Dynamic posttraumatic changes of monocyte subsets

Thesis

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”Success is not final, failure is not fatal: it is the courage to continue that counts.” –Winston S. Churchill

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Zusammenfassung

Die Überlebensrate von schwerverletzten PatientInnen verbesserte sich durch fortgeschrittene primäre Behandlungsalgorithmen, dennoch sind diese PatientInnen weiterhin anfällig für sekundäre Infektionen während ihres Krankenhausaufenthaltes. Infolge von einer traumatischen Verletzung wird eine proinflammatorische Immunantwort eingeleitet, deren Ausmaß und potenzielles Ungleichgewicht zu antiinflammatorischer Immunantwort trägt dazu bei, dass PatientInnen weiterreichende inflammatorische Komplikationen erleiden. Auf Grund dessen könnte die Analyse der Veränderungen von humoraler und zellulärer Immunantwort in schwerverletzten PatientInnen perspektivisch einen wichtigen therapeutischen Angriffspunkt darstellen.

Im Gewebe ansässige Makrophagen, die zirkulierenden Monozyten und neutrophil Granulozyten leiten auf der zellulären Ebene die unverzügliche Immunantwort ein, womit sie sich auf die Reparaturprozesse des Gewebes und dem Schutz vor eindringenden Pathogenen beteiligen. Unfallbedingte Immunsuppression und die anschließende Entwicklung von Multiorganversagen wurden mit anomaler Funktionalität der Monozyten assoziiert. Die Änderungen des Phänotyps von Monozyten spielen eine wichtige Rolle bei verschiedenen Krankheitsbildern. Dazu gibt es keine Daten bei schwerverletzten PatientInnen. Hier wurde untersucht, wie sich der Phänotyp der Monozyten (klassische $CD16^{-}CD14^{bright}$, proinflammatorische *‚intermediate‘* $CD16^{+}CD14^{bright}$ und nicht-klassische $CD16^{+}CD14^{low}$ Monozyten) und deren antimikrobielle Aktivität in schwerverletzten PatientInnen innerhalb der ersten 12 Stunden nach dem Unfall ändert.

Zusätzlich könnte die posttraumatische Behandlung wesentlich verbessert werden, wenn Zielmoleküle identifiziert würden, die den sekundären inflammatorischen Komplikationen entgegenwirken. Pulmonales Club-Zellprotein 16 (*club cell protein 16*; CC16) hat antiinflammatorische Eigenschaften. Abweichungen seiner Konzentration in der Lunge und im Blut sind mit verschiedenen Lungenerkrankungen assoziiert. Auf Grund dessen untersuchten wir die Bedeutung von CC16 auf die lokale und

systemische Plastizität von Phänotypen der Monozyten und Makrophagen (proinflammatorische Ly6C^{bright} und antiinflammatorische Ly6C^{low} Monozyten und Makrophagen) und seine antiinflammatorische Wirkung in einem Mausmodell der früh manifesten unfallbedingten Sepsis. Dieses Modell bestand aus einer stumpfen Thoraxverletzung und anschließender Ligation und Punktion des Zäkums (*cecal ligation and puncture*; CLP).

Unter Anderem sind bis zu 50% der in die Notaufnahme eingelieferten PatientInnen alkoholisiert, was zusätzlich das Risiko erhöht, eine sekundäre Infektion zu entwickeln. Eine akute Alkoholintoxikation geht mit einer Toll-like Rezeptor (TLR)4-Toleranz der Monozyten gegenüber Endotoxin und anschließender Herabregulation des NLRP3-Inflammasoms und der Sekretion von proinflammatorischen Zytokinen wie Interleukin-1β einher. Allerdings wurden dabei die unterschiedlichen Phänotypen der Monozyten noch nicht untersucht. Hierzu untersuchten wir im zeitlichen Verlauf den Einfluss von Rauschtrinken, einer der häufigsten Form des Alkoholmissbrauchs, auf die phänotypischen Änderungen der Monozyten und deren Aktivierung des NLRP3-Inflammasoms in gesunden ProbandInnen.

Es konnte gezeigt werden, dass schwerverletzte PatientInnen einen signifikant erhöhten Anteil an proinflammatorischen CD16^{+}CD14^{bright} Monozyten (*intermediate monocytes*) aufweisen, wobei alle Phänotypen deutlich höhere Mengen von Sauerstoffradikalen (*reactive oxygen species*; ROS) produzieren. Eine *ex vivo* Stimulation mit Phorbol-12-myristat-13-acetat erhöht weiter die intrazellulären Levels von ROS, allerdings deutlich weniger in proinflammatorischen CD16^{+}CD14^{bright} und nicht-klassischen CD16^{+}CD14^{low} Monozyten (*non-classical monocytes*) verglichen mit Monozyten der gesunden ProbandInnen. Dies deutet darauf hin, dass Monozyten von schwerverletzten PatientInnen eine Hyporesponsivität gegenüber einem zweiten Stimulus haben könnten. Übereinstimmend induziert eine stumpfe Thoraxverletzung mit anschließender CLP den systemischen und lokalen Anstieg von proinflammatorischen Ly6C^{bright} gegenüber den antiinflammatorischen Ly6C^{low} Monozyten und Makrophagen. Intrapulmonale Neutralisation von CC16 vor CLP verstärkt diesen Effekt und führt zu weiterem Anstieg von Lungenschäden und proinflammatorischen Mediatoren

Tumornekrosefaktor, Monozyten-chemoattraktives Protein 1 und Rezeptor für *advanced glycation end-products*. Zuletzt hat ein Alkoholintus von 1‰ einen wesentlichen Einfluss auf den Phänotyp und die Funktion der Monozyten. Zwei Stunden nach der ersten Alkoholeinnahme kommt es zu einem Anstieg der proinflammatorischen Monozyten im Blut. Dies ist gefolgt von deren Differenzierung zu antiinflammatorischen Monozyten weitere zwei und vier Stunden später. Ebenso ist die oberflächliche Expression von TLR4 anfänglich erhöht und wesentlich runterreguliert im Verlauf. Diese Ergebnisse deuten darauf hin, dass Alkohol einen biphasischen Effekt auf die Immunantwort hat. Funktionell setzt Alkohol die Aktivierung des NLRP3-Inflammasoms und die Adhäsionsvermögen der Monozyten herab.

Zusammenfassend induziert eine schwere Verletzung einen wesentlichen Anstieg von proinflammatorischen Monozyten im Blut der PatientInnen und deren intrazellulären Spiegel von ROS. Dies könnte einerseits die PatientInnen vor einer bakteriellen Infektion schützen, anderseits bei überschießender Immunantwort auch zu weiterreichender Gewebeschädigung beitragen. Zusätzlich könnte die Hyporesponsivität der Monozyten gegenüber einem zweiten Stimulus wie eine Operation oder Bakterienbelastung, dazu beitragen, dass schwerverletzte PatientInnen deutlich anfälliger für sekundäre inflammatorische Komplikationen sind. Da Monozyten eine TLR4-Toleranz und verminderte Aktivierung des NLRP3-Inflammasoms in der Folge von Alkoholintoxikation aufweisen, könnte Alkohol den immunsupprimierenden Effekt in schwerverletzten PatientInnen sogar verstärken. Nachdem wir das antiinflammatorische Potenzial von CC16 in früher unfallbedingten Sepsis bestätigten, könnte eine intrapulmonale Administration von CC16 relevant für die Entwicklung von neuartigen therapeutischen Maßnahmen bei Lungenverletzungen sein und sollte deswegen weiteruntersucht werden.

Abstract

Although the development of advanced treatment algorithms of severely injured patients has significantly increased the posttraumatic survival rates, these patients are still highly susceptible for the development of secondary infections during their clinical stay. Traumatic injury induces an exaggerated pro-inflammatory immune response that depending on the intensity and imbalance to anti-inflammatory response may have further reaching deteriorating effects on patient's health. Therefore, the analysis and the understanding of the ongoing humoral and cellular immunological changes in severely injured patients may prospectively represent a valuable therapeutic target.

At cellular level, tissue-resident macrophages, monocytes, and neutrophils initiate an immediate immune response to clear the tissue damage and protect the host from invading pathogens. Trauma-induced immunosuppression with subsequent development of multi organ failure has been associated with aberrant functionality of monocytes. Although their phenotypic shift has been linked to several pathologies, data about the posttraumatic shift of monocyte population were missing. Therefore, in this thesis, the redistribution of classical $CD16^{-}CD14^{bright}$, intermediate $CD16^{+}CD14^{bright}$, and non-classical $CD16^{+}CD14^{low}$ monocyte subsets and the function regarding their antimicrobial activity were evaluated within the first 12 hours posttrauma in severe injured patients.

Moreover, finding a potential therapeutic target that counteract the ongoing secondary inflammatory complications, would significantly improve the posttraumatic treatment management. Pulmonary club cell protein 16 (CC16) has anti-inflammatory properties and its abnormal occurrence in the lungs and in the blood has been associated with several lung diseases. Therefore, we evaluated the impact of CC16 on systemic and local distribution of pro-inflammatory $Ly6C^{bright}$ and anti-inflammatory $Ly6C^{low}$ monocyte and macrophage subsets and its anti-inflammatory potential in murine model

of early trauma-induced sepsis, consisting of blunt thoracic trauma and cecal ligation and puncture (CLP).

Finally, up to 50% of patients admitted to emergency department are alcohol intoxicated, making them additionally susceptible for secondary infections. Acute alcohol intoxication transiently leads in monocytes to Toll-like receptor (TLR)4 tolerance towards endotoxin and subsequent downregulation of NLRP3 inflammasome activation and the secretion of pro-inflammatory cytokines such an interleukin-1β, but without considering the monocyte subsets. Therefore, we evaluated the impact of binge drinking on the distribution of monocyte subsets and their NLRP3 inflammasome activation in healthy subjects in time-dependent manner.

The study has shown that severe traumatic injury induces phenotypic shift of monocytes toward the pro-inflammatory intermediate $CD16^{+}CD14^{bright}$ subset. This is paralleled by significantly enhanced generation of reactive oxygen species (ROS) by all subsets. *Ex vivo* stimulation with phorbol 12-myristate 13-acetate significantly increased those, however, significantly less in intermediate $CD16^{+}CD14^{bright}$ and non-classical $CD16^{+}CD14^{low}$ subsets compared to healthy subjects, suggesting a hyporesponsiveness to secondary hit. In line, thoracic trauma with subsequent CLP increases the ratio of pro-inflammatory $Ly6C^{bright}$ to anti-inflammatory $Ly6C^{low}$ monocyte and macrophage subsets systemically and locally. Intrapulmonary neutralization of CC16 before CLP induction enhances the pro-inflammatory phenotypes, pulmonary damage, and the level of pro-inflammatory mediators tumor necrosis factor, monocyte chemoattractant protein-1, and receptor for glycation of end-products. Lastly, alcohol intoxication of 1‰ has significant impact on the monocyte subsets and their functions. Two hours after the first alcohol shot, monocytes differentiate towards the pro-inflammatory phenotype with a later shift toward the anti-inflammatory phenotype further two and four hours later. Similarly, the initial upregulation of TLR4 expression is followed by significant decline, suggesting biphasic effect of alcohol on immune response. Functionally, alcohol downregulates the NLRP3 inflammasome activation and the adhesive capacity of monocytes in time-dependent manner.

Taking together, severe traumatic injury shifts the monocytes toward their pro-inflammatory subsets and enhances their generation of ROS. This may not only protect the patients from bacterial infections, but also contribute to deteriorated tissue damage in higher extent. On the other hand, the hyporesponsiveness of monocytes towards secondary hit such as surgery or bacterial load may contribute to the high susceptibility of severely injured patient for developing secondary complications. As shown by TLR4 tolerance and downregulation of NLRP3 inflammasome activation, an acute alcohol intoxication could even potentiate the immunosuppressing effects in severely injured patients. Since we confirmed the anti-inflammatory potential of CC16 in early trauma-induced sepsis, its local supplementation following traumatic injury may be highly relevant for the development of novel therapeutic intervention for patients with lung injuries and should be investigated in further studies.

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Abbreviations

AIS	abbreviated injury score
ALI	acute lung injury
ALOX5AP	5-lipoxygenase-activating protein
ARDS	acute respiratory distress syndrome
ATP	adenosine triphosphate
BALF	bronchoalveolar lavage fluid
Bcl-3	B-cell lymphoma 3-encoded protein
CARS	compensatory anti-inflammatory response
CC16	club cell protein 16
CCL	C-C motif ligand
CCR2	C-C chemokine receptor type 2
CD	cluster of differentiation
CLP	cecal ligation and puncture
COPD	chronic obstructive pulmonary disease
CX_3CR1	C-X_3-C motif chemokine receptor 1
CXCL	C-X-C motif chemokine ligand
DAMP	danger-associated molecular pattern
ED	emergency department
EMP	erythro-myeloid precursor
FABP-I	intestinal fatty-acid binding protein
fMLP	N-Formylmethionyl-leucyl-phenylalanine
HLA-DR	human leukocyte antigen complex - isotype DR
HMGB1	high mobility-box-1 protein
HSC	hematopoietic stem cell
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
ISS	injury severity score
LPS	lipopolysaccharide

MARS	mixed antagonist response
MCP-1	monocyte chemoattractant protein
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MOF	multiple organ failure
MPO	myeloperoxidase
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PMA	phorbol 12-myristate 13-acetate
PMNL	polymorphonuclear leukocyte
RAGE	receptor for advanced glycation end-products
rCD16	recombinant CD16
ROS	reactive oxygen species
sCD14	soluble CD14
SIRS	systemic inflammatory response syndrome
TBI	traumatic brain injury
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
VCAM	vascular cell adhesion molecule

Chapter 1 Introduction

1.1 Traumatic injury and its mechanisms

Severe traumatic injury with 5.8 million annual deaths has after cardiovascular diseases and cancer the third highest mortality rate worldwide.[1] However, in the first four decades of human life, trauma is actually the leading cause of death, hospitalization, and long-term disabilities.[2, 3] This impacts the individuals as well as the society on a physical, psychological, and economical level,[4] whereby the direct medical costs amount to at least €80 billion each year in Europe, not considering the additional costs of sick pay, rehabilitation, or work loss.[5] Nevertheless, without the development of advanced posttraumatic treatment algorithms, the most people with severe traumatic injury would bleed to death. The beginnings of medical care are dated back to the 16th century when French military surgeon Ambroise Paré first ligated arteries during an amputation.[6] The treatment improved gradually over the ensuing centuries and then more rapidly after the outbreak of World War 2. This advanced management of hemorrhage has resulted in survival of patients who previously would die.[6]

With the further development of the society, not only the posttraumatic treatment algorithms have been continuously improved, but also the traumatic injury patterns became more complex. Penetrating injuries and simple fractures have been replaced by multiple traumatic injuries. The term ‘polytrauma’ was used for the first time by Tscherne *et al.* in 1966 and was defined as a combination of at least two severe injuries of the head, chest, or abdomen or one of them in association with an extremity injury.[7] Over the decades, the definition was revised several times. According to the new Berlin definition, polytrauma are at the same time occurring serious injuries of at least two or more body regions in a conjunction with one or more additional variables from five physiologic parameters (hypotension, unconsciousness, acidosis,

coagulopathy, and age higher than 70).[8, 9] This refinement of the polytrauma definition increased the polytrauma-associated mortality from ~11% to 35-38%.[9]

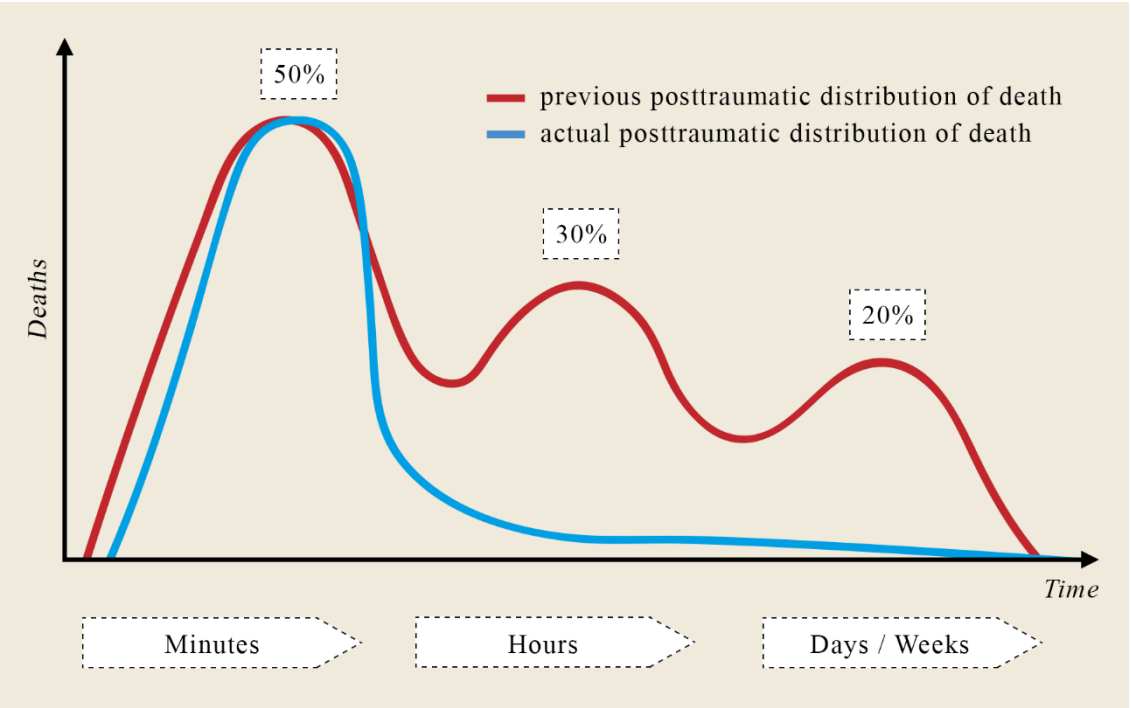

Figure 1 | Trimodal posttraumatic distribution of death is outdated. Posttraumatic distribution of death was previously trimodal. Deceased severely injured patients died either within minutes (50%) due to immediate and direct impact of injury or within the next hours (30%), mostly caused by hemorrhagic shock, or within next days or weeks (20%) due to secondary inflammatory complications. However, with the improved prehospital and critical care, 50% of deceased severely injured patients die within the first hour and in later course, no further peak of increased trauma-related mortality is existing.[10-13]

Trauma-related mortality is usually described as trimodal: immediate, early, and late.[10, 11] The first phase of death following traumatic injury is represented by immediate direct impact of injury, such as heart rupture, disruption of cervical spine, or massive exencephaly, causing death immediately or within minutes. The second phase of death occurs within hours after trauma and is mostly caused by hemorrhagic shock. The last and the third phase of death represents the indirect trauma-induced mortality caused by development of inflammatory complications within days or weeks.[10, 11] However, with the fast patient transport into emergency department (ED), improved prehospital care, early resuscitation, and critical care, the trimodal distribution of death

seems to be no more accurate. The actual analyses of data from in-hospital trauma facilities show that approximately 50% of all deaths occur within the first 24 hours.[12, 13] After this period, the incidence of trauma-induced mortality declines rapidly and does not show any further peak.[12, 13] Nevertheless, the posttraumatic care remains challenging, as the development of inflammatory complications and thus, late mortality still accounts for 10-30% of trauma-related deaths.[10, 14, 15]

1.2 Posttraumatic inflammatory complications

1.2.1 Clinical manifestation

The development of posttraumatic inflammatory complications and mortality has been clinically linked to the injury severity and pattern.[16-18] Injury severity is assessed on the basis of an anatomical scoring system that provides an overall injury score for patients with multiple injuries, called injury severity score (ISS). Here, each injury is assigned an abbreviated injury scale (AIS) score from one to six, that grades the injury from minor to unsurvivable, and is allocated to one of six body regions: head or neck, face, chest, abdomen, extremities, and external injury.[19, 20] Subsequently, the ISS is calculated as the sum of the squares of the highest AIS score of the three most severely injured body regions. The ISS ranges from one to 75, whereby ISS from 16 describes a severe traumatic injury.[19, 20] The current research has shown that ISS is in linear correlation with morbidity and incidence of infection.[16, 18] Additionally, patients with severe head injury show significantly higher prevalence of severe systemic inflammation and multiple organ failure (MOF) than patients with other injury patterns. In contrast, a severe injury of the abdomen reveals septic complications in both, mono- and polytrauma and higher risk for development of MOF, but without an impact on the mortality.[17] Polytrauma patients with extremity and pelvic injuries have been also shown to be at higher risk for development of septic complications.[17] Taking together, the more severe the patients are injured, the more they are prone to secondary posttraumatic complications with subsequent death, whereby the injury pattern plays here also a significant role.

1.2.2 Balancing the pro- and anti-inflammatory immune response

Trauma-induced immune response is the key factor contributing to development of inflammatory complications. Following traumatic injury, host-produced inflammatory signaling molecules, so-called danger-associated molecular patterns (DAMPs) or pathogen-derived inflammatory molecules, so-called pathogen-associated molecular patterns (PAMPs) are secreted by injured tissues or pathogens, respectively.[21, 22] These are recognized by tissue-resident immune cells such as alveolar macrophages through pattern recognition receptors as Toll-like receptors (TLRs), NOD-like receptors, receptor for advanced glycation end-products (RAGE), purinergic receptors or complement receptors.[23-25] Upon activation, tissue-resident macrophages release soluble factors to attract other immune cells. To those belong e.g. pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β or interferon (INF)γ.[24, 25] Neutrophils, as one of the first immune cells that are recruited to the sites of infection and tissue damage, egress from the bone marrow into circulation und migrate to the site of injury toward the chemoattractant gradients.[24, 26] They are immediately followed by monocytes that after transmigration into tissue differentiate into monocyte-derived macrophages.[24] Recruited phagocytes release antimicrobial molecules, pro-inflammatory cytokines, reactive oxygen species (ROS), growth factors, and phagocytose the pathogens and cell debris with the aim to clear tissue damage and provide protection from invading pathogens.[27-30] Although this immune surveillance and injury control is critical for timely resolution of inflammation and recovery from the injury,[31, 32] an exaggerated posttraumatic pro-inflammatory response may lead to collateral tissue damage and systemic inflammatory response syndrome (SIRS), which has been associated with MOF and increased mortality up to 50-80%.[33]

To restore the equilibrium of the excessive pro-inflammatory immune response, a counterpart anti-inflammatory immune response is parallelly initiated. Dependent on the balance of pro- and anti-inflammatory factors, the response returns to baseline or progress to persistent inflammation or immunosuppression. An excessive anti-inflammatory response can lead to a compensatory anti-inflammatory response

(CARS), which is characterized by raised anti-inflammatory cytokines and immunoparesis, or to

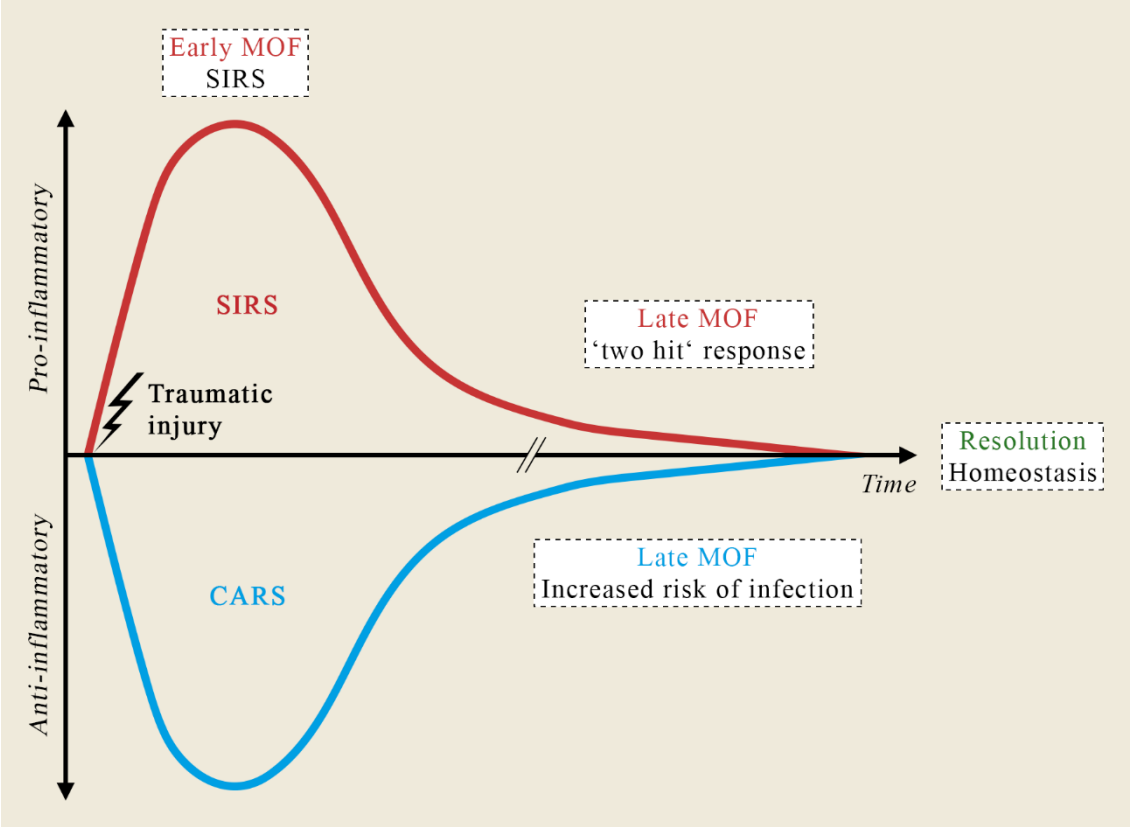

Figure 2 | Traumatic injury induces an imbalance in immune system homeostasis. Traumatic injury disrupts immune system homeostasis that leads to the development of systemic inflammatory response syndrome (SIRS) and anti-inflammatory response syndrome (CARS). The pro-inflammatory immune response is mediated by cells and mediators of innate immune system, whereas the anti-inflammatory immune response is driven by adaptive immune system. If opportunistic infections occur in at the time point when the immune homeostasis is in wide disbalance, the injured patient will be highly susceptible for development of secondary inflammatory complications such as sepsis, septic shock, and multi organ failure (MOF). Resolution of the inflammatory response equals the restoration of immune system homeostasis in trauma patients who survived. Figure adapted from [11, 36].

mixed antagonist response (MARS), which counterbalances both responses. By both, CARS and MARS, severely injured patients are extremely susceptible for microbial infections due to immunoparesis, which has been associated with an increased risk of sepsis.[34] There is an increasing evidence that the cells of innate as well as the cells of adaptive immune system are involved in the posttraumatic immune response during

SIRS and CARS, showing significant alterations in the composition, phenotype, and function of the circulating immune cell pools.[35] For example, significant alterations have been described in the antimicrobial functions of neutrophils, the surface phenotype of monocytes, and the absolute numbers of circulating lymphocytes.[35]

1.3 Monocytes

Monocytes are cells of innate immune system that form together with dendritic cells and macrophages the mononuclear phagocyte system. Before the development of hematopoietic stem cells (HSCs) *in utero*, monocytes are derived from erythro-myeloid precursors (EMPs) in the fetal liver and give arise to the majority of tissue-resident macrophages.[37] These are e.g. alveolar macrophages in the lungs, Kupffer cells in the liver, and Langerhans cells in the skin.[38] The existence of independent monocyte and macrophage pools is supported by the study by Bigley *et al.*, where the patients with a GATA-binding protein 2 mutation totally lack circulating monocytes but the populations of resident dermal and pulmonal macrophages remain unaffected.[39] After the birth, bone marrow-derived HSCs differentiate into monocytes that are rapidly released into the circulation upon their maturation.[37] Circulating monocytes constitute about 5-10% in human and less than 2% in mice of total blood leukocytes [37, 40] and exert different functions depending on their phenotype and surrounding environment.

1.3.1 Monocyte subsets and their functions

Circulating monocytes were originally classified by their irregular cell morphology and density.[41, 42] However, the morphological features of monocytes, such as size, nuclear morphology, and granularity, are considerably variable and thus, a distinction between the monocytes by only histological criteria has not been accurate.[43, 44] Almost four decades ago, two different populations of circulating human monocytes were identified by the morphology and a specific expression pattern of the antigenic

surface markers cluster of differentiation (CD)16 and CD14. This indicated for the first time that monocytes might possess different physiological functions.

CD16, also called FcγRIII, belongs to the Fcγ receptors, which are expressed by neutrophils, natural killer cells, macrophages, distinct population of monocytes, and activated endothelial cells.[45-47] The binding of their Fc region to immunoglobulin (Ig)G immune complexes transduces a wide array of cellular responses. To those count cell activation, release of pro-inflammatory mediators, lymphokine production, cytotoxic triggering, regulation of antibody production, and phagocytosis.[45, 48]

CD14 is a ~55 kDa protein that is expressed on the surface of myeloid linage cells, such as monocytes, macrophages, dendritic cells, and neutrophils.[49] This glycoprotein plays a fundamental role in the phagocytic clearance of apoptotic cells and in the reactivation and immune recognition of microbial cell wall compounds from Gram-positive and Gram-negative bacteria.[50] In association with TLR4 and MD-2, CD14 binds to lipopolysaccharide (LPS) that induces a cascade of inflammatory signaling, triggering the synthesis and release of pro-inflammatory chemokine (C-X-C motif chemokine ligand (CXCL)10) and cytokines (TNF-α, IL-6, and IL-1β).[51, 52] The physiological relevance of CD14 was approved in a knockout mouse, that did not respond to low dose LPS in the secretion of these chemokine and cytokines.[53] However, the ingestion of CD14 in its soluble form (sCD14), which is formed by the shedding of membrane-bound CD14 from monocytes, macrophages, and neutrophils, can restore the ability to induce the inflammatory response.[54] In context of acute respiratory distress syndrome (ARDS), patient's bronchoalveolar lavage fluid (BALF) contains elevated level of sCD14 and using CD14 neutralizing antibodies has shown a decreasing trend towards neutrophil numbers and cytokine concentrations.[55]

Monocytes have been subdivided into three major populations according their CD16 and CD14 expression: $CD16^{-}CD14^{bright}$, $CD16^{+}CD14^{bright}$, and $CD16^{+}CD14^{low}$ monocyte subsets.[29, 47, 56] $CD16^{-}CD14^{bright}$ monocytes have been termed as 'classical' subset since this monocyte population represents approximately 85% of circulating human monocyte pool in homeostasis.[56] $CD16^{+}CD14^{bright}$ and

$CD16^{+}CD14^{low}$ monocytes characterize the 'intermediate' and 'non-classical' subsets, which are represented in the circulation by ~5% and ~10%, respectively.[56] It is generally accepted that classical $CD16^{-}CD14^{bright}$ monocytes are precursors for intermediate $CD16^{+}CD14^{bright}$ monocytes that in turn differentiate into non-classical $CD16^{+}CD14^{low}$ monocytes.[56] Patel *et al.* have shown using human *in vivo* deuterium labeling that classical monocytes are released from the bone marrow into circulation after a postmitotic interval of 1.6 days and remain in the circulation for approximately one day. Subsequently, classical monocytes undergo one of two different fates: they either differentiate into intermediate monocytes or disappear from the circulation by transmigration or apoptosis.[56] The transmigrated monocytes have been found to dwell in the subcapsular red pulp of the spleen, being a secondary classical monocyte reservoir and fulfilling the urgent demand of monocytes during an inflammation.[57] Thus, the 'splenic' monocytes provide an emergency source that extends the time to generate more monocytes by the bone marrow under inflammatory conditions.[37, 57] Intermediate monocytes remain in the circulation for approximately 4.3 days until they give a rise to non-classical monocytes with circulating half-life of approximately 7.4 days.[56] Cross-species transcriptome data analysis revealed that human monocyte subsets have their counterparts in mouse. It has been shown that human $CD16^{-}CD14^{bright}$, $CD16^{+}CD14^{bright}$, and $CD16^{+}CD14^{low}$ monocyte subsets are equivalent to the murine $Ly6C^{+}$ C-X_3-C motif chemokine receptor 1 $(CX_3CR1)^{intermediate}$ C-C chemokine receptor type 2 $(CCR2)^{+}$, $Ly6C^{intermediate}CX_3CR1^{bright}CCR2^{-}$, and $Ly6C^{-}CX_3CR1^{bright}CCR2^{-}$ monocytes, respectively.[58, 59] However, the nomenclature and also the division of murine monocytes into subsets is not consistent in the literature. The most actual literature describes instead of three only two murine monocyte subsets according their either high or low expression of Ly6C.[60-62] Whereas the $Ly6C^{low}$ monocyte subset has been determined to be the counterpart of human non-classical monocytes, the $Ly6C^{bright}$ monocyte subsets comprises both, classical and intermediate human monocyte subsets.[60] In steady state, the $Ly6C^{bright}$ and $Ly6C^{low}$ populations are present in the circulation approximately in the same ratio.[63] Similar to human monocytes, $Ly6C^{bright}$ monocytes are the precursors for the $Ly6C^{low}$ monocytes, confirmed by single-cell transcriptomics [58] and the repopulation

kinetics after monocyte depletion [64]. A brief summary of human and murine monocyte subsets is shown in **Figure 3** and **Table 1** and their specific functions in homeostasis and in inflammatory environment are described below.

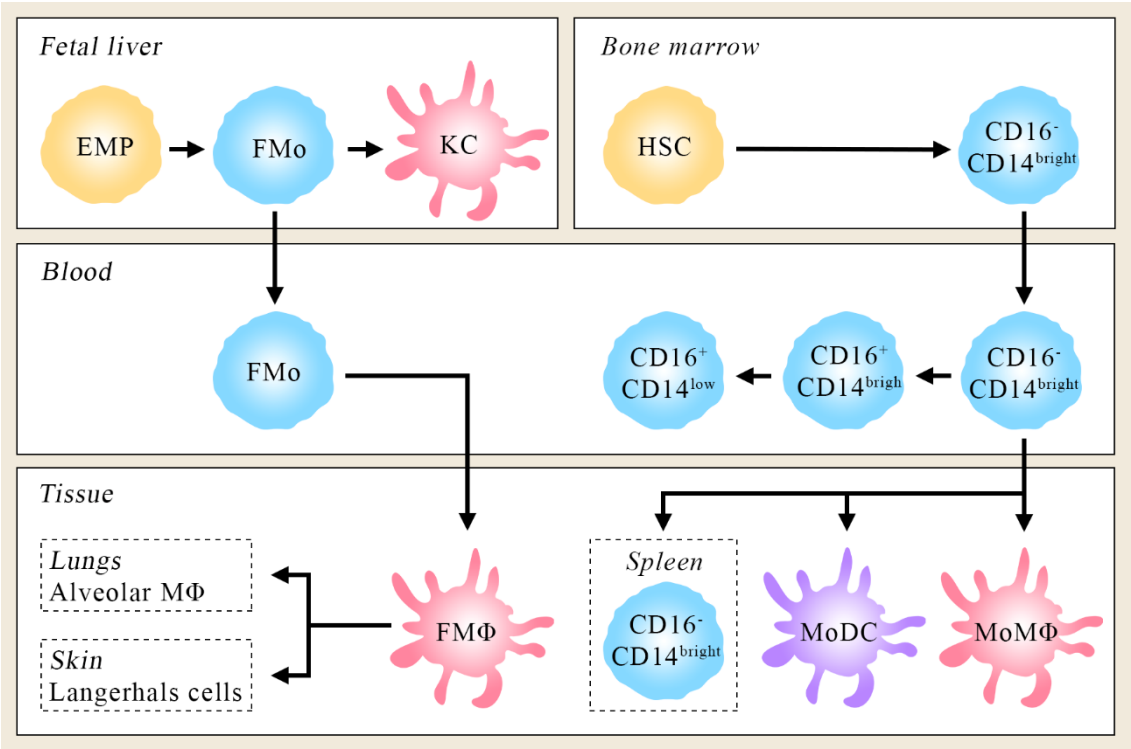

Figure 3 | Monocyte development and compartment in human. Erythro-myeloid precursors (EMPs) generated in the fetal liver give arise to fetal liver-derived monocytes (FMo) before birth, which in turn differentiate into tissue-resident macrophages (FMΦ) like Kupffer cells (KC), alveolar macrophages, and Langerhals cells. Hematopoietic stem cells (HSCs) differentiate *via* monocyte progenitor intermediates into classical $CD16^{-}CD14^{bright}$ monocytes. After maturation, these are released into circulation. Classical $CD16^{-}CD14^{bright}$ monocytes either differentiate over intermediate $CD16^{+}CD14^{bright}$ monocytes to non-classical $CD16^{+}CD14^{low}$ monocytes in the circulation or they transmigrate into tissues and give arise to monocyte-derived Dendritic cells (MoDC) and macrophages (MoMΦ). In steady state, classical $CD16^{-}CD14^{bright}$ monocytes have been found in the spleen as a secondary monocyte reservoir.

Table 1 | Monocyte subsets and their functions

Species	*Ratio*	*Subset*	*Markers*	*Function*
Human	85%	Classical	$CD16^{-}CD14^{bright}$	Phagocytosis
	5%	Intermediate	$CD16^{+}CD14^{bright}$	Pro-inflammatory
	10%	Non-classical	$CD16^{+}CD14^{low}$	Survey endothelial integrity, antiviral
Mouse	50%	Inflammatory	$Ly6C^{bright}$	Pro-inflammatory, pathogen defense
	50%	Patrolling	$Ly6C^{low}$	Patrolling, promotion of tissue repair

Human classical $CD16^{-}CD14^{bright}$ and murine inflammatory $Ly6C^{bright}$ monocytes. Human classical $CD16^{-}CD14^{bright}$ and murine inflammatory $Ly6C^{bright}$ monocytes are rapidly released into circulation after the last step of their maturation in the bone marrow.[37] Their egress and retention in the bone marrow is critically dependent on CCR2- and CXCR4-signaling, respectively.[37, 65-67] Confirmatively, *Ccr2*$^{-/-}$ mice display lower levels of circulating $Ly6C^{bright}$ monocytes and following an infection with *Listeria monocytogenes*, activated monocytes accumulate in the bone marrow.[66] However, *Ccr2*$^{-/-}$ monocytes are able to transmigrate to the site of infection, suggesting independence of CCR2 for the migration.[66] Chemokine monocyte chemoattractant protein (MCP)-1, also called C-C motif ligand (CCL)2, and MCP-3 are major chemokines toward those monocytes are recruited to the tissues.[67] Following *ex vivo* stimulation with LPS, classical monocytes secrete high amounts of pro-inflammatory mediators, such as MCP-1, CCL5, TNF-α, IL-6, IL-8, and IL-1β.[68] Interestingly, they release parallelly also anti-inflammatory cytokine IL-10, which was not observed in the intermediate and non-classical monocyte subset.[68] Additionally, they promote antimicrobial features, generating myeloperoxidase (MPO) and ROS that on the other hand may contribute to oxidative stress.[69, 70] An another mechanism to clear pathogens and apoptotic cells is phagocytosis. Classical monocytes have extraordinary phagocytic capacity, supported by the expression of phagocytosis-related genes like CD93, CD64, CD32, CD36, CD14, ficolin-1, and signal-regulatory protein α.[71, 72]

Human intermediate CD16^{+}CD14^{bright} monocytes. Classical CD16^{-}CD14^{bright} monocytes differentiate into intermediate CD16^{+}CD14^{bright} monocytes and the molecular mechanism of monocyte phenotype transition is still not clear.[56] However, some data indicates that CD16^{-} monocytes might differentiate into CD16^{+} monocytes through a direct interaction with platelets.[73] Intermediate monocytes represent the smallest population of circulating monocytes and have been suggested to share the features of classical and non-classical monocyte populations.[74, 75] That is also the reason, why they have been called 'intermediate'. For example, intermediate monocytes express CCR1, CCR2, and CXCR2 and thus, share these receptors with classical but not non-classical monocytes. Conversely, high co-expression of CX_3CR1 was found on non-classical monocytes, whereas classical monocytes express CX_3CR1 at low level.[72, 76] Upon LPS stimulation, intermediate monocytes display their high inflammatory potential by exaggerated secretion of TNF-α and IL-1β.[71, 72] Compared to classical and non-classical subsets, intermediate monocytes are suggested to generate the highest amount of ROS and display the highest surface presentation of major histocompatibility complex (MHC) class II molecules, encoded by human leukocyte antigen complex - isotype DR (HLA-DR).[68, 71, 72] HLA-DR is a crucial for antigen presentation that is supportive for clearing the pathogens and the cell debris by monocytes.[77] Increased ratio of intermediate monocytes has been shown in patients with angina [78], acute coronary syndrome [79], or rheumatoid arthritis [80], that supports the assumption about their pro-inflammatory character.

Human non-classical CD16^{+}CD14^{low} and murine patrolling Ly6C^{low} monocytes. Similar to human intermediate CD16^{+}CD14^{bright} monocytes, the molecular mechanism of their differentiation into non-classical CD16^{+}CD14^{low} subset still remain elusive, whereas the murine inflammatory Ly6C^{bright} monocytes are suggested to give arise to patrolling Ly6C^{low} monocytes in nuclear receptor subfamily 4 group A member 1-dependent manner.[81, 82] Both, circulating human non-classical and murine patrolling monocytes crawl along the luminal side of the endothelium and constantly patrol and monitor blood vessels to maintain endothelial integrity.[82] This patrolling behavior is partially mediated by Mac-1, a complement receptor consisting of CD11b and CD18,

and is highly dependent on CX_3CR1 signaling and lymphocyte function-associated antigen 1/intercellular adhesion molecule (ICAM)-1 or ICAM-2 interaction with endothelial cells.[82-84] Under homeostasis, non-classical monocytes generate only low levels of ROS, MPO, and lysozyme. Interestingly, toward bacterial stimuli, this phenotype do not response in the synthesis and release of pro-inflammatory TNF-α and IL-1β, however, they show exaggerated production of those in response to viruses and complexes containing nucleic acids in TLR7- and TLR8-dependent manner.[68, 71] Furthermore, non-classical monocytes have been associated with wound healing and the resolution of inflammation in damaged tissues, showing high expression of cytoskeletal rearrangement genes.[68, 83, 84] Nevertheless, non-classical monocytes also seem to have detrimental role in autoimmune disorders: In active lupus nephritis, human and murine patrolling monocytes have been identified to contribute to the glomerular inflammation and disease progression [85, 86], whereas patients with rheumatoid arthritis have elevated levels of non-classical monocytes, correlating with C-reactive protein and rheumatoid factor [87]. Additionally, in a murine model of inflammatory arthritis, patrolling monocytes have been shown to be major mediators of joint tissue damage.[88]

1.3.2 Monocytes in trauma

Monitoring cell-specific responses to traumatic injury is challenging, however, also promising for the development of future posttraumatic treatment strategies additionally to the constantly improving surgical treatments. Analysis of whole blood counts reveals an immediate and persistent leukocytosis, including monocytes, neutrophils, and also lymphocytes.[35, 89] However, although the numbers of circulating monocytes significantly increase within the first hour postinjury and remain elevated for at least three days, their ratio within the leukocyte pool is decreased and neutrophils represent the major increased population.[35, 89] It has been shown that severe traumatic injury leads to immediate monocyte activation, reflecting the initial posttraumatic pro-inflammatory phase, but this is rapidly followed by substantial paralysis of monocyte function.[90, 91] Significantly less pro-inflammatory cytokines such as IL-1β, IL-6, IL-

8, and TNF-α are secreted by monocytes 24 hours after severe trauma compared to the healthy subjects and the cytokine levels at the admission to emergency department.[91] The substantial lower levels of pro-inflammatory mediators also negatively correlate with the extent of MOF.[91]

The study by Laudanski *et al.* clearly demonstrates how the cell-specific transcriptome and pathway analyses can reveal signaling pathways that lead to immunological dysfunction in hospitalized patients with severe blunt trauma and manifested MOF.[89] Genomic analysis of 22,411 genes has shown an alteration in gene expression of 2,801 genes in monocytes obtained from severely injured patients at the postinjury day 10 and compared with matched healthy subjects.[89] Using a knowledge-based gene network analysis of the biological relationships, monocytes from patients with severe trauma display decreased expression of several genes required for T cell co-stimulation like *HLA-DR* and *CD86*, whereas the genes such as *PD-L2*, *CD47*, and *immunoglobulin-like transcript-4*, required for potential monocyte and T cell reciprocal inhibitory activation, were expressed at significantly higher levels.[89] This suggests that posttraumatic transcriptional modification of monocytes is associated with decreased monocyte antigen presentation, inhibition of monocyte and T cell activation, and reduced T cell proliferation.[89]

HLA-DR is also a marker for decreased cellular activity and has been extensively studied on trauma patient's monocytes.[90] Within the first 48 hour after trauma, the expression of HLA-DR is significantly decreased and the timing of its recovery is predictive for the outcome.[90] Whereas the HLA-DR level in patients with uneventful posttraumatic course recovers within one week after trauma, a delayed HLA-DR recovery has been observed in patients who develop secondary infections and MOF.[90] A complete failure to recover the HLA-DR expression on monocytes has been associated with trauma-induced mortality in later time course.[90] Concurrently, monocyte deactivation positively correlates with the ISS.[92] Similarly, although TLR2 and TLR4 surface presentation is significantly elevated on monocytes of severely injured patients compared to healthy volunteers immediately after trauma, the patients

who develop secondary infections reveal significantly lower expression compared to those with uneventful course.[35, 93]

As can be gathered from the text above, monocyte deactivation is crucial component of trauma-induced immunosuppression, leading to secondary complications, MOF, and death. Although there is no direct proof, some data suggest that an impairment of inflammasome function may molecularly contributes to the trauma-induced immunosuppression. Inflammasomes are cytosolic multiprotein complexes expressed mainly by monocytes and macrophages that beyond the modulation of inflammation are involved in physiological processes such as cell metabolism and survival.[94] Relja *et al.* have shown that gene expression of the NLRP1, an intracellular receptor implicated in inflammasome assembly, in monocytes of severely injured patients is downregulated upon the admission to the emergency department and persist over the entire observation period of ten days.[95] An *ex vivo* stimulation of monocytes with LPS revealed decreased *NLRP1* levels compared to healthy subjects, suggesting a hyporesponsiveness to secondary hit. Additionally, the diminished posttraumatic release of IL-1β could be restored by NLRP1 transfection.[95] Moreover, lung damage caused by mechanical ventilation of severely injured patients is assumed to be mediated by increased serum levels of DAMPs, such as adenosine triphosphate (ATP) and ROS, that in turn activates the NLRP3 inflammasome.[96]

Interestingly, although the capacity of phagocytes to recognize and eliminate bacteria and cell debris is critical for a timely resolution inflammation and recovery from the injury,[31, 32] posttraumatic studies on antimicrobial function as phagocytic capacity and generation of ROS have been done almost exclusively in neutrophils. Bao *et al.* evaluated the oxidative activity of human monocytes after spinal cord injury and severe fractures of vertebrae or long bone and compared them with uninjured subjects.[97] Whereas the monocytes of patients with fractures display increasing trend in oxidative burst over the entire observation period of two weeks, the levels were 4- to 6-fold higher following a spinal cord injury with the peak at 12 and 24 hours.[97] Furthermore, polytrauma patients' monocytes display continually increasing oxidative burst over the observation period of three days following *ex vivo* phorbol 12-myristate

13-acetate (PMA) stimulation, whereas the *ex vivo* intake of *Staphylococcus aureus* remain comparable with healthy subjects.[98] An interesting experiment approach has been shown by Vollrath *et al.*.[99] In experimental porcine polytrauma model, the authors investigated the phagocytic capacity of different pathogen strains. The polytrauma consisted of lung contusion, liver laceration, tibial fracture with subsequent external fixation, and hemorrhagic shock with fluid resuscitation, that mimic the typical injury pattern of a heavy car crash.[99] Interestingly, porcine monocytes prefer more to phagocyte *Escherichia coli* and less *S. aureus*. Compared to these both, less than a half of monocytes are positive for engulfment of *Saccharomyces cerevisiae*. In line with the previous study, the phagocytic capacity of monocytes regarding the *S. aureus* did not change over 72 hours. However, the capacity to phagocyte *E. coli* initially decreased directly after the trauma and subsequently increased between 24-72 hours. Inversely, the phagocytic capacity of *S. cerevisiae* did not change over the first 24 hours posttrauma and significantly decreased between 48-72 hours.[99] This indicates that the development and the severity of infectious complications following traumatic injury might be also dependent on the involved pathogen strain, making the research even more demanding.

Although the relevance of the conversion of monocytes in their subsets has been shown in several pathophysiological processes as infections [29, 56, 70], autoimmune diseases [85, 87, 88], atherosclerosis [100], and cancer [101, 102], no study describes phenotypical changes of human monocytes following traumatic injury. In a murine model of traumatic brain injury (TBI), monocytes constitute a significant proportion of the initial brain infiltrate.[103] Monocyte depletion studies have revealed that the patrolling and not the inflammatory monocytes mediate brain oedema, worsen the neurocognitive outcome, and contribute to the neutrophil recruitment to the site of injury.[103] On the other hand, patrolling monocytes are recruited to the site of skin injury, where they differentiate into alternatively activated, $CD206^{+}$ wound healing macrophages, contributing to the skin regeneration.[59] In a model of acute renal injury, the cross-talk between monocytes and neutrophils induce the generation of ROS in glomerulus by neutrophils.[104] Interestingly, patrolling monocytes and not

neutrophils secrete TNF during the inflammation, whereby TNF inhibition before inducing renal injury reduce neutrophil dwell time in glomerulus, their ROS production, as well as the renal injury.[104] This indicates that the interaction between patrolling monocytes and neutrophils might be critical for mediation of secondary injury. On the other hand, inflammatory monocytes have an impact on the progression of ventilation-induced lung injury.[105] It is known that an excessive lung stretch during mechanical ventilation induces ventilator-induced lung injury, leading to pulmonary oedema and inflammation, that have impact of the outcome of acute lung injury (ALI) and ARDS.[105] It has been shown that a high-stretch ventilation mediates recruitment of inflammatory monocytes and neutrophils into lungs, whereas patrolling monocytes seem to not cross the endothelium.[105] Since elevated level of MCP-1, which binds to CCR2 that is preferentially expressed on inflammatory monocytes, is found in BALF of mechanically ventilated animals, [106] this might be the reason why preferentially the inflammatory and not the patrolling monocytes are recruited to the lungs. Moreover, transmigrated inflammatory monocytes display high activation state according to their increased expression of CD11b and decreased expression of CD62L.[105] Additionally, lung-infiltrating inflammatory monocytes secrete IL-1β, which in turn downregulate the expression of the tight junction-associated protein Zonula occludens-2 in pulmonary vascular endothelial cells, leading to loss of vascular integrity and neutrophil extravasation in MyD88-dependent manner.[107] Similar to the TBI described above, monocyte depletion attenuates the ventilation-induced lung injury.[105]

The data described in this section indicate that although the posttraumatic studies focus mainly on neutrophil functions and there are no data on human monocyte subsets, monocytes clearly contribute to the posttraumatic course and the development of secondary injury. The varying involvement of inflammatory and patrolling monocytes following traumatic injury do not have to be contradictory but can be dependent on the injury pattern. Thus, a further evaluation of monocyte subsets and their functions do not only reveal an injury-dependent posttraumatic dynamic of monocytes, but also may be

prospectively a potential target for the amelioration of secondary complications and injury in human settings.

1.4 Potential therapeutic target with a prognostic value for posttraumatic inflammatory complications

The term ‘biomarker’ characterizes a homogenous phenotype within a given situation, risk rank, and predicts specific result like e.g. mortality. They are also used to assess the degree of a disease, its progression, and the therapeutic response.[108] In context of multiple traumatic injuries, approximately 55% of polytrauma patients suffer from severe chest injury, leading to a direct lung tissue damage on the one hand and to activation of inflammatory response on the other hand, resulting in alveolocapillary membrane breakdown.[109] These patients are at high risk for disseminated intravascular coagulation, pneumonia, as well as ALI and its more severe form ARDS.[109] ARDS is associated with an overall mortality between 35% and 50% and treatment of those patients is limited.[110] Since ARDS cannot be diagnosed by simple laboratory test, finding of reliable biomarker may contribute to following critical aspects: (I) to predict the development of ARDS; (II) to stratify the disease severity into more accurate groups; (III) to monitor the response to the treatment; (IV) to predict the outcome; and (V) to provide new insights into its pathogenesis with the aim to develop new therapeutic approaches.[111]

On cellular level, alveolar macrophages are one of the most important immune cells in lung inflammation, contributing to the development and the resolution of ARDS.[112] Thus, alveolar macrophage-related gene and protein expression represent a potential therapeutic target with a prognostic value. Transcriptional analyses by Morrell *et al.* have shown that pro-inflammatory M1-like alveolar macrophages obtained from BALF of ARDS patients who survived or were already extubated within 28 days express pro-inflammatory genes involved in IL-6/Janus kinase/signal transducer and activator of transcription 5 signalling at day 1, followed by rapid downregulation at day 4 and 8.[113] Conversely, patients who died or were still intubated after 28 days display

progressive upregulation of the pro-inflammatory genes over the course of ARDS.[113] Additionally, upregulation of the expression of anti-inflammatory M2-like genes at day 4 or 8 has been associated with 28-days survival.[113] In line with trauma-induced infectious complications and septic shock,[90, 114, 115] HLA-DR expression on circulating monocytes is significantly decreased in ARDS patients, however, concurrently has not been associated with ARDS-related mortality.[116]

Recently, we identified pulmonary club cell protein 16 (CC16) as a circulating biomarker for secondary pneumonia in patients with severe chest trauma.[117] Additionally, anti-inflammatory and immunosuppressive features of CC16 contribute to the maintenance of pulmonary integrity in several pulmonary diseases.[118-120] This suggests that CC16 may contribute to worsening lung injury and inflammatory complications following severe traumatic injury in case of its aberrant occurrence. As described in the example of ARDS given above, a reliable biomarker should predict the development of inflammatory complications, stratify the disease severity, monitor the response to the treatment, and predict the outcome. The markers described in this section fulfill this purpose so far. However, the last point, namely, to provide new insights into the pathogenesis with the scope to develop new therapeutic strategies, cannot be fulfilled by monitoring of universal pro-inflammatory markers. Such a marker and/or mediator that is also prospectively applicable in the disease treatment should be specific to the affected organ and/or tissue, as lungs and pulmonary endothelium in case of ARDS. Therefore, CC16 could be a potential therapeutic target with a prognostic value for posttraumatic inflammatory complications.

1.4.1 Club cell protein 16

CC16, also called uteroglobin, is a 15.8 kDa protein secreted primarily by non-ciliated club cells along the tracheobronchial epithelium, especially in distal respiratory and terminal bronchioles. [121, 122] In humans, approximately 17-27% and 8-14% of club cells are localized in respiratory and terminal bronchioles, respectively, whereas more than 95% of non-ciliated cells in murine pulmonary epithelium consist of club cells.[123, 124] CC16 exerts anti-inflammatory and immunosuppressive properties and

there is a rising evidence that its aberrant appearance in the lungs and the circulation contributes to the pathogenesis of several pulmonary diseases, such as chronic obstructive pulmonary disease (COPD), asthma, sarcoidosis, pulmonary infections, and ARDS.[118-120]

In healthy individuals, systemic concentration of CC16 is approximately 5-6 ng/ml.[117, 120] Whereas this level does not change in severely injured patients (ISS ≥ 16) without thoracic injury, an additional severe chest trauma leads to significant elevation of CC16 in circulation (11.2 ng/ml) immediately after the trauma. This systemic CC16 level either normalizes within 12-24 hours and remain at the basic level or it would subsequently increase if the patients would develop secondary pneumonia.[117] In an another clinic study, serum level of CC16 is significantly elevated in critically ill patients with ARDS compared to those without ARDS.[125] Moreover, systemic CC16 level is significantly more increased in ARDS non-survivors, compared to ARDS survivors.[126, 127] In contrast, BALF obtained from ARDS non-survivors contains significantly less CC16 than ARDS survivors.[128] Decreased local and increased systemic levels of CC16 have been associated with pulmonary epithelium breakdown and intravascular leakage, leading to crossing the bronchoalveolar-blood-barrier of CC16 with subsequent accumulation in the circulation.[118, 119, 129, 130]

The anti-inflammatory and immunosuppressive properties of CC16 have been also investigated in *in vitro* and *in vivo* studies. Administration of recombinant CC16 (rCC16) prior LPS stimulation of murine RAW264.7 macrophages attenuates the LPS-induced TNF-α, IL-6, and IL-8 expression at mRNA and protein level in dose-dependent manner, inhibiting the transcriptional and DNA binding activity of NF-κB.[131] In a murine model of acute lung infection in COPD, *Cc16*$^{-/-}$ mice infected with *Mycoplasma pneumoniae* display enhanced airway hyperresponsiveness during methacholine challenge, whereas oropharyngeal and also intravenous administration of rCC16 before *M. pneumoniae* infection restores the lung function.[132] The authors suggest that CC16 has only protective features, if the leucine-valine-asparagine integrin binding site of CC16 is intact, inhibiting leukocyte adhesion and thus, limiting inflammatory cell recruitment into the lungs.[132] This is in line with a study by Xu *et*

al., where rCC16 inhibited the migratory capacity of polymorphonuclear leukocytes (PMNLs) *ex vivo* toward IL-8.[133] In context of traumatic injury, *ex vivo* stimulation of PMNLs isolated from healthy individuals with sera from severely injured patients significantly decreases their migratory capacity.[133] Prior neutralization of CC16 in those sera reverses this effect and is paralleled by enhanced CD62L expression, indicating higher activation state of PMNLs.[133] Interestingly, in a murine ALI model with early sepsis, CC16 neutralization after thoracic blunt injury and before sepsis induction by cecal ligation and puncture (CLP) leads initially to increased neutrophil lung infiltration and increased pulmonary injury and is reversed 24 hours after sepsis induction.[134] However, CC16 neutralization only delays and does not improve the mortality rate.[135] Additionally, CC16 neutralization after sepsis induction first does not have any beneficial effects.[134, 135] These data indicate that CC16 is a potent marker for determining lung injury and however, its pathophysiological role – also with regard to the timing – during lung injury is not fully understood, modification of CC16 availability in the lungs could became a reliable therapeutic tool in trauma-induced lung injury.

1.5 Acute alcohol intoxication – common coincidence at ED

Alcohol is one of the oldest and nowadays one of the most common addictive substances worldwide,[136] however, otherwise than other addictive drugs such as narcotics, alcohol consumption is legal. Although low-to-moderate alcohol consumption is associated with decreased risk for a development of cardiovascular diseases and cardiovascular disease-related mortality [137], an alcohol abuse contributes to the development of alcoholic liver disease, pancreatitis, and further pathologies [136]. 2018, an epidemiological survey of abuse substances in Germany has revealed that 71.6% of respondents stated that they had consumed alcohol in the previous 30 days, whereby 34.5% of those reported to have at least one episode of binge drinking.[138] In other words, approximately 7.8 million adults consume excessive amounts of alcohol [139], which leads to 74,000 deaths yearly [140]. However, this statistic includes deaths, which are directly related to alcohol, such as

alcohol poisoning and alcoholic liver disease with subsequent cancer development, and not physical traumatic injury-related deaths that occurred under the influence of alcohol.[140] It is remarkable, that up to 50% of injured patients are admitted to the emergency department with an acute alcohol intoxication. [141, 142] The majority of these patients are adults (53.5%; 18-64 years old), whereby 20.5% of elderly patients (≥ 65 years old) and 13.3% of paediatric patients (≤ 17 years old) have positive blood alcohol level.[143] Assaults, falls, and biking accidents have been shown to be the most frequent causes of traumatic injury of alcohol intoxicated patients.[144] In turn, these injury mechanisms are linked to higher prevalence of TBI.[144] Since TBI is a major cause of injury-related deaths,[145-147] the numbers of alcohol-related deaths may be underestimated.

1.5.1 Immunological changes in alcohol abuse

Research over the last decades has shown that alcohol intake has biphasic effect on the innate immune response in a dose- and time-dependent manner [148-151]. In human studies, chronic alcohol abuse is linked to increased vulnerability to infections, postoperative complications, slow recovery, and poor outcome after traumatic injury as well as to significant organ damage as indicated by higher incidence of ARDS, alcohol liver disease, certain cancers, and sepsis.[152-155] This is associated with an exaggerated pro-inflammatory response and activation of monocytes and macrophages, leading to an excessive release of pro-inflammatory mediators, that can contribute to alcohol-related tissue damage.[136, 153] It has been shown that peripheral blood mononuclear cells (PBMCs) and alveolar macrophages obtained from heavy drinkers secrete elevated amount of pro-inflammatory TNF-α, IL-1β, IL-2, interferon γ and IL-6 in dose-dependent manner and their *ex vivo* stimulation with LPS potentiated this increase.[153, 156] This suggests that chronic alcohol abuse leads to a sensitization of pro-inflammatory pathways.

Binge drinking is defined as an episodic excessive alcohol intake and it is the most common form of alcohol abuse.[157] Compared to chronical alcohol intake, binge drinking modulates the immune response in different manner. In the first 20 minutes of

drinking, the numbers of circulating leukocytes, monocytes, and natural killer cells increase, whereas an *ex vivo* whole blood stimulation with LPS leads to elevated TNF-α level in plasma, suggesting a pro-inflammatory state in the very early phase of binge drinking. The numbers of monocytes and natural killer cells as well as the level of systemic IL-1β, IL-6, and MCP-1 decline thereupon and along with elevated systemic IL-10, it suggests that binge drinking has anti-inflammatory effect in the later course.[148, 150, 158] This is in line with studies on severely injured patients with an excessive alcohol intoxication who have shown significantly decreased systemic levels of IL-6 and IL-12 compared to non-intoxicated patients.[151, 159] However, severely injured patients have increased systemic level of anti-inflammatory cytokine IL-10 in parallel compared to those with an alcohol intoxication and to uninjured subjects.[159] Moreover, lower blood alcohol concentration do not induce pro-inflammatory acute phase proteins such as serum amyloid A and serum amyloid P.[150] Pro-inflammatory mediators are essential for the recruitment of the cells of innate immune system and the subsequent initiation of adaptive immune response.[149] Thus, acute alcohol intoxicated patients are prone to infections due to immunosuppression and imbalance between pro- and anti-inflammatory mediators.

1.5.2 Role of monocytes in acute alcohol intoxication

In monocytes and macrophages, the pro- and anti-inflammatory effects of alcohol intake are based on so-called TLR sensitization and tolerance, respectively.[136] TLR4 is mainly expressed on monocytes, macrophages, and dendritic cells and only sparsely on other cell types like e.g. endothelial cells.[160] In complex with CD14 and MD-2, TLR4 specifically recognizes LPS and induces NF-κB translocation and expression of pro-inflammatory cytokines and chemokines in MyD88-dependent manner.[160] Chronic alcohol abuse-mediated sensitization of immune cells to gut-derived LPS is an elemental factor in the pathogenesis of alcoholic liver disease and alcoholic pancreatitis, empathizing the biological and clinical relevance of TLR4 signaling in chronic alcohol abuse.[154, 161-163] Conversely, acute alcohol intake attenuates TLR-mediated signaling in monocytes. It has been shown that an *ex vivo* and *in vitro* alcohol

administration to human monocytes and murine RAW264.7 macrophages prior LPS stimulation results in decrease of TNF-α release.[164, 165] Parallelly, acute alcohol induces B-cell lymphoma 3-encoded protein (Bcl-3) expression and its association with NF-κB p50 homodimer, that is a negative regulator of NF-κB transcription.[164] This has been also confirmed in murine model of binge drinking, where the mice had decreased systemic level of TNF-α and Bcl-3 expression was increased in the liver.[164] Moreover, in human *in vivo* and *ex vivo* binge alcohol model, the expression of TLR4-MyD88-dependent cytokines *Tnfα* and *Il6*, as well as the TLR4-TRIF-depedent cytokines and chemokines *Infβ*, *Ip10*, and *Ccl5* are inhibited in monocytes that were exposed to alcohol prior LPS administration.[166] Since *Tlr4* expression is significantly decreased in liver of mice from two to six hours after intragastric application of ethanol (5 mg/g body weight) that is gradually increasing to the basal level within 48 hours,[167] it seems that acute alcohol intoxication directly inhibit the TLR4 expression that in turn leads to attenuation of pro-inflammatory response toward LPS. Tolerance toward inflammatory mediators has been associated with diminished functionality of monocytes and neutrophils, reducing their capability to migrate into tissues, to generate ROS, and to phagocytose.[160, 168-170] In context of traumatic injury, patients with immune incompetent monocytes and neutrophils display higher incidence of secondary complications such as sepsis, multiorgan dysfunction syndrome, and subsequent death.[91, 171, 172]

In addition to direct inhibition of pro-inflammatory cytokines and chemokines by TLR4 tolerance induced by acute alcohol administration, attenuation of the inflammasome activation was detected in lymphoid cells such as murine J774 macrophages, murine bone marrow-derived dendritic cells, murine neutrophils, and human PBMCs.[173] The authors have shown that ethanol specifically inhibit the activation of the NLRP3 inflammasome, resulting in ameliorated caspase-1 cleavage and secretion that in turn is required for cleavage of pre-IL-1β to its active form.[173] Moreover, LPS- and ATP-induced activation of caspase-1 and secretion of IL-1β is inhibited dose-dependently by ethanol administration in human THP1 macrophages.[174] However, since *NLRP3* and *IL1B* expression is not attenuated by alcohol, it indicates that ethanol inhibits

inflammasome activation and not the synthesis of pro-IL-1β.[174] This is in line with experimental traumatic injury model, where rats that underwent thoracic injury and hemorrhagic shock with resuscitation following acute alcohol intoxication. Here, Franz *et al.* have shown that the increased inflammasome activation, evaluated by caspase-1 activity, is reversed by intragastric ethanol administration two hours prior injury.[170] This is paralleled by diminished pulmonary infiltration by PMNLs, *Il6* expression, and total protein concentration in BALF, whereas the trauma-induced alveolar disruption with alveolar wall thickening is not altered by acute alcohol intoxication.[170] However, the data were evaluated two hours after resuscitation and thus, no conclusion can be drawn regarding the protective or deteriorating effect of alcohol in later course following alcohol intoxication.

The outcome of alcohol intoxicated patients following severe traumatic may also depend on the distribution of the three monocyte subsets that have been described above since they have different functions, and their phenotypic shift is involved in several pathological processes. However, the only reference about alcohol-induced phenotypic shift of monocyte subsets has been shown in chronic excessive abuse.[175] Here, the numbers of classical monocytes are significantly reduced and the non-classical increased, whereas these changes particularly restore two weeks after alcohol withdrawal.[175] Thus, the distribution and the role of monocyte subsets in acute alcohol intoxication remains elusive.

1.6 Aim of this study

As already described in the section ‘Balancing the pro- and anti-inflammatory immune response’, traumatic injury induces an exaggerated pro-inflammatory immune response that dependent on the intensity and imbalance to anti-inflammatory response may have further reaching deteriorating effects on patient’s health. In the posttraumatic inflammatory complications, monocyte deactivation is critical for trauma-induced immunosuppression, contributing to development of MOF and death. However, although the shift of monocyte subsets and their altered functions are associated with

several pathologies, no data from severely injured patients are available. Moreover, finding a reliable mediator its modulation could counteract the posttraumatic inflammatory complications would significantly improve the posttraumatic treatment management. Thereby, acute alcohol intoxication, which affects up to 50% of trauma patients, should be also considered in development of novel therapeutic algorithms since it has immunomodulating effects.

Therefore, the aim of the present thesis is to evaluate:

1. The distribution of monocyte subsets in severely injured patients and the function regarding their antimicrobial activity in the first 12 hours after trauma. We hypothesize that severe traumatic injury shifts the monocyte subsets toward the pro-inflammatory phenotype early after trauma. We expect that severely injured patients' monocytes generate significantly more ROS compared to healthy subjects but also that an *ex vivo* stimulation with PMA could mitigate the differences between those.
2. The impact of pulmonary CC16 on systemic and local distribution of monocyte and macrophage subsets and its anti-inflammatory potential in murine double hit model consisting of thoracic trauma and CLP, mimicking early septic posttraumatic complications. We hypothesize that a local intrapulmonary neutralization of CC16 is associated with phenotypic shift of monocytes and macrophages toward the pro-inflammatory subsets. Additionally, we expect that this is paralleled by an early upregulation of pro-inflammatory mediators such as TNF, MCP-1, and RAGE and aggravated lung damage.
3. The impact of binge drinking on healthy subjects in time-dependent manner regarding the distribution of monocyte subsets and their inflammasome activation. We hypothesize that acute alcohol intoxication shifts monocytes toward their anti-inflammatory phenotype, paralleled by TLR4 tolerance. The expected alcohol-induced immunosuppressive modulation of immune response could be associated with reduced activation of NLRP3 inflammasome in monocytes and their reduced adhesive capacity, impairing the transmigration into tissues.

Chapter 2 Publications

2.1 Severe traumatic injury induces phenotypic and functional changes of neutrophils and monocytes

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Reference [176]

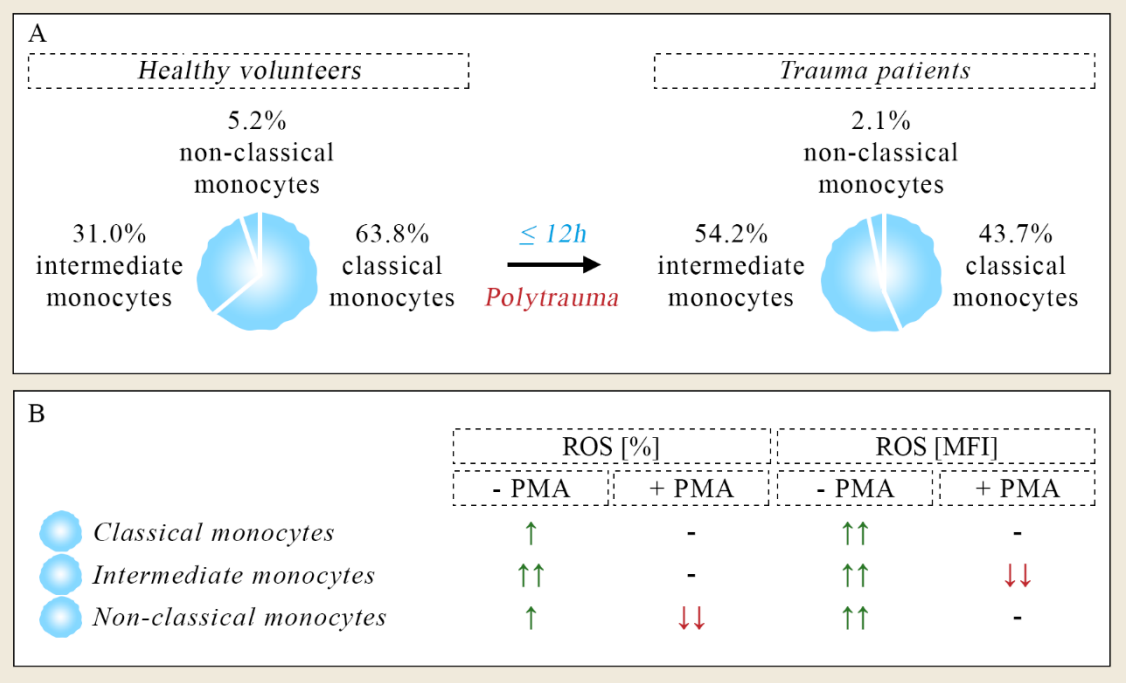

Figure 4 | Monocytes shift towards the pro-inflammatory phenotype following traumatic injury. Polytrauma patients display significant increase of intermediate monocytes within 12 hours after injury (A). The subset-specific generation of reactive oxygen species (ROS) was evaluated (B). - no change, ↑ increasing tendency, and ↑↑/↓↓ significant increase/decrease compared to unstimulated (- PMA) and *ex vivo* stimulated (+ PMA) sample of healthy volunteers, respectively, are shown. MFI, mean fluorescence intensity; PMA, phorbol 12-myristate 13-acetate.

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Severe Traumatic Injury Induces Phenotypic and Functional Changes of Neutrophils and Monocytes

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Abstract: Background: Severe traumatic injury has been associated with high susceptibility for the development of secondary complications caused by dysbalanced immune response. As the first line of the cellular immune response, neutrophils and monocytes recruited to the site of tissue damage and/or infection, are divided into three different subsets according to their CD16/CD62L and CD16/CD14 expression, respectively. Their differential functions have not yet been clearly understood. Thus, we evaluated the phenotypic changes of neutrophil and monocyte subsets among their functionality regarding oxidative burst and the phagocytic capacity in severely traumatized patients. Methods: Peripheral blood was withdrawn from severely injured trauma patients (TP; $n = 15$, ISS ≥ 16) within the first 12 h post-trauma and from healthy volunteers (HV; $n = 15$) and stimulated with fMLP and PMA. $CD16^{dim}CD62L^{bright}$ (immature), $CD16^{bright}CD62L^{bright}$ (mature) and $CD16^{bright}CD62L^{dim}$ ($CD62L^{low}$) neutrophil subsets and $CD14^{bright}CD16^{-}$ (classical), $CD14^{bright}CD16^{+}$ (intermediate) and $CD14^{dim}CD16^{+}$ (non-classical) monocyte subsets of HV and TP were either directly analyzed by flow cytometry or the examined subsets of HV were sorted first by fluorescence-activated cell sorting and subsequently analyzed. Subset-specific generation of reactive oxygen species (ROS) and of *E. coli* bioparticle phagocytosis were evaluated. Results: In TP, the counts of immature neutrophils were significantly increased vs. HV. The numbers of mature and $CD62L^{dim}$ neutrophils remained unchanged but the production of ROS was significantly enhanced in TP vs. HV and the stimulation with fMLP significantly increased the generation of ROS in the mature and $CD62L^{dim}$ neutrophils of HV. The counts of phagocyting neutrophils did not change but the mean phagocytic capacity showed an increasing trend in TP. In TP, the monocytes shifted toward the intermediate phenotype, whereas the classical and non-classical monocytes became less abundant. ROS generation was significantly increased in all monocyte subsets in TP vs. HV and PMA stimulation significantly increased those level in both, HV and TP. However, the PMA-induced mean ROS generation was significantly lower in intermediate monocytes of TP vs. HV. Sorting of monocyte and neutrophil subsets revealed a significant increase of ROS and decrease of phagocytic capacity vs. whole blood analysis. Conclusions: Neutrophils and monocytes display a phenotypic shift following severe injury. The increased functional abnormalities of certain subsets may contribute to the dysbalanced immune response and attenuate the antimicrobial function and thus, may represent a potential therapeutic target. Further studies on isolated subsets are necessary for evaluation of their physiological role after severe traumatic injury.

Keywords: traumatic injury; reactive oxygen species; phagocytosis; CD14; CD16; CD62L; fMLP; PMA

1. Introduction

Severe traumatic injury is with 5.8 million annual deaths one of the most common causes of death worldwide [1]. Although the survival rates have improved globally in the past decades due to advanced post-traumatic treatment, the development of immune-related secondary complications such as systemic inflammatory response syndrome, nosocomial infections, or sepsis, remains the major contributing factor in trauma-associated mortality [2–4]. Approximately 55% of polytrauma patients suffer from severe chest injury, leading to a direct lung tissue damage on the one hand and to activation of inflammatory response on the other hand, resulting in alveolocapillary membrane breakdown [5]. These patients are highly susceptible for disseminated intravascular coagulation, pneumonia, as well as acute lung injury, and its more severe form acute respiratory distress syndrome (ARDS) [5]. ARDS is associated with a dysregulated immune response, an overall mortality between 35% and 50%, and treatment of those patients is limited [6]. An excessive generation of ROS by the injured endothelium and epithelium as well as recruited leukocytes play a major role in ARDS progression and lung damage [7]. Moreover, a dysregulated immune response after trauma has been associated with post-traumatic phenotypical and functional aberrations of certain cells of the innate immune system such as neutrophils and monocytes [7–10].

Neutrophils and monocytes represent the first line of immune defense following an infection or a sterile injury [11,12]. Both are released from the bone marrow into the circulation and are recruited to sites of infection or tissue damage towards the pathogen-derived signaling molecules (pathogen-associated molecular patterns) or host-produced inflammatory signaling molecules (damage-associated molecular patterns), respectively [11,12]. The ability to recognize and eliminate bacteria and debris is critical for timely resolution of inflammation and recovery from the injury [13,14]. An impairment of phagocytic activity ex vivo has been observed in patients with community-acquired pneumonia [15], spinal cord injury [16] and traumatic brain injury [17,18] compared to healthy individuals. Moreover, neutrophil depletion impedes the clearance of the debris from necrotic sites in sterile hepatic injury, leading to impairment of regeneration and revascularization of the focal injury [10]. As some neutrophils in human peripheral neutrophil pool phagocyte their targets better than others [19], certain neutrophil populations may have differential functionality in this process.

Although the production of reactive oxygen species (ROS) is essential for the pathogen clearance, an exaggerated release of proteases and oxygen radicals may lead to collateral tissue damage and contribute to the development of secondary complications [20,21]. In the context of acute lung injury or its most severe form ARDS, excessive generation of ROS leads to the loss of junctional integrity of vascular microvessels, promoting the migration of polymorphonuclear leukocytes and the transition of fluids in the alveolar lumen, causing pulmonary edema [7].

Three different subsets of neutrophils were described according to their expression of the cluster of differentiation (CD)16 and CD62L expression [22]. $CD16^{dim}CD62L^{bright}$ subset shows the characteristics of immature neutrophils with banded nuclei, $CD16^{bright}$ $CD62L^{bright}$ population represents the mature neutrophils with prototypically segmented nuclei, and $CD16^{bright}CD62L^{dim}$ neutrophils are characterized by hypersegmented nuclei. The latter have been shown to directly suppress lymphocyte proliferation by ROS release into the immunological synapse between neutrophil and T-cell, suggesting its immunosuppressive features [22]. Although all neutrophil subsets enter the circulation almost immediately after trauma, their precise post-traumatic function still remains elusive [8].

Similarly, three different subsets of monocyte can be distinguished according to their CD16 and CD14 surface expression [23,24]. Classical monocytes, characterized by

$CD16^{-}CD14^{bright}$ expression contribute to bacterial clearing and immune sensing, whereas non-classical $CD16^{+}CD14^{dim}$ monocytes patrol along the blood vessel walls [25]. Intermediate $CD16^{+}CD14^{bright}$ monocytes share particularly the functions of classical and non-classical subsets but have more pro-inflammatory features and have been linked to the regulation of apoptosis and transendothelial migration [23,24]. Following human experimental endotoxemia, all monocyte subsets are lost within one to two hours after lipopolysaccharide administration and recover from classical, over intermediate to non-classical monocytes within 8–24 h [9]. This clearly confirms the generally accepted theory, that monocyte subsets differentiate from classical to intermediate and non-classical monocytes in a dynamic process. There is no evidence about the subset dynamics including specific kinetics and functions following traumatic injury in humans.

Considering that a well-orchestrated immune response is essential for the initiation and subsequent resolution of inflammation and an adverse phenotype and functional transition of neutrophils and monocytes may negatively affect the post-traumatic outcome, we evaluated the phenotypic shift of neutrophils and monocytes as well as their phagocytic capacity and ROS production in the currently known subsets of those cell types.

We hypothesized that major traumatic injury shifts the cell subset ratios toward the pro-inflammatory phenotypes early after trauma and that along with this, the circulatory cells will exert modified functions regarding phagocytosis and oxidative burst.

2. Materials and Methods

2.1. Ethics

The current study was performed at the University Hospital of the Goethe-University Frankfurt in accordance with the Declaration of Helsinki and following the Strengthening the Reporting of Observational studies in Epidemiology-guidelines [26].

2.2. Patient Cohort

Fifteen healthy volunteers and 15 severely injured patients between 18 and 50 years of age were enrolled. Trauma patients with an injury severity score (ISS) $\geq$ 16 were included and the samples were collected and analyzed within the first 12 h post-injury. Exclusion criteria included an acute infection, pre-existing chronic inflammatory diseases, immunological disorders, human immunodeficiency virus infection, infectious hepatitis, immunosuppressive medication, and pregnancy.

2.3. Staining of Neutrophil and Monocyte Subsets for Flow Cytometry

The blood of trauma patients and healthy volunteers was withdrawn into Li-Heparin blood collection tube (Sarstedt, Nümbrecht, Germany), stored on ice and processed within 30 min. A total of 100 µL of heparinized blood was transferred into FACS tube (Corning, New York, NY, USA). For neutrophils, heparinized blood was stimulated with 1×10^{-3} mM N-formyl-methionyl-leucyl-phenylalanine (fMLP) on ice for 15 min. Subsequently, samples were washed with 2 mL ice-cold FACS buffer (0.5% bovine serum albumin in 1× phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+}), gently vortexed, and centrifuged at 350× *g* and at 4 °C for 7 min. After the supernatant was discarded, 5 µL Alexa Fluor 647-conjugated anti-human CD16 antibody (clone 3G8; BD Biosciences, Franklin Lakes, NJ, USA) and 20 µL PE-conjugated anti-human CD62L antibody (clone SK11; BD Biosciences, USA) were added. For monocytes, heparinized blood was stimulated with 1×10^{-4} mM phorbol 12-myristate 13-acetate (PMA) on ice for 15 min. Subsequently, the samples were washed. 5 µL Alexa Fluor 647-conjugated anti-human CD16 antibody (clone 3G8; BD Biosciences, USA) and 5 µL PE-conjugated anti-human CD14 antibody (clone M5E2; BioLegend, San Diego, CA, USA) were added, all samples gently vortexed and incubated in the dark on ice for 30 min. Subsequently, the samples were washed. Then, the protocols for functional analyses regarding the phagocytic capacity (see Section 2.4) and ROS generation (see Section 2.5) followed as described below.

2.4. Analysis of Bacterial Intake by Neutrophils by Flow Cytometry

FITC-labeled *E. coli* bioparticles were reconstituted in 1× PBS without Mg^{2+} and Ca^{2+} according to the manufacturer's instructions (*Escherichia coli* (K-12 strain) BioParticles E-2861; Thermo Fisher Scientific, Waltham, MA, USA), aliquoted and stored at −20 °C and in dark until use. In the experiment, 10 bioparticles per leukocyte were added to the sample and incubated at 37 °C and 5% CO_2 for 60 min. Following "bacterial" loading, cells were washed with 2 mL ice-cold FACS buffer and centrifuged at 350× *g* and at 4 °C for 7 min. For red blood cells lysis, 2 mL cold lysis buffer were added, and the samples were incubated in the dark at 4 °C for 10 min. Washing step with 2 mL ice-cold FACS buffer was repeated. Cells were resuspended in 500 µL ice-cold FACS buffer and immediately evaluated by flow cytometry. Granulocytes were defined by gating the corresponding forward and side scatter scan. From each sample a minimum of 5.0×10^4 cells were measured, which were subsequently analyzed. The percentage of $CD16^{dim}CD62L^{bright}$ (immature), $CD16^{bright}CD62L^{bright}$ (mature) and $CD16^{bright}CD62L^{dim}$ (hypersegmented) neutrophils as well the percentage and mean fluorescent units of phagocytosis-positive out of the respective subsets were assessed by flow cytometric analyses using a BD FACSCanto 2™ and FACS DIVA™ software (BD Biosciences, USA).

2.5. Analysis of Reactive Oxygen Species Production in Neutrophils and Monocytes by Flow Cytometry

Totals of 90 µL warm PBS and 2 µL 100 µM CM-H_2DCFDA (Thermo Fisher Scientific, USA) were added to samples (see Section 2.3) and incubated at 37 °C and 5% CO_2 for 30 min. 2 mL warm RPMI 1640 medium (Gibco, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal calf serum (Gibco, Carlsbad, CA, USA), 100 IU/mL penicillin (Gibco, USA), 10 µg/mL streptomycin (Gibco, Carlsbad, CA, USA) and 20 mM HEPES buffer (Sigma Aldrich, St. Lois, MO, USA) were added and samples were centrifuged at 350× *g* and at room temperature for 7 min. Supernatant was discarded and subsequently, 1 mL medium with supplements was added and the samples were incubated at 37 °C and 5% CO_2 for further 60 min. Following the recovery step, samples were centrifuged at 350× *g* and at room temperature for 7 min and the supernatant was discarded. For red blood cells lysis, 2 mL lysis buffer (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM ethylenediaminetetraacetic acid in distilled water) were added and incubated in the dark at 4 °C for 10 min. Washing step with 2 mL ice-cold FACS buffer was repeated. Cells were resuspended in 500 µL ice-cold FACS buffer. Granulocyte and monocyte populations were defined by gating the corresponding forward and side scatter scan. Their ratio out of leukocyte population was assessed after excluding the cell debris. From each sample a minimum of 5.0×10^4 cells were measured, which were subsequently analyzed. For neutrophils, the percentage of $CD16^{dim}CD62L^{bright}$ (immature), $CD16^{bright}CD62L^{bright}$ (mature) and $CD16^{bright}CD62L^{dim}$ ($CD62L^{dim}$) neutrophils and for monocytes, the percentage of CD14brightCD16$^-$ (classical), $CD14^{bright}CD16^+$ (intermediate) and $CD14^{dim}CD16^+$ (non-classical) as well the percentage and mean fluorescent units of ROS-positive out of the respective subsets were assessed by flow cytometric analyses using a BD FACS Canto 2™ and FACS DIVA™ software (BD Biosciences, USA).

2.6. Fluorescence-Activated Cell Sorting of Neutrophil and Monocyte Subsets

For each neutrophil and monocyte subsets, the blood of healthy volunteers was freshly withdrawn into Li-Heparin blood collection tubes (Sarstedt, Germany), stored on ice and processed within 30 min. For each neutrophil subset, 200 µL of heparinized blood was transferred into FACS tube (Corning, USA). For neutrophils, 10 µL Alexa Fluor 647-conjugated anti-human CD16 antibody (clone 3G8; BD Biosciences, USA) and 40 µL PE-conjugated anti-human CD62L antibody (clone SK11; BD Biosciences, USA) were added. The sample was gently mixed and incubated in the dark and on ice for 30 min. For washing, 2 mL ice-cold FACS buffer were added, and samples were subsequently centrifuged at 350× *g* and at 4 °C for 7 min. The supernatant was discarded. For red blood cells lysis, 2 mL

of cold red blood cell lysis buffer were added and incubated in the dark at 4 °C for 10 min. Washing step with 2 mL ice-cold FACS buffer was repeated. Cells were resuspended in 1000 µL ice-cold FACS buffer and immediately sorted by using BD FACSCalibur (BD Biosciences, USA).

For monocyte subsets, peripheral blood mononuclear cells (PBMCs) were isolated first by a density-gradient centrifugation (Biocoll separation solution, 1.077 g/mL density; Biochrom, Berlin, Germany). Here, 10 mL of Biocoll separation solution (room temperature) were carefully overlaid with 10 mL of at room temperature tempered heparinized blood and centrifuged at 800× *g* and at room temperature for 25 min. PBMCs in the interphase were transferred into FACS tube and washed with 3 mL ice-cold FACS buffer. Remaining red blood cells were lysed by 500 µL cold red blood cell lysis buffer at 4 °C for 10 min. After further washing step, PBMCs were resuspended in 200 µL ice-cold FACS buffer. Next, 20 µL Alexa Fluor 647-conjugated anti-human CD16 antibody (clone 3G8; BD Biosciences, USA) and 20 µL PE-conjugated anti-human CD14 antibody (clone M5E2; BioLegend, USA) were added. The sample was gently mixed and incubated in the dark and on ice for 30 min. Subsequently, cells were washed with 2 mL ice-cold FACS buffer and resuspended in 1000 µL ice-cold FACS buffer and immediately sorted by using BD FACSCalibur (BD Biosciences, USA).

Granulocyte and monocyte populations were defined by gating the corresponding forward and side scatter scan and the doublets were excluded. Neutrophil subsets were sorted as $CD16^{bright}CD62L^{bright}$ (mature) and $CD16^{bright}CD62L^{dim}$ ($CD62L^{dim}$) using BD FACSCalibur™ (BD Biosciences, USA). Monocyte subsets were sorted as CD14brightCD16^{-} (classical), $CD14^{bright}CD16^{+}$ (intermediate) and $CD14^{dim}CD16^{+}$ (non-classical). Cell number and cell viability of the sorted populations were determined by Türk's solution exclusion assay (Merck, Darmstadt, Germany). Sorted populations were reanalyzed and typically >95% pure. The generation of ROS (see Section 2.4; excluding the red blood cells lysis step) and the phagocytic capacity (see Section 2.5; excluding the red blood cells lysis step) were assessed.

2.7. Statistics

GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) was used to perform the statistical analysis. Data are given as mean ± standard error of the mean (SEM). The differences between the healthy volunteers and trauma patients were analyzed by Mann–Whitney *U*-test. The Kruskal–Wallis test with a Dunn's post hoc test was applied to compare the differences between the subsets. A *p*-value below 0.05 was considered statistically significant.

3. Results

3.1. Patient Cohort

Fifteen patients with severe trauma and 15 healthy volunteers were enrolled in this study. The mean age of the patients was 38.2 ± 4.99 years of age. Two thirds of patients were male. All patients were substantially injured with an ISS of 27.7 ± 2.27. The mean stay in the intensive care unit was 15.5 ± 4.15 days, and the total duration of the in-hospital stay was 29.0 ± 7.32 days. The mean time of artificial ventilation was 8.4 ± 2.97 days. No patients developed ARDS, sepsis, or died. One patient developed pneumonia two days after admission to emergency department. The mean count of leukocytes in blood was 8.81/nL ± 0.92. The mean ratios of granulocytes and monocytes out of leukocytes were 67.8% ± 3.79 and 6.8% ± 0.98, respectively in trauma patients and 43.4% ± 2.35 and 6.6% ± 0.36, respectively in healthy subjects. The data are summarized in Table 1.

Table 1. Patient cohort.

	Parameter	SEM
Age	38.2 years old	4.99
Gender	10 men/5 women	-
ISS	27.7	2.27
Hospital stay	29.0 days	7.32
ICU stay	15.5 days	4.15
Ventilation	8.4 days	2.97
Death	0	-
Leukocytes	8.81/nL *	0.92
Neutrophils	67.8% of leukocytes	3.79
Monocytes	6.8% of leukocytes	0.98
Pneumonia	1 patient	-
ARDS	0	-
Sepsis	0	-

* Normal range: 3.92–9.81/nL. ARDS, acute respiratory distress syndrome; ICU, intensive care unit; ISS, injury severity score; SEM, standard error of mean.

3.2. Severe Trauma Modulates the Distribution of CD16⁺ Neutrophil Subsets

As it is known that severe traumatic injury has modulating effects on the immune system, we investigated the distribution of three neutrophil subsets according their CD16 and CD62L expression. Circulatory neutrophils were stained ex vivo and evaluated by flow cytometry. The gating strategy as well as the representative figures of $CD16^{dim}CD62L^{bright}$ (immature), $CD16^{bright}CD62L^{bright}$ (mature) and $CD16^{bright}CD62L^{dim}$ ($CD62L^{dim}$) neutrophil subsets in healthy volunteers and trauma patients are shown in Figure 1A. The main population of neutrophils in healthy volunteers and trauma patients is formed by mature neutrophils (Figure 1B). Immature and $CD62L^{dim}$ neutrophil populations are significantly less present compared to mature neutrophils (Figure 1B, $p < 0.05$). Comparing healthy volunteers to severely injured patients, the numbers of immature neutrophils significantly increase following major injury (Figure 1B, $p < 0.05$), whereas the counts of mature neutrophils do not change, and a decreasing trend is shown in $CD62L^{dim}$ neutrophil subset (Figure 1B).

3.3. Severe Traumatic Injury Causes a Phenotypic Shift of CD14⁺ Monocytes

Similar to neutrophils, we evaluated the phenotypic redistribution of $CD14^{+}$ monocytes following traumatic injury. The gating strategy as well as the representative figures of $CD16^{-}CD14^{bright}$ (classical), $CD16^{+}CD14^{bright}$ (intermediate) and $CD16^{+}CD14^{dim}$ (non-classical) monocyte subsets in healthy volunteers and trauma patients are shown in Figure 2A. In healthy volunteers, classical monocytes present the most abundant monocyte population, whereas intermediate and non-classical monocytes are significantly less present compared to this subset (Figure 2B, $p < 0.05$). The population of non-classical monocytes is also significantly smaller than the intermediate monocyte population (Figure 2B, $p < 0.05$). In trauma patients, intermediate subset presents the most abundant monocyte population and the classical monocyte counts have a decreasing trend compared to this subset (Figure 2B). Non-classical monocytes of trauma patients are significantly less present than classical and intermediate monocytes (Figure 2B, $p < 0.05$). Comparing healthy volunteers to trauma patients, classical monocyte population decreases in trauma patients, whereas intermediate monocyte population becomes significantly more abundant (Figure 2B, $p < 0.05$). The numbers of the non-classical monocytes significantly decrease in trauma patients (Figure 2B, $p < 0.05$).

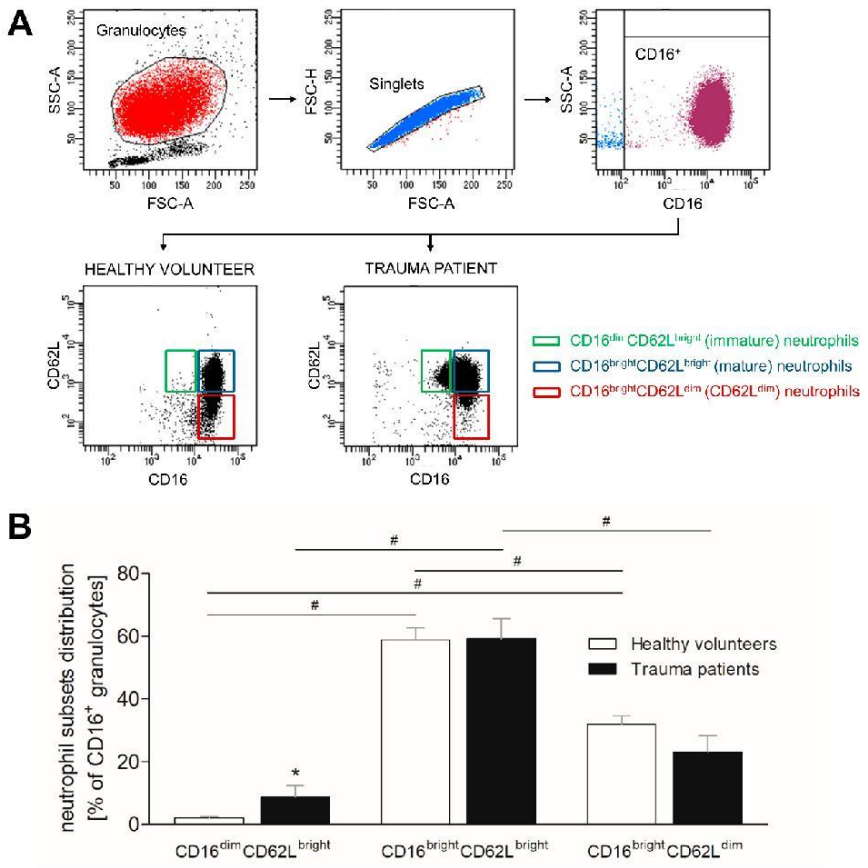

Figure 1. Impact of severe traumatic injury on neutrophil subset distribution. (**A**) Representative gating strategy for phenotyping human neutrophil subsets, including size discrimination, doublet exclusion, selection of $CD16^+$ cells, and separation according to expression of CD16 and CD62L. (**B**) The percentage distribution of $CD16^{dim}CD62L^{bright}$ (immature), $CD16^{bright}CD62L^{bright}$ (mature) and $CD16^{bright}CD62L^{dim}$ ($CD62L^{dim}$) neutrophils out of $CD16^+$ granulocytes was determined in healthy subjects (white bars) and severely injured patients (black bars) within 12 h postinjury. Data are presented as mean ± standard error of the mean. *: $p < 0.05$ vs. healthy volunteers; #: $p < 0.05$ vs. respective subset.

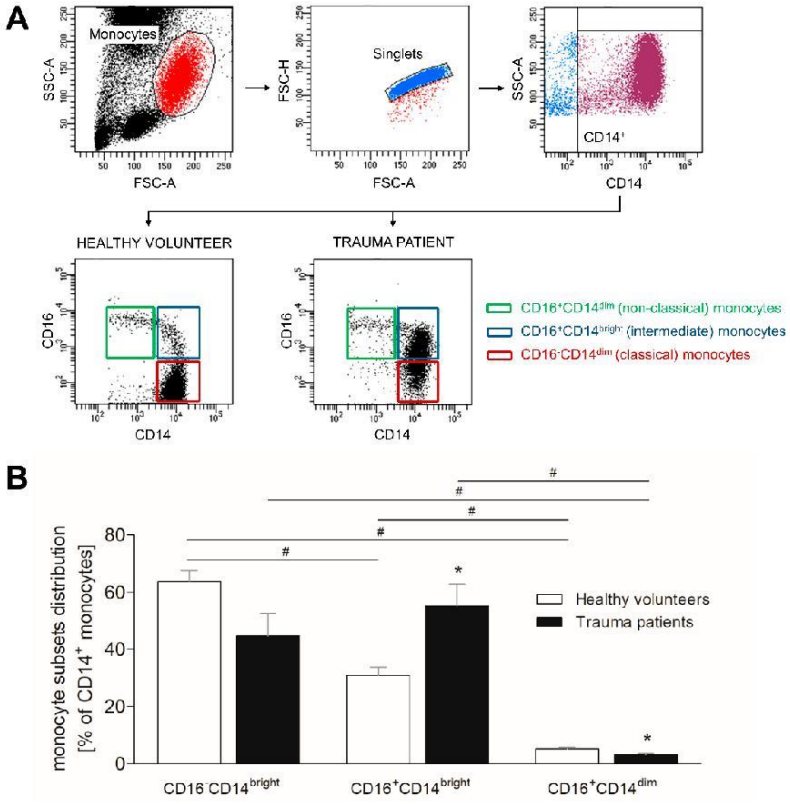

Figure 2. Impact of severe traumatic injury on monocyte subset distribution. (**A**) Representative gating strategy for phenotyping human monocyte subsets, including size discrimination, doublet exclusion, selection of $CD14^+$ cells, and separation according to expression of CD16 and CD14. (**B**) The percentage distribution of $CD14^{bright}CD16^-$ (classical), $CD14^{bright}CD16^+$ (intermediate) and $CD14^{dim}CD16^+$ (non-classical) monocytes out of $CD14^+$ monocytes was determined in healthy subjects (white bars) and severely injured patients (black bars) within 12 h postinjury. Data are presented as mean ± standard error of the mean. *: $p < 0.05$ vs. healthy volunteers; #: $p < 0.05$ vs. respective subset.

3.4. Severe Trauma Does Not Affect Phagocytic Capacity of Neutrophils at Early Time Point

Severely injured patients often develop secondary infectious complications, which may be caused by reduced phagocyting capacity [16]. Therefore, we evaluated the bacterial intake of neutrophils following traumatic injury. All three neutrophil populations obtained from healthy volunteers incorporate comparable numbers of FITC-labeled *E. coli* bioparticles, with slight increase in mature and $CD62L^{dim}$ neutrophils, however, without significance (Figure 3A). Within the first 12 h after injury, the counts of phagocyting cells do not significantly change compared to respective subsets in healthy volunteers (Figure 3A).

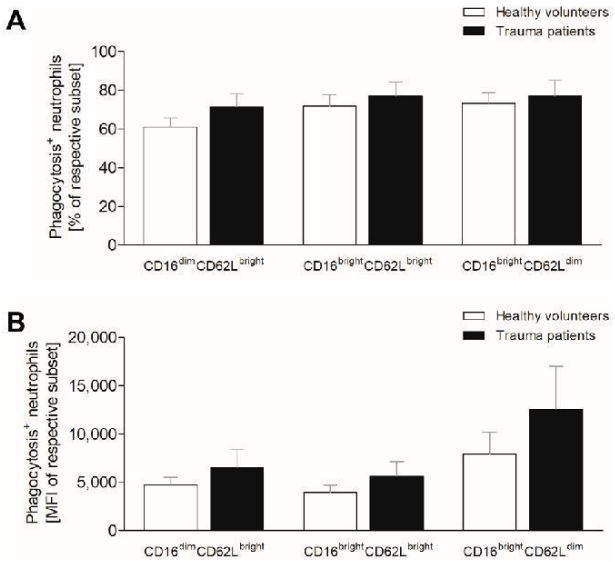

Figure 3. Impact of severe traumatic injury on phagocytic capacity of neutrophil subsets. The (**A**) percentage and (**B**) mean intensity of phagocytosis positive $CD16^{dim}CD62L^{bright}$ (immature), $CD16^{bright}CD62L^{bright}$ (mature) and $CD16^{bright}CD62L^{low}$ ($CD62L^{dim}$) neutrophils were determined in healthy subjects (white bars) and severely injured patients (black bars) within 12 h postinjury. Data are presented as mean ± standard error of the mean.

The mean bioparticle intake per cell is comparable in immature and mature neutrophils of healthy volunteers, whereas the $CD62L^{dim}$ neutrophils display higher phagocyting capacity compared to these two subsets (Figure 3B). Following traumatic injury, all three subsets exert increased mean phagocyting capacity without statistical significance compared to healthy volunteers (Figure 3B).

3.5. Severe Trauma Elevates the Production of Reactive Oxygen Species in Mature and $CD62L^{dim}$ Neutrophils

As it is known that enhanced ROS production contributes to endothelial dysfunction and tissue injury, we evaluated ROS levels in neutrophils. In healthy volunteers, significantly more immature neutrophils form ROS compared to mature and $CD62L^{dim}$ neutrophils (Figure 4A, $p < 0.05$), whereas in trauma patients the counts of ROS positive neutrophils are equal in all three subsets (Figure 4A). Following traumatic injury, the counts of ROS positive immature neutrophils are comparable with these in healthy volunteers (Figure 4A), whereas the mature and $CD62L^{dim}$ neutrophil subsets display significant increase of ROS positive neutrophils (Figure 4A, $p < 0.05$). Ex vivo stimulation of whole blood with fMLP does not affect the ratio of ROS positive neutrophils in the immature subset of healthy volunteers but significantly increases in the mature and $CD62L^{dim}$ subset (Figure 4A, $p < 0.05$). In trauma patients, the fLMP-induced generation of ROS tends to increase compared to unstimulated samples, however, without significance (Figure 4A).

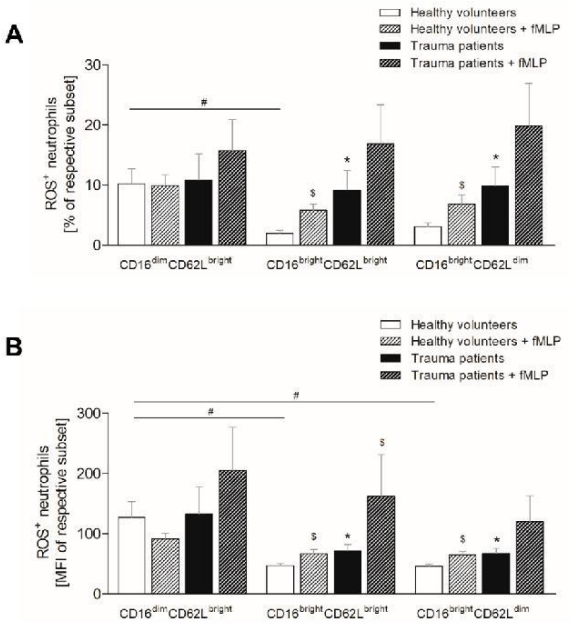

Figure 4. Impact of severe traumatic injury on generation of reactive oxygen species (ROS) by neutrophil subsets. The (**A**) percentage and (**B**) mean intensity of ROS positive $CD16^{dim}CD62L^{bright}$ (immature), $CD16^{bright}CD62L^{bright}$ (mature) and $CD16^{bright}CD62L^{low}$ ($CD62L^{dim}$) neutrophils were determined in healthy subjects (white bars) and severely injured patients (black bars) within 12 h postinjury. Whole blood was stimulated with PMA ex vivo (diagonally striped bars). Data are presented as mean ± standard error of the mean. *: $p < 0.05$ vs. healthy volunteers; #: $p < 0.05$ vs. respective subset; $: $p < 0.05$ vs. unstimulated corresponding subset.

Similarly, the mean ROS production intensity per cell is the highest in immature neutrophil subset in both, healthy volunteers and trauma patients (Figure 4B). Those levels are significantly lower in mature and $CD62L^{dim}$ neutrophils of trauma patients compared to immature neutrophils (Figure 4B, $p < 0.05$) and the levels in healthy volunteers are clearly lower as well, however, without statistical significance (Figure 4B). Comparing the ROS levels in trauma patients to healthy volunteers within the individual subsets, the mean ROS production of immature neutrophils does not change following traumatic injury (Figure 4B), whereas the levels in mature and $CD62L^{dim}$ neutrophil population significantly increase (Figure 4B, $p < 0.05$). Similar to ROS positive neutrophils, the mean ROS generation intensity per cell does not change in the immature neutrophils of healthy volunteers following ex vivo fMLP stimulation and is significantly higher in mature and CD62L subsets (Figure 4B, $p < 0.05$). fMLP stimulation of trauma patient's blood increases the mean generation of ROS compared to that of healthy subjects in the mature neutrophils, whereas the immature and $CD62L^{dim}$ subsets show only increasing tendency (Figure 4B, $p < 0.05$).

3.6. Severe Traumatic Injury Increases the Production of Reactive Oxygen Species in Monocytes

Similar to neutrophils, monocytes display subset specific differences in ROS production following traumatic injury. In healthy volunteers, the same ratio of classical and intermediate monocytes generates ROS, whereas non-classical monocyte population dis-

plays significantly higher number of ROS positive cells (Figure 5A, $p < 0.05$). The monocytes obtained from trauma patients have increasing ratios of ROS positive cell from classical over intermediate to non-classical monocytes, whereby the difference between classical and non-classical subset is significant (Figure 5A, $p < 0.05$). When comparing trauma patients with healthy volunteers, there is an increasing ratio of ROS positive monocytes in all subsets, with significance in the intermediate subset (Figure 5A, $p < 0.05$). Ex vivo stimulation of whole blood of both healthy and injured subjects with PMA significantly increases the ratio of ROS positive monocytes within all three subsets compared to the unstimulated samples (Figure 5A, $p < 0.05$). However, only the non-classical monocytes of injured patients generate significantly less ROS following PMA stimulation compared to the equivalent subsets in healthy subjects (Figure 5A, $p < 0.05$).

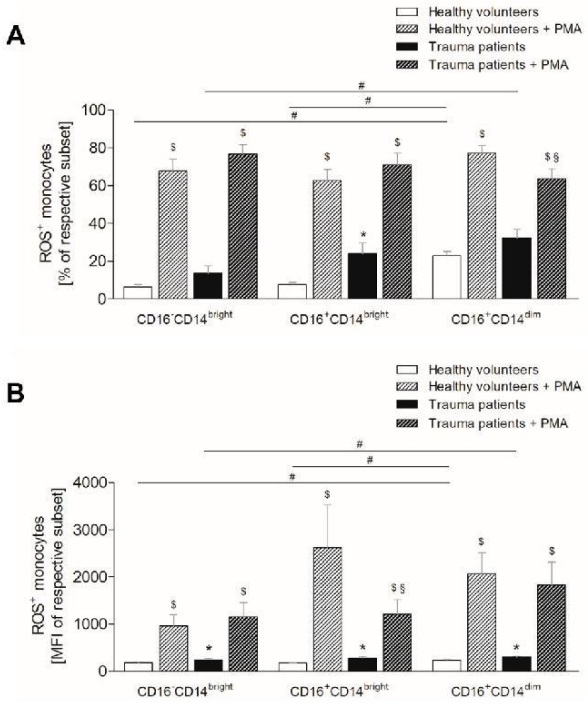

Figure 5. Impact of severe traumatic injury on generation of reactive oxygen species (ROS) by monocyte subsets. The (**A**) percentage and (**B**) mean intensity of ROS positive CD14brightCD16$^-$ (classical), CD14^{bright}CD16$^+$ (intermediate) and CD14^{dim}CD16$^+$ (non-classical) monocytes were determined in healthy subjects (white bars) and severely injured patients (black bars) within 12 h postinjury. Whole blood was stimulated with PMA ex vivo (diagonally striped bars). Data are presented as mean ± standard error of the mean. *: $p < 0.05$ vs. healthy volunteers; #: $p < 0.05$ vs. respective subset; $: $p < 0.05$ vs. unstimulated corresponding subset; §: $p < 0.05$ vs. healthy volunteers + PMA.

The mean ROS production per cell is the highest in the non-classical subset, whereas classical and intermediate monocyte population is significantly less positive for ROS compared to non-classical monocytes (Figure 5B, $p < 0.05$). In trauma patients, classical monocytes produce significantly less ROS than non-classical monocytes (Figure 5B,

$p < 0.05$), whereas the mean ROS production intensity of intermediate subset is comparable with that of non-classical monocyte population (Figure 5B). Comparing the respective subsets, all monocyte populations display significantly increased mean ROS production intensity following traumatic injury compared to healthy volunteers (Figure 5B, $p < 0.05$). Ex vivo stimulation with PMA leads similarly to the ratios of ROS positive monocytes, and also to a significant increase of mean ROS generation intensity in both healthy and injured subjects compared to untreated samples (Figure 5B, $p < 0.05$). This increase is significantly lower in the intermediate subset of severely injured patients compared to healthy subjects, whereas the mean intensity does not change in the classical and non-classical monocytes (Figure 5B, $p < 0.05$).

3.7. Fluorescence-Activated Cell Sorting Reveals an Exhaustion of Neutrophils and Monocytes and Aligns the Functional Differences between the Subsets

The individual neutrophil subsets of healthy volunteers were sorted by fluorescence-activated cell sorting (FACS) and subsequently analyzed for ROS production and phagocytic capacity. Nearly 100% of mature and $CD62L^{dim}$ neutrophils were positive for ROS (Figure S1A) and the mean ROS production did not differ between the subsets as well (Figure S1B). Compared to whole blood analyses (see Figure 3), the production of ROS approximately increased 30-fold. The counts of phagocytosis positive neutrophils (Figure S1C) and the mean phagocytic capacity (Figure S1D) did not vary between the mature and $CD62L^{dim}$ subsets. The bacterial intake is significantly impeded compared to whole blood analyses (see Figure 4). The immature neutrophil subset was not analyzed due to its low numbers and nearly absence in HV.

Similarly, nearly 100% of classical, intermediate, and non-classical monocytes were positive for ROS following FACS (Figure S2A). The mean production of ROS did not differ between the subsets (Figure S2B). Compared to whole blood analyses (see Figure 5), all monocyte subsets produce significantly higher amounts of ROS.

4. Discussion

Although the clinical treatment algorithms of severely injured trauma patients have improved over the last decades, leading to higher post-traumatic survival rates, those patients are still highly vulnerable to secondary infections during their clinical stay. The development of infectious complications has been associated with a dysregulated immune response, wherefore a mapping and characterization of immunological changes and intercellular interactions may predict post-traumatic vulnerability to secondary complications and potentially represent promising therapeutic targets in future [2–4]. Neutrophils and monocytes have been shown to initiate an immediate innate immune response with the aim to clear tissue damage and provide protection from invading pathogens [7–10]. Therefore, we investigated the phenotypical changes of different neutrophil and monocyte subsets in severely injured trauma patients as well as their functionality regarding the ROS generation and phagocytic capacity.

In this study, severely injured patients' leukocyte counts remain in the normal range, whereas the ratio of granulocytes significantly increases compared to healthy subjects. Emergency granulopoiesis, which has been extensively described following traumatic injury and microbial challenge [27,28], is a part of the first line of cellular defense that crucially modulate subsequent repair processes after tissue damage [29]. However, on the other hand, an exaggerated release of neutrophils leads to bone marrow exhaustion and in turn, can impair the innate immune response to secondary hit such as surgery or infection [21].

Subset-specifically, we observed that in both, healthy volunteers and severely injured patients mature neutrophils ($CD16^{brigh}CD62L^{bright}$) represent the most abundant neutrophil subset. Although it has been reported that only a homogenous population of mature neutrophils can be found in healthy individuals, we detected additionally the $CD62L^{dim}$ population ($CD16^{brigh}CD62L^{dim}$). Comparing healthy volunteers with severely injured patients, the counts of $CD16^{brigh}CD62L^{dim}$ neutrophils did not differ between those.

A significantly increased frequency and absolute cell numbers of $CD16^{brigh}CD62L^{dim}$ neutrophils in trauma patients is extensively described in the literature and thus, contradictory to our results [30]. $CD16^{brigh}CD62L^{dim}$ neutrophils occur within the first hour after the injury [30] and locally release hydrogen peroxide into the immunological synapse between the neutrophils and T cells in Mac-1-dependend manner, leading to the suppression of T cell activation [22]. This suggests that a neutrophil subset with dimmer CD62L expression exhibits immunosuppressive features. Although CD62L is rapidly shed from the cell surface upon neutrophil activation [31], also changes in osmotic pressure, pH value, or hemodynamic shear stress can cause CD62L shedding [32–34]. Thus, regarding our results, $CD16^{brigh}CD62L^{dim}$ population in healthy volunteers may not constitute of $CD62L^{dim}$ neutrophils but rather we might potentially detect the mature neutrophils that underwent CD62L shedding ex vivo caused by mechanical stress caused by the isolation and analysis methods. This, however, should be the case for both healthy donors and trauma patients.

Further, we observed a massive presence of immature ($CD16^{dim}CD62L^{bright}$) neutrophils in severely injured patients, whereas healthy individuals lack this population. It is assumed that immature neutrophils have impaired functional ability [21]. Moreover, Spijkerman et al. have shown that the ratio of immature neutrophils positively correlates with the severity of the injury and the development of infectious complication [28]. This indicates that immature neutrophils have an inadequate immune response towards traumatic stimuli depending on the injury severity and wherefore, the ratio of immature neutrophils may become a potent tool in the prediction of infectious complications following traumatic injury.

For a timely resolution of inflammation and recovery from injury, phagocytes such as neutrophils and monocytes recognize and eliminate microbes and cell debris [13,14]. Thus, if the phagocytes fail to engulf and clear their targets, the tissue inflammation is prolonged, causing tissue damage with subsequent infectious complications [10]. In the present study, we did not observe any significant differences between the severely injured patients and healthy individuals and the individual neutrophil subsets regarding the phagocytosis in the first twelve hours after trauma. Actually, the references about aberrated phagocytic capacity of neutrophils following traumatic injury are not consistent [17,35,36]. However, it must be considered that the most studies evaluate the intake of fluorochrome-conjugated bacteria or bacterial bioparticles by neutrophils and not directly their killing potential. That an accurate engulfment of bacteria does not necessarily mean that the pathogens are also adequate killed is underlined by the study by Leliefeld et al., analyzing the incorporation of bacteria and the capacity of killing the bacteria by neutrophils in human experimental endotoxemia model [37]. Interestingly, immature neutrophils exhibit a superior engulfment of bacteria and killing capacity. However, even though mature and $CD62L^{dim}$ subsets incorporate bacteria at comparable level to immature neutrophils, they are incapable to kill the bacteria, that has been associated with higher intraphagosomal pH, subsequent intracellular bacterial growth and escape of the pathogens from the neutrophils [37]. This indicates that the above-described positive correlation between the elevated ratio of immature neutrophils and the development of inflammatory complications does not depend on defective phagocytosis. Moreover, as severe traumatic injury leads to neutrophilia with subsequent bone marrow exhaustion and the $CD62L^{dim}$ subset appears within the first hour after the injury [21,27,28,30], this may together with the incapability of $CD62L^{dim}$ neutrophils to adequately kill the bacteria [37] contribute to the development of infectious complications in later time course, because the immune system might not adequate respond to the bacterial escape from the $CD62L^{dim}$ neutrophils.

The generation of ROS is an elemental mechanism to clear the pathogens within the phagosome, but it also acts as a chemoattractant for immune cells to clear and repair the tissue. However, an exaggerated release of free radicals can have detrimental consequences to the host such a loss of junctional integrity of vascular microvessels that contributes to the development of pulmonary edema and has been associated with the development of ARDS, systemic inflammatory response syndrome and multiorgan failure (MOF) [7,20,21,38–40].

Although the extent of the generation of ROS seems to be injury severity-dependent [17] and the contribution of neutrophil-induced exaggerated oxidative burst in the development of secondary complications after trauma is generally accepted, there is no evidence about neutrophil subset-specific generation of ROS.

We have shown that the oxidative burst significantly increases in mature and $CD62L^{dim}$ neutrophils of severely injured patients, but its level remains stable in immature neutrophils compared to healthy individuals. However, immature neutrophils have the highest ratio of ROS compared to the other subsets. This supports the suggestion that immature neutrophils may be the key players in development of secondary post-traumatic complications. In line, the mean generation of ROS in the immature neutrophils noticeable increases in severely injured patients following ex vivo stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLP) compared to stimulated blood samples from healthy subjects. It has been shown that immature neutrophils are apoptosis resistant [41], whereas an exaggerated oxidative burst to secondary hit post-trauma has been associated with uncontrolled inflammatory response, resulting in endothelial permeability and tissue damage [42–44]. This along with our data indicates that the ROS-induced collateral tissue damage and subsequent infectious complications following traumatic injury could be primarily caused by immature neutrophil subset. Taken together, neutrophils undergo phenotypical and functional changes in severely injured patients dependently on the injury severity and contribute to the development of secondary complications. Therefore, a consequent monitoring of those during the whole period of hospital stay along with the incidence of infectious complications could provide data about the prediction of post-traumatic vulnerability to secondary infections.

Similar to neutrophils, monocytes have a multi-faced role in maintaining the tissue homeostasis and responding to inflammatory stimuli in order to clear the pathogens and cellular debris with subsequent restoration of tissue integrity [45]. However, the initial monocyte activation after trauma is rapidly followed by substantial paralysis of monocyte function, reflected by the decreased surface presentation of human leukocyte antigen (HLA)-DR [46,47]. Delayed recovery of HLA-DR expression and decreased release of pro-inflammatory mediators such as interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α) have been associated with the development of secondary infections and MOF [46,47]. However, these studies focus mainly on aberrated monocyte functions from 24 h after trauma and there are no data about potential phenotypical changes of human monocytes following traumatic injury at early time point. Therefore, we analyzed the redistribution of monocyte subsets within the first twelve hours after severe trauma.

It is generally accepted that classical $CD16^{-}CD14^{bright}$ monocytes, which is the main monocyte population in healthy individuals, are precursors for pro-inflammatory intermediate $CD16^{+}CD14^{bright}$ monocytes that in turn differentiate into patrolling non-classical $CD16^{+}CD14^{low}$ monocytes [48]. In trauma patients, the numbers of circulating intermediate monocytes significantly increase, whereas we have observed a decrease of classical and non-classical subsets, suggesting a phenotype switch toward the pro-inflammatory phenotype. Classical monocytes have anti-microbial features with superior phagocytic capacity and after entering the tissues, they differentiate into monocyte-derived macrophages or dendritic cells [24]. Thus, the initial decrease of circulating classical monocytes after severe trauma might be caused on the one hand by a differentiation into intermediate monocytes and on the other hand by their transmigration to injury site in order to shape and resolve the inflammation. Regarding the intermediate monocytes, such an exaggerated elevation has been shown in severely injured patients 48 h post-trauma [49] and under inflammatory conditions such as sepsis or bacterial and viral infections [9], paralleled by sequestration of high amounts of TNF-α, IL-1β, and IL-6 [50]. Such an exaggerated release of pro-inflammatory cytokines has been associated with a so-called cytokine storm, that in turn can lead to blood pressure collapse, coagulopathy, up to MOF and death [51]. Therefore, the extent of the intermediate subset may prospectively provide an information about secondary post-traumatic complications.

Although an excessive oxidative burst has been already shown in monocytes of severely injured patients [52,53], there is no evidence whether the monocyte subsets generate ROS in a different extent and thus, may differently contribute to the pathogen and tissue clearance. In the present study, all monocyte subsets have significantly increased mean capacity to produce ROS compared to healthy individuals, which is comparable between the subsets. The ratio of ROS positive monocytes was elevated also in all subsets; however, a significant increase has been observed only in the pro-inflammatory intermediate monocytes. This along with the significant increase of the intermediate monocyte numbers support the assumption that the very early post-traumatic phase is characterized by a pro-inflammatory immune response of monocytes. Interestingly, ex vivo stimulation of whole blood with phorbol 12-myristate 13-acetate (PMA) leads to significant increase of mean generation of ROS compared to unstimulated samples, but this increase is significantly lower in the intermediate subset of severely injured patients compared to healthy volunteers. Once a pathogen is phagocytized, ROS contribute to the elimination of ingested pathogen and in the case of their not sufficient intracellular level, pathogen can escape and survive [54]. Thus, this inadequate intracellular oxidative burst of intermediate monocyte subset upon secondary stimuli may contribute to the susceptibility to infectious complications.

Interestingly, intermediate monocytes display the highest expression of HLA-DR in human experimental endotoxemia model compared to another two subsets over the whole observation period of 24 h [9]. Considering the post-traumatic monocyte deactivation within the first 48 h [9], it would be reasonable to follow the redistribution of monocyte subsets of severely injured patients and the subset-specific HLA-DR expression and the generation of ROS over the entire intensive care unit stay. We assume that the extent and the timing of the initial pro-inflammatory phase followed by the immunosuppressive phase in combination with the monocyte subsets distribution might provide valuable insight into post-traumatic monocyte kinetics and prospectively also a potent tool for counteracting the secondary infections.

For the evaluation of the physiological role of the neutrophil and monocyte subsets, studies on isolated subsets are necessary. Thus, we isolated the subsets by FACS. Although we obtained viable and clearly defined populations verified by flow cytometry, the above discussed functional differences between the subsets were no longer visible. Whereas nearly 100% of the cells were positive for oxidative burst and the mean levels elevated extraordinary, the bacterial incorporation was significantly impaired compared to whole blood analyses. We used BD FACSCalibur flow cytometer (BD Biosciences, USA), which is the first multicolor benchtop flow cytometry system capable of analyzing and sorting cells of interest for further study. The cell sorting rate and consequently the velocity, in which we obtained the requested cell counts, were extremely low. As both neutrophils and monocytes respond overly sensitive to their environment, so prolonged isolation led to cell exhaustion and impaired functionality. Thus, we have not been successful in isolating the individual subsets and subsequent analyzing their physiological roles and further studies under optimum conditions are necessary.

5. Limitations

This study provides a solid fundament for understanding the early post-traumatic phenotypic shift of neutrophils and monocytes and their antimicrobial functions, however, it also has several limitations. First, we only included fifteen polytrauma patients. Although the results shown are conclusive, increasing the number of study participants would enable a more precise group allocation according to the trauma pattern, and also correlation analyses of the evaluated neutrophil and monocyte subsets with clinical parameters such as ISS, bacterial complications, or ARDS. Second, we evaluated the phenotypic and functional changes only at the early time point. As severely injured patients develop secondary inflammatory complications in later time course, it would be reasonable to follow-up on the changes during the entire stay at intensive care unit. Third, as we did not use counting beads during the flow cytometry measurements, we obtained only relative

fractions of neutrophil and monocyte subsets. Additionally, the percentage of neutrophils and monocytes out of leukocytes was evaluated by flow cytometry and, thus, this is not so precise as the blood analysis by hospital laboratory would be. Therefore, for achieving the absolute numbers, counting beads must be included in the upcoming studies as well as the blood work performed by hospital laboratory for evaluation of the ratios between the different leukocyte subpopulations. Lastly, FITC-labeled *E. coli* BioParticles were used for the phagocytic assay. This is a proper assay for the evaluation of bacterial intake; however, it is not possible to make a statement regarding the bacterial killing. A combination of the assessment of the bacterial intake and killing by neutrophils and monocytes would provide an immerse improvement of understanding the post-traumatic antimicrobial kinetics of leukocytes.

6. Conclusions

Severe traumatic injury induces an immediate phenotype shift of neutrophils as well as monocytes accompanied by their alterations in ROS generation compared to healthy subjects. In the circulation of trauma patients, the ratio of immature neutrophils is immensely elevated, whereas numbers of mature and $CD62L^{dim}$ neutrophils do not change. All three subsets display an increasing tendency in phagocytic capacity and the mature and $CD62L^{dim}$ neutrophils produce significantly more free radicals than those in healthy individuals. Thereby, an ex vivo stimulation with fMLP increases mean generation of ROS by trauma patients' immature neutrophils compared to healthy subjects. Similarly, monocytes shift toward the pro-inflammatory intermediate phenotype and the classical and non-classical subsets becomes less abundant in trauma patients. All monocyte subsets generate high levels of ROS after severe traumatic injury. However, the intermediate and non-classical monocytes of severely injured patients generate significantly less ROS following ex vivo stimulation with PMA compared to healthy subjects. The here presented post-traumatic dynamic changes of those cells of innate immune system provide a solid fundament for functional studies of the individual subsets. The data following the ex vivo stimulation suggest that neutrophils may more contribute to the endothelial permeability and tissue damage, whereas monocytes seem to more contribute to higher susceptibility to secondary infections by their hyporesponsiveness to secondary hit. Future directions will include a larger cohort of severely injured patient and the analysis of trauma-induced phenotypical and functional changes of neutrophils and monocytes over an observation period of two weeks. We assume that the appearance and the antimicrobial functions of immature neutrophils and intermediate monocytes may be decisive for the development of secondary infectious complications in severely injured patients. The gained findings may improve the therapeutic approach or even contribute to a prevention of developing life-threatening infections.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10184139/s1, Figure S1: Generation of reactive oxygen species and phagocytic capacity in isolated neutrophil subsets obtained from healthy volunteers, Figure S2: Generation of reactive oxygen species in isolated monocyte subsets obtained from healthy volunteers.

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Abbreviations

ARDS	acute respiratory distress syndrome
CD	cluster of differentiation
FACS	fluorescence-activated cell sorting
fMLP	N-formyl-methionyl-leucyl-phenylalanine
IL	interleukin
ISS	injury severity score
MOF	multi organ failure
PBS	phosphate-buffered saline
PBMCs	peripheral blood mononuclear cells
PMA	phorbol 12-myristate 13-acetate
ROS	reactive oxygen species

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2.2 Endogenous Uteroglobin as Intrinsic Anti-inflammatory Signal Modulates Monocyte and Macrophage Subsets Distribution Upon Sepsis Induced Lung Injury

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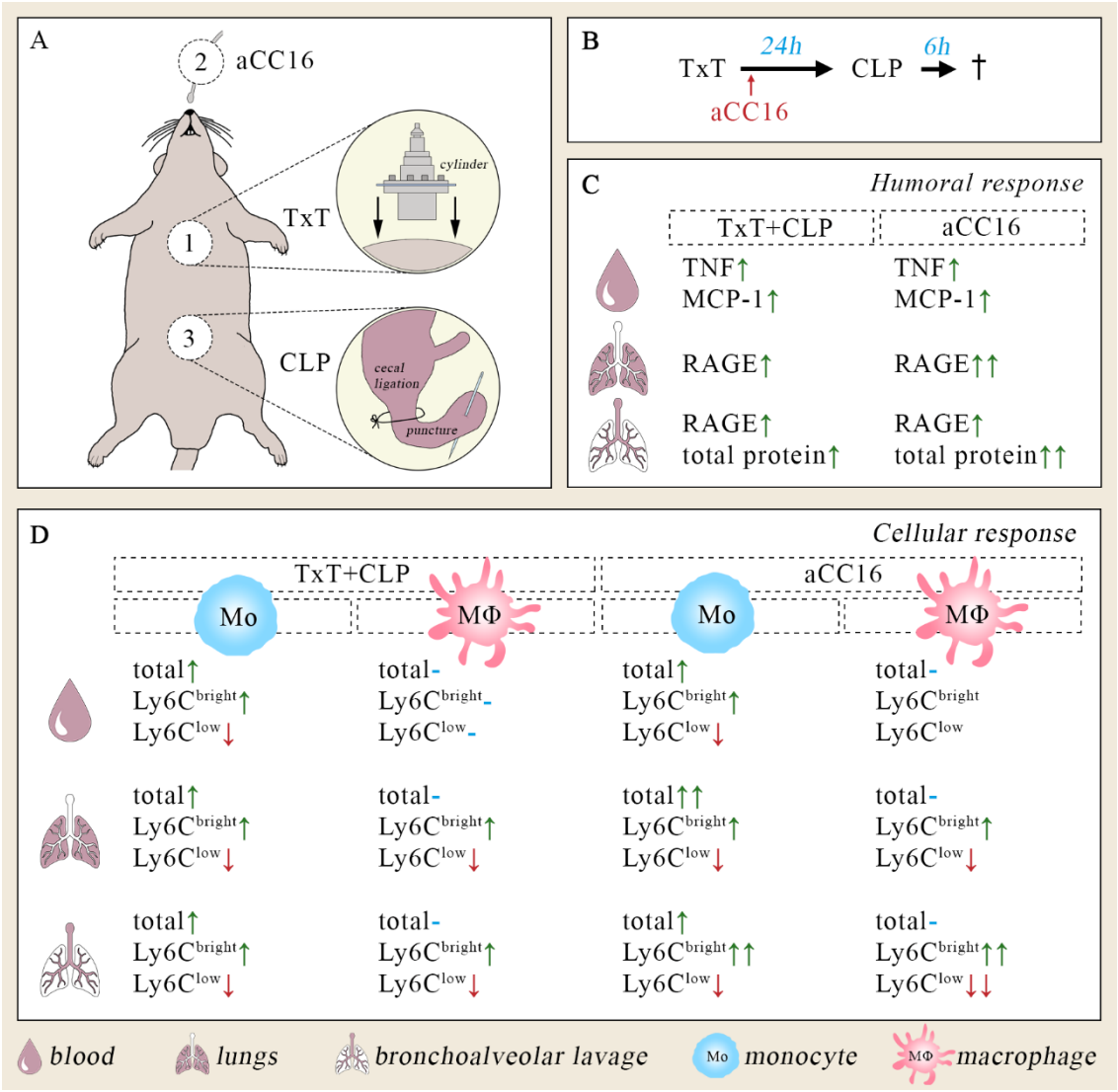

Figure 5 | Impact of thoracic injury with cecal ligation and puncture and pulmonary CC16 neutralization on humoral and cellular immune response. Experimental design of blunt thoracic injury (TxT) with cecal ligation and puncture (CLP) and local club cell protein 16 (CC16) neutralization by intratracheal application of CC16-neutralizing antibody (aCC16) is shown (A, B). Level of tumor necrosis factor (TNF), monocyte chemoattractant protein-1 (MCP-1), receptor for advanced glycation end-product (RAGE), and total protein was analyzed in blood, lungs, and bronchoalveolar lavage (C). The distribution of total, pro-inflammatory Ly6C^{bright}, and anti-inflammatory Ly6C^{low} monocytes and macrophages was evaluated in blood, lungs, and bronchoalveolar lavage. - no change, ↑/↓ increasing/decreasing tendency, and ↑↑/↓↓ significant increase/decrease compared to sham operated animals are shown.

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Endogenous Uteroglobin as Intrinsic Anti-inflammatory Signal Modulates Monocyte and Macrophage Subsets Distribution Upon Sepsis Induced Lung Injury

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Sepsis is a serious clinical condition which can cause life-threatening organ dysfunction, and has limited therapeutic options. The paradigm of limiting excessive inflammation and promoting anti-inflammatory responses is a simplified concept. Yet, the absence of intrinsic anti-inflammatory signaling at the early stage of an infection can lead to an exaggerated activation of immune cells, including monocytes and macrophages. There is emerging evidence that endogenous molecules control those mechanisms. Here we aimed to identify and describe the dynamic changes in monocyte and macrophage subsets and lung damage in CL57BL/6N mice undergoing blunt chest trauma with subsequent cecal ligation and puncture. We showed that early an increase in systemic and activated Ly6C$^+$CD11b$^+$CD45$^+$Ly6G$^-$ monocytes was paralleled by their increased emigration into lungs. The ratio of pro-inflammatory Ly6C^{high}CD11b$^+$CD45$^+$Ly6G$^-$ to patrolling Ly6C^{low}CD11b$^+$CD45$^+$Ly6G$^-$ monocytes significantly increased in blood, lungs and bronchoalveolar lavage fluid (BALF) suggesting an early transition to inflammatory phenotypes during early sepsis development. Similar to monocytes, the level of pro-inflammatory Ly6C^{high}CD45$^+$F4/80$^+$ macrophages increased in lungs and BALF, while tissue repairing Ly6C^{low}CD45$^+$F4/80$^+$ macrophages declined in BALF. Levels of inflammatory mediators TNF-α and MCP-1 in blood and RAGE in lungs and BALF were elevated, and besides their boosting of inflammation via the recruitment of cells, they may promote monocyte and macrophage polarization, respectively, toward the pro-inflammatory phenotype. Neutralization of uteroglobin increased pro-inflammatory cytokine levels, activation of inflammatory phenotypes and their recruitment to lungs; concurrent with increased pulmonary damage in septic mice. In *in vitro* experiments, the influence of uteroglobin on monocyte functions including migratory behavior, TGF-β1 expression, cytotoxicity and viability were proven. These results highlight an important role of endogenous uteroglobin as intrinsic anti-inflammatory signal upon sepsis-induced early lung injury, which modules the early monocyte/macrophages driven inflammation.

SHORT SUMMARY

Blunt chest injury is the third largest cause of death following major trauma, and ongoing excessive pro-inflammatory immune response entails high risk for the development of secondary complications, such as sepsis, with limited therapeutic options. In murine double hit trauma consisting of thoracic trauma and subsequent cecal ligation and puncture, we investigated the cytokine profile, pulmonary epithelial integrity and phenotypic shift of patrolling $Ly6C^{low}CD11b^{+}CD45^{+}Ly6G^{-}$ monocytes and $Ly6C^{low}CD45^{+}F4/80^{+}$ macrophages to pro-inflammatory $Ly6C^{high}CD11b^{+}CD45^{+}Ly6G^{-}$ monocytes and $Ly6C^{high}CD45^{+}F4/80^{+}$ cells in blood, lungs and bronchoalveolar lavage fluid (BALF). Pro-inflammatory mediators and phenotypes were elevated and uteroglobin neutralization led to further increase. Enhanced total protein levels in BALF suggests leakage of respiratory epithelium. *In vitro*, uteroglobin inhibited the migratory capacity of monocytes and the TGF-β1 expression without affecting the viability. These results highlight an important role of endogenous uteroglobin as an intrinsic anti-inflammatory signal upon sepsis-induced early lung injury, which modulates the early monocyte/macrophages driven inflammation.

Keywords: uteroglobin, CC16, chest injury, acute lung injury, CLP, sepsis, monocytes, macrophages

INTRODUCTION

Trauma is one of leading causes of death worldwide (1). Twenty to twenty-five percent of patients with multiple injuries suffer from severe lung contusion, whereas thoracic trauma represents the third most frequent cause of death after major trauma (2, 3). Thoracic injury contributes significantly to the development of acute respiratory distress syndrome (ARDS) (4), while infectious complications such as e.g., sepsis constitute a serious risk factors for up to 50% of mortalities occurring upon ARDS (5). Thus, the development of secondary complications is still a major contributing factor in trauma-associated mortality (6–8).

In general, traumatic injury-related tissue damage induces a release of endogenous damage-associated molecular patterns to initiate the resolution of non-pathogenic and pathogenic inflammation with subsequent tissue repair (9). This systemic inflammatory response syndrome triggers an excessive release of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α (9), interleukin (IL)-6 (9) and chemokines, such as monocyte chemoattractant protein (MCP)-1 (10, 11), which is a potent factor for monocyte and macrophage migration and infiltration (12).

Monocytes play a pivotal role in pathogen recognition and killing, and although their functional and phenotypic alterations provide a good base for prediction of complications after trauma or of poor prognosis in septic patients, exact pathophysiologic mechanisms and solid biomarkers still remain to be elucidated (13–17). Murine monocytes expressing high levels of lymphocyte antigen (Ly)6C have pro-inflammatory and anti-microbial features and have been shown to be precursors for patrolling monocytes, which survey the vasculature and contribute to the early response of inflammation and tissue repair, and which are characterized by a low expression of Ly6C (18, 19). Next to monocytes, resident alveolar macrophages initiate the inflammatory cascade and secrete pro-inflammatory mediators during acute lung injury (ALI) (20–22). While the pro-inflammatory M1 macrophages release e.g., nitric oxide, TNF-α, interferon-γ and IL-12 and critically contribute to pathogen clearance, their apoptosis during the process of pathogen clearance simultaneously contributes to downregulation of the pro-inflammatory phase and transition of M1 to tissue-repairing M2 macrophages (23). With regards to specific roles of monocytes/macrophages during lung injury and/or sepsis, both beneficial or detrimental effects of each cell type have been reported. While an early depletion of circulating monocytes before lipopolysaccharide (LPS) administration deteriorated lung injury (24), later monocyte depletion ameliorated lung injury (25, 26). Furthermore, macrophage polarization into M1 phenotype improved organ dysfunction and reduced mortality in lethal sepsis (27), while an intratracheal administration of M2 macrophages after CLP reduced mortality (28). These results indicate that an early balance of the pro-inflammatory and the anti-inflammatory response is required for ameliorating lung injury.

Uteroglobin (club cell protein (CC)16), is a 15.8 kDa protein secreted primarily by non-ciliated club cells along the tracheobronchial epithelium, especially in distal respiratory and terminal bronchioles (29, 30). Next to its biomarker character to indicate the development of secondary pulmonary complications after trauma, CC16 exerts anti-inflammatory and immunosuppressive properties (31–33). Its anti-inflammatory

Abbreviations: Ab, antibody; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; CC, club cell protein; CLP, cecal ligation and puncture; HV, healthy volunteers; ISS, injury severity score; LPS, lipopolysaccharide; Ly, lymphocyte antigen; MCP, monocyte chemoattractant protein; MPP, matrix metalloproteinase; RAGE, receptor for advanced glycation endproducts; TNF, tumor necrosis factor; TP, trauma patient; TxT, thoracic trauma.

biology has been confirmed in tracheal epithelial cells, isolated human mononuclear cells and murine macrophages (34–36). Due to this, CC16 has been described as being protective in the development of chronic obstructive pulmonary disease in human (37) and mouse (33).

Considering that functional and phenotypic alterations of monocytes/macrophages play an important role in sepsis development and due to the potent anti-inflammatory biology of CC16, we hypothesize that an early upregulation of the pro-inflammatory response by local CC16 neutralization will deteriorate the dynamic changes in monocyte and macrophage subsets and early lung damage in a murine trauma model of sepsis after blunt chest trauma.

MATERIALS AND METHODS

Ethics

The *in vitro* study was performed in the University Hospital Frankfurt, Goethe-University, Germany, with the institutional ethical committee approval (312/10) in accordance with the Declaration of Helsinki and following STROBE-guidelines (38). In this experimental trial, twenty severely injured trauma patients (TP) with a history of acute blunt or penetrating trauma with an injury severity score (ISS) of $\geq$ 16 were enrolled, along with and 8 healthy volunteers. All individuals who were <18 or >80 years of age, suffering from a severe burn injury, acute myocardial infarction, cancer or chemotherapy, HIV, infectious hepatitis, acute CMV infection and/or thromboembolic events, or receiving immunosuppressive drug therapy were excluded. The ISS was calculated according to the abbreviated injury scale (39) upon arrival to the emergency department. The signed written informed consent form was obtained from all patients or their legally authorized representatives, as well as from all included healthy volunteers (HV).

Animal experiments were conducted at the Zentrale Forschungseinrichtung of the University Hospital Frankfurt in accordance with the German Federal Law in regard of protection of animals with the approval of the responsible government authority, the Veterinary Department of the Regional Council in Darmstadt, Germany (Regierungspräsidium Darmstadt, Hessen, Germany; AZ: FK 1068). All experiments were performed in accordance with the ARRIVE Guidelines (40).

Animals and Experimental Model

Forty male CL57BL/6N mice (25 ± 5 g, 6–8 weeks old) were included (Janvier Labs, France) (41). Before and after experimental procedures, all animals had access to water and food *ad libidum*. Blunt chest trauma was performed under general mask anesthesia as described before (41). Briefly, the animals were placed in a supine position and a blunt bilateral thoracic trauma (TxT) was induced by a standardized pressure wave provided directly to the chest (41). After 24 h, a median laparotomy with moderate cecal ligation and puncture (CLP) followed as described before (41). Eight animals underwent only TxT. Twenty-four animals underwent the double hit consisting of TxT and CLP. Eight animals in the sham control group underwent anesthesia without performing any surgical procedures. After 6 h, euthanasia was done to facilitate sampling.

Group Allocation Based on the Administration of CC16 Neutralizing Antibody

Animals were randomly assigned to different experimental groups for local antibody (Ab) application to the lungs. Administration of Uteroglobin/SCGB1A1 (CC16 Ab, 10 µg/mL, LS Biosciences) or IgG Control (IgG) Antibody (10 µg/mL, R&D Systems) was performed immediately after the induction of thoracic trauma. For this procedure, mice were placed in a supine position and the tongue was thoroughly kept aside. A buttoned cannula was placed at the beginning of the trachea and 50 µL of the Ab solution were carefully administered. Then, mice were kept in a reverse trendelenburg position for 30 s to ensure the Ab distribution inside the lungs.

Sampling and Quantification of Protein Expression Levels in Lungs, Plasma, and BALF

The *vena cava* was punctured by a heparinized syringe for blood withdrawal at 6 h after CLP. After centrifugation at 1,164 g for 15 min at 4°C, the plasma was stored at –80°C for the subsequent measurements of pro-inflammatory mediators. MCP-1 and TNF-α were measured in plasma with the CBA Mouse Inflammation Kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, 50 µL of the Capture Beads were added into polystyrene FACS tubes (BD Pharmingen™) to 50 µL of plasma. To each FACS tube, 50 µL of the Mouse Inflammation PE Detection Reagent were added and incubated at room temperature in the dark for 2 h. Subsequently, samples were washed with 1 mL of Wash Buffer and centrifuged at 200 g for 5 min. Supernatant was discarded and pellet resuspended in 300 µL of Wash buffer. Analysis was performed using a BD FACS Canto 2™ and FCAP Array™Software (BD).

After blood withdrawal, the trachea was punctured, intubated and the lungs were flushed with 1.2 mL phosphate buffered saline (PBS) to gain the bronchoalveolar lavage fluid (BALF) for analysis. BALF samples were centrifuged at 1,164 g at 4°C for 5 min and the supernatant was used for the detection of the receptor for advanced glycation endproducts (RAGE DuoSet® ELISA Kit; R&D Systems, Minneapollis, US). Quickly, a microplate (Sarstedt, Nümbrecht, Germany) was coated with 100 µL Capture Antibody overnight at room temperature. Following a washing step, 300 µL of Reagent Diluent was added for 1 h to block the microplate. After another washing step, samples were loaded and incubated for 2 h at room temperature. Subsequently, the microplate was washed again and incubated with 100 µL Detection Antibody for 2 h. Microplate was washed again and incubated with 100 µL Streptavidin-HRP solution for 20 min in the dark at room temperature. Following the last washing step, 100 µL Substrate Solution was added to the wells and incubated in the dark at room temperature until a color reaction occurred. Subsequently, the reaction was stopped by adding 50 µL Stop Solution. The optical density was measured with the Infinite M200 microplate reader (Tecan, Männedorf, Switzerland, 450 nm absorbance, 570 nm reference wavelength; software Magellan).

The cell pellets from BALF were resuspended in 100 µL PBS supplemented with 0.5% bovine serum albumin (FACS buffer), and 40 µL were transferred into polystyrene FACS tubes (BD Pharmingen™) for subsequent cell staining as described below.

Then the animals were perfused with 20 mL PBS via the caudal *vena cava*, and, subsequently, the lungs were removed. One lung lobe was snap-frozen using liquid nitrogen for later protein isolation, and the other one was used for flow cytometric analyses. For protein isolation, lung tissue was homogenized in protein lysis buffer at 4°C, followed by centrifugation for 30 min at 4°C at 20,000 g. Supernatants were stored at −80°C for later analysis. Protein concentrations of pulmonary RAGE were determined using a mouse RAGE DuoSet® ELISA Kit (R&D Systems) as described above.

Analysis of Monocyte and Macrophage Subsets by Flow Cytometry

Lung tissue was processed as described in the Minute Single Cell Isolation protocol (Invent Biotechnologies, Minnesota, US). Briefly, 25 mg of fresh lung tissue were placed into a filter cartridge where 100 µL ice-cold Buffer A were subsequently added. The tissue was grinded with a plastic rod for 50–60 times. After adding further 400 µL Buffer A, sample was mixed by inverting the closed filter cartridge and centrifuged at 1,200 g and 4°C for 5 min. The pellet was resuspended and centrifuged again at 400 g and 4°C for 5 min. Subsequently, supernatant was discarded and pellet was resuspended in 100 µL FACS buffer. Forty microliter were transferred into each polystyrene FACS tubes (BD Pharmingen™) and stained for flow cytometry analysis as described below.

Thirty microliters of whole blood was transferred into each polystyrene FACS tubes (BD Pharmingen™) and stained for flow cytometry analysis as described below.

The cell pellets from BALF were resuspended in 100 µL of FACS buffer, and 40 µL was transferred into polystyrene FACS tubes (BD Pharmingen™).

Then, the samples were incubated with Pacific Blue-conjugated anti-mouse Ly-6G/Ly6C antibody (Ab) (Clone RB6-8C5; BioLegend, San Diego, California, US), APC/Fire 750 conjugated anti-mouse CD45 Ab (Clone 30-F11; BioLegend), Alexa Fluor 647-conjugated anti-mouse CD11b Ab (Clone M1/70; BioLegend), Brilliant Violett 510-conjugated anti-mouse F4/80 Ab (Clone BM8; BioLegend), and Phycoerythrin-Cyanine7-conjugated anti-mouse Ly6C Ab (Clone RB6-8C5; BioLegend). Control stainings with the corresponding isotype antibodies were applied for the settings. After 30 min on ice, 5 µL of 7-AAD (BD Biosciences, Franklin Lakes, USA) were added, and samples were incubated for further 15 min. Then, the samples were washed with 2 mL FACS buffer [7 min at room temperature (RT) and 423 g]. Supernatants were removed and cell pellets were homogenized in 1 mL of BD FACS Lysing Solution for an additional 10 min (RT). Then, samples were centrifuged at 400 g for 7 min and washed twice with 2 mL of FACS buffer. After removal of supernatants, cells were diluted in 80 µL FACS buffer and stored on ice until measurement. Each cell population was defined by gating the corresponding forward and side scatter scan as well as the viable cells by applying 7-AAD for gating. From each sample a minimum of 3.0×10^4 cells was measured, which were subsequently analyzed. The percentage of $Ly6C^+$ out of $CD11b^+Ly6G^-CD45^+$ and $F4/80^+CD45^+$ viable cells was assessed by flow cytometric analyses using a BD FACS Canto 2™ and FACS DIVA™ software (BD). The gating is shown in **Figure 2**.

Quantification of Uteroglobin in Sera From Healthy Volunteers and Trauma Patients

Collected sera from healthy volunteers and trauma patients were analyzed using human Uteroglobin Quantikine ELISA Kit (R&D Systems, Minneapolis, US) according to the manufacturer's instructions. Briefly, 100 µL of Assay Diluent were added to each well with subsequent addition of 50 µL of each sample and incubated at room temperature for 2 h. Then, each well was washed with 400 µL Wash Buffer. Subsequently, wells were incubated with 200 µl of Human Uteroglobin Conjugate for 2 h. After the next washing step, 200 µL of Substrate Solution were added into the wells for 30 min. The reaction was stopped by addition of 50 µl of Stop Solution to each well. The optical density was measured with the Infinite M200 microplate reader (Tecan, Männedorf, Switzerland, 450 nm absorbance, 570 nm reference wavelength; software Magellan).

Isolation of $CD14^+$ Monocytes

Isolation of peripheral blood mononuclear cells was performed by a density-gradient centrifugation (Bicoll separating solution, Biochrom, Berlin, Germany) according to manufacturer's instructions. Briefly, 25 mL of Bicoll separating solution (density: 1.077 g/mL) was carefully overlaid with an equal volume of heparinized whole blood from HV and centrifuged at 800 g for 30 min. Interphase containing peripheral blood mononuclear cells was transferred to another tube and washed with PBS w/o Ca^{2+} and Mg^{2+} (Invitrogen, Carlsbad, California, US). The remaining red blood cells were lysed by lysis buffer (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM EDTA) and washed with MACS buffer (0.5% BSA, 2 mM EDTA). For CD14 labeling, cell pellet was resuspended in 75 µL of MACS buffer and incubated with 25 µL magnetic CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min. After washing, $CD14^+$ monocytes were isolated by magnetic isolation with LS columns (Miltenyi Biotec) according to the manufacturer's protocol. Cell number and cell viability were determined by Türk's solution exclusion assay (Merck, Darmstadt, Germany). Only cell cultures with a purity of ≥95% were used for further experiments. The cells were cultured in RPMI 1640 medium (Seromed, Berlin, Germany), supplemented with 10% heat-inactivated fetal calf serum (Gibco, Karlsruhe, Germany), 100 IU/mL penicillin (Gibco), 10 µg/mL streptomycin (Gibco) and 20 mM HEPES buffer (Sigma) at 37°C and 5% CO_2.

Monocyte Treatment

Ex vivo, $CD14^+$ monocytes isolated from HV were treated with sera from HV and TP that were obtained at the admission to the emergency department. Prior to the experiment, the sera were incubated with or without anti-CC16-antibody (1 µg/mL;

R&D Systems) for CC16 neutralization or corresponding isotype control antibody (1 μg/mL; R&D Systems), respectively, for 1 h at 37°C and 5% CO_2, slightly slewing every 15 min. For monocyte stimulation, cell culture media was supplemented with 20% sera for 2 h at 37°C and 5% CO_2 and used for further analysis.

Migration Assay

Alterations in migratory capacity were determined by CytoSelect™ Cell Migration Assay (3 μm pores; Cell Biolabs, San Diego, US). 100,000 cells were plated in the upper chamber and treated as described in the Monocyte treatment section. MCP-1 (10 ng/mL; R&D Systems) was added to the lower chamber. After 3 h at 37°C and 5% CO_2, the upper chamber was removed and cells in the lower chamber were lysed and quantified using CyQuant® GR Fluorescent Dye (Cell Biolabs, San Diego, US) according to the manufacturer's instructions. Briefly, the cells containing supernatant from the feeder tray was transferred into a black-walled, clear bottom microplate. CyQuant® GR Dye was diluted 1:75 in 4x Lysis Buffer and subsequently added to each well to reach a 1x concentration. Samples were incubated at RT for 20 min. Fluorescence intensity was measured by Twinkle LB 970 Microplate Fluorometer (490 nm excitation/520 nm emission; software MikroWin 2000).

Measurement of TGF-β1 Expression in Monocytes by Flow Cytometry

100,000 cells per polystyrene FACS tube (BD Pharmingen™) were treated according to the Monocyte treatment section with slight change. After 1 h of treatment with sera, Brefeldin A (Invitrogen) was added to each tube to 1x concentration and monocytes were incubated for further 2 h at 37°C and 5% CO_2. Subsequently, monocytes were incubated with Phycoerythrin-conjugated anti-human CD14 antibody (2 μL; Clone M5E2; BioLegend) and fixable yellow dead cell stain (2 μL; Invitrogen, Carlsbad, California, US). After 30 min at RT, cells were washed with FACS buffer and centrifuged at 400 g for 5 min. Supernatant was removed and monocytes were fixed with Fix and Perm Medium A at room temperature for 15 min. After further washing step, cells were permeabilized with Fix and Perm Medium B (both Invitrogen, Carlsbad, California, US) and incubated with PerCP/Cyanine5.5-conjugated anti-human TGF-β1 antibody (2 μL; Clone TW4-2F8; BioLegend) at room temperature for 30 min. Following the washing step, monocytes were resuspended in 50 μL FACS buffer and analyzed using BD FACS Canto 2™ and FACD DIVA™ software (BD). Monocytes were gated by the corresponding forward and side scatter scan and as shown in **Figures 5A,B**. The percentage of TGF-β1 expression of viable $CD14^+$ monocytes was analyzed.

Quantification of IL-6 and TNF-α Levels in Monocyte Supernatants

100,000 cells were seeded in flat-bottom 96-well plate (Sarstedt) and treated as described in the Monocyte treatment section. The supernatants were collected to detect the IL-6 (Diaclone, Besançon cedex, France) or TNF-α (R&D Systems, Minneapollis, US) levels using ELISA kits according to the provider's instructions. For a brief IL-6 protocol, see equivalent RAGE measurement protocol in the Sampling and quantification of protein expression levels in lungs, plasma and BALF section. The protocol for TNF-α is equivalent to the Uteroglobin Quantikine ELISA Kit described in the section Quantification of uteroglobin in sera from healthy volunteers and trauma patients.

Cell Viability Assay

100,000 cells per well were plated in a clear bottom, black-walled 96-well plate (BD Biosciences) and left to adhere for 30 min at 37°C and 5% CO_2. Subsequently, monocytes were treated as described in the Monocyte treatment section. For cell viability measurement, Calcein AM reagent (1 μg/mL; Cayman Chemical, Michigan, US) was added to the cells and incubated at 37°C and 5% CO_2 for 30 min. Fluorescence intensity was measured by Twinkle LB 970 Microplate Fluorometer (490 nm excitation/520 nm emission; software MikroWin 2000).

LDH Assay

100,000 cells per well were plated in a flat bottom 96-well plate (Sarstedt) and let to adhere at 37°C and 5% CO_2 for 30 min. Subsequently, media was replaced with phenol-free RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 100 IU/mL penicillin, 10 μg/mL streptomycin and 20 mM HEPES buffer and monocytes were treated as described in Monocyte treatment section.

For cell cytotoxicity detection, 100 μL of monocyte supernatant was transferred to a fresh 96-well plate and incubated with LDH reaction mixture (Cytotoxicity Detection Kit, Roche, Mannheim, Germany) according to the manufacturer's instructions in dark at RT for 30 min. Absorbance was measured by Infinite M200 microplate reader (490 nm absorbance, 600 nm reference wavelength; software Magellan).

Statistical Analysis

GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA) was used to perform the statistical analyses. Normality of all data was analyzed by the D'Agostino-Pearson normality test. Differences between the groups were determined by non-parametric Kruskal-Wallis test which does not assume a normal distribution of the residuals followed by Dunn's *post hoc* test for the correction of multiple comparisons. A p-value below 0.05 was considered significant. Data are given as box-whisker plot and min to max.

RESULTS

Pro-inflammatory Mediators and Lung Damage Significantly Increase in the Early Phase of Sepsis

TxT in mice increased TNF-α and MCP-1 levels in plasma, whereby the TxT+CLP group showed a significant increase compared to the control group ($p < 0.05$, data not shown). RAGE protein levels were significantly higher in TxT+CLP compared to control, both in BALF and lungs ($p < 0.05$, data not shown). Following TxT and TxT+CLP, the total protein content in bronchoalveolar lavage which is associated with the extent of

lung damage significantly increased compared to control ($p < 0.05$, data not shown).

The Ratio of Inflammatory and Patrolling Monocytes Increases in Blood, Lungs, and BALF in the Early Phase of Sepsis

For detailed examination of monocyte and macrophage subset distributions, the constituent phenotypes were characterized by their specific surface protein markers. In blood, TxT alone slightly increased levels of activated $Ly6C^+CD11b^+Ly6G^-CD45^+$ monocytes, whereas TxT+CLP resulted in a significant elevation compared to control ($p < 0.05$, data not shown). Regarding the subset distribution, inflammatory $Ly6C^{hi}CD11b^+Ly6G^-CD45^+$ monocyte subset expanded significantly in TxT+CLP animals ($p < 0.05$, data not shown), and, in parallel, patrolling $Ly6C^{lo}CD11b^+Ly6G^-CD45^+$ monocytes showed an equivalent decrease vs. control ($p < 0.05$, data not shown). Thus, a significant increase in the ratio between inflammatory and patrolling monocytes was observed in early phase of sepsis ($p < 0.05$, data not shown).

Similarly, activated $Ly6C^+CD11b^+Ly6G^-CD45^+$ monocytes were markedly more abundant in lungs following TxT+CLP compared to control lungs ($p < 0.05$, data not shown). Whereas a significant increase of the inflammatory $Ly6C^{hi}CD11b^+Ly6G^-CD45^+$ phenotype was observed in TxT+CLP group compared to control group ($p < 0.05$, data not shown), the number of patrolling $Ly6C^{lo}CD11b^+Ly6G^-CD45^+$ monocytes was equally reduced ($p < 0.05$, data not shown). Therefore, comparable to systemic monocytes, the ratio of inflammatory to patrolling monocytes increased markedly in TxT+CLP vs. control ($p < 0.05$, data not shown).

Furthermore, significantly higher emigration rates of activated $Ly6C^+CD11b^+Ly6G^-CD45^+$ monocytes to BALF were found in TxT and TxT+CLP compared to control ($p < 0.05$, data not shown). Regarding the inflammatory phenotypes in BALF, the inflammatory $Ly6C^{hi}CD11b^+Ly6G^-CD45^+$ monocyte subset increased in TxT and TxT+CLP compared to control ($p < 0.05$, data not shown), whereas the patrolling $Ly6C^{lo}CD11b^+Ly6G^-CD45^+$ monocyte population was reduced ($p < 0.05$, data not shown). Concurrent with this data, an increased ratio between pro-inflammatory and patrolling monocytes was found in both, TxT and TxT+CLP groups, vs. control group ($p < 0.05$, data not shown).

The Number of Pro-inflammatory Macrophages Increases in Lungs and BALF

No significant systemic changes in $Ly6C^+F4/80^+CD45^+$ cell counts were found after TxT and TxT+CLP compared to control (data not shown). The pro-inflammatory $Ly6C^{hi}F4/80^+CD45^+$ phenotype slightly increased in TxT with further expansion in TxT+CLP vs. control but both without significance (data not shown). A decline of patrolling $Ly6C^{lo}F4/80^+CD45^+$ phenotype was observed in TxT and TxT+CLP vs. control (data not shown).

Whereas, no differences in total macrophage counts were shown in the lungs of TxT and TxT+CLP, a significant increase of pro-inflammatory $Ly6C^{hi}F4/80^+CD45^+$ macrophages compared to control was detected ($p < 0.05$, data not shown). The cell number of tissue repairing $Ly6C^{lo}F4/80^+CD45^+$ macrophages in lungs did not change after TxT and TxT+CLP vs. control (data not shown).

Following TxT, mice displayed a slight increase of $Ly6C^+F4/80^+CD45^+$ macrophage counts in BALF, whereas TxT with subsequent CLP did not markedly affect the cell numbers compared to control (data not shown). The inflammatory $Ly6C^{hi}F4/80^+CD45^+$ phenotype expanded significantly in both, TxT and TxT+CLP group in comparison to control group ($p < 0.05$), while a significant decline of tissue repairing $Ly6C^{lo}F4/80^+CD45^+$ macrophages was observed in BALF of TxT and TxT+CLP compared to control ($p < 0.05$, data not shown).

CC16 Neutralization Is Associated With an Increase of Inflammatory Markers and Lung Damage

To investigate the impact of CC16 on inflammatory changes and lung injury, CC16 was neutralized (CC16 Ab) in mice undergoing TxT and subsequent CLP. TxT+CLP induced a significant systemic increase of pro-inflammatory TNF-α and MCP-1 levels compared to control ($p < 0.05$) with a trend to a further increase in animals that underwent CC16 neutralization (**Figures 1A,B**). Whereas, protein concentrations of RAGE in both lungs (C) and BALF (D) were significantly increased after TxT+CLP vs. control, CC16 neutralization significantly increased RAGE in the lungs after TxT+CLP ($p < 0.05$, **Figures 1C,D**). With regards to lung tissue damage, total protein content, that was measured in the BALF, and was significantly increased after TxT+CLP vs. control, with a further significant increase in the TxT+CLP group after CC16 neutralization vs. reference TxT+CLP group ($p < 0.05$, **Figure 1E**).

CC16 Modulates Phenotypic Distribution of Monocytes and Macrophages

The effect of CC16 on the subset distribution of monocytes and macrophages was analyzed. The representative gating for the data analyses is shown in **Figure 2**. Total counts of activated $Ly6C^+CD11b^+Ly6G^-CD45^+$ monocytes in blood increased significantly after TxT+CLP compared to control, while CC16 neutralization did not show any significant impact on this increase compared with the TxT+CLP group ($p < 0.05$, **Figure 3A**). Similarly, although inflammatory $Ly6C^{hi}CD11b^+Ly6G^-CD45^+$ monocytes became significantly more abundant, and the patrolling $Ly6C^{lo}CD11b^+Ly6G^-CD45^+$ monocytes displayed a significant decline in TxT+CLP animals vs. control, CC16 neutralization did not affect this subset distribution ($p < 0.05$, **Figures 3B,C**). The ratio of pro-inflammatory to patrolling monocytes was significantly increased in both TxT+CLP groups vs. control ($p < 0.05$, **Figure 3D**).

TxT+CLP induced a significantly increased migration of $Ly6C^+CD11b^+Ly6G^-CD45^+$ monocytes to the lungs ($p < 0.05$, **Figure 3E**). CC16 neutralization markedly reinforced this effect, and significantly enhanced the presence of

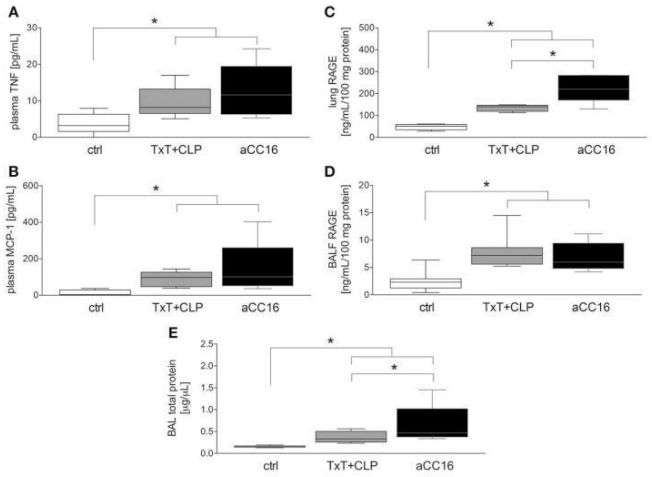

FIGURE 1 | Impact of CC16 neutralization on expression levels of pro-inflammatory mediators **(A–D)** and the total pulmonary protein amount **(E)** following thoracic trauma (TxT) with cecal ligation and puncture (CLP). Plasma levels of TNF **(A)** and MCP-1 **(B)**, and RAGE protein levels in lungs **(C)** and BALF **(D)** were measured. Total protein amount was determined in BALF. Data are represented as box-whisker plot and min to max, $^*p < 0.05$ vs. control.

$Ly6C^+CD11b^+Ly6G^-CD45^+$ monocytes in the lungs after TxT+CLP compared to the TxT+CLP reference group ($p < 0.05$, **Figure 3E**). However, compared to control, the significant increase of pro-inflammatory $Ly6C^{hi}CD11b^+Ly6G^-CD45^+$ and a respective decrease of patrolling $Ly6C^{lo}CD11b^+Ly6G^-CD45^+$ monocytes were not significantly modulated by CC16 neutralization after TxT+CLP ($p < 0.05$, **Figures 3F,G**). Thus, the ratio between $Ly6C^{hi}CD11b^+Ly6G^-CD45^+$ to $Ly6C^{lo}CD11b^+Ly6G^-CD45^+$ monocytes was significantly increased in both TxT+CLP and the TxT+CLP group undergoing CC16 neutralization compared to the control (**Figure 3H**).

In BALF, a significant expansion of activated $Ly6C^+CD11b^+Ly6G^-CD45^+$ monocytes after TxT+CLP was detected ($p < 0.05$, **Figure 3I**). CC16 neutralization after TxT+CLP did not change this increase compared to control ($p < 0.05$, **Figure 3I**). The pro-inflammatory $Ly6C^{hi}CD11b^+Ly6G^-CD45^+$ phenotype was significantly more abundant in TxT+CLP vs. control ($p < 0.05$, **Figure 3J**), while CC16 neutralization further enhanced the migration of inflammatory monocytes into the BALF showing a significant increase compared with the TxT+CLP group ($p < 0.05$, **Figure 3J**). The counts of patrolling $Ly6C^{lo}CD11b^+Ly6G^-CD45^+$ monocytes significantly declined in TxT+CLP vs. control, while CC16 neutralization did not significantly further impact this monocyte subset decrease after TxT+CLP ($p < 0.05$, **Figure 3K**). TxT+CLP induced a significant increase on the ratio of pro-inflammatory to patrolling monocytes compared to control ($p < 0.05$, **Figure 3L**), while a further significant increase after CC16 neutralization vs. TxT+CLP reference group was detected ($p < 0.05$, **Figure 3L**).

Systemic $Ly6C^+F4/80^+CD45^+$ cells were not markedly changed after TxT+CLP or intervention with aCC16 (**Figure 4A**). Neither the pro-inflammatory $Ly6C^{hi}F4/80^+CD45^+$ phenotype (**Figure 4B**) nor the patrolling $Ly6C^{lo}F4/80^+CD45^+$ phenotype (**Figure 4C**) were changed.

With regards to local influence of CC16 after TxT+CLP, in lungs, $Ly6C^+F4/80^+CD45^+$ macrophage levels remained stable in TxT+CLP compared to control (**Figure 4D**). However, the inflammatory macrophage subset expanded significantly after TxT+CLP vs. control ($p < 0.05$, **Figure 4E**), whereas the anti-inflammatory phenotype decreased significantly after TxT+CLP ($p < 0.05$, **Figure 4F**). CC16 neutralization did not have a significant impact on macrophage subset redistribution after TxT+CLP in lungs (**Figures 4D–F**).

Total macrophage counts in BALF did not change after TxT+CLP, neither did CC16 neutralization change their levels (**Figure 4G**). However, pro-inflammatory $Ly6C^{hi}F4/80^+CD45^+$ macrophages elevated significantly, while patrolling $Ly6C^{lo}F4/80^+CD45^+$ macrophages declined significantly in TxT+CLP or TxT+CLP with CC16 neutralization compared to control ($p < 0.05$, **Figures 4H,I**). CC16 neutralization significantly increased the percentage of pro-inflammatory macrophages and reduced significantly the percentage of patrolling macrophages after TxT+CLP compared to the TxT+CLP reference group ($p < 0.05$, **Figures 4H,I**).

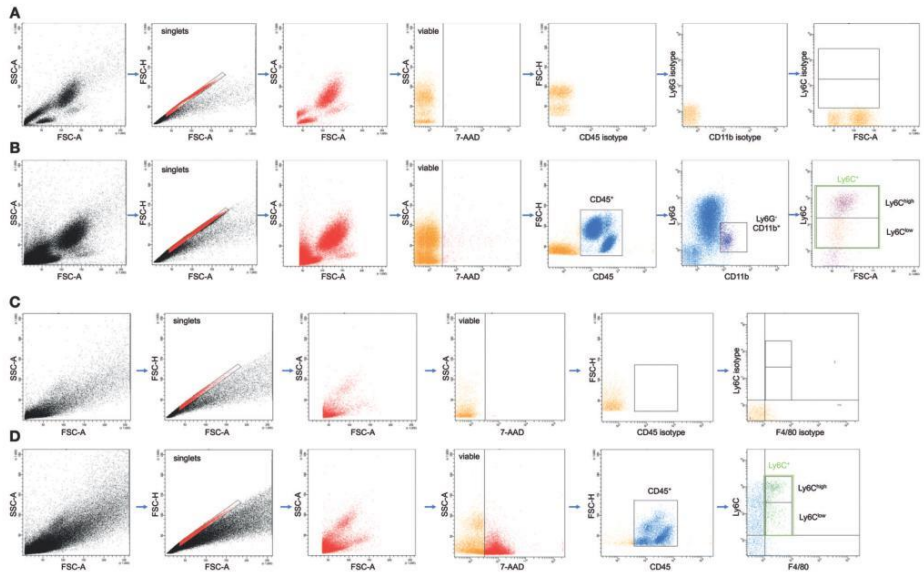

FIGURE 2 | Representative gating strategy for the flow cytometric analyses and evaluation of different monocyte subsets in whole blood **(A,B)** and macrophage subsets in lungs **(C,D)** as dot plot analyses is shown. **(A,C)** are showing the gating upon staining with isotype control antibodies, while **(B,D)** show results from the staining with specific antibodies as described in the material and method section.

CC16 Inhibits Migratory Capacity and TGF-β1 Expression in CD14$^+$ Monocytes *ex vivo*

To examine the impact of CC16 on monocytes under septic conditions, systemic monocytes were isolated from healthy volunteers and subsequently stimulated with sera from HV or TP (with as well as without septic complications), since the last are known to contain higher levels of CC16 compared to control or trauma patients without complications (42). We have determined CC16 concentrations in samples of trauma patients and healthy volunteers. We found that CC16 was significantly increased in sera obtained from traumatized patients compared to those obtained from healthy volunteers (28.09 ± 4.60 vs. 15.18 ± 1.25 ng/mL, $p < 0.05$; data not shown). Regarding the migratory rate, stimulation of CD14$^+$ monocytes with sera from HV did not show any changes compared to control and the neutralization of CC16 or application of isotype antibody in these sera did not affect the migration either (**Figure 5C**). Although the migration of CD14$^+$ monocytes treated with sera from TP remained unchanged vs. control and HV, CC16 neutralization resulted in significantly higher migration rates toward MCP-1 compared to control, stimulation with sera alone or with IgG ($p < 0.05$, **Figure 5C**). Intracellular TGF-β1 expression showed no significant changes following treatment with sera from HV vs. control (**Figure 5D**). Stimulation of monocytes with sera from TP significantly declined TGF-β1 expression, and administration of CC16 neutralizing antibodies recovered TGF-β1 level to the baseline ($p < 0.05$, **Figure 5D**). Furthermore, TNF-α and IL-6 levels in the supernatants obtained from human monocytes that were treated with sera were determined. Stimulation of CD14$^+$ monocytes with sera obtained from HV and TP did not induce any significant impact on TNF-α nor IL-6 levels (data not shown). TNF-α concentration of the control was 30.06 ± 4.60 pg/mL. Following treatment with sera from HV, TNF-α level was comparable at 32.42 ± 6.90 pg/mL, whereas CC16 neutralization in those sera did not lead to a significant decrease (24.70 ± 5.306 pg/mL). Supernatants from cells that were stimulated with TP sera have shown comparable concentrations of TNF-α to those obtained after incubation with TP sera upon CC16 neutralization (22.01 ± 3.20 vs. 30.18 ± 3.66 pg/mL). Control IL-6 concentration was 46.96 ± 12.31 pg/mL. Treatment with sera from HV did not change the IL-6 level, which was 50.13 ± 19.57 pg/mL and which also stayed stable after CC16 neutralization (40.48 ± 13.14 pg/mL). Comparable results were found in supernatants from TP samples (40.97 ± 6.436 pg/mL) and the corresponding CC16 neutralized sample also (45.84 ± 6.66 pg/mL). Further, we examined the cytotoxic potential of CC16 analyzing the release of LDH. Here, the stimulation

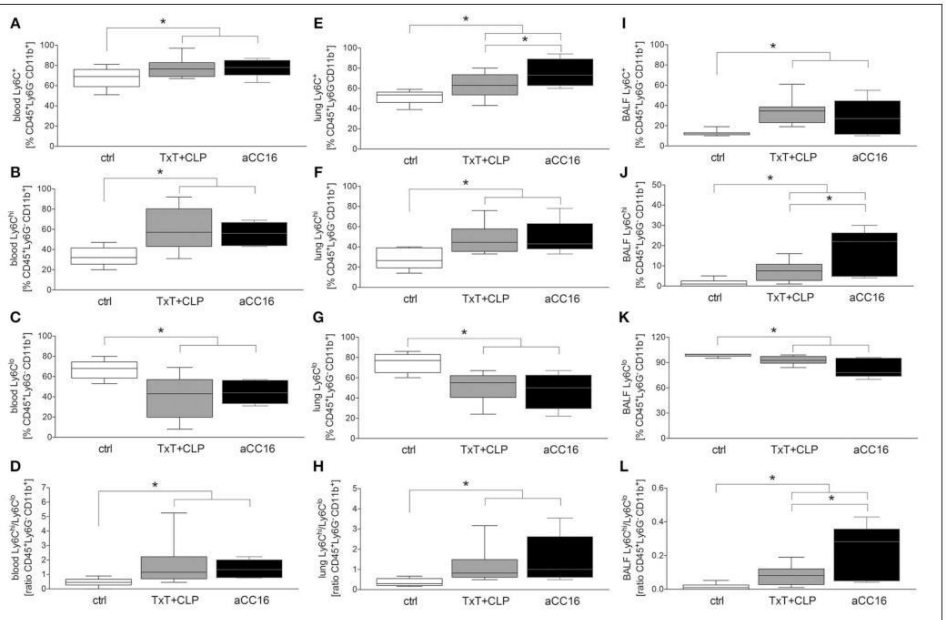

FIGURE 3 | Impact of CC16 neutralization on distribution of monocyte subsets following thoracic trauma (TxT) with cecal ligation and puncture (CLP). Activated monocyte count (Ly6C$^+$) as well the pro-inflammatory (Ly6C^{hi}) and patrolling (Ly6C^{lo}) subsets and the ratio between them were assessed in blood **(A–D)**, lungs **(E–H)** and BALF **(I–L)** by flow cytometry. Data are represented as box-whisker plot and min to max, $^*p < 0.05$ vs. control.

with neither sera from HV nor TP changed the LDH release compared to untreated control (**Figure 5E**). Finally, treatment with both sera and sera with neutralized CC16 or IgG did not show significant changes in the viability of CD14$^+$ monocytes (**Figure 5F**).

DISCUSSION

Since blunt chest injury with ongoing excessive pro-inflammatory immune response entails high risk for the development of secondary complications with limited therapeutic options, the unveiling of underlying mechanisms is necessary (43, 44). Here, we discuss the dynamic changes in monocyte and macrophage subsets and uncover the potentially protective role of the anti-inflammatory endogenous CC16 in the very early phase of sepsis development following thoracic injury. We confirmed the anti-inflammatory potential of CC16 in the early phase of sepsis-induced ALI following blunt chest injury. Its local neutralization after thoracic trauma increased the immigration of pro-inflammatory cell phenotypes to the lungs, and was accompanied by increased total protein levels in BALF, indicating the loss of epithelial lung integrity, and thus lung damage. Concurrently, systemic elevation of humoral pro-inflammatory mediators was observed. This is in line with our recent study, where early increased lung infiltration with neutrophils and lung injury in this model was shown (45). However, in that study, 24 h post-CLP, the lung injury was ameliorated and the lungs have exhibited no further increase in neutrophilic infiltration after CC16 neutralization (45). Thus, CC16 may first reduce a necessary early pro-inflammatory immune response for tissue repair, and at a later time point, may contribute to the amelioration of the lung injury. Although the mechanism is still not clear, the observed lung injury could be caused by the paralleled enhanced lung infiltration with neutrophils. This assumption is supported by Lerman et al. where neutrophil extravasation and tissue infiltration in murine CLP-induced sepsis were inhibited by blocking or deletion of α3β1 and paralleled by significantly reduced mortality (46).

Following infectious or non-infectious stimuli, alveolar macrophages are, among other cells, the first to be involved in the early immune response, initiating the inflammatory cascade and secretion of pro-inflammatory mediators (20–23). We have shown that thoracic injury followed by CLP increased systemic levels of TNF-α; which is an important indicator of sepsis development (47). Neutralization of endogenous CC16 forced further increase of TNF-α, indicating anti-inflammatory capacity

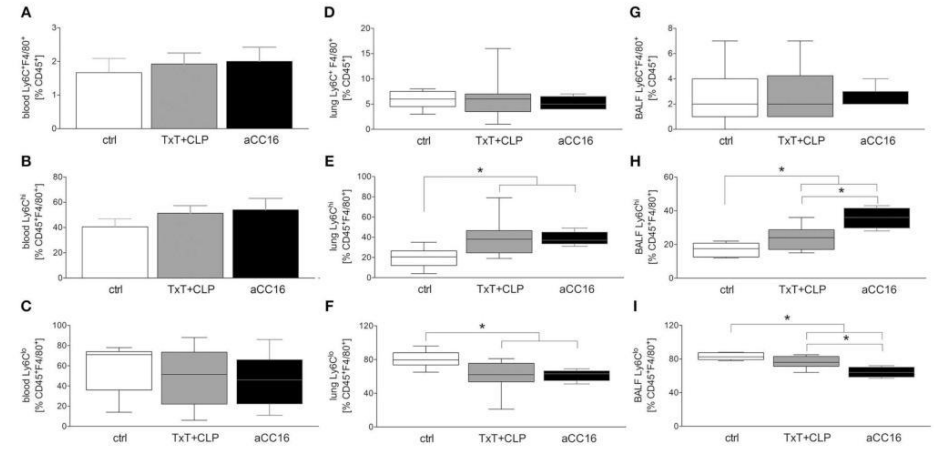

FIGURE 4 | Impact of CC16 neutralization on distribution of macrophage subsets following thoracic trauma (TxT) with cecal ligation and puncture (CLP). Amount of macrophages ($Ly6C^+F4/80^+$) as well as of the pro-inflammatory ($Ly6C^{hi}$) and patrolling ($Ly6C^{lo}$) subsets was assessed in blood **(A–C)**, lungs **(D–F)** and BALF **(G–I)** by flow cytometry. Data are represented as box-whisker plot and min to max, $^*p < 0.05$ vs. control.

of CC16 in the present model, and confirming in general its anti-inflammatory character. In turn, elevated TNF-α level is also one of the factors inducing MCP-1 expression by a variety of cell types (48). Consistent with literature, we have shown that TNF-α increase after thoracic trauma and CLP was paralleled by a systemic MCP-1 increase. CC16 neutralization led similarly to TNF-α to a further systemic increase of MCP-1. Whether this further MPC-1 increase is caused directly by CC16 or indirectly by an increase of TNF-α or other factors still remains to be elucidated in future studies.

Further, we have shown that expression of RAGE significantly increased in lungs and BALF of septic mice, whereas CC16 neutralization led to further significant increase in the lungs. In a clinical study, both CC16 and RAGE were identified as plausible biomarkers for ARDS in patients with severe sepsis (49), but whether RAGE positively or negatively regulates the immune response seems to differ according to the inflammatory mechanism (50, 51). Moreover, we have shown that blunt chest injury itself increased the protein levels in BALF and subsequent CLP did not cause a further increase after 6 h. This does not mean that sepsis did not have an impact on lung epithelial integrity, but lungs are at this time point affected by thorax trauma directly and abdominal-induced sepsis may take longer to affect the lung epithelial integrity than could be seen in the observation period. Upon CC16 neutralization, total protein level in BALF further increased, suggesting a positive impact of CC16 in lung epithelial injury.

Following sepsis-induced ALI after blunt chest trauma, a significant increase of monocyte counts was observed in blood and lungs, as well as in the BALF. The characterization of monocytes has uncovered significantly more pro-inflammatory monocytes compared to a marked decline of the patrolling phenotype. Since MCP-1 is the pivotal regulator of monocyte recruitment to the site of injury (12), the observed elevated MCP-1 levels in septic mice may be the key factor for the excessive infiltration of lungs with pro-inflammatory monocytes, whereby the disrupted lung epithelial integrity may contribute to the higher monocyte content in BALF as well. Upon CC16 neutralization, increased monocyte emigration to the lungs was observed, indicating the anti-migratory potential of CC16. To substantiate our assumption, we isolated monocytes from healthy volunteers and stimulated them with sera obtained from healthy volunteers or from trauma patients, since we have shown in previous studies that increased systemic concentrations of CC16 correlate with the development of secondary respiratory complications following traumatic injury in patients and is lowered in healthy individuals (31, 42, 52). CC16 neutralization in patient's sera before monocyte stimulation led to a significant increase of monocyte chemotaxis toward MCP-1 but the mechanism still remains to be elucidated. However, although CC16 was neutralized, monocytes from TP+aCC16 display elevated migration compared to controls. Serum from trauma patients contains other mediators beside CC16, which may change the migratory behavior of monocytes, thus further mediators such as IL-6 or RANTES that potentially increase the migratory capacity of monocytes are probably concurrently present in the blood from trauma patients (53–56). In a further *ex vivo* experiment, we have shown unaffected viability of isolated monocytes and their release of lactate dehydrogenase following treatment with sera, suggesting no cytotoxic effects of CC16.

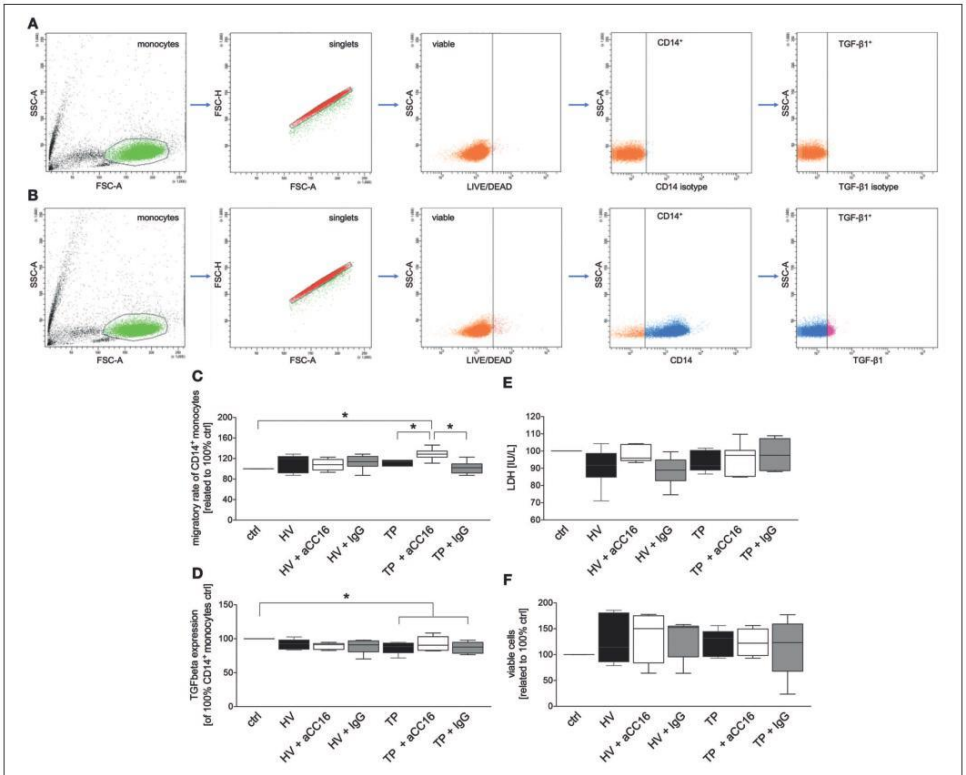

FIGURE 5 | Representative gating strategy for the flow cytometric analysis and evaluation of isolated CD14$^+$ monocytes with regard to their TGF-β1 expression as dot plot analysis is shown. **(A)** is showing representative isotype staining controls, while **(B)** shows representative gating after staining with specific antibodies. **(C–F)** show effects of serum samples obtained from healthy volunteers (HV) or trauma patients (TP) and CC16 on CD14$^+$ monocytes behavior. CD14$^+$ monocytes isolated from HV were incubated with sera obtained from HV or TP. Cell culture medium was applied alone (control) or with/without prior intervention/neutralization with either CC16 antibody (aCC16) or IgG control antibody (IgG) in sera from HV or TP. Migratory capacity toward MCP-1 **(C)**, TGF-β1 expression **(D)**, and the impact of sera on monocyte cytotoxicity **(E)** and viability **(F)** were evaluated. Data are represented as box-whisker plot and min to max, *$p < 0.05$ vs. indicated.

Interestingly, stimulation of isolated human monocytes with sera obtained from trauma patients led to a significant decrease of TGF-β1 expression, whereas neutralization of CC16 recovered the levels to the baseline. Although this was unexpected, it was already reported in rodent models of lung fibrosis that CC16 contributes to diminished TGF-β1 levels, however, the mechanism still remains elusive (37, 57). Here, some studies indicate that CC16 suppresses TGF-β1 expression via MPP-9 inhibition (34, 58).

Regarding macrophage distribution, we observed elevated levels of pro-inflammatory macrophages paralleled by decline of anti-inflammatory phenotype in lungs and BALF. CC16 neutralization reinforced the observed changes, whereas lung infiltration remained unaffected. Since resident alveolar macrophages have been described to have anti-inflammatory properties in steady state and, upon infection or injury, they display a phenotypic shift and gain pro-inflammatory features (18), we hypothesized that CC16 neutralization may contribute to macrophage polarization toward the pro-inflammatory phenotype. Whether CC16 in fact suppresses an exaggerated transition to pro-inflammatory macrophages remains to be further elucidated by future studies.

LIMITATIONS

Showing an isolated CLP group with performed interventions would further increase the relevance of our results. However,

since the scope of the present study was to elaborate the role of CC16 in the underlying double-hit model, this approach was not considered. In humans, it is well-known that secondary stimuli (e.g., surgeries and infections) following chest trauma contribute to the development of secondary pulmonary complications, including ALI and ARDS (44). In mice, it was already shown that pulmonary contusion primes the systemic innate immune response to the LPS challenge, increasing inflammation and worsening lung injury compared to injury or LPS application alone (59). Moreover, recently we have shown that isolated blunt chest trauma in mice was not enough to mimic human conditions since the ongoing pro-inflammatory response decreased to baseline within 24 h, and that combining blunt chest trauma with CLP led to pulmonary changes that were characteristic for ALI (41). However, the mechanism is still unknown, and it remains to be elucidated whether either direct local tissue injury and the subsequent pro-inflammatory response, or the second hit with excessive pro-inflammatory response and remote organ damage contribute more to the ALI development (41, 60). However, literature indicates that the combination of both hits contributes to the increased pro-inflammatory response following double-hit trauma (61–64). Thus, the question of whether CC16 neutralization would affect the isolated CLP animals in the same way as in the TxT+CLP group remains unanswered. Furthermore, showing CC16 levels in all experimental groups at the timing of therapy would support our findings. Yet, due to ethical reasons with regard to animal protection, such analyses were not possible. Following the principle of 3Rs (Replacement, Reduction, Refinement) the number of animals per each group was limited to 8. Following severe thoracic injury, human trauma patients mostly require mechanical ventilation, whereas mice were spontaneously breathing in our experimental settings. Thus, the impact of mechanical ventilation following chest injury could not be considered. Moreover, although mouse models are key tools for studying different pathophysiologies, the immune response between mouse and human differs and the applied treatment cannot be directly translated into human settings. We could not show the impact of CLP on lung integrity, and we consider the short observation period as a further limitation that could lead to negative results. Similarly, monocytes and recruited and resident alveolar macrophages seem to have specific functions in a time-dependent manner. Thus, the right time frame for the examination of monocyte and macrophage function is essential and has to be examined further. In flow cytometric analysis, the chosen markers did not distinguish between recruited and resident alveolar macrophages and this has to be examined in future studies also. Furthermore, although CC16 neutralization increased pro-inflammatory monocyte and macrophages phenotypes, whether CC16 directly contributes to exaggerated transition to pro-inflammatory macrophages still remains to be elucidated. A longer observation period of up to 7 days would bring clarity to the beneficial or negative effects of CC16. Additionally, the distribution of neutrophil and monocyte/macrophage subsets should be evaluated as well. Comparing this with the extent of the lung injury would clarify whether CC16 has either a negative or positive effect on outcomes. It would be interesting to know whether in case of positive effects, CC16 would improve only the lung injury and pro-inflammatory immune response or whether the survival would be improved as well. To confirm the above-discussed potential results, recombinant CC16 therapy should be applied as well. In *in vitro* studies, we pooled sera from only ten trauma patients without secondary complications and 10 trauma patients who developed sepsis in a later course, and thus, a larger sample size may clarify the results. Although CC16 is known to have anti-inflammatory properties, we have shown recovered TGF-β1 protein expression levels in $CD14^+$ monocytes following treatment with a trauma patient's sera. It is possible that TGF-β1 expression is inhibited indirectly by another mechanism and this has to be evaluated in further studies as well.

DATA AVAILABILITY STATEMENT

All relevant datasets for this study are contained in the manuscript.

ETHICS STATEMENT

This studies involving human participants were reviewed and approved by Institutional Ethical Committee of the University Hospital Frankfurt, Goethe-University, Germany. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Veterinary Department of the Regional Council in Darmstadt, Germany (Regierungspräsidium Darmstadt, Hessen.

AUTHOR CONTRIBUTIONS

BR: conceptualization, supervision, and project administration. AJ, PS, NB, BX, and BR: methodology. AJ: validation, data curation, and writing—original draft preparation. BR and AJ: formal analysis. AJ, PS, NB, and BX: investigation. BR, SW, FH, and IM: resources. AJ, JV, SE, and BR: writing—review and editing. AJ and BR: visualization. BR, FH, and SW: funding acquisition.

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2.3 Acute alcohol intoxication modulates monocyte subsets and their functions in a time-dependent manner in healthy volunteers

Janicova A., F. Haag, B. Xu, A.P. Garza, I.R. Dunay, C. Neunaber, A.J. Nowak, P. Cavalli, I. Marzi, R. Sturm, B. Relja

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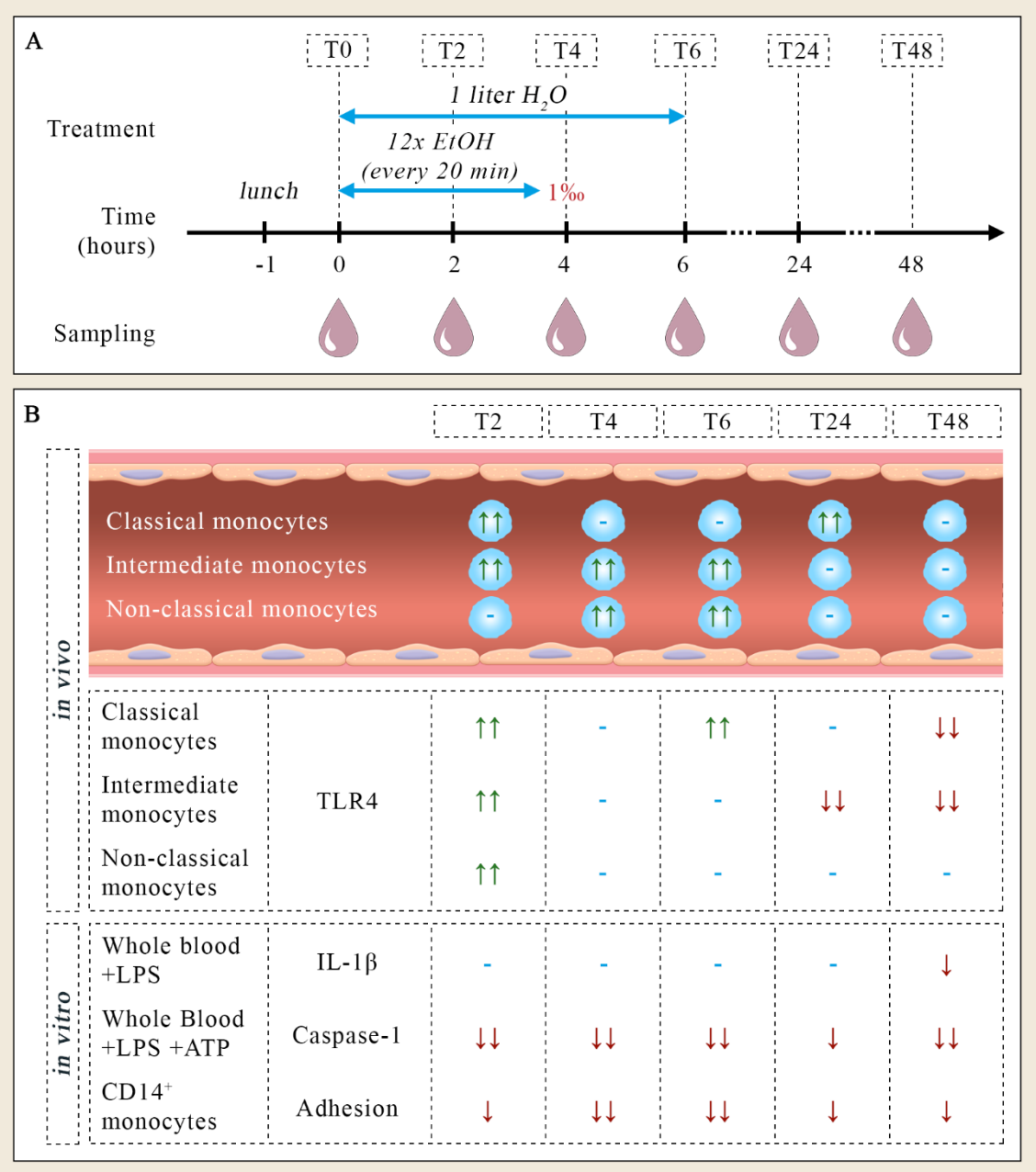

Figure 6 | Impact of alcohol intoxication on monocyte subsets and their functionality. Experimental design is shown (A). Redistribution of monocyte subsets and their Toll-like 4 (TLR4) expression, caspase-1 activation, and adhesive capacity were evaluated as well as interleukin-1β (IL-1β) release following whole blood stimulation. - no change, ↓ decreasing tendency, and ↑↑/↓↓ significant increase/decrease compared to non-alcohol intoxicated subjects are shown. Figure adapted from [46]. ATP, adenosine triphosphate; CD, cluster of differentiation; EtOH, ethanol/alcohol; H_2O, water; LPS, lipopolysaccharide.

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Acute Alcohol Intoxication Modulates Monocyte Subsets and Their Functions in a Time-Dependent Manner in Healthy Volunteers

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Background: Excessive alcohol intake is associated with adverse immune response-related effects, however, acute and chronic abuse differently modulate monocyte activation. In this study, we have evaluated the phenotypic and functional changes of monocytes in acutely intoxicated healthy volunteers (HV).

Methods: Twenty-two HV consumed individually adjusted amounts of alcoholic beverages until reaching a blood alcohol level of 1‰ after 4h (T4). Peripheral blood was withdrawn before and 2h (T2), 4h (T4), 6h (T6), 24h (T24), and 48h (T48) after starting the experiment and stained for CD14, CD16 and TLR4. $CD14^{bright}CD16^{-}$, $CD14^{bright}CD16^{+}$ and $CD14^{dim}CD16^{+}$ monocyte subsets and their TLR4 expression were analyzed by flow cytometry. Inflammasome activation *via* caspase-1 in $CD14^{+}$ monocytes was measured upon an *ex vivo in vitro* LPS stimulation. Systemic IL-1β and adhesion capacity of isolated $CD14^{+}$ monocytes upon LPS stimulation were evaluated.

Results: The percentage of $CD14^{+}$ monocyte did not change following alcohol intoxication, whereas $CD14^{bright}CD16^{-}$ monocyte subset significantly increased at T2 and T24, $CD14^{bright}CD16^{+}$ at T2, T4 and T6 and $CD14^{dim}CD16^{+}$ at T4 and T6. The relative fraction of TLR4 expressing $CD14^{+}$ monocytes as well as the density of TLR4 surface presentation increased at T2 and decreased at T48 significantly. $TLR4^{+}CD14^{+}$ monocytes were significantly enhanced in all subsets at T2. TLR4 expression significantly decreased in $CD14^{bright}CD16^{-}$ at T48, in $CD14^{bright}CD16^{+}$ at T24 and T48, increased in $CD14^{dim}CD16^{+}$ at T2. IL-1β release upon LPS stimulation decreased at T48, correlating with TLR4 receptor expression. Alcohol downregulated inflammasome activation following *ex vivo in vitro* stimulation with LPS between T2 and T48 *vs*. T0. The adhesion capacity of $CD14^{+}$ monocytes decreased from T2 with significance at T4, T6 and T48. Following LPS administration, a significant reduction of adhesion was observed at T4 and T6.

Conclusions: Alcohol intoxication immediately redistributes monocyte subsets toward the pro-inflammatory phenotype with their subsequent differentiation into the anti-inflammatory phenotype. This is paralleled by a significant functional depression, suggesting an alcohol-induced time-dependent hyporesponsiveness of monocytes to pathogenic triggers.

Keywords: ethanol, drinking, innate immunity, inflammasome, LPS, IL-1β, CD14, TLR4

INTRODUCTION

Alcohol is one of the oldest and nowadays one of the most common addictive substances worldwide (1). Although low-to-moderate alcohol intake is associated with decreased risk of cardiovascular diseases and subsequent mortality (2), alcohol abuse contributes to the development of alcoholic liver disease, pancreatitis and further pathologies (1). In Germany, approximately 7.8 million adults drink excessive amounts of alcohol (3), causing 74,000 deaths every year (4). However, these numbers do not include physical trauma-related deaths that frequently occur under the influence of alcohol (4). Since up to 50% of physically traumatized patients are admitted to the emergency departments with an acute alcohol intoxication (5, 6), the numbers of alcohol-related deaths may be underestimated.

There is a rising evidence that alcohol intake has a biphasic effect on the innate immune response in a dose- and time-dependent manner (7–10). Chronic alcohol intake is associated with increased susceptibility to infections and sterile inflammation, which may cause severe tissue damage and poor outcomes after injury (1, 11). Following binge drinking, defined as an episodic excessive alcohol intake and the most common form of alcohol abuse, the counts of circulating leukocytes, monocytes and natural killer cells as well as the release of tumor necrosis factor (TNF)-α after an *ex vivo in vitro* whole blood stimulation with lipopolysaccharide (LPS) increase in the first 20 minutes after drinking, suggesting an early pro-inflammatory response. Subsequent decline of monocytes, natural killer cells and interleukin (IL)-1β, IL-6 and monocyte chemoattractant protein (MCP)-1 levels in circulation and the elevation of systemic IL-10 level suggest an anti-inflammatory state in the later course (7, 9, 12). In line with this data, leukocytes and IL-6 decrease in circulation was observed in severely injured patients with excessive alcohol intoxication (10). However, lower alcohol concentrations do not seem to induce the pro-inflammatory acute phase proteins (9). Those pro-inflammatory mediators are essential for the cell recruitment of the innate immune system and the initiation of the adaptive immune response (8), why acutely intoxicated patients with alcohol are potentially more vulnerable to infections.

Human monocytes exposed to moderate amount of alcohol *in vivo* or *in vitro* express significantly lower levels of pro-inflammatory cytokines in Toll-like receptor (TLR)4-MyD88 and TLR-TRIF-dependent manner when stimulated with LPS *in vitro* (13). Additionally, activation of the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) is downregulated p65/p50 dependently (14). The above described studies deal with monocytes as a single population, however, three monocyte subsets can be distinguished according to their expression levels of cluster of differentiation (CD)14 and CD16: Classical monocytes ($CD14^{bright}CD16^{-}$) are released into circulation from bone marrow and act as a precursor for intermediate monocytes ($CD14^{bright}CD16^{+}$), which in turn differentiate into non-classical monocytes ($CD14^{dim}CD16^{+}$) (15). Classical subset has anti-microbial and innate immune sensing features, contributing to phagocytosis, adhesion and migration (15). Once they enter tissues, classical monocytes differentiate into monocyte-derived macrophages or dendritic cells, where they are crucial for shaping and subsequent resolution of inflammation (15). Intermediate monocytes regulate apoptosis and transendothelial migration (15), and their high occurrence in patients with systemic infections suggests a defensive role against invading pathogens (16). Non-classical monocytes provide complement- and FcR-mediated phagocytosis and are linked with anti-viral response to human immunodeficiency virus (15). In chronic excessive alcohol abuse, the numbers of classical monocytes are significantly reduced, whereas the non-classical monocytes display an increase in their counts, while the changes particularly restore two weeks following alcohol withdrawal (17). Although accumulating evidence indicates a shift in monocyte subsets under several pathological conditions, their distribution and function in acutely intoxicated subjects with alcohol remain elusive.

Considering that phenotypic and functional alterations of monocytes are substantially involved in the initiation and subsequent resolution of inflammation, we evaluated the phenotypic shift of monocytes following episodic excessive alcohol intake in healthy volunteers (HV). We hypothesized that acute alcohol intoxication shifts the monocytes toward the anti-inflammatory phenotype and ameliorates LPS-induced pro-inflammatory response *ex vivo in vitro* by suppressing the inflammasome activation.

Abbreviations: BSA, bovine serum albumin; Bcl-3, B-cell lymphoma 3-encoded protein; CD, cluster of differentiation; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HV, healthy volunteers; IL, interleukin; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; NLRP3, NLR family pyrin domain containing 3; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; PBS, phosphate-buffered saline; TLR, Toll-like receptor; TNF, tumor necrosis factor.

MATERIALS AND METHODS

Ethics

The current study was performed pursuant to ethics committee approval (255/14) from the University Hospital of the Goethe-University Frankfurt and in accordance with the Declaration of

Helsinki and following the Strengthening the Reporting of Observational studies in Epidemiology-guidelines (18).

Patient Cohort

Twenty-two HV between 18 and 50 years of age were enrolled. Exclusion criteria included a history of chronic alcohol abuse, acute infection, pre-existing chronic inflammatory diseases, immunological disorders, human immunodeficiency virus infection, infectious hepatitis, medication and pregnancy. Renal insufficiency and changes in transaminases to identifying chronic liver diseases were excluded by blood test.

Study Design

Healthy volunteers received a standardized lunch (1 pizza) one hour before starting the experiment. Subsequently, HV consumed individually calculated amount of alcohol until reaching blood alcohol level of 1 ‰ after 4 hours. In detail, HV received whisky-coke cocktail (Tennessee Whiskey Jack Daniels, 40%; Coca-Cola) in a mixing ratio of 1:2 every 20 minutes for 4 hours. Then, the HV were monitored for further 2 hours without alcohol intake. For the individual calculation of the amount of alcohol to reach 1 ‰ after 4 hours, the modified Widmark equation including age, sex, height and weight was applied. Blood alcohol concentration (BAC) was monitored hourly until T6 and at T24 and T48. At every time point, a blood sample for the determination of the BAC was taken. Blood samples were obtained in prechilled ethylenediaminetetraacetic acid tubes (BD vacutainer; Becton Dickinson Diagnostics, Belgium) and kept on ice. Then, blood was centrifuged at 2,000 × g and at 4°C for 15 minutes and the supernatant was immediately used for the determination of BAC using the diagnostic set serum EtOH by Cobas 8000 Modular Analyzer (Roche Diagnostic, Germany). The participants leaved the research facility after T6 and came back at T24 and T48. During the time out of the institute, participants were allowed to eat and drink with restriction of drinking alcohol. A brief overview of the study design and sampling is shown in **Figure 1**.

Blood Sampling

Peripheral blood was withdrawn in serum-gel and Li-Heparin collection tubes (Sarstedt, Germany) before (T0) and 2 (T2), 4 (T4), 6 (T6), 24 (T24) and 48 (T48) hours after starting the alcohol consumption. Subsequently, the samples collected in serum-gel collection tubes were centrifuged directly after the collection at 2,000 × g and at 4°C for 15 minutes. Serum was stored at -80°C until further analysis. Blood in Li-Heparin collection tubes were directly processed for flow cytometry and adhesion assay.

Analysis of Monocyte Subsets and TLR4 Expression by Flow Cytometry

100 µl heparinized blood was transferred into polystyrene FACS tubes (BD Pharmingen™). The samples were incubated with 5 µl CD14 (APC/Cy7; Clone M5E2; BioLegend), 5 µl CD16 (PE/Cy7; Clone 3G8; BioLegend) and 5 µl TLR4 (APC; Clone HTA125; BioLegend) in the dark at 4°C for 10 minutes. Subsequently, the samples were washed with 2 ml FACS buffer (0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)) and centrifuged at 350 × g at 4°C for 5 minutes. For red blood cells lysis, 3 ml lysis buffer (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM ethylenediaminetetraacetic acid (EDTA)) were added and incubated in the dark at 4°C. Washing step was repeated. Monocyte population was defined by gating the corresponding forward and side scatter scan. From each sample a minimum of 3.0×10^4 cells were measured, which were subsequently analyzed. The percentage of $CD14^{bright}CD16^{-}$ (classical subset), $CD14^{bright}CD16^{+}$ (intermediate subset) and $CD14^{dim}CD16^{+}$ (non-classical subset) as well the percentage and mean fluorescent units of TLR4 out of $CD14^{+}$ viable cells were assessed by flow cytometric analyses using a BD FACS Canto 2™ and FACS DIVA™ software (BD Bioscience). Unspecific binding of the antibodies was excluded by using isotype controls.

Whole Blood Stimulation With LPS

50 µl heparinized blood was added to 450 µl RPMI 1640 media with supplements [100 UI/ml penicillin, 100 µg/ml streptomycin, 0.1 mg/ml

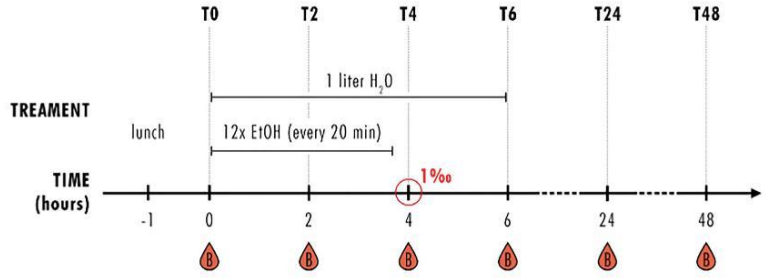

FIGURE 1 | Study design. Healthy volunteers (HV) received a standardized lunch one hour before starting the experiment and had one liter water at hand during the first observation period of 6 hours (T0-T6). Every 20 minutes, HV received an individually calculated amount of whisky-coke cocktail (EtOH) for four hours, reaching blood alcohol concentration of 1‰ after 4 hours (T4). Blood samples were collected before (T0) and 2 (T2), 4 (T4), 6 (T6), 24 (T24) and 48 (T48) hours after starting the alcohol consumption. This is the same study design as published by Sturm et al. (19).

gentamycin, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10% heat-inactivated fetal bovine serum (FBS)] and LPS at final concentration of 10 μg/ml (20). Subsequently, the samples were incubated at 37°C and 5% CO_2 for 24 hours. After stimulation, the samples were centrifuged at 2,100 × g and at room temperature for 10 minutes. Supernatants were stored at -80°C until the further analysis.

Measurement of IL-1β

For the detection of IL-1β levels in directly collected serum (see Blood Sampling) and the supernatants after whole blood stimulation with LPS (see Whole Blood Stimulation With LPS), ELISA DuoSet kit (#DY201, R&D, USA) was used according to the provider's instructions.

Caspase-1 Activity

Active caspase-1 was quantified by using a FAM-FLICA Caspase-1 (YVAD) Assay Kit (ImmunoChemistry, USA) (21). 50 μl blood and 240 μl RPMI 1640 media with supplements was transferred into polystyrene FACS tubes. Samples were incubated with 1 μg/ml LPS (*E. coli* O127:B8 strain; Sigma Aldrich, Germany), 100 μM BzATP (ATP; Sigma-Aldrich, Germany) (22) and 10 μl 30X FAM-FLICA caspase-1 inhibitor) at 37°C and 5% CO_2 for 90 minutes. Subsequently, cells were washed with RPMI 1640 media supplemented with 10% FBS 2 times. For red blood cell lysis, 3 ml lysis buffer (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM EDTA) were added and incubated in the dark at 4°C for 10 minutes. Cells were washed with 2 ml FACS buffer and centrifuged at 200 × g at 4°C for 5 minutes. Following pellet resuspension in 500 μl FACS buffer, caspase-1 expression was assessed by flow cytometric analyses using a BD FACS Canto 2™ and FACS DIVA™ software (BD).

Adhesion Assay

The isolation of $CD14^+$ monocyte we described already elsewhere (23). Peripheral blood monocytes were isolated by a density-gradient centrifugation (Bicoll separation solution, 1.077 g/ml density; Biochrom, Germany). Here, 20 ml Bicoll separating solution was carefully overlaid with 20 ml of heparinized blood and centrifuged at 800 × g at room temperature for 30 minutes. Peripheral blood mononuclear cells from interphase were transferred into fresh tubes and washed with 10 ml FACS buffer and centrifuged at 350 × g at room temperature for 5 minutes. Remaining red blood cells were lysed by 10 ml lysis buffer in dark for 10 minutes and subsequently centrifuged. Following a further washing step with 15 ml MACS buffer (0.5% BSA, 2 mM EDTA in 1x PBS w/o Mg^{2+} and Ca^{2+}), cells were resuspended in 500 μl RPMI 1640 media with supplements. Mononuclear cells were stimulated with 1 μg/ml LPS at 37°C and 5% CO_2 for 90 minutes and subsequently washed with MACS buffer.

For CD14 labeling, cell pellet was resuspended in 75 μl MACS buffer and the cell suspension was incubated with 25 μl magnetic CD14 microbeads (Miltenyi Biotec, Germany) at room temperature for 15 minutes. Following a washing step with 3 ml MACS buffer, $CD14^+$ labeled monocytes were magnetically isolated by using LS columns (Miltenyi Biotec, Germany) according to the manufacturer's protocol. Following a further washing step with 10 ml FACS buffer, cell pellet was resuspended in 500 μl of RPMI 1640 media with supplements. Only cell cultures with a purity and viability of ≥95% were used for further experiments. Cell population purity was evaluated by flow cytometry according its size and CD14 expression. Cell viability was proved by Türk's solution exclusion assay (Merck, Darmstadt, Germany).

The adhesion assay was performed as described before (24). 200,000 $CD14^+$ monocytes were seeded in 6-well flat-bottom plate (Sarstedt, Germany), where adherent A549 pulmonary epithelial cells to the density of 80% were seeded a day before, and, incubated at 37°C and 5% CO_2 for 35 minutes. Cells were washed twice with RPMI 1640 media with supplements and following fixation by 1 ml 1% glutaraldehyde for 3 minutes, monocytes were washed once more with PBS. Adherent monocytes on plates were stored in 2 ml PBS in the dark and at 4°C until evaluation. The adherent monocytes were then counted in 5 different fields of a defined size (5×0.25 mm^2) using a phase contrast microscope (×20 objective). The mean cellular adhesion rate was calculated.

Statistics

GraphPad Prism 6.0 software (GraphPad Software Inc. San Diego, CA, USA) was used to perform the statistical analysis. Data are given as mean ± standard error of the mean (SEM). The data distribution was tested by the D'Agostino-Pearson test, and the data is not normally distributed. Since we have 6 groups with matched or repeated measures, thus paired data, the non-parametric Friedman's test was applied. Dunn-Bonferroni post-hoc test for multiple comparisons was used. T0 is the base condition to which the others were compared. The Wilcoxon matched-pairs signed rank test as a nonparametric statistical tool comparing two paired groups in **Figure 6** was used to determine if two sets of pairs are different from one another in a statistically significant manner. Spearman's correlation coefficient was calculated to determine correlations. A p value below 0.05 was considered statistically significant.

RESULTS

Characteristics of the Study Participants

From 22 healthy volunteers, 12 female and ten male with a mean age of 25 (± 4) years were enrolled. All included individuals exerted normal ranges of transaminases excluding chronic e.g. liver diseases, since the rages at T0 were: 15-20 U/l GGT, 22 U/l GPT and 23 U/l GOT. Thus, no individuals with chronic liver diseases or malfunction were included. Furthermore, the BAC increased to 0.46±0.02‰ at T2, with further at T4 (1.11±0.05‰) reaching the target BAC of 1‰. At T6 BAC decreased to 0.83±0.06‰ and was not further detectable at T24 and T48.

Acute Alcohol Intake Modulates the Distribution the $CD14^+$ Monocyte Subsets

Since it is known that alcohol misuse has modulating effects on the immune system, we investigated the distribution of different

monocytes subsets in healthy volunteers following excessive acute alcohol intake. Fluorescently *ex vivo in vitro* labeled circulating monocytes were gated as it is shown in the **Figure 2**. Following alcohol intake, the percentage of $CD14^+$ monocytes did not change during the whole observational period of 48 hours compared to the counts before the experiment start (T0) (**Figure 3A**). Regarding the subsets, the classical monocytes defined as $CD14^{bright}CD16^-$ cells became significantly more abundant at T2 and T24 after starting drinking alcohol compared to T0 (**Figure 3B**, $p < 0.05$). The intermediate $CD14^{bright}CD16^+$ monocytes displayed a significant increase in the first 6 hours (T2, T4, T6) with subsequent decrease to the baseline at T24 and T48 (**Figure 3C**, $p < 0.05$). We observed an increase of non-classical $CD14^{dim}CD16^+$ subset at T4 and T6 hours compared to the counts at T0, followed by decrease from T24 (**Figure 3D**, $p < 0.05$).

Combining the absolute cell numbers of leukocytes per microliter (25) with the relative fractions of monocytes and their subsets, we converted the percentages of monocyte subsets into absolute cell numbers. These are summarized in **Supplementary Table 1**.

TLR4 Expression Is Upregulated in the Early Phase and Downregulated in the Later Phase of Acute Alcohol Intake

Following the determination of monocyte subsets, we analyzed the expression of surface receptor TLR4. The representative gating strategy of TLR4-positive monocytes is shown in the **Figure 2**. The abundance of TLR^+ $CD14^+$ monocytes significantly increased at T2 while decreasing to base level at T6 until T24 (**Figure 4A**, $p < 0.05$). At T48, TLR4 expression was significantly decreased compared to

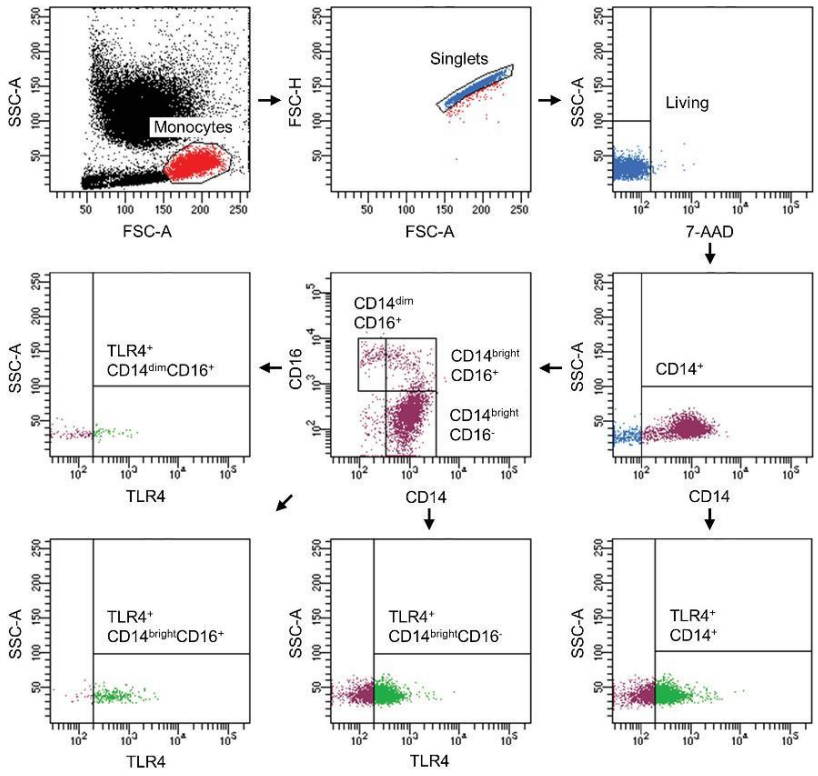

FIGURE 2 | Representative gating strategy for the flow cytometric analyses and evaluation of monocyte subsets with regard to their TLR4 expression.

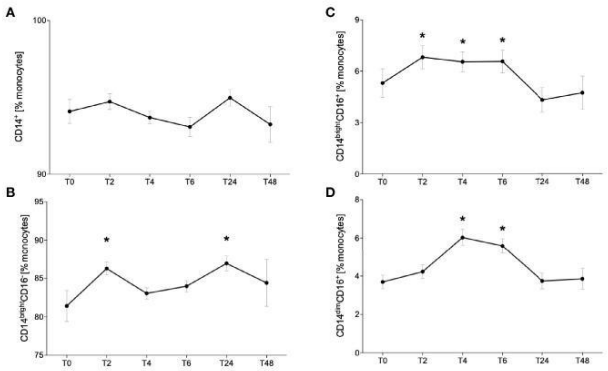

FIGURE 3 | Impact of acute alcohol intoxication on monocyte subset distribution. Percentage distribution of $CD14^+$ **(A)** classical ($CD14^{bright}CD16^-$) **(B)**, intermediate ($CD14^{bright}CD16^+$) **(C)** and non-classical ($CD14^{dim}CD16^+$) **(D)** was determined in blood of healthy volunteers (n=22) before (T0) and 2 (T2), 4 (T4), 6 (T6), 24 (T24) and 48 (T48) hours after starting the alcohol consumption. Data are presented as mean ± standard error of the mean. *$p < 0.05$ *vs*. T0.

T0 (**Figure 4A**, $p < 0.05$). The relative fraction of $TLR4^+$ $CD14^{bright}CD16^-$ classical monocytes increased significantly at T2 and T6 *vs*. T0 (**Figure 4B**, $p < 0.05$). $CD14^{bright}CD16^+$ intermediate and $CD14^{dim}CD16^+$ non-classical subsets showed a significant elevation of $TLR4^+$ monocytes at T2 compared to T0 (**Figures 4C, D**, $p < 0.05$).

Additionally, to the relative fraction of $TLR4^+$ monocytes, we evaluated the mean TLR4 expression on monocytes. $CD14^+$ monocytes displayed significantly higher expression at T2 and significantly reduced TLR4 receptor density at T48 compared to T0 (**Figure 4E**, $p < 0.05$). Rising trend was indicated at T2 and T6 in classical monocyte subset and the density of TLR4 decreased significantly at T24 *vs*. T0 (**Figure 4F**, $p < 0.05$). Intermediate monocytes showed increased TLR4 expression at T2 and a significant decline at T24 and T48 compared to T0 (**Figure 4G**, $p < 0.05$). The non-classical subset displayed significant elevation of TLR4 receptor intensity at T2 *vs*. T0 (**Figure 4H**, $p < 0.05$).

IL-1β Release Following *Ex Vivo In Vitro* Whole Blood Stimulation With LPS Is Diminished Two Days After Acute Alcohol Intake Correlating With TLR4 Receptor Density on Monocytes

Further, since it is known that ethanol inhibits the NLRP3 inflammasome activation, we evaluated the levels of circulating IL-1β and following *ex vivo in vitro* stimulation with LPS. IL-1β was not detectable in serum directly obtained from the participants at all time points (detection limit of 3.91 pg/mL; data not shown). Following LPS stimulation, we have shown that the acute ethanol intoxication did not have an impact on IL-1β concentration at T2, T4 and T6 compared to IL-1β concentration before starting binge drinking (T0) (**Figure 5A**). At T24, we observed a slight decrease that continued and became significant at T48 *vs*. T0 (**Figure 5A**, $p < 0.05$). This IL-1β level positively correlated with TLR4 receptor density on monocytes at T48 (**Figure 5B**).

Ethanol Intake Leads to Hyporesponsiveness to Secondary Hit

A brief overview about *ex vivo in vitro* experimental design and representative gating strategy is shown in **Figures 6A, B**. Level of active caspase-1 did not show significant changes in $CD14^+$ monocytes during the entire observation period of 48 hours compared to the level before alcohol intoxication (T0) (**Figure 6C**). The *ex vivo in vitro* stimulation of $CD14^+$ monocytes with 1 μg/mL LPS and 100 μM ATP led to significant increase of caspase-1 expression at all time points compared to equivalent unstimulated monocytes (**Figure 6C**, $p < 0.05$). At T2, T6 and T48, caspase-1 expression significantly decreased following LPS and ATP stimulation compared to stimulated monocytes at T0 (**Figure 6C**, $p < 0.05$).

Furthermore, we found that the adhesion capacity of $CD14^+$ monocytes is impaired by alcohol intake. *Ex vivo in vitro*, $CD14^+$ monocytes displayed less adhesion to A549 cells from T2 until T48 compared to T0, with significance at T4, T6 and T48 (**Figure 6D**, $p < 0.05$). Following LPS administration, monocyte adhesion decreased from T6 until T48 *vs*. stimulated monocytes at T0, with significances at T4 and T6 (**Figure 6D**, $p < 0.05$). At T48, significant increase in adhesion rates of LPS stimulated monocytes compared to unstimulated monocytes is shown (**Figure 6D**, $p < 0.05$). At T6, there is no difference in adhesion rates between stimulated und unstimulated monocytes (**Figure 6D**).

DISCUSSION

Excessive alcohol drinking is associated with adverse immune response-related effects such as susceptibility to nosocomial

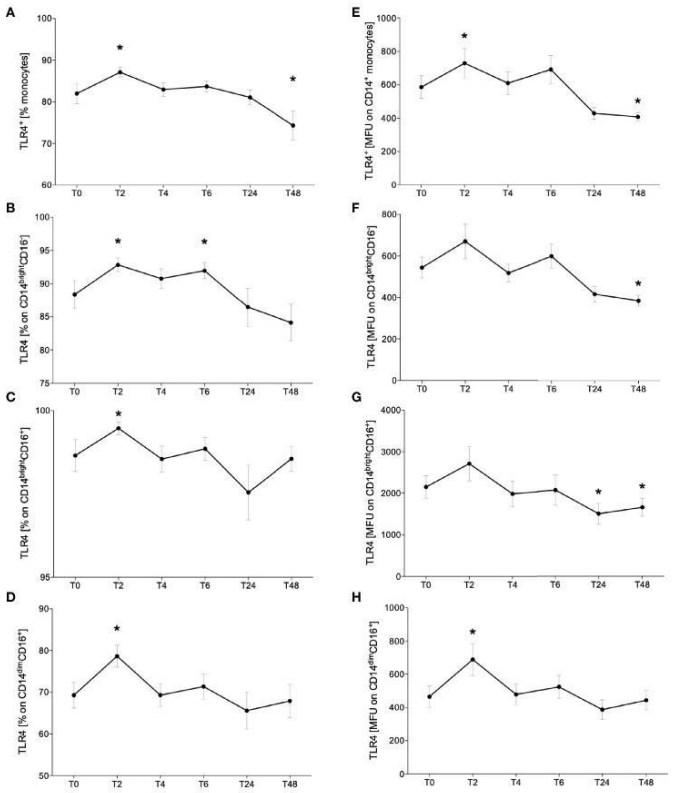

FIGURE 4 | Impact of acute alcohol intoxication on Toll-like receptor 4 (TLR4) surface presentation on monocytes. Percentage and mean intensity expression of TLR4 on $CD14^+$ monocytes **(A, E)** and the classical ($CD14^{bright}CD16^-$) **(B, F)**, intermediate ($CD14^{bright}CD16^+$) **(C, G)** and non-classical ($CD14^{dim}CD16^+$) **(D, H)** monocyte subsets were determined in blood of healthy volunteers (n=22) before (T0) and 2 (T2), 4 (T4), 6 (T6), 24 (T24) and 48 (T48) hours after starting the alcohol consumption. Data are presented as mean ± standard error of the mean. *$p < 0.05$ *vs.* T0.

infections with possible further progression to acute respiratory distress syndrome or sepsis (26). Following traumatic injury, monocytes show a shift toward the pro-inflammatory phenotype (23), and whereas chronic alcohol intake causes an exaggerated differentiation into the anti-inflammatory phenotype (17), the effect of acute alcohol intake still remains elusive. Since there is a frequent coincidence of alcohol misuse and traumatic injuries (5, 6), understanding of their interaction and the mechanisms of ongoing misbalanced immune response after trauma may contribute to the development of advanced therapeutic strategies for alcohol-intoxicated severely injured patients. Therefore, we evaluated the phenotypic and functional changes of monocytes in intoxicated HV. The key results are summarized in **Figure 7**.

In this study, HV consumed calculated amount of whisky-coke cocktail every 20 minutes during the first 4 hours of the experiment, reaching a blood alcohol level of 1‰. Over the whole observation period of 48 hours, the relative fraction of $CD14^+$ monocytes remained stable, whereas the individual monocyte subsets showed significant alterations. Classical monocytes, characterized by $CD14^{bright}CD16^-$ expression, provide phagocytosis contributing to immune defense against invading pathogens and are equated with murine pro-inflammatory $Ly6C^{bright}$ monocytes (15, 27). Two hours after

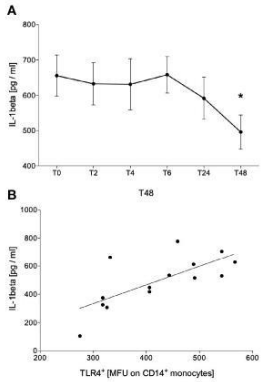

FIGURE 5 | Impact of acute alcohol intoxication on IL-1β release after stimulation with lipopolysaccharide (LPS). IL-1β was determined in blood of healthy volunteers (n=22) that was stimulated with LPS for 24 h before (T0) and 2 (T2), 4 (T4), 6 (T6), 24 (T24) and 48 (T48) hours after starting the alcohol consumption **(A)**. Spearman analysis of correlation between IL-1β and TLR4 at T48 is shown **(B)**. Data are presented as mean ± standard error of the mean. *$p < 0.05$ *vs.* T0.

drinking, the ratio of classical subset significantly increased. This was paralleled by increased abundance of intermediate subset that lasted for 6 hours and by enhanced levels of non-classical monocytes between 4 and 6 hours after alcohol intake. It is generally accepted that classical monocytes are precursors for the intermediate subset, which expresses the highest levels of antigen presentation-related molecules and has been linked with rapid immune defense in systemic infections (15, 16). Alcohol intoxication has been shown to enhance the expression of MCP-1 and C-C chemokine receptor type 2 in pancreas and microglia, contributing to the extent of tissue injury (28, 29). Therefore, binge alcohol drinking may cause a release of classical monocytes from the bone marrow within the first 2 hours after drinking, which in turn may differentiate into intermediate monocytes. Regarding the delayed increase of the relative non-classical monocyte fraction, it seems, that alcohol intoxication converts the intermediate into non-classical monocytes and thereby, switch their pro-inflammatory character into an immunosuppressive cell type. Under the same experimental settings, it has been shown that absolute leukocyte numbers significantly increase two and four hours after starting alcohol drinking, suggesting an early mobilization of immune system (25). The percentage of granulocytes out of all leukocytes decreases significantly at four and six hours, whereas the monocyte percentage decreases first significantly at six hours with subsequent significant elevation at 24 and 48 hours post-drinking (25). The current literature describes changes in leukocyte subpopulations almost exclusively in chronic alcohol abuse, although these changes are not less relevant in acute setting of alcohol drinking. Gacouin et al. analyzed leukocytes in critically ill patients, which have been acutely intoxicated with alcohol (30). They have shown that the circulating neutrophils and classical monocytes are less present in those patients, whereas the values for B lymphocytes, cytotoxic and noncytotoxic lymphocytes were significantly higher. Since these results persist in patients with and without infection, they suggest that these changes may be induced by alcohol.

The initial pro-inflammatory immune response is paralleled by elevated TLR4 expression 2 hours after starting the alcohol intake. Following an alcohol binge, the intestinal barrier is disrupted, enhancing the mucosal penetration of intestinal luminal toxic substances, pathogens and antigens, that in turn can lead to intestinal mucosal injury and inflammation (31). An increase of TLR4 in ileum and colon has been linked with increased intercellular permeability by the disruption of tight junctions between epithelial cells (32). Alcohol-induced loss of tight junctions allows the intestinal pathogens or pathogen-derived molecules to translocate into circulation, where they trigger the inflammatory cascade and activate immune cells such as monocytes and macrophages in TLR4-dependent manner, leading to a secretion of pro-inflammatory cytokines (31–33). Moreover, the intensity of alcohol intoxication positively correlates with serum levels of LPS and markers of monocyte activation (33). However, 24 hours after binge drinking, we have shown that the TLR4 expression on monocytes is downregulated and positively correlates with systemic IL-1β levels measured at 48 hours following *ex vivo in vitro* LPS stimulation. The relative fraction of non-classical monocytes, which are comparable with murine anti-inflammatory $Ly6C^{low}$ monocytes (27), recovered to the baseline at 24 hours. However, the immunosuppressive effect of alcohol seems to affect the functionality of individual monocyte subsets without changing their ratios at T48. In line with this, less monocytes are positive for reactive oxygen species at 48 hours after binge drinking, suggesting lower antimicrobial competence (25). Acute alcohol intoxication-related decrease in TLR4 response, so-called TLR4 tolerance, is induced by B-cell lymphoma 3-encoded protein (Bcl-3) that negatively regulates TNF-α transcription (34). Treatment of human monocytes and murine macrophages with 25 mM and 50 mM ethanol, respectively induces Bcl-3 expression that in turn enhances p50 homodimer stabilization. Bcl-3-p50 homodimer complex binds to TNF-α promotor region, inhibiting the TNF-α and consequently the NF-κB transcriptional activity (34, 35). However, repeated alcohol intoxication such as chronic alcohol abuse abolishes the initial anti-inflammatory effect and leads to the loss of TLR4 tolerance (1), supporting the theory about the biphasic effect of alcohol.

We have shown that systemic IL-1β level is below the detection limit following alcohol intoxication and that the amount of active caspase-1 does not significantly change in monocytes during the entire observation period. The release of IL-1β upon inflammasome activation involves 2 major steps: 1) Synthesis of inactive pro-IL-1β, 2) enzymatic cleavage of pro-IL-1β into active IL-1β by caspase-1 with subsequent secretion of IL-1β into extracellular space (36). Since LPS-induced IL-1β

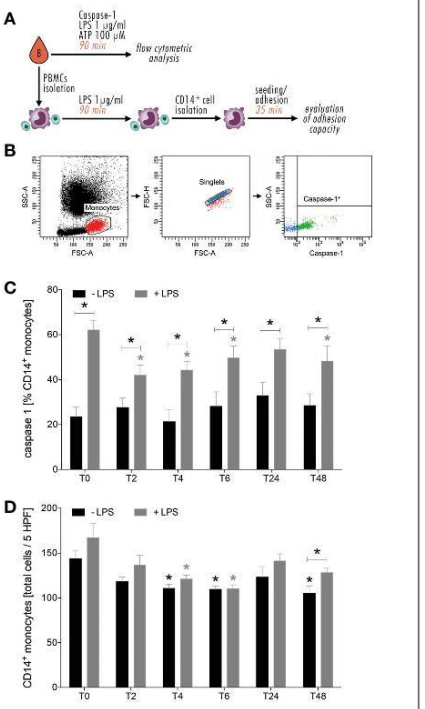

FIGURE 6 | Impact of acute alcohol intoxication on inflammasome activation in monocytes and their adhesive capacity upon LPS and ATP or only LPS stimulation *ex vivo*. Experimental design and representative gating strategy for caspase-1 is shown in **(A, B)**. Percentage expression of caspase-1 in CD14+ monocytes **(C)** and their adhesive capacity **(D)** following *ex vivo* LPS stimulation was determined in blood of healthy volunteers (n=22) before (T0) and 2 (T2), 4 (T4), 6 (T6), 24 (T24) and 48 (T48) hours after starting the alcohol consumption. Data are presented as mean ± standard error of the mean. *p < 0.05 *vs.* T0 and indicated.

decrease positively correlates with TLR4 surface presentation at T48, monocytes appear less responsible toward TLR4 ligands such as LPS and may mitigate the transcription and translation of pro-IL-1β in NF-κB-dependent manner. No alteration in caspase-1 levels suggests that inflammasome assembly was not impeded and the lower IL-1β may be caused by the lack of inactive pro-IL-1β. Although it is well known that IL-1β is mainly produced by monocytic immune cells in NLR family pyrin domain containing 3 (NLRP3)/Caspase-1-dependent manner, there is increasing evidence that IL-1β can be produced by neutrophils or inflammasome-independently as well (37–39). Thus, a monocyte-independent production of IL-1β following alcohol intoxication should be further investigated.

Ex vivo in vitro stimulation of monocytes with LPS and ATP significantly increases their caspase-1 level. During and following the excessive alcohol intake, caspase-1 expression is upregulated but significantly less induced compared to non-intoxicated HV. This is in line with an *in vitro* study, that we have published recently showing that ethanol pre-treatment (170 mM) of human HepG2 liver cells downregulated caspase-1 expression following LPS and ATP administration (22). This may be caused by innate immune memory (40), whereby the first hit (here alcohol) may reprogram the innate immune cells resulting in an adaptation of the response to the subsequent second hit (here LPS and ATP). However, the underlying mechanism remains elusive. Some studies show that this adaptation may be caused by the downregulation of TLR surface expression and an upregulation of Toll-interacting protein with subsequent hypophosphorylation of IL-1 receptor-associated kinase and downregulation of canonical NF-κB pathway (41, 42). Moreover, acute alcohol intoxication leads to impaired adhesion of monocytes, whereby the subsequent *ex vivo in vitro* stimulation with LPS cannot at all or only barely increase the adhesive capacity compared to monocytes without endotoxin challenge. This may be caused by the lower surface presentation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 as shown in an *in vitro* study with polyphenols from red wine by Ferrero et al. (43). Polyphenols are non-alcoholic compounds of alcoholic beverages such as wine or whisky (44). Since de-alcoholized wine inhibits the monocyte migration *ex vivo* (45), we should consider that the effect we attribute to ethanol/alcohol may be also caused by other non-alcoholic compounds.

Limitations

This study has several strengths including pharmacologically relevant model for binge drinking and a novel approach to understanding the variability in monocyte subsets and the dynamic responses of these subsets as well as their functions following acute alcohol intoxication. However, this study also has several limitations. First, although we have shown a clear phenotypical shift of monocytes toward the anti-inflammatory phenotype in time-dependent manner, an analysis of the expression pattern of pro- and anti-inflammatory genes and of the altered functionality (e.g. phagocytosis and bacterial killing, generation of reactive oxygen species) would significantly improve these findings. This was not possible for technical and time reasons. Second, including a placebo group would immensely enhance the validity of the results. Until now, there are no short-term *in vivo* studies showing the impact of cola or its ingredients (sugar, phosphoric acid, carbonic acid etc.) on monocytes and the most *in vitro* studies focus on the long-term impact of glucose on monocyte functionality (diabetes models). Afshar et al. have shown in line with the data presented in this study that acute alcohol intoxication by ethanol mixed with chilled sugar-free flavored seltzer water led an early pro-inflammatory immune response 20 minutes after reaching blood alcohol concentration of 1‰ (7). Two and five hours later, an anti-inflammatory state was observed with reduced numbers of monocyte and natural killer cells, attenuated LPS-induced IL-1β levels and a trend toward increased IL-10 levels (7). Further, *in vitro* studies with THP-1

			T0	T2	T4	T6	T24	T48
in vivo	classical monocytes	TLR4		↑↑	-	↑↑	-	↓↓
	intermediate monocytes			↑↑	-	-	↓	↓↓
	non-classical monocytes			↑↑	-	-	-	-
ex vivo	whole blood + LPS	IL-1β		-	-	-	-	↓
	whole blood + LPS + ATP	caspase-1		↓↓	↓↓	↓↓	↓	↓↓
	CD14$^+$ monocytes	adhesion		↓	↓↓	↓↓	↓	↓

FIGURE 7 | Summary of phenotypical and functional changes of monocyte subsets following an acute alcohol intoxication. -, no change; ↑↑, significant increase; ↓↓, significant decrease; ↓, decreasing trend; ATP, adenosine triphosphate; CD, cluster of differentiation; IL-1β, Interleukine-1β; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4.

describe increased secretion of IL-1β in glucose dose- and time-dependent manner, that is paralleled by enhanced adhesion capacity (46, 47). Although these opposite results to our study cannot definitely exclude that cola might have anti-inflammatory features on monocyte subsets and functions, it considerably supports our conclusion that these effects are attributed to alcohol. Third, the calculated absolute cell numbers of monocyte subsets are artificial. Since we did not use cell counting beads, we obtained only the relative fractions of monocyte subsets. Thus, for getting the absolute cell numbers, we calculated those out of leukocytes counted by automated blood cell counter (Sysmex Europe GmbH, Germany). Lastly, although density gradient centrifugation by using Bicoll as a separation medium is a standard method for immune cell isolation for diverse immunological investigations, such as evaluation of the inflammasome activation in monocytes (48, 49), we cannot exclude a monocyte activation by the density gradient centrifugation itself or by direct or indirect interactions with lymphocytes and platelets, which have been shown to undergo phenotypical and functional changes following a density gradient centrifugation (50–52).

CONCLUSIONS

Taking together, an acute intoxication with alcohol induces monocyte conversion toward their pro-inflammatory phenotype in the very early phase upon drinking. In the later time course, they differentiate into anti-inflammatory monocytes, findings that are paralleled by downregulation of TLR4 expression and IL-1β release. Our results suggest that an acute intoxication with alcohol has immunosuppressive effects in a time-dependent manner. Therefore, along with the endotoxin tolerance, excessively alcoholized subjects may be susceptible to the development of secondary infections. *Ex vivo in vitro* LPS stimulation of monocytes shows a downregulation of NLRP3 expression in samples obtained from severely injured patients compared to healthy subjects (53). This in turn elicits the synthesis and release of active IL-1β (53), whereby the monocyte deactivation correlates with the injury severity (54). In a rat model of blunt chest thorax trauma and hemorrhagic shock with subsequent resuscitation, monocytes express lower levels of caspase-1 in response to ethanol administration 2 hours before injury (21). Accordingly, alcohol intoxication may synergize with later trauma-induced immunosuppression leading to further enhanced vulnerability to infectious complications in the clinical course and has to be elaborated in further studies. It remains controversial whether an acute intoxication with alcohol has an impact on the outcome after a major injury, however, an adjustment of the timing of life-saving intervention according to the immune response may improve the therapeutic approach.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee approval (255/14) from the University Hospital of the Goethe-University Frankfurt. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BR designed the study, obtained the ethical approval, performed statistical analyses, and revised the manuscript. RS, FH, BX, and AJ collected the samples and performed the experiments. AJ and RS performed the statistical analyses, and AJ wrote the original draft. AG, ID, CN, AN, and PC contributed to analyses and revised the manuscript. ID, CN, and IM contributed intellectually to the completion of the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.652488/full#supplementary-material

Supplementary Table 1 | Calculated absolute monocyte numbers and their subsets.

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Chapter 3 Discussion

Although the development of advanced treatment algorithms of severely injured patients has significantly increased the posttraumatic survival rates, these patients are still highly susceptible for the development of secondary infections during their clinical stay. This has been associated with a dysregulated immune response. Therefore, the analysis and the understanding of the ongoing humoral and cellular immunological changes may prospectively represent a valuable therapeutic target.[177-179] At the cellular level, tissue-resident macrophages, neutrophils, and monocytes have been shown to initiate an immediate immune response in order to clear the tissue damage and protect the host from invading pathogens.[27-30] Phenotypic shift of certain monocyte populations has been associated with several pathologies such as infections [29, 56, 70], autoimmune diseases [85, 87, 88], atherosclerosis [100], and cancer [101, 102]. However, despite the high clinical relevance, no studies describe the distribution of monocyte subsets and their functions following severe traumatic injury in humans. Therefore, we evaluated the phenotypic shift of monocyte subsets in polytrauma patients in the first twelve hours postinjury as well as their capacity to generate ROS.

As described above, severely injured patients are prone to secondary infectious complications. Previously, we identified pulmonary CC16 as a biomarker for secondary pneumonia in severely injured patients.[117] CC16 has anti-inflammatory and immunosuppressive properties and its aberrant appearance in the lungs and the circulation is associated with development of several pulmonary diseases, such as COPD, asthma, sarcoidosis, pulmonary infections, and ARDS.[118-120] This suggests that lacking CC16 may contribute to worsening lung injury and inflammatory complications following severe traumatic injury. Therefore, we evaluated the lung damage, the humoral inflammatory response, and the monocyte and macrophage subsets distribution in the blood, lungs, and BALF following local intrapulmonary CC16 neutralization in a murine model of posttraumatic early sepsis.

Moreover, approximately 50% of patients who are admitted to the emergency department have positive blood alcohol level.[141, 142] Since alcohol has immunomodulating effects, either pro- or anti-inflammatory depending on the drinking behaviour, it may contribute to the development of secondary inflammatory complication in severely injured patients. [148-151]. Therefore, we evaluated the distribution of monocyte subsets and the inflammatory response regarding their TLR4 expression and caspase-1 activation in time-dependent manner in healthy volunteers who were acutely alcohol intoxicated as a baseline for pursuing alcohol studies on severely injured trauma patients.

3.1 Phenotypical and functional changes of monocyte subsets following severe traumatic injury

3.1.1 Monocytes differentiate towards the pro-inflammatory phenotype in severely injured patients

Monocytes have multifunctional role in maintaining the tissue integrity and responding to inflammatory mediators to clear the cellular debris and pathogens with subsequent restoration of tissue homeostasis.[180] Traumatic injury leads to immediate and persistent leukocytosis whereby monocytes are rapidly activated.[35, 89-91] In this study, we have shown that the distribution of the classical $CD16^{-}CD14^{bright}$, intermediate $CD16^{+}CD14^{bright}$, and non-classical $CD16^{+}CD14^{low}$ monocytes is significantly shifted compared to healthy uninjured individuals.[176] It is generally accepted that classical monocytes, which is the main monocyte population in healthy subjects, are precursors for pro-inflammatory intermediate monocytes that in turn differentiate into patrolling non-classical monocytes [56]. The classical and non-classical monocytes become less abundant in the circulation of severely injured patients within of the first 12 hours after trauma, whereas the numbers of circulating intermediate monocytes significantly increase.[176] This suggests that the monocyte population switches towards the pro-inflammatory phenotype in the early posttraumatic

phase. The decreased abundance of classical monocytes in the circulation may be caused on the one hand by their differentiation into intermediate monocytes or on the other hand by their extravasation into tissues and to the site of injury, where they differentiate either into macrophages or dendritic cells, contributing to shaping and resolving the inflammation [75]. Classical monocytes are the only subset that highly expresses CCR2.[181] Since the major chemokine toward that monocytes are recruited into tissues is MCP-1,[67] this can be the reason why predominantly classical monocytes migrate into tissues. Although the monocyte trafficking across the vessel wall is well investigated,[63] the exact mechanism along the subsets still remains to be elucidated.

As described above, intermediated monocyte subset becomes significantly abundant in the circulation of severely injured patients within the first twelve hours after trauma.[176] Such an exaggerated elevation has been also shown under inflammatory conditions such as sepsis or bacterial and viral infections [29] that has been paralleled by release of high amounts of TNF-α, IL-1β, and IL-6 [182], clearly showing the pro-inflammatory potential of intermediate monocyte subset. Such an exaggerated sequestration of these pro-inflammatory mediators has been related to so-called cytokine storm in septic patients, leading to blood pressure collapse, coagulopathy, up to MOF, and death.[183] It has been reported that intermediate monocytes have the highest expression of HLA-DR from the three monocyte subsets in human experimental endotoxemia model over the whole observation period of 24 hours.[29] Parallelly, monocyte deactivation is reflected by extended downregulation of HLA-DR that in turn is associated with development of secondary infectious complications and MOF in severely injured patients.[29, 90] If we would consider that monocytes are deactivated within the first 48 hours after trauma and their prolonged downregulation of HLA-DR expression is linked to posttraumatic secondary complications, we should follow the redistribution of monocyte subsets and subset-specific HLA-DR expression over the entire intensive care unit stay. It might be that the initial high abundance of intermediate monocytes would decrease, whereby also the surface presentation of HLA-DR allocated to the remained monocytes would be attenuated, reflecting the

posttraumatic immunosuppression. We assume that the extent and the timing of the initial pro-inflammatory phase followed by the immunosuppressive phase in combination with the monocyte subsets distribution might provide valuable insight into posttraumatic monocyte kinetics and prospectively also a potent tool for counteracting the secondary infections.

3.1.2 The level of ROS is predominantly increased in intermediate monocytes of severely injured patients

There is a rising evidence that the posttraumatic development of infectious complications is strongly related to dysregulated immune response [27-30]. For a timely resolution of inflammation and recovery from injury, phagocytes such as monocytes and neutrophils recognize and eliminate microbes and cell debris [31, 32]. Thus, if the phagocytes fail to engulf and clear their targets, the tissue inflammation is prolonged, that in turn causes tissue damage with subsequent infectious complications [30]. Phagocytosis and the generation of ROS are one of the fundamental mechanisms for clearing the debris and the pathogens and those not adequate formation has been linked to secondary infections.[184, 185]

The aim of this thesis was also to evaluate the phagocytic capacity of monocyte subsets obtained from severely injured patients. However, we have not been successful in establishing this method. Following the CD14 and CD16 staining *ex vivo*, we administrated ten *E. coli* BioParticles (K-12 strain; Thermo Fisher Scientific, #E2861) per leukocyte to the sample and after the incubation of one hour, we assessed the phagocytic capacity using flow cytometry. The *E. coli* BioParticles, which were conjugated with fluorescein fluorochrome, did give such a massive signal on monocyte population that performing proper compensation was not possible. That the monocytes have provided phagocytosis in such a strong extent could be excluded, because granulocytes, which show generally higher phagocytosis rates compared to monocytes,[186, 187] did not show such a strong signal and spectral overlap. It might be that the BioParticles were stacking extracellularly on the monocyte surface. Here, using a pH-dependent fluorochrome-conjugated bacteria could be useful, which

fluoresce first after reaching the phagosome with low pH. This has to be evaluated in further studies.

As described already above, the generation of ROS is an elemental mechanism to clear the pathogens within the phagosome, but it also acts extracellularly as a chemoattractant for immune cells to clear and repair the tissue. However, exaggerated release of free radicals can have detrimental consequences to the host [188-190]. Such an excessive generation of ROS has been shown in monocytes of severely injured patients,[97, 98] but there is no evidence until now, whether the individual monocyte subsets generate ROS in different extent.

In this study, the mean intracellular content of ROS is significantly higher in all monocyte subsets of severely injured patients compared to healthy individuals.[176] Regarding the monocytes that are positive for ROS, we observed an increase in all subsets, but this increase was significant only in the pro-inflammatory intermediate monocytes.[176] Along with the elevated ratio of intermediate monocytes, this suggests that the very early posttraumatic phase is modulated by pro-inflammatory immune response of monocytes. We examined the intracellular generation of ROS. Thus, we could only evaluate the extent of the generated ROS within the cell but not their release. There are two possible scenarios how to interpret this data. Firstly, the intracellular content of ROS may also reflect their exaggerated extracellular release. Extracellular oxidative burst leads to disruption of endothelial barrier by opening the inter-endothelial junctions, that in turn facilitates the immune cells to transmigrate to the site of infection or injury. Dependent on the concentration of ROS, the migrated inflammatory cells do not only clear but also can damage the tissue.[191] It has been shown that the major sources of ROS are lipooxygenases.[192] The 5-lipooxynase, which is activated by 5-lipoxygenase-activating protein (ALOX5AP), has an essential role in monocyte adhesion to vascular endothelium and converts arachidonic acid into leukotriene B4.[192, 193] Liepelt *et al.* found out that the mRNA encoding ALOX5AP is significantly upregulated in $CD14^+$ monocytes of only intensive care unit patients with septic complications.[194] In previous studies, we revealed elevated systemic level of leukotriene B4 as an indicator for pulmonary complications following severe

injury.[195, 196] If we would consider the intermediate monocyte subset as a source of ALOX5AP in the early posttraumatic phase, subsequent excessive generation and release of ROS and leukotriene B4 might contribute to the pulmonary damage and inflammatory complications in ALOX5AP-depenedent manner.

Secondly, the exaggerated generation of ROS may not reflect the extracellular release and the free radicals may remain in the cell. It is well known that whereas intracellular ROS at low level act as a signalling molecule and contribute to the cell survival Janus kinase-dependently, high intracellular level of ROS causes cell death, activating the apoptotic signalling pathways.[191] Therefore, an exaggerated amount of ROS in intermediate monocytes might cause their subsequent death in later time course. As described in previous section, intermediate monocytes have the highest expression of HLA-DR of all three monocyte subsets in human experimental endotoxemia model over the entire observation period of 24 hours.[29] Therefore, ROS-induced cell death of intermediate monocytes in the later time course might reflect the loss of HLA-DR surface presentation on residual monocytes that has been linked to posttraumatic inflammatory complications. [29, 90] This is also in line with our finding showing hyporesponsiveness of intermediate monocytes towards the *ex vivo* PMA stimulation.[176] Due to our study design, we could not evaluate the effects of ROS generated by the individual monocyte subsets on the patient's outcome or development of secondary complications. To answer this, the future experiments should include evaluation of the phenotypic shift of monocyte subsets over the entire intensive care unit stay, their capacity to generate ROS, and the activation of apoptotic pathways. Additionally, evaluation of extracellular generation of ROS should be directed in further studies as well.

3.2 Impact of CC16 on humoral and cellular immune response in murine model of posttraumatic early sepsis

3.2.1 Reliable murine model for early sepsis following thoracic injury

Blunt chest injury is the third cause of death after major trauma and excessive pro-inflammatory immune response possesses a high risk for development of secondary inflammatory complications such a sepsis, with limited therapeutic options.[197, 198] Therefore, uncovering of the underlying molecular mechanism could substantially contribute to the development of novel therapeutic strategies. However, an excision of injured lung tissue following severe chest injury is not always indicated. Additionally, bronchoalveolar lavage is performed for diagnostic and therapeutic applications, which require evaluation of microbiological and/or cellular components [199] and thus, is also not indicated in the very early posttraumatic phase. Therefore, for the initial examination of early sepsis following thoracic injury, animal models are the tool of choice.

However, researchers are faced by great challenges. Although mouse models are the key tools for studying the pathophysiology and subsequent life-saving strategies, the immune response between mouse and human differs and the applied treatment cannot be directly translated into human settings. Moreover, there is no single mouse model that can capture all clinical aspects of complicated pathology of sepsis. Generally, three types of models are commonly used to induce experimental sepsis: (I) administration of a toxic agent, (II) administration of live pathogens, and (III) disruption of the endogenous protective barrier.[200, 201] Administration of toxic agents such as LPS, zymosan, and lipoteichoic acid induces a strong immediate immune response, mimicking the activation of innate immune system.[201] This sepsis model is low invasive, standardized, controllable, and reproducible, however, since the immune system does not have to eliminate the pathogen, it does not mimic the host-pathogen interactions and the development of polymicrobial sepsis.[200, 202] Administration of live pathogens such as Gram-positive and -negative bacteria is comparably low

invasive and reproducible as the administration of sepsis-inducing toxins.[200, 201] It is well applicable for studying the host-pathogen interactions and immune response to a particular pathogen. Therefore, this model is more clinically relevant for studying nosocomial infections, which are mostly monomicrobial, than sepsis.[200, 201, 203, 204] The missing point of different pathogens and local focus of infection where from bacteria continuously spread is fulfilled by the disruption of the endogenous protective barriers.

Following traumatic injury, intestinal perforation is common and leads to inflammatory complications. Therefore, CLP as a method for the disruption of the endogenous protective barrier represents a convincing model causing tissue necrosis and polymicrobial sepsis that in turn has been observed in patients with sepsis.[201] In this model, both pro- and anti-inflammatory immune responses are activated.[200] We have shown in our recent study that the lung tissue injury with moderate inflammatory changes after blunt chest trauma is quickly reversed, and therefore, might be caused by mechanical lung injury.[205] In contrast, lung injury in the blunt chest trauma group with subsequent CLP did not show such a recovery and was closely associated with significant changes of inflammatory mediators, mostly mimicking the human posttraumatic sepsis.[205] Therefore, we applied this model in the current study.

3.2.2 Local CC16 neutralization is associated with increased pro-inflammatory mediators and lung damage.

Alveolar macrophages have high phenotypic and functional plasticity in order to orchestrate the innate and the adaptive immune responses. Following infection or non-infectious stimuli like a thoracic injury, alveolar macrophages are one of the first cells involved in the early immune response, initiating the inflammatory cascade and the secretion of pro-inflammatory mediators.[206-209] Increased systemic level of TNF is a well-known indication for sepsis.[210] Confirmatively, mice that underwent thoracic injury with subsequent CLP have significantly higher TNF level compared to sham operated animals.[61] Local CC16 neutralization by intratracheal application of CC16 neutralizing antibodies after thoracic trauma induction forces a further elevation of TNF

in the circulation.[61] This indicates that CC16 has an anti-inflammatory capacity in the present model of early sepsis following thoracic injury and we confirmed its general anti-inflammatory character. Interestingly, stimulation of human bronchiolar epithelial cells *in vitro* and *ex vivo* with TNF-α (20 ng/ml) led to increased *CC16* mRNA level after 8 - 36 hours.[211] It could be assumed that TNF-α may upregulate counterregulatory molecules in order to suppress an exaggerated pro-inflammatory response as balancing mechanism. The modulation of the TNF-α level is a promising therapeutic approach for sepsis, however, the trials are impeded by its complex signaling and general pro-inflammatory character. Moreover, the efficacy of neutralizing anti-TNF-α antibodies in patients who developed sepsis seems to be also contradictory.[212, 213] However, the not uniform patient cohorts between the studies and different genetic background of the patients may lead to this data discrepancy.[212-214] Recently, single nucleotide polymorphism of *TNF-α* +489G/A A-allele has been shown to be associated with sepsis in terms of lower risk and severity but not outcome.[214]

TNF-α is also a signaling molecule, inducing the expression of MCP-1 by variety of cells, including monocytes, macrophages, and endothelial cells.[215] In line, the systemic level of MCP-1 in mice that underwent thoracic injury and CLP is also significantly increased. Similarly, the neutralization of endogenous CC16 leads to further increase of MCP-1 in circulation.[61] However, whether this further increase is directly caused by CC16 neutralization or indirectly by the TNF increase or other factors could not be clarified and should be directed in further studies. Laucho-Contreras *et al.* have shown that MCP-1 spontaneously accumulate in the lungs of *Cc16*$^{-/-}$ mice.[216] Since enhanced MCP-1 level in plasma reflects the injury severity and is associated with an increased vulnerability to developing sepsis, CC16 deficiency might represent a contributing factor for developing posttraumatic inflammatory complications.

Further, the expression of RAGE is significantly elevated in the lungs and BALF of mice with thoracic injury and CLP, whereas the local neutralization of endogenous CC16 results in a further increase of RAGE in the lungs.[61] In a clinical study, CC16

and RAGE were identified as reasonable biomarkers for ARDS in severe septic patients [217] and RAGE in its soluble form seems to reflect activity of RAGE signaling pathway.[218] Intranasal administration of CC16 in murine model of asthma reveals a reduction of airway inflammation and damage, suppressing the airway epithelial cell apoptosis in a high mobility-box-1 protein (HMGB1)-dependent manner.[219] This is on contrary to our study, showing attenuation of caspase-3-dependent apoptosis following CC16 neutralization.[46] However, this does not necessarily mean that CC16 has reversed apoptotic effect on lung tissue in asthma and traumatic injury. Less mature $CD16^{+}CD62L^{bright}$ and more immature $CD16^{-}CD62L^{bright}$ neutrophils transmigrate into lungs following blunt thoracic injury with subsequent CLP in mice, which underwent local intrapulmonary CC16 neutralization before trauma induction compared to animals with no CC16 modulation.[46] Since neutrophil-induced tissue injury with subsequent epithelial cell apoptosis is one of the pathomechanisms in ALI [220] and it is assumed that immature neutrophils have impaired functional ability [189], it may be that the attenuated apoptosis in the lung tissue is mediated by lower ratio of mature neutrophils and thus, not directly by CC16 itself. In context of endotoxemia and CLP-induced sepsis, HMGB1 is required for caspase-11-mediated pyroptosis, a lytic form of cell death, and lethality.[221] Mechanistically, HMGB1 delivers LPS into cytosol through RAGE-mediated internalization that in turn subsequently activates caspase-11. This activation and the associated death are reversed in RAGE knockout mice.[221] Moreover, caspase-11 mediates the activation of caspase-3 involved in the apoptosis.[222] Another studies have shown that RAGE either positively or negatively regulates the bacterial clearance depending on the bacterial strain.[223, 224] This indicates that the effect of RAGE is dependent on the inflammatory conditions.

Total protein level in BALF negatively reflects the lung epithelial integrity. Thoracic injury significantly increases the total protein content in the BALF and the additional CLP does not have any effect regarding the protein content.[61] However, this does not necessarily mean that sepsis does not have an impact on pulmonary barrier breakdown. Lungs are directly affected by the blunt thoracic injury and the sacrifice six hours post-CLP may be too early to show any effect on the lung epithelial integrity. Upon intrapulmonary CC16 neutralization, the total protein content significantly

increases.[61] Patients who were diagnosed with non-trauma-related ALI or ARDS have lowered CC16 level in pulmonary edema fluid.[225] This suggests that CC16 has a positive impact on the lung epithelial integrity. This is in line with our recent study by Störmann *et al.*, showing aggravated lung injury six hours post-CLP in animals with neutralized CC16.[134] However, 24 hours post-CLP, the lung injury is attenuated compared to mice without CC16 neutralization. Additionally, when the CC16-neutralizing antibodies are administrated first after the CLP, there is no effect on the pulmonary damage six hours post-CLP, whereas at 24 hours, significant worsening can be observed.[134] This suggests that dependent on the timing, CC16 may first reduce the necessary early pro-inflammatory response and at later time point, may contribute to the amelioration of the lung injury.

Since several studies suggest that the key driver of pulmonary damage in ALI is an exaggerated pro-inflammatory immune response, neutralization studies are a potent tool to investigate the involvement of concrete pro- or anti-inflammatory mediators. Aminoprocalcitonin, a bioactive peptide of procalcitonin, seems to mediate the pro-inflammatory response in sepsis *via* activation of hypothalamic prostaglandin-dependent pathways and corticotrophin and melanocortin neutrons.[226-229] Its neutralization by intraperitoneal application of aminoprocalcitonin-neutralizing antibodies decreases the secretion of pro-inflammatory cytokines IL-1β and TNF-α, increases the secretion of anti-inflammatory factors IL-10 and adrenocorticotropic hormone, reduces neutrophil infiltration, bacterial invasion, lung inflammation and damage, and reduces the mortality of rats in septic models.[228, 230] Furthermore, cytokines such as IL-6 and IL-8 predict the outcome of severe sepsis although they are not independent markers.[231] Intravenous injection of anti-IL-6 immediately after CLP significantly improves dose-dependently survival rates, possibly mediated by blocking of the upregulation of C5a receptor in septic mice.[232] Similarly, neutralization of IL-8 before and after hydrochlorid acid-induced lung injury improves the oxygenation and lung oedema, whereas the extravascular protein and neutrophil accumulation is significantly reduced in lungs.[233] Taking together, neutralization of pro-inflammatory mediators is effective for amelioration of sepsis-induced lung injury and inflammation. Nevertheless, a local application of anti-inflammatory factors may

also show comparable effects. Therefore, to confirm the anti-inflammatory effect of CC16, an intrapulmonary administration of rCC16 should be considered.

3.2.3 CC16 modulates the distribution of monocyte and macrophage subsets *in vivo* and their functionality *ex vivo*

Beside the effects on humoral factors, these by alveolar macrophages secreted cytokines and chemokines induce the egress of monocytes from the bone marrow and their subsequent transmigration to the site of injury, where they differentiate into inflammation-associated M1 macrophages.[234] In the current model, the relative monocyte counts significantly increase in the blood, lungs, and BALF of mice that underwent thoracic injury, whereas an additional sepsis induction amplify the monocyte appearance in all three compartments.[61] Regarding their pro- and anti-inflammatory character, septic mice have significantly more pro-inflammatory $Ly6C^{bright}$ monocytes compared to the significant decline of anti-inflammatory $Ly6C^{low}$ monocytes.[61] This indicates that $Ly6C^{bright}$ monocytes are released into circulation and migrate into affected tissues without their transition to $Ly6C^{low}$ monocytes in the early phase of trauma-induced sepsis.[235] Moreover, since MCP-1 is the pivotal regulator of monocyte recruitment to the site of injury,[236] elevated MCP-1 level in septic mice may significantly contribute to the exaggerated lungs infiltration with pro-inflammatory monocytes that in turn may contribute to the extent of the tissue injury. In line, the deficiency of CCR2, that is the receptor for MCP-1 and the major factor for monocyte egress from the bone marrow, protects the mice from CLP-induced sepsis.[237] The authors have shown that CCR2-deficient mice release significantly less pro-inflammatory monocytes into circulation and the organs. This is paralleled by attenuated organ damage and systemic cytokine production. Interestingly, the numbers of neutrophils significantly increase, suggesting a counterbalance to the absence of pro-inflammatory monocytes.[237] Upon CC16 neutralization before CLP, $Ly6C^{+}$ monocytes migrate extensively into lungs, suggesting an anti-migratory character of CC16.[61] This is in line with an *in vitro* study showing a CC16-induced inhibition of chemotaxis of human monocytes towards N-Formylmethionyl-leucyl-phenylalanine

(fMLP).[238] Additionally, since CC16 contributes to the maintenance of the pulmonary epithelial integrity,[225] its neutralization may contribute to the enhanced level of pro-inflammatory monocytes in the BALF.[61]

Further, we investigated the phenotypic changes of macrophages following thoracic injury and CLP. In blood, neither total macrophages nor their Ly6C^{bright} F4/80^{+} pro-inflammatory and Ly6C^{low} F4/80^{+} anti-inflammatory subsets change in their ratios.[61] This is reasonable because macrophages are tissue-specific cells. Differently, the abundance of pro-inflammatory and anti-inflammatory macrophages in the lungs and the BALF significantly increases and decreases, respectively in mice that underwent thoracic injury with subsequent CLP.[61] As described above, the blood level of pro-inflammatory monocytes significantly increases in injured septic mice. These monocytes may transmigrate into tissues along the chemoattractant gradient and differentiate into pro-inflammatory macrophages.[75] However, CC16 seems to not have such a significant impact on macrophage biology compared to the monocytes. The ratios of pro- and anti-inflammatory macrophages in the lungs do not change by CC16 neutralization, whereas BALF reveals significantly fewer anti-inflammatory macrophages.[61] In steady state, resident alveolar macrophages have anti-inflammatory properties or so-called M2 phenotype. Upon infectious or traumatic stimuli, alveolar M2 macrophages polarize toward the pro-inflammatory phenotype, which is also called M1 phenotype.[234] Thus, CC16 neutralization might have an impact on the polarization of macrophages toward the pro-inflammatory phenotype. However, whether CC16 directly suppresses the polarization of M2 to M1 macrophages must be evaluated in further studies.

Taking together, we have shown that thoracic injury with subsequent CLP leads to a significantly higher abundance of pro-inflammatory monocytes and macrophages six hours after the sepsis induction, that is also paralleled by increase of pro-inflammatory mediators and lung damage.[61] Thereby, CC16 negatively modulates monocytes- and macrophages-driven inflammation.[61]

Patients who initially survived sepsis are frequently rehospitalized due to late pulmonary complications.[239] This is associated with an immunoparalysis that lasts considerably longer than the clinical manifestation of the initial sepsis.[239-242] Murine alveolar macrophages and intravascular lung monocytes display impaired activation and phagocytic capacity several weeks after systemic inflammation, leading to an inefficient protection against a secondary infection.[240] In human, alveolar macrophages and circulating monocytes show six months after sepsis an epigenetic reprogramming associated with high susceptibility to develop secondary infections.[242] So far, the impact of CC16 on the phagocytic activity of monocytes or macrophages has not been studied. Few studies have shown that neither a neutralization nor an application of recombinant CC16 affects the phagocytic activity of PMNLs.[133, 243] Interestingly, *SCGB1A1* genes in the equid family are triplicated and two of these genes encode for distinct SCGB/CC16 proteins.[243-245] The variations SCGB 1A1 and SCGB 1A1A differ by 13 of 70 amino acids and 7 of them are in the region that forms the hydrophobic pocket.[243, 245] Cote *et al.* have shown that *ex vivo* incubation of horse neutrophils with recombinant SCGB 1A1A but not SCGB 1A1 enhances the phagocytic capacity and generation of ROS, whereby the underlying mechanism could not be identified.[243] Although no such as different variants have been found in human, it might be that a modification of the hydrophilic pocket would also change the antimicrobial properties of human CC16. If we would assume that an application of SCGB 1A1A would also positively impact the antimicrobial biology of monocytes, its local post-septic application might attenuate the immunoparalysis and the associated infectious pulmonary complications.

3.2.4 CC16 inhibits the migratory capacity of $CD14^+$ human monocytes and the TGF-β expression *ex vivo*

We assume that CC16 has anti-migratory potential towards monocytes and its neutralization, in turn, leads to an enhanced recruitment into the lungs from the circulation. In line, Vasanthakumar *et al.* have shown an inhibited chemotaxis of human monocytes towards fMLP *in vitro* in CC16-dependent manner, being the only

publication of the last 30 years regarding the anti-migratory effects of CC16.[238] Therefore, we isolated $CD14^+$ monocytes from healthy subjects and stimulated them with sera obtained from healthy volunteers and severely injured patients who developed sepsis in later clinical course. Additionally, we neutralized CC16 in patient's sera as we have shown in previous studies, where an increased systemic concentration of CC16 correlates with the development of secondary respiratory complications following traumatic injury and its level is lower in healthy subjects.[117, 119, 127] The CC16 neutralization of CC16 before the stimulation of $CD14^+$ monocytes significantly increases their chemotaxis towards MCP-1.[61] Interestingly, they migrate even more than the controls. However, this is not contradictory since serum from severely injured patients contains beside CC16 also other mediators such as IL-6 or CCL5, which can contribute to the enhanced migratory capacity.[246-249] Thereby, CC16 has no cytotoxic effects on monocytes, shown in their unaffected viability and release of lactate dehydrogenase following treatment with sera.[61] In a further *ex vivo* experiment we have shown that the isolated $CD14^+$ monocytes express significantly less amount of TGF-β1 following stimulation with sera obtained from severely injured patients.[61] Since CC16 has anti-inflammatory properties, this result has been unexpected. However, it has been reported that CC16 contributes to the diminished level of TGF-β in rodent model of lung fibrosis although the underlying mechanism remains elusive.[216, 250] Following *in vitro* LPS stimulation of rat tracheal epithelial cells, CC16 suppresses the expression of matrix metalloproteinase (MMP)-9.[251] In turn, MMP-9 proteolytically activates TGF-β.[252] Therefore, it might be that CC16 suppresses the TGF-β1 *via* MPP-9 inhibition.

3.3 Impact of binge drinking on monocyte phenotype and function in healthy volunteers

3.3.1 Acute alcohol intoxication redistributes monocyte subsets time-dependently

It is well known that alcohol abuse is associated with adverse immune response-mediated inflammatory effects such a vulnerability of the patients for development of nosocomial infections, which can progress to ARDS or sepsis.[253] This even increases the risk of severely injured patients for secondary inflammatory complications. The understanding of ongoing misbalanced immune response in alcohol intoxicated severely injured patients is highly clinically relevant because up to 50% of patients admitted to emergency department have positive blood levels.[141, 142] As we showed phenotypic shift of monocytes toward the pro-inflammatory subset in severely injured patients[176] and in murine model of posttraumatic early sepsis[61], it would be of importance to evaluate how alcohol intoxication would impact this shift and their function. Therefore, we evaluated the phenotypic and functional changes of acutely intoxicated healthy subjects in time-dependent manner to provide a solid fundament for further studies on alcohol intoxicated severely injured patients.

Healthy subjects who consumed calculated amount of whisky-cola cocktail every 20 minutes for four hours and reached the blood alcohol level of 1‰ display biphasic phenotypic shift of monocytes in time-dependent manner, whereby the relative numbers of $CD14^+$ monocytes do not significantly change over the entire observation period of 48 hours.[47] The ratio of classical $CD16^-CD14^{bright}$ monocytes in the circulation is significantly increased two hours after the first whisky-cola shot (T2). Parallelly, the relative fraction of intermediate classical $CD16^+CD14^{bright}$ monocytes increases and lasts until T6. Four and six hours after starting the binge drinking (T4, T6), the ratio of non-classical $CD16^+CD14^{low}$ monocytes significantly increases in the circulation.[47] It is well known that classical monocytes, which predominantly have antimicrobial properties, egress the bone marrow in CCR2- and CXCR4-depenent manner,

respectively.[37, 56, 65-67]. In the circulation, they either enter the tissues or differentiate into pro-inflammatory intermediate monocytes that in turn are precursors for anti-inflammatory non-classical monocytes.[56] Following acute alcohol intoxication, immune system is mobilized early, which is reflected by elevated absolute leukocyte numbers two and four hours after the starting alcohol drinking.[254] Thereby, the percentage of monocytes out of all leukocytes decreases significantly at six hours and granulocytes four and six hours post-drinking.[254] Since it has been shown that alcohol intoxication enhances the expression of MCP-1 and its receptor CCR2 in pancreas and microglia,[255, 256] it suggests that acute alcohol intoxication causes a release of classical monocytes into circulation within the first two hours of alcohol drinking. Subsequently, circulating classical monocytes may differentiate into intermediate monocytes or migrate into tissues, substantiating the decreased monocyte numbers at six hours. Additionally, monocytes and granulocytes possess significantly elevated levels of caspase-3 and caspase-7 two hours after the first whisky-cola shot.[254] Since caspase-3 and caspase-7 are associated with increased apoptosis rate,[257] monocytes and granulocytes may undergo apoptosis in later time course that in turn would contribute to their decreased cell numbers also. Lastly, the delayed increase of the relative non-classical monocyte fraction may occur due to acute alcohol-induced conversion of intermediate into non-classical monocyte subset, suggesting their switch from pro-inflammatory to immunosuppressing character. This is in line with the current literature, that describes an increase of circulating leukocytes, monocytes, and natural killer cells as well as pro-inflammatory mediators in the very early phase of binge drinking, whereas those levels are subsequently decreased in later time course.[148, 150, 158] As described above, excessive alcohol intake increases the risk for development of infections.[253] That reduced numbers of circulating neutrophils and classical monocytes, paralleled by higher abundance of B lymphocytes, cytotoxic, and noncytotoxic lymphocytes, are caused directly by alcohol itself and not by the concurrent infection has been confirmed by Gacouin *et al.*, who evaluated alcohol intoxicated critically ill patients with and without infectious complications.[258]

3.3.2 Acute alcohol intoxication initially upregulates TLR4 expression on monocytes with subsequent downregulation in later time course

The pro- and anti-inflammatory effects of alcohol on monocytes and macrophages are attributed to TLR sensitization and tolerance, respectively.[136] Parallelly to the elevated ratio of pro-inflammatory monocytes, alcohol binge significantly increases the TLR4 expression on all monocyte subsets two hours after the first whiskey-cola shot, suggesting an initial pro-inflammatory response toward the alcohol.[47] Acute alcohol intoxication is associated with an intestinal barrier breakdown that leads to mucosal penetration of intestinal luminal toxic substances, pathogens, and antigens. This in turn can result in intestinal mucosal injury and inflammation.[259] Moreover, elevated expression of TLR4 in ileum and colon correlates with increased intercellular permeability by the disruption of tight junctions between the epithelial cells.[259] Thus, alcohol-induced loss of tight junctions enables the intestinal pathogens and pathogen-derived molecules to cross the epithelial barrier and enter the circulation, where they trigger the inflammatory cascade and activate immune cells such as monocytes and macrophages TLR4-dependently.[259-261] Additionally, serum levels of LPS and markers of monocyte activation increase in alcohol dose-dependent manner.[261] In the study by Sturm *et al.*, we evaluated the serum level of intestinal fatty-acid binding protein (FABP-I) in acute alcohol intoxicated individuals.[262] FABP-I is an intracellular protein of the small and large intestine that is specifically and abundantly expressed in the epithelial cells of the mucosal layer.[263] If the intestinal mucosal barrier would be broken through, FABP-I would be released and accumulate in the circulation.[263] We have shown that the FABP-I concentration in serum initially decreases and elevates from six hours after starting binge drinking, confirming the intestinal barrier breakdown in acute alcohol intoxication.[262] In context of traumatic injury, intestinal barrier breakdown facilitates the development of sepsis and impacts negatively the posttraumatic outcome.[264, 265]

From 24 hours after binge drinking, TLR4 expression on monocytes is downregulated and positively correlates with IL-1β concentration at 48 hours in serum following *ex*

vivo stimulation of whole blood with LPS.[47] Parallelly, the relative numbers of anti-inflammatory non-classical monocytes drop to the baseline from 24 hours.[47] This indicates that for the immunosuppressive effect of alcohol, an increase of immunosuppressive monocyte fraction is not necessary and alcohol affects the functionality of individual monocyte subsets without changing their ratios. Supportively, in the study by Haag *et al.*, less monocytes generate ROS at 48 hours after binge drinking, indicating diminished cell activation and antimicrobial competence.[254] As described above, the pro- and anti-inflammatory effects of alcohol on monocytes are attributed to TLR sensitization and tolerance, respectively.[136] The binge drinking-dependent attenuation of TLR4-mediated response is induced by Bcl-3.[164] In a murine model of binge drinking, the *Bcl3* and *Tnfα* expression is significantly increased and decreased, respectively in the liver following intraperitoneal LPS challenge. Knockdown of Bcl-3 in murine RAW 264.7 macrophages using siRNA reveal an abrogation of the alcohol-induced inhibition of TNF-α expression at mRNA and protein level.[164] Thus, Bcl-3 negatively regulates TNF-α transcription. Mechanistically, increased expression of Bcl-3 enhances p50 homodimer stabilization. Subsequently, Bcl-3-p50 homodimer complex binds to TNF-α promotor region that in turn inhibits the TNF-α and consequently the NF-κB transcriptional activity.[164, 266] In contrast, long term *ex vivo* exposition of human monocytes to ethanol (7 days; 25 mM) leads to enhanced TNF-α mRNA and protein expression.[267] This data support the thesis that alcohol has biphasic effect on the immune response depending on the extent of alcohol intake or abuse, respectively.

3.3.3 Acute alcohol intoxication causes hyporesponsiveness of monocytes to secondary hit

Alcohol-induced immunosuppressive modulation of immune response could lead in context of abdominal or penetrating injury to an immediate spreading of pathogens and pathogen-derived molecules through the body that in turn could cause e.g. sepsis. An evaluation of inflammasome activation is another approach to test the immunocompetence of monocytes. Initial and subsequent secondary tissue damage

initiate systemic and local release of endogenous DAMPs and exogenous PAMPs.[10, 264] These trigger the assembly of intracellular multiprotein complexes called inflammasomes. In monocytes, NLRP3 inflammasome can be activated by well-studied two-steps inflammasome pathway and alternative one-step inflammasome pathway, however, in macrophages and Dendritic cells, only the two-steps inflammasome activation is known.[268, 269] The two-steps NLRP3 inflammasome activation runs followingly: First, Through TLR4- or TNF receptor-mediated transduction, DAMPs and PAMPs activates the NF-κB signaling, which results in increased expression of NLRP3, inactive pro-IL-1β, and pro-IL-18. Second, NLRP3 is activated by wide range of stimuli following the priming step, including ATP, ROS, mitochondrial DNA, or dsDNA. This leads to NLRP3 inflammasome complex assembly and activation of caspase-1. In turn, active caspase-1 proteolytically cleaves the inactive pro-IL-1β and pro-IL-18 to their active forms that are subsequently released in extracellular space, where they activate and recruit further immune cells.[10, 268, 270, 271]

We have shown that acute alcohol intoxication does not change the level of active caspase-1 in $CD14^+$ monocytes during the entire observation period.[47] An *ex vivo* stimulation of these monocytes with LPS and ATP results in significant increase their caspase-1 level. However, this upregulation is significantly higher in the non-alcohol intoxicated subjects, indicating an impairment of NLRP3 inflammasome activation by impact of alcohol.[47] This is in line with our recent *in vitro* study by Hörauf *et al.*, where we have shown a downregulation of caspase-1 expression in ethanol pretreated (170 mM) human HepG2 liver cells following LPS and ATP administration.[272] Alcohol-induced tolerance towards different PAMPs has been also demonstrated in other studies.[164, 166, 273] This may be caused by innate immune memory,[274] whereby the first hit (here alcohol) may reprogram the innate immune cells resulting in an adaptation of the response to the subsequent second hit (here LPS and ATP). Although the underlying mechanism still remain to be elucidated, some studies indicate that this adaptation may be induced by downregulation of TLR surface expression and upregulation of Toll-interacting protein with subsequent hypophosphorylation of IL-1 receptor-associated kinase and downregulation of canonical NF-κB pathway.[275, 276]

Concurrent to the evaluation of caspase-1 activation, we evaluated the systemic IL-1β and its level following whole blood stimulation with LPS.[47] We have shown that the systemic IL-1β level has been below the detection limit of 3.91 pg/ml over the entire observation period. On the other hand, stimulating the blood *ex vivo* with LPS leads to significant increase of IL-1β. This increase remains stable over the first six hours of the experiment inclusive the non-alcohol intoxicated control (T0). However, the IL-1β level decreases from 24 hours and is significantly lower at 48 hours compared to T0, suggesting an immunosuppressive effect of acute alcohol intoxication in later time course, when the blood alcohol level is negative. Additionally, LPS-induced decrease of IL-1β level at 48 hours positively correlates with the TLR4 surface presentation on monocytes. Thus, monocytes may response to a lesser extent toward TLR4 ligands such a LPS and mitigate the transcription and translation of pro-IL-1β in NF-κB-dependent manner.[47] The concurrent stable level of active caspase-1 indicates that the inflammasome assembly has not been inhibited. Reduced IL-1β level may be caused either by lack of inactive pro-IL-1β or by inhibition of IL-1β secretion. Mitra and Sarkar found purinergic receptor P2X7 to regulate the secretion of bioactive mature IL-1β.[277] Additionally, although IL-1β is mainly produced by monocytic immune cells in NLRP3/caspase-1-dependent manner, there is increasing evidence that IL-1β can be generated by neutrophils or inflammasome independently.[278-280] Taking together, acute alcohol intoxication diminishes the pro-inflammatory immune response to the secondary hit as LPS, which has been used in the current study.[47] Under normal conditions, LPS concentration is the highest in the intestinal lumen, where the gut bacteria reside, and very low or undetectable in the circulation.[281-283] In context of severe traumatic injury, bacterial translocation and their metabolites into circulation of acutely alcohol intoxicated patients possess a higher risk for the development of inflammatory secondary complications due to alcohol-induced diminished immunocompetence. However, the underlying mechanism of attenuated caspase-1 and IL-1β following LPS stimulation remain elusive. Future acute alcohol studies should include the evaluation of intracellular level of pro-IL-1β and IL-1β as well as the IL-1β secretion mechanism from the cell. Monocyte-independent production of IL-1β should be investigated also.

3.3.4 Acute alcohol intoxication diminishes monocyte adhesion *ex vivo*

In context of traumatic injury or infection, monocytes are activated, leave the circulation, and migrate to the site of injury or infection to clear the tissue. Thereby, the adhesion step is critical because without the adhesion and arrest of monocytes on the endothelium, monocytes are not able to extravasate.[284] This in turn, would impede the local immune response and lead to further tissue damage and spreading of the infection. Since alcohol intake has immunosuppressive properties, it can be assumed that that ethanol might affect the adhesive capacity of monocytes. Thus, we isolated $CD14^+$ monocytes from the alcohol intoxicated subjects and evaluated their adhesive capacity to lung carcinoma cell line A549 *ex vivo*.[47] We have shown that the $CD14^+$ monocytes significantly less adhere to the cells four, six, and 48 hours after starting drinking alcohol. These were the time points, when the participants reached the highest alcohol blood levels (1.11 ± 0.05‰ and 0.83 ± 0.06‰, respectively) and on the other hand, when no more alcohol was detectable in blood.[47] This suggests that an acute alcohol intoxication not only impairs the monocyte adhesion acutely with increased blood alcohol levels but also sustainably when the individuals are no more alcohol intoxicated. Study by Imhof *et al.* shows similar results.[285] Monocytes obtained from male and female subjects who drank daily 30 and 20 grams of ethanol per day over the period of three weeks migrate significantly less towards MCP-1 *ex vivo* and express significantly less E-selectin, an adhesion molecule.[285] Using a carrageenan air pouch model in mice, intravenous administration of 1.5 gram ethanol per kilogram of body weight inhibits the leukocyte recruitment to the site of locally induced inflammation and the endothelial cell activation, reflected by downregulation of E-selectin and vascular cell adhesion molecule (VCAM)-1 expression.[286] *In vitro*, isolated human monocytes that were added to ethanol-pretreated (0.5%) and TNF-stimulated primary cultures of adult human dermal microvascular endothelial cells reveal up to 80% lower adhesion rate, whereby the endothelial cells secrete less MCP-1, CCL5, and IL-8 compared to non-stimulated endothelial cells.[286] This suggests that alcohol compromises monocyte adhesion on both levels - inhibiting the monocyte and endothelium activation. However, we should consider that alcohol beverages also

contain non-alcoholic compounds such as polyphenols in wine and whiskey.[287] *Ex vivo* stimulation of isolated $CD14^+$ monocytes not at all or only barely increases the adhesion to lung epithelial cells compared to monocytes without endotoxin challenge in our model of acute alcohol intoxication.[47] Treatment of human umbilical vein endothelial cells and human saphenous vein endothelial cells with resveratrol, a polyphenol contained in red wine, reveals a significant downregulation of ICAM-1 and VCAM-1 induced by TNF-α and LPS, respectively.[288] Additionally, de-alcoholized wine significantly diminishes monocyte migration toward MCP-1 *ex vivo*.[285] Therefore, this indicates that the effects we attribute to the ethanol/alcohol may be also caused by non-alcoholic compounds and should be further evaluated.

3.4 Limitations

This study provides solid fundament for understanding the early post-traumatic phenotypic shift of monocytes and their antimicrobial capacity in severely injured patients as well as the impact of CC16 on local and systemic dynamics of monocyte and macrophage subsets, lung damage, and inflammatory mediators in murine model of early trauma-induced sepsis. Moreover, the pharmacologically relevant and realistic model for binge drinking allowed the evaluation of the plasticity of monocyte subsets and functions following acute alcohol intoxication. However, this study has also several limitations.

Although the changes in ratios of circulating monocyte subsets obtained from severely injured patients are significant, by increasing the number of study participants, correlation analyses of monocyte subsets with injury pattern und clinical parameters such as ISS, inflammatory complications, and ARDS. Additionally, phenotypic and functional changes were evaluated only at one time point. It would be reasonable to monitor the patients during their entire hospital stay as the secondary inflammatory complications occur first in later time course.

As discussed in the section ’Reliable murine model for early sepsis following thoracic injury’, there is no single mouse model that can capture all clinical aspects of

complicated pathology of sepsis. Unlike in humans, blunt thoracic injury is not sufficient to induce sepsis in mice.[205] Additionally, patients with severe thoracic injury mostly require artificial respiration, whereas the mice are spontaneously breathing. Therefore, although blunt thoracic injury with subsequent CLP in mice seems to reliably mimic human traumatic settings, a direct comparison should be treated with caution. The immune response between mouse and human differs and thus, results from animal studies cannot be directly translated into human settings. Moreover, immune response is synchronized and coordinated in time and space. Including further time points would provide a valuable insight into kinetics of monocyte and macrophage subsets.

To study the impact of alcohol intoxication on monocyte subsets and the NLRP3 inflammasome activation, healthy subjects drank whisky-coke cocktail every 20 minutes until reaching blood alcohol level of 1‰ after 4 hours. Despite the clear results, including a placebo group would considerably enhance the validity of data. Coke and its ingredients such as sugar, phosphoric acid, or carbonic acid may contribute to the shown immunological changes. Nevertheless, Afshar *et al.* have shown that experimental alcohol intoxication by ethanol mixed with chilled sugar-free flavored seltzer water leads to an early and transient pro-inflammatory state followed by an anti-inflammatory state.[148] This supports our conclusion that the observed effects are attributed to alcohol. Moreover, investigation of alcohol-induced changes in gene expression of pro- and anti-inflammatory mediators and in antimicrobial features (e.g. phagocytosis and generation of ROS) of monocytes would gain the informative value of biphasic effect of alcohol. However, this was not possible by technical and time reasons.

3.5 Conclusions and outlook

This thesis clearly demonstrates that severe traumatic injury induces a phenotypic shift of monocytes towards the pro-inflammatory phenotype in human settings and in murine model of blunt thoracic trauma with CLP-induced sepsis. An acute alcohol intoxication,

which is diagnosed in approximately 50% of patients admitted to emergency department, leads initially to higher abundance of pro-inflammatory phenotype that in turn differentiates into a subset with anti-inflammatory features. The elevated generation of ROS by the monocyte subsets following severe traumatic injury supports the pathogen clearance on the one hand, may also contribute to the tissue damage on the other hand. However, the data following *ex vivo* stimulation with PMA indicate that intermediate monocytes may also contribute to higher susceptibility to secondary infections by their hyporesponsiveness to secondary hit. Whether the ratios of the subsets to each other and the subset-specific extent of the oxidative burst contribute more to tissue injury or increased risk for infections should be evaluated in further studies. Firstly, significantly more severely injured patients should be included. This would allow performing correlation analyses between the clinical parameters and outcome and the monocytes. Since we have shown that acute alcohol intoxication has immunosuppressive properties, those severely injured patients should be included as well to proof the clinical relevance of alcohol abuse in traumatological settings. Moreover, the patients should be consequently monitored during the entire period of hospital stay. Altogether could provide solid data about the prediction of posttraumatic vulnerability to secondary infections. Additionally, we confirmed the anti-inflammatory and lung tissue-protecting properties of CC16 by its intrapulmonary neutralization following thoracic injury. However, to proof its clinical relevance, a local intrapulmonary supplementation of recombinant CC16 following traumatic injury should be evaluated. If the intrapulmonary application would protect the pulmonary tissue against the damage caused by direct tissue injury or indirectly by exaggerated pro-inflammatory response, this could be prospectively a potent therapeutic approach in everyday clinical practice.

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Declaration

“I hereby declare that I prepared this thesis without the impermissible help of third parties and that none other than the aids indicated have been used; all sources of information are clearly marked, including my own publications. In particular I have not consciously:

- fabricated data or rejected undesirable results,
- misused statistical methods with the aim of drawing other conclusions than those warranted by the available data,
- plagiarized external data or publications,
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