL supernatant [pkat/mg]	0.8 ± 1.1	2 ± 1.2	0.002
Total protein concentration in BAL supernatant [mg/mL]	3.8 ± 6.4	0.06 ± 0.01	<0.001
Albumin concentration in BAL supernatant [mg/mL]	1.5 ± 3	0.03 ± 0.01	0.0011
LDH in BAL supernatant [U/L]	342 ± 779	28 ± 9.7	0.024
LDH in plasma [U/L]	821 ± 1104	184 ± 53	<0.001
Cell count in cell pellet [10^6/mL]	330 ± 994	8.6 ± 2.5	0.007
Macrophages [%]	29.1 ± 27.4	92.6 ± 3.4	<0.001
Neutrophile granulocytes [%]	65.1 ± 27.7	2.8 ± 2.5	<0.001
Lymphocytes [%]	5.1 ± 7.9	6.3 ± 2.9	0.056

Data are means ± SD.

4. Discussion

Our data show that the extracellular alveolar space in ARDS patients contains (1) an altered proteasomal composition with a distinct proteasomal subtype pattern, and (2) immunoproteasome proteins, that is, a different type of proteasome when compared to healthy subjects. Most likely, these alterations are evoked by pulmonary inflammation.

Intracellularly, three subpopulations of 20S proteasomes are known, that is, standard proteasome, immunoproteasome, and intermediate-type proteasomes. These subpopulations can only be separated and characterized by high-resolution anion exchange chromatography, as used in our study, and not by ELISA or western blotting techniques.

Detection of the immunoproteasomal subunits LMP 2 and LMP 7 in the extracellular alveolar space in all ARDS patients but not in healthy controls was associated with a change in the ratio of proteasomal enzyme activities as revealed following purification. This is consistent with reports that intermediate-type proteasomes or immunoproteasomes, at least in cell cultures, show an altered ratio of peptidyl-glutamyl peptide-hydrolyzing to trypsin-like activity and of chymotrypsin-like to trypsin-like activity when compared to the standard 20S proteasome [11, 13, 14, 36, 37]. Presumably, this is caused by a decrease in size and charge of the S1 pocket of β_{i1} as compared to that of β_1 [38]. Thus, the observed change of proteasomal composition and activity in BAL supernatant of ARDS patients may be caused by replacement of standard 20S proteasome proteins by catalytic subunits β_{1i} (LMP2), β_{2i} (MECL-1), and β_{5i} (LMP7) that are incorporated into a newly synthesized intermediate type and/or immunoproteasome.

Despite detection of immunoproteasome proteins LMP 2 and LMP 7 by western blot and of MECL-1 by 2-D gelelectrophoresis it remains unclear whether it is pure immunoproteasome and/or intermediate-type proteasomes that are found in the extracellular alveolar space of ARDS patients. However, data obtained in cells [32] suggest that the proteasomal subtype pattern seen in BAL supernatant of our ARDS patients represents intermediate-type proteasome as a dominant proteasome fraction. The higher molecular weight of the Immuno β catalytic subunits in the cell pellet lysate of ARDS patients presumed the existence of proproteins as described elsewhere [39–41]. These findings suggest that the Immuno β catalytic subunits were built in the cell pellet, and the completed immunoproteasomes were transported into the alveolar space. This mechanism of extracellular transport of the immunoproteasome is unclear and further work had to be done to clarify this question.

In any case, that immunoproteasome proteins were detected in the BAL supernatant of ARDS patients but not in healthy individuals, which likely represents a biological reaction in response to alveolar inflammation. ARDS results in a marked proinflammatory response with high IFN-γ and TNF-α [21, 42, 43] concentration in the alveolar space. While we did not measure alveolar cytokine concentrations one may speculate that high IFN-γ concentrations induce the assembly of immunoproteasome proteins. In this context,

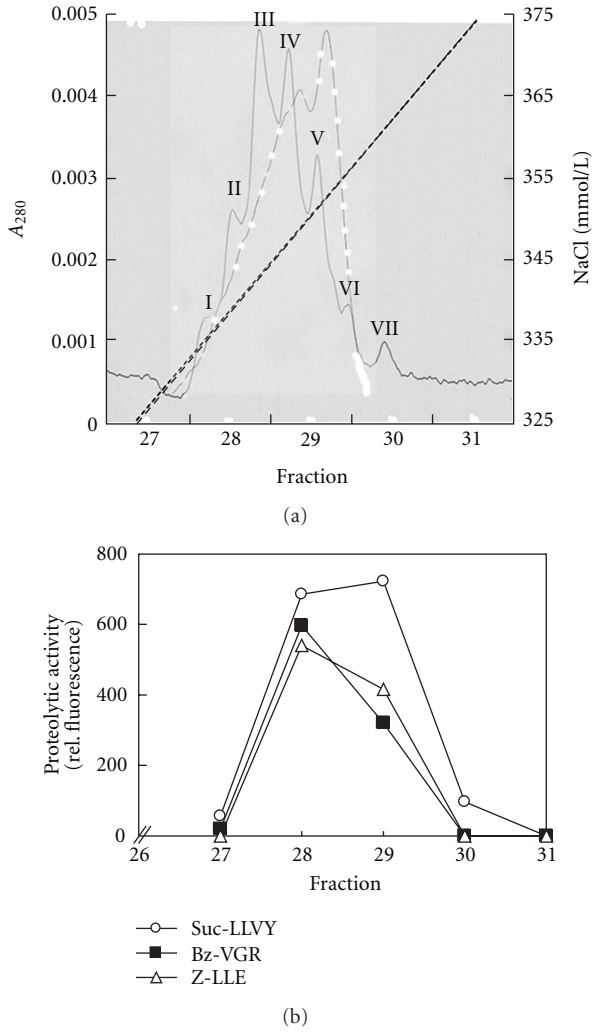

Figure 5: Subtype pattern of the extracellular alveolar proteasome of patients with ARDS (continuous line) and of plasma from healthy subjects (white points). (a) 20 μg of 20S proteasome from pooled BAL supernatant of five ARDS patients were purified, subjected to chromatography on Mini Q, and separated into their subtypes by elution with increasing concentrations of NaCl. Subtypes detected by absorption at 280 nm are designated by roman figures (I–VII) according to the order of their elution from the column. All subtypes elute at NaCl concentrations (dashed line) between 330 and 370 mmolNaCl/L, and only this detail of the chromatograms is shown. (b) All collected fractions of the subtype pattern chromatography of the extracellular alveolar proteasome of patients with ARDS were measured by the highly specific proteasomal fluorogenic peptides Suc-LLVY-AMC (open points), BZ-VGR-AMC (black rectangle), and Suc-LLE-AMC (Z-LLE-AMC) (open triangle). Only in the fractions 28–30, proteasomal enzyme activity could be observed. Analysis of alveolar proteasome revealed a new proteasomal subtype pattern in the extracellular alveolar space of ARDS patients that differs from that of healthy subjects' plasma, suggesting that the extracellular alveolar proteasome in ARDS does not derive from plasma.

the greater molecular weight of the immuno β catalytic subunits found in the cell pellet lysate of ARDS patients suggests the existence of immunoproteasome pro-proteins (13–15) that by a yet undefined mechanism apparently gain access to the extracellular space.

In this study, we identified for the first time a new proteasomal subtype pattern in the alveolar space of ARDS patients that differs from that of proteasomes in blood cells. Therefore, the extracellular alveolar immunoproteasome and/or intermediate-type proteasome found in ARDS patients is unlikely to derive from cytolysis of blood cells and sequestration of their contents into alveoli across leaky endothelial and epithelial barriers. This is supported by the finding that no significant correlation between the proteasome concentration in plasma and in BAL supernatant was seen. Thus, while endothelial and epithelial damage as well as basement membrane destruction is a feature of ARDS [20, 44] extravasation of circulating proteasomes alone cannot be responsible for the presence of extracellular alveolar 20S proteasomes.

By the same token, it is unlikely that alteration of proteasomal composition in the alveolar space in ARDS patients resulted from lysis of cells of the alveolar wall. This appears to be ruled out by the fact that PA28 proteasomal caps, normally present intracellularly, were not found in western blots from BAL supernatant of patients with ARDS. In addition, masked PA28 proteasomal caps (by proteins or protein complexes) might not be accessible using western blot analysis so that this conclusion has to be verified by MS analysis. Furthermore, no significant correlation between total proteasomal concentration in BAL supernatant and LDH activity, a marker of cell lysis, or with the BAL cell count was observed. Thus, the presence of immunoproteasome proteins likely relates to the inflammatory process in lung tissue rather than to cell lysis.

Since no 19S and PA28 proteasomal cap proteins were detected by western blot of BAL supernatant, 26S proteasome and/or hybrid proteasome were not present in the alveolar space of patients with ARDS. However, since the detection limit of our method is in the range of 0.5–1 μg protein/μL we cannot exclude the presence of lesser extracellular concentrations of 26S proteasome.

Our data showing the presence of immunoproteasome proteins and a distinct proteasomal subtype pattern in BAL supernatant from patients with ARDS extend our previous work [19] reporting increased total proteasome concentrations but lesser proteasomal activities when compared to healthy subjects.

Different types of proteasomes are known to have different cleavage repertoires [45] and to yield different peptides for antigen presentation [16]. Possibly, a function of the extracellular immunoproteasome, evoked by inflammation, could be to cleave epitopes different from that of the standard 20S proteasome. It is unknown which extracellular proteins are degraded by the standard proteasome and which ones by the immunoproteasome or the intermediate-type proteasome. However, the presence of immunoproteasome proteins may suggest an altered extracellular protein degradation [26]. In any case, the presence of immunoproteasome proteins in the BAL supernatant of ARDS patients raises the provocative question whether antigen processing and hence

part of the immunological response could also take place in the extracellular alveolar space.

To our knowledge, this study is the first to address the presence of immunoproteasome proteins in lung disease and the activity of extracellular alveolar proteasome in ARDS patients. Fluorogenic substrates, used in combination with epoxomicin, the most potent, selective, and irreversible proteasome inhibitor currently available, and an ELISA are accepted methods for analyzing proteasomal existence and activity [30, 46, 47]. In this study, we used an ELISA technique for the measurement of proteasomal concentration in the BAL supernatant. This technique does not allow to discriminate quantitatively between the 20S proteasome and the immunoproteasome. The western blots directed against LMP2 and LMP7, however, showed high signal intensity of the immunoproteasome proteins, likely reflecting a high concentration of immunoproteasome proteins in the BAL supernatant, in patients with ARDS but not in healthy controls.

It is conceivable, therefore, that quantitative immunoproteasome measurements in BAL might provide discrimination between disease activity, clinical scores, predictable survival, and efficacy of therapy. Obviously, this should be addressed in further studies.

In summary, we identified immunoproteasome proteins in the extracellular alveolar space of patients with ARDS, which are absent in healthy controls, and we discovered a distinct, previously undescribed alveolar proteasome subtype pattern that differs from the 20S proteasomes found in various blood cells. This may alter cleavage of alveolar proteins existing in the alveolar space during pulmonary inflammation seen in ARDS.

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Clinical Study

Deoxyribonuclease Is a Potential Counter Regulator of Aberrant Neutrophil Extracellular Traps Formation after Major Trauma

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Introduction. Neutrophil extracellular traps (NET) consist of a DNA scaffold that can be destroyed by Deoxyribonuclease (DNase). Thus DNases are potential prerequisites for natural counter regulation of NETs formation. In the present study, we determined the relationship of NETs and DNase after major trauma. *Methods*. Thirty-nine major trauma patients, 14 with and 25 without sepsis development were enrolled in this prospective study. Levels of cell-free (cf)-DNA/NETs and DNase were quantified daily from admission until day 9 after admission. *Results*. Levels of cf-DNA/NETs in patients who developed sepsis were significantly increased after trauma. In the early septic phase, DNase values in septic patients were significantly increased compared to patients without sepsis ($P < 0.05$). cf-DNA/NETs values correlated to values of DNase in all trauma patients and patients with uneventful recovery ($P < 0.01$) but not in septic patients. Recombinant DNase efficiently degraded NETs released by stimulated neutrophils in a concentration-dependent manner in vitro. *Conclusions*. DNase degrades NETs in a concentration-dependent manner and therefore could have a potential regulatory effect on NET formation in neutrophils. This may inhibit the antibacterial effects of NETs or protect the tissue from autodestruction in inadequate NETs release in septic patients.

1. Introduction

Major trauma is associated with the induction of systemic inflammation, development of sepsis, and multiple organ failure (MOF) [1–3]. Neutrophils are the main types of effector cells in the innate immune system and are the first line against infection [4–7]. Activated polymorphonuclear neutrophils play a pivotal role in the systemic inflammatory response syndrome (SIRS) and development of sepsis after major trauma [8].

In addition to the more traditional mechanism of phagocytosis to kill bacteria, it has recently been shown that activation of neutrophils can cause the release of neutrophil extracellular traps (NETs) [9]. NETs are large DNA-associated molecule complexes carrying nucleic and cytoplasmic proteins such as histones, elastase, myeloperoxidase (MPO), pentraxin, lactoferrin, and bactericidal/permeability-increasing protein (BPI) [10, 11]. Each of them has got strong antimicrobial and/or immunomodulating properties. Upon activation (by e.g., IL-8, lipopolysaccharide, bacteria, fungi, or activated platelets) neutrophils start a program that leads to the formation of NETs [12–14]. The formation of NETs, recently termed "NETosis", is an active process and is distinct from neutrophil apoptosis and necrosis involving *postmortem* antimicrobial and proinflammatory immune responses [15]. NETs provide a high local concentration of antimicrobial components and bind, disarm, and kill microbes extracellularly as an emergency first line defense mechanism [9]. NET trapping in the tissue may allow the host to confine an infection and thus reduce the likelihood for the pathogens to spread into the bloodstream [12]. On the other hand, the high local concentration of NETs-associated effector molecules may contribute to severe tissue damage and organ dysfunction and/or failure [16].

Deoxyribonuclease (DNase) I, a Ca^{2+}/Mg^{2+}-dependent endonuclease, is the major nuclease found in body fluids such as serum and urine. Its primary function has been assumed to be the degradation of dietary DNA within the alimentary tract. Moreover, it has been shown that extracellular

DNase may account for the chromatin breakdown during necrosis as a basis of protection against anti-DNA autoimmunity. DNA is the major structural component of NETs with granule proteins attached to this DNA backbone [9]. Thus, the DNA scaffold of NETs can be destroyed by DNase. So, aberrant NET formation in combination with lack of patient's DNases degrading NETs might contribute to their prolonged persistence with subsequent tissue damage and/or autoimmune diseases [17–20]. Furthermore, DNases are expressed by several bacterial pathogens. Bacterial DNases act as a virulence determinant by counteracting NETs-mediated trapping, thereby promoting bacterial spread from local sites to the bloodstream [17, 18].

Recently it has been shown that NETs kinetics followed the inflammatory course after severe trauma [21]. In the current study we aimed to determined cfDNA/NETs and DNase in the serum of critically ill patients during the early posttraumatic phase. We further provide evidence for a DNase-mediated dissolution of NETs in vitro.

2. Materials and Methods

2.1. Patients. Thirty-nine patients were enrolled in this prospective study. Study approval was obtained from the Ethics Review Board of the University of Duesseldorf, Germany. Patients with blunt or penetrating multiple injuries who were admitted to our Level I Trauma Center with an Injury Severity Score (ISS) >16, and aged 18 years and older were enrolled in this study. Written informed consent was obtained from all participants or their legal representatives if the patients lacked consciousness. Exclusion criteria were death of the patient on day of admission or within the first two days on ICU and withdrawal of patient consent. In addition, patients with known preexisting immunological disorders or systemic immunosuppressive medication were excluded. The severity of injury was assessed by the ISS, based on the Abbreviated Injury Scale (AIS) [19]. SIRS and sepsis were defined using the criteria outlined 2005 from the International Sepsis Forum [20]. Patients were determined as septic if they fulfilled criteria for systemic inflammatory response syndrome and had a proven source of infection. Systemic inflammatory response syndrome was defined by two or more of the following criteria: temperature >38°C or <36°C; heart rate >90 beats per minute; respiratory rate >20 breaths per minute or arterial carbon dioxide tension ($PaCO_2$) <32 mmHg; white blood cell count >12.000 cells/mm^3 or <4.000 cells/mm^3, or with >10% immature (band) forms. In order to evaluate organ dysfunction/failure, the Sequential Organ Failure Assessment (SOFA) and Multiple Organ Dysfunction (MOD) score were determined prospectively every day. In addition clinical laboratory data, including red and white blood counts, electrolytes, creatinine, blood urea nitrogen, C-reactive protein (CRP), and liver enzymes were monitored daily. Serum and EDTA blood were collected on admission to the emergency room (ER) and on days 1 to 10 after injury. Samples were centrifuged, immediately frozen, and stored at −80°C until further analysis.

2.2. cfDNA/NETs Assay. cfDNA/NETs were quantified using the Quant-iT PicoGreen dsDNA assay (Invitrogen GmbH, Darmstadt, Germany). This assay used to label neutrophil-derived NETs by targeting the NET containing cf-DNA directly within serum has been recently developed [22]. The fluorescence intensity reflects the amounts of DNA and was measured at excitation and emission wavelengths of 485 nm and 530 nm, respectively, in a microplate reader (Victor3, PerkinElmer, Waltham, USA) A standard calibration curve by means of defined calf thymus DNA (Sigma) amounts ranging from 0 to 2 μg/mL has been used in all analyses.

2.3. Quantification of Desoxyribonuklease (DNase) by ELISA. Desoxyribonuklease (DNase) levels in serum samples were measured by using ORG 590 DNase Activity Immunometric Enzyme Immunoassay for the Quantitative Determination of DNase Activity (ORGENTEC, Mainz, Germany) according to the manufacturer's instructions. Additionally, the concentration of DNase in human sera was quantified using known concentrations of the standard provided with rh-DNase1 (0.75 up to 12.5 ng/mL).

2.4. Isolation of Human Neutrophils. Human neutrophils were isolated by discontinuous density-gradient centrifugation on Percoll (Biochrom, Berlin, Germany) as previously described [23]. After hypotonic lysis to remove contaminating erythrocytes, cells were suspended in phosphate-buffered saline (PBS). Purity and viability were routinely >95% as assessed by forward and side scatter characteristics of FACScan (BD, Heidelberg, Germany) and trypan blue exclusion, respectively.

2.5. Stimulation of Neutrophils and NETs Release. Freshly isolated neutrophils from healthy volunteers were resuspended in RPMI 1640 containing 2 mM glutamine supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat inactivated fetal calf serum to a final concentration of 2×10^6/mL. Cells were stimulated with 5 nM PMA for 3 h at 37°C in a humidified atmosphere containing 5% CO_2. Then cfDNA/NETs release was quantified in the supernatant. In addition, supernatants were incubated with 0, 0.02, 0.2, 2.0, and 10 μg/mL recombinant DNase (Pulmozyme, Roche, Swisse) for 3 h at 37°C before cfDNA/NETs quantification.

2.6. Immunofluorescence Staining of NETs. For immunofluorescence, freshly isolated neutrophils were seeded on poly-D-lysin-coated coverslips, allowed to adhere, and stimulated with 50 nM PMA for 3 h at 37°C. Cells were further fixed with 4% PFA and blocked with 5% NGS, 0.3% Triton X-100 in PBS for 30 min. To stain NETs, samples were incubated with a monoclonal mouse antimyeloperoxidase antibody (1 : 300) and a secondary FITC-conjugated antibody (1 : 200; both Dako, Hamburg, Germany). After staining of DNA with DAPI, specimens were mounted in Dako fluorescent mounting medium (Dako). Neutrophil-derived NET formation was visualized by immunofluorescence microscopy (Axiovert 100, Zeiss, Goettingen, Germany).

Table 1: Infection site of sepsis and microbiological pathogens.

Subject	Site of infection	Pathogen	Evidence for sepsis, d
1	Pneumoniae	n.d.	8
2	Pneumoniae	*Escherichia coli*	7
3	Pneumoniae	*Klebsiella pneumoniae*	5
4	Pneumoniae	*Klebsiella pneumoniae*	4
5	Pneumoniae	*Morganella morganii*	6
6	Pneumoniae	*Enterobacter cloacae*	4
7	Soft tissue	*Enterobacter cloacae*	8
8	Pneumoniae	*Morganella morganii*	4
		Klebsiella oxytoca	
9	Pneumoniae	*Escherichia coli*	7
10	Pneumoniae	*Klebsiella pneumoniae*	7
11	Pneumoniae	*Klebsiella oxytoca*	4
12	Pneumoniae	*Klebsiella pneumoniae*	4
13	Pneumoniae	*Enterococcus faecalis*	5
	Soft tissue	*Enterococcus faecalis*	
14	Pneumoniae	*Enterobacter cloacae*	8

d indicates days after trauma, n.d.: not determined.

2.7. Statistical Analyses. To evaluate differences between the study groups, a Kruskal-Wallis test with Dunn's post hoc test was performed. Correlation between numerical values was evaluated by Spearman's rank correlation coefficient (r). The Mann-Whitney rank sum test was performed to compare two groups of values at the same time. Analyses were performed using GraphPad Prism software (version 5; GraphPad Software, San Diego, CA, USA). Data were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Demographics. The 39 patients (28 male, 11 female) enrolled into the study had an ISS of 38.8 ± 2.6 (mean ± SEM, range 16–75). The mean age was 45.2 ± 3.1 years (range 19–82 years). From all patients, 14 developed sepsis (sepsis group) within 5.8 ± 0.4 days (range 4–8 days) after admission. Infection site of sepsis and microbiological pathogens for each patient are depicted in Table 1. Three patients died posttraumatically after 55.2 ± 23.3 days (range 24–147 days). The mean ICU stay was 16.9 ± 2.4 days (range 2–74 days). The mean age of the 14 patients (3 female, 11 male) who subsequently developed sepsis was 50.3 ± 5.8 years (range, 21–82 years). The mean ISS in this patient group was 45.6 ± 4.9 (range, 16–75). Mean ISS in patients without sepsis was 35.0 ± 2.7 (range, 16–66). The mean ICU stay in the sepsis group was 25.5 ± 3.4 days (range 7–50 days) and in the group without development of a sepsis 12.1 ± 2.9 days (range 2–74 days).

3.2. cfDNA/NETs and DNase in Sepsis versus No Sepsis Group. cfDNA/NETs and DNase values were determined from admission to the emergency room (ER) until day 9 after trauma. In order to define normal values, blood samples from 10 healthy volunteers were analyzed for cfDNA/NETs (median, 116.3; interquartile range (IQR), 102.8 to 131.7 ng/mL) and DNAse (median, 3.15; IQR, 2.61 to 3.80 ng/mL).

Early after major trauma cf-DNA/NETs values were significantly enhanced in comparison to healthy donors, decreased within the next days but remain elevated above normal values of volunteers for the entire period (Figure 1(a)). However, cfDNA/NETs values of patients who subsequently developed sepsis increased again at day 5 until day 9 after trauma (day 5, median, 334.0; interquartile range (IQR), 209.4 to 482.2 ng/mL), whereas the values for those patients without development of sepsis remained on the same level (day 5, median, 178.3; interquartile range (IQR), 125.8 to 223.4 ng/mL; Figure 1(b)). Significant intergroup differences were detectable between sepsis and nonsepsis patients on days 5 to 9 as shown in Figure 1(b).

DNase values were also significantly increased on admission (heathly volunteers: median, 3.145; interquartile range (IQR), 2.614 to 3.799; all patients at day 0: median, 5.715; interquartile range (IQR), 3.400 to 7.119 ng/mL. Figure 2(a)). Regardless of the development of a sepsis or not, DNase values decreased within the next days for both groups and remained on the control level throughout the entire period.

Patients developed sepsis in the mean 5.8 ± 0.4 days (range 4–8 days) after trauma. In order to investigate the potential role of cfDNA/NETs and DNase values as markers for the onset of sepsis, we determined the highest values of both parameters on the day before (−1), the day of (0), and the day after (+1) diagnosis of sepsis (Figure 3). These values were compared to highest values between day 4 and 6 of patients without development of sepsis and healthy volunteers. During the early phase of a sepsis, cf-DNA/NETs and DNase in patients were significantly elevated compared

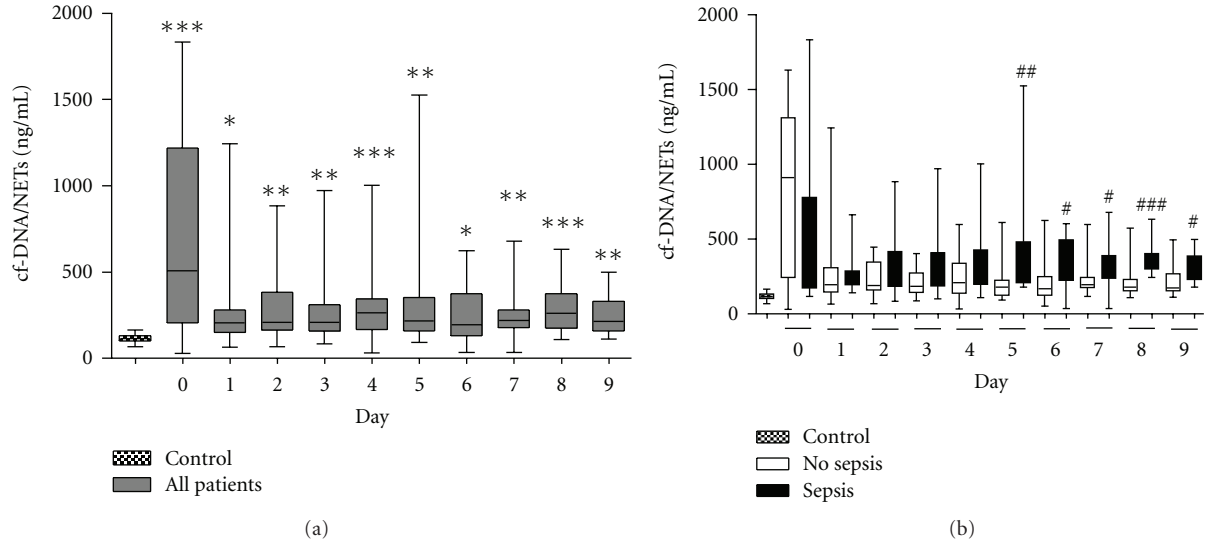

Figure 1: Kinetics of cfDNA/NETs after major trauma. (a) cf-DNA/NETs levels in patients ($n = 39$) were significantly enhanced after trauma ($P < 0.01$), versus control group (healthy volunteers). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.0001$, (b) cfDNA/NETs levels in patients who developed sepsis during the first 10 days after trauma ($n = 14$) were significantly elevated when compared to the values determined in patients with uneventful recovery ($n = 25$). The horizontal line across the boxplots represents the median, and the lower and upper ends of the boxplots are the 25th and 75th percentiles, respectively. Whiskers indicate the minimum and maximum values, respectively versus no sepsis group at the same time. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.0001$.

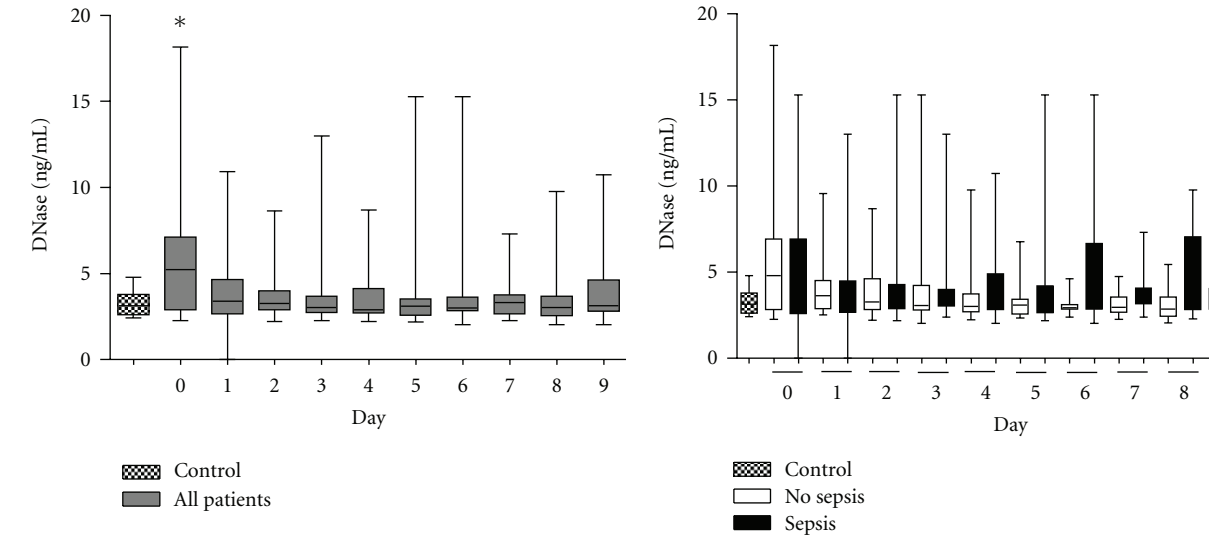

Figure 2: Kinetics of DNase after major trauma. (a) DNase values in patients ($n = 39$) were significantly elevated early after trauma ($^{*}P < 0.05$ versus control group). (b) DNase levels in patients who developed sepsis during the first 10 days after trauma ($n = 14$) did not show any differences compared to the values determined in patients with uneventful recovery ($n = 25$) or control group (healthy volunteers). The horizontal line across the boxplots represents the median, and the lower and upper ends of the boxplots are the 25th and 75th percentiles, respectively. Whiskers indicate the minimum and maximum values, respectively.

to patients without development of sepsis after major trauma ($P < 0.05$) and healthy volunteers ($P < 0.0001$).

cf-DNA/NETs values strongly correlate to DNase values ($r = 0.2523$, $P < 0.0001$) after trauma. Furthermore, cf-DNA/NETs concentrations determined in patients without sepsis development after severe trauma strongly correlated with DNase ($r = 0.207$, $P < 0.01$). However, values did not correlate in patients who subsequently developed sepsis ($r = 0.4771$; $P > 0.05$).

3.3. Degradation of NETs by DNase Treatment. In order to prove the ability of DNase to degrade the scaffold of NETs in

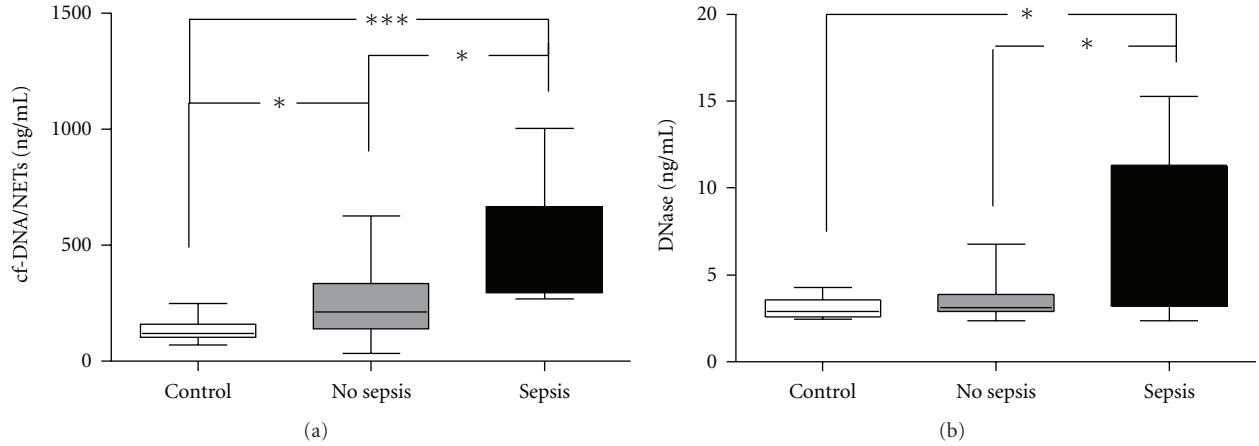

Figure 3: Values of cfDNA/NETs and DNase in the early septic phase. Highest values of cf-DNA/NETs and DNase in the range between the day before (−1), the day of (0), and the day after (+1) diagnosis of sepsis after major trauma. (a) cf-DNA/NETs values of patients with sepsis were significantly enhanced compared to patients with uneventful recovery ($P < 0.05$) or healthy volunteers ($P < 0.0001$). (b) Likewise values of DNase of patients who developed sepsis after major trauma were significantly enhanced compared to patients with uneventful recovery ($P < 0.05$) or healthy volunteers ($P < 0.05$). The horizontal line across the boxplots represents the median, and the lower and upper ends of the boxplots are the 25th and 75th percentiles, respectively. Whiskers indicate the minimum and maximum values, respectively. $^{*}P < 0.05$ and $^{***}P < 0.0001$.

vitro, supernatants of stimulated neutrophils were incubated with DNase, and NETs were quantified by cf-DNA/NETs assay. As depicted in Figure 4(a), NETs were disintegrated in a concentration-dependent manner. Furthermore, qualitative evidence of DNase-mediated NET degradation has been shown by immunofluorescence (Figure 4(b)). Viable unstimulated neutrophils from healthy volunteers and neutrophils stimulated with PMA were fixed and stained for NET components (blue = DNA, green = MPO). The image of unstimulated neutrophils shows the nuclear localization of DNA and the granular pattern of MPO (top right). After stimulation, morphological changes during NET formation could be determined with loss of nuclear lobules and granular integrity of MPO (left bottom). Exposure of fixed NETs with rhDNase resulted in the disintegration of NETs with loss of DNA structures (right bottom).

4. Discussion

Formation of NETs has been discussed as an effective mechanism of the innate immune system and as relevant in infections, sepsis, and autoimmune diseases. NETs have been shown to trap various types of pathogens [21, 24–26]. Although NETs have been demonstrated to effectively enhance bacterial trapping, this antibacterial mechanism occurs at the expense of injury to endothelium, tissue, and organs [21, 27].

We could confirm previously published results that cf-DNA/NETs are enhanced in the serum of trauma patients, especially those who later developed sepsis [28]. In this study we could show that particularly cfDNA/NETs values were enhanced in the very early phase of sepsis or even before clinical manifestation. This suggests a certain regulatory importance of this finding. Furthermore, in this phase DNase values in septic patients were also significantly increased compared to patients without sepsis and healthy volunteers. Moreover, NETs released by stimulated neutrophils in vitro were efficiently degradated by recombinant DNase in a concentration-dependent manner. All the data obtained in this study provide indication for an important pathophysiological role of cf-DNA/NETs and their relationship to DNase in the early phase of sepsis after trauma. The release of DNase may on one hand inhibit the antibacterial effects of NETs on the other hand DNase could protect the tissue from autodestruction caused by inadequate NETs release in septic patients. Furthermore DNase itself may have a regulatory function in NETs formation of neutrophils.

As their structural backbone is composed of chromatin, NETs are destroyed by DNase. The endonuclease DNase is the major nuclease normally produced by the pancreas and salivary glands and is a physiological constituent of human plasma at concentrations of approximately 3 ng/mL [29]. Brinkmann et al. have already shown in vitro that brief treatment of activated neutrophils with DNase abolishes microbial killing by NETs [9]. In our study this DNase-related dissociation of NETs in vitro has been confirmed by immunofluorescence. Furthermore DNase degrades NETs in a concentration-dependent manner. As after trauma DNase levels of all patients correlate to DNase values and both were significantly elevated in the early phase of sepsis after major trauma, an immunological-based interaction is possible. Given that NETs play a role in the pathogenesis of diverse immune disorders, the formation and activity of endogen-released DNases are prerequisites for natural counter regulation. It ought to be taken into account that adequate DNase release may inhibit the antibacterial effects of NETs or protect the tissue from autodestruction in inadequate NETs release in septic patients. Physiologic amounts of NETs are likely to be important in anti-infectious innate immune responses. In contrast, aberrant amounts of

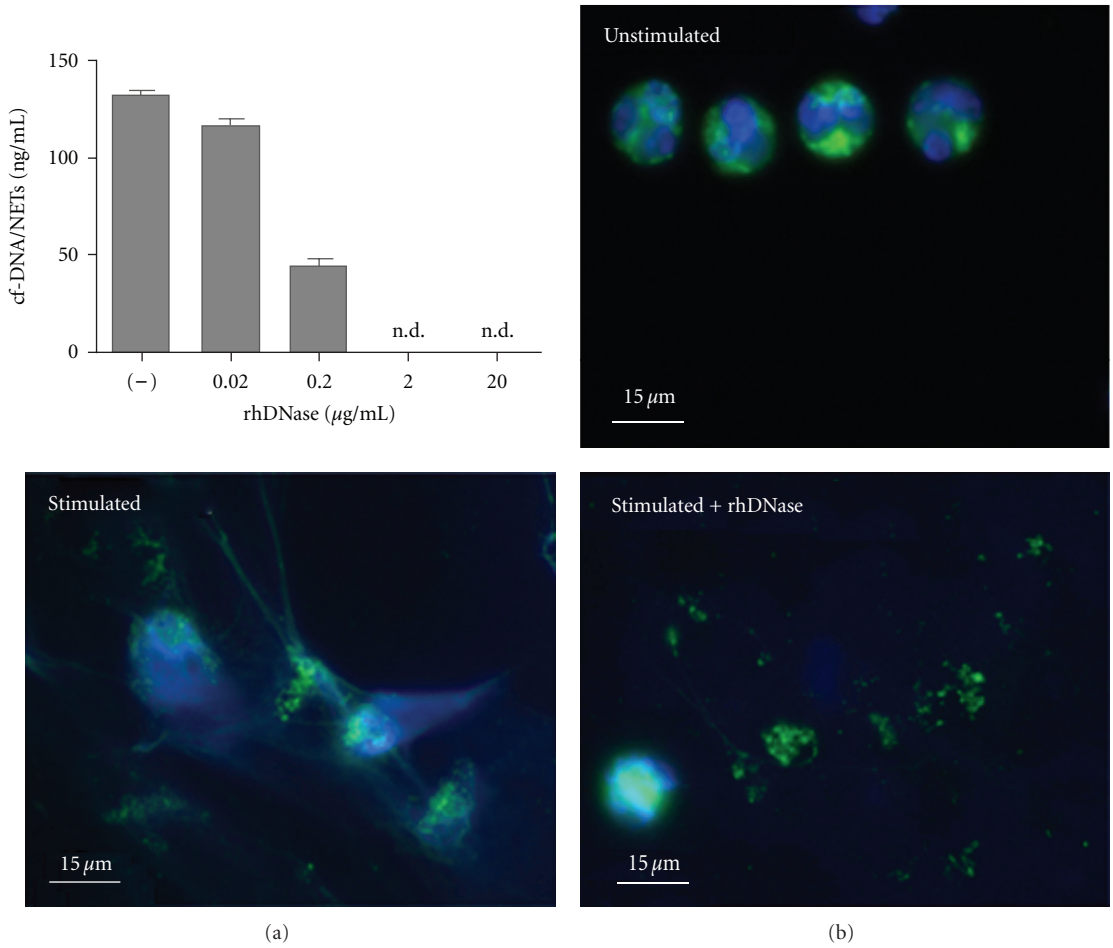

Figure 4: Degradation of NETs by DNase in vitro. (a) Degradation of NETs in a concentration-dependent manner. Neutrophils were isolated from healthy volunteers ($n = 5$) and stimulated 3 h with 50 nM PMA. Then, the supernatants were incubated with 0, 0.02, 0.2, 2.0, and 20.0 μg/mL rhDNase for 30 minutes and values of cfDNA/NETs were determined (n.d.: not detected). Data are presented as mean ± SEM. (b) Immunofluorescence staining of NETs. Neutrophils were stimulated with 30 nM PMA for 3 h and stained for DNA (blue) and MPO (green). Cells were further incubated with 20 μg/mL rhDNase.

NETs, not sufficiently degraded by DNAses within the blood, may occlude capillaries, impair microcirculation, enzymatically damage tissues, and strongly promote inflammation. Patients who have low amounts of DNases in their blood are not able to adequately control NET formation and thus may have a higher risk to develop severe sequelae than patients with normal amounts of DNases.

Beside physiological production of DNases from pancreas and salivary gland cells, some bacteria protect themselves from trapping by degrading the NETs via endogenous DNase [22]. DNases are expressed by many Gram-positive bacterial pathogens [30], but their role in virulence has been unclear until the discovery of NETs. Beiter et al. showed that the surface endonuclease A (EndA) of *Streptococcus pneumoniae* enabled the bacteria to escape the immune system by degrading the DNA scaffolds of NETs [18]. Upon degradation by extracellular DNases excessively accumulated NETs within tissues or capillaries release NET-associated effector molecules enzymes such as neutrophil-derived elastase which may entail severe tissue damage. However, the effects of these proteins when released from NETs after degradation by natural or therapeutic DNase are unclear.

The assay used to label neutrophil-derived NETs by targeting the NET containing cf-DNA directly within serum has been recently developed. It has been shown to be specific for neutrophils since stimulation of other blood cells or other cells did not result in production of NETs or in increased fluorescence signals [22, 31]. The assumption that the cfDNA is a component of floating NETs has been supported by previous studies, in which MPO, as a granule component of NETs, strongly correlates to cfDNA/NETs values [22, 32]. However, as rightly remarked by the reviewer the definite proof in vivo that cfDNA largely derives from hyperactivated neutrophils in patients after multiple trauma has to be done.

5. Conclusions

Values of cf-DNA/NETs and DNase are significantly increased in the early phase of sepsis after major trauma. DNase degrades NETs in a concentration-dependent manner

and may have a regulatory effect on NET formation in neutrophils. This may inhibit the antibacterial effects of NETs or protect the tissue from autodestruction in inadequate NETs release in septic patients. Furthermore, therapeutic strategies that limit NETs activity, for example, by DNase or DNase inhibitors treatment might prevent neutrophil-derived pathological effects possibly resulting in posttraumatic organ failure.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Inhalative IL-10 Attenuates Pulmonary Inflammation following Hemorrhagic Shock without Major Alterations of the Systemic Inflammatory Response

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Several studies report immunomodulatory effects of endogenous IL-10 after trauma. The present study investigates the effect of inhalative IL-10 administration on systemic and pulmonary inflammation in hemorrhagic shock. Male C57/BL6 mice (8 animals per group) were subjected to pressure-controlled hemorrhagic shock for 1.5 hrs followed by resuscitation and inhalative administration of either 50 μL PBS (Shock group) or 50 μg/kg recombinant mouse IL-10 dissolved in 50 μL PBS (Shock + IL-10 group). Animals were sacrificed after 4.5 hrs of recovery and serum IL-6, IL-10, KC, and MCP-1 concentrations were measured with ELISA kits. Acute pulmonary inflammation was assessed by pulmonary myeloperoxidase (MPO) activity and pulmonary H&E histopathology. Inhalative IL-10 administration decreased pulmonary inflammation without altering the systemic concentrations of IL-6, IL-10, and KC. Serum MCP-1 levels were significantly reduced following inhalative IL-10 administration. These findings suggest that inhalative IL-10 administration may modulate the pulmonary microenvironment without major alterations of the systemic inflammatory response, thus minimizing the potential susceptibility to infection and sepsis.

1. Introduction

Hemorrhagic shock initiates a systemic inflammatory response which is thought to be responsible for the development of ARDS and MOF [1]. The pharmacological modulation of this excessive and uncontrolled release of proinflammatory cytokines is considered a promising therapeutic strategy [2]. Previous studies have shown that the administration of IL-10 following hemorrhagic shock is capable of reducing systemic and pulmonary inflammation [3, 4]. However, an early inhibition of the systemic inflammatory response may result in increased rates of infection and sepsis [5–10]. This concern may be diminished by immunomodulating strategies that target hyperinflammation in specific organs, but leave systemic inflammatory responses unabated. In view of these demands, we hypothesized that inhalative administration of IL-10 following hemorrhagic shock would attenuate the pulmonary but not the systemic inflammatory response.

2. Materials and Methods

2.1. Animal Care. This research protocol complied with the regulations regarding the care and use of experimental animals published by the NIH and was approved by the Institutional Animal Use and Care Committee of the RWTH Aachen University. Male C57/BL6 mice (Charles Rivers Laboratories, Germany), 6–10 weeks old and weighing 20–30 g, were used in the experiments. The animals were maintained in the Animal Research Center of the RWTH Aachen University with a 12:12 h light-dark cycle and free access to standard laboratory feed and water. Animals were anesthetized with inhaled isoflurane (Abbott Laboratories,

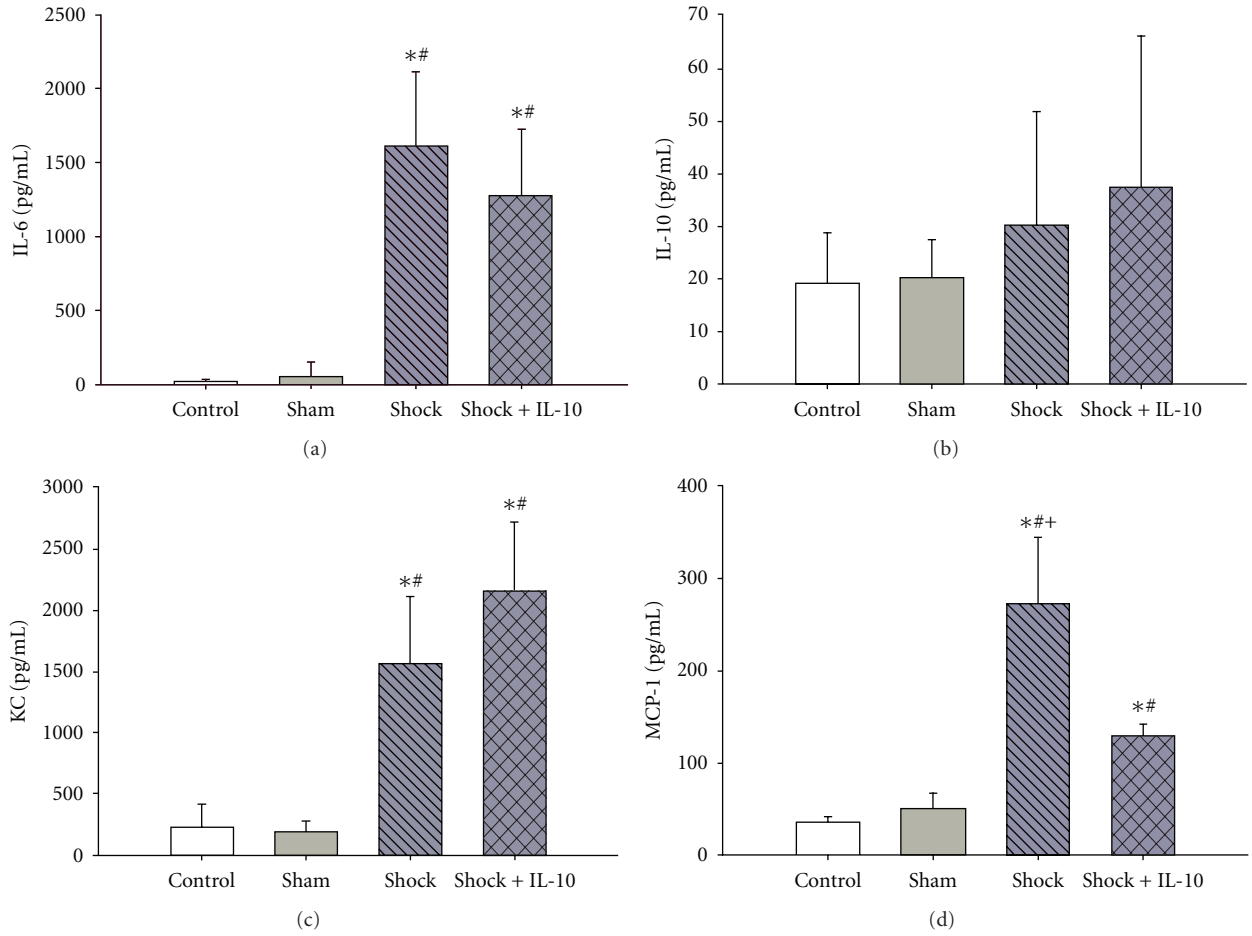

Figure 1: Comparison of serum IL-6 (a), IL-10 (b), KC (c), and MCP-1 (d) levels in C57/BL6 mice following hemorrhagic shock with (Shock + IL-10) or without (Shock) inhalative administration of IL-10. Results are expressed as means ± SD of 8 animals per group ($^{*}P < 0.05$ versus Control; $^{\#}P < 0.05$ versus Sham; $^{+}P < 0.05$ versus Shock + IL-10).

Wiesbaden, Germany), 70 mg/kg i.p. pentobarbital (Merial GmbH, Hallbergmoos, Germany), and 0.5 mg/kg buprenorphine (Reckitt-Benckiser, Bergheim, Germany).

2.2. Groups. C57/BL6 mice were divided into four groups. In the Control group ($n = 8$), animals were euthanized after the induction of anaesthesia to obtain physiological baseline levels. Animals in the Sham group ($n = 8$) were subjected to femoral artery catheterization, 1.5 hrs of anaesthesia, and 4.5 hrs of recovery before euthanasia. Animals of the Shock ($n = 8$) or Shock + IL-10 ($n = 8$) group were subjected to femoral artery catheterization, 1.5 hrs of anaesthesia with pressure-controlled hemorrhagic shock followed by inhalative administration of either 50 μL phosphate buffered saline (PBS) or 50 μg/kg recombinant mouse IL-10 (Cys 149 Typ, R&D Systems) dissolved in 50 μL PBS. The animals were sacrificed after 4.5 hrs of recovery.

2.3. Femoral Artery Cannulation and Induction of Hemorrhagic Shock. Animals were subjected to anesthesia as described above. A sterile technique was used to perform a left groin exploration, and the left femoral artery was cannulated with tapered polyethylene-10 tubing. The catheter was connected to a digital blood pressure monitor (TSE Systems, Bad Homburg, Germany) and the mean arterial pressure (MAP) was recorded. Pressure-controlled hemorrhagic shock was performed by withdrawing blood over a period of 15 min in a syringe with 0.07 mL of heparin (1000 USP units/mL) until a MAP of 35 mmHg was reached. Hemorrhagic shock was maintained for 1.5 hrs followed by resuscitation with shed blood and an equal volume of 0.9% saline. The catheter was removed, the artery ligated, and the skin incision closed. After a recovery phase of 4.5 hrs the animals were sacrificed.

2.4. Administration of Inhalative IL-10. The inhalative administration of PBS or IL-10 was performed using a MicroSprayer Aeroliser (Penn-Century, Philadelphia, Pa, USA) connected to a high-pressure syringe (FMJ-250, Penn-Century, Philadelphia, Pa, USA). The endotracheal intubation was carried out as described by Bivas-Benita et al. [11]. Briefly, the mouth was opened with a blunt forceps and the tongue was pulled out and moved to the left in order to visualise the trachea. The MicroSprayer was inserted into

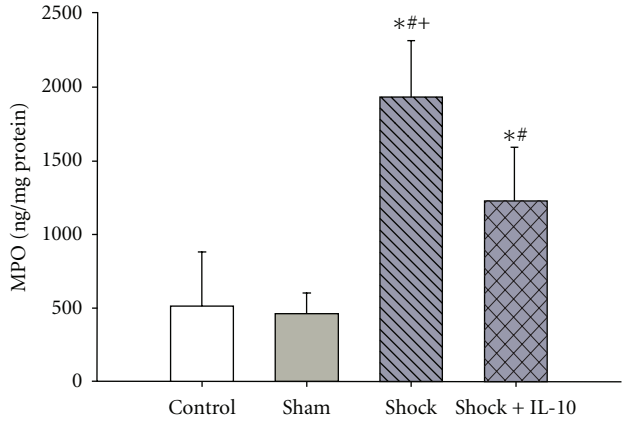

Figure 2: Pulmonary myeloperoxidase (MPO) activity in C57/BL6 mice following hemorrhagic shock with (Shock + IL-10) or without (Shock) inhalative administration of IL-10. Results are expressed as means ± SD of 8 animals per group ($^{*}P < 0.05$ versus Control; $^{\#}P < 0.05$ versus Sham; $^{+}P < 0.05$ versus Shock + IL-10).

trachea. Once intubation was completed PBS or IL-10 was administered as aerosol. The deposition of aerosols into the small airways using this method has been validated with fluorescent nanoparticles [11].

2.5. Blood Collection for Serum Cytokines. Following thoracotomy, cardiac blood was withdrawn under deep anesthesia as part of the procedure of exsanguination for euthanasia. Plasma samples were allowed to clot at 4°C and then were centrifuged at 7000 rpm for 7 min in order to separate the serum from cellular blood components. Serum was stored at −20°C until thawed for further evaluation. Serum IL-6, IL-10, MCP-1, and KC levels were quantified with ELISA kits (R&D System Inc., Minneapolis, Minn, USA) as per manufacturer's specifications.

2.6. Pulmonary Myeloperoxidase (MPO) Activity. To minimize background MPO activity by remaining nonadherent intravascular polymorphonuclear cells, a needle was inserted into the beating right ventricle, after withdrawal of cardiac blood, and the circulation was perfused with 1.5 mL of PBS. The left lung was harvested and immediately snapped-frozen in liquid nitrogen and stored at −80° Celsius. To determine tissue MPO activity, the samples were thawed and homogenized in a lysis buffer according to the manufacturer's protocol. The MPO activity was measured using an MPO-ELISA kit (Hycultec GmbH, Beutelsbach, Germany) and normalized to the protein concentration of the sample (BCA Protein Assay Kit, Pierce, Rockford, Ill, USA).

2.7. Pulmonary Histopathology. For the detection of pulmonary inflammation and lung injury the right lung was harvested and immediately fixed in buffered formalin. Paraffin-embedded blocks were cut at 5 μm thickness and stained with H&E (Hematoxylin and Eosin).

2.8. Statistical Analysis. All results in this paper are expressed as the mean ± SD of eight animals per group. Data were transformed by the BoxCox transformation (JMP 5.0.1 for Windows). In normally distributed variables group comparisons were assessed using ANOVA followed by Tukey's HSD test. Nonnormally distributed parameters were tested using the Kruskal-Wallis-Test. The null hypothesis was rejected for $P < 0.05$. Data were analysed using SPSS Version 18 (SPSS, Chicago, Ill, USA).

3. Results

3.1. Serum Cytokine Levels. Serum IL-6 levels were significantly higher in animals subjected to hemorrhagic shock as compared to Control and Sham animals. However, the serum IL-6 concentration was indistinguishable between Shock and Shock + IL-10 animals, thus inhalative IL-10 did not significantly alter systemic IL-6 levels (Figure 1(a)).

Serum IL-10 concentrations showed an increase following hemorrhagic shock; however this did not reach statistical significance. Inhalative IL-10 was not associated with a significant increase in systemic IL-10 levels (Figure 1(b)).

Serum KC levels were significantly increased in Shock and Shock + IL-10 animals as compared with Control and Sham mice. There was no significant difference in serum KC levels between Shock and Shock + IL-10 mice (Figure 1(c)).

Serum MCP-1 levels were significantly higher following hemorrhagic shock as compared to Control and Sham animals. Furthermore, the MCP-1 concentration was significantly reduced in animals with inhalative IL-10 administration, thus inhalative IL-10 alters systemic MCP-1 levels (Figure 1(d)).

3.2. Pulmonary Inflammation. Pulmonary MPO activity was significantly higher in the Shock and Shock + IL-10 group as compared with Control and Sham animals. Inhalative IL-10 significantly reduced the pulmonary MPO activity as compared to Shock animals (Figure 2).

This effect was confirmed by histology which showed a reduced pulmonary infiltration with inflammatory cells (Figure 3).

4. Discussion

The immunoregulatory potential of IL-10 is well recognized and a potential role of IL-10 as a therapeutic agent is increasingly investigated in various animal models [3, 4, 12–14] and human studies [12]. Beneficial effects of early systemic IL-10 release following injury have been reported [3, 15], mainly related to the inhibition of proinflammatory cytokine synthesis [16, 17] and leukocyte recruitment [18]. However, especially in the later course following trauma, the incidence of infection is strongly associated with systemic IL-10 concentrations [5–10]. Thus, systemic IL-10 is a double-edged sword in treating severe trauma: potentially beneficial in the early but deleterious in the later phase. This conundrum raises the possibility whether locally applied IL-10 strategy could possibly protect end organs by changing their inflammatory microenvironment without altering the systemic inflammatory response and the susceptibility to

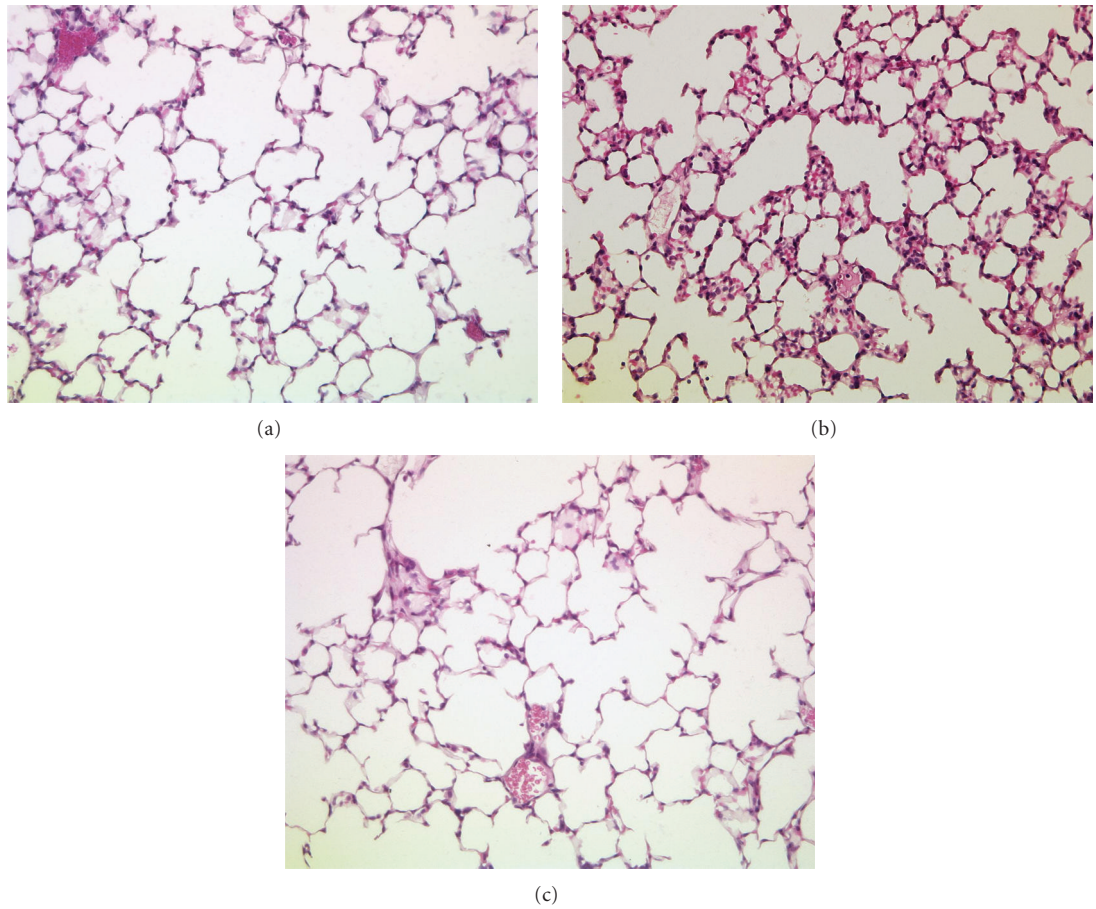

Figure 3: Representative H&E (Hematoxylin and Eosin) lung histology (20x) of the Control (a), Shock (b), and Shock + IL-10 (c) group 4.5 hrs after resuscitation. Inhalative IL-10 reduces the pulmonary influx of inflammatory cells.

infection and sepsis. The lung is frequently affected by systemic inflammation and the development of acute lung injury seems to be associated with local and systemic IL-10 concentrations, examples are hemorrhage [3, 4], peritonitis [19], or major surgery [20]. Low IL-10 concentrations in the BAL of patients already suffering from ARDS were correlated with an increased mortality rate [21], whereas patients having an IL-10 polymorphism with increased IL-10 release showed a decreased incidence of ARDS [22]. Given that hemorrhagic shock impairs the induction of IL-10 expression by alveolar macrophages [23] and that systemic IL-10 administration attenuates pulmonary but not hepatic injury [4, 13], we hypothesized that a local repletion of pulmonary IL-10 following hemorrhagic reduces pulmonary inflammation without altering the systemic inflammatory response.

Trauma hemorrhage is well known to lead to pulmonary inflammation and leukocyte infiltration, that is at least in part mediated by KC [24]. In the present study, we observed significant increases of systemic IL-6 and KC concentrations following hemorrhagic shock and both effects were not altered by inhalative IL-10 administration. These findings are in contrast to studies reporting significantly reduced systemic IL-6 and KC levels in hemorrhagic shock following intra-arterial, intraperitoneal, or subcutaneous IL-10 administration [3, 13, 25]. These findings support our hypothesis that inhalative administered IL-10 mainly affects the lung without major alterations of the systemic inflammatory response. This is in accordance with no detectable differences of systemic IL-10 concentrations between animals subjected to hemorrhagic shock with or without inhalative IL-10, although we cannot exclude an earlier systemic IL-10 peak, due to the short half-life of IL-10.

Interestingly, we observed a significant reduction of systemic MCP-1 following inhalative IL-10 application. MCP-1 is a major attractant for macrophages and monocytes and is upregulated following trauma hemorrhage [26]. An explanation for reduced systemic MCP-1 concentrations may lie in the role of alveolar macrophages which secrete MCP-1 and thereby potentiate inflammatory triggered lung injury [27, 28]. Thus systemic measured MCP-1 levels following hemorrhagic shock may be derived from the secretion of alveolar macrophages.

We have previously observed these specific modulations of chemokine release in a model of IL-10 KO mice [4]. Herein, IL-10 deficiency significantly increased systemic MCP-1 but not KC concentrations. Yet, we are not able to explain this selective modulation of chemokines by IL-10, but it may be speculated that, in contrast to the systemic

MCP-1 concentration, the alveolar macrophages are not the main source for systemic measured KC levels.

The influx of inflammatory cells in the lung following hemorrhagic shock has been well described and is thought to be a major contributor to the development of remote organ dysfunction following trauma [29, 30]. Our data show that inhalation of IL-10 significantly reduces pulmonary myeloperoxidase activity, an established marker for pulmonary neutrophil infiltration, in shocked animals. Pulmonary histopathology showed a trend towards a decreased influx of inflammatory cells; however signs of severe pulmonary injury were not detectable in this early phase. This is in line with other studies detecting histological evidence of ARDS usually not before 24 hrs after hemorrhagic shock [4]. Overall, the reduced pulmonary inflammation is in accordance with other studies showing that IL-10 administration significantly decreased neutrophil infiltration in the lung [3, 31, 32]. The beneficial pulmonary effect of endogenous IL-10 has also been shown in other animal models [13, 33] and is further supported by clinical studies which report that nonsurvivors of ARDS had significantly lower levels of IL-10 in the BAL-fluid as compared to survivors [34].

Several features and limitations of our study merit further comment. We did not investigate later time points and one may criticize that inhalative IL-10 administration may delay but not prevent pulmonary inflammation following hemorrhagic shock. Further, the unchanged systemic concentrations of IL-6, KC, and IL-10 do not rule out that other important mediators in the posttraumatic inflammatory cascade may be altered by inhalative IL-10 and thereby the susceptibility to infection may again be increased.

In conclusion, this study demonstrates that inhalative IL-10 reduces pulmonary inflammation following hemorrhagic shock without major alterations in the systemic inflammatory response. This may be a valuable therapeutic strategy because inhalative IL-10 administration may solve the dilemma of the two-edged sword: end organ protection is possible without altering the systemic inflammatory response and the susceptibility to infection and sepsis. Nonetheless, further studies are required to elucidate the complex immunomodulatory action of IL-10 under the condition of hemorrhagic shock.

Abbreviations

ARDS: Adult Respiratory Distress Syndrome
IL-6: Interleukin-6
IL-10: Interleukin-10
KC: Keratinocyte-derived Cytokine
MCP-1: Macrophage/Monocyte Chemotactic Protein-1
MOF: Multiple Organ Failure
MPO: Myeloperoxidase
PBS: Phosphate Buffered Saline.

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