

Systemic and tissue neutrophil responses to experimental trauma



Michel Paul Johan Teuben

Systemic and tissue neutrophil responses to experimental trauma

Michel Paul Johan Teuben

Systemic and tissue neutrophil responses to experimental trauma

PhD Thesis, Utrecht University, The Netherlands.

@ M.P.J. Teuben, 2022

All rights reserved. No part of this thesis may be reproduced, stored or transmitted in any form or by any means without prior written permission from the author. The copyright of the articles that have been published or have been accepted for publication has been transferred to the respective journals.

The research in this thesis was financially supported by: Van Walree Fund (The Royal Netherlands Academy of Arts and Sciences), Alexandre Suerman Stipendium, Girard de Miolet van Coehoorn Foundation, Prof. Michaël-van Vloten Fund, Deutscher Akademischer Austauschdienst, Anna Fund for Orthopedic Research, RWTH Uniklinik Aachen (Start-Up Grant) and the AO (Arbeitsgemeinschaft für Osteosynthesefragen) Foundation.

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged. Financial support by the Anna Fonds, Stichting Arbeidsongevallen, and ChipSoft B.V. is also greatly acknowledged.

ISBN: 978-94-6469-216-7

Cover design, Layout and printed by: Proefschriftmaken.nl

Systemic and tissue neutrophil responses to experimental trauma

Systemische en weefsel reacties van neutrofielen op experimentele trauma

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
maandag 16 januari 2023 des middags te 4.15 uur

door

Michel Paul Johan Teuben

geboren op 21 augustus 1986
te 's-Hertogenbosch

Promotoren:

Prof. dr. L.P.H. Leenen

Prof. dr. L. Koenderman

Prof. dr. med. H.C. Pape (University Hospital Zürich, Zwitterland)

voor mijn lieve

(groot)ouders, broertje en Alba

TABLE OF CONTENTS

<i>Chapter 1</i>	General introduction and outline of the thesis <i>In part adapted from: Patient Saf Surg 2020;14,28.</i>	p. 8
------------------	--	-------------

Part i. Systemic neutrophil responses to injury

<i>Chapter 2</i>	Invasive surgery reduces infarct size and preserves cardiac function in a porcine model of myocardial infarction in pigs <i>Journal of Cell Mol Med. 2015;19(11):2655-63.</i>	p. 44
<i>Chapter 3</i>	Standardized porcine unilateral femoral nailing is associated with changes in PMN activation status, rather than aberrant systemic PMN prevalence <i>European Journal of Trauma Emerg Surg. 2022;48(3):1601-11.</i>	p. 66
<i>Chapter 4</i>	Instant intra-operative neutropenia despite the emerge of banded CD16^{dim}/CD62L^{bright} neutrophils in peripheral blood – an observation in a model of extensive surgery for acute trauma <i>Injury 2021;52(3):426-33.</i>	p. 88
<i>Chapter 5</i>	Altered cell surface receptor dynamics and circulatory occurrence of neutrophils in a small animal fracture model <i>Pathol Res Pract. 2020 Oct;216(10):153108.</i>	p. 116

Part ii. Remote organ and local neutrophil responses to injury

<i>Chapter 6</i>	The impact of intramedullary nailing on the characteristics of the pulmonary neutrophil pool in rodents <i>International Orthopaedics 2020;44, 595–602.</i>	p. 148
<i>Chapter 7</i>	Shift of neutrophils from blood to bone marrow upon extensive experimental trauma surgery <i>Frontiers of Immunology (accepted for publication)</i>	p. 172

<i>Chapter 8</i>	Reamed Irrigation-Aspiration (RIA) evoked transient local hypothermia is associated with enhanced early fracture hematoma immune cell viability and decreased neutrophil activation in porcine intramedullary nailing <i>Journal of Orthopaedic Research (accepted for publication)</i>	p. 200
<i>Chapter 9</i>	General Discussion and future perspectives	p. 226
<i>Addendum</i>	Summary in Dutch (Nederlandse samenvatting)	p. 264
	Review committee	p. 273
	Acknowledgments (Dankwoord)	p. 274
	List of publications/presentations	p. 281
	Curriculum vitae auctoris	p. 299

1

General introduction and outline of the thesis

General introduction and outline of the thesis

In part adapted from:

Lessons learned from the mechanisms of posttraumatic inflammation extrapolated to the inflammatory response in COVID-19: a review

Michel Paul Johan Teuben^{1,2}

Roman Pfeifer²

Henrik Teuber³

Leon Leonardus De Boer^{4,5}

Sascha Halvachizadeh²

Alba Shehu²

Hans-Christoph Pape²

¹ Department of Traumatology, University Medical Centre Utrecht, Utrecht, The Netherlands

² Department of Traumatology, University Hospital Zurich, Zurich, Switzerland

³ Department of Surgery, Cantonal Hospital Thurgau, Frauenfeld, Switzerland

⁴ The Francis Crick Institute, London, United Kingdom

⁵ Imperial College London, London, United Kingdom

Epidemiology and mortality of severe trauma

Trauma is the injuries suffered when a person experiences a blunt or penetrating force. Traumatic injury or trauma is still among the leading causes of death worldwide [1]. More specifically, in the 'WHO Global Status Report on Road Safety' it was reported that due to traumatic injuries, over 1.2 million people die on a yearly basis [2]. In addition, trauma is the number one killer in younger populations [3-5].

Traditionally, trauma mortality is believed to follow a trimodal distribution pattern. This includes an immediate peak of trauma fatalities, which occurs mostly due to catastrophic cardiovascular or neurological injuries, and within the first 60 minutes upon trauma and representing about 45% of overall trauma mortality. Then, a second peak of early deaths (representing 34% of overall fatalities) is seen within the first 4 hours after injury. Also, in this group of patients, mortality is mainly caused by neurological or cardiovascular injury involvement. Finally, the third (late) peak corresponds to fatalities distributed over the subsequent days or weeks and this cohort entails approximately 20% of trauma mortality. This latter group of patients die, in the hospital, as a result of systemic complications including sepsis or multiple organ dysfunction syndrome [6,7].

Due to dramatic advances in both pre- and in-hospital trauma care, outcome of severe trauma patients has improved markedly during the last decades [8-11]. In parallel, documented causes of death and previously described mortality distribution patterns have changed accordingly [12].

In short, during the last decades, the trauma field managed to overcome the burden of fatalities due to early exsanguination [13]. Patients with blunt trauma, dying due to massive blood loss, became seldom in the Western world [13]. Unfortunately, a similar improvement was not seen in patients developing inflammatory complications after severe trauma and modulatory treatment options for involved patients are limited [14,15]. Assuming that currently little can be done to ameliorate the outcome of severe craniocerebral injury, it is tempting to direct the focus of trauma research towards optimization of the care for the severely injured which survived the early peaks of mortality, namely those patients prone to develop inflammatory complications. In order to do so, it is critical to gain insights into the interplay between severe trauma and inflammatory homeostasis and related life-threatening complications. In addition, aberrant immune response to trauma do not only endanger severely injured patients but also contribute to impaired outcome in less severely injured patients due to prolonged hospitalization, concomitant infections and delayed fracture/wound healing.

Trauma ‘=’ inflammation

Trauma victims belong to a heterogeneous population of patients and trauma severity correlates with mortality rates [16]. Mortality in trauma patients with isolated minor injuries is considered as low, whereas mortality rates can rise rapidly in the case of combined injuries [17]. Treatment goals of those patients with minor injuries can be summarized as follows: *to enable optimal restoration of functionality, as soon as possible*. The primary treatment aim of a subgroup of more severely injured patients, known as polytrauma patients, is to prevent mortality. The combination of a specific patient and trauma characteristics determines the risk for in-hospital mortality. According to the Berlin Definition, which is based on an international consensus process, these polytrauma cases are defined as those individuals with significant injuries of three or more abbreviated injury score (AIS) points in at least two different anatomic AIS regions in conjunction with one or more additional variable from five specific physiological parameters (hypotension, level of consciousness, acidosis, coagulopathy and age) [18,19]. As mentioned previously, in-hospital mortality in polytraumatized patients is mainly related to inflammatory/infectious complications such as acute respiratory distress syndrome and multiple organ dysfunction syndrome (MODS) [6,7,12,14]. However, single organ failure such as ARDS, and MODS are the end-station of a complex interplay between inadequate immune homeostasis at different levels. Three different levels of immune homeostasis may be affected and an inadequate transition between these levels and subsequent cumulative decompensation dictates outcome (figure 1):

Figure 1. **Different levels of immune homeostasis**

- i. **Local immune homeostasis**
- ii. **Systemic immune homeostasis**
- iii. **Immune homeostasis in remote organs**

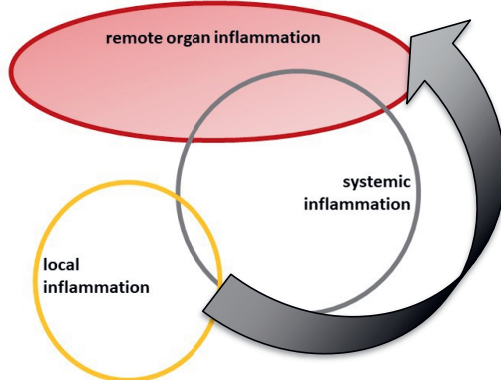


Fig. 1. Overview of interplay between different levels of inflammation. Of note, in specific cases (such as thoracic trauma) an interplay between local and remote organ inflammation may occur as well. In other cases end-organ involvement occurs via systemic inflammation following a local immune response.

Aberrant transition from local immune reactions into systemic and remote organ immune reactions play an essential role in the development of ARDS and MODS. It is tempting to speculate that the human body allows for these mismatches in immune homeostasis because our physiology is not adapted properly to survive high-impact trauma. As evolution stopped long before the introduction of advanced trauma care, essential feedback-mechanisms have not been implemented and selected into our immune response to extreme tissue damage, and immune-mediated auto-destruction may occur.

Pathophysiology of polytrauma

In trauma, tissue damage activates the immune response, with extensive tissue damage invoking systemic inflammation [20-22]. Alterations in local and systemic immune responses after severe trauma are recognized as a physiological reaction to restore homeostasis. The magnitude of these immunological changes correlates with the degree of local and systemic tissue damage [20, 23].

Necrotic cells rapidly release alarmins (Damage Associated Molecular Patterns (DAMPs)), which are endogenous molecules. As their name suggests, alarmins alert the immune system and their ultimate function is to restore homeostasis by promoting regeneration of damaged tissue [20,22,24,25].

In trauma, various relevant alarmins have been identified and characterized. One such alarmin, High Mobility Group Box 1 (HMGB1), has shown chemotactic effects on monocytes, macrophages, and neutrophils, and is a very potent stimulator of immune cell maturation [26]. In addition, Heat Shock Protein (HSP) interacts with several receptors including toll-like receptors and stimulates the secretion of proinflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-1 β [27]. These cytokines are early regulators of the pro-inflammatory immune response to trauma, and both of them induce the release of secondary cytokines, such as IL-6 and IL-8 [20,28]. These cytokines, which are predominantly produced by monocytes and macrophages, mediate a variety of frequently overlapping effects, and their actions can be additive [20,28]. DAMPS and cytokines further activate different immune cells including neutrophils and monocytes via DAMP-receptors [29].

The balance and interplay of these different endogenous molecules dictate the clinical course in trauma patients. Overexpression of either pro-inflammatory or anti-inflammatory mediators

may induce organ dysfunction. Whereas a predominantly pro-inflammatory response leads to the systemic inflammatory response syndrome (SIRS), a predominantly anti-inflammatory reaction leads to the compensatory anti-inflammatory response syndrome (CARS). In the absence of inflammatory complications, concurrent SIRS/CARS responses should be considered a physiological process and balance each other out. However, excessive SIRS can result in immune overreaction, while CARS may lead to immune suppression or paralysis, with a subsequent increased risk of infectious complications [20,22,24,25]. Interestingly, in severe blunt trauma, it has been demonstrated that the early immune response is consistent with simultaneously increased expression of both pro-inflammatory genes (SIRS-response) and anti-inflammatory genes (CARS-response) [30]. Together, this response induces an increase in circulating cytokines resulting in a cytokine storm [31]. Moreover, the development of systemic inflammatory complications, such as organ failure, are associated with the intensity of this cytokine storm [24,24,28,30,31].

Extrapulmonary inflammatory processes affect the pulmonary compartment via the systemic rise of these cytokines [20,32]. One crucial step in the pathophysiology of distant organ damage is the adherence of activated polymorphonuclear leukocytes to post-capillary venules and capillary endothelial cells [33]. This is characterized by leukocyte diapedesis into organ tissue, with subsequent potential release of oxygen radicals and proteases after cell activation [34]. These consequently damage the endothelial layer, resulting in increased capillary permeability, interstitial edema and finally distant organ damage [20,22,34]. Further inflammatory activation causes local collateral damage to parenchymal cells and results in subsequent lung dysfunction [20-22,35]. The collaboration of humoral immune factors and immune cells plays an essential role in the transition from an appropriate to an overwhelming, dysregulated immune response [20-22,35,36].

These pathophysiological inflammatory cascades result in significant changes in the anatomy, mechanics and function of the lung [37]. An initial increase in pulmonary capillary permeability stimulates alveolar flooding and edema. The consequence is the loss of surfactant function. This causes atelectasis and subsequent alveolar instability, resulting in repetitive alveolar collapse and expansion, and finally impaired gas exchange with local and systemic hypoxia [36,37]. The sequential threshold in trauma-evoked inflammation and end-organ involvement are described in figure 2.

Figure 2. Sequential threshold in trauma-evoked inflammation

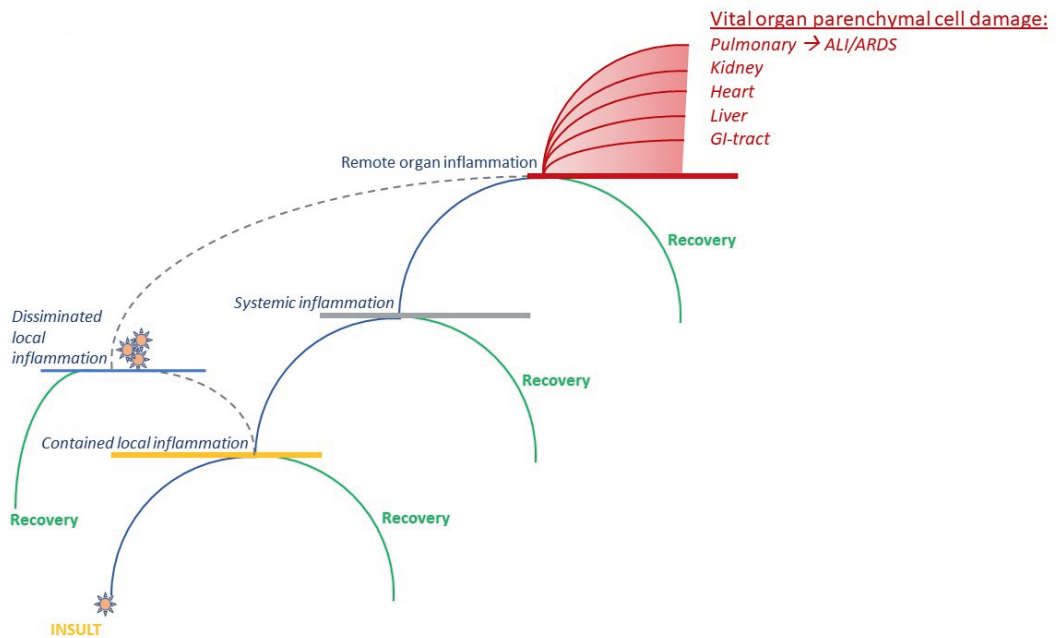


Fig 2. Sequential thresholds in trauma-evoked inflammation. A hypothetical multifactorial model of progression of inflammation upon trauma is presented. Two specific pathways have been described, and an interplay is likely to occur. At all phases restoration of homeostasis is possible and will lead to recovery. The development of differentiated treatment concepts may benefit from this model, guide tailored interventions at different stages of disease progression. *Abbreviations:* ALI, acute lung injury; ARDS, acute respiratory distress syndrome.

First and second hit mechanisms in severe trauma

'First hit'

In traumatology, the term 'first hit' is used to describe the initial insult condition, or trauma, the patient faces. The intensity of the first hit is based on the trauma load: the degree of initial tissue damage, including organ injury, fractures, burn injury, soft tissue injury, and hypovolemic shock. Consequently, local and systemic release of both pro-inflammatory and anti-inflammatory mediators is dependent on the severity of the first hit of the trauma itself [38]. These insults determine the initial trauma load and result in activation of the innate and adaptive immune system that stimulates the local and systemic inflammatory reactions. Moreover, genetic predisposition seems to affect the immune response as well [20]. The subsequent activation of immune cells (polymorphonuclear granulocytes, monocytes and lymphocytes) trigger multifocal processes leading to tissue regeneration and repair. Excessive tissue damage, however, may magnify the extent of local and systemic activation leading to organ dysfunction. Whereas a predominantly pro-inflammatory response leads to an excessive SIRS-response, a predominantly anti-inflammatory reaction may result in an intensified CARS-response and with subsequent immunoparalysis and a consequent increased risk of infectious complications [20-22].

'Second and consecutive hits'

In trauma cases, the secondary activation of various molecular cascades due to an additional insult is known as the second hit- phenomenon. This secondary immune cell activation, or priming, is stimulated by a variety of triggers [20-22,35]. These triggers may be iatrogenic (e.g. mechanical ventilation [39], transfusion of blood products, surgical intervention [24,25]) or non-iatrogenic (e.g. secondary infection [40], thromboembolic complications [41], ischemia/reperfusion injury [42]). Each insult further catalyses the immune response, and depending on severity, may cause an excessive inflammatory response. This process initiates a vicious cycle of local tissue damage, additionally aggravated by systemic hyperactivation ultimately leading to a life-threatening, overwhelming immune response. The consecutive hit principle is summarized in figure 3.

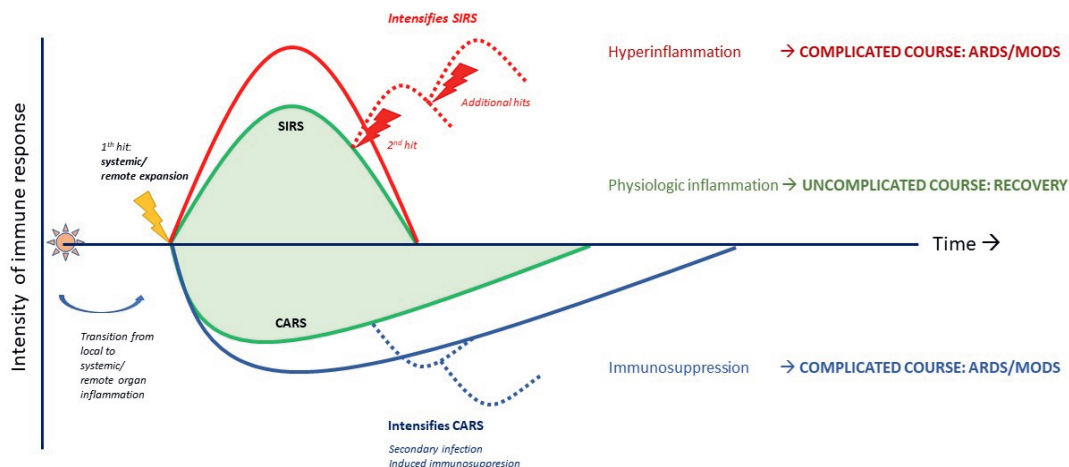
Figure 3. **Model of consecutive insult conditions and outcome determination in trauma**

Fig. 3. Model of consecutive insult conditions and systemic inflammatory responses to trauma. Systemic inflammation may contribute to the restoration of homeostasis and should be considered a physiological process. However, altered inflammatory response may lead to hyperinflammation or a pathological hypo-inflammatory immune response. See text for details and explanations. Abbreviations: SIRS, systemic inflammatory response syndrome; CARS, compensatory anti-inflammatory response syndrome.

Neutrophil dysregulation as a cornerstone of organ dysfunction upon trauma

Neutrophils are the most abundant cells in circulation and autopsy studies after fatal trauma have shown large amounts of neutrophils in vital organs [43-45]. Even in the absence of infection or direct trauma involvement of the specific vital organ. These findings underline the essential role of excessive neutrophil organ invasion in the development of life-threatening inflammatory complications after trauma. [43-46].

Of note, massive pooling of neutrophils in vital organs does not result in organ damage on itself. Additional excessive activation of these cells, however, is required to cause collateral damage to organs parenchymal cells, eventually leading to organ dysfunction [20,22,38,46]. Therefore, prerequisites for maintenance of adequate neutrophil homeostasis include regulation of both (i) neutrophil numbers in different compartments and (ii) neutrophil activation at all

different levels (local, systemic and remote organ). Injury-evoked regulatory mismatches of these processes eventually form the basis for inflammatory complications.

Regulation of circulatory neutrophil numbers after trauma

Neutrophils belong, together with eosinophils and basophils, to a subtype of leukocytes referred to as polymorphonuclear leukocytes (PMNs) or granulocytes as these cells have a characteristic multilobulated shaped nucleus. In circulation, neutrophils largely outnumber eosinophils and basophils, as well as all other leukocytes. More specifically, under homeostatic conditions neutrophils account for 60-70% of circulating leukocytes, whereas during inflammation this number can rise up to 90% of all systemic leukocytes [47]. Neutrophils are produced in the bone marrow and approximately 60 percent of bone marrow immune cells comprise of granulocytes and their precursors [48]. After mobilization, cells stay in the circulation, and thereafter neutrophils migrate into the tissue compartment and/or are cleared [49]. So, regulation of circulatory neutrophil numbers depends on adequate balancing between the mobilization of cells into circulation and withdrawal of cells due to cell death or tissue migration. In the case of acute systemic inflammation, however, striking shifts in neutrophil numbers in different compartments occur [20, 21, 50-53]. Clinical studies have shown that the intensity of the initial systemic immune response largely dictates outcome in severely injured trauma victims [54]. Altered leukocyte, and more specifically neutrophil numbers in peripheral blood is associated with impaired outcome. A prompt decline in circulatory neutrophil numbers upon trauma is linked with the development of inflammatory complications [51,52]. However, not only leukopenia, but also persistent leukocytosis upon trauma (both reflecting a state of inadequate innate immune homeostasis) is associated with impaired outcome [55,56].

Circulatory neutrophil receptor expression dynamics and diversity after trauma

Besides quantitative fluctuations within the circulatory neutrophil pool after trauma, qualitative alterations have been identified as well. Flow analysis and especially cell surface receptor expression analysis, enables not only the determination of (I) neutrophil activation status but also further characterization of populations such as (II) maturation status, (III) mobilization and (IV) viability. Gaining insights into receptor expression dynamics after insult is essential to further understand the cellular basis of immune deregulation after severe trauma. *In vivo*

analysis and previous studies on different models of inflammation led to basic knowledge of neutrophil receptor alterations after insult. Neutrophil receptor status dynamics in trauma, however, are currently relatively unexplored. Relevant human (and specific porcine markers without correlating analogue human receptors) have been described hereafter and are summarized in figure 4.

Figure 4. Overview of neutrophil markers to determine neutrophil characteristics

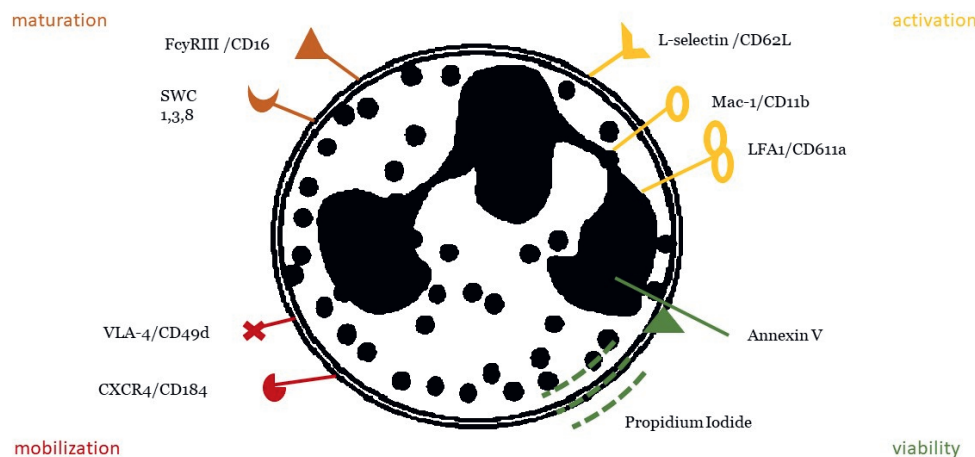


Fig. 4. An overview of key neutrophil markers to study cell and population characteristics. Maturation, mobilization, activation and viability markers are displayed.

I. Neutrophil maturation: FcγRIII, ITGB-1, VLA-4, CD54, CD44, Swine Workshop Cluster antibodies

Granulopoiesis has been described to occur in the bone marrow and neutrophils are derived from multipotent hematopoietic stem cells, as are other leukocytes, erythrocytes and platelets [57]. Myeloblasts are considered the first committed cell type in the granulocyte lineage and these cells have the capacity to divide and differentiate. Further differentiation may occur into promyelocytes and later proceed into myelocytes. As all these precursor cells have the capacity

to divide and proliferate, these subtypes are considered the bone marrow neutrophil mitotic pool. However, myelocytes may lose their capacity to undergo mitosis, and thereby to proliferate. From this stage on, only cell maturation processes can occur. Neutrophil maturation is reflected by altered shaping of the nucleus (banded vs. segmented and hypersegmented neutrophils) [58]. Of note, different PMNs/polymorphonuclear leukocytes (neutrophils, basophils and eosinophils) share common progenitors. An overview of granulopoiesis is provided in Figure 5.

Figure 5. Overview of stem cell differentiation granulopoiesis and neutropoiesis

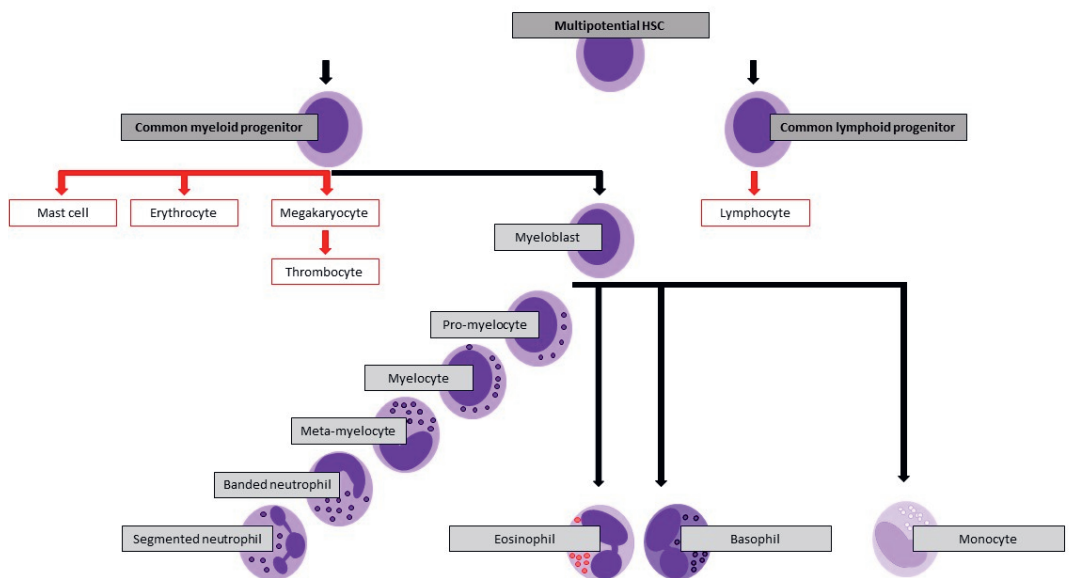


Fig 5. Different leukocyte subtypes as well as their progenitors are displayed. Under homeostatic conditions stem cell differentiation occurs in the bone marrow and cells at different development stages are present in the bone marrow. The different granulocyte types in the peripheral blood were originally named after their characteristics after staining with different dyes and can be identified by differences in morphology (microscopic) and differences in cell surface receptor expression profiles (flowcytometry). Abbreviations: HSC, hematopoietic stem cell.

It has also been demonstrated that FcγRII/CD16 expression rises with maturation. On the other hand, VLA-4/CD49d and ITGB-1/CD29 receptors disappear from the cell surface upon

ongoing maturation [59,60].

HCAM/CD44 expression alterations on bone marrow cells has been studied in pigs and display a biphasic expression pattern during maturation. Initially, during the myeloid progenitor phase high expression levels are seen, whereas cell surface expression levels drop and return to baseline at most mature stages [61]. These findings are in accordance with findings results from human studies on bone marrow hematopoietic cells [60].

The pan leukocyte-marker CD45 also tends to rise during neutrophil maturation [62]. Interestingly, the bone marrow compartment of pigs has been studied in detail as well and resulted in the identification of several specific receptors (named Swine Workshop Cluster) without analogue human receptors [61]. These markers allow for further characterization of cells' maturation status as the expression of SWC-3 and SWC-8 rise during maturation. Furthermore, forward, and sideward scatter profiles, respectively FSC (reflecting cell size) and SSC (reflecting cell complexity), also seem to change upon maturation. More specifically, during the initial stages of neutrophil development signals fluctuate however, after cell-proliferation capacities have been lost, SSC and FSC-signals decrease [60,62].

So, in order to study neutrophil maturation several parameters can be analyzed: (i) functional capacity to proliferate or differentiate by performing *in vitro* experiments, (ii) morphology by executing cytological studies and (iii) expression of neutrophil cell characteristics and surface receptors by the utilization of flow analysis.

II. Neutrophil mobilization and bone marrow retention CXCR4/VLA-4-axis

VLA-4 (CD49d/ $\alpha 4\beta 1$ - integrin) and CXCR4 (CD184) play an essential role in the retention and controlled release from (mature) neutrophils into circulation [63,64]. Neutrophils and progenitors are retained in the bone marrow compartment via VLA-4-receptor functioning and subsequent adherence to vascular adhesion molecule-1 (VCAM-1) which is present on endothelium and bone marrow stroma [63]. In addition, CXCR4 enhances VLA-4/VCAM-1 mediated adhesion and is considered as an other key receptor responsible for intramedullary retention of neutrophil progenitors [63-66]. During medullary maturation, the cell surface expression of CXCR4 on neutrophils diminishes and mobilization is eventually enabled [64]. VLA-4 expression has been described on neutrophil progenitor cells in the bone marrow [60,67,68]. VLA-4 is not expressed on circulatory neutrophils under homeostatic conditions [60,69]. Although, VLA-4-positive neutrophils (progenitors) have been identified in blood in

cases of severe sepsis [70,71]. The origin and functionality of this population, however, is currently unknown, as is their exact pattern of appearance in trauma-induced inflammation.

III. Neutrophil activation and migration: selectins and integrins

Circulatory neutrophils have to extravasate to reach inflammatory sites. The adhesion cascade regulates this process and two receptor families are essential: selectins and integrins. Selectins are type I membrane glycoproteins and this family entails three specific molecules with specific expression profiles and functional characteristics [72]. L(eukocyte)-selectin (CD62L) is expressed on most circulating leukocytes, including neutrophils, and is involved in leukocyte recruitment upon inflammation [73]. *In vitro* studies showed that after activation, shedding of the receptor occurs due to proteolytic cleavage near the surface of cells [74]. Selectins play an important role in the transmigration processes of neutrophils from circulation into the tissue compartment. Neutrophil capturing and rolling along the endothelial surface, an essential step prior to direct neutrophil interaction with the endothelium, highly depends on proper selectin signaling. Additionally, neutrophils can also bind to each other via L-selectin-mediated secondary tethering [75]. The importance of selectin-signaling in neutrophil extravasation is demonstrated by the observation that selectin knockout mice have major neutrophil migration deficits [76].

Neutrophils rolling along the vascular wall, become activated by chemokines on the surface of endothelial cells and this modifies inside-out activation of integrins [73].

The integrin receptor-family is also essential in neutrophil migration processes. These receptors are named integrins as they typically integrate extracellular and intracellular environments, by binding to ligands outside the cell and transmitting signals to intracellular components of cells [77].

Integrins are noncovalently associated heterodimeric cell surface adhesion molecules. Neutrophils express four beta2-integrins, of whom Mac-1 (CD11b/ α M β 2) and LFA-1 (CD11a/ α L β 2) are the most essential and well described [77]. Integrins mediate slow rolling (LFA-1) and subsequent firm arrest (by both LFA-1 and Mac-1) of neutrophils by inside-out activation of these receptors [73]. Eventually, after achievement of firm arrest, neutrophil crawling over the endothelial occurs, until a decent spot for transmigration is reached. This final process is also mediated by Mac-1 receptor dynamics [73,78].

So, selectins are considered as *main brakes* of neutrophils as they are key to mediate slow rolling of cells. Integrin function (e.g. beta2-integrins) however, is reflected by the *handbrake* and determines the final neutrophil adhesion and arrest phase. And upon neutrophil activation, the expression of L-selectin (CD62L) typically decreases due to shedding [79], whereas the expression of integrins (CD11a/CD11b) increases [59,79-81].

A schematic representation of the neutrophil migration and selectin as well as integrin receptor dynamics is provided in figure 6.

Figure 6. **Selectin and integrin receptor dominance in stepwise neutrophil extravasation**

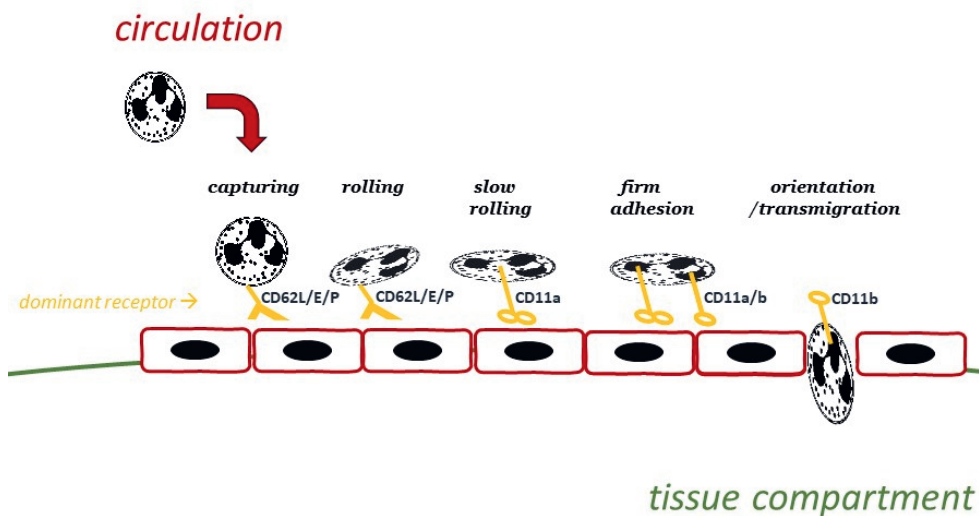


Fig. 6. An overview of different steps in neutrophil extravasation and involved receptors. These essential neutrophil receptors have been the focus of several experiments in the current thesis.

Circulatory neutrophils, on themselves, are not harmful. However, after tissue migration, cells may evoke damage to parenchymal cells. Therefore, behaviour of tissue neutrophils is of upmost interest and unfortunately, data on these cells are relatively scarce as most research has been focussed on circulatory cells. For trauma, pulmonary neutrophils and fracture hematoma neutrophils are of special interest. Altered regulation of pulmonary neutrophils forms the basis

for the development of ARDS, whereas inadequate neutrophil homeostasis in fracture hematoma is associated with impaired fracture healing. Flowcytometry (FACS)-studies allow for analysis of tissue neutrophils as well and the same markers are feasible to determine cell or population characteristics. Furthermore, as with circulatory neutrophils, the concept of neutrophil diversity has been established in tissue neutrophils as well. During the last decade, specific subpopulations have been identified and partly characterized. However, gaining more insight into the (immune modulatory) role and interplay of different neutrophil subsets under homeostatic and inflammatory conditions is essential and may form the basis for more tailored immune modulatory future interventions in critically ill patients.

The concept of neutrophil diversity

It has recently been recognized that neutrophils do not belong to a homogeneous population of immune cells. Both under homeostatic and inflammatory conditions different neutrophil subtypes have been identified. Furthermore, additional subsetting experiments demonstrated specific functional capacities and morphological characteristics. Besides different circulatory neutrophil subsets, tissue-specific populations have been identified as well [82]. The origin, specific functionality and kinetics of the currently identified subsets as well as their overlap are to be explored.

The concept of neutrophil diversity/heterogeneity has relatively recently also been adopted by the field of trauma. Under homeostatic conditions the blood neutrophil pool is very homogenous, however upon systemic inflammation neutrophils are released into circulation and population heterogeneity as well as circulating cell numbers increase [52,83,84].

In short, in trauma and lipopolysaccharide-induced systemic inflammation different systemic neutrophil subsets can be identified by specific CD16 and CD62L-cell surface expression profiles. These subsets have specific morphological features and functionality [83,85]. Altered systemic appearance of myeloid-derived suppressor cell populations with specific functional capacities during acute systemic inflammation has also been observed and linked with impaired clinical outcome [86].

Furthermore, a specific subset of OLFM4-positive neutrophils have been identified in mice and plays a role in ischemia/reperfusion-injury [87,88]. Moreover, increased presence of Olfactomedin 4 (OLFM4)-positive neutrophils has also been observed upon hemorrhagic shock and high OLFM4 expression on circulatory neutrophils is associated with inferior

outcome [87].

Additionally, in mice, both proangiogenic neutrophils (CD49d⁺VEGFR1^{hi}CXCR4^{hi}) as well as antigen-presenting neutrophils (CD11b⁺CD15^{hi}CD10⁻CD16^{lo}) have been identified. [89]

In addition to circulatory neutrophil subsets, novel tissue neutrophil populations with specific functionality have been identified as well. A specific population of neutrophils is margined in the microcirculation of the lung by CXCR4 signaling [90], most likely in order to respond rapidly to pulmonary pathogens [91]. Alternatively, immunosuppressive neutrophils have been identified as well in both pancreatic cancer modelling (P2RX-1-negative neutrophils) and in lipopolysaccharide-induced systemic inflammation (CD16^{bright}/CD62L^{dim}-neutrophils) [50,58,83].

Low-density neutrophils (LDN), which can easily be isolated by density gradient centrifugation, have also been further classified into specific subgroups according to their in vitro capacities: immunosuppressive and pro-inflammatory LDNs [92,93]. CD10- cell surface receptor expression specific neutrophil diversity is also associated with specific T-cell interaction profiles [93].

Furthermore, a specific splenic neutrophil population (CD62L^{low}CD11b^{high}/ICAM-1^{high}), residing in the marginal zone are able to produce cytokines, affect B-cell differentiation and antibody production and also modify neutrophil extracellular traps formation [94,95].

The interplay and potential overlap between currently identified neutrophil subtypes in different species (humans, rodents, pigs) as well as different tissues (blood vs. organs) and different study conditions (homeostasis, acute inflammation and chronic inflammation) is also relatively unclear.

Moreover, according to Ng et al. adequate characterization of neutrophil subsets should include epigenetic regulation/transcriptional profiling, and determination of proliferative capacities, phenotypic profile, tissue localization, effector function, site of origin and maturation status [96]. For the previously addressed neutrophil subsets, most of these essential parameters have not been investigated yet.

Regulation of local and remote neutrophil homeostasis

Tissue damage due to injury evokes a local immune response and neutrophils play an important role in this initial immune activation. Suboptimal local neutrophil responses after trauma are associated with impaired outcome by several mechanisms.

First, due to the direct detrimental effects of inadequate neutrophil homeostasis on local processes such as fracture/wound healing or conquering potential invaders and the risk of infections. Experimental and clinical studies have demonstrated that aberrant local neutrophil influx or functionality are linked with impaired fracture and wound healing [97-100].

Secondly, due to the interplay with systemic neutrophil homeostasis because local inflammation influences the systemic inflammatory response and vice versa. In the specific case of orthopedic trauma, fracture haematoma can alter, and more specifically, increase systemic levels of inflammatory mediators. These mediators could also activate circulating polymorphonuclear neutrophils (PMNs) [20, 22].

Moreover, following the transition from local to systemic inflammation, there is a risk of remote organ involvement as inflammatory responses in end organs such as lungs, liver and kidneys occur. Remote organ involvement, and more specifically damage to organs' parenchymal cells due to immune responses and associated collateral damage form the basis for the development of (multiple) organ failure [101-103].

In order to gain more insight in local and remote organ inflammatory responses to trauma, there is a high need to focus on neutrophil populations and related alterations in vital organs. Especially as histological analysis in trauma patients who died from ARDS revealed substantially increased pulmonary neutrophil influx [44]. And experimental neutrophil depletion in trauma-induced ARDS was associated with improved outcome [104-105].

The lung is a unique organ with respect to neutrophil homeostasis and high neutrophil numbers are encountered even in healthy individuals. It has even been suggested that the lungs are involved in neutrophil de-priming and clearance [106]. In the case of ARDS, however, boosted neutrophil homing and uncontrolled immune cell activation are considered crucial processes, but the exact pathogenesis of ARDS and the specific role of neutrophils remains unclear [22,102]. Despite interesting developments in other fields of medicine, interventions or therapies targeting neutrophil kinetics upon trauma have not been developed yet [107].

Neutrophil modelling in trauma and the rationale for experimental animal experiments

In order to develop potential treatment strategies aimed to modify neutrophil kinetics upon trauma, more knowledge on neutrophil physiology and pathology after trauma is required. Heterogeneity of patient and trauma characteristics, treatment protocols, and complex ethical considerations in acute care situations, make the execution of standardized clinical studies in

trauma is incredibly challenging. Hence, as standardization is a prerequisite for successful experimental research, aimed to identify novel pathways and testing potential future interventions, alternatives for human studies are highly needed in the field of trauma. Studies with suboptimal standardization results in false or scattered results, rather than conclusive findings [108-110].

Animal experimentation allows for the execution of hypothesis-driven studies in a controlled setting. Different models, using various species have been introduced in the past and it is up to the investigators to choose the model which fits best to their research question [111, 112]. In general, animal models are divided into two groups: small animal models (mainly rodents) and large animal models (sheep/pig).

In our view, trauma studies with short (<24hrs) and mid-term (24-72hrs) observation periods are preferably performed in large-animal models, whereas long-term trauma (>72hrs) studies require rodent models. For the current thesis, different new trauma models have been developed, validated and utilized to test specific hypotheses on systemic, local and remote immune homeostasis. These projects are multidisciplinary and multi-institutional, and mostly international. For experiments with short-term observation periods (3-72 hrs after insult), we utilized different pig models and long term observation studies (2 weeks) have been performed in rats. The rationale for these decisions are briefly described hereafter.

In short, owing to their physiological and anatomical resemblance to human, swine models have been extensively used in biomedical research. [113, 114]. Furthermore, due to optimal similarities with respect to cellular bone composition and remodeling, pig models are also frequently utilized for orthopedic experiments [115]. A large number of well standardized pig models of acute systemic inflammation, triggered by ischemia-reperfusion, have been developed by cardiovascular research groups. Moreover, acute systemic inflammation in pigs has also been described upon standardized bacteremia (lipopolysaccharide infection) [116], hypovolemic shock [117], burn-injury [and of course mechanic injuries due to blunt trauma [118], penetrating trauma [119] and blast injuries [120]. Chronic systemic inflammation is studied in the field of oncology, transplant surgery and infectiology. Polytrauma requires – by definition – a combination of injuries and during the last decade, a standardized large animal model with a combined ISS of 27 has been developed and improved over time by the TREAT-Research consortium [121-125]. However, in addition to polytrauma, monotrauma conditions (unilateral midshaft femur fracture) are also studied in a controlled setting by this research

group [126]. Both mono- and polytrauma animals have been observed for 72 hours after insult in a porcine intensive care situation [126]. Furthermore, in order to study even more severe trauma cases, a pre-lethal trauma model has been developed in collaboration with Definitive Surgical Trauma Care (DSTC)TM-courses in Homburg/Saarland (Germany) and Nijmegen (The Netherlands). Long-term observation studies in rats have been performed in Aachen (Germany). These models enabled the author to execute several investigations on neutrophil alterations after different insult conditions.

Next to the logistic difficulties of these complex novel animal models, the development of proper protocols for laboratory analysis of the cellular innate immune response in different species is challenging as well. Especially due to the fact that specific markers for the identification of neutrophils in rats and pigs have relatively been unexplored. In contrast to mice, in which specific markers and antibodies have been developed and validated to identify PMN-populations (e.g. Gr-1 and Ly6G) [127] these markers are less well studied in other species. For blood analysis this is not a major limitation as flowcytometry-based identification of neutrophils by specific forward-sideward scatter profiles by flowcytometry allows for specific neutrophil identification. Although, FSC/SSC-profiles are less prominent in tissue samples and therefore the author had to focus on this issue as well. The introduction and subsequent validation of granulocyte specific markers such as RP-1 in rats [128] and Swine Workshop Cluster antibodies 1,3 and 8 [129-131] in pigs, paved the way for unique studies on the tissue compartment in rats and pigs. The development, validation and utilization of novel animal models as well as unique laboratory analysis on the cellular immune system in traumatized rats and pigs gave us the opportunity to test specific hypotheses in a controlled setting. These different models, which have been described in detail in the method sections of the different chapters in this thesis, are very suitable to perform future proof-of-concept studies on (immune) modulatory interventions in severe trauma.

Scope and outline of this thesis:

As mentioned before, clinical studies in trauma patients highlight the importance of neutrophil homeostasis and regulation after trauma, however, these studies are suboptimal due to standardization issues and ethical limitations. Therefore, the aim of this thesis was to develop, validate and utilize preclinical experimental neutrophil modelling and to determine systemic, remote and local neutrophil responses upon trauma related insult conditions in a controlled setting. Given the similarities in anatomy, physiology and immunology between human and pigs a well established model of myocardial infarction in pigs was utilized to develop and validate *in vitro* methods to study porcine circulatory neutrophils. Cell processing methods, antibody staining protocols as well as flow cytometry analysis plus sorting procedures have been developed and utilized to study systemic neutrophil responses in the field of cardiovascular research first (*Chapter 2*).

These novel research tools have thereafter been implemented in the field of traumaresearch and the impact of different specific insult conditions on systemic neutrophil homeostasis was studied. First, a monotrauma model including standardized intramedullary nailing of a femur fracture, was developed and enabled us to determine neutrophil receptor expression dynamics within the first 72 hours after trauma (*Chapter 3*).

In addition, a more severe model of extensive combined thoraco-abdominal trauma surgery, developed in collaboration with the DSATCTM and executed at the Radboud University Nijmegen (The Netherlands), was used to study specific mobilization patterns of neutrophil subsets in blood. Due to the severity of this trauma model, observation periods were limited to the first 4 hours of intervention (*Chapter 4*).

In order to further enhance observation periods after trauma, and to study long-term neutrophil kinetics upon insult, rodent modelling was mandated. A rat model was developed to study the long-term systemic neutrophil response to standardized intra-medullary nailing and a femur fracture. Neutrophil occurrence as well as cell surface receptor dynamics have been studied during the first 14 days upon intervention (*Chapter 5*).

As organ failure is a result of damage to parenchymal cells of vital organs, and therefore local and remote immune responses in vital organs dictate outcome rather than systemic alterations. However, an interplay between circulatory and remote/local immune responses occur and peripheral blood should be considered as an adequate readout for the systemic immune response. Studying the tissue compartment is challenging due to more complicated cell isolation and processing.

Therefore, after establishing experimental trauma models with different insult conditions and subsequent intensities in both pigs and rats to investigate circulatory neutrophil responses, the focus was expanded to tissue site neutrophil homeostasis.

First, the monotrauma long-term observation model in rats was used to investigate the pulmonary remote neutrophil response to intramedullary nailing for a femur fracture in a 14-day observational study (*Chapter 6*).

Additionally, the interplay between circulatory neutrophil homeostasis and instant bone marrow neutrophil alterations was investigated in a severe model of polytrauma (*Chapter 7*).

After studying the circulatory and remote neutrophil response, local neutrophil responses were studied in a preclinical monotrauma model. More specifically, the impact of different treatment strategies for femur fractures on immune cell viability and neutrophil cell surface receptor kinetics was determined (*Chapter 8*). Finally, **Chapter 9** provides a summary and general discussion of preceding chapters as well as directives of upcoming international projects. This thesis is split up in two parts. The first part describes experiments focussed on clinical and preclinical circulatory neutrophil responses in trauma, whereas the second part describes preclinical studies on remote and local neutrophil responses to trauma.

The previously mentioned experiments have been executed at different facilities and initiated fruitful collaborations with the following research groups:

Chapter 2: Dept. of Cardiology, University Medical Center Utrecht, The Netherlands.

Chapter 3: TREAT-Research Consortium & Harald Tscherne Laboratory/ Dept. of Trauma and Orthopedic Surgery, University Hospital RWTH Aachen, Germany.

Chapter 4: DSTC™-Course and International Association for trauma and Intensive Care/Dept. of Trauma, Radboud University Medical Center, The Netherlands.

Chapter 5: Harald Tscherne Laboratory/ Dept. of Trauma and Orthopedic Surgery, University Hospital RWTH Aachen, Germany.

Chapter 6: Harald Tscherne Laboratory/ Dept. of Trauma and Orthopedic Surgery, University Hospital RWTH Aachen, Germany.

Chapter 7: DSTC™-Course and International Association for trauma and Intensive Care

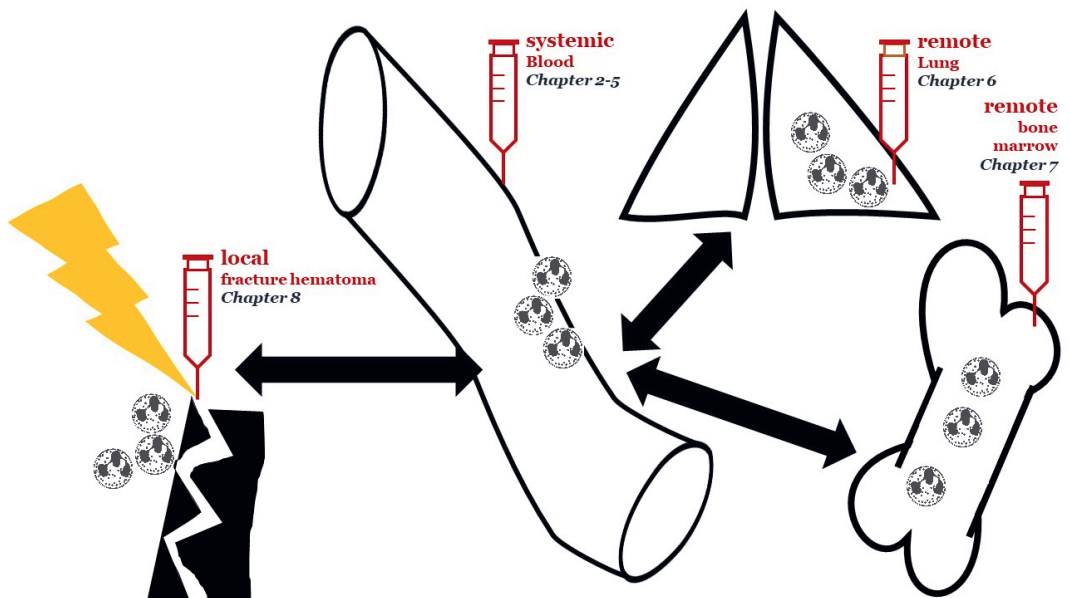
Dept. of Trauma, Hand and Reconstructive Surgery, Saarland University,
Homburg/Saarland, Germany/ Dept. of Trauma and Orthopedic Surgery, University
Hospital RWTH Aachen, Germany.

Chapter 8: TREAT-Research Consortium & Department of Traumatology, University
Hospital Zürich, Switzerland

The TREAT-Research Consortium is a collaboration between the trauma laboratories of the
following institutions:

University Hospital RWTH Aachen, University Hospital Düsseldorf, University Hospital
Frankfurt, University Hospital Ulm, Technical University München/Rechts der Isar Clinic,
University Hospital Zürich, University Hospital Wien.

An overview of the different chapters of this thesis and investigated compartments is
displayed hereafter:



References

1. World Health Organization. Global burden of disease, 2004 update. Geneva: WHO Press; 2008.
2. World Health Organization. Global status report on road safety 2013: supporting a decade of action. Geneva: WHO Press; 2013.
3. Finkelstein E, Corso PS, Miller TR. The incidence and economic burden of injuries in the United States. Oxford; NY: Oxford University Press; 2006.
4. Institute of Medicine Committee on Injury Prevention and Control: Reducing the burden of injury: advancing prevention and treatment. 1999, National Academy Press, Washington, DC.
5. Sakran JV, Greer SE, Werlin E, et al. Care of the injured worldwide: trauma still the neglected disease of modern society. *Scand J Trauma Resusc Emerg Med.* 2012;20: 64.
6. Trunkey DD, Lim RC. Analysis of 425 Consecutive Trauma Fatalities. *J Am Coll Emerg Phys* 1974;Nov/Dec:368-371.
7. Baker CC, Oppenheimer L, Stephens B, Trunkey DD. Epidemiology of Trauma Deaths. *Am J Surg* 1980;140:144–148.
8. Celso B, Tepas J, Langland-Orban B et al. A systematic review and meta-analysis comparing outcome of severely injured patients treated in trauma centres following the establishment of trauma systems. *J Trauma.* 2006;60(2):371-378.
9. MacKenzie EJ, Rvara FP, Jurkovich GJ et al. A national evaluation of the effect of trauma-center care on mortality. *N Eng J Med.* 2006;354(4):366-378.
10. Ali J, Adam R, Butler AK, Chang H, Howard M, Gonsalves D, Pitt-Miller P, Stedman M, Winn J, Williams JI. Trauma outcome improves following the advanced trauma life support program in a developing country. *J Trauma.* 1993 Jun;34(6):890-8; discussion 898-899.
11. Teuben M, Löhr N, Jensen KO, Brüesch M, Müller S, Pfeifer R, Mica L, Pape HC, Sprengel K. Improved pre-hospital care efficiency due to the implementation of pre-hospital trauma life support (PHTLS®) algorithms. *Eur J Trauma Emerg Surg.* 2020 Dec;46(6):1321-1325.
12. Pfeifer R, Teuben M, Andruszkow H, Barkatali BM, Pape HC. Mortality Patterns in Patients with Multiple Trauma: A Systematic Review of Autopsy Studies. *PLoS One.* 2016 Feb 12;11(2):e0148844.
13. Jochems D, Leenen LPH, Hietbrink F, Houwert RM, van Wessem KJP. Increased reduction in exsanguination rates leaves brain injury as the only major cause of death in blunt trauma. *Injury.* 2018 Sep;49(9):1661-1667.
14. Birkner DR, Halvachizadeh S, Pape HC, Pfeifer R. Mortality of Adult Respiratory Distress Syndrome in Trauma Patients: A Systematic Review over a Period of Four Decades. *World J Surg.* 2020 Jul;44(7):2243-2254.

15. Han S, Mallampalli RK. The acute respiratory distress syndrome: from mechanism to translation. *J Immunol.* 2015 Feb 1;194(3):855-60. Erratum in: *J Immunol.* 2015 Jun 1;194(11):5569.
16. Baker SP, O'Neill B, Haddon W, Long WB. The Injury Severity Score: a method for describing patients with multiple injuries and evaluating emergency care. *J Trauma* 1974;14 (3): 187–196.
17. Copes WS, Champion HR, Sacco WJ, Lawnick MM, Keast SL, Bain LW. The Injury Severity Score revisited. *J Trauma.*1988;28 (1): 69–77.
18. The Abbreviated Injury Scale (AIS) 1990 revision, update 1998. Association for the Advancement of Automotive Medicines. Barrington, IL.
19. Pape HC, Lefering R, Butcher N, Peitzman A, Leenen L, Marzi I, Lichte P, Josten C, Bouillon B, Schmucker U, Stahel P, Giannoudis P, Balogh Z. The definition of polytrauma revisited: An international consensus process and proposal of the new 'Berlin definition'. *J Trauma Acute Care Surg.* 2014 Nov;77(5):780-786.
20. Keel M, Trentz O. Pathophysiology of polytrauma. *Injury.* 2005;36(6):691–709.
21. Lord JM, Midwinter MJ, Chen YF, Belli A, Brohi K, Kovacs EJ, Koenderman L, Kubers P, Lilford RJ. The systemic immune response to trauma: an overview of pathophysiology and treatment. *Lancet.* 2014 Oct 18;384(9952):1455–1465.
22. Griensven van M, Krettek C, Pape HC. Immune reactions after trauma. *Eur J Trauma.* 2003;29:181–192.
23. Okeny PK, Ongom P, Kituuka O. Serum interleukin-6 level as an early marker of injury severity in trauma patients in an urban low-income setting: a cross-sectional study. *BMC Emerg Med.* 2015;15:22.
24. Pape HC, Schmidt RE, Rice J, van Griensven M, das Gupta R, Krettek C, Tscherne H. Biochemical changes after trauma and skeletal surgery of the lower extremity: quantification of the operative burden. *Crit Care Med.* 2000;28(10):3441–3448.
25. Pape HC, Grimme K, Van Griensven M, Sott AH, Giannoudis P, Morley J, Roise O, Ellingsen E, Hildebrand F, Wiese B, Krettek C, EPOFF Study Group. Impact of intramedullary instrumentation versus damage control for femoral fractures on immunoinflammatory parameters: prospective randomized analysis by the EPOFF study group. *J Trauma.* 2003;55(1):7–13
26. Cohen MJ, Brohi K, Calfee CS, Rahn P, Chesebro BB, Christiaans SC, Carles M, Howard M, Pittet JF. Early release of high mobility group box nuclear protein 1 after severe trauma in humans: role of injury severity and tissue hypoperfusion. *Crit Care.* 2009;13(6):R174.
27. Zhang Z, Zhang ZY, Wu Y, Schluesener HJ. Immunolocalization of toll-like receptors 2 and 4 as well as their endogenous ligand, heat shock protein 70, in rat traumatic brain injury. *Neuroimmunomodulation.* 2012;19(1):10–19.
28. Hildebrand F, Pape HC, Krettek C. The importance of cytokines in the posttraumatic inflammatory reaction. *Unfallchirurg.* 2005;108(10):793–4 796-803.

29. Prince LR, Whyte MK, Sabroe I, Parker LC. The role of TLRs in neutrophil activation. *Curr Opin Pharmacol.* 2011;11(4):397–403.
30. Xiao W, Mindrinos MN, Seok J, Cuschieri J, Cuenca AG, Gao H, Hayden DL, Hennessy L, Moore EE, Minei JP, Bankey PE, Johnson JL, Sperry J, Nathens AB, Billiar TR, West MA, Brownstein BH, Mason PH, Baker HV, Finnerty CC, Jeschke MG, López MC, Klein MB, Gamelli RL, Gibran NS, Arnoldo B, Xu W, Zhang Y, Calvano SE, GP MD-S, Schoenfeld DA, Storey JD, Cobb JP, Warren HS, Moldawer LL, Herndon DN, Lowry SF, Maier RV, Davis RW, Tompkins RG. Inflammation and Host Response to Injury Large-Scale Collaborative Research Program. A genomic storm in critically injured humans. *J Exp Med.* 2011;208(13):2581–2590.
31. Clark IA, Vissel B. The meteorology of cytokine storms, and the clinical usefulness of this knowledge. *Semin Immunopathol.* 2017;39(5):505–516.
32. Hudson LD, Milberg JA, Anardi D, Maunder RJ. Clinical risks for development of the acute respiratory distress syndrome. *Am J Respir Crit Care Med.* 1995;151(2 Pt 1):293–301.
33. Eppihimer MJ, Granger DN. Ischemia/reperfusion-induced leukocyte-endothelial interactions in postcapillary venules. *Shock.* 1997;8(1):16–25.
34. Roumen RM, Hendriks T, van der Ven-Jongekrijg J, et al. Cytokine patterns in patients after major vascular surgery, hemorrhagic shock, and severe blunt trauma. Relation with subsequent adult respiratory distress syndrome and multiple organ failure. *Ann Surg.* 1993;218(6):769–776.
35. Adams JM, Hauser CJ, Livingston DH, Lavery RF, Fekete Z, Deitch EA. Early trauma polymorphonuclear neutrophil responses to chemokines are associated with development of sepsis, pneumonia, and organ failure. *J Trauma.* 2001;51(3):452–456.
36. Nieman G, Satalin J, Andrews P, Wilcox K, Aiash H, Baker S, Kollisch-Singule M, Madden M, Gatto L, Habashi N. Preemptive mechanical ventilation based on dynamic physiology in the alveolar microenvironment: novel considerations of time-dependent properties of the respiratory system. *J Trauma Acute Care Surg.* 2018;85(6):1081–1091.
37. Bellington GJ. The pulmonary physician in critical care 6: the pathogenesis of ALI/ARDS. *Thorax.* 2002;57(6):540–546.
38. Moore FA, Sauaia A, Moore EE, Haenel JB, Burch JM, Lezotte DC. Postinjury multiple organ failure: a bimodal phenomenon. *J Trauma.* 1996;40(4):501–512.
39. van Wessem KJ, Hennis MP, Heeres M, Koenderman L, Leenen LP. Mechanical ventilation is the determining factor in inducing an inflammatory response in a hemorrhagic shock model. *J Surg Res.* 2013;180(1):125–132.
40. Vicente DA, Bradley MJ, Bograd B, Leonhardt C, Elster EA, Davis TA. The impact of septic stimuli on the systemic inflammatory response and physiologic insult in a preclinical non-human primate model of polytraumatic injury. *J Inflamm (Lond).* 2018;15:11..
41. Mukhopadhyay S, Johnson TA, Duru N, Buzza MS, Pawar NR, Sarkar R, Antalis TM. Fibrinolysis and inflammation in venous Thrombus resolution. *Front Immunol.* 2019;10:1348.

42. van Hout GP, Teuben MP, Heeres M, de Maat S, de Jong R, Maas C, Kouwenberg LH, Koenderman L, van Solinge WW, de Jager SC, Pasterkamp G, Hoefer IE. Invasive surgery reduces infarct size and preserves cardiac function in a porcine model of myocardial infarction. *J Cell Mol Med*. 2015;19(11):2655–2663.
43. Windsor AC, Mullen PG, Fowler AA, Sugerman HJ. Role of the neutrophil in adult respiratory distress syndrome. *Br J Surg*. 1993 Jan;80(1):10-7.
44. Pape HC, Remmers D, Kleemann W, Goris JA, Regel G, Tscherne H. Posttraumatic multiple organ failure--a report on clinical and autopsy findings. *Shock*. 1994 Sep;2(3):228-234.
45. Störmann P, Auner B, Schimunek L, Serve R, Horst K, Simon TP, Pfeifer R, Köhler K, Hildebrand F, Wutzler S, Pape HC, Marzi I, Relja B; This study was performed within the study consortium of the TREAT Research Group. Leukotriene B4 indicates lung injury and on-going inflammatory changes after severe trauma in a porcine long-term model. *Prostaglandins Leukot Essent Fatty Acids*. 2017 Dec;127:25-31.
46. Goris RJ, te Boekhorst TP, Nuytinck JK, Gimbrère JS. Multiple-organ failure. Generalized autodestructive inflammation? *Arch Surg*. 1985 Oct;120(10):1109-1115.
47. Kobayashi SD, DeLeo FR. Role of neutrophils in innate immunity: a systems biology-level approach. *Wiley Interdiscip Rev Syst Biol Med*. 2009;1:309–333.
48. Dancy J, Deubelbeiss K, Harker L, Finch C. Neutrophil kinetics in man. *J Clin Invest* 1976;58:705-715.
49. Vietinghoff von S, Ley K. Homeostatic regulation of blood neutrophil counts. *J Immunol* 2008;181:5183-5188.
50. Hesselink L, Spijkerman R, Wessens van K, Koenderman L, Leenen L, Huber-Lang M, Hietbrink F. Neutrophil heterogeneity and its role in infectious complications after severe trauma. *World J Emerg Surg* 2019;14(24).
51. Pallister I, Dent C, Topley N: Increased neutrophil migratory activity after major trauma: a factor in the etiology of acute respiratory distress syndrome? *Crit Care Med* 2002, 30(8):1717-1721.
52. Botha AJ, Moore FA, Moore EE, Sauaia A, Banerjee A, Peterson VM. Early neutrophil sequestration after injury: a pathogenic mechanism for multiple organ failure. *J Trauma*. 1995;39:411–417.
53. Wengner A, Pitchford S, Furze R, Rankin S. The coordinated action of G-CSF and ELR + CXC chemokines in neutrophil mobilization during acute inflammation *Blood* 2008;181:5183-5188.
54. Napolitano LM, Ferrer T, McCarter RJ Jr, Scalea TM. Systemic inflammatory response syndrome score at admission independently predicts mortality and length of stay in trauma patients. *J Trauma* 2000;49: 647–652.
55. Paladino L, Subramanian RA, Bonilla E, Sinert RH. Leukocytosis as prognostic indicator of major injury. *West J Emerg Med*. 2010 Dec;11(5):450-455.

56. Bochicchio GV, Napolitano LM, Joshi M, Knorr K, Tracy JK, Ilahi O, Scalea TM. Persistent systemic inflammatory response syndrome is predictive of nosocomial infection in trauma. *J Trauma*. 2002 Aug;53(2):245-50; discussion 250-251.
57. Boll IT, Fuchs G. A kinetic model of granulocytopoiesis. *Exp Cell Res* 1970;61(1):147-152.
58. Tak T, Tesselaar K, Pillay J, Borghans JA, Koenderman L. What's your age again? Determination of human neutrophil half-lives revisited. *J Leukoc Biol*. 2013 Oct;94(4):595-601.
59. Elghetany MT. Surface antigen changes during normal neutrophilic development: a critical review. *Blood Cells Mol Dis* 2002;28(2):260-274.
60. Lund-Johansen F, Terstappen LW. Differential surface expression of cell adhesion molecules during granulocyte maturation. *J Leukoc Biol*. 1993 Jul;54(1):47-55.
61. Summerfield A, McCullough K. Porcine bone marrow myeloid cells: phenotype and adhesion molecule expression. *J Leuk Biol* 1997;62(2):176-185.
62. Francis WR, Ireland RE, Spear AM, Jenner D, Watts SA, Kirkman E, Pallister I. Flow Cytometric Analysis of Hematopoietic Populations in Rat Bone Marrow. Impact of Trauma and Hemorrhagic Shock. *Cytometry A*. 2019 Nov;95(11):1167-1177.
63. Petty JM, Lenox CC, Weiss DJ, Poynter ME, Suratt BT. Crosstalk between CXCR4/stromal derived factor-1 and VLA-4/VCAM-1 pathways regulates neutrophil retention in the bone marrow. *J Immunol* 2009;182(1):60406-12.
64. Lawrence SM, Corriden R, Nizet V. The Ontogeny of a Neutrophil: mechanisms of granulopoiesis and homeostasis. *Microbiol Mol Biol Rev*. (2018) 82:e00057–17.
65. Ma Q, Jones D, Springer TA. The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity* 1999;10(4):463-471.
66. Iyer CV, Evans RJ, Lou Q, Lin D, Wang J, Kohn W, et al. Rapid and recurrent neutrophil mobilization regulated by T134, a CXCR4 peptide antagonist. *Exp Hematol* 2008;36(9):1098-1109.
67. Soligo D, Schiro R, Luksch R, Manara G, Quirici N, Parravicini C, et al. Expression of integrins in human bone marrow. *Br J Haematol* 1990;76(3):323-32.
68. Kerst JM, Sanders JB, Slaper-Cortenbach IC, Doorakkers MC, Hooibrink B, van Oers RH, et al. Alpha 4 beta 1 and alpha 5 beta 1 are differentially expressed during myelopoiesis and mediate the adherence of human CD34+ cells to fibronectin in an activation-dependent way. *Blood* 1993;81(2):344-351.
69. Bochner BS, Luscinskas FW, Gimbrone MA, Jr., Newman W, Sterbinsky SA, Derse-Anthony CP, et al. Adhesion of human basophils, eosinophils, and neutrophils to interleukin 1-activated human vascular endothelial cells: contributions of endothelial cell adhesion molecules. *J Exp Med* 1991;173(6):1553-1557.

70. Ibbotson GC, Doig C, Kaur J, Gill V, Ostrovsky L, Fairhead T, et al. Functional alpha4-integrin: a newly identified pathway of neutrophil recruitment in critically ill septic patients. *Nat Med* 2001;7(4):465-470.
71. Lewis SM, Treacher DF, Bergmeier L, Brain SD, Chambers DJ, Pearson JD, et al. Plasma from patients with sepsis up-regulates the expression of CD49d and CD64 on blood neutrophils. *Am J Respir Cell Mol Biol* 2009;40(6):724-32.
72. Kansas GS. Selectins and their ligands: current concepts and controversies. *Blood*. 1996 Nov 1; 88(9):3259-3287.
73. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7:678–689.
74. Smalley DM, Ley K. L-selectin: mechanisms and physiological significance of ectodomain cleavage. *J Cell Mol Med*. 2005 Apr-Jun; 9(2):255-266.
75. Alon, R., Fuhlbrigge, R. C., Finger, E. B. & Springer, T. A. Interactions through L-selectin between leukocytes and adherent leukocytes nucleate rolling adhesions on selectins and VCAM-1 in shear flow. *J Cell Biol* 1996;135, 849-865.
76. Collins RG, Jung U, Ramirez M, Bullard DC, Hicks MJ, Smith CW, Ley K, Beaudet AL Dermal and pulmonary inflammatory disease in E-selectin and P-selectin double-null mice is reduced in triple-selectin-null mice. *Blood*. 2001 Aug 1; 98(3):727-735.
77. Luo BH, Carman CV, Springer TA. Structural basis of integrin regulation and signaling. *Annu Rev Immunol*. 2007;25:619-647.
78. Chesnutt BC, Smith DF, Raffler NA, Smith ML, White EJ, Ley K. Induction of LFA-1-dependent neutrophil rolling on ICAM-1 by engagement of E-selectin. *Microcirculation*. 2006 Mar;13(2):99-109.
79. Neeley S, Hamann K, White S, Baranowski S, Burch R, Leff A. Selective regulation of expression of surface adhesion molecules Mac-1, L-selectin, and VLA-4 on human eosinophils and neutrophils. *Am J Respir Cell Mol Biol* 1993;8:633-639.
80. Maekawa K, Futami S, Nishida M, Terada T, Inagawa H, Suzuki S, et al. Effects of trauma and sepsis on soluble L-selectin and cell surface expression of L-selectin and CD11b. *J Trauma*. 1998; 44:460–468.
81. Kuijpers TW, Tool AT, van der Schoot CE, Ginsel LA, Onderwater JJ, Roos D, Verhoeven AJ. Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood*. 1991 Aug 15; 78(4):1105-1111.
82. Silvestre-Roig C, Fridlender ZG, Glogauer M, Scapini P. Neutrophil Diversity in Health and Disease. *Trends Immunol*. 2019 Jul;40(7):565-583.
83. Pillay J, Kamp VM, Van Hoffen E, Visser T, Tak T, Lammers J, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest*. 2012;122:327–336.

84. Jol S, Hietbrink F, Leenen LPH, Koenderman L, van Wessem KJP. Similar change in platelets and leucocytes 24 h after injury is associated with septic shock a week later. *ANZ J Surg.* 2017;87:190–194.
85. Hampson P, Dinsdale RJ, Wearn CM, Bamford AL, Bishop JRB, Hazeldine J, et al. Neutrophil dysfunction, immature granulocytes, and cell-free DNA are early biomarkers of sepsis in burn-injured patients. *Ann Surg.* 2017;265:1241–1249.
86. Cheng L, Xu J, Chai Y, Wang C, Han P. Dynamic changes in trauma-induced myeloid-derived suppressor cells after polytrauma are associated with an increased susceptibility to infection. *Int J Clin Exp Pathol.* 2017;10:11063–11068.
87. Kassam AF, Levinsky NC, Mallela JP, Angel K, Opoka A, Lahni P, Sahay RD, Fei L, Nomellini V, Wong HR, Alder MN. Olfactomedin 4-Positive Neutrophils Are Upregulated after Hemorrhagic Shock. *Am J Respir Cell Mol Biol.* 2021 Feb;64(2):216-223.
88. Levinsky NC, Mallela J, Opoka AM, Harmon K, Lewis HV, Zingarelli B, Wong HR, Alder MN. The olfactomedin-4 positive neutrophil has a role in murine intestinal ischemia/reperfusion injury. *FASEB J.* 2019 Dec;33(12):13660-13668.
89. Christoffersson G, Vågesjö E, Vandooren J, Lidén M, Massena S, Reinert RB, Brissova M, Powers AC, Opdenakker G, Phillipson M. VEGF-A recruits a proangiogenic MMP-9-delivering neutrophil subset that induces angiogenesis in transplanted hypoxic tissue. *Blood.* 2012 Nov 29;120(23):465346-62.
90. Devi S, Wang Y, Chew WK, Lima R, A-González N, Mattar CN, Chong SZ, Schlitzer A, Bakocevic N, Chew S, Keeble JL, Goh CC, Li JL, Evrard M, Malleret B, Larbi A, Renia L, Haniffa M, Tan SM, Chan JK, Balabanian K, Nagasawa T, Bachelier F, Hidalgo A, Ginhoux F, Kubes P, Ng LG. Neutrophil mobilization via plerixafor-mediated CXCR4 inhibition arises from lung demargination and blockade of neutrophil homing to the bone marrow. *J Exp Med.* 2013 Oct 21;210(11):2321-2336.
91. Yipp BG, Kim JH, Lima R, Zbytnuik LD, Petri B, Swanlund N, Ho M, Szeto VG, Tak T, Koenderman L, Pickkers P, Tool ATJ, Kuijpers TW, van den Berg TK, Looney MR, Krummel MF, Kubes P. The Lung is a Host Defense Niche for Immediate Neutrophil-Mediated Vascular Protection. *Sci Immunol.* 2017 Apr 28;2(10):eaam8929.
92. Scapini P, Marini O, Tecchio C, Cassatella MA. Human neutrophils in the saga of cellular heterogeneity: insights and open questions. *Immunol Rev.* 2016;273:48–60.
93. Marini O, Costa S, Bevilacqua D, Calzetti F, Tamassia N, Spina C, De Sabata D, Tinazzi E, Lunardi C, Scupoli MT, Cavallini C, Zoratti E, Tinazzi I, Marchetta A, Vassanelli A, Cantini M, Gandini G, Ruzzenente A, Guglielmi A, Missale F, Vermi W, Tecchio C, Cassatella MA, Scapini P. Mature CD10+ and immature CD10- neutrophils present in G-CSF-treated donors display opposite effects on T cells. *Blood.* 2017 Mar 9;129(10):1343-1356.
94. Puga I, Cols M, Barra CM, He B, Cassis L, Gentile M, Comerma L, Chorny A, Shan M, Xu W, Magri G, Knowles DM, Tam W, Chiu A, Bussel JB, Serrano S, Lorente JA, Bellosillo B, Lloreta J, Juanpere N, Alameda F, Baró T, de Heredia CD, Torán N, Català A, Torrealbadell M, Fortuny C, Cusi V, Carreras C, Diaz GA, Blander JM, Farber CM, Silvestri G, Cunningham-Rundles C, Calvillo M, Dufour C, Notarangelo LD, Lougaris V, Plebani A, Casanova JL, Ganai SC, Diefenbach A, Aróstegui JJ, Juan M, Yagüe J, Mahlaoui N, Donadieu J, Chen K, Cerutti A. B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat Immunol.* 2011 Dec 25;13(2):170-180.

95. Cerutti A, Puga I, Magri G. The B cell helper side of neutrophils. *J Leukoc Biol.* 2013 Oct;94(4):677-682.
96. Ng LG, Ostuni R, Hidalgo A. Heterogeneity of neutrophils. *Nat Rev Immunol.* 2019;19, 255–265.
97. Bastian O, Pillay J, Alblas J, Leenen L, Koenderman L, Blokhuis T. Systemic inflammation and fracture healing. *J Leukoc Biol.* 2011 May;89(5):669-673.
98. Hesselink L, Bastian OW, Heeres M, Ten Berg M, Huisman A, Hoefer IE, van Solinge WW, Koenderman L, van Wessem KJP, Leenen LPH, Hietbrink F. An increase in myeloid cells after severe injury is associated with normal fracture healing: a retrospective study of 62 patients with a femoral fracture. *Acta Orthop.* 2018 Oct;89(5):585-590.
99. Bastian OW, Croes M, Alblas J, Koenderman L, Leenen LPH, Blokhuis TJ. Neutrophils Inhibit Synthesis of Mineralized Extracellular Matrix by Human Bone Marrow-Derived Stromal Cells In Vitro. *Front Immunol.* 2018 May 1;9:945.
100. Canesso MC, Vieira AT, Castro TB, Schirmer BG, Cisalpino D, Martins FS, Rachid MA, Nicoli JR, Teixeira MM, Barcelos LS. Skin wound healing is accelerated and scarless in the absence of commensal microbiota. *J Immunol.* 2014 Nov 15;193(10):5171-5180.
101. Maier M, Geiger EV, Wutzler S, Lehnert M, Wiercinski A, Buurman WA, Marzi I. Role of lung contusions on posttraumatic inflammatory response and organ dysfunction in traumatized patients. *Eur J Trauma Emerg Surg.* 2009 Oct;35(5):463-469.
102. Ning J, Mo L, Yi B, Gu J, Lu K, Zhou Y, Lai X, Zhao H, Ma D. Therapeutic Whole-body Hypothermia Protects Remote Lung, Liver, and Kidney Injuries after Blast Limb Trauma in Rats. *Anesthesiology.* 2016 Jun;124(6):1360-1371.
103. Ning JL, Mo LW, Lu KZ, Lai XN, Wang ZG, Ma D. Lung injury following lower extremity blast trauma in rats. *J Trauma Acute Care Surg.* 2012 Dec;73(6):1537-1544.
104. Perl M, Hohmann C, Denk S, Kellermann P, Lu D, Braumüller S, Bachem MG, Thomas J, Knöferl MW, Ayala A, Gebhard F, Huber-Lang MS. Role of activated neutrophils in chest trauma-induced septic acute lung injury. *Shock.* 2012 Jul;38(1):98-106.
105. Matute-Bello G, Liles WC, Radella F 2nd, Steinberg KP, Ruzinski JT, Hudson LD, Martin TR. Modulation of neutrophil apoptosis by granulocyte colony-stimulating factor and granulocyte/macrophage colony-stimulating factor during the course of acute respiratory distress syndrome. *Crit Care Med.* 2000 Jan;28(1):1-7.
106. Singh NR, Johnson A, Peters AM, Babar J, Chilvers ER, Summers C. Acute lung injury results from failure of neutrophil de-priming: a new hypothesis. *Eur J Clin Invest.* 2012 Dec;42(12):1342-1349.
107. Németh T, Sperandio M, Mócsai A. Neutrophils as emerging therapeutic targets. *Nat Rev Drug Discov.* 2020 Apr;19(4):253-275.
108. Relja B, Huber-Lang M, van Griensven M, Hildebrand F, Maegele M, Nienaber U, Brucker DP, Sturm R, Marzi I. A nationwide fluidics biobank of polytraumatized patients: implemented by the Network

- "Trauma Research" (NTF) as an expansion to the TraumaRegister DGU® of the German Trauma Society (DGU). *Eur J Trauma Emerg Surg.* 2020 Jun;46(3):499-504.
109. Tsukamoto T, Pape HC. Animal models for trauma research: what are the options? *Shock.* 2009 Jan;31(1):3-10.
110. Harvey EJ, Giannoudis PV, Martineau PA, Lansdowne JL, Dimitriou R, Moriarty TF, Richards RG. Preclinical animal models in trauma research. *J Orthop Trauma.* 2011 Aug;25(8):488-493.
111. Haffner-Luntzer M, Hankenson KD, Ignatius A, Pfeifer R, Khader BA, Hildebrand F, van Griensven M, Pape HC, Lehmcke M. Review of Animal Models of Comorbidities in Fracture-Healing Research. *J Orthop Res.* 2019 Dec;37(12):2491-2498.
112. Bigam-Sadegh A, Oryan A. Selection of animal models for pre-clinical strategies in evaluating the fracture healing, bone graft substitutes and bone tissue regeneration and engineering. *Connect Tissue Res.* 2015 Jun;56(3):175-194.
113. Schook LB, Collares TV, Darfour-Oduro KA, De AK, Rund LA, Schachtschneider KM, Seixas FK. Unraveling the swine genome: implications for human health. *Annu Rev Anim Biosci.* 2015;3:219–244.
114. Gutierrez K, Dicks N, Glanzner WG, Agellon LB, Bordignon V. Efficacy of the porcine species in biomedical research. *Frontiers in genetics.* 2015;6:293.
115. Heino TJ, Alm JJ, Moritz N, Aro HT. Comparison of the osteogenic capacity of minipig and human bone marrow-derived mesenchymal stem cells. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society.* 2012;30:1019–1025.
116. Wyns H, Plessers E, De Backer P, Meyer E, Croubels S. In vivo porcine lipopolysaccharide inflammation models to study immunomodulation of drugs. *Vet Immunol Immunopathol.* 2015 Aug 15;166(3-4):58-69.
117. Hannon J: Hemorrhage and hemorrhagic-shock in swine – a review; in Swindle M (ed): *Swine as Models in Biomedical Research.* Ames, Iowa State University Press, 1992, pp 197–245.
118. Grottke O, Braunschweig T, Philippen B, Gatzweiler KH, Gronloh N, Staat M, Rossaint R, Tolba R. A new model for blunt liver injuries in the swine. *Eur Surg Res.* 2010;44(2):65-73.
119. Chen J, Zhang B, Chen W, Kang JY, Chen KJ, Wang AM, Wang JM. Local and distant trauma after hypervelocity ballistic impact to the pig hind limb. *Springerplus.* 2016 Sep 7;5(1):1497.
120. Axelsson H, Hjelmqvist H, Medin A, Persson JK, Suneson A. Physiological changes in pigs exposed to a blast wave from a detonating high-explosive charge. *Mil Med.* 2000 Feb;165(2):119-126.
121. Hildebrand F, Andruszkow H, Huber-Lang M, Pape HC, van Griensven M. Combined hemorrhage/trauma models in pigs-current state and future perspectives. *Shock.* 2013 Oct;40(4):247-273.

122. Hildebrand F, Weuster M, Mommsen P, Mohr J, Fröhlich M, Witte I, Keibl C, Ruchholtz S, Seekamp A, Pape HC, Flohe S, van Griensven M. A combined trauma model of chest and abdominal trauma with hemorrhagic shock--description of a new porcine model. *Shock*. 2012 Dec;38(6):664-670.
123. Fröhlich M, Hildebrand F, Weuster M, Mommsen P, Mohr J, Witte I, Raeven P, Ruchholtz S, Flohé S, van Griensven M, Pape HC, Pfeifer R. Induced hypothermia reduces the hepatic inflammatory response in a swine multiple trauma model. *J Trauma Acute Care Surg*. 2014 Jun;76(6):1425-1432.
124. Eschbach D, Steinfeldt T, Hildebrand F, Frink M, Schöller K, Sassen M, Wiesmann T, Debus F, Vogt N, Uhl E, Wulf H, Ruchholtz S, Pape HC, Horst K. A porcine polytrauma model with two different degrees of hemorrhagic shock: outcome related to trauma within the first 48 h. *Eur J Med Res*. 2015 Sep 4;20(1):73.
125. Horst K, Simon TP, Pfeifer R, Teuben M, Almahmoud K, Zhi Q, Santos SA, Wemmers CC, Leonhardt S, Heussen N, Störmann P, Auner B, Relja B, Marzi I, Haug AT, van Griensven M, Kalbitz M, Huber-Lang M, Tolba R, Reiss LK, Uhlig S, Marx G, Pape HC, Hildebrand F. Characterization of blunt chest trauma in a long-term porcine model of severe multiple trauma. *Sci Rep*. 2016 Dec 21;6:39659.
126. Störmann P, Wagner N, Köhler K, Auner B, Simon TP, Pfeifer R, Horst K, Pape HC, Hildebrand F, Wutzler S, Marzi I, Relja B. Monotrauma is associated with enhanced remote inflammatory response and organ damage, while polytrauma intensifies both in porcine trauma model. *Eur J Trauma Emerg Surg*. 2020 Feb;46(1):31-42.
127. Fleming TJ, Fleming ML, Malek TR. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J Immunol*. (1993) 151:2399–2408.
128. Barnett-Vanes A, Sharrock A, Birrell MA, Rankin S (2016) A Single 9-Colour Flow Cytometric Method to Characterise Major Leukocyte Populations in the Rat: Validation in a Model of LPS-Induced Pulmonary Inflammation. *PLoS ONE* 2016; 11(1): e0142520.
129. Haverson K, Saalmüller A, Alvarez B, Alonso F, Bailey M, Bianchi AT, Boersma WJ, Chen Z, Davis WC, Dominguez J, Engelhardt H, Ezquerro A, Grosmaire LS, Hamilton MJ, Hollemweguer E, Huang CA, Khanna KV, Kuebart G, Lackovic G, Ledbetter JA, Lee R, Llanes D, Lunney JK, McCullough KC, Molitor T, Nielsen J, Niewold TA, Pescovitz MD, de la Lastra JM, Rehakova Z, Salmon H, Schnitzlein WM, Seebach J, Simon A, Sinkora J, Sinkora M, Stokes CR, Summerfield A, Sver L, Thacker E, Valpotic I, Yang H, Zuckermann FA, Zwart R. Overview of the Third International Workshop on Swine Leukocyte Differentiation Antigens. *Vet Immunol Immunopathol*. 2001 Jul 20;80(1-2):5-23.
130. Saalmüller A, Pauly T, Lunney JK, Boyd P, Aasted B, Sachs DH, Arn S, Bianchi A, Binns RM, Licence S, Whyte A, Blecha F, Chen Z, Chu RM, Davis WC, Denham S, Yang H, Whittall T, Parkhouse RM, Dominguez J, Ezquerro A, Alonso F, Horstick G, Howard C, Zuckermann F, et al. Overview of the Second International Workshop to define swine cluster of differentiation (CD) antigens. *Vet Immunol Immunopathol*. 1998 Jan 30;60(3-4):2072-28.
131. Summerfield A, Haverson K, Thacker E, McCullough KC. Differentiation of porcine myeloid bone marrow haematopoietic cell populations. *Vet Immunol Immunopathol*. 2001 Jul 20;80(1-2):121-129.

i.

Systemic neutrophil responses to injury

Chapter 2

Invasive surgery reduces infarct size and preserves cardiac function in a porcine model of myocardial infarction in pigs

Gerardus P.J. van Hout¹
Michel P.J. Teuben²
Marjolein Heeres²
Steven de Maat³
Renate de Jong⁴
Coen Maas³
Lisanne H. J. A. Kouwenberg¹
Leo Koenderman²
Wouter W. van Solinge³
Saskia C. A. de Jager¹
Gerard Pasterkamp^{1, 3}
Imo E. Hoefer^{1, 3}

¹ Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht, The Netherlands

² Department of Respiratory Medicine, University Medical Center Utrecht, Utrecht, The Netherlands

³ Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, The Netherlands

⁴ Department of Anesthesiology, Erasmus Medical Center, Rotterdam, The Netherlands

Abstract:

Reperfusion injury following myocardial infarction (MI) increases infarct size (IS) and deteriorates cardiac function. Cardioprotective strategies in large animal MI models often failed in clinical trials, suggesting translational failure. Experimentally, MI is induced artificially and the effect of the experimental procedures may influence outcome and thus clinical applicability. The aim of this study was to investigate if invasive surgery, as in the common open chest MI model affects IS and cardiac function. Twenty female landrace pigs were subjected to MI by transluminal balloon occlusion. In 10 of 20 pigs, balloon occlusion was preceded by invasive surgery (medial sternotomy). After 72 hrs, pigs were subjected to echocardiography and Evans blue/triphenyl tetrazoliumchloride double staining to determine IS and area at risk. Quantification of IS showed a significant IS reduction in the open chest group compared to the closed chest group (IS versus area at risk: $50.9 \pm 5.4\%$ versus $69.9 \pm 3.4\%$, $P = 0.007$). End systolic LV volume and LV ejection fraction measured by echocardiography at follow-up differed significantly between both groups (51 ± 5 ml versus 65 ± 3 ml, $P = 0.033$; $47.5 \pm 2.6\%$ versus $38.8 \pm 1.2\%$, $P = 0.005$). The inflammatory response in the damaged myocardium did not differ between groups. This study indicates that invasive surgery reduces IS and preserves cardiac function in a porcine MI model. Future studies need to elucidate the effect of infarct induction technique on the efficacy of pharmacological therapies in large animal cardioprotection studies.

Introduction

Myocardial infarction (MI) and heart failure (HF) remain the most important cardiovascular causes of death worldwide [1, 2]. Because of improved medical care and revascularization therapy, survival after MI has increased considerably during the past decades [3, 4]. This improved survival increases the risk to develop HF as patients survive with a severely deteriorated cardiac function. To prevent progression into HF and preserve cardiac function post-MI, investigators aim to modulate the predominant mechanisms involved in MI and post-MI healing (reperfusion injury, adverse cardiac remodelling) [5,6].

Before clinical testing of therapeutics is considered, thorough testing in clinically relevant animal models should be performed [7]. In this perspective, large animal MI models are required to confirm findings from studies performed in small animals, as the development of tissue damage post-MI differs in small animals compared to larger mammals [8, 9]. Additionally, large animal models show great similarity with humans regarding hemodynamics, cardiac anatomy (coronary) pharmacokinetics and –dynamics [10, 11]. Large animal models also allow the use of clinical treatment regimens and administration routes along with identical function-related read-outs [12, 13]. Despite these advantages, many compounds that were successful in large animal models eventually failed in clinical trials, suggesting optimization of experimental protocols remains necessary [14].

Numerous methods of inducing myocardial ischemia in large animals have been proposed. Despite abundant variations of ischemia induction, there are two main approaches [11]. The first requires invasive surgery, including lateral or medial sternotomy, to reach the coronary arteries and induce ischemia from an external approach. The second method mimics percutaneous coronary intervention and follows a transluminal approach. The invasive method induces bone trauma and alters intra-thoracic pressure, possibly influencing cardiac physiology whereas trauma induced by the minimal-invasive method is much less extensive [11-13, 15].

Previous reports from small and large animal studies indicate that abdominal skin injury prior to MI limits infarct size (IS) after cardiac ischemia [16, 17]. It has also been established that tissue damage, as can be observed during MI and traumatic injury, attracts circulating leucocytes to the site of injury [18-20]. Furthermore, surgery can cause a decreased *ex vivo* responsiveness of leucocytes [21, 22]. As additional injury is known to influence myocardial IS and temporary immune suppression occurs in patients undergoing surgery, we hypothesized

that medial sternotomy required for external MI induction decreases myocardial IS and preserves cardiac function post-MI. Additionally, we aimed to determine if sternotomy influences the local inflammatory response in the myocardium. This may have significant implications for the interpretation of experimental studies on therapeutic compounds tested in either model.

Material and methods

All animal experiments were approved by the institutional animal welfare committee of the University Medical Center Utrecht and were executed conforming to the ‘Guide for the Care and Use of Laboratory Animals’. Twenty female landrace pigs were evaluated in this study (Van Beek, Lelystad, The Netherlands). Pigs (69.8 ± 1.0 kg) were subjected to MI by transluminal balloon occlusion of the left anterior descending artery (LAD) followed by invasive pressure-volume measurements; 3D-Echocardiography and Evans blue/triphenyl tetrazoliumchloride (TTC) double staining to determine IS and area at risk (AAR) at 72 hrs follow-up. In 10 of 20 pigs, balloon-occlusion was preceded by medial sternotomy.

Surgical procedure

Pre-treatment and anaesthesia protocols have been described elsewhere [12, 23]. In short, all animals were pre-treated with amiodaron for 10 days (1200 mg loading dose, 800 mg/day maintenance), clopidogrel for 3 days (75 mg/day) and acetylsalicylic acid for 1 day (320 mg loading dose, 80 mg/day maintenance). All medication was continued until the end of the study. To prevent unnecessary stress and discomfort, animals were anaesthetized with an intramuscular injection of 10 mg/kg ketamine, 0.4 mg/kg midazolam and 0.014 mg/kg atropine. Venous access was obtained by insertion of an 18G cannula in the ear vein for intravenous administration of 5 mg/kg sodiumthiopental. Depth of anaesthesia was then determined by checking eyelid reflex, response to skin stimulus and laryngeal reflex followed by intubation with an endotracheal tube and balloon-ventilation. Pigs were transported to the operating room where anaesthesia was maintained by intravenous infusion of 0.5 mg/kg/hr midazolam, 2.5 µg/kg/hr sufentanyl and 0.1 mg/kg/hr pancuronium. Pigs were mechanically ventilated and arterial blood pressure and heart rate were checked when performing surgical actions to determine the depth of anaesthesia. Pre-operatively, animals received a fentanyl patch (25 µg/hr). Arterial access was obtained by introduction of an 8F sheath into the carotid artery after surgical exposure. Pigs were then randomly allocated to either an open or closed chest procedure.

Closed chest group

After arterial and venous access were obtained, the closed chest animals were left untouched for 20 min. This corresponds to the time required to perform the sternotomy in the open chest group.

A coronary angiogram of the left coronary tree was acquired using an 8F JL4 guiding catheter (Boston Scientific, Natick, MA, USA). An adequately sized balloon was placed distal to the second diagonal branch and inflated for 75 min. After reperfusion, a catheter was temporarily placed in the coronary sinus via an introducer sheath in the jugular vein to draw blood 60 min. after reperfusion. Animals were observed for 3 hrs post-reperfusion and blood samples were drawn at various time-points. A dwelling catheter was placed in the jugular vein in 3 pigs of each experimental group to allow additional venous blood sampling at 4 and 8 hrs reperfusion. The surgical wound was closed and animals were weaned from anaesthesia. Animals were defibrillated in case of ventricular fibrillation (VF). Body temperature was kept constant between 37.0 and 38.5°C throughout the experiment. Heart rate and arterial blood pressure were measured continuously and documented every 30 min.

Open chest group

The open chest group was treated identical to the closed chest group with the exception that in the 20 min. period after the insertion of the arterial and venous sheaths, a medial sternotomy was performed. After performing the myocardial ischemia/reperfusion protocol as described above, the sternum and the surgical wound were closed and animals were weaned from anaesthesia.

Pressure–volume measurements

Pressure–volume measurements were performed as described recently [23, 24]. In short, animals were again anaesthetized 72 hrs after infarct induction. Arterial access was obtained by introducing an 8F sheath into the carotid artery. A 7F tetra-polar admittance catheter (7.0 VSL Pigtail/no lumen; Transonic Scisense, London, ON, Canada) was inserted into the left ventricle (LV) through the sheath in the carotid artery under fluoroscopic guidance.

Echocardiography

Three-dimensional echocardiography was performed as described before [12, 24, 25]. In short, an X3-1 transducer on an iE33 ultrasound device (Philips, Eindhoven, The Netherlands) was used 72 hrs after reperfusion. Immediately after PV measurements, a medial (re)sternotomy was performed and a gel-filled flexible sleeve was placed directly on the apex of the heart. The depth and sector size were adjusted to fit the complete ventricle. The images were analysed offline using QLab 10.1 (Philips, Eindhoven, The Netherlands) (3DQ advanced) analysis

software.

Area at risk and infarct size

Before exsanguination, the leukocyte adhesion deficiency (LAD) was externally ligated at the exact site of balloon occlusion. The LV was punctured with a sterile needle attached to a 50 ml syringe filled with 2% Evans blue dissolved in 50 ml 0.9% NaCl. The aortic root was clamped distal to the origin of the coronary arteries. Evans blue was infused at a rate of 10 ml/sec. Animals were then killed by exsanguination under anaesthesia. The heart was excised and the LV was cut into 5 equal slices from apex to base. Slices were incubated in 1% TTC (Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands) in 37°C 0.9% NaCl for 10 min. to discriminate between infarct tissue and viable myocardium. After incubation, photographs of the slices were taken at the remote area, AAR and infarcted tissue were quantified using ImageJ software (NIH, Bethesda, MD, USA). Following quantification, infarcted tissue, tissue from the border zone and tissue from the remote area were collected and either conserved in 4% formaldehyde for histological quantification of neutrophils, or snap frozen in liquid nitrogen for cytokine measurements.

Neutrophil numbers, macrophage numbers and inflammatory parameters

Circulating leucocyte numbers from blood drawn at multiple time-points after reperfusion were measured by whole-blood analysis using an automated haematological cell-counter (Cell-Dyn Sapphire; Abbott, Santa Clara, CA, USA). The Cell-Dyn Sapphire is a routine haematology analyzer, which uses spectrophotometry, electrical impedance and laser light scattering to classify blood cells (platelets, erythrocytes and leukocytes).

Neutrophil numbers, macrophage numbers and active caspase-3 positive cells in myocardial tissue were measured in paraffin-embedded histological biopsies that were conserved in 4% formaldehyde for at least 7 days. Histological samples were cut into 5 µm sections using a microtome and sections were deparaffinized. To assess neutrophil numbers, sections were incubated with a porcine-specific monoclonal mouse anti-pig antibody against porcine neutrophils (Clone PM1; BMA Biomedicals, Augst, Switzerland) for 60 min. followed by incubation with Brightvision Poly-AP-antimouse (ImmunoLogic, Duiven, The Netherlands) for 30 min. To determine macrophage numbers, sections were incubated with a monoclonal mouse anti-pig CD107a antibody (Serotec, Raleigh, NC, USA) followed by incubation with the same secondary antibody. Caspase-3 positive cells were assessed using a purified rabbit

anti-active caspase-3 antibody (BD Pharmingen, San Diego, CA, USA) followed by incubation with Brightvision Poly-AP-anti-rabbit (Immunologic) for 30 min. For microscopic visualization, incubation with liquid permanent red (DAKO, Heverlee, Belgium) for 10 min. was performed. For every animal, 10 random pictures were made at 200× magnification and quantified using CellSens software (Center Valley, PA, USA).

Malondialdehyde (MDA) is a marker of lipid peroxidation and oxidative stress and was measured to determine if sternotomy induced less oxidative stress in the myocardium during reperfusion. Malondialdehyde was measured from plasma obtained by centrifugation at $1850 \times g$ for 10 min. at 4°C. To quantify MDA, we used a lipid peroxidation – MDA – kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions.

To assess the inflammatory response in the myocardium, the expression of 9 different cytokines [interleukin (IL)-1 β , IL-4, IL-6, IL-8, IL-10, IL-12p40, tumour necrosis factor (TNF)- α , interferon (IFN)- α and IFN- γ] was measured using a luminex immunoassay (Procarta™ Multiplex; eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Western blotting

High molecular weight kininogen (HMWK) was measured as a proxy of bradykinin release. Plasma samples drawn at baseline and 15 min. reperfusion were diluted 20 times in reducing sample buffer (15.5% Glycerol, 96.8 mM Tris-HCL, 3.1% SDS, 0.003% bromophenol blue, 25 mM Dithiothreitol) and incubated for 10 min. at 95°C. Samples were separated on a 4–12% Bis-Tris gel at 165 V for 65 min. in 3-(N-morpholino)propanesulfonic acid buffer. Proteins were transferred onto Immobilon-FL membranes at 125 V for 60 min. in blotting buffer (14.4 g/l glycine, 3.03 g/l Tris-HCL, 20% ethanol) and blocked with blocking buffer (0.5× Odyssey blocking reagent in Tris-buffered saline) for 1 hr at RT. High molecular weight kininogen was detected by overnight incubation at 4°C with polyclonal affinity purified sheep anti-human HMWK antibody (Affinity Biologicals Inc., Ancaster, ON, Canada). Blots were washed with 0.005% TBS-Tween (TBST), and primary antibodies were detected with Alexa Fluor® 680 Donkey Anti-Sheep IgG (1:7500 in blocking buffer; Life Technologies, Carlsbad, CA, USA). Blots were extensively washed with TBST, followed by washing with water and analysed on a near infra-red Odyssey scanner (LI-COR Biotechnology, Lincoln, NE, USA).

Statistical analysis

All data are expressed as mean \pm SE unless stated otherwise. Differences in mortality were

tested using a Fisher's exact test. Myocardial IS, LV ejection fraction (LVEF) and all other outcome were compared using a Student's t-test for unrelated measurements. All statistical analyses were performed in SPSS statistics version 20.0 (IBM software, Armonk, NY, USA). A two-sided P-value of <0.05 was regarded statistically significant in all analyses.

Results

Mortality and hemodynamics

Twenty pigs were subjected to 75 min. myocardial ischemia-reperfusion injury. Four pigs died before the follow-up period (1 in the closed chest and 3 in the open chest group, all because of the persistent VF during cardiac ischemia). Fisher's exact testing showed that this difference was statistically not significant ($P = 0.292$). This allowed a comparison of nine animals in the closed chest and seven animals in the open chest group.

During the 3-hr observation period post-MI, heart rate did not significantly differ between both groups (Fig. 1A). However, from the onset of ischemia onwards, mean arterial pressure (MAP) in the open chest group was significantly lower than in the closed chest group (Fig. 1B). This difference in MAP reached its peak at 60 min. post-occlusion (71 mmHg versus 107 mmHg, $P = 0.006$) and gradually returned to equal levels by the end of the observational period of 180 min. (85 mmHg versus 84 mmHg, $P = \text{ns}$).

Figure 1

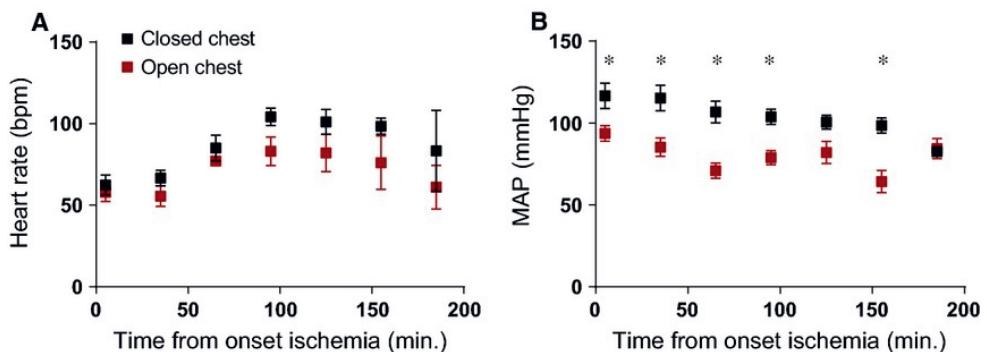


Fig. 1. Hemodynamic parameters were recorded every 30 min. during the experiments. **(A)** Heart rate did not differ significantly from the onset of ischemia to 180 min. post-reperfusion. **(B)** A significant decrease in MAP was observed at multiple time-points during ischemia and reperfusion, peaking at 60 min. of ischemia. Bpm: beats per minute; MAP: mean arterial pressure, * $P < 0.05$.

Myocardial infarct size and cardiac function

Cardiac function and myocardial IS were determined after a follow-up period of 72 hrs. Figure 2A shows representative pictures of the remote area (blue), AAR (red) and infarcted myocardium (white). Quantification of the AAR showed a similar AAR as a percentage of the LV in the open chest and closed chest group ($19.1 \pm 3.0\%$ versus $19.4 \pm 1.3\%$, $P = \text{ns}$; Fig. 2B). Quantification of IS showed a significant reduction in the open chest group compared to the closed chest group when measured as percentage of the AAR ($50.9 \pm 5.4\%$ versus $69.9 \pm 3.4\%$, $P = 0.007$; Fig. 2C) and using the total LV as reference ($9.2 \pm 1.3\%$ versus $13.6 \pm 1.2\%$, $P = 0.024$; Fig. 2D).

Figure 2

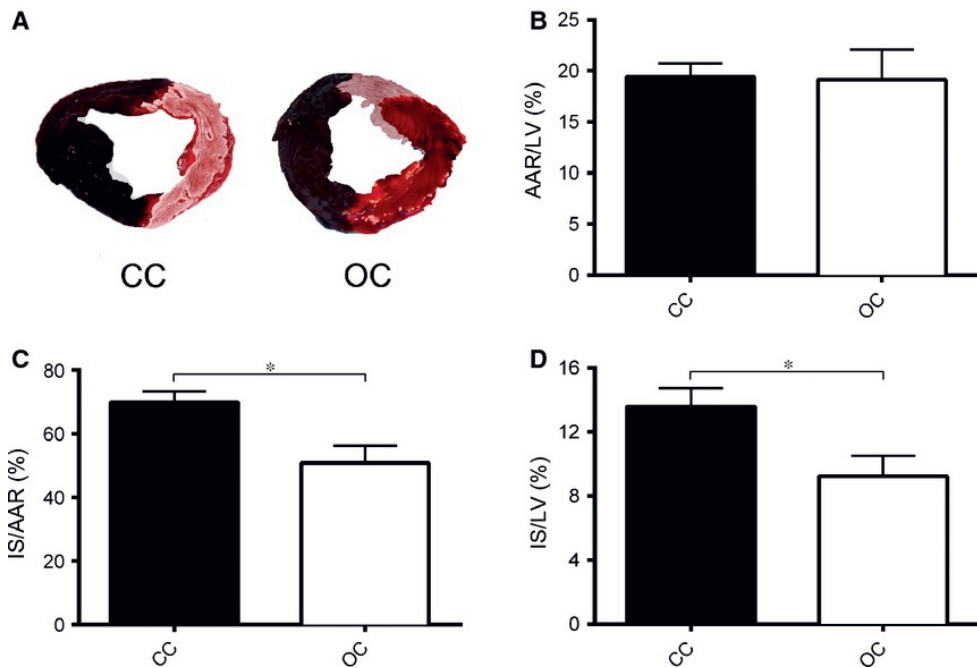


Fig. 2. After 72 hrs of reperfusion, the AAR and IS were determined. (A) Representative pictures of an apical slice of a pig that was subjected to MI without (CC) and with sternotomy (OC). (B) The AAR as a percentage of the total LV was equal between the closed chest and open chest group. (C) The IS as a percentage of the AAR differed significantly between the closed chest and open chest group. (D) The IS as a percentage of the total LV differed significantly between the closed chest and open chest group. CC: closed chest; OC: open chest; AAR: area at risk; IS: infarct size; LV: left ventricle, * $P < 0.05$.

Cardiac function also differed between the two experimental groups. While end diastolic volume did not differ between the open chest and the closed chest group (99 ± 7 ml versus 106 ± 4 ml, $P = \text{ns}$), end systolic volume (51 ± 5 ml versus 65 ± 3 ml, $P = 0.033$) and LVEF ($47.5 \pm 2.6\%$ versus $38.8 \pm 1.2\%$, $P = 0.005$) differed significantly between both groups (Fig. 3A–C).

Figure 3

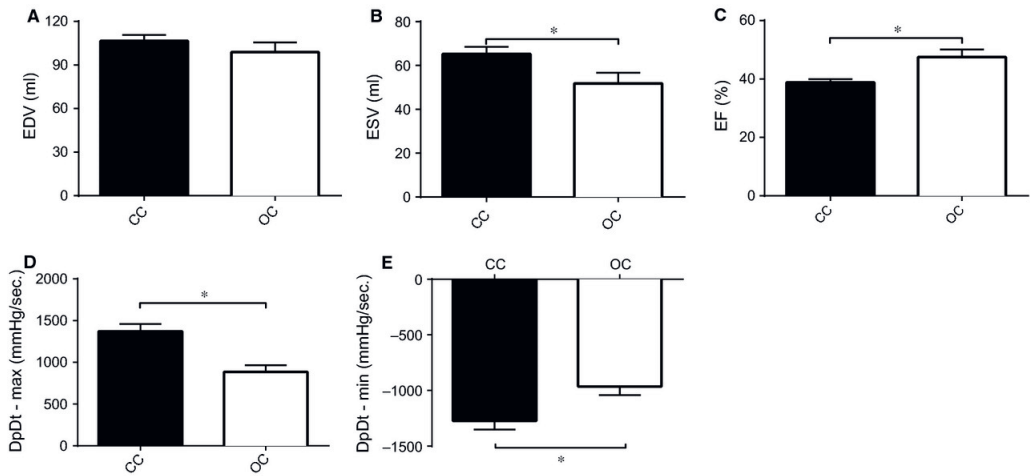


Fig. 3. LV function was measured 72 hrs after reperfusion. (A) End diastolic volume was not significantly lower in pigs subjected to medial sternotomy. (B) End systolic volume was significantly lower in pigs subjected to medial sternotomy. (C) Ejection fraction was significantly higher in the open chest compared to the closed chest group. (D) dPdt-max was lower in the open chest group. (E) dPdt-min was less negative in the open chest compared to the closed chest group. EDV: end diastolic volume; ESV: end systolic volume; EF: LV ejection fraction; ml: milliliter; dPdt-max: rate in pressure change over time during systole; dPdt-min: rate in pressure change over time during diastole; CC: closed chest; OC: open chest, $*P < 0.05$.

Interestingly, the load-dependent rate of pressure increase (dPdt-Max) during systole was lower in the open chest group than in the closed chest group despite a smaller IS and better global cardiac function (886 ± 80 mmHg/sec. versus 1373 ± 87 mmHg/sec., $P = 0.002$). The load-dependent rate of pressure decrease (dPdt-Min) during diastole was less negative in the open chest group compared to the closed chest group (-964 ± 78 mmHg/sec. versus -1273 ± 77 mmHg/sec., $P = 0.018$; Fig. 3D and E). Troponin was measured 2 hrs after reperfusion

and was significantly lower in the open chest compared to the closed chest group (251 ± 44 ng/ml versus 1256 ± 281 ng/ml, $P = 0.008$; Fig. 4A).

Figure 4

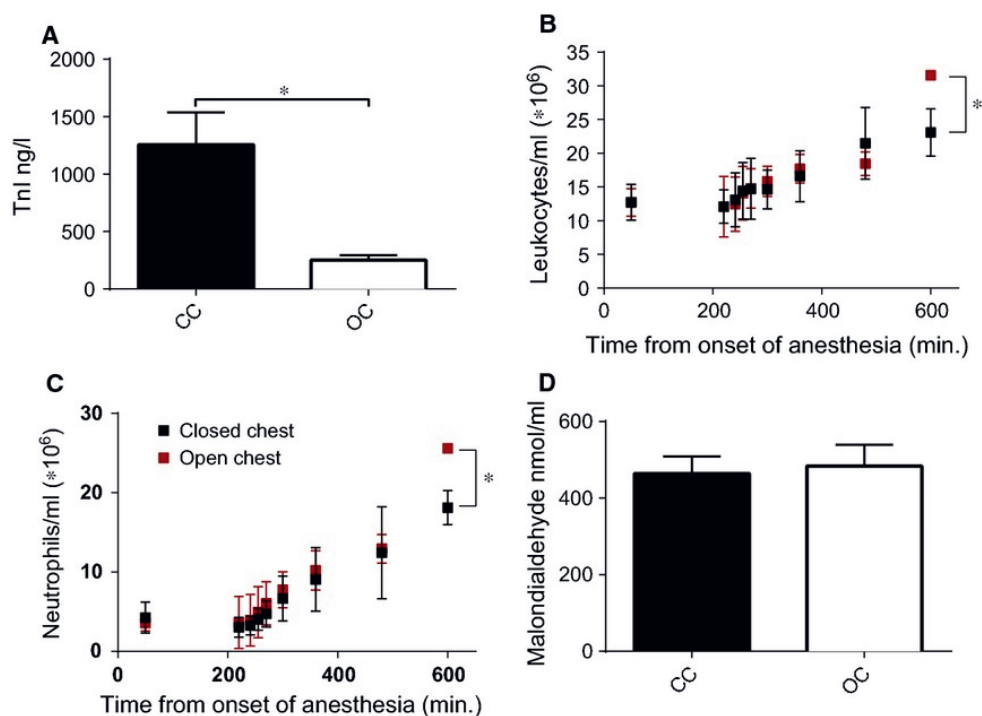


Fig. 4. Different measurements were performed in the systemic circulation. (A) Troponin was significantly higher in the closed chest group compared to open chest group. (B) Systemic leucocyte numbers were measured at baseline and different time-points during ischemia and reperfusion. (C) Systemic neutrophil numbers were measured at baseline and different time-points during ischemia and reperfusion. (D) Lipid peroxidation measured by malondialdehyde concentration in coronary sinus blood was not different between the open chest and closed chest group. TnI: troponin I; ng: nanogram; l: litre; ml: millilitre; nmol: nanomol; CC: closed chest; OC: open chest, * $P < 0.05$.

Inflammatory markers, oxidative stress and bradykinin release

To determine if the two models differed in the systemic inflammatory response post-MI, we measured systemic leucocyte numbers up to 2 hrs reperfusion. As no differences in leucocyte numbers were detected during ischemia and the first 2 hrs of reperfusion (Fig. 4B and C), we also measured systemic leucocyte numbers 4 and 8 hrs post-reperfusion in a subset of pigs

(n = 3 in each group). In the open chest group, circulating leucocyte numbers were significantly higher at 8 hrs reperfusion in the open chest compared to the closed chest group ($31.6 \pm 0.2 \times 10^6$ versus $23.1 \pm 2.0 \times 10^6$ leucocytes/ml, $P = 0.014$; Fig. 4B). Neutrophil numbers were also significantly increased in this group ($25.6 \pm 0.1 \times 10^6$ neutrophils/ml versus $18.1 \pm 1.2 \times 10^6$ neutrophils/ml, $P = 0.040$; Fig. 4C) 8 hrs post-reperfusion. Other leucocyte subtypes did not differ significantly between both groups at any time-point (data not shown). As inflammatory cells are an important source of free radical scavengers at the site of injury, MDA, a marker of oxidative stress, was measured in blood directly drawn from the coronary sinus 1 hr after reperfusion in both groups. However, there was no significant difference in MDA levels between both groups (MDA concentration: 483 ± 55 nmol/ml versus 465 ± 45 nmol/ml, $P = \text{ns}$; Fig. 4D).

To investigate potential differences in the severity of the local immune response, we measured a variety of cytokines known to play a role in the inflammatory phase post-MI. We did not observe any significant differences in any of the measured cytokines in the myocardium. As examples, IL-6 (infarct area, $P = 0.15$; border area $P = 0.40$) and IL-1 β (infarct area, $P = 0.9$; border area $P = 0.25$) concentrations are shown in Figure 5A and B. Similarly, we did not observe any significant difference between open and closed chest procedure animals regarding neutrophil and macrophage numbers in the myocardium 72 hrs after reperfusion (Fig. 5C–F). Finally, we determined cardiomyocyte apoptosis 72 hrs after reperfusion. There were no significant differences between the open chest and closed chest group in caspase-3 positive cells (Fig. 5G and H).

As bradykinin has previously been reported to mediate the cardioprotective effects of abdominal skin incision on reperfusion injury, we measured the bradykinin precursor HMWK. High molecular weight kininogen fragmentation serves as a proxy for bradykinin, with low HMWK levels reflecting fragmentation and high bradykinin release. Compared to baseline, absolute HMWK concentrations were higher in the closed chest compared to the open chest group ($P = 0.043$) and a trend was observed for relative expression levels between groups ($P = 0.095$), indicating that bradykinin release was higher in the open chest group (Fig. 5I and J).

Figure 5

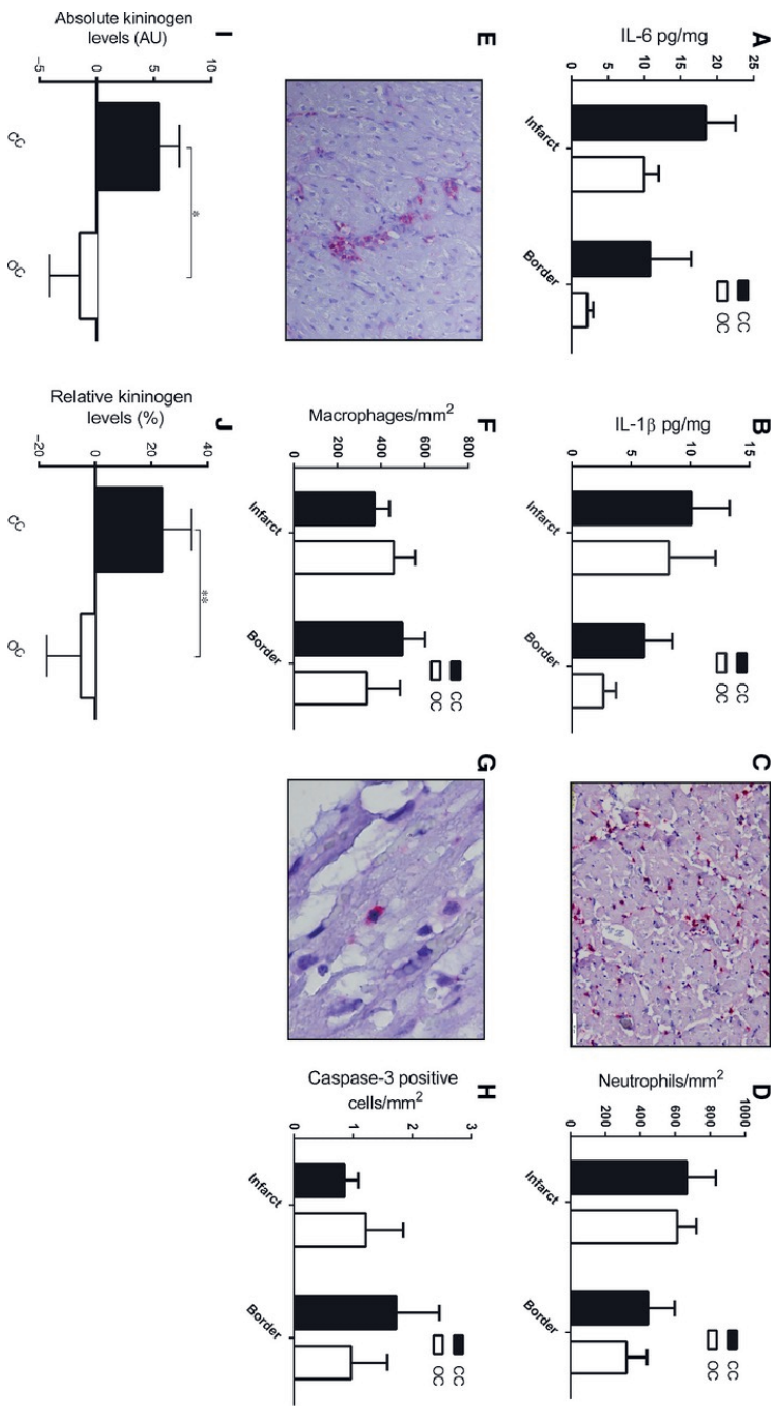


Fig. 5. Local markers of the inflammatory response were measured in the myocardium. **(A)** Myocardial IL-6 content corrected for protein concentration in both the border and infarct zone differed non-significantly between the open chest and closed chest group. **(B)** Myocardial IL-1 β content corrected for protein concentration in both the border and infarct zone differed non-significantly between the open chest and closed chest group. **(C)** Representative picture of histological section of infarcted myocardial tissue containing neutrophils (red cells) 72 hrs post-reperfusion. **(D)** Neutrophil numbers in both the border and infarcted zone of the myocardium are similar between the open chest and closed chest group. **(E)** Representative picture of macrophages (red) resided in the infarcted myocardium. **(F)** Macrophage numbers in both the border and infarcted zone of the myocardium are similar between the open chest and closed group. **(G)** Representative picture of an active caspase-3 positive cell (red). **(H)** Apoptosis of myocardial cells was similar between open chest and closed chest pigs. **(I and J)** Absolute and relative kininogen levels are higher in the closed chest compared to the open chest group. pg: picogram; mg: milligram; AU: arbitrary units; CC: closed chest; OC: open chest, * $P < 0.05$, ** $0.05 < P < 0.1$.

Discussion

For the development and validation of novel cardioprotective strategies aiming at reduced ischemia reperfusion injury, new compounds need to be tested in models that resemble the clinical situation as closely as possible [7, 26]. This study indicates that medial sternotomy reduces IS and preserves cardiac function in a porcine MI model, possibly confounding the effect of pharmacological therapies in this model.

Our observation may partly be explained by the effect of open chest surgery on cardiac pressure, as MAP decreased during the first 3 hrs after cardiac ischemia. Cardiac pressure analysis over time revealed a significant decrease in load dependent dPdt-max and dPdt-min 72 hrs after surgery despite smaller IS and better cardiac function. This indicates that sternotomy unloads the LV and this effect was still measurable after 3 days. However, a reduction in myocardial workload is unlikely to be the only responsible mechanism for the observed IS reduction as previous research reported similar effects of additional trauma on IS, which persisted after blood pressure normalization by saline infusion [27].

Various small and large animal studies have shown that abdominal skin injury preceding experimental MI results in reduced IS [27, 28]. This observed reduction seems to be mediated through nociceptive bradykinin-mediated pathways and can be abolished by pharmacologically inhibiting these pathways [16, 17, 29, 30]. Because of its very short half-life, we were unable to detect circulating bradykinin levels in this study. However, levels of the bradykinin precursor HMWK were significantly lower in the open chest group compared to the closed chest group, possibly reflecting a higher bradykinin release. This suggests that medial sternotomy exerts its cardioprotective effects through increased bradykinin release.

A third, potentially relevant, mechanism is the inflammatory response after MI. Traumatic injury triggers a very complex immune reaction [31, 32] and could therefore alter the inflammatory response that is involved in myocardial reperfusion injury and post-MI repair. However, the number of circulating leucocytes during reperfusion was not decreased in the open chest group, but actually higher at 8 hrs reperfusion. Moreover, after 72 hrs, we neither observed any significant difference in neutrophil and macrophage accumulation in the myocardium nor myocardial cytokine content, two major determinants of post-MI infarct expansion [33-35]. Furthermore, no differences in cardiomyocyte apoptosis were found between the groups.

The free radical burst that occurs within the first hours after reperfusion depends on the activation of inflammatory cells during reperfusion in the damaged myocardium [20]. Malondialdehyde levels in blood drawn from the coronary sinus 1 hr after reperfusion did not differ between the open chest and closed chest group. Although this suggests sternotomy does not significantly influence the inflammatory response post-MI, decreased *ex vivo* cellular responsiveness that has been observed in patients after different surgical procedures could also play a role [21, 22, 36].

Regardless of the mechanisms at hand, possible confounders that may obscure the effect of cardioprotective agents in studies of experimental MI should be avoided as much as possible. Better clinical resemblance of animal models will increase translational value and improve reproducibility and translatability towards clinical application.

Mortality between the 2 groups was not significantly different. However, this lack of statistical significance might be attributable to insufficient power. Mortality is an essential parameter in translational research and survival bias could be a confounder in cardioprotection studies that have high mortality rates. Unfortunately, the cause of the malignant arrhythmias in the non-surviving animals remains unknown, but something that would be worth investigating. However, in this study we were unable to clarify this issue, as we did not store any plasma or tissue of the animals that died before the follow-up time was completed.

Despite our best efforts, we could not evade introducing limiting factors ourselves. First of all, experiments were performed under general anaesthesia, which cannot be avoided in animal models. The anaesthetics used may have a protective effect on the myocardium, although the IS compared to the AAR in the minimally invasive group was reasonably high (approximately 70%). Theoretically, differences in blood loss because of the type of intervention may affect blood pressure and thus IS. However, blood loss in our experiments was minimal (<200 ml) for animals with a circulating blood volume of approximately 5l. Furthermore, we did not find any evidence for a modulation of myocardial inflammation by medial sternotomy, indicating that the reduction in IS cannot be ascribed to this mechanism. However, to fully conclude this, follow-up studies need to be performed that specifically focus on the inflammatory response (e.g. other leucocyte subsets, cellular responsiveness). Finally, we did not administer any pharmacological agent to block bradykinin-mediated pathways or pro-inflammatory pathways to conclusively determine whether any of the two mechanisms could (partly) be held

responsible for the IS reduction after medial sternotomy.

Determining whether these or other key mechanisms play a role in the reduction in IS after medial sternotomy will allow for specifically targeting these pathways. Pharmacological inhibitors could be developed to inhibit these newly discovered cellular signals and determine whether the phenomenon that we have observed in this study, can directly be translated into clinical applications to salvage myocardium in post-MI patients. As this was not the primary purpose of this study, and therefore remains to be elucidated in future studies.

In conclusion, this study shows that medial sternotomy preceding MI in a porcine model reduces IS and preserves cardiac function. As the mechanisms responsible for this observation are not fully elucidated, it remains unclear how our findings affect the outcome of cardioprotection studies with open chest surgery. The ever-evolving techniques of percutaneous coronary interventions enable animal models to increasingly resemble the clinical situation and in combination with our results call for minimally invasive cardiac ischemia models. This may reduce the possibility of false-positive and –negative studies and improve translational value of preclinical research.

References

1. Go AS, Mozaffarian D, Roger VL, et al. Heart disease and stroke statistics - 2014 update: a report from the American Heart Association. *Circulation*. 2014; 129: e28–292.
2. Mendis S, Abegunde D, Yusuf S, et al. WHO study on prevention of recurrences of myocardial infarction and stroke (WHO-PREMISE). *Bull World Health Organ*. 2005; 83: 820–8.
3. Peterson MC, Syndergaard T, Bowler J, et al. A systematic review of factors predicting door to balloon time in ST-segment elevation myocardial infarction treated with percutaneous intervention. *Int J Cardiol*. 2012; 157: 8–23.
4. Lawesson SS, Alfredsson J, Fredrikson M, et al. Time trends in STEMI—improved treatment and outcome but still a gender gap: a prospective observational cohort study from the SWEDEHEART register. *BMJ Open*. 2012; 2: e000726.
5. Mudd JO, Kass DA. Tackling heart failure in the twenty-first century. *Nature*. 2008; 451: 919–28.
6. Sutton MG, Sharpe N. Left ventricular remodeling after myocardial infarction: pathophysiology and therapy. *Circulation*. 2000; 101: 2981–8.
7. Lecour S, Botker HE, Condorelli G, et al. ESC working group cellular biology of the heart: position paper: improving the preclinical assessment of novel cardioprotective therapies. *Cardiovasc Res*. 2014; 104: 399–411.
8. Skyschally A, Van Caster P, Boengler K, et al. Ischemic postconditioning in pigs: no causal role for risk activation. *Circ Res*. 2009; 104: 15–8.
9. Seok J, Warren HS, Cuenca AG, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci USA*. 2013; 110: 3507–12.
10. Munz MR, Faria MA, Monteiro JR, et al. Surgical porcine myocardial infarction model through permanent coronary occlusion. *Comp Med*. 2011; 61: 445–52.
11. Lukacs E, Magyari B, Toth L, et al. Overview of large animal myocardial infarction models (review). *Acta Physiol Hung*. 2012; 99: 365–81.
12. De Jong R, van Hout GPJ, Houtgraaf JH, et al. Intracoronary infusion of encapsulated glucagon-like peptide-1-eluting mesenchymal stem cells preserves left ventricular function in a porcine model of acute myocardial infarction. *Circ Cardiovasc Interv*. 2014; 7: 673–83.
13. McCall FC, Telukuntla KS, Karantalis V, et al. Myocardial infarction and intramyocardial injection models in swine. *Nat Protoc*. 2012; 7: 1479–96.
14. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *N Engl J Med*. 2007; 357: 1121–35.
15. Ishikawa K, Aguero J, Tilemann L, et al. Characterizing preclinical models of ischemic heart failure: differences between LAD and LCx infarctions. *AJP Hear Circ Physiol*. 2014; 307: H1478–86.

Chapter 2

16. Gross GJ, Baker JE, Moore J, et al. Abdominal surgical incision induces remote preconditioning of trauma (RPCT) via activation of bradykinin receptors (BK2R) and the cytochrome P450 epoxigenase pathway in canine hearts. *Cardiovasc Drugs Ther.* 2011; 25: 517–22.
17. Gross GJ, Hsu A, Gross ER, et al. Factors mediating remote preconditioning of trauma in the rat heart: central role of the cytochrome P450 epoxigenase pathway in mediating infarct size reduction. *J Cardiovasc Pharmacol Ther.* 2012; 18: 38–45.
18. Timmers L, Pasterkamp G, De Hoog VC, et al. The innate immune response in reperfused myocardium. *Cardiovasc Res.* 2012; 94: 276–83.
19. Arslan F, de Kleijn DP, Pasterkamp G. Innate immune signaling in cardiac ischemia. *Nat Rev Cardiol.* 2011; 8: 292–300.
20. Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. *Cardiovasc Res.* 2004; 61: 481–97.
21. Versteeg D, Hoefer IE, Schoneveld AH, et al. Monocyte toll-like receptor 2 and 4 responses and expression following percutaneous coronary intervention: association with lesion stenosis and fractional flow reserve. *Heart.* 2008; 6: 770–6.
22. Versteeg D, Dol E, Hoefer IE, et al. Toll-like receptor 2 and 4 response and expression on monocytes decrease rapidly in patients undergoing arterial surgery and are related to preoperative smoking. *Shock.* 2009; 31: 21–7.
23. Van Hout GPJ, Jansen of Lorkeer SJ, Gho JMIH, et al. Admittance-based pressure-volume loops versus gold standard cardiac magnetic resonance imaging in a porcine model of myocardial infarction. *Physiol Rep.* 2014; 2: e00287.
24. Van Hout GPJ, de Jong R, Vrijenhoek JEP, et al. Admittance-based pressure-volume loop measurements in a porcine model of chronic myocardial infarction. *Exp Physiol.* 2013; 98: 1565–75.
25. Koudstaal S, Jansen of Lorkeers SJ, Gho JMIH, et al. Myocardial infarction and functional outcome assessment in pigs. *J Vis Exp.* 2014; 86: 1–10.
26. Sluijter JPG, Condorelli G, Davidson SM, et al. Novel therapeutic strategies for cardioprotection. *Pharmacol Ther.* 2014; 144: 60–70.
27. Ren X, Wang Y, Jones WK. TNF-alpha is required for late ischemic preconditioning but not for remote preconditioning of trauma. *J Surg Res.* 2004; 121: 120–9.
28. Jones WK, Fan GC, Liao S, et al. Peripheral nociception associated with surgical incision elicits remote nonischemic cardioprotection via neurogenic activation of protein kinase C signaling. *Circulation.* 2009; 120: S1–9.
29. Heusch G, Bøtker HE, Przyklenk K, et al. Remote ischemic conditioning. *J Am Coll Cardiol.* 2015; 65: 177–95.
30. Hausenloy DJ, Yellon DM. “Conditional conditioning” in cardiac bypass surgery. *Basic Res Cardiol.* 2012; 107: 258.

31. Valparaíso AP, Vicente D, Bograd B, et al. Modeling acute traumatic injury. *J Surg Res.* 2015; 194: 220–32.
32. Robertson CM, Coopersmith CM. The systemic inflammatory response syndrome. *Microbes Infect.* 2006; 8: 1382–9.
33. Carbone F, Nencioni A, Mach F, et al. Pathophysiological role of neutrophils in acute myocardial infarction. *Thromb Haemost.* 2013; 110: 501–14.
34. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc Res.* 2002; 53: 31–47.
35. Marx N, Neumann FJ, Ott I, et al. Induction of cytokine expression in leukocytes in acute myocardial infarction. *J Am Coll Cardiol.* 1997; 30: 165–70.
36. Scholtes VPW, Versteeg D, de Vries J-PPM, et al. Toll-like receptor 2 and 4 stimulation elicits an enhanced inflammatory response in human obese patients with atherosclerosis. *Clin Sci.* 2011; 121: 205–14.

Chapter 3

Standardized porcine unilateral femoral nailing is associated with changes in PMN activation status, rather than aberrant systemic PMN prevalence

Michel Paul Johan Teuben^{1,2,3,4}

Roman Pfeifer^{2,4}

Klemens Horst⁵

Tim-Philipp Simon⁶

Marjolein Heeres^{1,3}

Yannik Kalbas^{2,4}

Taco Blokhuis⁷

Frank Hildebrand⁵

Leo Koenderman^{3,8}

Hans-Christoph Pape^{2,4}

Luke Leenen¹

TREAT-Research Collaboration

¹ Department of Traumatology, University Medical Center Utrecht, The Netherlands

² Department of Traumatology, University Hospital Zurich, Zurich, Switzerland

³ Laboratory for Translational Immunology, Utrecht, The Netherlands

⁴ Harald Tscherne Laboratory for Orthopaedic and Trauma Research, Zurich, Switzerland

⁵ Department of Trauma and Reconstructive Surgery, RWTH University Hospital Aachen, Aachen, Germany

⁶ Department of Intensive Care and Intermediate Care, RWTH University Hospital Aachen, Aachen, Germany

⁷ Department of Surgery, University Hospital Maastricht, Maastricht, The Netherlands

⁸ Department of Pulmonology, University Medical Center Utrecht, Utrecht, The Netherlands

Abstract:

Purpose

Intramedullary nailing (IMN) of fractures is associated with increased rates of inflammatory complications. The pathological mechanism underlying this phenomenon is unclear. However, polymorphonuclear granulocytes (PMNs) seem to play an important role. We hypothesized that a femur fracture and standardized IMN in pigs is associated with altered appearance of PMNs in circulation and enhanced activation status of these cells.

Methods

A porcine model including a femur fracture and IMN was utilized. Animals were randomized for control [anesthesia+ mechanical ventilation only (A/MV)] and intervention [A/MV and unilateral femur fracture (FF) + IMN] conditions. PMN numbers and responsiveness, integrin (CD11b), L-selectin (CD62L) and Fc γ -receptor (CD16 and CD32)-expression levels were measured by flowcytometry of blood samples. Animals were observed for 72 h.

Results

Circulatory PMN numbers did not differ between groups. Early PMN-responsiveness was retained after insult. PMN-CD11b expression increased significantly upon insult and peaked after 24 h, whereas CD11b in control animals remained unaltered ($P = 0.016$). PMN-CD16 expression levels in the FF + IMN-group rose gradually over time and were significantly higher compared with control animals, after 48 h ($P = 0.016$) and 72 h ($P = 0.032$). PMN-CD62L and CD32 expression did not differ significantly between conditions.

Conclusion

This study reveals that a femur fracture and subsequent IMN in a controlled setting in pigs is associated with enhanced activation status of circulatory PMNs, preserved PMN-responsiveness and unaltered circulatory PMN-presence. Indicating that monotrauma plus IMN is a specific and substantial stimulus for the cellular immune system. Early alterations of circulatory PMN receptor expression dynamics may be predictive for the intensity of the post traumatic response.

Introduction

Trauma patients treated by intramedullary nailing (IMN) for a femoral shaft fracture are at increased risk to develop inflammatory complications such as in severe cases acute respiratory distress syndrome (ARDS) [1]. Despite improvements in trauma care, no changes in ARDS incidences in trauma cases occurred over the last three decades [2]. Nevertheless, a large number of studies indicate an association between intramedullary nailing of fractures and the occurrence of ARDS. Albeit underlying pathomechanisms are still poorly understood [3]. Previous trauma studies mainly determined the effect of humoral immune processes on ARDS development [1–6]. Plasma interleukin-6 (IL-6) levels are considered as a reliable marker for injury severity and it has been shown that femoral shaft fractures are associated with increased plasma IL-6 levels [6]. However, despite being investigated thoroughly, data on interleukins lack prognostic value for trauma conditions [7, 8]. To predict outcome and to gain insight in the pathomechanisms of orthopedic trauma related inflammatory complication, it is key to also investigate cellular immunological parameters. Cytokines, including interleukin-6, are involved in regulation of essential innate effector cells, namely polymorphonuclear leukocytes (PMNs) [7]. Previously, it has been demonstrated that post-trauma inflammation leads to mobilization and activation of circulatory PMNs. Activated circulatory PMNs migrate into the tissue compartment, including highly vascularized lung-tissue. Upon activation, tissue residing PMNs release radical oxygen species (ROS) and proteases, which can cause collateral damage to parenchymal cells and thereby contribute to organ dysfunction [4, 7]. Due to standardization issues in clinical trauma studies, the impact of IMN of long-bone fractures on PMN homeostasis remains unclear. We aimed to evaluate the impact of IMN of a femur fracture on appearance and activation status of the circulatory PMN population, in a standardized long-term large animal experiment, as it has previously been demonstrated that experimental IMN of a unilateral femur fracture is associated with enhanced pulmonary PMN pooling [9]. A porcine model was utilized due to the close similarity of anatomical, physiological, genetical and immunological properties to human [10]. We hypothesized that standardized IMN of a unilateral femur fracture in pigs is associated with increased appearance of PMNs in circulation and enhanced activation status of these cells.

Material and methods

Experiments were executed in accordance with the German legislation governing animal studies following The Principles of Laboratory Animal Care [11]. The study was approved by the regional authority: Landesamt für Natur-, Umwelt- und Verbraucherschutz, LANUV NRW, Germany under application number: AZ 84-02.04.2014.A265. For these experiments, male *German landrace* pigs were included with a body weight between at 30 ± 5 kg. This study presents partial results obtained from a large animal porcine multiple trauma model. The model has been previously described in detail by Horst et al. [12].

Study groups

For the purpose of the study two groups were composed:

- i. Control group: anesthesia/mechanical ventilation only (A/MV), $n = 6$.
- ii. Intervention group (FF + IMN): A/MV + femur fracture (FF) and IMN, $n = 6$.

Fracture model and operative intervention

Intramuscular premedication via injection of 4 mg/kg azaperone (Stresnil™, Janssen, Germany) was applied to animals prior to the experiments. Animals were pre-oxygenized with 100% O₂/min. Anesthesia was maintained via continuous infusion of Propofol and Sufentanil. After intubation animals received volume controlled ventilation with lung-protective ventilation parameters (6–8 ml/kg/BW); i.e., inspiratory oxygen fraction (FiO₂) of 0.5; positive end-expiratory pressure (PEEP) 8 mmHg (plateau pressure < 28 mmHg) and targeting a pCO₂ of 35–45 mmHg (Draeger, Evita, Lubeck, Germany) as recommended for the chest trauma [13]. Ventilation was optimized based on continuous capnometry and frequent blood gas analyses. An arterial line (Vygon, Aachen, Germany) was introduced in the femoral artery for continuous blood pressure assessment and a central venous catheter (Four-Lumen Catheter, 8.5 Fr., Arrow Catheter, Teleflex Medical, Germany) was placed in the external jugular vein for administration of fluids, anesthesia. The animals received a suprapubic urine catheter and fluid management could therefore be monitored on urine production measurements. The animal's temperature was measured continuously and was kept between 38.7 and 39.8 °C, according to a physiologic temperature for this age group. Volume status was maintained by the infusion of Sterofundin®. Animals received antibiotics on a daily basis (Ceftriaxon® 2 g, i.v.). All vitals were documented routinely during the observation period. Control animals were instrumented

and also kept under these conditions throughout the whole experiment but did not receive any trauma or surgery. In the intervention group, a unilateral femur fracture was set using a bolt gun machine (Blitz-Kerner, turbocut JOBB GmbH, Germany) of which the bolt hit a custom made plate positioned on the mid third of the femur. To produce a reproducible and standardized fracture, prior to fracturing a 5-cm skin incision was made and a channel towards the femur was created. Fracture placement was confirmed by X-ray imaging. To simulate the rescue time, animals were observed for 90 min after fracturing the femur. During that time, volume management was reduced, O₂ fraction was set to 0.21 to simulate ambient air and warming of the animals was suspended. Thereafter, resuscitation was started in accordance with established trauma guidelines [14]. After a resuscitation time of 60 min, the operative phase started, and fracture fixation was performed by the placement of an intramedullary nail. All procedures were accomplished by one experienced orthopedic surgeon and under sterile conditions. Before nail placement, intramedullary reaming was performed, and adequate nail placement and fracture reduction were again confirmed by control X-ray imaging.

Sampling

For blood PMN analysis, we collected peripheral blood from a central venous at several time points. The first sample (baseline) was drawn directly after the induction of general anesthesia. Thereafter we draw blood 6 h after intramedullary nailing and after 24, 48, and 72 h of observation.

Peripheral blood PMN isolation and flowcytometry analysis

Analysis was performed with whole blood samples collected in a Vacutainer® which was anticoagulated with ethylenediaminetetraacetic acid (EDTA). An icecold isotonic NH₄Cl-lysis buffer was utilized to lyse erythrocytes. Thereafter cells were washed with FACS-buffer (Dulbecco phosphate-buffered saline supplemented with 2% heat inactivated fetal calf serum, 5 mM EDTA). Antibody mixes were added, and samples had to incubate in a dark room for 45 min on 4 °C. After two wash steps with FACS, buffer cells were fixed by BD Cellfix (BD, Mountain View, CA, USA). All samples were analyzed within 8 h after collection using a FACS Canto II flowcytometer (BD, Mountain View, CA, USA). Data from individual experiments are depicted as fluorescence intensity as the median fluorescence intensity (MFI) of at least 20,000 PMNs. PMNs were identified by specific forward- and sideward-scatter characteristics on CD45⁺-cells after exclusion of doublets (Supplement 1). Additionally, FL-8 (unstained

Pacific Blue/autofluorescent)-positive cells were excluded. Sample processing and gating strategy have been validated before [15] and was further confirmed by pilot experiments including cell sorting experiments and subtype-specific co-expression analysis (including Swine Workshop Cluster (SWC)-8⁺/CD16).

Determination of PMN-responsiveness to ex vivo LPS-stimulation prior and after trauma

In a different set of experiments ($N = 8$) from our research group (TREAT-consortium/ethical approval: ZH138/2017), early PMN receptor expression alterations upon *ex vivo* whole blood stimulation with the bacterial component lipopolysaccharide (LPS) was determined. A similar trauma model was utilized in these experiments as the initial model and the protocol is described in detail elsewhere [16]. For the *ex vivo* experiments, samples of monotrauma conditions (unilateral femur fracture + IMN) were obtained at baseline (BL) and 6 h after insult. Blood was collected using pyrogen-free heparinized Vacutainer®-tubes. Thereafter, whole blood was incubated with 10ug/ml (LPS, Sigma, Escherichia coli O55:B5) for 60 min at 37 °C. Unstimulated samples were analyzed to verify the effect of the LPS-stimulation. After processing of samples (as previously described), the single cell solutions were analyzed by flow cytometry. *PMN-responsiveness* was defined as the difference in PMN-CD11b expression between unstimulated and stimulated conditions at different timepoints. *Peak-PMN activation status* was defined as the level of PMN-CD11b expression upon *ex vivo* LPS-stimulation at different timepoints.

Monoclonal antibodies utilized to determine activation status of blood PMNs

For the analysis of PMN receptor expression by flow measurements the following anti-pig antibodies were commercially purchased: CD45 (clone K252.1E4, AbD Serotec, Kidlington, UK), SWC8 (clone MIL-1, AbD Serotec, Kidlington, UK), CD11b(RIII) (clone 2F4/11, AbD Serotec, Kidlington, UK), CD16 (clone G7, AbD Serotec, Kidlington, UK), CD62L (clone produced by Laboratory for Translational Research, Utrecht) and CD32 (Clone AT10, AbD Serptec, Kidlington, UK).

Absolute cell counts

CountBright counting beads (Invitrogen, Waltham, Massachusetts, USA) were utilized to calculate absolute cell numbers.

Statistical analysis

Pooled data from individual experiments were described by mean and standard deviation (SD) or by median and interquartile range (IQR). For comparisons, a Student's *T*-test, Paired-Samples *T*-tests or Mann-Whitney-*U*-tests were used as appropriate. Furthermore, changes over time were analyzed using repeated measurement analysis by the non-parametric hypothesis Wilcoxon signed-rank test. A *P* value < 0.05 is considered statistically significant. Data were analyzed using software programs SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Results

All experimental animals survived the 72 h observation period. Furthermore, a mid-shaft fracture and adequate fracture fixation was confirmed by macroscopic analysis after termination in all intervention animals. No complications were diagnosed during the observation period.

Absolute PMN-numbers in peripheral blood over time

No statistically significant differences in circulatory PMN numbers were found between groups prior, and immediately after intervention [control 10.5 (IQR 4.8–15.0) $\times 10^6$ cells/ml vs. FF + IMN 10.3 (IQR 7.4–12.7) $\times 10^6$ cells/ml, $P = 1.00$]. In addition, no differences between the groups were present 6 h after the intervention timepoint [control 13.6 (IQR: 8.4–18.1) $\times 10^6$ PMNs/ml and fracture + IMN 16.3 (IQR 6.4–25.1) $\times 10^6$ cells/ml, $P = 0.73$]. Thereafter a gradual and statistically significant decrease in circulatory PMN numbers was seen in both groups ($P < 0.01$). After 3 days of observation polymorphonuclear leukopenia was seen in both groups, however PMN counts did not differ between control [3.0 (IQR 2.1–5.7) $\times 10^6$ cells/ml] and FF + IMN-conditions [2.0 (IQR 1.6–2.2) $\times 10^6$ cells/ml, $P = 0.11$]. Circulatory PMN numbers are shown in **Fig. 1a**. As displayed in **Fig. 1b**, under homeostatic conditions. No statistical differences were seen between control and intervention-groups, with respectively 58.9 (IQR 33.0–62.1)% blood white blood cells (WBCs) were identified as PMNs and 57.3 (IQR 44.8–58.0)% PMNs/WBCs ($P = 0.69$). After intervention, the percentage of circulatory PMNs/all leukocytes peaked in both control ($P = 0.03$) and the FF + IMN ($P = 0.008$) groups. No differences between groups were seen at this timepoint. Thereafter, the percentage of circulatory PMNs dropped in both study groups. At 2 days of observation, lowest circulatory PMN percentages were encountered and again, percentages between control [24.0 (IQR 23.2–44.0)%] and FF + IMN [25.0 (IQR 17.6–25.9)%] conditions did not differ significantly.

Figure 1

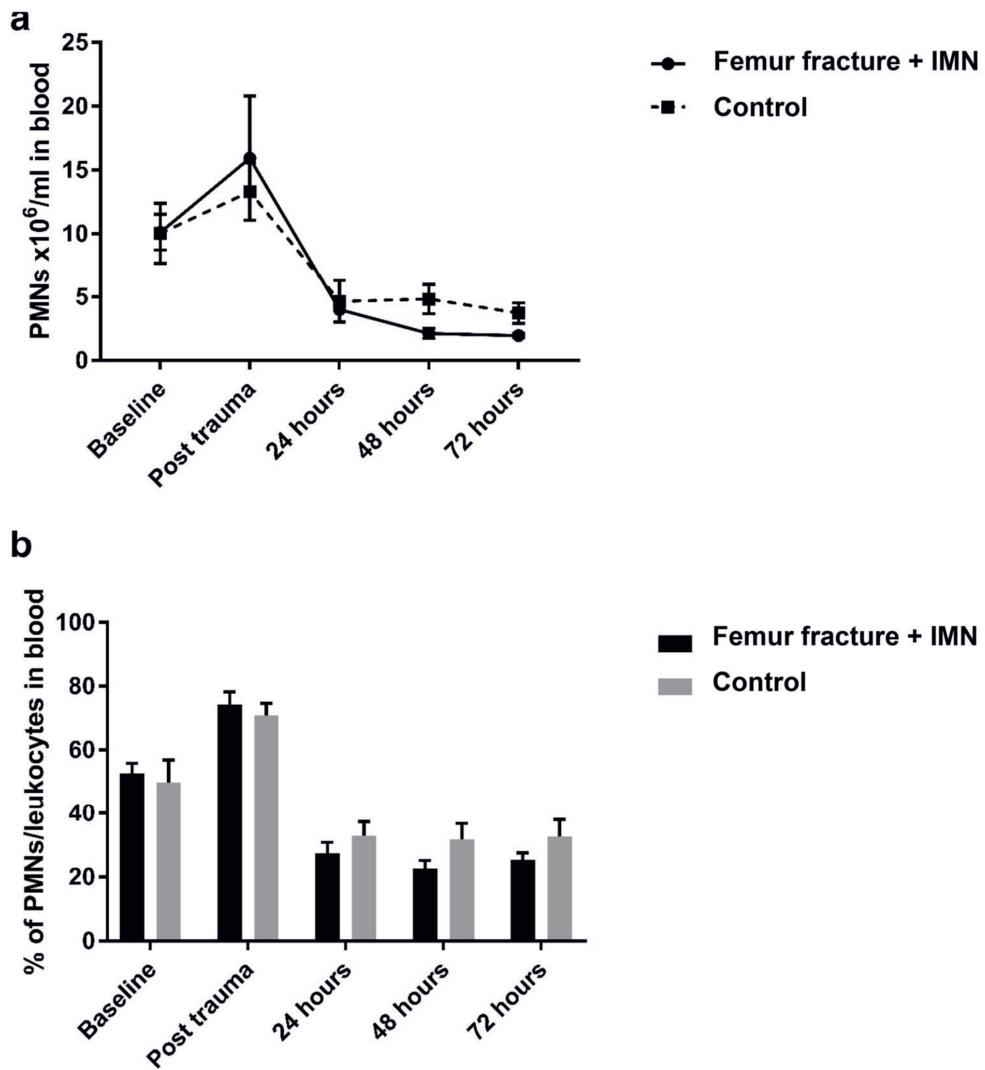


Fig. 1a. Absolute neutrophil numbers in peripheral blood over time. **b.** Alterations in percentages of PMNs/leukocytes in circulation.

Membrane receptor expression of Mac-1 (CD11b) and L-selectin (CD62L) on circulatory PMNs over time

Figure 2a displays alterations in membrane receptor expression of CD11b on circulatory PMNs over time. Significantly increased blood PMN surface expression of CD11b was found in animals exposed to FF + IMN compared with control conditions. Upon intramedullary femoral nailing, expression levels of CD11b on blood PMNs increased significantly during the first day and peaked at 24 h of observation. Thereafter, PMN-CD11b decreased gradually over time and returned to baseline values within 3 days after insult. In contrast, cell surface CD11b expression on circulatory PMNs in control animals did not change significantly over time. After 1 day of observation, CD11b expression levels on blood PMNs were twice as high in intervention animals as in control conditions ($P = 0.016$). A non-significant trend of gradual increasing L-selectin cell surface expression on circulatory PMNs over time occurred in both control and IMN groups, however no statistical differences between groups were found at any timepoint (Fig. 2b).

Figure 2

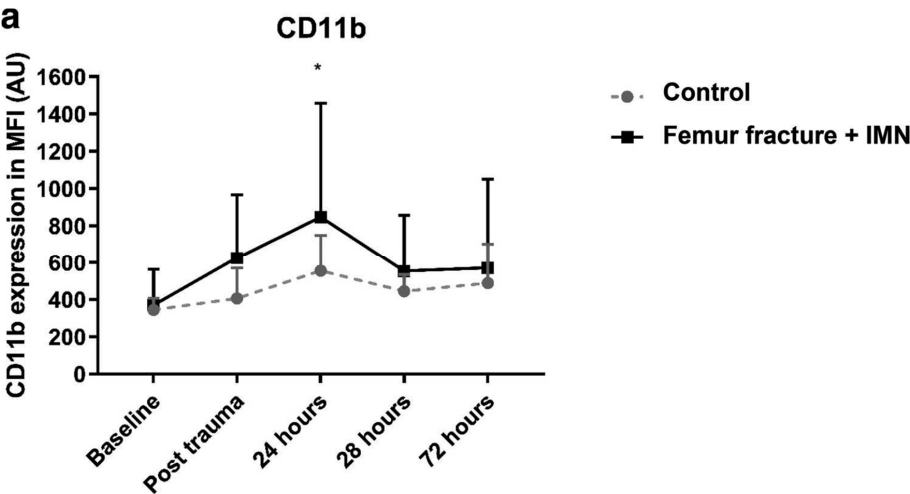


Fig. 2a. Circulatory neutrophil Mac-1 expression changes over time. Receptor expression on neutrophils over time.
* $P < 0.05$.

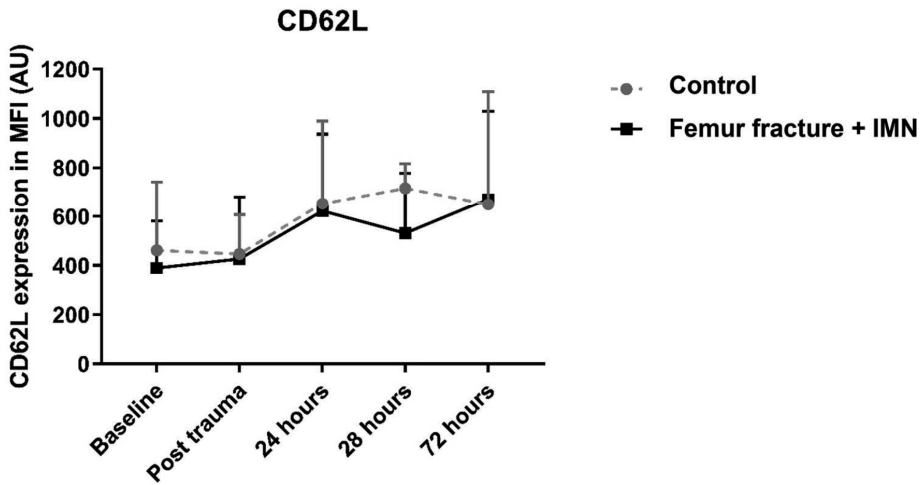
b

Fig. 2b. Circulatory neutrophil L-Selectin expression alterations over time. Receptor expression on neutrophils over time. Data in mean (SD). * $P < 0.05$, control vs. isolated femur fracture and IMN

Membrane receptor expression of Fcγ-receptors on blood PMNs

Blood PMN-CD16 (FcγIII) expression significantly increased 2 and 3 days after trauma (FF + IMN-group) ($P = 0.016$ and $P = 0.032$, respectively), whereas PMN-CD16 expression in the control group was found to be unaltered over time. During the course of the experiment, cell surface expression levels of CD32 (FcγII) on circulatory PMNs did not differ significantly between control and intervention groups. Fcγ-receptor changes over time are shown in Fig. 3.

Figure 3

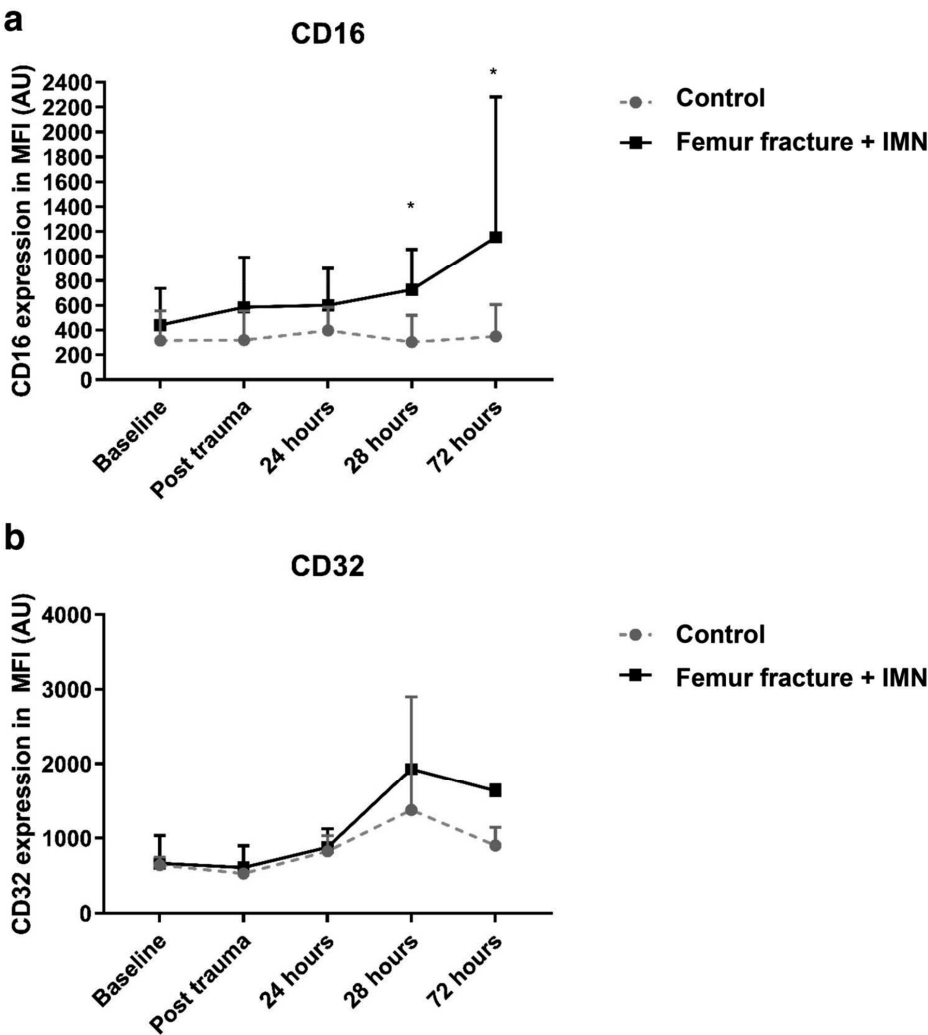


Fig. 3a. Circulatory neutrophil Fc γ RIII expression alterations over time.

Fig. 3b. Circulatory neutrophil Fc γ RII expression alterations over time. Receptor expression on neutrophils over time. Data in mean (SD).

*P < 0.05, isolated femur fracture and IMN vs. control.

PMN-responsiveness to ex vivo lipopolysaccharide stimulation at baseline and after insult

No statistically significant differences were seen between unstimulated conditions. PMN-responsiveness was preserved over time as *ex vivo* LPS-stimulation evoked a statistically significant increase of PMN-CD11b expression after stimulation both at baseline (mean \pm SD increase of CD11b-MFI: $127.5 \pm 107.6\%$, $P = 0.002$) and 6 h after insult (mean \pm SD increase of CD11b-MFI: $130.7 \pm 71.1\%$, $P = 0.0002$). Moreover, peak-PMN activation status, reflected by the amplitude of PMN-CD11b expression after *ex vivo* LPS-stimulation was significantly higher after insult than at baseline (with a mean \pm SD difference of CD11b-MFI: $21.0 \pm 19.2\%$, $P = 0.03$). Results of *ex vivo* LPS-stimulation of peripheral blood samples at baseline and after insult are displayed in Fig. 4.

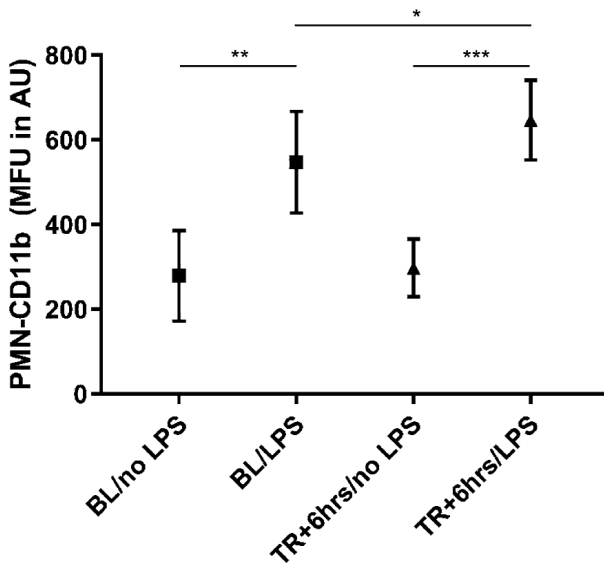
Figure 4

Fig. 4. PMN-Mac 1 expression following *ex vivo* LPS-stimulation prior and post instrumentation. Data in mean (SD). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Discussion

Orthopedic trauma and intramedullary nailing are associated with increased incidences of inflammatory complications [1]. Polymorphonuclear leukocytes are believed to play an important role in the development of these complications after trauma. Clinical trauma studies, however, lack standardization due to (i) heterogeneous insult condition types and severity, (ii) suboptimal timing of sampling/ procedures, (iii) variation in patient characteristics (genetic background, co-morbidities, physiology, age, gender, medication). Therefore, the current standardized large animal long-term observation study was executed, and the results may be summarized as follows:

1. General anesthesia and mechanical ventilation in pigs in a controlled context is associated with changes in circulatory PMN numbers and cell surface receptor dynamics over time.
2. Standardized experimental femur fracturing and subsequent intramedullary nailing evoke a more intensified systemic PMN response which is characterized by increased early activation status of circulatory PMNs, rather than altered circulatory PMN counts or early impaired PMN-responsiveness.

This indicates that a unilateral femur fracture and subsequent intramedullary femoral nailing should be considered as a substantial trigger for the innate cellular immune system. In addition, we previously managed to demonstrate with this porcine monotrauma model that, compared with control conditions, IMN of an isolated femur fracture is associated with increased pulmonary PMN occurrence after 72 h [9]. This indicates that early alterations of circulatory PMN activation status (current study) precede pulmonary innate immune cell accumulation [9]. It is tempting to speculate that monitoring of circulatory PMN alterations could assist in the prediction of remote organ inflammation. Post-insult variations in circulatory PMN numbers over time in our study mimic both clinical and experimental findings [17–19]. Furthermore, in line with a rodent trauma study, we found altered circulatory PMN presence over time in both the control (A/MV) and intervention study groups. This suggests that general anesthesia and mechanical ventilation shape circulatory PMN kinetics and that the additional impact of trauma/surgery on changes in circulatory PMN presence is limited [20]. Despite the presumed limited additional impact of a unilateral fracture and subsequent fracture fixation on circulatory PMN numbers, the current study does reveal that cell surface expression profiles of relevant

receptors are largely affected by this trigger (FF + IMN). This makes it tempting to hypothesize that the interplay between (i) trauma anesthesia/mechanical ventilation settings and (ii) fracture (fixation standards) dictates the innate immune response in these trauma patients. All these factors and their interplay should therefore be considered as potential targets for immune modulation and this requires further study. Nowadays, cell surface receptor expression, in contrast to immune cell functionality, can easily be determined by modern automatized flow systems [21]. Determination of these parameters in a controlled setting is of great relevance and may form the basis for future routine diagnostic, prognostic and therapeutic investigations in the clinical setting. Therefore the current study focuses on PMN activation status by studying well-established PMN markers with appropriately defined biological function [22], rather than *ex vivo* characterization of PMN functionality. CD11b is a well-established activation marker and *in vitro* studies demonstrate that cell activation is associated with neutrophil-CD11b upregulation [23, 24]. Clinical studies on systemic inflammation further showed that circulatory neutrophil-CD11b expression rise upon insult [25–27]. Like the findings of the current study, Johansson et al. demonstrated increased PMN-CD11b expression in burn patients [25] and others demonstrated an early rise of neutrophil- CD11b cell surface expression in trauma patients [26]. In addition, a similar pattern of Mac-1 expression kinetics was seen by Botha et al. [27]. They showed that neutrophil- CD11b expression levels in trauma patients peaked between 6 and 18 h after admission [27]. In the current porcine experiment, PMN-CD11b expression reached peak levels later, namely after 24 h. We believe that this discrepancy may mainly be due to differences in trauma load between both studies. Trauma victims in the study from Botha et al. had ISS scores over 20 [27], whereas our porcine trauma model represents a monotrauma model with an ISS of 9. This might imply that trauma severity is associated with PMN-integrin expression. A study from Bathia et al. further found that PMN-CD11b expression started to decrease and return to homeostatic levels, as late as 72 h after severe trauma [28]. A similar pattern of restoration was observed in our experimental study. Timing of definitive fracture fixation for orthopedic trauma is a topic of considerable debate. Operative intervention is an additional trigger for the immune system and thereby leads to an upsurge of the post-trauma inflammatory response [29]. As integrins are considered as essential receptors in PMN adhesion and migration processes, changes in circulatory PMN-CD11b expression may potentially be utilized to guide timing of definitive fracture fixation. Especially as homogeneous blood PMN-CD11b response was found in all previously mentioned trauma studies and our experimental data. This response entails peak PMN-Mac-1 expression levels

within the first 24 h and restoration to homeostatic levels within 72 h post-insult. Therefore, it is tempting to speculate that postponing IMN for the first 72 h in the critically ill is beneficial as integrin levels are allowed to normalize first, which is in line with clinical studies. L-selectin plays an critical role in neutrophil transmigration as this receptor binds to its ligands on vascular endothelium and thereby facilitates slowing down, or ‘rolling’ of neutrophils [30]. Neutrophil activation is typically characterized by shedding of L-selectin [31] and higher trauma load is associated with more profound decreased neutrophil L-selectin expression [32]. In the current study, L-selectin expression on PMNs did not significantly change over time after insult. A similar observation was made in a study by Mommson et al. on patients undergoing elective surgery of the lower extremities [33] and in a cohort of burn patients [25]. Striking alterations in blood neutrophil L-selectin expression over time, however, have been shown in a study performed by Maekawa et al. on more severely injured trauma patients [34]. These discrepancies might be partly explained by differences in trauma load and the intensity of the post-insult inflammatory response and are thereby in line with the observations from Seekamp et al. [32]. Fc γ -receptors are involved in activation of neutrophils as they bind to immunoglobins (IgG) either in aggregates or attached to pathogens. Binding induces phagocytosis or promotes oxidative burst [35]. Interestingly, expression of Fc γ III (CD16) on blood PMNs increased over time in our model. This contradicts kinetics in burn injuries [25, 36] and polytrauma patients studied by White-Owen et al. in which overall PMN-CD16 expression dropped after trauma and remained decreased for more than 48 h [37]. We believe that our porcine monotrauma insult-condition did not cause an immune response that was intense enough to evoke the mobilization of novel subsets, and more specifically CD16^{low}-PMNs in mobilization [38, 39]. Consequently, altered blood PMN-pool constitution-related reduction of PMN-CD16 expression did not occur in our model. The paradoxical increase of PMN-CD16 expression in our trauma study might reflect a specific activation profile of the blood neutrophil pool as regulation of the PMN-CD16-receptor in trauma is affected by different processes including altered PMN-phenotype appearance, delayed apoptosis, potentially modified balance between receptor shedding/ biosynthesis and mobilization of the PMNs/ internal pool of pre-formed CD16 [38–41]. However most likely an interplay between these mechanisms occurs and, in our view, PMN-CD16 receptor dynamics upon trauma should be a focus of upcoming studies. Our findings regarding PMN-Fc γ RII (CD32) expression changes are in accordance with a study from Visser et al. Both studies showed no evident alterations in PMN-CD32 expression within the first 24 h after trauma [42]. The current study

demonstrates that a more profound blood PMN CD11b/CD16-response was found in the group subjected to orthopedic trauma/IMN than in animals exposed to anesthesia and mechanical ventilation only. Thereby our experimental study reveals that orthopedic trauma and subsequent IMN elicit a specific pattern of enhanced PMN-CD11b/Mac-1 cell surface expression. This may be an important factor in the association between increased occurrence of inflammatory complications and intramedullary nailing in orthopedic trauma. It is tempting to speculate that IMN for long-bone fractures induces a specific systemic PMN activation profile due to increased release of DAMPs, local cytokines, pro-inflammatory bone particles or angiogenic/growth factors [5–7, 43, 44]. Upcoming experimental studies should focus on the identification of involved pathways. In contrast to studies on polytrauma [45, 46] we did not observe impaired PMN-responsiveness as LPS-induced upregulation of CD11b on PMNs was retained after controlled monotrauma and fracture fixation. Interestingly, there even seems to be a trend towards intensified early PMN-responsiveness upon trauma and subsequent nailing as peak-PMN activation status, reflected by the amplitude of PMNCD11b expression after LPS-stimulation, is significantly greater after trauma than under homeostatic conditions. This phenomenon is in contrast with observations in polytrauma studies. As in these clinical investigations, impaired PMN-responsiveness, and the occurrence of refractory neutrophils in circulation are linked with severe trauma and complicated courses. [45–47]. The specific impact of instrumentation on early PMN-receptor alterations was studied as well. In short, additional samples were collected prior to fracture fixation and 2 h after fracture fixation. In line with Hietbrink et al. [26] no differences in circulatory PMN-integrin/selectin/Fc γ -receptor expression were seen between pre- and post-instrumentation conditions. (Data are displayed in Supplementary file 2). The current standardized porcine trauma model, and human trauma studies are (except for CD16-data) remarkably similar regarding PMN receptor dynamics trends. Given these similarities, the current porcine trauma model is very suitable for proof-of-principle interventions with novel therapeutic strategies for trauma. Integral translational effects are summarized in Supplementary file 3. Limitations of this study include its sample size, however relevant effects reached statistical significance and, therefore, we conclude that there was no need to include/sacrifice more experimental animals. We further decided not to include additional study conditions without clinical importance or translational value. So, unconventional fracture fixation techniques for cardiopulmonary compensated monotrauma scenarios (such as external fixation/plating/splinting) or IMN without fracture induction were not investigated. Finally, as porcine immune cells, in contrast to rodent models [48], do allow

for adequate analysis of the key Fc γ -receptor family, we decided to focus with our antibody panel on these specific markers plus integrins and selectins, rather than adding antibodies aimed to investigate other circulatory immune cell populations. In conclusion, the current standardized large-animal study demonstrates that general anesthesia and mechanical ventilation in pigs is associated with changes in circulatory PMN numbers and activation status. Furthermore, an isolated femur fracture and intramedullary nailing is associated with more intensified activation of circulatory PMNs and preserved PMN-responsiveness. Indicating that monotrauma plus IMN should be considered as a substantial stimulus for the cellular immune system with a specific profile of enhanced circulatory PMN-integrin/Fc γ RIII-receptor expression. This mechanism may play an important role in the development of inflammatory complications upon IMN for long-bone fractures and requires further research. Moreover, analysis of early circulatory PMN receptor expression profiles may be useful to predict the intensity of the long-term post-insult immune response and may thereby be an interesting tool to guide therapy decisions in trauma patients.

References

1. Pape HC, Auf'm Kolk M, Paffrath T, et al. Primary intramedullary femur fixation in multiple trauma patients with associated lung contusion—a cause of posttraumatic ARDS? *J Trauma*. 1993;34(4):540–7 (discussion 547–8).
2. Pfeifer R, Heussen N, Michalewicz E, et al. Incidence of adult respiratory distress syndrome in trauma patients: a systematic review and meta-analysis over a period of three decades. *J Trauma*. 2017;83(3):496–506.
3. Hietbrink F, Koenderman L, van Wessem KJP, et al. The impact of intramedullary nailing of tibia fractures on the innate immune system. *Shock*. 2015;44(3):209–14.
4. Abraham E. Neutrophils and acute lung injury. *Crit Care Med*. 2003;31(4 Suppl):S195–9.
5. Maier B, Lefering R, Lehnert M, et al. Early versus late onset of multiple organ failure is associated with differing patterns of plasma cytokine biomarker expression and outcome after severe trauma. *Shock*. 2007;28(6):668–74.
6. Giannoudis PV, Harwood PJ, Loughenbury P, et al. Correlation between IL-6 levels and the systemic inflammatory response score: can an IL-6 cutoff predict a SIRS state? *J Trauma*. 2008;65(3):646–52.
7. Lenz A, Franklin GA, Cheadle WG. Systemic inflammation after trauma. *Injury*. 2007;38(12):1336–45.
8. Visser T, Pillay J, Koenderman L, et al. Post injury immune monitoring. Can multiple organ failure be predicted? *Curr Opin Crit Care*. 2008;14(6):666–72.
9. Stormann P, Wagner N, Kohler K, et al. Monotrauma is associated with enhanced remote inflammatory response and organ damage, while polytrauma intensifies both in porcine trauma model. *Eur J Trauma Emerg Surg*. 2020;46(1):31–42.
10. Hildebrand F, Andruszkow H, Huber-Lang M, et al. Combined hemorrhage/trauma models in pigs-current state and future perspectives. *Shock*. 2013;40(4):247–73.
11. National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the care and use of laboratory animals. Washington: National Academies Press; 2011.
12. Horst K, Simon TP, Hildebrand F, et al. Characterization of blunt chest trauma in a long-term porcine model of severe multiple trauma. *Sci Rep*. 2016;6:39659
13. Arora S, Singh PM, Trikha A. Ventilatory strategies in trauma patients. *J Emerg Trauma Shock*. 2014;7:25–31.
14. ATLS Subcommittee: International ATLS Working group. Advanced trauma life support (ATLS): the ninth edition. *J Trauma*. 2013;74(5):1363–6.
15. van Hout GP, van Solinge WW, Gijsberts CM, et al. Elevated mean neutrophil volume represents altered neutrophil composition and reflects damage after myocardial infarction. *Basic Res Cardiol*. 2015;110(6):58.

16. Halvachizadeh S, Teuben M, Lempert M, et al. Protective effects of new femoral reaming techniques (Reamer irrigator aspirator, RIA I and II) on pulmonary function and posttraumatic contusion (CT morphology)—results from a standardized large animal model. *Injury*. 2021;52(1):26–31.
17. McManus AT. Examination of neutrophil function in a rat model of decreased host resistance following burn trauma. *Rev Infect Dis*. 1983;5(Suppl 5):S898–907.
18. Visser T, Pillay J, Pickkers P, et al. Homology in systemic neutrophil response induced by human experimental endotoxemia and by trauma. *Shock*. 2012;37(2):145–51.
19. Bastian OW, Kuijter A, Koenderman L, et al. Impaired bone healing in multitrauma patients is associated with altered leukocyte kinetics after major trauma. *J Inflamm Res*. 2016;9:69–78.
20. Van Wessel KJ, Hennus MP, Heeres M, et al. Mechanical ventilation is the determining factor in inducing an inflammatory response in a hemorrhagic shock model. *J Surg Res*. 2013;180(1):125–32.
21. Spijkerman R, Hesselink L, Bongers S, et al. Point-of-care analysis of neutrophil phenotypes: a first step toward immunobased precision medicine in the trauma ICU. *Crit Care Explor*. 2020;2(7):e0158
22. Pillay J, Hietbrink F, Koenderman L, Leenen LP. The systemic inflammatory response induced by trauma is reflected by multiple phenotypes of blood neutrophils. *Injury*. 2007;38(12):1365–72.
23. Ley K. Integration of inflammatory signals by rolling neutrophils. *Immunol Rev*. 2002;186:8–18.
24. van Griensven M, Krettek C, Pape HC. Immune reactions after trauma. *Eur J Trauma*. 2003;29:181–92.
25. Johansson J, Sjogren F, Bodelsson M, et al. Dynamics of leukocyte receptors after severe burns: an exploratory study. *Burns*. 2001;37(2):227–33.
26. Hietbrink F, Koenderman L, Leenen LP. Intramedullary nailing of the femur and the systemic activation of monocytes and neutrophils. *World J Emerg Surg*. 2011;31:6–24.
27. Botha AJ, Moore FA, Moore EE, et al. Base deficit after major trauma directly relates to neutrophil CD11b expression: a proposed mechanism of shock-induced organ injury. *Intensive Care Med*. 1997;23(5):504–9.
28. Bhatia RK, Pallister I, Dent C, et al. Enhanced neutrophil migratory activity following major blunt trauma. *Injury*. 2005;36(8):956–62.
29. Morley JR, Smith RM, Pape HC, et al. Stimulation of the local femoral inflammatory response to fracture and intramedullary nailing. *J Bone Joint Surg Br*. 2008;90(3):393–9.
30. Seely AJ, Pascual JL, Christou NV. Science review: cell membrane expression (connectivity) regulates neutrophil delivery, function and clearance. *Crit Care*. 2003;7(4):291–307.
31. Neeley SP, Hamann KJ, White SR, et al. Selective regulation of expression of surface adhesion molecules Mac-1, L-selectin, and VLA-4 on human eosinophils and neutrophils. *Am J Respir Cell Mol Biol*. 1993;8:633–9.
32. Seekamp A, van Griensven M, Hildebrandt F, Brauer N, Jochum M, Martin M. The effect of trauma on neutrophil L-selectin expression and sL-selectin serum levels. *Shock*. 2001;15(4):254–60.

33. Mommsen P, Barkhausen T, Hildebrand F, et al. Regulation of L-selectin expression by trauma-relevant cytokines. *Pathol Res Pract*. 2011;207(3):142–7.
34. Maekawa K, Futami S, Nishida M, et al. Effects of trauma and sepsis on soluble L-selectin and cell surface expression of L-selectin and CD11b. *J Trauma*. 1998;44(3):460–8.
35. Huizinga TW, Roos D, von dem Borne AE. Neutrophil Fcγ receptors: a two-way bridge in the immune system. *Blood*. 1990;75(6):1211–4.
36. Vindenes H, Bjerknes R. Activation of polymorphonuclear neutrophilic granulocytes following burn injury: alteration of Fc-receptor and complement-receptor expression and of opsonophagocytosis. *J Trauma*. 1994;36(2):161–7.
37. White-Owen C, Alexander JW, Babcock GF. Reduced expression of neutrophil CD11b and CD16 after severe traumatic injury. *J Surg Res*. 1992;52(1):22–6.
38. Pillay J, Kamp VM, van Hoffen E, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest*. 2012;122(1):327–36.
39. Orr Y, Taylor JM, Bannon PG, et al. Circulating CD10-/CD16low neutrophils provide a quantitative index of active bone marrow neutrophil release. *Br J Haematol*. 2005;131(4):508–19.
40. Dransfield I, Buckle AM, Savill JS, McDowall A, Haslett C, Hogg N. Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. *J Immunol*. 1994;153(3):1254–63.
41. Tosi MF, Zakem H. Surface expression of Fc gamma receptor III (CD16) on chemoattractant-stimulated neutrophils is determined by both surface shedding and translocation from intracellular storage compartments. *J Clin Invest*. 1992;90(2):462–70.
42. Visser T, Hietbrink F, Groeneweld KM, et al. Isolated blunt chest injury leads to transient activation of circulating neutrophils. *Eur J Trauma Emerg Surg*. 2011;37(2):177–84.
43. Keel M, Trentz O. Pathophysiology of polytrauma. *Injury*. 2005;36(6):691–709.
44. Li H, Liu J, Yao J, et al. Fracture initiates systemic inflammatory response syndrome through recruiting polymorphonuclear leucocytes. *Immunol Res*. 2016;64(4):1053–9.
45. Hietbrink F, Koenderman L, Althuisen M, et al. Kinetics of the innate immune response after trauma: implications for the development of late onset sepsis. *Shock*. 2013;40(1):21–7.
46. Groeneweld KM, Koenderman L, Warren BL, et al. Early decreased neutrophil responsiveness is related to late onset sepsis in multitrauma patients: an international cohort study. *PLoS ONE*. 2017;12(6):e0180145.
47. Hesselink L, Spijkerman R, van Wessem KJP, et al. Neutrophil heterogeneity and its role in infectious complications after severe trauma. *World J Emerg Surg*. 2019;29(14):24.
48. Casey E, Bournazos S, Mo G, et al. A new mouse expressing human Fcγ receptors to better predict therapeutic efficacy of human anti-cancer antibodies. *Leukemia*. 2018;32(2):547–9.

Chapter 4

Instant intra-operative neutropenia despite the emergence of banded (CD16^{dim}/CD62L^{bright}) neutrophils in peripheral blood - An observational study during extensive trauma surgery in pigs

Michel P.J. Teuben^{1,2,3}

Marjolein Heeres^{1,2}

Taco B. Blokhuis⁴

Arne Hollman^{1,2}

Nienke Vrisekoop²

Edward Tan⁵

Roman Pfeifer³

Hans-Christoph Pape³

Leo Koenderman²

Luke P.H. Leenen¹

¹ University Medical Centre Utrecht, Department of Trauma, Utrecht, the Netherlands

² University Medical Centre Utrecht, Laboratory for Translational Research, Utrecht, the Netherlands

³ University Medical Center Zurich, University Hospital Zurich, Zurich, Switzerland

⁴ Maastricht University Medical Centre, Department of Surgery, Maastricht, the Netherlands

⁵ Radboud University Medical Center, Department of Surgery, Nijmegen, the Netherlands

Abstract:

Introduction

Deregulation of polymorphonuclear neutrophils (PMNs) is an essential step in the development of inflammatory complications upon trauma. Different neutrophil subtypes have been identified recently, however, the role of neutrophil subtypes in immunoregulation upon trauma is unclear. We hypothesize that extensive trauma surgery causes instant progressive heterogeneity of the blood neutrophil pool, and increased appearance of young ($\text{CD16}^{\text{dim}}/\text{CD62L}^{\text{bright}}$) neutrophils in peripheral blood.

Material and methods

A standardized extensive thoraco-abdominal porcine trauma surgery model was utilized, and 12 animals were included. Blood was collected at defined timepoints and neutrophil numbers and subtypes were studied by flowcytometry. Neutrophil subtypes were identified by differences in cell surface expression levels of CD16 (Fc γ RIII) and CD62L (L-selectin). Porcine neutrophil subtypes were further characterized after flow sorting.

Results

Eleven animals survived the 3-hour surgical protocol. Neutrophil numbers dropped significantly from a mean of $8,6 \pm 3,5 \times 10^6$ to $2,4 \pm 1,8 \times 10^6$ cells/ml during 180 min, ($p < 0.001$). Simultaneously, the blood PMN population became increasingly heterogeneous due to the appearance of new neutrophil subtypes. Cell sorting experiments and cytological analysis revealed that these porcine subtypes had specific morphological characteristics, mimicking their human counterparts. At baseline, $88\% \pm 1$ percent of circulatory PMNs comprised of mature ($\text{CD16}^{\text{bright}}/\text{CD62L}^{\text{bright}}$) PMNs, while at 3 h the blood PMN pool consisted of $59\% \pm 2$ percent of mature subtypes ($p < 0.001$). Despite a marked drop in neutrophil levels during surgery, absolute and relative numbers of banded ($\text{CD16}^{\text{dim}}/\text{CD62L}^{\text{bright}}$) neutrophils continued to rise throughout surgery.

Conclusion

Standardized extensive trauma surgery was associated with instant progressive neutropenia and increased heterogeneity of the blood neutrophil pool. Furthermore, three different neutrophil subsets in peripheral porcine blood were identified over the course of surgery. Further studies should clarify their precise role in the development of early organ failure upon extensive trauma

surgery. This for the first time exemplifies experimentally the time constraints and impact of damage control surgery after severe trauma.

Introduction

Surgical intervention activates the innate immune system. A local immune response following tissue damage is mandatory to stimulate wound healing and to prevent infection. However, in the case of extensive surgery this immune reaction is not restricted to the surgical site, but rather stimulates a systemic inflammatory response syndrome (SIRS) [1,2].

Polymorphonuclear neutrophils (PMNs) are important effector cells in both local and systemic early responses to tissue damage. Neutrophils are equipped with several mechanisms to protect the host from harmful pathogens [3,4]. However, excessive pathogen invasion combined with uncontrolled activation of the innate immune system can cause severe collateral damage to tissue cells. This phenomenon forms the basis for severe inflammatory complications such as Acute Respiratory Distress Syndrome (ARDS) and Multiple Organ Dysfunction Syndrome (MODS) [1–9]. Under homeostatic conditions, the blood neutrophil pool is homogeneous. However, it has been shown that different phenotypes emerge in peripheral blood after the induction of acute systemic inflammation [10]. These blood neutrophil subtypes can be identified by differences in membrane receptor expression levels of Fc γ RIII (CD16) and L-selectin (CD62L) [10]. Experimental *in vitro* studies have demonstrated that these subtypes have differential functional capacities [10–12]. These observations therefore support the hypothesis that the different subsets are unique phenotypes [10–13]. Hypersegmented neutrophils (CD16^{bright}/CD62L^{dim}) have been shown to be inferior in executing bactericidal tasks [11], exhibit T-cell suppressive capacities and, thereby, can modify the adaptive immune response during inflammation [10]. On the other hand, neutrophils with a band-shaped nucleus (CD16^{dim}/CD62L^{bright}) are younger neutrophils [14] with specific functionality, including a very strong capacity to contain bacteria such as methicillin-resistant *S. aureus* (MRSA) [10–12]. Neutrophil deregulation is believed to be an essential factor in the development of early pro-inflammatory and late infectious complications upon trauma surgery [1,8,15,16]. However, the underlying mechanisms remain largely unclear. As clinical studies on extensive/extreme trauma surgery lack adequate standardization due to heterogeneous patient and trauma characteristics, treatment concept and timing of interventions we decided to determine the impact of extensive trauma surgery on systemic neutrophil homeostasis in a controlled setting in pigs. We hypothesized that extensive thoraco-abdominal trauma surgery causes instant progressive heterogeneity of the blood neutrophil pool characterized by the appearance of young (CD16^{dim}/CD62L^{bright}) neutrophils in peripheral blood.

Material and methods

Experimental animals

The experimental animals were cared for in accordance with the guidelines of the Institutional Animal care and Use guidelines at Radboud University Nijmegen Animal Care Committee (ID-number: 2013-122 2013-122). Female Landrace pigs were utilized that weighed 50–60 kgs at the beginning of the experiments.

Experimental procedure

Prior to all interventions, animals received intramuscular premedication including midazolam (1 mg/kg), ketamine (20 mg/kg) and atropine (50ug/kg). Thereafter, they were pre-oxygenated with 100 percent Oxygen at 10 L/min in prone position. The animals were intubated orotracheally and received volume-controlled ventilation. After intubation, anesthesia was maintained with isoflurane 0,25–0,50%, continuous midazolam infusion (0,6 mg/kg/hour), and sufentanil infusion (15ug/kg/hour). FiO₂ was 0.3, Positive end-expiratory pressure was 0 cm H₂O, and an inspiration-to expiration ratio of 1:2 was applied to all animals. Ventilation settings were guided by endtidal CO₂ measurements, pulse oximetry and by frequent blood gas analyses.

Hypothermia (<38°C) was prevented by controlling room temperature. Normovolemia was maintained by continuous saline (sodium chloride solution (NaCl)) infusion. A triple lumen central venous line was placed via the right jugular vein (HD, Arrow, PA, USA). For monitoring purposes, an arterial line and urinary catheter were placed as well. Standardized thoraco-abdominal trauma surgery was performed as surgical summarized in Supplement 1. Therapy was in line with both Definitive Surgical Trauma Care-guidelines and the German S3-guidelines [17,18]. After 180 min of ongoing trauma surgery, all animals were euthanized by pentobarbital infusion.

Sampling

For blood neutrophil analysis, 9 mL of peripheral blood was collected from a central venous catheter in an ethylenediaminetetraacetic acid (EDTA)-anticoagulated Vacutainer at several time points. The first sample (baseline) was drawn directly after induction of general anesthesia. Thereafter, blood was drawn at 60, 120 and 180 min of surgery.

Monoclonal antibodies, staining and flow cytometry measurements

For the flow cytometry analysis of neutrophil receptor expression, the following commercially available anti-pig monoclonal antibodies were obtained: FITC-labelled CD11b (clone 2F4/11, Abd Serotec, Kidlington, UK), RPE-labelled CD16 (clone G7, Abd Serotec, Kidlington, UK), unconjugated CD62L (clone produced by Center for Translational Research, Utrecht) and Alexa647-labelled CD45. Samples were stained in consecutive steps. Each incubation step took 45 min and was performed at 4 °C in dark conditions. After each incubation step, cells were twice washed with a PBS2+ (phosphate-buffered saline with added sodium citrate [0.38% wt/vol] and pasteurized plasma proteins [10% vol/vol] solution. Finally, flow cytometry analysis was performed with the Gallios flow cytometer (Beckman Coulter, Indianapolis, IN, USA). Measurements were performed within 15 min of the final wash.

Leukocyte count and differentiation

CountBright counting beads (Invitrogen, Waltham, Massachusetts, USA) were utilized to count and compare absolute cell numbers over time.

Leukocyte and neutrophil subtype identification

Prior to this experiment, a new gating strategy to identify porcine neutrophils in peripheral blood was developed and validated. In short, granulocytes were identified by their specific forward-sideward scatter signal from CD45 positive cells. Additionally, non-singlets and FL-8 (unstained Pacific Blue/autofluorescent)-positive cells were excluded. The neutrophil gating procedure and related validation process has been summarized in Supplement 2. Data from individual experiments are expressed in fluorescence intensity by median fluorescence intensity (MFI) of at least 20,000 neutrophils (per subtype).

Cell sorting experiments

To visualize and analyse the morphological characteristics of the neutrophil subtypes, cell sorting experiments were performed after 180 min of observation in a total of 4 samples. Subtypes were identified through differences in the membrane receptor expression of CD16 and CD62L and a BD FACSAria I Cell Sorter (BD, Mountain View CA, USA) was utilized to identify a minimum of 50,000 cells per subset.

Cytology and visualization of cell morphology

Differential cell counts of lysed blood samples as well as cell-sorted samples were enumerated on cytopsin-prepared slides, fixed with methanol and finally stained with May-Grunwald & Giemsa. Images of cytopsin were made by a Leica light microscope (Leica, Wetzlar, Germany) at 100x magnification.

Data analysis and statistics

The data was analyzed using software programs SPSS version 20.0 (SPSS Inc., Chicago, IL, USA), GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA), Microsoft Excel 2019 (Microsoft Redmond, Washington, USA) and FCS Express Flow Cytometry Data Analysis 4.0 (De Novo Software, Glendale, CA, USA). Results are expressed as means (SEM) unless otherwise mentioned. For comparisons, Student's T-tests/ Paired T-tests or Mann Whitney U-tests were applied as appropriate to compare values over time with baseline measurements. Normality of variance was tested using Lavene's test. Furthermore, changes over time for related data were analyzed using repeated measures ANOVA. A p-value < 0.05 was considered statistically significant.

Results

Eleven out of 12 animals survived three hours of continuous surgery. One animal died due to hemodynamic instability and exsanguination two hours into the procedure.

Three hours of extensive thoraco-abdominal trauma surgery leads to deterioration of hemodynamic status and metabolic homeostasis

As surgery progressed, increasingly abnormal hemodynamic parameters were observed in all experimental animals. Despite volume resuscitation with balanced saline solutions, both systolic and diastolic blood pressure decreased from 110 ± 5 mmHg at baseline to 83 ± 10 at 180 min ($p = 0.026$) and 69 ± 4 at baseline to 44 ± 6 ($p = 0.0024$) at 180 min, respectively. Additionally, there was a non-significant trend towards an increasing heart rate between baseline and 150 min of surgery with heart rate increasing from 76 ± 3 to 106 ± 9 , respectively. Fig. 1 summarizes relevant hemodynamic parameters.

Figure 1

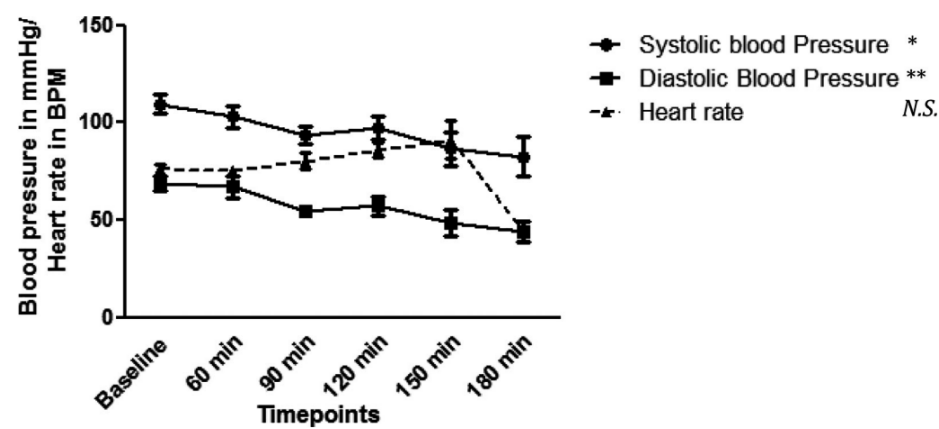


Fig 1. Alterations in hemodynamic parameters during the course of the experiment Ongoing extensive thoraco-abdominal trauma surgery leads to deterioration of hemodynamic status (reflected by decreasing blood pressure and the development of tachy- cardia) and metabolic homeostasis. All data are presented as mean \pm SEM. Blood pressure in mmHg and heart rate in beats per minute (BPM). Different time points were compared by repeated-measures ANOVA. Significance was displayed as: * $p < 0.05$; ** $p < 0.01$. N.S., non-significant.

Arterial blood gas analysis revealed a significant drop in blood pH over the course of the three-hour surgery (7.48 ± 0.03 vs. 7.37 ± 0.05 , $p = 0.03$). Furthermore, base excess decreased significantly from $+5 \pm 0.7$ at baseline to -5 ± 1.6 ($p < 0.001$) at 180 min. Renal filtration also worsened with a strong trend towards increased blood urea concentration from 4.4 ± 0.27 at baseline to 5.4 ± 0.28 at 3 h ($p = 0.056$). Severe hemodilution and suboptimal ventilation were prevented as no statistically significant changes in haematocrit, pO₂, pCO₂ levels were seen. Laboratory test results are summarized in Table 1.

Table 1. Peripheral blood laboratory measurements over time

	Baseline	60 min	120 min	180 min
<i>Metabolic (peripheral blood)</i>				
Sodium (mmol/L)	140 (0.38)	142 (0.75) *	143 (1.44) *	145 (2.54) *
Potassium (mmol/L)	4.1 (0.06)	4.1 (0.08)	4.2 (0.20)	3.7 (0.31)
Ionised Calcium (mmol/L)	1.57 (0.29)	1.58 (0.30)	1.27 (0.07)	1.16 (0.07)
Ureum (mmol/L)	4.4 (0.27)	4.4 (0.30)	4.6 (0.33)	5.4 (0.28)
Glucose (mmol/L)	3.9 (0.36)	3.8 (0.49)	4.5 (0.81)	4.3 (1.09)
<i>Coagulation and Hematocrit</i>				
APTT (seconds)	40.3 (6.1)	32 (3.1)	77 (43.6)	35 (3.5)
PT (seconds)	14.9 (0.5)	15.9 (0.5)	16.5 (1.7)	16.9 (2.3)
Hematocrit (L/L)	0.27 (0.01)	0.26 (0.02)	0.21 (0.02)	0.24 (0.11)
<i>Metabolic (arterial blood gas)</i>				
pH	7.48 (0.025)	7.43 (0.024)	7.41 (0.035)	7.37 (0.047) *
pCO ₂ (kPa)	5.3 (0.37)	5.5 (0.31)	4.7 (0.59)	4.7 (0.49)
pO ₂ (kPa)	29.2 (4.00)	36.5 (4.59)	38.4 (3.47)	35.9 (5.51)
HCO ₃ (mEq/L)	28.8 (0.73)	27.1 (0.67)	22.3 (1.54) ***	20.3 (2.40) ***
Base Excess	5 (0.7)	2.5 (0.8) *	-2 (1.6) ***	-5 (2.8) ***
Saturation (%)	95 (3.9)	97 (2.8)	100 (0.0)	99 (0.7)

Table 1. All data in Mean (SEM). For comparisons, a Student's T-tests or Mann Whitney U test was applied as appropriate to compare values over time with baseline measurements. Normality of variance was tested by the Lavene's test. Significance was displayed as: * indicates $p < 0.05$ vs. Baseline value; ** indicates $p < 0.01$ vs. Baseline value, *** indicates $p < 0.001$ vs. baseline value.

Extensive thoraco-abdominal trauma surgery was associated with a marked progressive neutropenia throughout the three hour surgery

Circulating neutrophil counts at baseline ranged from 4,4 to 14×10^6 PMNs/ml with a mean neutrophil count of $8,6 \pm 3,5 \times 10^6$ cells/ml. After 3 h of surgery, a markedly decreased mean neutrophil count of $2,4 \pm 1,8 \times 10^6$ cells/ml was measured ($p < 0.001$, Fig. 2). The observed decrease in neutrophil count (PMN) followed a near linear, time dependent pattern. The mean PMN or PMN-slope between baseline, and subsequent measurements did not differ significantly over time and ranged between $-1,9 \pm 2,9 \times 10^6$ cells/ml in the first hour, $-2,6 \pm 1,9 \times 10^6$ cells/ml during the second hour and $-2,6 \pm 1,5 \times 10^6$ cells/ml during the last hour. Absolute neutrophil counts during all time points are shown in Fig. 2.

Figure 2

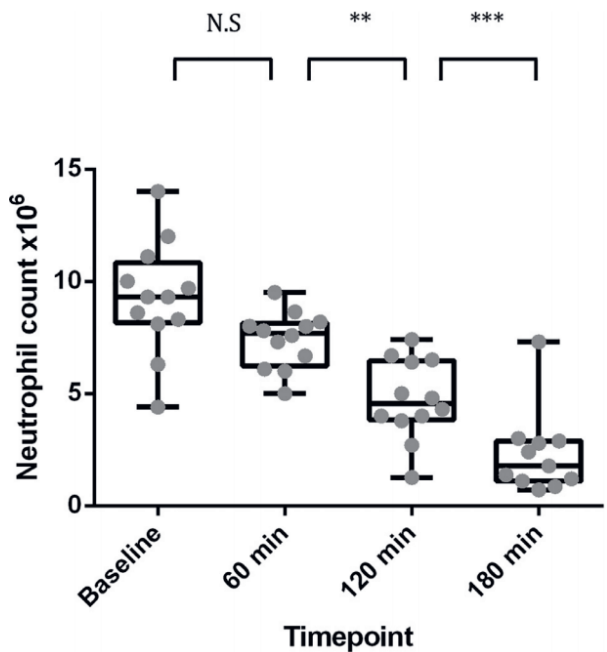


Fig. 2. Changes in neutrophil counts in peripheral blood over time Progressive neutropenia was observed throughout the three-hour thoraco- abdominal trauma surgery. All data are presented as mean \pm range. Neutrophil counts are measured and displayed as PMNs $\times 10^6$ / ml peripheral blood. Different time points were compared by Repeated-measures ANOVA. Significance was displayed as: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. N.S., non-significant.

Different neutrophil subtypes were identified in peripheral blood of pigs subjected to extensive thoraco-abdominal trauma surgery

We identified three different porcine neutrophil subtypes in blood after 180 min of continuous surgery by staining neutrophils for CD16 (Fc γ RIII) and CD62L (L-selectin). Flow cytometry at baseline showed one homogenous blood neutrophil pool. After 180 min of surgery, however, the neutrophil population developed heterogeneity with three different subtypes being observed (Fig. 3a, Supplement 3,4). Microscopic examination of sorted subsets (n = 4), revealed clear differences in morphological characteristics (Fig. 3b): CD16^{dim}/CD62L^{bright} cells exhibited a band shaped nucleus analogous to human banded PMNs [10]. The CD16^{bright}/CD62L^{bright} cells observed in this study have a more complex nuclear architecture with multiple lobules corresponding to mature neutrophils, while the CD16^{bright}/CD62L^{dim} cells exhibited even greater complexity with greater than 3 nuclear lobules. Thereby, porcine PMN-subset morphologic characteristics mimic microscopic properties of their human counterparts [10]. In addition, PMN-subset specific Mac-1/CD11b co-expression profiles in pigs also align with human data, as cell surface expression of the activation marker CD11b is significantly lower on CD16^{dim}/CD62L^{bright}-PMNs compared with other subsets [10,12]. (Fig. 3c).

Figure 3

a

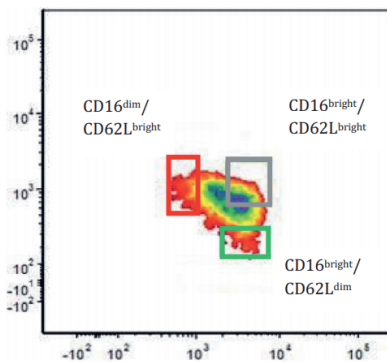


Fig. 3a. Cell sorting strategy of porcine neutrophil subsets and specific morphological characteristics of sorted subsets after 180 min of ongoing trauma surgery. At baseline one homogenous blood neutrophil pool was seen. However, three different porcine neutrophil subtypes were identified in blood after 180 min of continuous surgery. PMN-Fc γ RIII (CD16) expression on the cell membrane in MFI (AU) is depicted on the X-axis. L-selectin (CD62L) expression in MFI (AU) is depicted on the Y-axis. For sorting experiments a total of 50.000 cells/subset were sorted. The experiment shown is representative for 3 additional experiments.

b

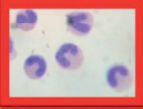

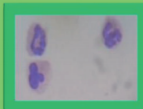
Membrane receptor expression level per subtype	Nuclear morphology	Subsequent definition (in line with human counterparts)
CD16 ^{dim} /CD62L ^{bright}		Banded neutrophils (kidney shaped nuclei)
CD16 ^{bright} /CD62L ^{bright}		Mature neutrophils (max of 3 lobes)
CD16 ^{bright} /CD62L ^{dim}		Hypersegmented neutrophils (>3 lobes)

Fig. 3b. Subset-specific morphological characteristics were seen, and they are in line with observations from human studies.

c

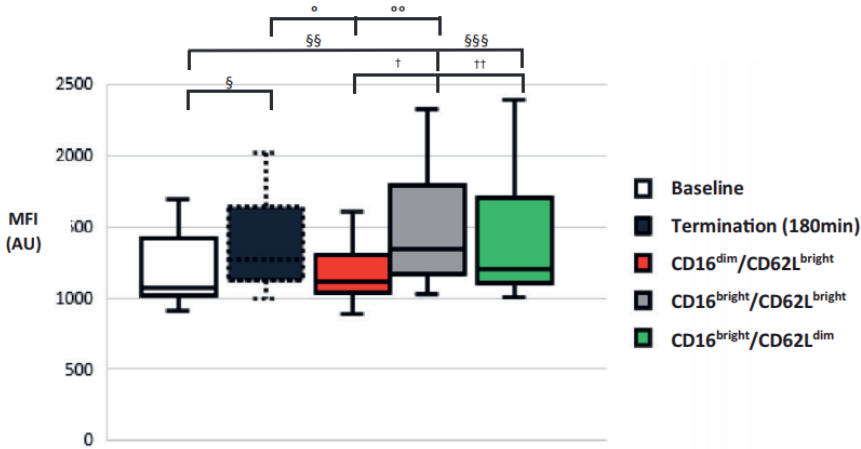


Fig. 3c. Subset-specific Mac-1/CD11b co-expression patterns were observed, and they are in line with observations from human studies. Boxplots include the minimum, first quartile, median, third quartile and maximum. Neutrophil CD11b expression was compared between the following conditions by paired T-tests: §, Baseline (all PMNs) vs. Termination 180 min (all PMNs), $p = 0.002$ §§, Baseline (all PMNs) vs. Mature PMNs 180 min, $p < 0.001$ §§§, Baseline (all PMNs) vs. Hypersegmented PMNs 180 min, $p = 0.006$ °, Termination (all PMNs) vs. Banded PMNs 180 min, $p = 0.002$ °°, Termination (all PMNs) vs. Mature PMNs 180 min, $p = 0.02$ †, Banded PMNs 180 min vs. Mature PMNs 180 min, $p = 0.005$ ††, Banded PMNs 180 min vs. Hypersegmented PMNs 180 min, $p = 0.03$.

An absolute increase in CD16^{dim}/CD62L^{bright} peripheral neutrophil count was observed during ongoing extensive trauma surgery, despite progressive overall neutropenia

The composition of the blood neutrophil population shifted gradually during the course of the experiment from a homogenous PMN pool with almost exclusive expression of mature CD16^{bright}/CD62L^{bright} cells at baseline under homeostatic conditions to a heterogeneous PMN pool (Fig. 4a and Supplement 3). Prior to the insult, there is a virtual absence of banded and hypersegmented neutrophils in peripheral blood with $88\% \pm 1\%$ of neutrophils comprised of mature CD16^{bright}/CD62L^{bright} cells at baseline. After 180 min of surgery, however, the fraction of mature PMNs shows a statistically significant drop to $59\% \pm 2\%$ ($p < 0.001$). A statistically significant increase in the hypersegmented CD16^{bright}/CD62L^{dim} neutrophil-fraction was observed with the fraction rising from $6\% \pm 1\%$ at baseline to $13\% \pm 1\%$ at 180 min after the start of insult induction ($p < 0.0001$). However, the most pronounced increase was seen with the banded CD16^{dim}/CD62L^{bright} PMN-subpopulation with the neutrophil fraction at baseline rising from $5\% \pm 1\%$ up to $25\% \pm 1\%$ at 180 min ($p < 0.0001$). Despite the absolute drop in overall neutrophil count, absolute numbers of banded CD16^{dim}/CD62L^{bright} neutrophils significantly increased during the first 2 h of surgery from $0.5 \pm 0.1 \times 10^6$ (mean \pm IQR) cells/ml at baseline to $0.8 \pm 0.1 \times 10^6$ (mean \pm IQR) cells/ml at 120 min ($p = 0.013$). The increase showed a consistent pattern over time and occurred simultaneously with an absolute drop in the circulating mature neutrophil count (Fig. 4b). At 180 min also the banded PMN count decreased when compared with 120 min, which is consistent with the other PMN subtypes. Supplement 4 provides an overview of changes in PMN-subtype cell counts over time.

Figure 4

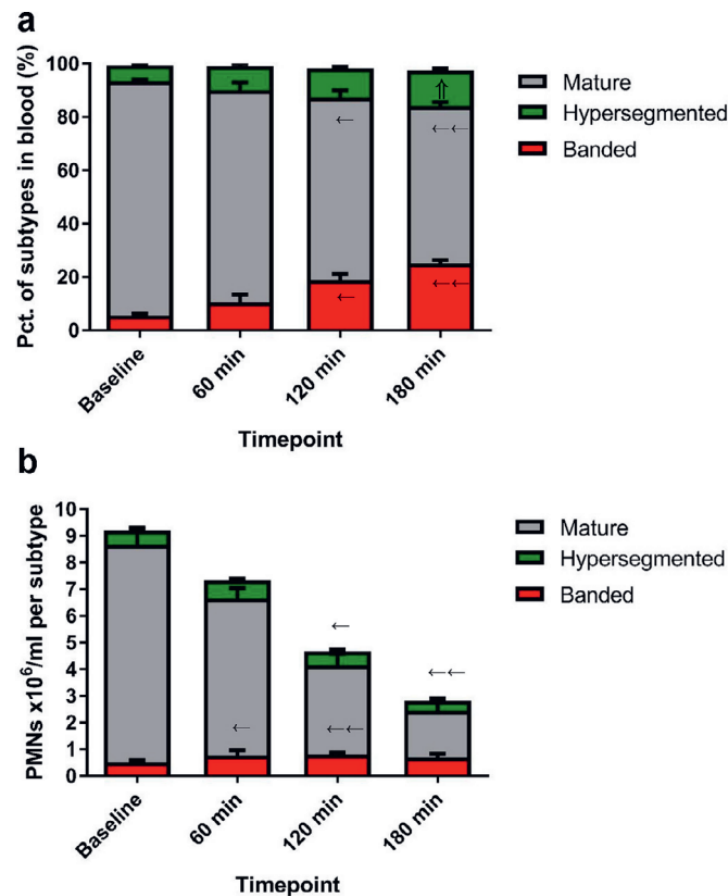


Fig. 4. Relative and absolute changes in the constitution of the blood neutrophil pool over time Analysis of PMN subset-specific kinetics in peripheral blood reveals that an absolute increase in CD16^{dim}/CD62L^{bright} peripheral neutrophil count occurred during the course of the study, despite progressive overall neutropenia. Fig. 4a: All data are presented as mean \pm SEM. Neutrophil counts were normalized and subsets measured in percent were compared over time. Different time points were compared by Repeated-measures ANOVA. †, Banded PMNs 60 min vs. 120 min, $p = 0.002$ ††, Banded PMNs 120 vs. 180 min, $p < 0.001$ ‡, Mature PMNs 60 min vs. 120 min, $p = 0.003$ ‡‡, Mature PMNs 120 min vs. 180 min, $p < 0.001$ *, Hypersegmented PMNs 120 min vs. 180 min, $p = 0.012$. Indicators of significance levels are displayed under the involved barchart. Fig. 4b: All data are presented as mean \pm SEM. Neutrophil counts were measured and displayed as PMNs $\times 10^6$ / ml peripheral blood. Different time points were compared by Repeated-measures ANOVA. Significance is displayed as: †, Banded PMNs BL vs. 60 min, $p = 0.044$ ††, Banded PMNs 60 min vs. 120 min, $p = 0.013$ ‡, Mature PMNs 60 min vs. 120 min, $p < 0.001$ ‡‡, Mature PMNs 120 min vs. 180 min, $p = 0.008$ Kinetics and significant differences of hypersegmented neutrophils are shown in Supplement 4. Indicators of significance levels are displayed above the involved barchart.

Discussion

This study describes the early impact of extensive thoracoabdominal trauma surgery on the composition of the circulatory PMN pool in a porcine controlled trauma model. Our analysis revealed the following findings:

1. continuous extensive surgery causes immediate, progressive neutropenia during a 3-hour intervention.
2. In a setting of increasing neutrophil depletion during surgical intervention, blood PMNs shift to a heterogeneous pool.
3. Different neutrophil subsets appear almost immediately after insult.
4. Porcine PMN subsets have specific morphological characteristics, activation status (reflected by Mac-1/CD11b co-expression levels) as well as kinetic characteristics.

Patients subjected to varying insults, such as trauma, sepsis, burn injuries, ischemic reperfusion injuries, and extensive surgery are at risk of complications such as ARDS and MODS [1,3,5,6,13]. The exact pathogenesis that causes the immune deregulation in patients developing these life-threatening conditions is unclear. However, deregulation of neutrophil homeostasis is thought to play an essential role [1,5–9]. In the current study, animals were subjected to an extensive, standardized series of thoraco-abdominal interventions, and injury severity was reflected by the worsening of the metabolic status. A statistically significant decrease in peripheral PMN count was encountered in all experimental animals throughout the thoracoabdominal procedure. Clinical studies on surgical patients have identified early neutropenia, and subsequent leukopenia as risk factors for increased morbidity and mortality rates [19,20]. This makes it tempting to hypothesize that instant progressive neutropenia, as observed in the current study, represents a pathological process, rather than a protective reactive response. The model clearly showed a uniform response of the circulatory PMN pool to the surgical insults with a pronounced and continuous drop in neutrophil count throughout the procedure. Interestingly, the composition of the peripheral PMN blood pool changed from a primarily homogenous cell population with almost exclusively normal mature cells being present in blood at baseline (homeostatic condition) into a heterogeneous cell population with several distinct neutrophil subsets. A possible explanation for the neutropenia observed in this study is that activated neutrophils leave the circulation and migrate into tissue compartments such as lung [21] or into the marginated pool [22,23]. In addition, it might be that in parallel

the bone marrow is unable to release a sufficient number of cells to compensate for the instantly circulatory neutrophil deprivation [24]. The observed early decrease in neutrophil count in this study resembles most 'pathogen associated molecular pattern' (PAMP)-driven models of immune activation, such as lipopolysaccharide induced systemic inflammation and models for abdominal surgery [25–27]. Since this phenomenon, as opposed to early leukocytosis, is not observed in primarily 'damage associated molecular pattern' (DAMP)-related innate immune activation in models of major vascular [28] or thoracic surgery [29], it is tempting to speculate that this phenomenon is specific for thoraco-abdominal surgery. The contaminated nature with translocation of microorganisms liberated during abdominal surgery as opposed to the relatively sterile conditions during isolated thoracic surgical component would then dictate the overall cellular innate immune response. This further suggests that our model with extensive thoraco-abdominal trauma surgery, as well as emergency surgery on severely injured trauma patients evokes a combined DAMP and (predominantly) PAMP-driven, instant systemic neutrophil response [30]. Previous analyses in humans have shown that the presence of systemic inflammation is associated with in the emergence of different neutrophil subtypes in blood [10,31,32]. Extensive surgery of pigs in this study resulted in similar changes in the constitution of the blood neutrophil pool with progressive heterogeneity over the course of surgery. These findings, including the temporal aspect of neutrophilic changes are in line with findings from Pillay et al. in a standardized model of human endotoxemia [10]. Interestingly however, during the development of progressive heterogeneity, human endotoxemia evokes prominent leukocytosis upon insult induction, whereas porcine thoraco-abdominal trauma induces leukopenia during the first 180 min after insult. Thereby, the current study is the first to demonstrate concurrent systemic leukopenia and the increasing circulatory neutrophil heterogeneity upon insult. In addition to decreased levels of CD16^{bright}/CD62L^{bright} neutrophils in blood, a constant increase in CD16^{dim} neutrophils was observed. These cells have previously been described in the setting of systemic inflammation as young neutrophils [10,12,33]. The assumption that these neutrophils are younger than other subsets is supported by the cell sorting experiments. These showed that CD16^{dim}/CD62L^{bright} cells were characterized by band-shaped nuclei. The human counterparts of this band-cell subpopulation have been well described [10–12] and have been shown to be younger cells [14]. Another important consideration that helps explain the increase in CD16^{dim} neutrophil fraction and absolute count observed in this study is the neutrophilic left shift in peripheral blood in the setting of acute systemic inflammation; thought to be an 'emergency release' of banded CD16^{dim} neutrophils [33,34]. Moreover,

previous experimental profiling studies have shown differences in functional capacities between different neutrophil subsets. In contrast to regular, mature neutrophils, hypersegmented (CD16^{bright}/CD62L^{dim}) neutrophils are characterized by impaired *in vitro* endothelial adhesion and reduced Reactive Oxygen Species (ROS)-production [35,36]. These cells are further capable of suppression of T-cell proliferation, have inferior bacterial killing capacities and a lower phagocytotic index [10,11,36]. Conversely, banded/CD16^{dim}-neutrophils showed enhanced capacity to contain MRSA intracellularly, whereas these cells exhibit impaired interaction with *S. epidermis*, and are less capable of generating a respiratory burst compared with CD16^{bright}-neutrophils [11,12]. Of note, these subset specific properties are based on subsetting experiments in non-surgical studies [10–12,36]. Therefore, unambiguous translation to the trauma field might not be accurate and more studies are mandated to define subset specific characteristic in trauma-induced acute inflammation. Nevertheless, both the current study in pigs and previously mentioned human experiments indicate a clear neutrophil subset heterogeneity. Currently, it is unclear whether the mobilization of these subsets should be considered as harmful, a bystander effect or evolutionary protective. However, functional capacities of the two novel subsets seem to be altered compared to regular/mature neutrophils and this may contribute to deregulation of immune homeostasis after trauma. In the current study none of the following factors was associated with altered outcome: (i) a more profound drop in neutrophil numbers, (ii) a lower initial neutrophil count, (iii) a more profound shifts in neutrophil subsets (*data not shown*). Nevertheless, the ratio between different neutrophil subsets might influence the course of innate immune homeostasis and outcome of surgical patients. More studies are mandated to further determine the potential prognostic value of neutrophil subset alterations in trauma/surgery for identifying patients at risk for major altered cellular immune status and inflammatory complications. In addition, targeting neutrophil subset alterations during extensive trauma surgery may form the basis for novel treatment concepts in extensive trauma/surgery and should be focus of upcoming studies as well. Several characteristics of our study should be taken into account. Firstly, since vasopressors have been shown to markedly interfere with neutrophil kinetics and the systemic immune response, the experimental model was designed without use of vasopressors [37]. Secondly, autologous blood transfusion was not conducted as not to interfere with the early phase analysis of peripheral blood during shock and surgery [38]. Furthermore, a porcine trauma model was preferred over alternative study designs given its' similarities in anatomical, physiological, genetical and immunological

properties to human [39,40]. Moreover, clinical studies on extensive/pre-lethal trauma likely lack standardization with respect to timing of blood sampling/interventions, injury severity and specific patient characteristics.

Conclusions

The current study reveals that extensive thoraco-abdominal trauma surgery in a controlled setting in pigs is associated with progressive neutropenia, depletion of regular mature neutrophils and increased heterogeneity of the blood neutrophil pool. Three different neutrophil subsets in peripheral blood were identified during the course of surgery. These subsets have similar morphological characteristics, receptor co-expression of Mac-1 and kinetics of mobilization to their human counterparts and further studies should clarify their precise role in the development of early organ failure during surgically induced acute systemic inflammation.

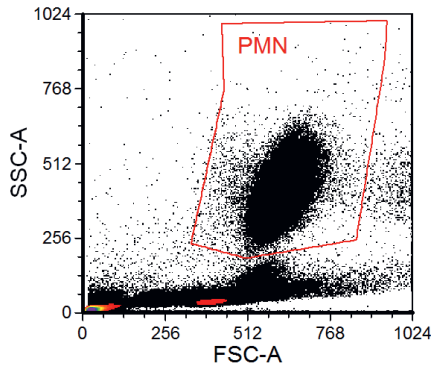
Supplement 1: Standardized porcine model of extensive thoraco-abdominal trauma surgery

First, a midline laparotomy was performed and a liver laceration plus 5 small bowel injuries were created and treated according to trauma guidelines. Thereafter, the diaphragm, stomach, spleen, pancreas and left kidney were injured. After achieving haemostasis, the diaphragm and stomach injuries were repaired, a splenectomy and distal pancreatectomy as well as a left sided nephrectomy were performed. Next, a second stellate liver laceration was induced and subsequently treated with abdominal packing strategies. A left anterior thoracotomy was performed and a penetrating cardiac injury above the phrenic nerve and away from the artery was treated with clamshell thoracotomy and staples in combination with intra-cardiac Foley catheter insertion. Additionally, the left lung was injured and treated through hilar clamping and non-anatomical resection. Finally, the infrarenal inferior vena cava and the right kidney were lacerated, and bleeding was subsequently controlled. All surgical procedures were completed within 180 minutes.

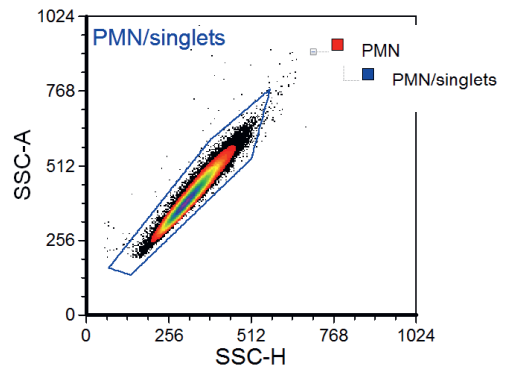
Supplement 2:

Validation of PMN-gating in porcine blood samples

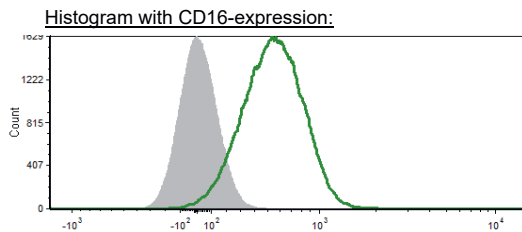
S1.1



S1.2

**Validation of PMN-gating by co-expression levels on control populations:**

S1.3

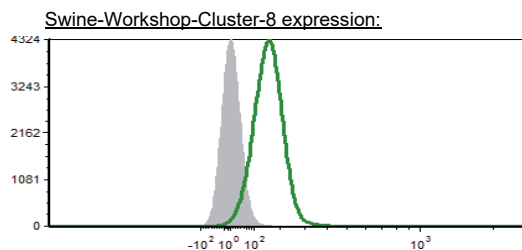


on Lymphocytes

on Neutrophils

(CD16⁺/CD45⁺/FSC^{high}/SSC^{high}/PB⁻)

S1.4



on Lymphocytes

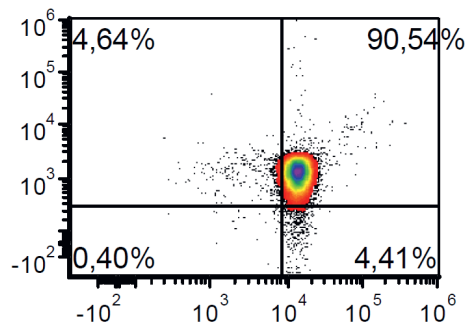
on Neutrophils

(CD16⁺/CD45⁺/FSC^{high}/SSC^{high}/PB⁻)

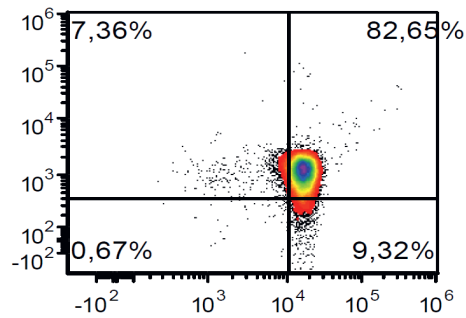
Stepwise porcine neutrophil gating strategy: Step 1: CD45 positive cells were selected and blood granulocytes were identified by their specific forward-sideward scatter signal (densityplot S1.1). Step 2: Non-singlets were excluded by comparing the forward-scatters signal area-measurement with the corresponding height-values densityplot S1.2). In order to exclude autofluorescent macrophages and eosinophils from our PMN-gate a negative gating strategy was applied, in which all positive cells in the FL-8 (Pacific Blue) channel were excluded. This gating strategy was validated by sorting experiments and comparing the co-expression of CD16 (S1.3) and SWC-8 (S1.4) antibodies on both neutrophil (green lines in histogram) and non-neutrophil populations (grey lines in histogram).

Supplement 3: **Changes in PMN-heterogeneity over time**

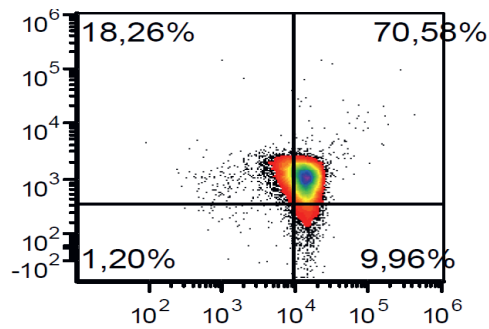
T=0



T=120 min



T=180 min

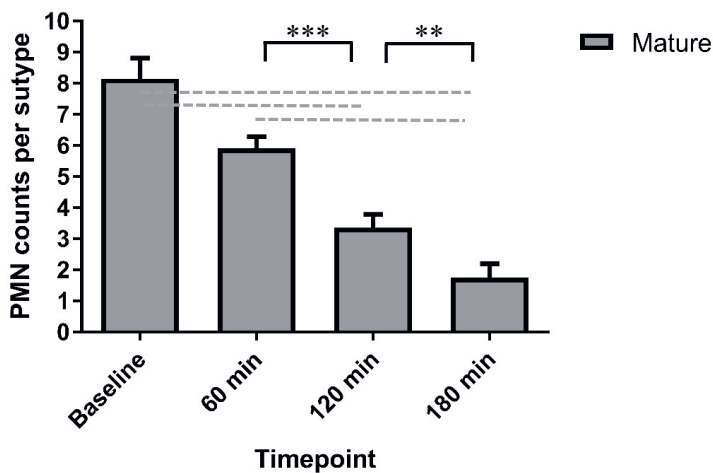


x-axis: PMN-Fc γ RIII (CD16) cell membrane expression levels in MFI (AU)

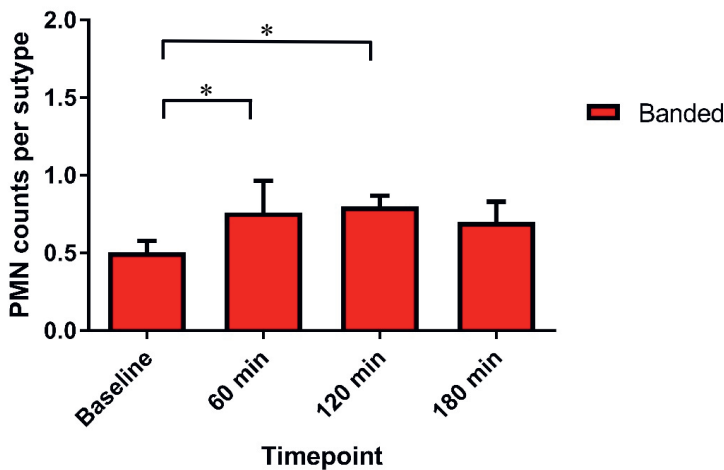
y-axis: L-selectin (CD62L) cell membrane expression levels in MFI (AU)

Supplement 4: **PMN-subset specific kinetics in peripheral blood over time**

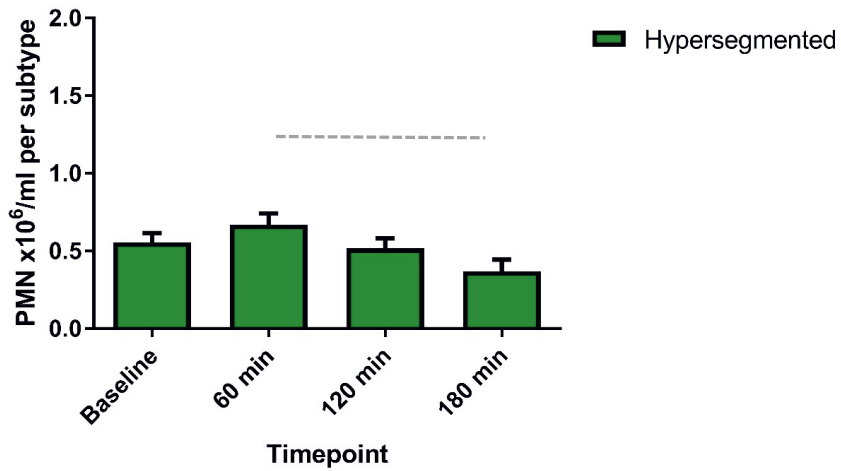
Fractions of PMN subtypes in blood over time



Fractions of PMN subtypes in blood over time



Fractions of PMN subtypes in blood over time



All data are presented as mean \pm SEM. Neutrophil counts are measured and displayed as PMNs $\times 10^6$ /ml peripheral blood. Different time points were compared by Repeated-measures ANOVA. Significance was displayed as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. N.S., non significant. Continuous black lines represent relevant statistically significant differences. Dotted grey lines indicate statistically significant differences that, according to the opinion of the authors, are of minor clinical relevance.

References

1. Keel M, Trentz O. Pathophysiology of polytrauma. *Injury* 2005;36(6):691–709.
2. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 1992;101(6):1644–55.
3. Flannagan RS, Cosio G, Grinstein S. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol* 2009;7(5):355–66.
4. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science* 2004;303(5663):1532–5.
5. Hietbrink F, Koenderman L, Althuisen M, Pillay J, Kamp V, Leenen LP. Kinetics of the innate immune response after trauma: implications for the development of late onset sepsis. *Shock* 2013;40(1):21–7.
6. Lord JM, Midwinter MJ, Chen YF, Belli A, Brohi K, Kovacs EJ, et al. The systemic immune response to trauma: an overview of pathophysiology and treatment. *Lancet* 2014;384(9952):1455–65.
7. Adams JM, Hauser CJ, Livingston DH, Lavery RF, Fekete Z, Deitch EA. Early trauma polymorphonuclear neutrophil responses to chemokines are associated with the development of sepsis, pneumonia, and organ failure. *J Trauma* 2001;50:792–800.
8. Brown KA, Brain SD, Pearson JD, Edgeworth JD, Lewis SM, Treacher DF. Neutrophils in development of multiple organ failure in sepsis. *Lancet* 2006;368(9530):157–69.
9. Windsor AC, Mullen PG, Fowler AA, Sugerman HJ. Role of the neutrophil in adult respiratory distress syndrome. *Br J Surg* 1993;80(1):10–17.
10. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers JW, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest* 2012;122(1):327–36.
11. Leliefeld PH, Pillay J, Vrisekoop N, Heeres M, Tak T, Kox M, et al. Differential anti-bacterial control by neutrophil subsets. *Blood Adv* 2018;2(11):1344–55.
12. Pillay J, Ramackers BP, Kamp VM, Loi AL, Lam SW, Hietbrink F, et al. Functional heterogeneity and differential priming of circulating neutrophils in human experimental endotoxemia. *J Leukoc Biol* 2010;88(1):11–220.
13. Rosales C. Neutrophil: a cell with many roles in inflammation or several cell types? *Front Physiol* 2018;9:113.
14. Tak T, Wijten P, Heeres M, Pickkers P, Scholten A, Heck AJ, et al. Human CD62Ldim neutrophils identified as a separate subset b proteome profiling and in vivo pulse-chase labeling. *Blood* 2017;129(26):3576–85.
15. Botha AJ, Moore FA, Moore EE, Sauaia A, Banerjee A, Peterson VM. Early neutrophil sequestration after injury: a pathogenic mechanism for multiple organ failure. *J Trauma* 1995;39(3):411–17.
16. Hesselink L, Spijkerman R, van Wessel KJP, Koenderman L, Leenen LPH, Huber-Lang M, et al. Neutrophil heterogeneity and its role in infectious complications after severe trauma. *World J Emerg Surg* 2019;14:24.

17. Manual of Definitive Surgical Trauma Care (DSTC): third edition. KD Boffard, International association of trauma surgery and intensive care. New York, USA. Oxford University, 2011.
18. Bouillon B, Marzi I. The updated german polytrauma-guideline: an extensive literature evaluation and treatment recommendation for the care of the critically injured patient. *Eur J Trauma Emerg Med* 2018;44(s1) 1-1.
19. Pallister I, Dent C, Topley N. Increased neutrophil migratory activity after major trauma: a factor in the etiology of acute respiratory distress syndrome. *Crit Care Med* 2002;30(8):1717–21.
20. Gulack BC, Englum BR, Lo DD, Nussbaum DP, Keenan JE, Scarborough JE, et al. Leukopenia is associated with worse but not prohibitive outcomes following emergent abdominal surgery. *J Trauma* 2015;79(3):437–43.
21. Summers C, Singh NR, White JF, MacKenzie IM, Johnston A, Solanki C. Pulmonary retention of primed neutrophils: a novel protective host response, which is impaired in the acute respiratory distress syndrome. *Thorax* 2014;69(7):623–9.
22. Reino DC, Palange D, Feketeova E, Bonitz RP, Xu DZ, Lu Q, et al. Activation of toll-like receptor 4 is necessary for trauma hemorrhagic shock-induced gut injury and polymorphonuclear neutrophil priming. *Shock* 2012;38:107–14.
23. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil kinetics in health and disease. *Trend Immunol* 2010;31:318–24.
24. Christensesn RB, Bradley PP, Rothstein G. The leukocyte left shift in clinical and experimental neonatal sepsis. *J Pediatrics* 1981;98(1):101–5.
25. Jacobi CA, Ordemann J, Zieren HU, Volk HD, Bauhofer A, Halle E. Increased systemic inflammation after laparotomy versus laparoscopy in an animal model of peritonitis. *Arch Surg* 1998;133(3):258–62.
26. Richardson RP, Rhyne CD, Fong Y, Hesse DG, Tracey KJ, Marano MA, et al. Peripheral blood leukocyte kinetics following in vivo lipopolysaccharide (LPS) administration to normal subjects. Influence of elicited hormones and cytokines. *Ann Surg* 1989;210(2):239–45.
27. Hawes AS, Fischer E, Marano MA, Zee van KJ, Rock CS, Lowry SF, et al. Comparison of peripheral blood leukocyte kinetics after live *Escherichia coli*, endotoxin, or interleukin-1 alpha administration. Studies using a novel interleukin-1 receptor antagonist. *Ann Surg* 1993;218(1):79–90.
28. Paterson IS, Klausner JM, Pugatch Allen P, Mannick JA, Valeri CR, et al. Noncardiogenic pulmonary edema after abdominal aortic aneurysm surgery. *Ann Surg* 1989;209(2):231–6.
29. Isitmangil G, Isitmangil T, Balkanli K, Cerrahoglu K, Kunter E. Detection of thoracotomy-induced alterations in cell- and humoral-mediated immune response. *Eur J Cardiothorac Surg* 2002;21(3):497–501.
30. Relja N, Land WG. Damage-associated molecular patterns in trauma. *Eur J Trauma* 2019;46:751–75.
31. Fraser JA, Kemp S, Young L, Ross M, Prach M, Hutchison GR, Malone E. Silver nanoparticles promote the emergence of heterogeneous human neutrophil sub-populations. *Sci Rep* 2018;8(7506).

32. Lecot P, Sarabi M, Pereira Abrantes M. Neutrophil heterogeneity in cancer: from biology to therapies. *Front Immunol* 2019;10:2155.
33. Orr Y, Taylor JM, Bannon PG, Geczy C, Kritharides L. Circulating CD10-/CD16low neutrophils provide a quantitative index of active bone marrow neutrophil release. *Br J Haematol* 2005;131:508–19.
34. Drifte G, Dunn-Siegrist I, Tissieres P, Pugun J. Innate immune functions of immature neutrophils in patients with sepsis and severe systemic inflammatory response syndrome. *Crit Care* 2013;41(3):820–32.
35. Kamp VM, Pillay J, Lammers JW, Pickkers P, Ulfman LH, Koenderman L. Human suppressive neutrophils CD16bright/CD62Ldim exhibit decreased adhesion. *J Leuk Biol* 2012;92(5):1011–20.
36. Sauce D, Dong Y, Campillo-Gimenez L, Casulli S, Bayard C, Autran B, et al. Reduced oxidative burst by primed neutrophils in the elderly individuals is associated with increased levels of the CD16bright/CD62Ldim immunosuppressive subset. *J Geront A Biol Sci Med Sci* 2017;72(2):163–72.
37. Davis JM, Albert JD, Tracy KJ, Calvano SE, Lowry SF, Shires GT, et al. Increased neutrophil mobilization and decreased chemotaxis during cortisol and epinephrine infusions. *J Trauma* 1991;31(6):725–31.
38. Kessler U, Grau T, Gronchi F, Berger S, Brandt S, Bracht H, et al. Comparison of porcine and human coagulation by thromboelastometry. *Thromb Res* 2011;128(5):477–82.
39. Swindle MM. Comparative anatomy and physiology of the pig. *Scan J Labor Anim Science* 1998;23:1–10.
40. Hildebrand F, Andruszkow H, Huber-Lang M, Pape HC, Griensven van M. Combined hemorrhage/trauma models in pigs- current state and future perspectives. *Shock* 2013;40(4):247–73.

Chapter 5

Altered cell surface receptor dynamics and circulatory occurrence of neutrophils in a small animal fracture model

Michel Paul Johan Teuben¹

Martijn Hofman²

Johannes Greven²

Alba Shehu²

Henrik Teuber¹

Roman Pfeifer¹

Hans-Christoph Pape¹

Frank Hildebrand²

¹ Department of Traumatology and Harald Tscherne Research Laboratory, University Hospital Zurich, Zurich, Switzerland

² Department of Orthopaedic Trauma and Reconstructive Surgery, University of Aachen Medical Center, Aachen, Germany

Abstract:

Introduction

Excessive activation of the immune response after femoral fractures and fracture fixation is potentially associated with the development of systemic and local complications, particularly in multiple trauma patients. A dysregulated function of neutrophils, the most prevailing immune cells in circulation, has been discussed as a central pathophysiological background for these unfavourable post-traumatic courses. Our aim was to investigate alterations in activity and functionality as expressed by the cell surface receptor dynamics of circulatory neutrophils after femoral fracture and intramedullary stabilization.

Material and Methods

After intramedullary stabilization, an isolated femur fracture was induced in 18 Sprague Dawley rats. Animals were terminated at different time points, i.e. after 3 (n = 5, group 3d), 7 (n = 5, group 7d) and 14 (n = 5, Group 14d) days and grouped accordingly. Additionally, baseline measurements were performed in one control animal per study group (n = 3) after anaesthesia induction and termination, without prior intramedullary nailing and fracture induction. The numbers and cell surface expression of CD11b, CD11a, CD62L, and CD49d of circulating neutrophils were compared between groups.

Results

Neutrophil numbers were significantly reduced at 3 days compared with baseline measurements (1.2×10^5 vs. 6.3×10^5 cells/mL, $p < 0.01$). By day 7, neutrophil counts significantly increased back to homeostatic levels ($p < 0.05$). At day 3, CD11b-expression was significantly reduced, whereas CD11a-expression was increased compared with the baseline measurements ($p < 0.05$). At day 7, the circulatory neutrophil pool exhibited a unique CD11b^{high}/CD11a^{high}-neutrophil subset showing a significantly increased co-expression of CD49d. The expression of CD62L did not change significantly throughout the experiment compared with baseline measurements.

Conclusions

This descriptive small animal fracture study is the first to show that an intramedullary stabilized femur fracture is associated with a temporary reduction in circulatory neutrophil count and concurrent changes in circulatory neutrophil function. Moreover, we demonstrated that the

restoration to homeostatic neutrophil activation status occurs concomitantly with the appearance of a novel neutrophil subtype (CD11b^{high}/CD11a^{high}) in circulation. Our fundamental new findings of the changes in circulatory neutrophil count and functionality after trauma form an excellent basis for future studies to further elucidate the role of neutrophils as activators and regulators of different post-traumatic processes, potentially resulting in local (e.g., fracture healing disturbances) or systemic (e.g., MODS) complications. This might result in the development of specific therapies to reduce adverse outcomes after trauma.

Introduction

It is well-known that particularly in subgroups of multiple trauma patients, femur fractures and their intramedullary stabilization might result in a higher incidence of post-traumatic complications [1,2]. Posttraumatic activation of the immune response has been shown to play a central role in these unfavourable events over the clinical course. It is assumed that neutrophils, the most prevailing immune cells in the human circulation system, play a central role in pathophysiological processes, potentially leading to systemic and local complications [3,4]. At the local level at the fracture site, neutrophils are one of the first cells that permeate into the fracture hematoma. Here, they differentiate into different subsets, synthesize an “emergency extra cellular matrix,” and initiate the inflammation stage of fracture healing [5,2]. At this stage, recruited immune cells clear the fracture area from pathogens and cell debris, restricting tissue damage [6]. On the other hand, if this stage is not terminated adequately, damage of even uninjured tissue because of an excessive stimulation of neutrophils can occur [6]. In this way, even systemic complications can occur because of the infiltration of neutrophils into lung parenchyma, with the subsequent formation of oedema, leading to tissue and, eventually, organ damage [7–9]. Neutrophil tissue homing is regulated by alterations in the expression of cell surface receptors. Various selectins, including L-selectin (CD62L), are initially upregulated and later shed from the cell surface to enable vessel wall adherence and to initiate deceleration and rolling. In addition, integrin upregulation, including macrophage-1 antigen (Mac-1 or CD11b) and lymphocyte function-associated antigen-1 (LFA1 or CD11a), achieves actual neutrophil adhesion and extravasation [10]. However, *in vitro* studies suggest that there is a functional overlap and that integrins are not only involved in adhesion and transmigration, but also in rolling [11]. The aforementioned changes of the local release and/or differentiation of diverse neutrophil subsets have also been described after fractures [5]. These subsets present with various surface receptor expressions, which, in turn, result in different neutrophil characteristics. However, knowledge of the specific influence of an isolated stabilized femoral fracture on the systemic neutrophil pool, especially throughout the later post-traumatic phases, is sparse. Indeed, the alterations in circulatory neutrophil number and their cell surface expression of selectins and integrins following fracture and intramedullary nailing have never been investigated. Knowledge of the time course and specific pattern of neutrophil surface receptor expression after trauma is of the utmost importance for the future development of therapeutic options to modulate the role and homing of neutrophils in injured tissue.

Therefore, we investigated if femoral fractures and intramedullary nailing are associated with alterations in circulatory neutrophil counts and circulatory neutrophil surface receptor expression of selectins and integrins.

Material and methods

Housing

Our study cohort consisted of 21 adult female Sprague-Dawley rats weighing approximately 250 g; the rats were obtained from Envigo B.V. (Horst, Netherlands). All the animals were specifically pathogen free according to the Federation of European Laboratory Animal Science Associations' recommendations. The animals were housed and the experiments performed at the Institute of Laboratory Animal Science, University of Aachen Medical Center, Germany, with the approval of the Governmental Animal Care and Use Committee (Landesamt für Natur, Umwelt und Verbraucherschutz, North Rhine-Westphalia, Recklinghausen, Germany; Protocol No. 84-02.04.2015.A078). The animals were housed under controlled temperature (20 ± 2 °C) and air humidity (45–65 %), with a 12 h light–dark cycle and a light intensity of < 200 lx. Food and water were offered ad libitum. Prior to study inclusion, all animals were kept in groups for 1 week in the laboratory premises to allow for acclimatization. Throughout the entire experiment, the rats underwent physical examinations according to a “score sheet” documentation [12] and the “body condition scoring” according to Hickman [13] to obtain the general health status. In order to optimize standardization of this long-term observation study, female animals were utilized, as variations in animal weight over time are smaller than observed in their male counterparts. Because of the protective effects of female hormones in cases of inflammatory stimuli, all animals were confirmed to be in the same “metestrus” phase of the menstrual cycle because this phase is characterized by low oestrogen and progesterone levels [14,15]. The menstrual cycle phase was identified by the assessment of vaginal swabs according to Marcondes et al. [16].

Experimental design and study groups

In order to minimize usage of experimental animals in the current study, a baseline measurement was performed after anaesthesia and termination after 3 days ($n = 1$), 7 days ($n = 1$), and 14 days ($n = 1$), without prior intramedullary nailing and fracture induction. Data of control animals was pooled and analysed as Group control. In all other animals ($n = 18$), after anaesthesia, an intramedullary Kirschner wire was inserted, and a standardized femoral fracture was induced at the right side. The animals were then randomly divided into three study groups according to the observation period: Group 3d: 3 days of observation after fracture induction Group 7d: 7 days of observation after fracture induction Group 14d: 14 days of observation

after fracture induction. The reasons for excluding animals were as follows: death from anaesthetic complications, open fracture, comminuted fracture, implant failure, wound dehiscence, or infection.

Anaesthesia and pain management

The animals received buprenorphine hydrochloride (0.03–0.05 mg/kg s.c.) as a multimodal analgesic at 30 min before operation. The operative procedures were performed under general anaesthesia induced with ketamine (100 mg/kg i.p.) and xylazine (2 %; 10 mg/kg i.p.) and, if necessary, extended with isoflurane inhalation (2.0–2.5 Vol. %). The toe pinch reflex was used to ensure adequate anaesthesia. Postoperative analgesia was ensured with buprenorphine hydrochloride (0.03–0.05 mg/kg s.c.) every 6 h for the first 24–48 h. Subsequently, it was given twice daily during the entire period of the experiment. All animals were allowed to mobilize freely directly after the operative procedure. The animals were evaluated three times per day using the score sheet evaluation [12]. In the potential case of clinical signs of uncompensated/enhanced pain, animals are to be withdrawn from the study. Similar painkilling protocols were applied to control animals during the observation period.

Standardized femoral fracture

Sterilized implants (autoclave processing) and surgical equipment was used. Furthermore, procedures were performed using sterile gowns, surgical mask and theatre caps. After anaesthesia induction, the animals were placed on a heated pad (37 °C), and their eyes were covered with a moistening ointment. Their right hind leg was shaved, disinfected, and draped. Then, a para-patellar incision was made, the patella was everted laterally, and a 1 mm stainless-steel intramedullary Kirschner wire (Königsee Implantate GmbH, Allendorf, Germany) was inserted in a retrograde manner. Its placement was confirmed by fluoroscopy. The sharp end of the Kirschner wire was placed to the proximal end of the femur. The Kirschner wire was cut flush with the intercondylar notch, and the proximal end was bent over the greater trochanter, cut, and hidden subcutaneously. The distal end of the Kirschner wire was then shortened underneath the cartilaginous surface. The patella was repositioned, and the wounds were sutured (Ethicon Inc., Somerville, NJ, USA) in layers. Surgical procedures have been described in detail by Klueter et al. [17]. A transverse fracture was induced with a blunt guillotine according to the method by Bonnarens and Einhorn [18]. A fluoroscopic evaluation of the

fractured side was performed directly after fracture induction. An example of fracture fixation adequacy and radiographic imaging is provided in the Appendix, Fig. A1.

Blood collection and euthanasia

In accordance with animal grouping, animals were euthanized at three different time points (3d, 7d, and 14d) by cardiac puncture with an ethylenediaminetetraacetic acid (EDTA)-coated syringe. Prior to cardiac puncture, animals were put under general anaesthesia induced with ketamine (100 mg/kg i.p.) and xylazine (2 %; 10 mg/kg i.p.). After cardiac puncture, an overdose of Isoflurane was provided and manual cervical dislocation was applied.

Sampling

Blood samples were lysed twice in an ice-cold isotonic NH₄Cl solution. Cells were then washed twice in a FACS (fluorescence-activated cell sorting) buffer solution (phosphate buffered saline supplemented with 0.5 % bovine serum albumin and 0.5 mM EDTA). Cell suspensions were then incubated with conjugated mouse anti-rat monoclonal antibodies. The following commercially available mouse anti-rat monoclonal antibodies were utilized: CD62L clone OX-85 (AbD Serotec, Düsseldorf, Germany) / fluorescein isothiocyanate (FITC), CD11b clone M1/70 (eBioscience Vienna, Austria) / R-phycoerythrin (RPE), CD49d (VLA-4) clone MRalpha4-1 (Becton & Dickinson, Mountain View, CA, USA) / peridinin-chlorophyll-protein-cyanine 5.5 (PerCP-Cy5.5), CD11a clone WT.1 (AbD Serotec, Düsseldorf, Germany) / allophycocyanin (APC), and Granulocytes mouse anti-rat clone RP-1 (Becton & Dickinson, Mountain View, CA, USA) / allophycocyanin-cyanine 7 (APCCy7). Details on flow analyses are provided in appendix, Fig. A2.

Neutrophil cell surface marker expression

Neutrophil cell membrane receptor expression levels of CD62L (L-selectin), CD11b (macrophage-1 antigen (Mac-1)), and CD11a (lymphocyte function-associated antigen-1 (LFA-1)) were measured by flow cytometry with a Canto II-device (Becton & Dickinson, Mountain View, CA, USA) and FACS Diva software (Becton & Dickinson, Mountain View, CA, USA). Neutrophils were identified using a four-step gating strategy (Appendix, Fig. A2.). In steps one and two, debris and doublets were excluded. In step three, cells with a neutrophil specific forward scatter/side scatter-pattern were included. Finally, RP-1 positive cells were included. The utilized gating strategy has previously been validated for analysis of both blood

and tissue neutrophils [19–22]. From each sample, a minimum of 200,000 cells were measured. Cell viability was confirmed in separate pilot experiments. Because cells were stained and measured within 2 h after blood collection, maximal neutrophil viability was ensured, and cell fixation was not indicated. The dynamics of cell surface receptors was compared between the groups. Additionally, the current study aimed to identify novel subsets that play a role in the inflammatory response to trauma.

Neutrophil count

The total cell count of viable neutrophils was determined using a Neubauer improved grid haemocytometer (LW Scientific, Lawrenceville, USA). This device is optimized for leukocyte versus non-leukocyte differentiation of cell suspensions. Two independent samples were taken from lysed blood samples. Cell suspensions were stained with Trypan blue (Thermo Fisher Scientific, Waltham, MA, USA) and then separately filled into two counting areas on the counting chambers. The total cell counts of large, viable cells were documented, and an average value was calculated. Thereafter, total neutrophil counts were determined by the ratio of RP-1 (a specific neutrophil marker in rats) positive cells: total gated white blood cells (cells) as measured by flow cytometry. This method has been described and validated by Krajnar et al. [21].

Endpoint and power analysis

The primary endpoint of our study was an alteration in circulatory neutrophil numbers in the early fracture healing period of 14 days. A secondary endpoint was an alteration in neutrophil activity, as measured by neutrophil cell surface expression of CD11a, CD11b, and CD62L. Our sample size calculation was based on alterations in circulatory neutrophil numbers over time in a rat study from McManus et al. [26], which demonstrates the raw data of circulatory neutrophil numbers from the control (1565 ± 387 cells / mm^3) and intervention groups (3999 ± 787 cells / mm^3) 9 days after insult. According to our power analysis, groups with at least four animals will provide 90 % power at an α -level of 0.05. To compensate for higher variability in our model and potential mortality, we decided to include six animals in each intervention group.

Data analysis

Statistical analysis was performed using SPSS (version 20.0; IBM Inc., Somers, NY, USA) and GraphPad Prism (version 7; San Diego, USA). All data are presented as the mean and standard deviation unless described otherwise. Comparison of the data with control values and between groups was performed using Mann-Whitney's U-test. In general, a two-sided p-value < 0.05 was considered significant.

Results

All animals survived the observation period, and no animals had to be excluded according to our exclusion criteria.

Circulatory neutrophil count

The absolute neutrophil count significantly decreased in Group 3d compared with the baseline conditions (1.2×10^5 vs. 6.3×10^5 cells / ml, $p < 0.01$). However, circulatory neutrophil numbers returned to homeostatic levels after 7 days of observation ($p < 0.05$) and remained stable at day 14 ($p < 0.05$) (Fig. 1).

Figure 1. Circulatory neutrophil count in early fracture healing

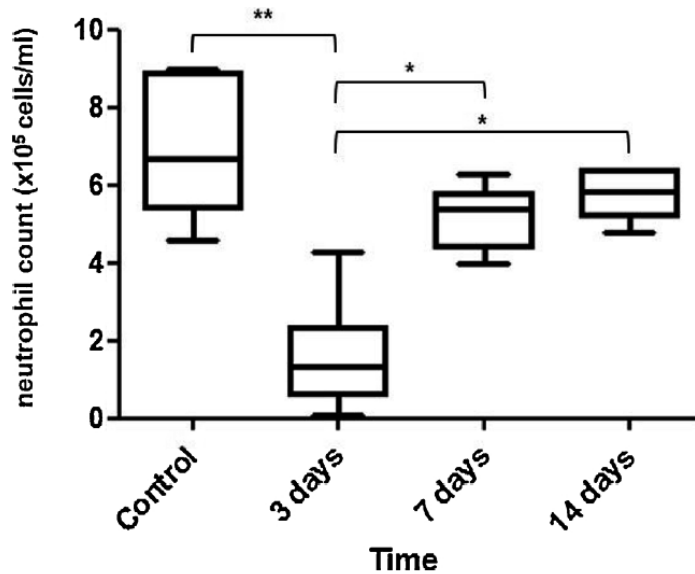


Fig. 1. A significant difference was seen between the baseline measurement (control) and animals terminated 3 days after surgery (** $p < 0.01$). Furthermore, a significant rise in neutrophil count was observed between day 3 and day 7 (* $p < 0.05$). No differences were found between the baseline measurement (control) and counts at day 7 and day 14.

Neutrophil cell surface expression of L-selectin (CD62L)

The expression of L-selectin (CD62L) at the surface of circulatory neutrophils decreased significantly in Group 7d and Group 14d compared with Group 3d, but no significant difference occurred between the baseline measurement and all three fracture groups (Fig. 2a).

Figure 2a. Cell surface expression of L-selectin (CD62L) on circulatory neutrophils

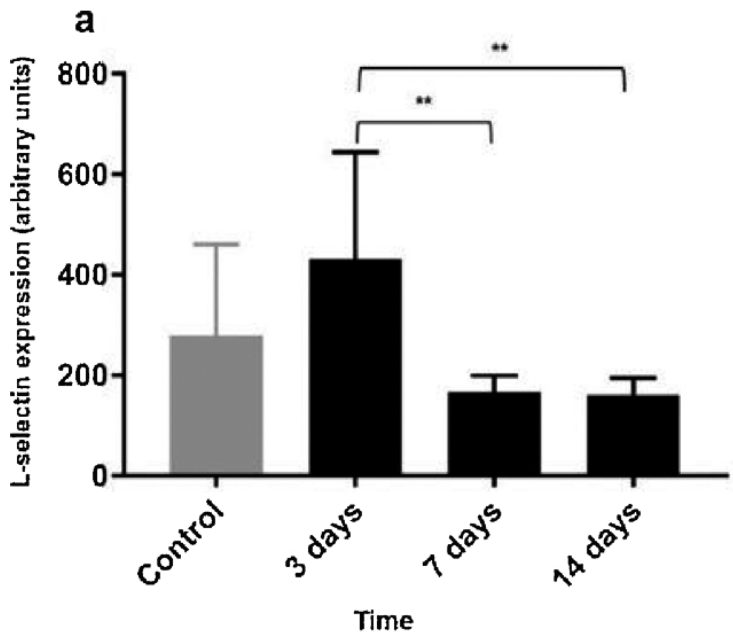


Fig. 2a. L-selectin (CD62 L) expression on circulatory neutrophils. Significant differences were seen between day 3 and 7, as well as at day 14, after intramedullary nailing and fracture induction (** $p < 0.01$). No significant differences were found between the intervention groups and the baseline measurement.

Neutrophil cell surface expression of Mac-1 (CD11b)

The expression of Mac-1 (CD11b) at the surface of circulatory neutrophils was significantly decreased in Group 3d compared with the baseline measurement ($p < 0.05$). In Group 7d and Group 14d, the expression of Mac-1 (CD11b) returned to baseline values again. These values were significantly increased compared with the values at day 3 (day 7: $p < 0.01$, day 14: $p < 0.05$) (Fig. 2b).

Figure 2b. Cell surface expression of Mac-1 (CD11b) on circulatory neutrophils

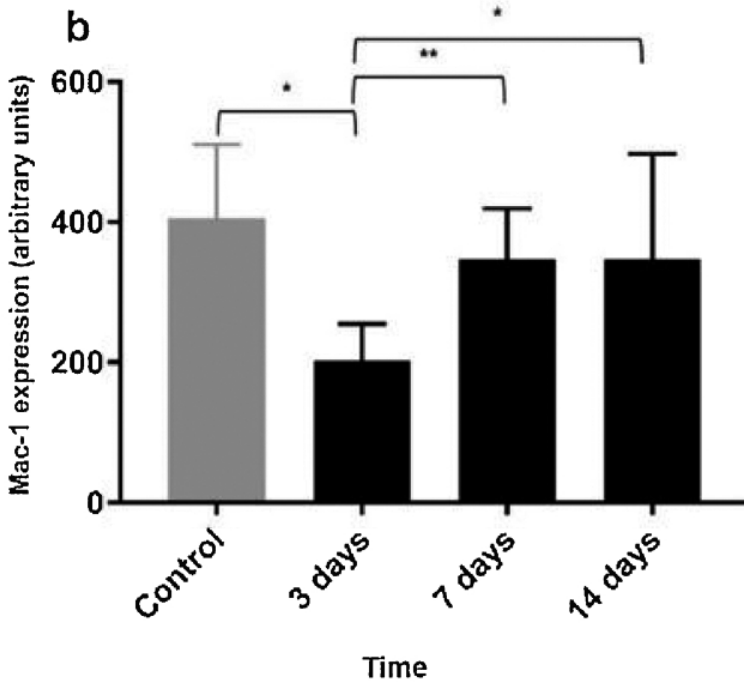


Fig. 2b. Mac-1 (CD11b) expression on circulatory neutrophils. A significant decrease of Mac-1 expression was seen at day 3 compared with the baseline measurement (* $p < 0.05$). After 7 days, the expression returned to baseline values and remained at this level throughout day 14.

Neutrophil cell surface expression of LFA-1 (CD11a)

The expression of LFA-1 (CD11a) at the surface of circulatory neutrophils significantly increased temporarily in Group 3d when compared with the baseline measurement ($p < 0.05$) and with Group 7d ($p < 0.01$). LFA-1 (CD11a) expression levels returned to homeostatic baseline levels by day 7 and remained at baseline until the end of the experiment (Fig. 2c).

Figure 2c. Cell surface expression of LFA-1 (CD11a) on circulatory neutrophils

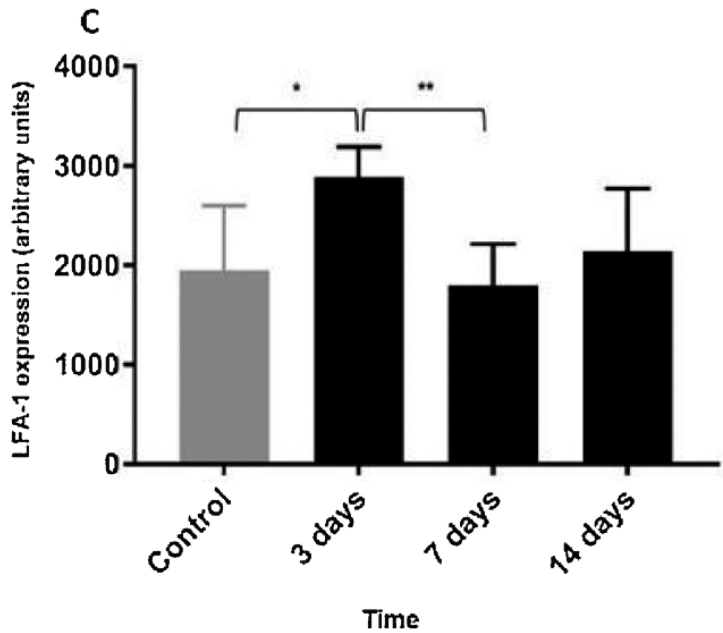


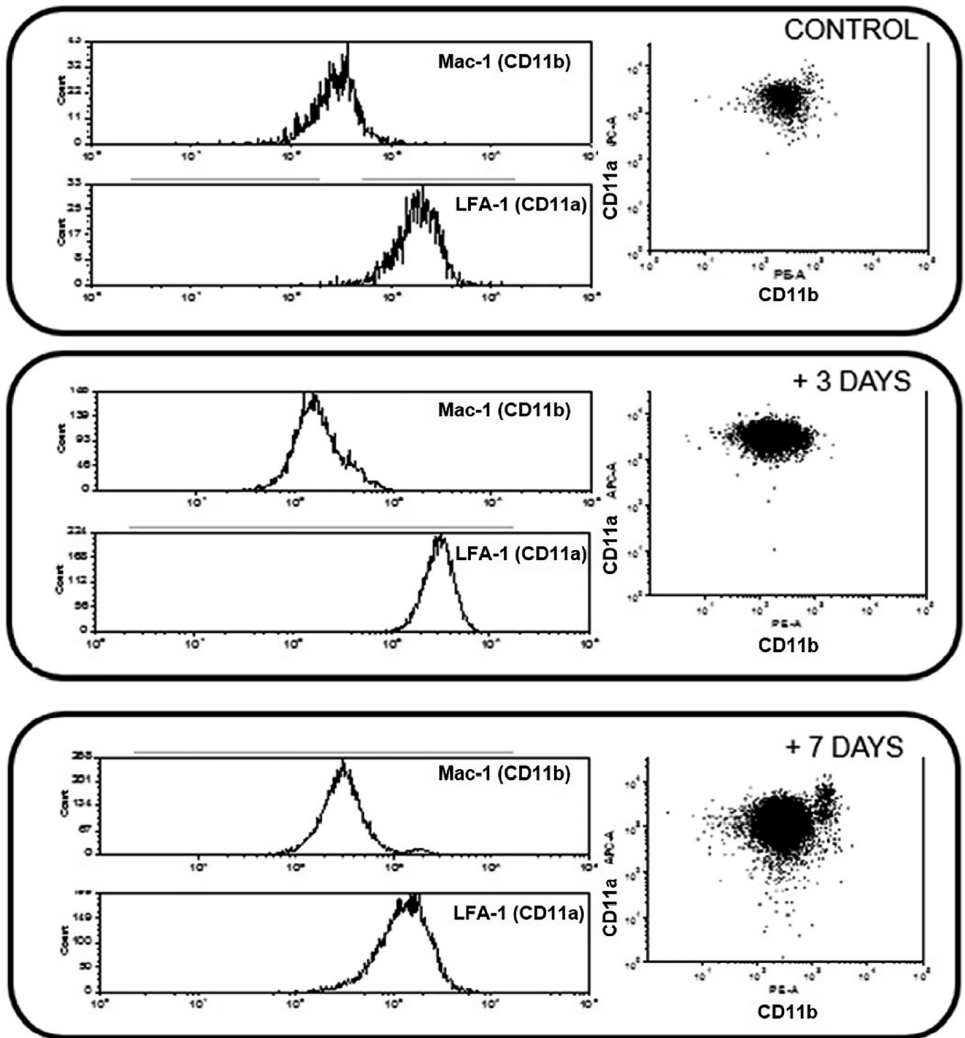
Fig. 2c. LFA-1 (CD11a) expression on circulatory neutrophils. Expression in median fluorescence intensity (MFI) in arbitrary units (AU) over time. Statistical significance: * $p < 0.05$; ** $p < 0.01$.

Co-expression patterns and the interplay between circulatory neutrophil LFA-1 (CD11a) and Mac-1 (CD11b) expression

Several specific alterations in neutrophil cell surface receptor expression levels of integrins over time were identified. Histograms and two-dimensional plots at different time points of all individual samples were analysed, showing increased circulatory neutrophil heterogeneity upon intervention. This contrasts with the control group, which exhibited a homogeneous neutrophil population consisting of CD11b^{intermediate}/CD11a^{intermediate}-neutrophils only (Fig. 3). More specifically, a novel CD11b^{high}/CD11a^{high}-neutrophil subtype appeared in circulation on postoperative day 7 (Figs. 3, 4). This neutrophil subset was encountered in five of six animals in the day 7 group, exhibiting a statistically significant fraction (average 7.62 %, range: 5.56–9.24 %, $p < 0.01$) of the overall circulatory neutrophil composition (Fig. 3b). This subset was

further characterized by a statistically significant increased co-expression of CD49d (VLA-4) on the cell surface compared with other regular neutrophils ($p < 0.01$).

Figure 3. Flow cytometric analysis of changes in circulatory neutrophil integrin cell surface expression



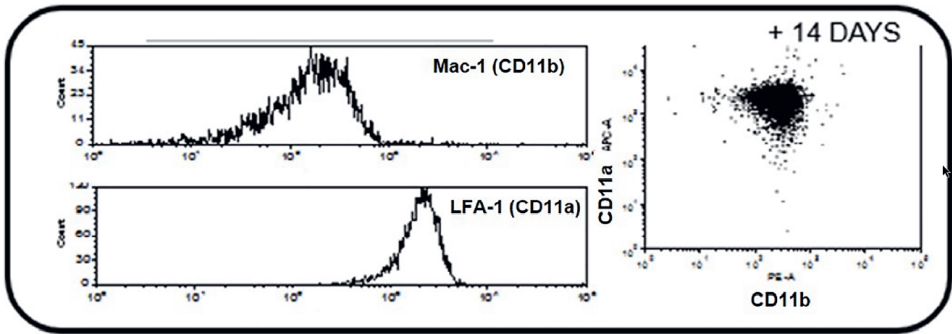


Fig. 3. Flow cytometric analysis of changes in circulatory neutrophil integrin cell surface expression. Representative examples of alterations in LFA-1 (CD11a) and Mac-1 (CD11b) expression during fracture healing were selected. Histograms (left): these show the cell surface expression levels of either Mac-1 (CD11b) or LFA-1 (CD11a) on the x-axis and cell count on the y-axis. Dot plot (right): A two-dimensional analysis was added to highlight the relationship between circulatory neutrophil Mac-1 (CD11b) and LFA-1 (CD11a) expression. Here, Mac-1 (CD11b)-PE expression in median fluorescence intensity (MFI) in arbitrary units (AU) is shown on the x-axis, and LFA-1 (CD11a)-APC median fluorescence levels in AU are plotted on the y-axis.

Figure 4. Fractions of neutrophil subtypes

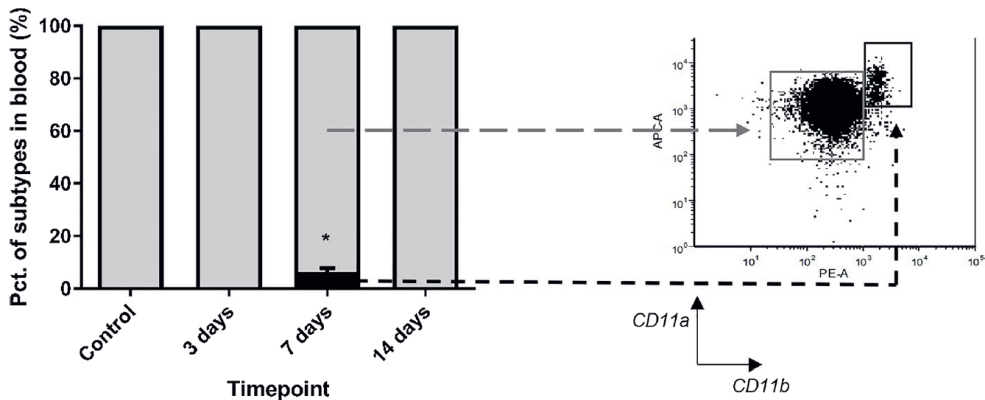


Fig. 4. Bar chart (left): The grey bars in the bar chart represent the percentage of regular non-CD11b^{high}/CD11a^{high} neutrophil subtypes, and the black bar represents the percentage (7.62%, range: 5.56-9.24%) of novel CD11b^{high}/CD11a^{high} cells in peripheral blood. The percentage of CD11b^{high}/CD11a^{high} neutrophils was significantly higher on postoperative day 7 compared with all other groups. Dot plot (right): A duplicate of a two-dimensional dot plot from a representative example provides an example of the utilized gating strategy. Statistical significance: ** p < 0.01.

A back-gating analysis of the novel CD11b^{high}/CD11a^{high}-neutrophil population showed that all cells were spread across the neutrophil gate with respect to forward and side-scatter profiles. Finally, no differences in L-selectin co-expression between CD11b^{high}/CD11a^{high}-neutrophils and regular neutrophils were seen (Fig. 5).

Figure 5. Differences in cell surface co-expression between C11b^{high}/CD11a^{high} vs. regular neutrophil subtypes

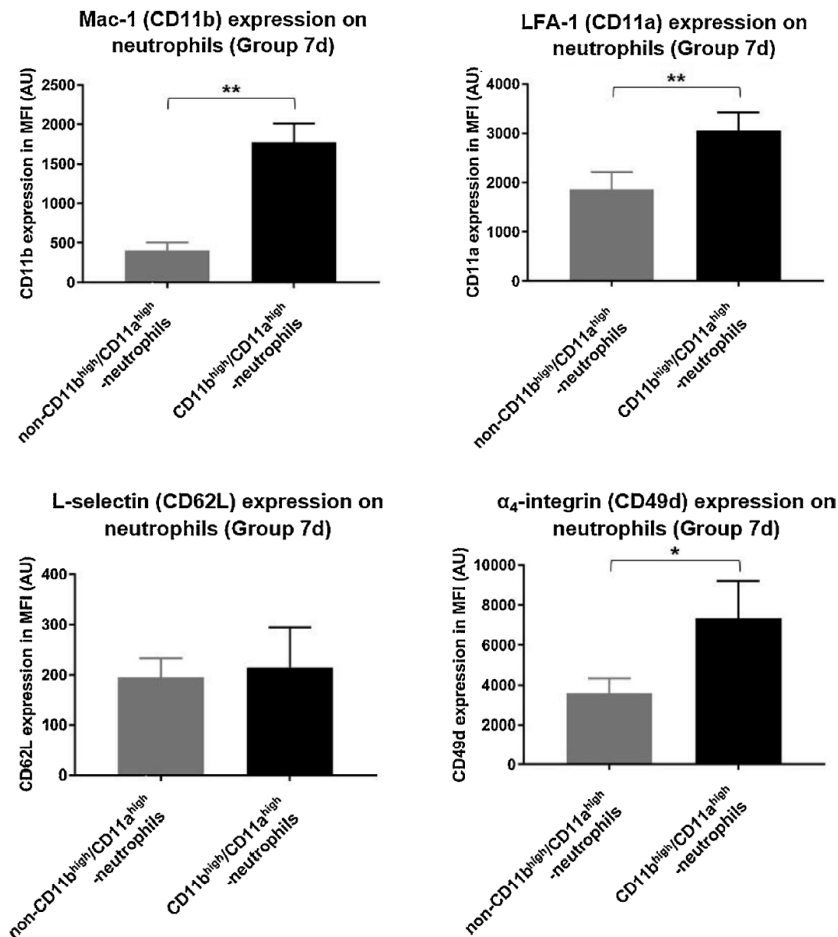


Fig. 5. Differences in cell surface co-expression of L-selectin (CD62L and α₄-integrin (CD49d) on C11b^{high}/CD11a^{high} vs. regular neutrophil subtypes. The grey bars represent co-expression of cell surface receptors on regular, non-CD11b^{high}/CD11a^{high}-neutrophils, and the black bars represent co-expression of cell surface receptors on novel CD11b^{high}/CD11a^{high}-neutrophils in Group 7d (n = 5). MFI: median fluorescence intensity, AU: arbitrary units. Statistical significance * p < 0.05.

Discussion

Intramedullary nailing of long bone fractures represents a standard procedure in femoral shaft fractures and is performed 100,000-fold worldwide. Both the fracture itself and the surgical procedure for stabilization have been shown to be associated with inflammatory changes, which are necessary for the normal fracture healing process. On the other hand, if these inflammatory changes are dysregulated, local and/or systemic hyper-inflammatory conditions can evolve, causing local and/or systemic complications, such as non-unions and acute respiratory distress syndrome (ARDS) [23,24]. Because neutrophils are the most abundant immune cells in the circulation and the primary first responders to trauma [25,26], a dysregulation of the neutrophil response plays a key role in the pathogenesis of these complications [10,27,1]. Indeed, severe trauma leads to changes in neutrophil counts and functionality [28]; however, research on the influence of isolated trauma (i.e., fracture) on circulatory neutrophils is scarce and ambiguous [4,3]. Especially, influences on the long-term course have not been investigated until now. Therefore, it is important to gain knowledge on the time course and the specific pattern of neutrophil surface receptor expression alterations in the circulatory neutrophil pool during the post-traumatic process.

The present study focused on the circulatory neutrophil pool, and we were the first to describe changes in the circulatory neutrophil population in a standardized long-term observation fracture model, primarily focusing on the later inflammatory phase (> 48 h after trauma) rather than the early/instant immune response. The results of the current study can be summarized as follows:

1. The number and activation of circulatory neutrophils is temporarily decreased at 3 days after femoral fracture and intramedullary nailing.
2. During the subsequent restoration of neutrophil homeostasis, the heterogeneity of the circulatory neutrophil pool increases. In this context, a new subset of CD11b^{high}/CD11a^{high}-neutrophils with co-expression of CD49d (VLA-4) occurs in systemic circulation at day 7.

One potential theory to explain this decrease in the number and activation of circulatory neutrophils 3 days after the described procedure is that homing of neutrophils into the fracture hematoma occurs. From the literature, it is known that in the very early phases of the

inflammatory stage of fracture healing, the neutrophil concentrations in the circulation and within the fracture hematoma remain equal for the first few hours [29,30]. However, within the first 24 h after fracture, because of the release of mitochondrial DAMPs (damage-associated molecular patterns) and the subsequent release of different chemo-attractants in the surrounding tissue, the homing of circulatory neutrophils into the fracture hematoma occurs, which are later replaced by macrophages [3,31]. Corresponding to our study, Grøgaard et al. find a depletion of the circulatory neutrophils in the first 2–5 days after fracture, demonstrating a correlation of this depletion with an enhanced fracture healing of a femur osteotomy in rats [4]. This indicates, in accordance with other experimental rodent fracture studies, that accurate neutrophil recruitment and function in the fracture hematoma is crucial to initiate and coordinate the downstream responses leading to bone regeneration [6,3]. The importance of neutrophil homing to the fracture hematoma is also demonstrated in the clinical situation in the study of Bastian et al., in which serum from human fracture hematoma significantly generates neutrophil chemotaxis during the inflammatory stage of fracture healing [1]. Besides the extent of the inflammatory response, its duration and the insufficient termination (hyper-activated neutrophils) can impair the fracture healing process [32,6,2].

In addition to the aforementioned recruitment into the fracture site, an enhanced migration of neutrophils into other organ systems after long bone fractures and intramedullary stabilization has also been described. In this context, the lungs have been identified as a primary target organ, in which neutrophils potentially damage parenchymal lung tissue [7] and cause complications such as acute lung injury and ARDS [8,9]. Clinical and experimental trauma studies have demonstrated that this pulmonary neutrophil infiltration peaks at 72 h after fracture fixation [8,33]. This could be a second explanation for our discovered decrease of circulatory neutrophils.

Besides neutrophil count, their functionality is also of the utmost importance to assess the competence of blood neutrophils. Neutrophil functionality is regulated by cell membrane receptor expression and is a complex, multistage process [34]. These cell membrane receptors, which can detect different chemo-attractants (e.g., chemokines, complement) [35], define different neutrophil subsets or phenotypes.

Among the neutrophil surface receptors, selectins and integrins are especially considered as essential for neutrophil activation and tissue migration. In the present study, the neutrophil

surface expression of integrins and selectins was investigated for the first time in an experimental model of an isolated intramedullary stabilized femoral fracture. Previous studies focusing on the heterogeneity and function of circulatory neutrophils mainly focused on either the postoperative setting or on multiple trauma and found highly divergent results.

The current study showed no significant differences in L-selectin (CD62L) surface expression levels compared with the control conditions. Similar findings can be found in clinical studies focusing on patients after elective surgery. After cardiac surgery, no significant shift in L-selectin expression was found in the first 5 days postoperatively [36]. Also, Mommsen et al. find no significant difference in L-selectin expression of isolated neutrophils in the first 48 h after elective lower limb surgery compared with preoperative expression levels. However, after stimulation with TNF- α , they show that there is a significant decrease of L-selectin expression in the first 24 h after surgery, indicating a role for the pro-inflammatory cytokine TNF- α in the neutrophil surface expression of L-selectin [37]. After multiple trauma, in contrast to our findings, Seekamp et al. find a significantly reduced level of L-selectin expression in the early phases (up to 24 h) after trauma. This reduction is associated with the complicated post-traumatic course (i.e., the development of multi-organ dysfunction syndrome (MODS)) [38]. Besides the differences of the underlying insults, time-related differences of the sample collection might explain the aforementioned differences. In this context, it is known that L-selectin mediates the very early stages (24–48 h after trauma) of the adhesion of neutrophils to endothelial cells. Therefore, we might have missed the early alterations in L-selectin expression in our study with the first time point being at 3 days after trauma [37,38,39]. For the current experiment only female animals have been utilized. This is of specific interest as L-selectin, in contrast to other receptors, responses in trauma are believed to be gender specific. Interestingly, in accordance with a study from Van Griensven et al. on gender related dimorphism in L-selectin receptor responses on circulatory PMNs in trauma, we also found an initial drop of L-selectin expression upon insult and later restoration to baseline levels [40]. This underlines the translational value of our model as well as the feasibility of our long-term fracture model in rats to perform proof-of-principle studies to test future immunomodulatory interventions. Future studies should focus on the occurrence of gender specific L-selectin responses upon trauma in rats as well.

In contrast to L-selectin, we demonstrated a relevant decrease of neutrophil CD11b expression 3 days after fracture induction. Thereafter, CD11b expression gradually increased for over 2 weeks after trauma. This recovery curve implies that restoration of CD11b expression on the

circulatory neutrophil pool takes at least 14 days. The results from a clinical study by Baehl et al. support our experimental findings, demonstrating that an isolated fracture is associated with reduced neutrophil cell surface expression of different integrins, such as CD11b, which are involved in binding and chemotaxis of neutrophils in the early phases after fracture. This decrease was followed by a gradual increase over 6 months of follow-up observation [41]. A decrease of CD11b expression in the initial phase after trauma has also been encountered in other clinical studies [42,43]. In two clinical studies of severely injured patients, an initially decreased peripheral neutrophil-CD11b expression after three days followed by a gradual increase is found, which is in line with our results [43].

We hypothesize that a transient decrease in neutrophil-CD11b expression is caused by a combination of (1) early extravasation of activated CD11b^{high}-neutrophils into different tissue compartments (e.g., fracture and lung) directly after trauma and (2) an inadequate supply of new potent neutrophils in the circulation.

The current investigation also showed that neutrophil-CD11a surface expression levels changed over time after fracture induction and intramedullary stabilization. In previous studies, the exact expression pattern of CD11a has been partly controversially discussed, mainly in clinical trauma studies. Our results of an increase in CD11a-expression on day 3 after fracture induction are supported by the findings of Maekawa et al., which also show an increased expression from 3 h up to 96 h after trauma. On the other hand, our results do not correspond with the findings from a clinical study in which no significant increase of CD11a expression was demonstrated at 3 days after severe trauma [43]. Lo et al. even find a downregulation of CD11a expression although early at 24 h after trauma [44]. Although based on our results we cannot comment on the early data from Lo et al., both CD11a and CD11b exhibit transient changes in adhesion- and migration-promoting activity. Because our first measuring time point was 3 days, we might have missed an early downregulation of CD11a expression.

The aforementioned results of our and previous studies underline the results of a review of Mortaz et al. that focuses on neutrophil phenotyping in trauma patients. Here, specific post-traumatic expression patterns with various dynamics for surface receptors of neutrophils are described. In line with our results, the study shows that neutrophil CD11a and CD11b cell membrane expression levels do not rise synchronously in response to a traumatic insult, suggesting the existence of independent integrin regulation pathways [43,44]. Both integrins, CD11a and CD11b, play a prominent role in regulating the transendothelial migration of neutrophils, which is essential for an adequate immune response. In this migration cascade,

these integrins partially have distinct roles [45–47], in that CD11a is critical at each step of the neutrophil extravasation and that CD11b is more involved in the adhesion of neutrophils to the endothelial cells [48]. However, there seems to be an interplay, even an overlap, between these two integrins because they share the same heterodimeric component CD18 (integrin β -2) and partially bind to the same ligands [39,48]. In this context, as an expression of this connection between both integrins, we were the first to identify a new subset of CD11b^{high}/CD11a^{high}-neutrophils in the circulation at day 7.

Because of the late post-traumatic appearance in peripheral circulation more than 3 days after the insult, these neutrophils exhibit a unique pattern of mobilization compared with the other subtypes. Therefore, it has been suggested that they also might play a role in the regulation of the anti-inflammatory phase following trauma. Because neutrophils of this subset show a significantly higher co-expression with very late antigen-4 (VLA-4/CD49d) than regular neutrophils, it is most likely that they represent a unique phenotype.

The expression of CD49d (VLA-4) has not been reported on circulating neutrophils under homeostatic conditions [49]. On the contrary, CD49d expression has been identified on neutrophil progenitor cells in the bone marrow; therefore, it is tempting to hypothesize that these CD11b^{high}/CD11a^{high}-neutrophils are released from the bone marrow [49,50]. CD49d (VLA-4) might play a significant role for the tissue migration of the new subset of neutrophils because it has been found to play a role in CD11b-independent neutrophil-adhesion pathways [51,52]. Although it is not clear yet if these subsets definitively belong to separate developing lineages or embody certain activation states of a common precursor, the origin, characteristics, and functionality of the specific CD11b^{high}/CD11a^{high}-neutrophil subset should be the focus of further research.

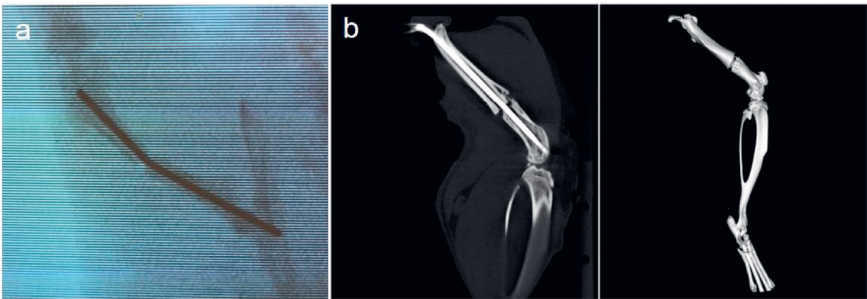
The current study also has some limitations. First, because our findings were derived from an animal model, they are not directly translatable to humans. We chose a rat model because this animal fracture model was established in our department, and it is suitable for this kind of research. Second, we used only one fixation method for the femur fractures, that is, intramedullary nailing; therefore, we cannot draw conclusions for other fixation methods based on these study results. Third, we planned to compare the data from the circulatory neutrophil pool with the neutrophils within the fracture hematoma, but we could not obtain enough fracture hematoma from the fracture site. Therefore, because the current descriptive study focused on the number and activation of neutrophils in the circulatory pool only, we could not

make assertions about the functional relevance of the new subset of CD11b^{high}/CD11a^{high}-neutrophils or their morphological characteristics yet. These important factors are planned to be addressed in subsequent studies in a larger animal model. Fifth, our study design does not enable the usage of statistical testing by repeated measure methods as all experimental animals have been terminated after sampling. In fact, as a total of 2 mL blood per sample was required, we were not allowed to sample four times and thereby we would not have been able to determine long-term kinetics. To overcome this issue, we decided to perform termination after sampling and to group animal according to termination time point. By doing so we avoided potential confounding of the impact of withdrawal of relevant amounts of blood from rodents on metabolic [53], hormonal [54] and even immunological [55] homeostasis. In addition, we preferred to use female rats only, as they have relatively stable weight alterations in comparison with their male counterparts. As a consequence, extrapolation to the male situation in trauma is limited. Especially, as gender specific cellular immune response, and more specifically, L-selectin alterations upon severe trauma have been reported by Van Griensven et al. [40].

Conclusions

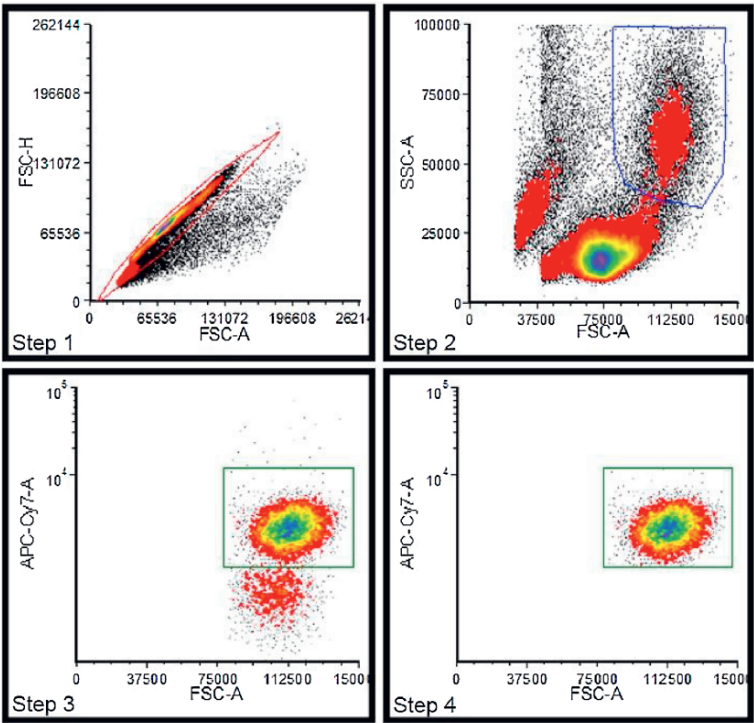
This descriptive, small animal fracture study is the first to show that an intramedullary stabilized femur fracture is associated with a temporary reduction in circulatory neutrophil count and concurrent changes in circulatory neutrophil function. Moreover, we demonstrated that the restoration to homeostatic neutrophil activation status occurs concomitantly with an increased heterogeneity of the circulatory neutrophil pool, which is accentuated by the appearance of a novel neutrophil subtype (CD11b^{high}/CD11a^{high}). Improved knowledge of neutrophil count and functionality after trauma form an excellent basis for future studies to further elucidate the role of neutrophils as activators and regulators of different post-traumatic processes, potentially resulting in local (e.g., fracture healing disturbances) or systemic (e.g., MODS) complications. This might result in the development of specific therapies to reduce adverse outcomes after trauma.

Appendix 1



Appendix 1. Fracture confirmation by X-ray and post-mortem micro-CT imaging (a) X-rax confirmation of a non-displaced midshaft femur fracture and adequate nail positioning. (b) Post-mortem micro-CT analysis of the fractured femur to evaluate fracture healing, rotation and displacement of the fracture or the nail.

Appendix 2



Appendix 2. 4-step neutrophil identification and gating strategy in peripheral murine blood samples. Neutrophils were identified through a 4-step gating strategy including (1,2) exclusion of debris and doublets, (3) inclusion of cells with a neutrophil specific FSC/SSC-pattern, and (4) inclusion of RP-1/APC-Cy7 positive cells. Cell viability was confirmed in separate pilot experiments. As cells were stained and measured within 2 hr after blood sampling there was maximal PMN viability and no need for cell fixation.

References

1. O.W. Bastian, M.H. Mrozek, M. Raaben, L.P.H. Leenen, L. Koenderman, T.J. Blokhuis, Serum from the human fracture hematoma contains a potent inducer of neutrophil chemotaxis, *Inflammation* 41 (3) (2018) 1084–1092.
2. L. Claes, S. Recknagel, A. Ignatius, Fracture healing under healthy and inflammatory conditions, *Nat. Rev. Rheumatol.* 8 (3) (2012) 133–143.
3. R. Chung, J.C. Cool, M.A. Scherer, B.K. Foster, C.J. Xian, Roles of neutrophilmediated inflammatory response in the bony repair of injured growth plate cartilage in young rats, *J. Leukoc. Biol.* 80 (6) (2006) 1272–1280.
4. B. Groggaard, B. Gerdin, O. Reikeras, The polymorphonuclear leukocyte: has it a role in fracture healing? *Arch. Orthop. Trauma Surg.* 109 (5) (1990) 268–271.
5. O.W. Bastian, L. Koenderman, J. Alblas, L.P. Leenen, T.J. Blokhuis, Neutrophils contribute to fracture healing by synthesizing fibronectin+ extracellular matrix rapidly after injury, *Clin. Immunol.* 164 (2016) 78–84.
6. A. Kovtun, S. Bergdolt, R. Wiegner, P. Radermacher, M. Huber-Lang, A. Ignatius, The crucial role of neutrophil granulocytes in bone fracture healing, *Eur. Cell. Mater.* 32 (2016) 152–162.
7. M.K.C. van Griensven, H. Pape, Immune reactions after trauma, *Eur J Trauma.* 29 (2003) 181–192.
8. P.W.N. Störmann, K. Köhler, B. Auner, T.P. Simon, R. Pfeifer, K. Horst, H.C. Pape, F. Hildebrand, S. Wutzler, I. Marzi, B. Relja, Monotrauma is associated with enhanced remote inflammatory response and organ damage, while polytrauma intensifies both in porcine trauma model, *Eur. J. trauma & emergency surgery: off. Publ. Eur. Trauma Soc.* 46 (February (1)) (2020) 31–42.
9. H.C. Pape, K. Grimme, M. Van Griensven, A.H. Sott, P. Giannoudis, J. Morley, et al., Impact of intramedullary instrumentation versus damage control for femoral fractures on immunoinflammatory parameters: prospective randomized analysis by the EPOFF Study Group, *J. Trauma* 55 (1) (2003) 7–13.
10. M. Keel, O. Trentz, Pathophysiology of polytrauma, *Injury* 36 (6) (2005) 691–709.
11. J.L. Dunne, R.G. Collins, A.L. Beaudet, C.M. Ballantyne, K. Ley, Mac-1, but not LFA- 1, uses intercellular adhesion molecule-1 to mediate slow leukocyte rolling in TNFalpha-induced inflammation, *J. Immunol.* 171 (11) (2003) 6105–6111.
12. D.B. Morton, P.H. Griffiths, Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment, *Vet. Rec.* 116 (16) (1985) 431–436.
13. D.L. Hickman, M. Swan, Use of a body condition score technique to assess health status in a rat model of polycystic kidney disease, *J. Am. Assoc. Lab. Anim. Sci. JAALAS* 49 (2) (2010) 155–159.
14. J.B. Becker, A.P. Arnold, K.J. Berkley, J.D. Blaustein, L.A. Eckel, E. Hampson, et al., Strategies and methods for research on sex differences in brain and behavior, *Endocrinology* 146 (4) (2005) 1650–1673.
15. M.R. Milad, S.A. Iggoe, K. Lebron-Milad, J.E. Novales, Estrous cycle phase and gonadal hormones influence conditioned fear extinction, *Neuroscience* 164 (3) (2009) 887–895.

16. F.K. Marcondes, F.J. Bianchi, A.P. Tanno, Determination of the estrous cycle phases of rats: some helpful considerations, *Braz. J. boil. Revista brasileira de biologia*. 62 (4A) (2002) 609–614.
17. T. Kluter, M. Weuster, S. Bruggemann, L. Menzdorf, S. Fitschen-Oestern, N. Steubesand, et al., Rivaroxaban does not impair fracture healing in a rat femur fracture model: an experimental study, *BMC Musculoskelet. Disord.* 16 (2015) 79.
18. F. Bonnarens, T.A. Einhorn, Production of a standard closed fracture in laboratory animal bone, *J. orthop. Res. Off. Publ. Orthop. Res. Soc.* 2 (1) (1984) 97–101.
19. S. Bolivar, R. Anfossi, C. Humeres, R. Vivar, P. Boza, C. Munoz, et al., IFN-beta plays both Pro- and anti-inflammatory roles in the rat cardiac fibroblast through differential STAT protein activation, *Front. Pharmacol.* 9 (2018) 1368.
20. E.A. Vander Top, G.A. Perry, M.J. Gentry-Nielsen, A novel flow cytometric assay for measurement of in vivo pulmonary neutrophil phagocytosis, *BMC Microbiol.* 6(2006) 61.
21. S. Skrajnar, M. Anzur Lasnik, A. Bedina Zavec, A flow cytometric method for determination of the blood neutrophil fraction in rats, *J. Am. Assoc. Lab. Anim. Sci. JAALAS* 48 (2) (2009) 152–156.
22. A. Barnett-Vanes, A. Sharrock, M.A. Birrell, S. Rankin, A single 9-Colour flow cytometric method to characterise major leukocyte populations in the rat: validation in a model of LPS-Induced pulmonary inflammation, *PLoS One* 11 (1) (2016) e0142520.
23. P.V. Giannoudis, H.C. Pape, A.P. Cohen, C. Krettek, R.M. Smith, Review: systemic effects of femoral nailing: from Kuntscher to the immune reactivity era, *Clin.Orthop. Relat. Res.* (404) (2002) 378–386.
24. H.C. Pape, M. Auf'm Kolk, T. Paffrath, G. Regel, J.A. Sturm, H. Tscherne, Primary intramedullary femur fixation in multiple trauma patients with associated lung contusion—a cause of posttraumatic ARDS? *J. Trauma* 34 (4) (1993) 540–547, discussion 7–8.
25. A.J. Botha, F.A. Moore, E.E. Moore, F.J. Kim, A. Banerjee, V.M. Peterson, Postinjury neutrophil priming and activation: an early vulnerable window, *Surgery* 118 (2) (1995) 358–364, discussion 64–65.
26. A.J. Botha, F.A. Moore, E.E. Moore, A. Sauaia, A. Banerjee, V.M. Peterson, Early neutrophil sequestration after injury: a pathogenic mechanism for multiple organ failure, *J. Trauma* 39 (3) (1995) 411–417.
27. J.M. Lord, M.J. Midwinter, Y.F. Chen, A. Belli, K. Brohi, E.J. Kovacs, et al., The systemic immune response to trauma: an overview of pathophysiology and treatment, *Lancet* 384 (9952) (2014) 1455–1465.
28. A. Kovtun, D.A.C. Messerer, K. Scharffetter-Kochanek, M. Huber-Lang, A. Ignatius, Neutrophils in tissue trauma of the skin, bone, and lung: two sides of the same coin, *J. Immunol. Res.* 2018 (2018) 8173983.
29. H. Li, J. Liu, J. Yao, J. Zhong, L. Guo, T. Sun, Fracture initiates systemic inflammatory response syndrome through recruiting polymorphonuclear leucocytes, *Immunol. Res.* 64 (4) (2016) 1053–1059.

30. K. Schmidt-Bleek, H. Schell, P. Kolar, M. Pfaff, C. Perka, F. Buttgereit, et al., Cellular composition of the initial fracture hematoma compared to a muscle hematoma: a study in sheep, *J. Orthop. Res.* 27 (9) (2009) 1147–1151.
31. J.G. Andrew, S.M. Andrew, A.J. Freemont, D.R. Marsh, Inflammatory cells in normal human fracture healing, *Acta Orthop. Scand.* 65 (4) (1994) 462–466.
32. T.A. Butterfield, T.M. Best, M.A. Merrick, The dual roles of neutrophils and macrophages in inflammation: a critical balance between tissue damage and repair, *J. Athl. Train.* 41 (4) (2006) 457–465.
33. B. Relja, R. Taraki, M.P. Teuben, K. Mors, N. Wagner, S. Wutzler, et al., Sera from severe trauma patients with pneumonia and without infectious complications have differential effects on neutrophil biology, *BMC Pulm. Med.* 16 (1) (2016) 171.
34. A. Seekamp, M. van Griensven, E. Dhondt, M. Diefenbeck, I. Demeyer, G. Vundelinckx, et al., The effect of anti-L-selectin (aselizumab) in multiple traumatized patients—results of a phase II clinical trial, *Crit. Care Med.* 32 (10) (2004) 2021–2028.
35. R. Stillie, S.M. Farooq, J.R. Gordon, A.W. Stadnyk, The functional significance behind expressing two IL-8 receptor types on PMN, *J. Leukoc. Biol.* 86 (3) (2009) 529–543.
36. Y.L. Fung, C.C. Silliman, R.M. Minchinton, P. Wood, J.F. Fraser, Cardiopulmonary bypass induces enduring alterations to host neutrophil physiology: a single-center longitudinal observational study, *Shock* 30 (6) (2008) 642–648.
37. P. Mommsen, T. Barkhausen, F. Hildebrand, C. Zeckey, C. Krettek, M. van Griensven, Regulation of L-selectin expression by trauma-relevant cytokines, *Pathol. Res. Pract.* 207 (3) (2011) 142–147.
38. A. Seekamp, M. van Griensven, F. Hildebrandt, N. Brauer, M. Jochum, M. Martin, The effect of trauma on neutrophil L-selectin expression and sL-selectin serum levels, *Shock* 15 (4) (2001) 254–260.
39. E. Mortaz, S.S. Zadian, M. Shahir, G. Folkerts, J. Garssen, S. Mumby, et al., Does neutrophil phenotype predict the survival of trauma patients? *Front. Immunol.* 10 (2019) 2122.
40. M. van Griensven, T. Barkhausen, F. Hildebrand, M. Grotz, L. Mahlke, R. Meier, et al., L-selectin shows time and gender dependency in association with MODS, *Injury* 35 (11) (2004) 1087–1095.
41. S. Baehl, H. Garneau, A. Le Page, D. Lorrain, I. Viens, A. Svoltelis, et al., Altered neutrophil functions in elderly patients during a 6-month follow-up period after a hip fracture, *Exp. Gerontol.* 65 (2015) 58–68.
42. G. Scannell, K. Waxman, N.D. Vaziri, J. Zhang, C.J. Kaupke, M. Jalali, et al., Effects of trauma on leukocyte intercellular adhesion molecule-1, CD11b, and CD18 expressions, *J. Trauma* 39 (4) (1995) 641–644.
43. A.J. Botha, F.A. Moore, E.E. Moore, V.M. Peterson, A.W. Goode, Base deficit after major trauma directly relates to neutrophil CD11b expression: a proposed mechanism of shock-induced organ injury, *Intensive Care Med.* 23 (5) (1997) 504–509.

44. S.K. Lo, G.A. Van Seventer, S.M. Levin, S.D. Wright, Two leukocyte receptors (CD11a/CD18 and CD11b/CD18) mediate transient adhesion to endothelium by binding to different ligands, *J. Immunol.* 143 (10) (1989) 3325–3329.
45. Z.M. Ding, J.E. Babensee, S.I. Simon, H. Lu, J.L. Perrard, D.C. Bullard, et al., Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration, *J. Immunol.* 163 (9) (1999) 5029–5038.
46. B. Heit, P. Colarusso, P. Kubes, Fundamentally different roles for LFA-1, Mac-1 and alpha4-integrin in neutrophil chemotaxis, *J. Cell. Sci.* 118 (Pt 22) (2005) 5205–5220.
47. N. Li, D. Mao, S. Lu, C. Tong, Y. Zhang, M. Long, Distinct binding affinities of Mac-1 and LFA-1 in neutrophil activation, *J. Immunol.* 190 (8) (2013) 4371–4381.
48. Y.M. Hyun, Y.H. Choe, S.A. Park, M. Kim, LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) distinctly regulate neutrophil extravasation through hotspots I and II, *Exp. Mol. Med.* 51 (4) (2019) 1–13.
49. F. Lund-Johansen, L.W. Terstappen, Differential surface expression of cell adhesion molecules during granulocyte maturation, *J. Leukoc. Biol.* 54 (1) (1993) 47–55.
50. D. Soligo, R. Schiro, R. Luksch, G. Manara, N. Quirici, C. Parravicini, et al. Expression of integrins in human bone marrow, *Br. J. Haematol.* 76 (3) (1990) 323–332.
51. J.M. Harlan, Leukocyte adhesion deficiency syndrome: insights into the molecular basis of leukocyte emigration, *Clin. Immunol. Immunopathol.* 67 (3 Pt 2) (1993) S16–24.
52. P.H. Reinhardt, J.F. Elliott, P. Kubes, Neutrophils can adhere via alpha4beta1-integrin under flow conditions, *Blood* 89 (10) (1997) 3837–3846.
53. M. Arnold, W. Langhans, Effects of anesthesia and blood sampling techniques on plasma metabolites and corticosterone in the rat, *Physiol. Behav.* 99 (5) (2010) 592–598.
54. R.H. Rao, Changes in insulin sensitivity from stress during repetitive sampling in anesthetized rats, *Am. J. Physiol.* 262 (6 Pt 2) (1992) R1033–1039.
55. C. Boudesco, T. Rattier, C. Garrido, G. Jego, Do not stress, just differentiate: role of stress proteins in hematopoiesis, *Cell Death Dis.* 6 (2015) e1628.

ii.

Remote organ and local neutrophil responses to injury

Chapter 6

The impact of intramedullary nailing on the characteristics of the pulmonary neutrophil pool in rodents

Michel Paul Johan Teuben^{1,2}

Martijn Hofman³

Alba Shehu³

Johannes Greven³

Zhi Qiao³

Kai Oliver Jensen^{1,2}

Frank Hildebrand³

Roman Pfeifer^{1,2}

Hans-Christoph Pape^{1,2}

¹ Department of Traumatology, University Hospital Zurich, Zurich, Switzerland

² Harald Tscherne Research Laboratory, University Hospital Zurich, Zurich, Switzerland

³ Department of Orthopedic Trauma and Reconstructive Surgery, University of Aachen Medical Center, Aachen, Germany

Abstract:

Purpose

Dysregulation of polymorphonuclear neutrophil (PMN) biology is associated with the development of inflammatory complications after trauma, such as acute respiratory distress syndrome (ARDS). It has been demonstrated that intramedullary nailing is both associated with altered pulmonary neutrophil deposition and the occurrence of ARDS. This standardized study aimed to characterize the long-term remote neutrophil response in the lungs in case of a femur fracture and intramedullary nailing.

Methods

A standardized rat model including intramedullary nailing and a femur fracture was utilized. Groups were terminated after observation times of three, seven and 14 days. Neutrophils were isolated from lung parenchyma and broncho-alveolar lavage fluid (BALF) and analyzed by flow cytometry. Absolute neutrophil numbers as well as membrane expression levels of CD11b, CD62L, and CD11a were compared.

Results

Pulmonary neutrophil numbers were increased 3 days after intervention. Membrane expression levels of CD11b ($P < 0.01$), CD62L ($P < 0.01$), and CD11a ($P = 0.06$) on parenchymal PMNs increased as well after 3 days. Thereafter, values restored gradually to physiological levels. Furthermore, neutrophil activation status patterns between parenchymal and BALF neutrophil pools did not correlate.

Conclusions

The current study demonstrates that IMN and a femur fracture are associated with transient increased pulmonary PMN deposition, as well as a specific pattern of activation characterized by temporary increased selectin and integrin receptor expression on pulmonary neutrophils. This phenomenon might play an important role in the pathomechanism of ARDS after IMN. Moreover, we found striking differences between parenchymal and BALF-neutrophil populations, demonstrating the limited readout potential of BALF analysis to investigate the entire pulmonary neutrophil pool.

Introduction

Pulmonary complications such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) occur after femoral fractures and intramedullary nailing (IMN). Additionally, a pulmonary entity called “fat embolism syndrome” (FES) has been described as well. This syndrome is difficult to distinguish from ALI/ARDS and also occurs in septic patients [1, 2]. Current concepts emphasize that femoral fracture haematomas can alter, and more specifically, increase systemic levels of inflammatory mediators. These mediators could also activate circulating polymorphonuclear neutrophils (PMNs), which have the potency to damage parenchymal lung tissue upon extravasation and subsequent activation when residing in the lungs [3].

It has well been demonstrated that systemic neutrophil dysregulation is an important process in the development of ARDS [4]. Histological analysis in trauma patients who died from ARDS revealed substantial increased pulmonary neutrophil influx [5]. Currently, the pathogenesis of these complications after orthopaedic trauma and subsequent IMN procedures are unclear, but there appears to be an important role for pulmonary polymorphonuclear neutrophils. Especially, as a recent investigation demonstrated increased pulmonary neutrophil presence and related parenchymal tissue damage upon fracture fixation in a porcine monotrauma model [6]. To date, however, specific characteristics of PMNs residing in the pulmonary compartment after orthopaedic trauma surgery have not been determined.

According to clinical and experimental trauma studies, pulmonary PMN infiltration seems to peak at 72 hours after fracture fixation [6, 7], although long-term pulmonary neutrophil kinetics upon trauma have not been investigated in an experimental setting yet and are currently unclear. Pulmonary tissue is not homogeneous and several compartments, namely lung parenchyma and the broncho-alveolar compartment, can be identified [8]. Subsequently, neutrophils are situated in different lung compartments as well and different pulmonary neutrophil pools can be distinguished. In addition, large pools of neutrophils appear to be marginated to the vascular wall, and the lung neutrophil population consists, under homeostatic conditions, mainly of cells that adhere to the endothelium of pulmonary blood vessels [9]. Upon activation, these marginated neutrophils can undergo rapid transendothelial and transepithelial migration into the interstitium and alveolar spaces. The insult-evoked influx of these cells into both lung parenchyma and broncho-alveolar spaces might alter the constitution and characteristics of the neutrophil pools in these compartments [10]. This process might play a relatively unexplored

role in the development of inflammatory complications in trauma.

Broncho-alveolar lavage was proposed as early as 1995 as a procedure to diagnose fat embolism syndrome. During this lavage, PMNs could be obtained for analysis [11]. In 2010, Blankstein described the combination of hemorrhagic shock, resuscitation, and fat embolism syndrome elicited neutrophil activation, infiltration of alveoli by PMNs, and inflammatory cytokine expression in broncho-alveolar lavage fluid [12]. However, characterization of neutrophils residing in different neutrophil compartments after trauma as well as alterations over time have not been studied before.

Pulmonary neutrophil homing and activation are multistep processes, orchestrated by alterations in cell surface expression and affinity of mainly selectin and integrin receptors. In order to obtain more insight into the pathogenesis of orthopaedic trauma-induced ARDS, the current study focuses on long-term kinetics, characteristics, and compartmentalization of pulmonary neutrophils upon orthopaedic trauma surgery. The following hypotheses were tested:

1. Standardized intramedullary nailing and a unilateral femur fracture is associated with the expansion of the pulmonary neutrophil population and increased activation status of both the parenchymal neutrophil pool and the broncho-alveolar neutrophil pool.
2. Cell surface expression profiles of activation markers on PMNs differ between distinct lung compartments (parenchymal vs. broncho-alveolar space).

Material and methods

Ethical approval

Prior to the start of the experiments the protocol was approved by the institutional animal committee and of the regulating authority: Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) Nordrhein-Westfalen, Recklinghausen, Deutschland (permit AZ 84-02.04.2015.A078). The utilized experimental animals are a part of a larger rodent study.

Experimental model

Adult female Sprague-Dawley rats (Harlan Industries, Indianapolis, IN, USA, 250–350 g) were subjected to standardized intramedullary nailing and a unilateral femur fracture, as previously described [13, 14]. Subcutaneous premedication included 0.03 mg/kg buprenorphine hydrochloride (Reckitt Benckiser Healthcare Ltd., UK). General anaesthesia was induced by 100 mg/kg ketamin (Pfizer, New York, USA) intraperitoneally and 2% 10 mg/kg xylazine (Xylapan, Vetoquinol, Ravensburg, Germany) intraperitoneally. Anaesthesia was maintained by 2–2.5% isoflurane inhalation. Post-operative animals received buprenorphine hydrochloride twice a day.

Study groups

Animals were sacrificed after three, seven and 14 days of observation (N = 6). In addition, one control group was added to define homeostatic values (N = 3). Animals were randomized for groups and terminated by cervical dislocation under isoflurane anesthesia.

The following groups were included:

Study group 1: Intramedullary nailing + unilateral femur fracture, observation period: three days.

Study group 2: Intramedullary nailing + unilateral femur fracture, observation period: seven days.

Study group 3: Intramedullary nailing + unilateral femur fracture, observation period: 14 days.

Control group: Anaesthesia (in line with study groups) and direct termination.

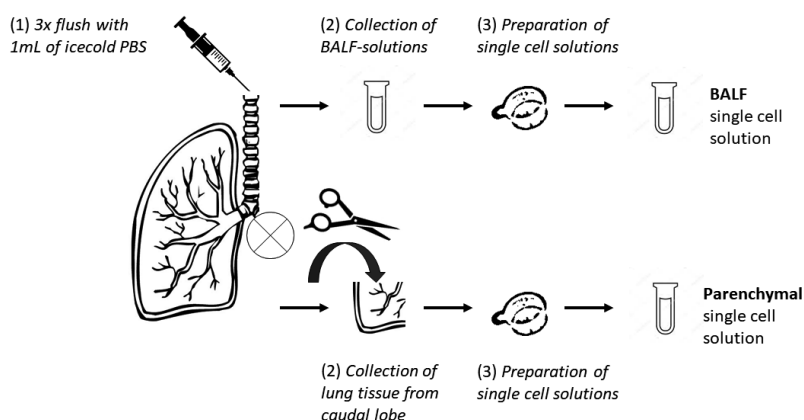
Isolation of single-cell solutions from different pulmonary compartments

Directly after termination, a thoracotomy was performed and lungs (including trachea) were isolated for further analysis. First, the left main bronchus was clipped and a blunt syringe was

utilized to flush the right lung. A total of three flushes with each 1 mL of ice-cold phosphate-buffered saline (PBS) were performed. The solution was then filtered using a 100 μ L cell strainer (BALF-single-cell solution).

Thereafter, the right caudal lobe was isolated. Lung tissue was crushed mechanically, and single-cell solutions were collected in a 50-mL Falcon tube (lung parenchymal single-cell solution). A schematic summary of our cell isolation process of distinct pulmonary compartments is provided in supplementary Fig. 1.

Supplement 1



Then, samples were lysed using a red blood cell (RBC) lysing buffer. After this lysis step, FACS-Buffer (phosphate-buffered saline enriched with 0.5% bovine serum albumin and 0.5 mM EDTA) was added to both samples, and two washing steps were performed. Thereafter, conjugated antibodies were added and allowed to incubate for 45 minutes. After staining, the single-cell solutions were washed twice and directly analyzed by flow cytometry (within 1 hour).

By doing so, neutrophil populations were isolated from two distinct compartments of uninjured lungs:

1. *Lung parenchymal neutrophils*
2. *Broncho-alveolar neutrophils*

Flow cytometry analysis and pulmonary neutrophil identification

Neutrophils have been implicated as having a pivotal role in the development of acute lung injury. Neutrophils were identified and differentiated from other white blood cells by characteristic CD45 and RP-1 fluorescence and light scatter properties as measured by flow cytometry. The following gating strategy was utilized: isolation of viable leukocytes (CD45^{high}), exclusion of doublets, and inclusion of RP-1^{high}/SSC^{high} cells. From each sample, a minimum of 10.000 RP1-positive cells were analyzed. This protocol has been validated by pilot experiments, and fluorescence levels were compared to negative control values as well as with fluorescence levels on blood and bone marrow cells.

In order to investigate characteristics of different pulmonary neutrophil populations and changes over time, membrane receptor expression levels of activation markers: integrin Mac-1 (CD11b), integrin LFA-1 (CD11a), and L-selectin (CD62L) were measured. Counting beads were added in order to calculate absolute cells counts of broncho-alveolar lavage fluid samples. Absolute neutrophil numbers and the neutrophil fraction were determined as described by Skrajnar et al. [15]. A Canto II-device (Becton & Dickinson, Mountain View, CA, USA) and FACS Diva Software (Becton & Dickinson, Mountain View, CA, USA) were utilized to analyze samples. In order to analyze differences between the tissue compartments we plotted correlations between all markers and calculated statistical significance.

Reagents

RP-1 clone RUO (Becton & Dickinson, Mountain View, CA, USA), CD11b clone M1/70 (eBioscience Vienna, Austria), CD62L clone OX-85 (AbD Serotec, Düsseldorf, Germany), CD11a clone WT.1 (AbD Serotec, Düsseldorf, Germany), CD45 clone 10558 (Abcam, Cambridge, Great Britain), CountBright counting beads (ThermoFisher Scientific, Waltham, USA). PBS (Sigma, Deisenhofen, Germany). RBC Lysis-Buffer (Bio-Rad, Hercules, USA), FACS-Buffer (phosphate-buffered saline enriched with 0.5% bovine serum albumin and 0.5 mM ethylenediaminetetraacetic acid (EDTA)).

Statistical analysis

Data are displayed as means and SEM, unless described otherwise. Groups were compared by using the Mann-Whitney U test. Differences were considered significant if $P < 0.05$. Data was analyzed with the following software programs: SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Results

All animals survived the observation period, and no signs of infection were seen. Furthermore, all animals were able to mobilize within 24 hours after the intervention. Single-cell solutions from both the *parenchymal compartment* and the *broncho-alveolar compartment* were prepared and analyzed as described previously. Pulmonary neutrophil numbers/deposition was determined by analysis of *broncho-alveolar fluid*. Additionally, specific characteristics of both the *parenchymal* as well as the *broncho-alveolar* neutrophil populations were determined and compared.

Intramedullary nailing is associated with temporarily increased pulmonary neutrophil deposition

As displayed in Fig. 1a, absolute neutrophil numbers in broncho-alveolar lavage samples were increased significantly three days post-insult (mean 35,026 PMNs/mL). Neutrophil counts thereafter decreased and equalled those under control conditions after both seven and 14 days. Furthermore, the percentage of PMNs out of total broncho-alveolar lavage fluid (BALF) leukocytes also peaked at 33.3% of leukocytes in the three day observation group (Fig. 1b). This was significantly higher than the percentages encountered under control conditions (mean PMN fraction 7.4%, $P = 0.036$) and in both animals terminated at seven and 14 days (respectively, mean PMN fraction 9.0%, $P < 0.01$ and mean PMN fraction 5.4%, $P < 0.01$).

Figure 1. Changes in neutrophil fraction and absolute neutrophil numbers in BALF between groups.

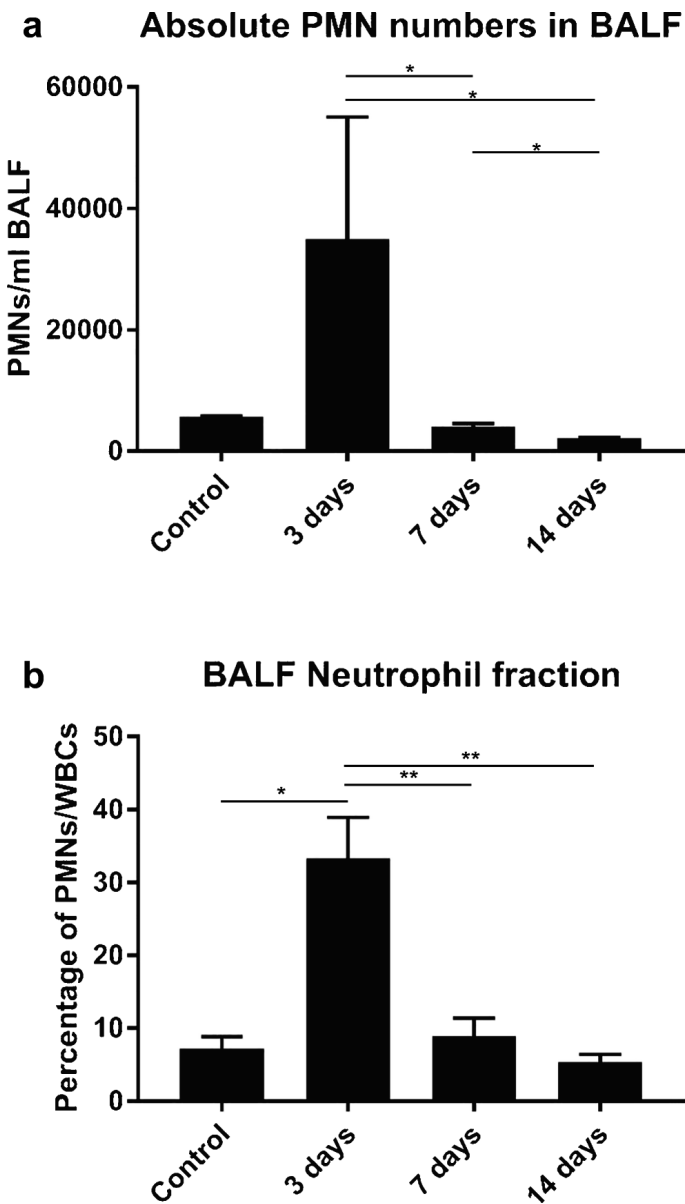


Fig. 1. Variations in absolute neutrophil numbers in BALF (a) and the neutrophil fraction in BALF analysis (b). Significance between groups is displayed as * $p < 0.05$; ** $p < 0.01$

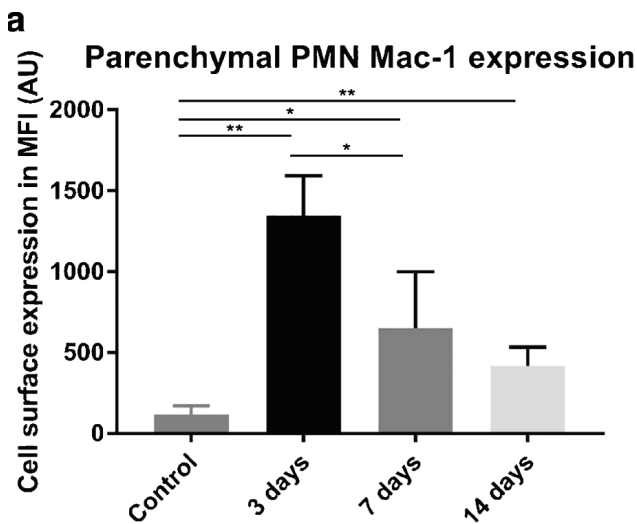
Alterations in activation status of the parenchymal neutrophil pool over time

A statistically significant increase in CD11b expression on parenchymal neutrophils was observed after 72 hours of observation compared with control conditions ($P < 0.01$) (Fig. 2a). Thereafter, PMN Mac-1 expression on the parenchymal PMN pool decreased gradually over time. However, Mac-1 expression did not recover fully to baseline levels within the first 14 days after surgery ($P < 0.01$).

Membrane receptor expression of CD11a on parenchymal PMNs (Fig. 2b) did not change significantly over time. Nevertheless, a notable statistically non-significant trend towards temporary peaking of LFA-1 expression levels at three days of observation was found ($P = 0.061$).

Pulmonary neutrophil L-selectin expression (Fig. 2c) was significantly increased after three days of observation ($P < 0.01$). Neutrophil L-selectin expression levels thereafter returned to baseline levels, and no statistically significant difference between control conditions and the group observed for 14 days was encountered ($P = 0.69$).

Figure 2. Changes in neutrophil cell surface expression of activation markers on parenchymal neutrophil



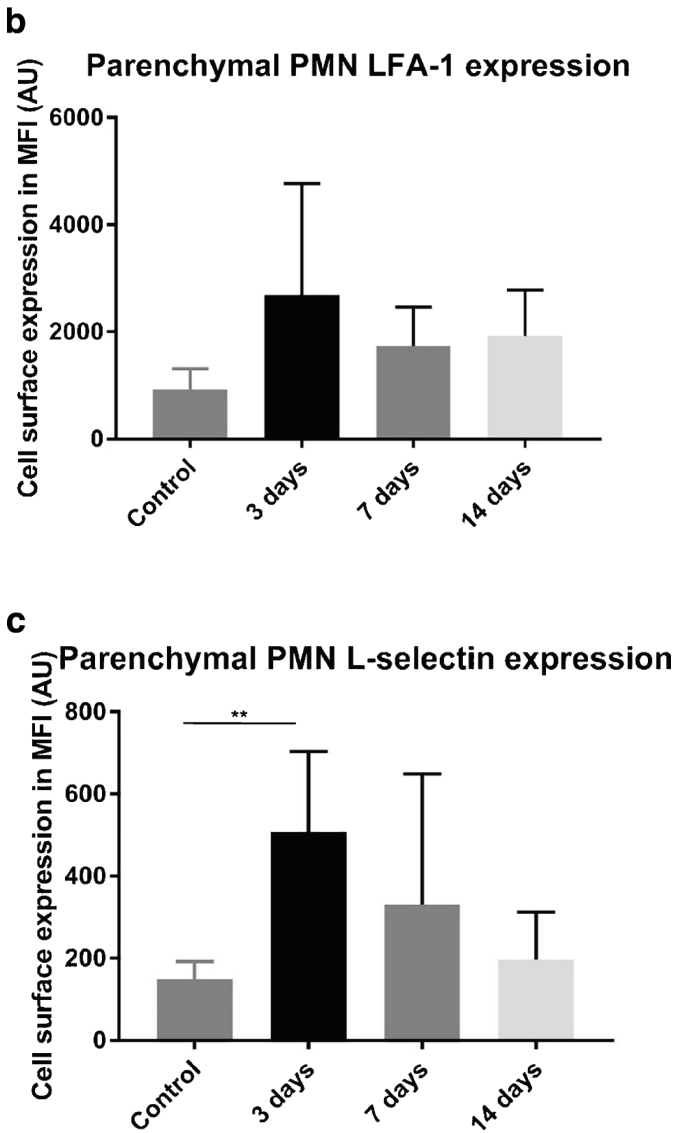
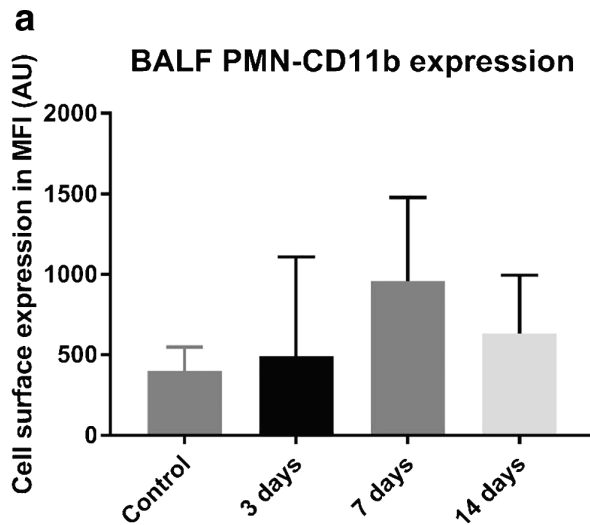


Fig. 2. Changes in neutrophil cell surface expression of activation markers on parenchymal neutrophils. Variations in cell surface expression levels of Mac-1 (a) and LFA-1 (b), and L-selectin (c). Data in median fluorescence intensities (MFI) in arbitrary units (AU). Significance between groups is displayed as * $p < 0.05$; ** $p < 0.01$

Changes in cell surface activation of the broncho-alveolar neutrophil pool following trauma

In contrast to the observed alterations of PMN-CD11b expression levels in the parenchymal neutrophil pool, no statistically significant changes in BALF-PMN expression levels over time occurred (Fig. 3a). LFA-1 expression levels on broncho-alveolar neutrophils, on the other hand, were significantly lower at three days of observation compared with control conditions ($P = 0.02$). However, at both seven and 14 days of observation, LFA-1 cell surface expression levels equalled those under control conditions again (Fig. 3b). As displayed in Fig. 3c, no statistically significant alterations in PMN-CD62L expression over time on BALF neutrophils were seen.

Figure 3. Changes in neutrophil cell surface expression of activation markers on broncho-alveolar neutrophils



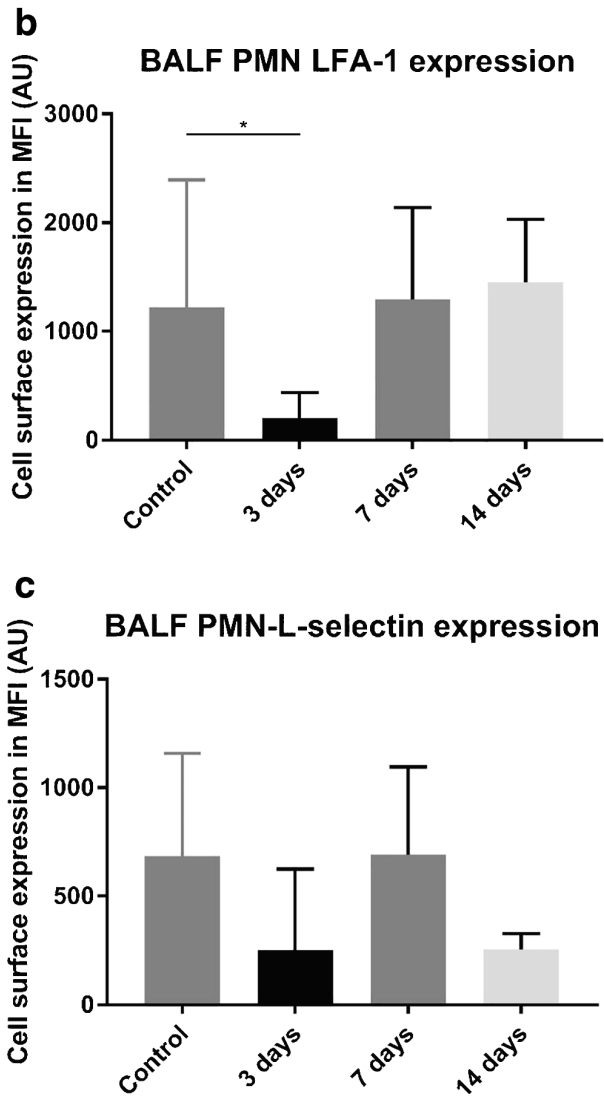
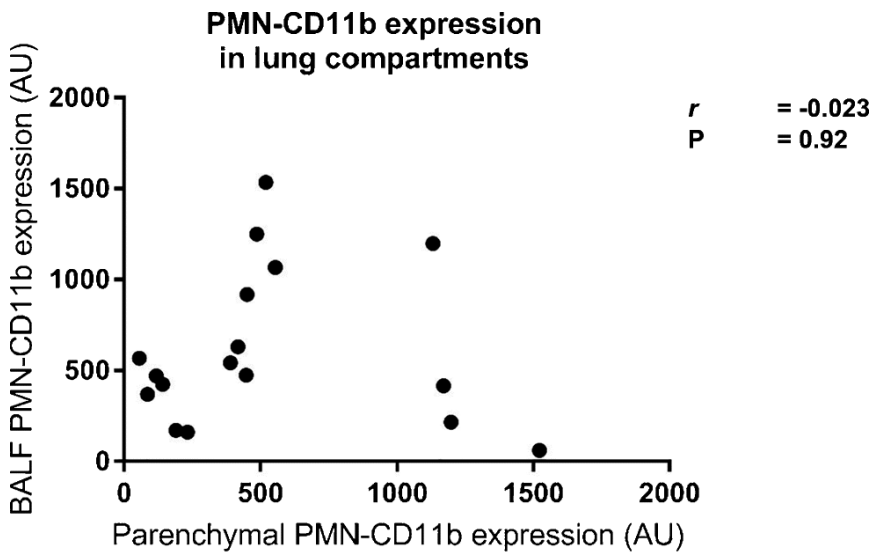


Fig. 3. Changes in neutrophil cell surface expression of activation markers on broncho-alveolar neutrophils. Variations in cell surface expression levels of Mac-1 (a) and LFA-1 (b), and L-selectin (c). Data in median fluorescence intensities (MFI) in arbitrary units (AU). Significance between groups is displayed as * $p < 0.05$

Activation status of parenchymal and broncho-alveolar neutrophils does not correlate when measured by flow cytometry

We also tested for differences between the cell surface expression levels of relevant neutrophil activation markers on both parenchymal and BALF neutrophils. When pooling all measurements, we found no statistically significant correlation of neutrophil CD11b ($P = 0.93$), CD11a ($P = 0.43$), or CD62L ($P = 0.20$) cell surface expression levels between parenchymal PMNs and BALF neutrophils (Fig. 4).

Figure 4. Correlation between parenchymal and broncho-alveolar neutrophil activation



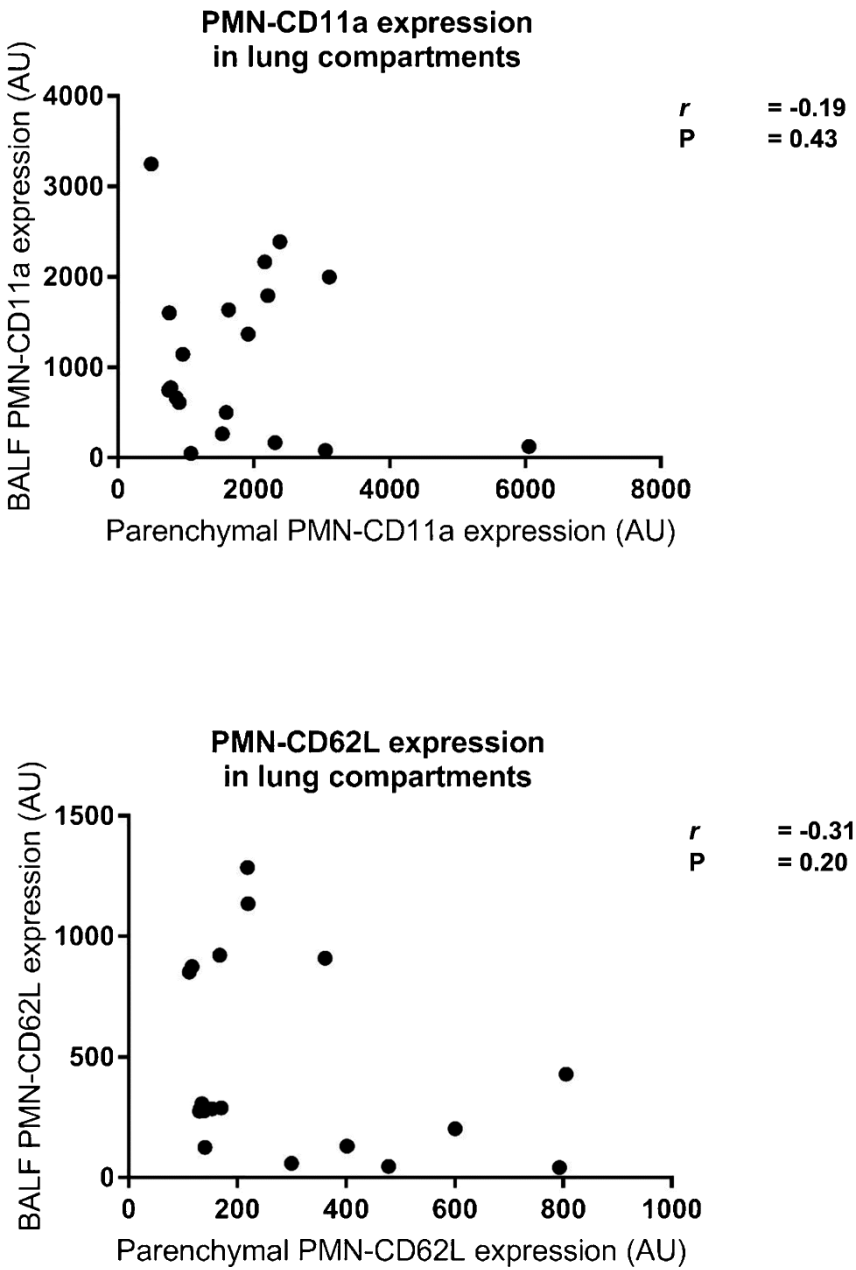


Fig. 4. Correlation between parenchymal and broncho-alveolar neutrophil activation

Discussion

Previous studies on the inflammatory cellular response after orthopaedic trauma focused mainly on alterations of blood neutrophils. However, circulatory neutrophils are, in contrast to tissue neutrophils, not believed to be harmful by themselves [2, 3]. The current study is the first to determine the specific neutrophil response to intramedullary nailing in the pulmonary tissue compartment. The key findings may be summarized as follows:

1. The current study demonstrates that intramedullary nailing and a unilateral fracture are associated with transient increases in pulmonary neutrophil deposition and contemporary increased activation status of the pulmonary neutrophil pool.
2. Moreover, this study revealed striking differences in the activation status of neutrophils belonging to the pulmonary parenchymal compartment and those belonging to the broncho-alveolar compartment and thereby demonstrates compartmentalization related to intra-pulmonary neutrophil heterogeneity.

Dysregulation of neutrophil activation and deposition in end-organ tissue compartments causes collateral damage to parenchymal cells and thereby contributes largely to the development of (multiple) organ failure and ARDS/ALI. Experimental studies showed that trauma results in increased pulmonary neutrophil influx [5, 6, 8]. In addition to these quantitative investigations of the pulmonary neutrophil response to trauma, we characterized the pulmonary parenchymal neutrophil pool and were the first to provide a qualitative description of pulmonary neutrophil populations and their characteristics. We revealed that cell surface receptor expression levels of Mac-1, LFA-1, and L-selectin on pulmonary neutrophils temporarily increased after trauma, and after that within the first two weeks after the trauma, then returned back to baseline levels.

Neutrophil activation status is generally studied by comparing cell surface expression levels of activation and migration markers. Relevant and validated markers include some that belong to the selectin and integrin receptor families [16]. Upon neutrophil activation, cells enter a primed state, and CD62L/L-selectin expression decreases due to the shedding of the receptor from the cell membrane [17]. On the other hand, neutrophil CD11b/Mac-1 expression increases in response to cell activation. These typical effects upon priming, however, have been identified and validated mainly in *in-vitro* studies of blood neutrophils [18]. In line with the *in vitro*

experiments on neutrophil activation, the current *in vivo* experiment also demonstrated a striking transient increase of CD11b/Mac-1 cell surface expression on parenchymal neutrophils after three days of observation. These findings are further in agreement with a previous trauma study by Van Wessem et al. in which hypovolaemic shock caused an increase in CD11b expression on circulatory neutrophils [19]. Interestingly, in the current investigation, the expression of CD11b on BALF-neutrophils decreased simultaneously with the encountered increase of CD11b on parenchymal neutrophils. This finding may be suggestive of a specific biological function of the PMN-Mac-1 receptor in pulmonary neutrophil compartmentalization and PMN migration processes between the interstitium and the alveolar space.

We also encountered increased neutrophil CD62L expression in the lungs upon activation. Increased pulmonary neutrophil-CD62L cell surface expression levels can be explained either by decreased shedding of the receptor from the PMN membrane [17] or by increased selective tissue infiltration of specific subtypes of CD62L^{high} neutrophils into the lung compartment [20]. *In vivo* studies on trauma also demonstrated increased CD62L receptor expression on blood neutrophils [21]. We hypothesize that the encountered increase in total PMN-CD62L expression was partly caused by alterations in the constitution of the pulmonary neutrophil pool due to the appearance of novel (CD16^{low}/CD62L^{high}) subsets in the circulation and subsequent tissue migration [17, 20, 21]. Unfortunately, we were unable to identify and characterize this specific subset in our model, as PMN Fcγ-receptor (CD16) expression cannot be investigated properly in rodent models due to significant biological differences with the human Fcγ-receptor family [22]. So it is tempting to hypothesize that the increased CD62L expression on parenchymal neutrophils in our study is related to increased selective pulmonary homing of this specific subset of neutrophils after trauma as well.

CD11a/LFA-1 is also of great importance in neutrophil tissue migration upon inflammation [23]. A trend towards higher parenchymal PMN-LFA-1-surface expression levels 72 hours after trauma was observed. Interestingly, a paradoxical effect was seen regarding LFA-1 expression on BALF neutrophils. This phenomenon suggests a potential specific biological role of the LFA receptor in neutrophil migration processes between the parenchymal compartment and the alveolar space. Following initial altered pulmonary neutrophil activation within the first three days after trauma, activation markers return to homeostatic levels. The gradual normalization of activation status of both the parenchyma and the broncho-alveolar

compartments is seen over time. This observed process of transient neutrophil activation and later restoration during the first weeks after trauma is in line with a clinical trauma study from Hietbrink et al. [24].

Broncho-alveolar lavage is a clinically useful and relatively non-invasive method to study the inflammatory milieu of distal airways and alveoli. Most studies in the field of pulmonology, however, have been performed on lung biopsy samples. Variations exist between both sampling techniques regarding the constitution of samples in chronic inflammation [25]. In addition, we also found striking differences between both sampling techniques in the cases of trauma-induced acute systemic inflammation. Cell surface expression of both L-selectin and integrins differ largely between the parenchymal neutrophil pool and the broncho-alveolar pool. These differences can be explained by potential biological functions of both receptors in neutrophil migration and compartmentalization processes and is likely to represent different neutrophil subsets/phenotypes [18]. As PMN-cell surface receptor profile correlations between compartments were lacking in our standardized study, utilization of BALF procedures to study the pulmonary compartment might not be the optimal method, and alternatives should be sought and further validated in upcoming studies.

This investigation has several limitations. Unfortunately, we were not able to investigate the functional capacities (such as potency of cells to form neutrophil extracellular traps, respiratory burst, and capacity to phagocytose) of different pulmonary neutrophil populations. It might be that the neutrophils residing in different compartments in fact represent different PMN phenotypes with specific functionality. Furthermore, we did not collect material for humoral or histological analysis as this would interfere with the quality of our cell isolation process.

In conclusion, the current study was the first to describe specific alterations in pulmonary neutrophil compartmentalization and activation patterns following intramedullary nailing. We demonstrated that our standardized rat model is feasible to study the long-term innate immune response to trauma in distinct pulmonary tissue compartments. As neutrophil cell surface activation levels between parenchymal and broncho-alveolar neutrophil pools in our study were not correlated, BALF analysis might not be an optimal readout to analyze characteristics of the heterogeneous pulmonary neutrophil pool. Therefore, it would be interesting to study alternative minimally invasive techniques for collecting representative lung neutrophils. Alternatively, discrepancy in cell surface receptor expression dynamics between distinct lung

neutrophil populations may represent the presence of pulmonary neutrophil subsets/phenotypes with specific compartmentalization profiles.

The current study determined the characteristics of pulmonary neutrophils upon orthopaedic trauma and further revealed a specific pattern of transiently increased neutrophil deposition and activation of lung neutrophils. The encountered differences in integrin and selectin expression profiles between distinct pulmonary PMN pools imply a relevant biological role of integrins and L-selectin in migratory lung processes. These insights might be interesting targets for upcoming therapies aimed to intervene in pulmonary neutrophil infiltration and compartmentalization mechanisms in patients at risk for inflammatory complications after orthopaedic trauma.

References

1. Pape HC, Auf'm'kolk M, Paffrath T, Regel G, Sturm JA, Tscherne H (1993) Primary intramedullary femur fixation in multiple trauma patients with associated lung contusion—a cause of posttraumatic ARDS? *J Trauma* 34(4):540–547 discussion 547–8.
2. Dunn RH, Jackson T, Burlew CC, Pieracci FM, Fox C, Cohen M, Campion EM, Lawless R, Mauffrey C (2017) Fat emboli syndrome and the orthopaedic trauma surgeon: lessons learned and clinical recommendations. *Int Orthop* 41(9):1729–1734.
3. van Griensven M, Krettek C, Pape HC (2003) Immune Reactions after Trauma. *Eur J Trauma* 29:181.
4. Brown KA, Brain SD, Pearson JD, Edgeworth JD, Lewis SM, Treacher DF (2006) Neutrophils in development of multiple organ failure in sepsis. *Lancet* 368(9530):157–169.
5. Pape HC, Remmers D, Kleemann W, Goris JA, Regel G, Tscherne H (1994) Posttraumatic multiple-organ failure—a report on clinical and autopsy findings. *Shock* 2(3):228–234.
6. Stoermann P, Wagner N, Koehler K, Auner B, Simon TP, Pfeifer R, Horst K, Pape HC, Hildebrand F, Wutzler S, Marzi I, Relja B (2019) Monotrauma is associated with enhanced remote inflammatory response and organ damage, while polytrauma intensifies both in porcine trauma model. *Eur J Trauma*.
7. Relja B, Taraki R, Teuben MP, Mörs K, Wagner N, Wutzler S, Hildebrand F, Perl M, Marzi I (2016) Sera from severe trauma patients with pneumonia and without infectious complications have differential effects on neutrophil biology. *BMC Pulm Med* 16(1): 171.
8. Barletta KE, Cagnina RE, Wallace KL, Ramos SI, Mehrad B, Linden J (2012) Leukocyte compartments in the mouse lung: distinguishing between marginated, interstitial, and alveolar cells in response to injury. *J Immunol Meth* 375(1-2):100–110.
9. Aulakh GK (2018) Neutrophils in the lung: 'the first responders'. *Cell Tissue Res* 371(3):577–588.
10. Granton E, Kim JH, Podstawka J, Yipp BG (2018) The lung microvasculature is a functional immune niche. *Trend Immunol* 39(11):890–899.
11. Roger N, Xaubet A, Agusti C, Zabala E, Ballester E, Torres A et al (1995) Role of bronchoalveolar lavage in the diagnosis of fat embolism syndrome. *Eur Respir J* 8:1275–1280.
12. Blankstein M, Byrick RJ, Nakane M, Bang KW, Freedman J, Richards RR et al (2010) Amplified inflammatory response to sequential hemorrhage, resuscitation, and pulmonary fatembolism: an animal study. *JBJS Am* 92(1):149–161.
13. Bonnarens F, Einhorn TH (1984) Production of a standard closed fracture in laboratory animal bone. *J Orthop Res* 2(1):97–101.
14. Kobbe P, Vodovotz Y, Kaczorowski DJ, Mollen KP, Billiar TR, Pape HC (2008) Patterns of cytokine release and evolution of remote organ dysfunction after bilateral femur fracture. *Shock* 30(1): 43–47.

15. Skrajnar S, Anzur Lasnik M, Bedina Zavec A (2009) A flow cytometric method for determination of the blood neutrophil fraction in rats. *J Am Lab Anim Sci* 48(2):145–156.
16. Simon SI, Rochon YP, Lynam EB, Smith CW, Anderson DC, Sklar LA (1993) Beta 2-integrin and L-selectin are obligatory receptors in neutrophil aggregation. *Blood* 82(4):1097–1106.
17. Ivetic A (2018) A head-to-tail view of L-selectin and its impact on neutrophil behavior. *Cell Tiss Res* 371(3):437–453.
18. Neeley SP, Hamann KJ, White SR, Baranowski SL, Burch RA, Leff AR (1993) Selective regulation of expression of surface adhesion molecules Mac-1, L-selectin, and VLA-4 on human eosinophils and neutrophils. *Am J Respir Cell Mol Biol* 8:633–639.
19. van Wessem KJP, Heeres M, Leliefeld PHC, Koederman L, Leenen LPH (2013) Lipopolysaccharide and hemorrhagic shock cause systemic inflammation by different mechanisms. *J Trauma* 74(1):37–44.
20. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers JW, Ulfman LH, Leenen LP, Pickkers P, Koederman L (2012) A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest* 122(1):327–336.
21. Cocks RA, Chan TY, Rainer TH (1998) Leukocyte L-selectin is upregulated after mechanical trauma in adults. *J Trauma* 45(1):1–6.
22. Bruhns P (2012) Properties of mouse and human IgG receptors and their contribution to disease models. *Blood* 119(24):5640–5649.
23. Lefort CT, Ley K (2012) Neutrophil arrest by LFA-1. *Front Immunol* 3:157.
24. Hietbrink F, Koederman L, Althuisen M, Pillay J, Kamp V, Leenen LPH (2013) Kinetics of the innate immune response after trauma: implications for the development of late onset sepsis. *Shock* 40(1):21–27.
25. Anderson BO, Brown JM, Harken AH (1991) Mechanisms of neutrophil-mediated tissue injury. *J Surg Res* 51(2):170–179.

Chapter 7

Shift of neutrophils from blood to bone marrow upon extensive experimental trauma surgery

Michel Paul Johan Teuben^{1,2,3}

Marjolein Heeres^{1,2}

Taco Blokhuis⁴

Roy Spijkerman^{1,5}

Eric Knot¹

Nienke Vrisekoop^{2,5}

Roman Pfeifer³

Hans-Christoph Pape³

Leo Koenderman^{2,5}

Luke Leenen¹

¹ Department of Trauma, University Medical Centre Utrecht, Utrecht, The Netherlands

² Center for Translational Immunology, University Medical Centre Utrecht, Utrecht, The Netherlands

³ Department of Traumatology, University Medical Center Zurich, Zurich, Switzerland

⁴ Department of Surgery, Maastricht University Medical Center, Maastricht, The Netherlands

⁵ Department of Respiratory Medicine, University Medical Center Utrecht, The Netherlands

Abstract:

Introduction

Extensive trauma surgery evokes an immediate cellular immune response including altered circulatory neutrophil numbers. The concurrent bone marrow (BM) response however is currently unclear. We hypothesize that these BM changes include (1) a relative reduction of the bone marrow neutrophil fraction and (2) increasing heterogeneity of the bone marrow neutrophil pool due to (3) the appearance of aged/returning neutrophils from circulation into the BM-compartment.

Material and methods

Eight pigs were included in a standardized extensive trauma surgery model. Blood and bone marrow samples were collected at baseline and after 3 hours of ongoing trauma surgery. Leukocyte and subtype counts and cell surface receptor expression levels were studied by flow cytometry.

Results

All animals survived the interventions. A significant drop in circulating neutrophil counts from 9.3 to 3.2×10^6 cells/ml ($P=0.001$) occurred after intervention, whereas circulatory neutrophil cell surface expression of CD11b increased. The concurrent bone marrow response included an increase of the BM neutrophil fraction from 63 ± 3 to 71 ± 3 percent ($P<0.05$). Simultaneously, the BM neutrophil pool became increasingly mature with a relative increase of a CXCR4^{high}-neutrophil subtype that was virtually absent at baseline.

Conclusion

The current study shows a shift in composition of the BM neutrophil pool during extensive trauma surgery that was associated with a relatively circulatory neutropenia. More specifically, under these conditions BM neutrophils were more mature than under homeostatic conditions and a CXCR4^{high}-neutrophil subset became overrepresented possibly reflecting remigration of aged neutrophils to the BM. These findings may contribute to the development of novel interventions aimed to modify the trauma-induced immune response in the BM.

Introduction

Trauma and subsequent surgery induce acute systemic inflammation and trigger activation of innate immune cells [1-3]. Extensive ongoing trauma surgery is associated with an increased activation state of circulatory neutrophils and enhanced neutrophil migration to the tissue [4,5]. Furthermore, a prompt decline in circulatory neutrophil numbers upon insult has been described in specific cases [4-7] and linked to the development of inflammatory complications [4,5]. To maintain homeostasis of circulatory neutrophils, compensatory mobilization of cells originating from the bone marrow (BM) occurs. Consequently, drastic shifts in the constitution of the blood neutrophil pool during systemic inflammation has been reported [8,9]. Enhanced mobilization of BM-neutrophils is considered to alter the content of the BM immune cell pool as well. To restore bone marrow immune reserves, enhanced hematopoiesis is mandated [10]. Granulopoiesis is, however, considered a time-consuming process as maturation in the post-mitotic pool takes >4 days [11,12]. The compensation mechanisms in the BM and kinetics in response to increased cell demands are poorly understood [10,13].

Profound inflammatory conditions have further been linked to the 'empty bone-marrow' phenomenon [14,15]. This BM-condition is defined as a deficit of both post-mitotic neutrophils and their progenitors. An empty bone marrow has been reported at least 24 hours post-insult after trauma due to a mismatch between circulatory demand and bone marrow synthesis capacity [14-16]. In addition to liberation of neutrophils from the bone marrow, remigration of relatively aged neutrophils from circulation into the bone marrow compartment has also been suggested [17,18]. The role of this phenomenon during a decrease in circulatory neutrophils during extensive surgery has not been studied before. To date, the exact interplay between changes of circulatory neutrophils after an acute trauma-induced inflammatory insult and the early bone marrow neutrophil-response has yet to be elucidated.

The hypothesis that is tested predicts that acute trauma-evoked depletion of systemic neutrophils is associated with altered composition of the bone marrow neutrophil pool. Such changes are characterized by (1) relative enhancement of the bone marrow neutrophil fraction and (2) increasing heterogeneity of the bone marrow neutrophil pool due to (3) the appearance of aged/returning neutrophils from circulation into the BM-compartment.

In order to test this hypothesis, a standardized porcine trauma model of extensive trauma surgery was utilized. The porcine model was chosen as this model allows for standardization of severe trauma and subsequent surgical interventions. Furthermore, porcine models have superior translational properties, as pigs are more closely related to humans in terms of their dimensions, anatomy, genetics, physiology and immunology compared to alternative non-primate animal models [19,20].

Materials and methods

All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use. The study protocol was approved by the Saarland University Hospital Animal Care Committee.

Experimental animals

All experiments were performed as described in the application and *Female Landrace* pigs were utilized (50-60 kilograms).

Experimental procedure

Premedication included intramuscular midazolam (1mg/kg), ketamin (20mg/kg) and atropine (50ug/kg). Intubation was performed following 2 minutes of pre-oxygenation with 100 percent Oxygen at 10L/min. A volume-controlled ventilation protocol was utilized. Maintenance of anesthesia was achieved by Isoflurane 0,25-0,50%, continuous midazolam infusion (0,6 mg/kg/hour), and sufentanil infusion (15ug/kg/hour). Ventilation rates were guided by end tidal CO₂-values and FiO₂ of 0.3, Positive end-expiratory pressure of 0cm H₂O and an I:E-ratio of 1:2 was preferred and aimed for in all animals. Frequent arterial blood gas analyses were performed to check ventilator and metabolic status. Antibiotics were not applied and hypothermia was prevented by altering room temperature. In accordance with the treatment concepts of the Definitive Surgical Trauma Care (DSTCTM)-course [21], hypovolemia was corrected by additional sodium chloride 0.9% fluid resuscitation. Continuous arterial line blood pressure measurements were available. All animals were exposed to standardized extensive trauma surgery. The protocol has been described previously [22]. In short, the following injuries were induced: liver laceration, 5 small bowel injuries, diaphragm injury, stomach, spleen, pancreas, left kidney. Furthermore, a thoracotomy was performed, and a cardiac injury was induced. Additionally, the left lung was injured and treated through hilar clamping and resection. Finally, the infrarenal inferior vena cava and right kidney were lacerated. Animals were euthanized after 3 hours of ongoing surgery by pentobarbital infusion.

Sampling

Blood and bone marrow samples were obtained at baseline and after 3 hours (immediately prior to euthanization). For blood neutrophil analysis, 9 mL of peripheral blood were collected from a central venous catheter in ethylenediaminetetraacetic acid (EDTA)-anticoagulated Vacutainers at baseline and directly after the animals were exposed to the final injury and subsequent therapy. An ice-cold isotonic NH_4CL -lysis buffer was utilized twice for the lysis of erythrocytes and cells were washed in between with FACS-buffer (Dulbecco phosphate buffered saline supplemented with 2% heat inactivated fetal calf serum, 5mM EDTA). White blood cell numbers for staining have been standardized by the utilization of a Neubauer Chamber to calculate the appropriate dilution factor. Antibody mixes were added, and samples were incubated in dark conditions for 45 minutes on 4°C. Then, samples were washed twice with PBS2+ (phosphate-buffered saline with added sodium citrate [0.38% wt/vol] and pasteurized plasma proteins [10% vol/vol; Sanquin, Amsterdam, The Netherlands]. Following the final wash steps, cells were fixed with BD Cellfix (BD, Mountain View, CA, USA), a premixed fixing concentrate containing 1% formaldehyde and 0,1% sodium azide. Prior to the experiments the stability of all antibodies was tested and validated. All fixed samples were analyzed within 24 hours with a FACS Canto II flowcytometer (BD, Mountain View, CA, USA). Data from individual experiments are depicted as fluorescence intensity as the median fluorescence intensity (MFI). A minimum of 20,000 neutrophils per sample was analyzed. Populations not expressing the used markers were used to set background fluorescence levels and compensation matrixes were composed by using beads and the automated setup system for compensation in BD FACSDiva software version 6.1.3 (BD, Mountain View, CA, USA).

Bone marrow material was harvested in accordance with recommendations for humans [23,24]. In short, the pig was placed in the supine position with both legs fixed. The appropriate extremity was prepared with antiseptic, scrubbed and draped, only exposing the site to be sampled. A 1.5cm longitudinal skin incision was made and bone-covering soft tissue was removed. Thereafter a 2 mm unicortical entry point was drilled with a sterile hand drill at the anterior-medial aspect of the proximal tibial metaphysis (10cm distal from the knee joint). Thereafter, an EDTA-coated 50 milliliter syringe with 1ml and a 25-Gauge needle were used to aspirate BM-content from the cavity.

After collection, BM-samples were directly and simultaneously processed with corresponding blood samples. At baseline, material was collected from the left tibia, whereas after intervention cells were gathered from the right tibia. In our model no extremity trauma was applied.

Flowcytometry analysis of porcine peripheral blood samples

A previously validated gating strategy was applied to distinguish between circulatory leukocyte subtypes. First, nucleated, viable singlet leukocytes (CD45⁺-cells) were included (see Figure 1a-c). Thereafter, forward/sideward scatter (FSC/SSC) profiles were utilized to identify different leukocyte subtypes, as previously described [25,26]. Neutrophils were identified through typical high sideward scatter profiles as observed both in human and porcine blood samples [25-29]. Furthermore, SWC 1 negative cells were excluded as this marker is expressed on porcine neutrophils and not on porcine eosinophils [29]. A representative example of the gating strategy of blood leukocytes is shown in Figure 1. To obtain leukocyte populations for determination of reference values, monocytes as well as lymphocytes and blasts (pooled) were gated as well. CountBright counting beads (Invitrogen, Waltham, Massachusetts, USA) were utilized to count and compare absolute cell numbers over time.

Figure 1. **Blood leukocyte subtype identification**

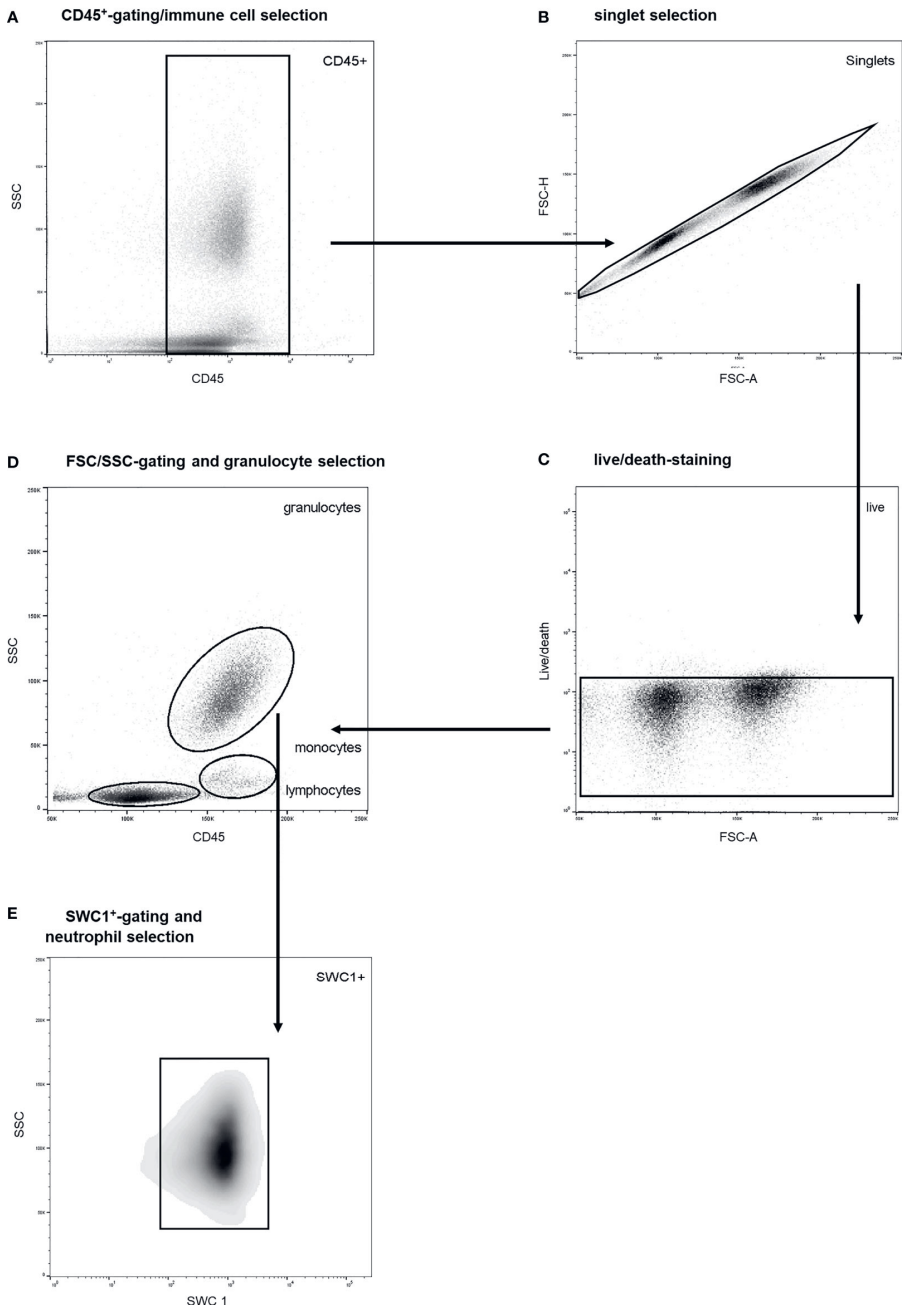


Fig. 1. Stepwise neutrophil gating protocol as utilized for identification of circulatory leukocyte subtypes and neutrophils a: Selection of CD45 positive cells and thereby the exclusion of debris and non-immune cells. b: Exclusion of doublets. c: life/death-stain and selection of viable immune cells. d: Identification of different leukocyte subtypes and subsequent selection of granulocytes using FSC/SSC-plots. e: Selection of SWC 1⁺ cells, and thereby exclusion of eosinophils. Abbreviations: SSC, sideward scatter signal; FSC, forward scatter signal.

Determination of bone marrow leukocyte subtypes and the neutrophil fraction

The bone marrow neutrophil fraction was defined as the proportion of marrow immune cells (CD45⁺) belonging to the neutrophil category, which was determined with flow studies [27-29]. More specifically, bone marrow immune cells were identified by a multistep gating protocol. A representative example of this gating strategy is shown in Figure 2.

First, vital BM-CD45⁺-cells were selected, and debris and doublets were excluded (see Figure 2a-2c). Thereafter, a SSC/CD45 gate was used to select bone marrow granulocytes (see Figure 2d). Basophils were gated out based on their lower SSC signal. This is an established gating strategy for both porcine and human bone marrow analysis [27-29]. Thereafter, SWC 1-positive cells were selected as this marker is exclusively expressed on porcine neutrophils and not on eosinophils (see Figure 2e) [29]. This gating strategy has been validated by co-expression analysis of different leukocyte subtypes. Furthermore, validation experiments including cell sorting studies with morphologic analysis demonstrated a neutrophil purity over 99%. The BM-neutrophil fraction was determined by calculating the percentage of BM-neutrophils among all BM-immune cells.

Figure 2. Bone marrow neutrophil fraction identification

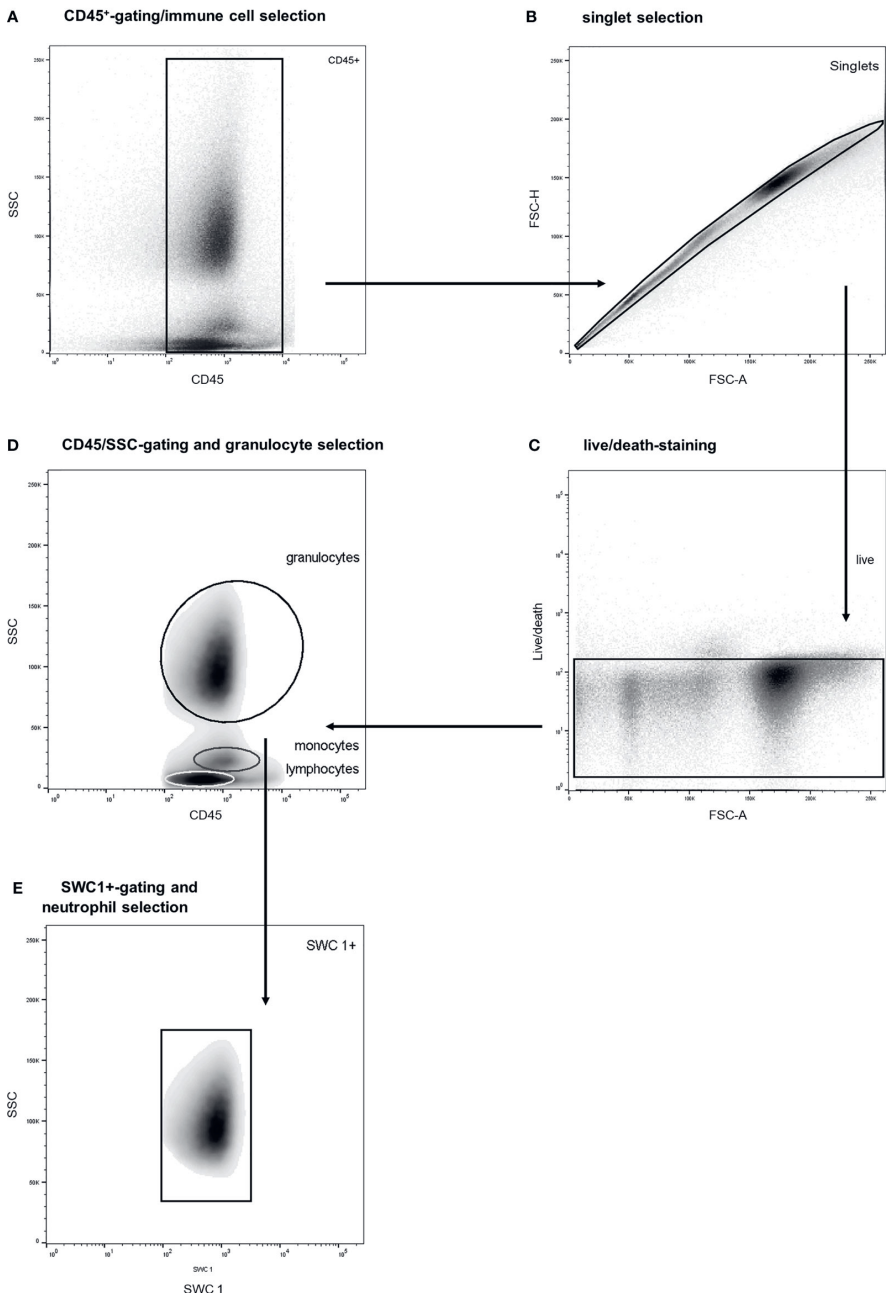


Fig. 2. Stepwise neutrophil gating protocol as utilized for identification of the bone marrow neutrophil fraction. a: Selection of CD45 positive cells and thereby the exclusion of debris and non-immune cells. b: Exclusion of doublets. c: life/death-stain and selection of viable immune cells. d: Identification of different leukocyte subtypes and subsequent selection of granulocytes (of note: basophils are not included in the granulocyte gate due to distinct side scatter signal). e: Selection of SWC 1+ cells, and thereby exclusion of potential contamination by eosinophils. Abbreviations: SSC, sideward scatter signal; FSC, forward scatter signal.

Monoclonal antibodies and flow cytometry measurements

For the flow cytometry analysis of neutrophil receptor expression, the following commercially available anti-pig monoclonal antibodies were obtained and when indicated conjugated by validated and commercially available antibody conjugation kits: SWC 1 (clone K263.3d7, Novus Biol, Centennial, Colorado, USA), SWC 3 (clone 74-22-15, Accurate Chemical, Westbury, New York, USA), SWC 8 (MIL-1, Abd Serotec, Kidlington, UK), CD11b (clone 2F4/11, Abd Serotec, Kidlington, UK), CD16 (clone G7, Abd Serotec, Kidlington, UK), CD29 (clone NaM160-1A3, BD, Mountain View, CA, USA), CD45 (clone K252.1E4, Abd Serotec, Kidlington, UK), CD45Ra (clone MIL-13, GenWay Biotech, San Diego, CA, USA), CD49D (clone L25, BD, Mountain View, CA, USA), CD184 (clone H-118, Santa Cruz Biotechnol, Santa Cruz, CA, USA). Lightning Link conjugation kits (Novus Biol, Centennial, Colorado, USA). A viability staining Vivid (Invitrogen, Waltham, USA) was added to exclude dead cells

Data analysis and statistics

Data was analyzed using software programs SPSS version 21.0 (SPSS Inc., Chicago, IL, USA), GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA) and FlowJo Version 10 (De Novo Software, Glendale, CA, USA). Results were expressed as means (SEM) unless otherwise mentioned. For comparisons, Student's T-tests, Paired T-testing or Mann Whitney U-tests were applied as appropriate. Normality of variance was tested using Lavene's test. A p -value < 0.05 was considered statistically significant.

Results

All subjects survived the interventions, and respiratory complications were not diagnosed. Bone marrow sampling was not successful in one experimental animal and, therefore, bone marrow analysis was not performed on cells from this animal. All other samples provided sufficient material for analysis.

Decreased circulatory leukocyte numbers and altered relative presence of leukocyte subtypes following extensive trauma surgery

As shown in Figure 3a, extensive trauma surgery is associated with a marked and statistically significant drop in leukocyte numbers in peripheral blood. At baseline a mean white blood cell count of $21.3 \pm 1.7 \times 10^6$ cells/ml was measured, and after intervention leukocyte numbers reduced to $9.6 \pm 1.6 \times 10^6$ cells/ml ($P < 0.001$). The identification of specific white blood cell subtypes is displayed in Figure 1. Absolute lymphocyte and neutrophil numbers both dropped significantly over time from 10.3 to 5.9×10^6 cells/ml ($P = 0.01$), and 9.3 to 3.2×10^6 cells/ml ($P = 0.001$), respectively. The monocyte population decreased from 1.7 to 0.5×10^6 cells/ml ($P = 0.01$; see Fig 13c). The mean percentage of blood neutrophils as part of all leukocytes decreased from 43% to 33% ($P = 0.07$), whereas the percentage of blood lymphocytes increased significantly ($P = 0.02$). This shows that extensive trauma surgery causes a shift in the constitution of the blood leukocyte pools characterized by decreased numbers of circulating immune cells and diminished neutrophil presence.

Figure 3a. **Altered blood leukocyte (CD45⁺) numbers upon insult**

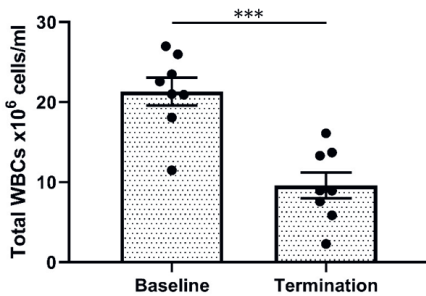


Figure 3b. **Shifts in leukocyte fractions in peripheral blood after trauma surgery**

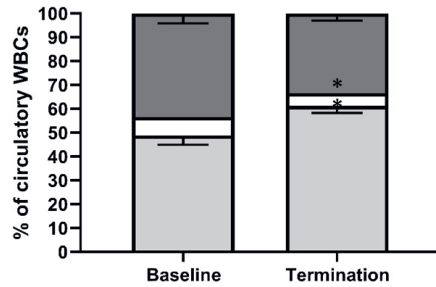


Figure 3c. **Absolute changes in blood-subtype appearance after trauma surgery**

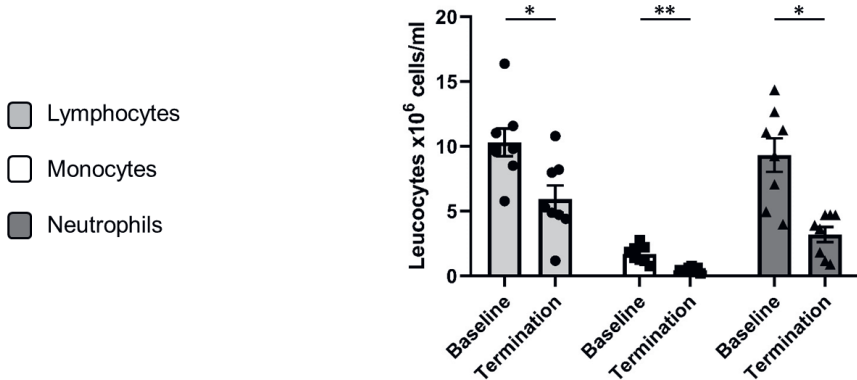


Fig. 3. Systemic leukocyte alterations after trauma surgery. a: Difference in white blood cell count between both conditions (baseline vs. termination). Relative (b) and absolute (c) differences in lymphocyte, monocyte and neutrophil numbers between baseline and termination, reflecting shifts in leukocyte fractions after trauma surgery. Abbreviations: S/FSC; sideward/forward scatter signal; N, neutrophils; M, monocytes; L, lymphocytes *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. Baseline samples were taken prior to intervention and termination sampling was performed directly after the final procedure.

Increase of the bone marrow neutrophil fraction during trauma was associated with a relative circulatory neutropenia

The composition of the bone marrow neutrophil fraction changed strikingly after extensive trauma surgery. Porcine bone marrow neutrophils have been identified according to a multistep gating protocol as described in Figure 2. The neutrophil bone marrow fraction increased in 6 out of 7 experimental animals upon insult. Under homeostatic conditions, 63 ± 3 percent of $CD45^+$ nucleated bone marrow cells were identified as neutrophils. However, following extensive surgery, this percentage increased up to 71 ± 3 percent ($P<0.05$, Figure 4).

Figure 4. Bone marrow neutrophil fraction expansion after trauma surgery

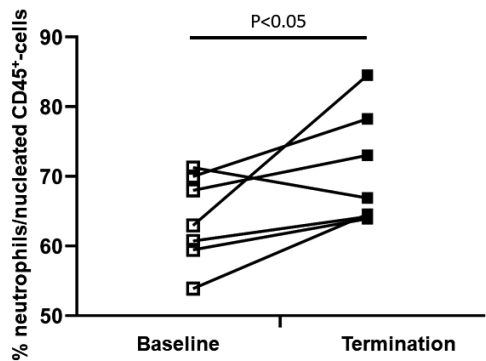


Fig. 4. Difference in the bone marrow neutrophil fraction between baseline and termination. The BM-neutrophil pool/fraction has been identified as described in Figure 2.

Dynamics in expression of cell surface receptors reflect an instantaneous shift of neutrophil populations from blood to bone marrow after trauma surgery

Next to a change in cell numbers of circulatory and bone marrow neutrophils, receptor expression profiles of neutrophils in both compartments changed as well. Changes were homogeneous across the different experimental animals as described in detail hereafter. Relevant receptors involved in porcine neutrophil maturation (Figure 5a), activation (Figure 5b) and bone marrow retention and homing (Figure 5c) were analyzed, using the previously described gating strategy (Figure 1 (blood samples) and 2 (bone marrow samples)).

At baseline, the population of peripheral blood neutrophils had significantly higher neutrophil surface expression levels of both SWC 3 ($P<0.01$) and CD16 ($P<0.01$) compared with bone marrow neutrophils. After trauma, a statistically significant increase of both neutrophil SWC 3 ($P<0.001$) and CD16 ($P<0.001$) expression was found on circulatory neutrophils, whereas a less prominent increase of SWC 3 (statistically non-significant) and CD16-expression levels ($P<0.05$) was measured on BM-cells. As anticipated, levels of cell surface expression of CD29 (integrin $\alpha 1$ -chain) were significantly higher on bone marrow neutrophils than on circulatory neutrophils. No differences were seen between homeostatic and post-insult conditions (Figure 5a).

As shown in Figure 5b, no statistically significant differences in cell surface expression levels of activation marker CD11b were seen between neutrophils from blood and BM. After extensive surgery, CD11b-expression on circulatory neutrophils significantly increased after insult ($P<0.05$).

In addition, in homeostasis expression levels of CD184 (CXCR4) and CD49d (VLA4) involved in neutrophil retention in the bone marrow were statistically significantly lower on circulatory neutrophils compared to bone marrow ($P<0.001$ and $P<0.05$, respectively). Following intervention, cell surface expression levels of CD184 ($P<0.05$) and CD49d ($P<0.001$) rose on bone marrow neutrophils. CD49d-expression on systemic neutrophils did not change after insult, whereas CD184-expression on circulatory neutrophils increased significantly ($P<0.01$) and VLA-4 on bone marrow neutrophils increased significantly after intervention. Cell surface receptor expression levels of CD49d and CXCR4 on blood and bone marrow neutrophils under different conditions are shown in Figure 5c.

Figure 5a. **Cell surface receptor dynamics of *maturation markers* on BM and blood neutrophils**

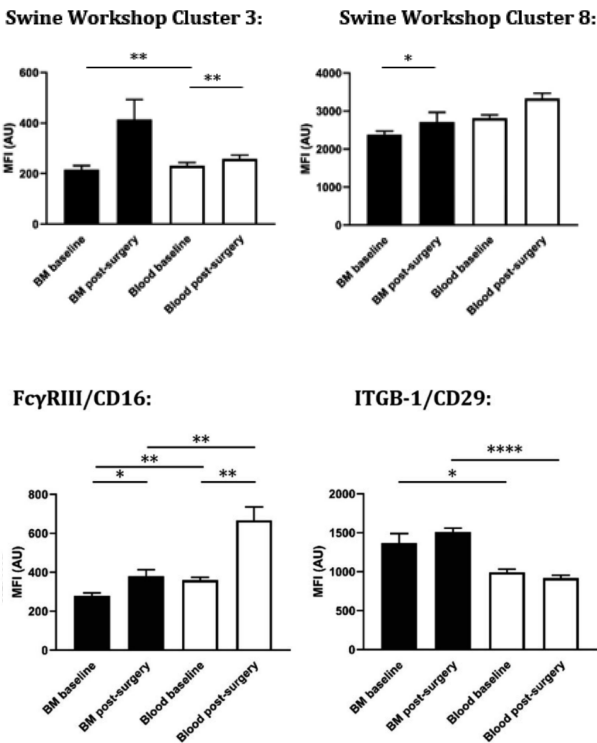


Figure 5b. Cell surface receptor dynamics of activation marker *Mac-1/CD11b* on BM and blood neutrophils

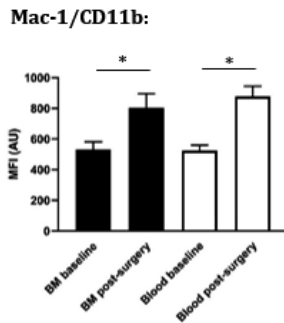


Figure 5c. Cell surface receptor dynamics of activation *bone marrow retention receptors* on BM and blood neutrophils

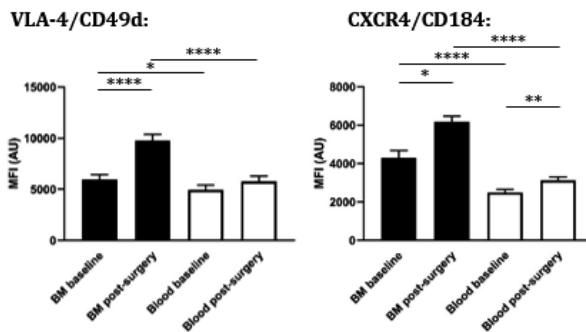


Fig. 5. Cell surface expression on circulating and bone marrow neutrophils at baseline and after extensive surgery. (a) BM-maturation markers, (b) activation markers, (c) BM retention receptors. Black bars represent bone marrow samples, white bars represent blood samples. Baseline samples were taken prior to intervention and termination sampling was performed directly after the final procedure. Data are presented as mean \pm SEM, MFI; median fluorescence intensity in arbitrary units. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Increased heterogeneity of the bone marrow neutrophil pool and overrepresentation of an FCS^{high}/CXCR4^{high}-neutrophil subset upon extensive porcine trauma surgery

As described previously, extensive trauma surgery is associated with striking changes in the cell-surface receptor expression profiles of the BM neutrophil pool (Figure 5a-c). Furthermore, the amount of variability of cell surface expression levels of relevant markers (Figure 5a-c) on BM neutrophils also increased after intervention, (Fig. 5a-c), reflecting increased heterogeneity of the bone marrow neutrophil population after trauma.

Additionally, overrepresentation of a specific neutrophil subset of FSC^{high}-neutrophils (BM-Neu2) after insult was demonstrated (Figure 6a). Forward and side scatter density plots of CD45⁺/SSC^{high}/SWC 1⁺ cells (neutrophils) allow for identifying this specific subset (see Figure 6a.6). Under homeostatic conditions this subset (BM-Neu2) is virtually absent in bone marrow and represents 2.7 ± 0.2 percent of bone marrow neutrophils. However, after extensive trauma surgery, BM2-Neu-cells comprise 9.2 ± 1.0 percent of BM-neutrophils (Figure 6a.6a). The relative increase of the size of the BM-Neu2 population as fraction of all BM-neutrophils after trauma was statistically significant ($P<0.001$). BM2-Neu cells were further characterized by a statistically significant higher co-expression of CXCR4/CD184 ($P<0.01$). A representative example of a histogram of CXCR4 expression on BM-Neu1 (regular neutrophils in BM), BM-Neu2 and a corresponding blood sample is displayed in Figure 6b. Table 1 provides an overview of additional co-expression receptor analyses of both neutrophil subsets.

Table 1. Comparison of receptor co-expression profiles of BM-Neu1 and BM-Neu2 cells

	BM-NEU1 (MFI)	BM-NEU2 (MFI)	P-VALUE
SWC3	457 ± 68	356 ± 52	0.20
SWC8	2376 ± 205	2236 ± 806	0.07
FcγRIII/CD16	298 ± 45	385 ± 34	0.16
ITGB-1/CD29	1430 ± 164	902 ± 313	0.31
Mac-1/CD11b	859 ±130	707 ± 70	0.52
VLA-4/CD49d	8497 ± 940	9777 ± 1156	0.70
CXCR4/CD184	5683 ± 530	14052 ± 1668	0.0074

Figure 6. Identification of different BM-neutrophil subset based on FSC/SSC signals

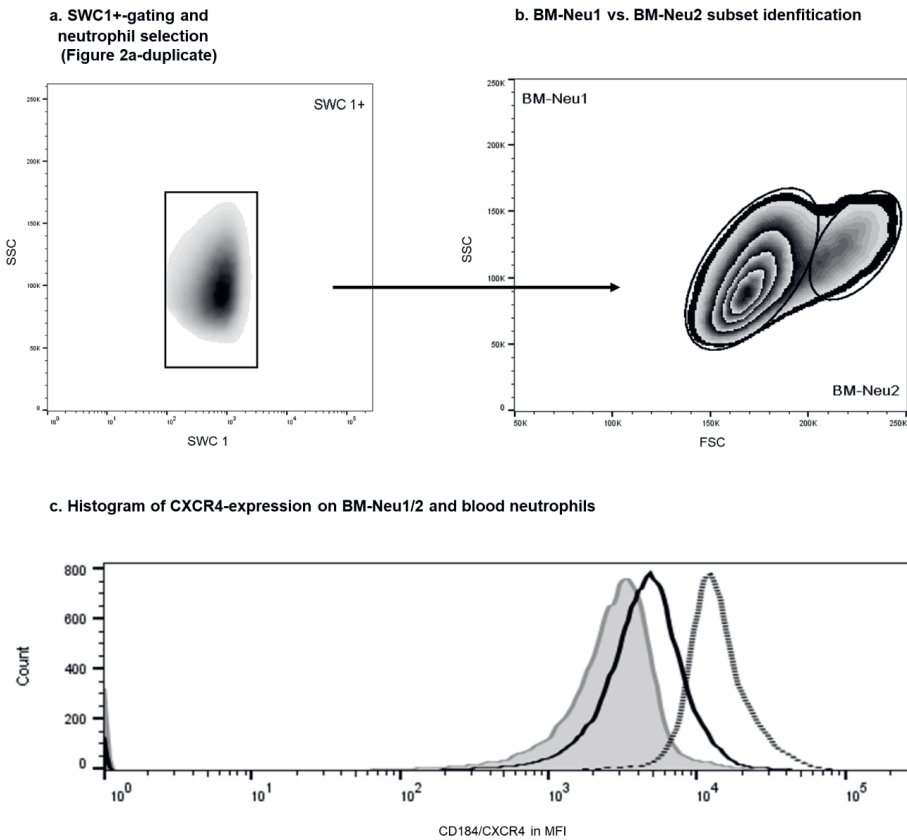


Fig. 6. Gating protocol for the identification of BM-neutrophil subsets after intervention. Initial gating steps have been displayed in Figure 2. (b) Representative example of FACS analysis of porcine bone marrow samples after intervention. (c) Histograms showing regular neutrophils (BM-Neu1, black line), novel FSC^{high} neutrophils (BM-Neu2, grey line) and reference blood neutrophils at termination (grey filled curve). Abbreviations: MFI; median fluorescence intensity.

Discussion

The key findings of the current study can be summarized as follows:

1. Extensive trauma surgery in a standardized setting in pigs is associated with a prompt (<3hrs) decrease in circulating leukocyte numbers, including neutrophils.
2. Extensive trauma surgery is associated with shifts in the composition of the bone marrow immune cell pools with an instantaneous relative increase of the BM-neutrophil fraction and an increased expression of neutrophil maturation markers on bone marrow neutrophils.
3. The post-traumatic BM-neutrophil pool is characterized by increased heterogeneity and overrepresentation of a unique CXCR4^{high} neutrophil subset.

Our study provides essential novel insights into the early cellular immune response to severe trauma surgery within the BM compartment. In line with other studies on extensive trauma surgery, an early increase of activation status (e.g. enhanced neutrophil CD11b/Mac-1 expression) and a systemic neutropenia were demonstrated following insult [30].

It has previously been demonstrated in clinical studies on surgical patients that aberrant circulatory neutrophil/leukocyte numbers after insult are linked with impaired clinical outcome [4,5,31-35]. Both situations of early decreased circulatory neutrophil numbers [4,5,31] as reported in the current study, but also situations of early elevated systemic immune cell numbers [32-35], are associated with inferior clinical outcome. These observations imply that specific patterns of (divergent) circulatory neutrophil kinetics, with either reduced or increased cell numbers, may represent an abnormal cellular immune response. A large number of neutrophils and their progenitors are present in the bone marrow under tight control of their production, differentiation and eventually mobilization [8-10,17]. Therefore, it is key to understand the interplay between the bone marrow, peripheral blood and distant tissues. To our knowledge this is the first study that has investigated the bone marrow's response to extensive trauma surgery and the related relative circulatory neutropenia in a controlled setting. Interestingly, and in contrast to the consensus [13-14,16], in the current study a relative increase of the BM-neutrophil fraction upon extensive surgical intervention was demonstrated, rather than depletion of the neutrophil bone marrow population [13,14,16].

The production and differentiation of granulocytes (granulopoiesis) takes mainly place in the bone marrow. Granulocytes and precursors make up 60% of BM-leukocytes [36]. It is estimated that bone marrow produces approximately $0.5-1.0 \times 10^{11}$ neutrophils a day and that there are approximately 50-100 times more neutrophils in the bone marrow than in circulation [36,37], but these issues are under current debate [38,39]. The bone marrow neutrophil population comprises of cells at different developmental stages. Three specific pools can be distinguished: the stem cell pool with self-renewal cell divisions, the mitotic pool (which includes cells (myeloblasts, (pro)myelocytes) that differentiate during proliferation) and the post-mitotic pool (with non-proliferating but maturing cells). The post-mitotic pool includes differentiated neutrophils (metamyelocytes, banded and mature cells) and forms a large bone marrow pool [40,41]. Mature neutrophils are released into circulation. After mobilization, neutrophils stay in circulation and may migrate into the tissue compartment before cells return to the bone marrow or other poorly defined tissue sites [17,42]. Under homeostatic conditions systemic neutrophil numbers remain constant due to a balance in production, compartmentalization, and cell death. However, in the case of acute systemic inflammation, neutrophil numbers in different compartments shift markedly [1-5,43]. Our research group has previously shown early neutropenia in a study on experimental extreme trauma surgery in pigs [22], which was reproduced in this study. Interestingly, these novel experiments reveal a simultaneous increase of the bone marrow neutrophil fraction. This is in contrast with several human studies in which the occurrence of an 'empty bone marrow phenomenon' upon extensive surgery has been suggested [13,14,16]. In these latter studies a marked decrease of the bone marrow neutrophil pool was seen after insult. This bone marrow neutropenia was explained by a putative exhaustion BM cell production. The differences between our results and these findings may partly be explained by differences in the timing of the different investigations. The current experiment focuses on the acute response to extensive surgery (first 3 hours) only, whereas other studies focus on later time-points (> 24 hours) [13,14,16]. A similar early relative neutropenia followed by later neutrophilia has been described in a model of human experimental endotoxemia [44].

Besides affecting neutrophil numbers, extensive trauma surgery also led to profound changes in the characteristics of both blood and BM neutrophils. Upon intervention, circulatory neutrophil CD11b expression almost doubled. Similar effects have been described in severe trauma and reflect the marked effect of our extensive surgical model on systemic immune homeostasis [30,45]. In parallel to the relative neutrophilia of the bone marrow, characteristics

of the BM-pool, determined by analysis of neutrophil surface expression profiles, changed as well after insult.

More specifically, cell surface expression levels of CD11b, CD16, CD184, SWC 8 on BM-neutrophils increased significantly after trauma surgery. In addition, a non-statistically significant trend towards increased SWC 3 expression was observed as well after intervention. CD11b, CD16, CD184, SWC 3 and SWC 8 have been identified as maturation markers for bone marrow neutrophils, whose expression levels on neutrophils rise during maturation [29,46,47]. Therefore, the findings from our study indicate increased overall maturation of the bone marrow neutrophil population [29,48]. Increased expression of CD184 on blood neutrophils after trauma may be secondary due to massive selective tissue migration of young cells under extreme inflammatory conditions. Alternatively, blood may function as a transport medium for tissue neutrophil returning back to the bone marrow. Of note, increased CD11b-expression on BM-neutrophils is most likely mainly due to increased cell activation [30,45], rather than due to more progressed maturation. Increased bone marrow maturation after surgery can be explained by four processes that are not mutually exclusive:

Firstly, an increase in older neutrophils in the bone marrow might be caused by selective release of young cells into the circulation. This hypothesis is supported by the massive release of banded neutrophils in the first hours after severe trauma [22,44].

Secondly, selective acceleration of processes of neutrophil proliferation, differentiation and maturation may occur upon extreme conditions; a situation generally coined as emergency granulopoiesis [49,50]. There are, however, no published studies supporting this phenomenon in trauma patients. Also, such emergency granulopoiesis after trauma would result in more immature, rather than aged neutrophils [49,50]. Thirdly, bone marrow neutrophil apoptosis may be affected or postponed after extensive trauma. As mentioned before, aged, but not necessarily apoptotic neutrophils are thought to be cleared in the bone marrow [17,42]. While bone marrow is thought to be involved in clearance of neutrophils, it is also known for its capacity to optimize cell survival by specific BM survival factors [51,52]. In cases of extensive trauma, regulation of cell survival in the bone marrow compartment may differ from regular conditions.

Lastly, enhanced selective influx of aged neutrophils into the bone marrow compartment may occur after trauma. Bone marrow homing of aged but not apoptotic neutrophils in murine models is thought to be a CXCR4 (CD184)-dependent process as cells can respond to stromal derived factor (SDF)-1alpha/CXCL12, the ligand for CXCR4 [47,53]. The bone marrow

compartment constitutively expresses SDF-1 α /CXCL12, and the BM is considered as the preferred homing compartment of CXCR4^{high}-cells [18,47]. As such, increased BM accumulation of aged neutrophils, which have been trafficking back from circulation after extensive trauma surgery likely contribute to overall aging of the BM neutrophil population. Additionally, the current study is the first to describe prominent increase of a specific CXCR4^{high}-neutrophil subset (termed BM-Neu2) in bone marrow after trauma. This increased population likely reflects returning neutrophils from circulation and has previously been described in non-trauma conditions as well [54,55]. The role and capacities of this subset are currently unclear.

Limitations:

Humoral factors upon standardized porcine trauma have been described in detail before and were not analyzed in the current study [56,57]. According to literature, systemic leukocyte neutrophil numbers in pigs range between 10 and 22 $\times 10^9/L$ and are higher than in humans [58]. Baseline leukocyte numbers in the current study were within ranges as described in literature. Therefore, we do not believe that stress-induced neutrophilia due to transportation or handling of the pigs played a relevant role [59]. Furthermore, as neutrophil subsets were identified after interpretation of the experiments, we were not able to perform *in vitro* studies on the novel BM-subset.

In conclusion, this study describes the early bone marrow response to extensive trauma surgery in a controlled setting. The current study shows for the first time that during trauma-induced neutropenia, a parallel increase in neutrophil numbers in the bone marrow occurs. This shift is characterized by relative enrichment of the bone marrow neutrophil fraction, increased maturation-status of the bone marrow neutrophils, and an increased number of a specific CXCR4^{high}-neutrophil subset in the bone marrow. This study also reveals that in pre-lethal trauma, aberrant neutrophil responses in blood and bone marrow go hand in hand. Hence, in order to design future immunomodulatory interventions for critically ill trauma patients it is important to acquire a better understanding of the pre-lethal bone marrow response. The porcine model is suitable to perform further studies on this issue and may also be utilized to perform future proof-of-principle interventions for pre-lethal trauma situations interventions.

References

1. Keel M, Trentz O. Pathophysiology of trauma. *Injury* (2005) 36(6):691-709.
2. Lord JM, Midwinter MJ, Chen YF, Belli A, Brohi K, Kovacs EJ, Koenderman L, Kubes P, Lilford RJ. The systemic immune response to trauma: an overview of pathophysiology and treatment. *Lancet* (2014) 384(9952):1455-65.
3. Hesselink L, Spijkerman R, Wessm van K, Koenderman L, Leenen L, Huber-Lang M, Hietbrink F. Neutrophil heterogeneity and its role in infectious complications after severe trauma. *World J Emerg Surg* (2019) 14(24).
4. Pallister I, Dent C, Topley N. Increased neutrophil migratory activity after major trauma: a factor in the etiology of acute respiratory distress syndrome? *Crit Care Med* (2002) 30(8):1717-21.
5. Botha AJ, Moore FA, Moore EE, Sauaia A, Banerjee A, Peterson VM. Early neutrophil sequestration after injury: a pathogenic mechanism for multiple organ failure. *J Trauma* (1995) 39:411-7.
6. Jacobi CA, Ordemann J, Zieren HU, Volk HD, Bauhofer A, Halle E, Müller JM. Increased systemic inflammation after laparotomy vs laparoscopy in an animal model of peritonitis. *Arch Surg* (1998) 133(3):258-62.
7. Richardson RP, Rhyne CD, Fong Y, Hesse DG, Tracey KJ, Marano MA, Lowry SF, Antonacci AC, Calvano SE. Peripheral blood leukocyte kinetics following in vivo lipopolysaccharide (LPS) administration to normal human subjects. Influence of elicited hormones and cytokines. *Ann Surg* (1989) 210(2):239-45.
8. Eash K, Means J, White D, Link D. CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. *Blood* (2009) 113(19):4711-9.
9. Furze RC, Rankin SM. Neutrophil mobilization and clearance in the bone marrow. *Immunology* (2008) 125:281-8.
10. Manz M, Boettcher S. Emergency granulopoiesis. *Nature Rev Immunol* (2014) 14:302-14.
11. Athens JW. Blood: leukocytes. *Annu Rev Physiol* (1963) 25:195-212.
12. Dancy JT, Deubelbeiss KA, Harker LA, Finch CA. Neutrophil kinetics in man. *J Clin Invest* (1976) 58(3):705-15.
13. Livingston D, Anjaria D, Wu J, Hauser C, Chang V, Deitch E, Rameshwar P. Bone marrow failure following severe injury in humans. *Ann Surg* (2003) 238(5):748-53.
14. Marsh C, Boggs D, Cartwright G, Wintrobe M. Neutrophil kinetics in acute infection. *J Clin Invest* (1967) 46(12):1943-53.
15. Rothstein G, Christensen RD, Nielsen BR. Kinetic evaluation of the pool sizes and proliferative response of neutrophils in bacterially challenged aging mice. *Blood* (1987) 70(6):1836-41.
16. Amos R, Deane M, Ferguson C, Jeffries G, Hinds C, Amess J. Observations on the haemopoietic response to critical illness. *J Clin Pathol* (1990) 43(10):850-6.
17. Rankin S. The bone marrow: a site of neutrophil clearance. *J Leukoc Biol* (2010) 88:241-251.

18. Furze RC, Rankin SM. The role of the bone marrow in neutrophil clearance under homeostatic conditions in mouse. *FASEB J* (2008) 22(9):3111-9.
19. Tsukamoto T, Pape HC. Animal models for trauma research: what are the options? *Shock* (2009) 31(1):3-10.
20. Mair K, Sedlak C, Käser T, Pasternak A, Levast B, Gerner W, Saalmüller A, Summerfield A, Gerdts V, Wilson HL, Meurens F. The porcine innate immune system: an update. *Dev Comp Immunol* (2014) 45(2):321-43.
21. Rotondo MF, Schwab CW, McGonigal MD, Phillips GR, Fruchterman TM, Kauder DR, Latenser BA, Angood PA. "Damage control": an approach for improved survival in exsanguinating penetrating abdominal injury. *J Trauma* (1993) 35(3):375-82.
22. Teuben M, Heeres M, Blokhuis T, Hollman A, Vrisekoop N, Tan E, Pfeifer R, Pape HC, Koenderman L, Leenen LPH. Instant intra-operative neutropenia despite the emergence of banded (CD16^{dim}/CD62L^{bright}) neutrophils in peripheral blood - An observational study during extensive trauma-surgery in pigs. *Injury* (2021) 52(3):426-33.
23. Schweinberger MH, Roukis TS. Percutaneous autologous bone marrow harvest from the calcaneus and proximal tibia: surgical technique. *J Foot Ankle Surg* (2007) 46(5):411-4.
24. Gillan E, Christensen R, Suen Y, Ellis R, Ven van de C, Cairo M. A randomized, placebo-controlled trial of recombinant human granulocyte colony-stimulating factor administration in newborn infants with presumed sepsis: significant induction of peripheral and bone marrow neutrophilia. *Blood* (1994) 84(5):1427-33.
25. Bréa D, Meurens F, Dubois AV, Gaillard J, Chevalere C, Jourdan ML, Winter N, Arbeille B, Si-Tahar M, Gauthier F, Attucci S. The pig as a model for investigating the role of neutrophil serine proteases in human inflammatory lung diseases. *Biochem J.* (2012) 1;447(3):363-70.
26. de Ruiter K, van Staveren S, Hilvering B, Knol E, Vrisekoop N, Koenderman L, Yazdanbakhsh M. A field-applicable method for flow cytometric analysis of granulocyte activation: Cryopreservation of fixed granulocytes. *Cytometry A* (2018) ;93(5):540-547.
27. Eleghetany M, Ge Y, Patel J, Martinez J, Uhrova H. Flow cytometric study of neutrophilic granulopoiesis in normal bone marrow using expanded panel of antibodies: correlation with morphologic assessments. *J Clin Lab Anal* (2004) 18:36-41.
28. van Lochem EG, van der Velden VH, Wind HK, te Marvelde JG, Westerdal NA, van Dongen JJ. Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts. *Cytometry B Clin Cytom.* (2004) 60(1):1.
29. Summerfield A, McCullough K. Porcine bone marrow myeloid cells: phenotype and adhesion molecule expression. *J Leuk Biol* (1997) 62(2):176-85.
30. Botha AJ, Moore FA, Moore EE, Peterson VM, Goode AW. Base deficit after major trauma directly relates to neutrophil CD11b expression: a proposed mechanism of shock-induced organ injury. *Intensive Care Med* (1997) 23(5):504-9.
31. Gulack BC, Englum BR, Lo DD, Nussbaum DP, Keenan JE, Scarborough JE, Shapiro ML. Leukopenia is associated with worse but not prohibitive outcomes following emergent abdominal surgery. *J Trauma* (2015) 79(3):437-43.
32. Rovlias A, Kotsou S. The blood leukocyte count and its prognostic significance in severe head injury. *Surg Neurol* (2001)55(4):190-6.

33. Waheed U, Williams P, Brett S, Baldock G, Soni N. White cell count and intensive care unit outcome. *Anaesthesia*. (2003) 58(2):180-2.
34. Lawrence YR, Raveh D, Rudensky B, Munter G. Extreme leukocytosis in the emergency department. *QJM* (2007) 100(4):217-23.
35. Hasjim BJ, Grigorian A, Stopenski S. Moderate to severe leucocytosis with vasopressor is associated with increased mortality in trauma patients. *J Intens. Care Soc* 2020.
36. Dancy J, Deubelbeiss K, Harker L, Finch C. Neutrophil kinetics in man. *J Clin Invest* (1976)58:705-15.
37. Semerad CL, Liu F, Gregory AD, Stumpf K, Link DC. G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood. *Immunity* (2002) 17(4):413-23.
38. Pillay J, den Braber I, Vrisekoop N, Kwast LM, de Boer RJ, Borghans JA, Tesselaar K, Koenderman L. In vivo labeling with ²H₂O reveals a human neutrophil lifespan of 5.4 days. *Blood*. (2010) 29;116(4):625-7.
39. Lahoz-Beneytez J, Elemans M, Zhang Y, Ahmed R, Salam A, Block M, Niederaal C, Asquith B, Macallan D. Human neutrophil kinetics: modeling of stable isotope labeling data supports short blood neutrophil half-lives. *Blood* (2016) 30;127(26):3431-8.
40. Orr Y, Wilson DP, Taylor JM, Bannon PG, Geczy C, Davenport MP, Kritharides L. A kinetic model of bone marrow neutrophil production that characterizes late phenotypic maturation. *Am J Physiol Regul Integr Comp Physiol* (2007) 292(4):R1707-16.
41. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil kinetics in health and disease. *Trends Immunol* (2010) 31(8):318-24.
42. Vietinghoff von S, Ley K. Homeostatic regulation of blood neutrophil counts. *J Immunol* (2008) 181:5183-8
43. Wengner A, Pitchford S, Furze R, Rankin S. The coordinated action of G-CSF and ELR + CXC chemokines in neutrophil mobilization during acute inflammation *Blood* (2008) 181:5183-8.
44. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers JW, Ulfman LH, Leenen LP, Pickkers P, Koenderman L. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest* (2012) 122(1):327-36.
45. Hietbrink F, Koenderman L, Leenen LP. Intramedullary nailing of the femur and the systemic activation of monocytes and neutrophils. *World J Emerg Surg* (2011)31;6:34.
46. Summerfield A, McCullough KC. Porcine bone marrow myeloid cells: phenotype and adhesion molecule expression. *J Leukoc Biol* (1997) 62(2):176-85.
47. Lund-Johannsen F, Terstappen L. Cell adhesion molecules during granulopoiesis *J Leuk Biol* (1993) 54(1):47-55.
48. Leitão L, Alves CJ, Alencastre IS, Sousa DM, Neto E, Conceição F, Leitão C, Aguiar P, Almeida-Porada G, Lamghari M. Bone marrow cell response after injury and during early stage of regeneration is independent of the tissue-of-injury in 2 injury models. *FASEB J* (2019) 33(1):857-872.
49. Fuchs A, Moonlish D, Ghosh S, Chang S, Bochicchio G, Schuettelpelz L, Turnbull I. Trauma Induces Emergency Hematopoiesis through IL-1/MyD88–Dependent Production of G-CSF. *J Immunol* (2019) 202(10):3020-2.

50. Benarafa C, LeCruyer TE, Baumann M, Stolley JM, Cremona TP, Remold-O'Donnell E. SerpinB1 protects the mature neutrophil reserve in the bone marrow. *J Leukoc Biol* (2011) 90:21-9.
51. Altnauer F, Martinelli S, Yousefi S, Thürig C, Schmid I, Conway EM, Schöni MH, Vogt P, Mueller C, Fey MF, Zangemeister-Wittke U, Simon UH. Inflammation-associated cell cycle-independent block of apoptosis by surviving in terminally differentiated neutrophils. *J Exp Med* (2004) 199:1943-54.
52. Nagase H, Miyamasu M, Yamaguchi M, Imanishi M, Tsuno NH, Matsushima K, Yamamoto K, Morita Y, Hirai K. Cytokine-mediated regulation of CXCR4 expression in human neutrophils. *J Leukoc Biol* (2002) 71(4):711-7.
53. Martin C, Burdon PC, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity* (2003) 19(4):583-93.
54. Martin C, Burdon PC, Bridger G; Gutierrez-Ramos JC, Williams TJ, Rankin SM. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their returning following senescence. *Immunity* (2003) 19:583-93.
55. De Filippo K, Rankin SM. CXCR4, the master regulator of neutrophil trafficking in homeostasis and disease. *Eur J Clin Invest* (2018) 48(Suppl. 2):e12949.
56. Horst K, Eschbach D, Pfeifer R, Hübenthal S, Sassen M, Steinfeldt T, Wulf H, Ruchholtz S, Pape HC, Hildebrand F. Local inflammation in fracture hematoma: results from a combined trauma model in pigs. *Mediators Inflamm*. (2015) 2015:126060.
57. Horst K, Greven J, Lüken H, Zhi Q, Pfeifer R, Simon TP, Relja B, Marzi I, Pape HC, Hildebrand F. Trauma Severity and Its Impact on Local Inflammation in Extremity Injury-Insights From a Combined Trauma Model in Pigs. *Front Immunol* (2020) 9:10:3028.
58. Fingerhut L, Dolz G, de Buhr N. What Is the Evolutionary Fingerprint in Neutrophil Granulocytes? *Int J Mol Sci* (2020);21(12):4523.
59. Dubreuil P, Courure Y, Faerman C, Petitclerc D. Hematological and biochemical changes following an acute stress in control and somatostatin-immunized pigs. *Can J Anim Sci* (1993) 73(2) :241-252.

Chapter 8

Cellular activation status in femoral shaft fracture hematoma following different reaming techniques – a large animal model

Michel Paul Johan Teuben^{1,2}

Sascha Halvachizadeh^{1,2}

Yannik Kalbas^{1,2}

Zhi Qiao³

Nikola Cesarovic^{4,5,6}

Miriam Lipiski⁴

Henrik Teuber^{1,2}

Miriam Kalbitz⁷

Paolo Cinelli^{1,2,4}

Roman Pfeifer^{1,2}

Hans-Christoph Pape^{1,2}

TREAT Research Group

¹ Dept. of Traumatology, University Hospital Zurich, Zurich, Switzerland

² Harald Tscherne Laboratory for Orthopedic Research, Zurich, Switzerland

³ Dept. of Trauma- and Reconstructive surgery, University Clinic RWTH Aachen, Germany

⁴ Division of Surgical Research, University of Zurich, Zurich, Switzerland

⁵ Translational Cardiovascular Technologies, Dept. of Health Sciences, ETH Zürich, Switzerland

⁶ Dept. of Cardiothoracic and Vascular Surgery, German Heart Institute Berlin, Germany

⁷ Dept. of Trauma and Orthopedic Surgery, University Hospital Erlangen, Friedrich-Alexander-University Nürnberg (FAU), Erlangen, Germany

Abstract:

The local inflammatory impact of different reaming protocols in intramedullary nailing has been sparsely investigated. We examined the effect of different reaming protocols on fracture hematoma (FH) immunological characteristics in pigs. To do so, a standardized midshaft femur fracture was induced in adult male pigs. Fractures were treated with conventional reamed femoral nailing (group RFN, n=6); unreamed femoral nailing (group UFN, n=6); reaming with a reamer irrigator aspirator device (group RIA, n=12). Animals were observed for 6 hours and FH was collected. FH-cell apoptosis and neutrophil receptor expression (Mac-1/CD11b and FcγRIII/CD16) were studied by flow cytometry and local temperature changes were analyzed. The study demonstrates that apoptosis-rates of FH-immune cells were significantly lower in group RIA ($3.50 \pm 0.53\%$) when compared with non-RIA groups: (group UFN $12.50 \pm 5.22\%$, $p=0.028$ UFN vs. RIA), (group RFN $13.30 \pm 3.18\%$, $P<0.001$, RFN vs. RIA). Further, RIA-FH showed lower neutrophil CD11b/CD16 expression when compared with RFN (median difference of 43.0% median fluorescence intensity (MFI), $P=0.02$; and mean difference of 35.3% MFI, $P=0.04$, respectively). Finally, RIA induced a transient local hypothermia and hypothermia negatively correlated with both FH immune cell apoptosis and neutrophil activation.

In conclusion, immunologic changes observed in FH appear to be modified by certain reaming techniques. Irrigation during reaming was associated with transient local hypothermia, decreased apoptosis, and reduced neutrophil activation. Further study is warranted to examine whether the rinsing effect of RIA, specific tissue removal by reaming, or thermal effects predominantly determine local inflammatory changes during reaming.

Introduction

The constitution and quality of fracture hematoma (FH) plays an important role in fracture healing. Removal of FH or repeated early FH debridement have shown deleterious effects on fracture healing [1,2]. Intramedullary reaming can also modify FH, most likely due to changes in intramedullary blood flow, intramedullary pressure, and intramedullary temperature [3-6]. Kinetics of humoral factors caused by changes in FH, have been studied before. Likewise, the importance of both local and systemic cellular factors on bone repair, has previously been explored [7-11]. After injury, circulating immune cells promptly infiltrate the rapidly changing FH and contribute to the regulation of local growth and differentiation factors, which have been shown to regulate the fracture healing process. Within hours, polymorphonuclear neutrophils (PMNs) within the FH outnumber all other immune cells [7-9]. Some authors have correlated an excessive influx of activated neutrophils into FH with compromised fracture healing [11]. Conventional reaming versus application of the Reamer Irrigator Aspirator (RIA) has been linked with more sustained systemic inflammatory changes [6]. Further, an association between inflammatory changes and systemic temperature alterations early after fracture was shown [12,13]. Finally, our group has previously demonstrated that RIA is associated with less fat intravasation, decreased systemic immunologic changes and reduced sequelae in concomitant acute chest trauma [14,15,16]. However, it is unclear whether the irrigating effects may also have a measurable influence on local inflammation or immune cell composition.

Our group has previously used a standardized large animal model to 1) Assess changes of the circulating immune response [17], 2) Create a standardized mid shaft femoral fracture [18], 3) Modulate immunologic changes by inducing hypothermia [12,13] and 4) Measure regional changes in circulation and inflammation [19].

We utilized this standardized porcine femur fracture model to test the following hypotheses with respect to direct local effects of RIA on FH in addition to other known changes induced by RIA:

- i. *Application of rinsing techniques, (Reamer Irrigator Aspirator (RIA)) is associated with regional immunological changes in fracture hematoma.*
- ii. *Among other known changes induced by RIA (blood flow, removal of tissue) the effect on local temperature changes and associated immune cell responses can be effective.*

Methods

The current study was approved by the local Official Veterinary Office (Kanton Zürich, Gesundheitsdirektion Veterinäramt under project number: ZH138/17). All animal experiments were designed and carried out in accordance with the 'Guide for Care and Use of Laboratory Animals' [20]. Data processing and documentation has been performed in line with the ARRIVE guidelines for reporting animal research [21].

A standardized animal protocol was applied, as previously described [10,12,18]. Briefly, experiments were performed using 24 male Swiss Large White pigs (4 months old animals weighing 50 ± 5 kg). Prior to the start of the experiment, animals received pre-medication by intramuscular injection of ketamine (Ketasol®-100, Dr.E.Graeb AG, Berne, Switzerland) 15mg/kg, midazolam (Dormicum®, Roche Pharma (Schweiz) AG, Reinach, Switzerland) 0.5mg/kg and atropine (Atropin 1%, Kantonsapotheke Zurich, Switzerland) 0.05mg/kg. Then, general anesthesia was induced and maintained with a mixture of propofol (Propofol-® Lipuro, B.Braun Medical AG, Sempach, Switzerland; 5-10mg/kg/h CRI) and sufentanil forte (Sufenta® Forte, Janssen-Cilag AG, Zug, Switzerland; 0.01mg/kg/h CRI). After intubation (Bivona®, ID: 9mm, OD: 12.4mm, 37FR, Length: 56cm, Balloon: 5cm), a lung protective volume-controlled ventilation regime was applied. Ventilation settings were frequently optimized based on routine blood gas analysis and capnometry to target a $p\text{CO}_2$ of 35-45mmHg. Percutaneous placement of an arterial line, 2-lumen central venous catheter (HighFlow Dolphin Catheter, 13F, Baxter International, Deerfield IL, USA) and a suprapubic catheter was performed after intubation. Crystalloids (Ringerfundin 2 ml/kg BW/h) were administered continuously and hemodynamic and metabolic parameters were assessed at set time points. Then, all animals underwent standardized unilateral femoral fracture as previously described [10,12,18]. Briefly, a bolt gun machine (Blitz-Kerner, turbocut JOBB GmbH, Germany) and a custom-made metal plate were applied to produce a standardized midshaft transverse femur fracture. Fracture location and morphology was confirmed by fluoroscopy. In the first 90 minutes after trauma the pre-clinical phase was mimicked with altered ventilator settings, no temperature correction, reduced fluid infusion of 10ml/h, and FiO_2 reduction to ambient air oxygen level of 0.21.

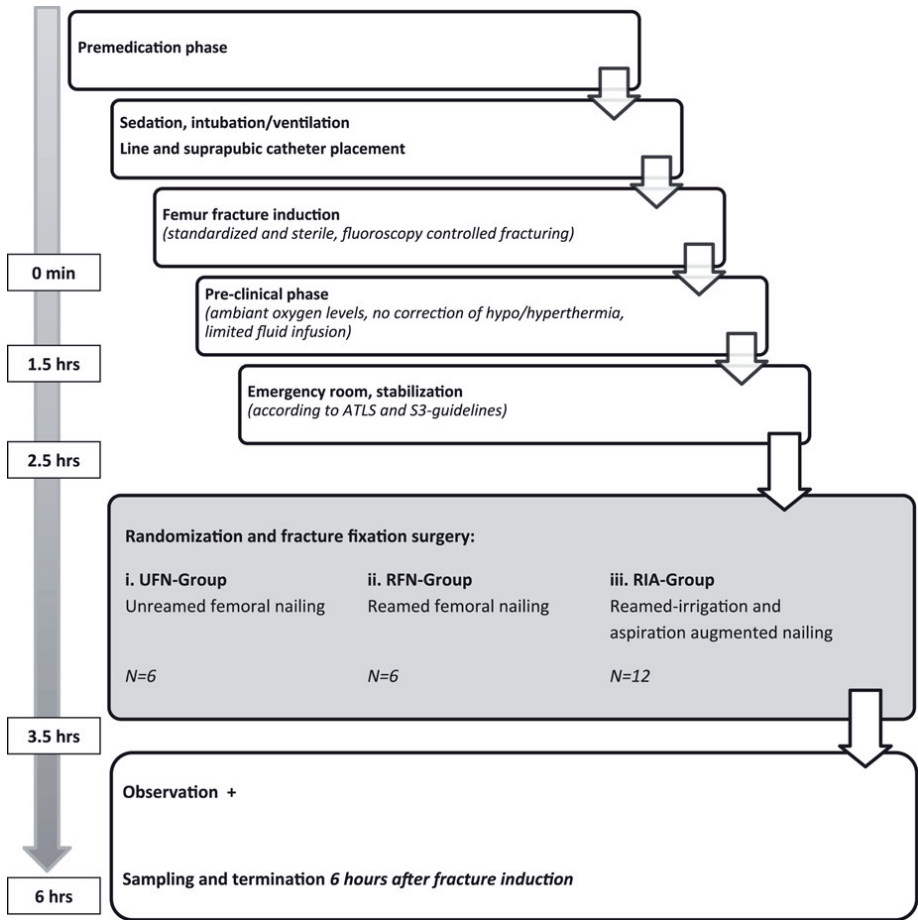
After circulatory stabilization, all 24 animals were randomly assigned to one of the following 3 study conditions:

- i. **unreamed femoral nailing/** (group UFN, $n=6$)
- ii. **reamed femoral nailing/** (group RFN, $n=6$) or,
- iii. **Reamer Irrigator Aspirator enhanced reamed femoral nailing/** (group RIA, $n=12$).

Surgical intervention

The standardized nailing system utilized a tailored 120 mm nail (cannulated DFN Ø 10.0 mm, DePuySynthes, Raynham, Massachusetts, United States). In animals randomized for RFN, standardized stepwise intramedullary reaming preceded nailing. The Synream intramedullary reaming system (DePuySynthes, Raynham, Massachusetts, United States) was utilized and reamer heads with a diameter up to 12 mm were used. In the RIA group, a Synthes Reamer/Irrigator/Aspirator System (DePuySynthes, Raynham, Massachusetts, United States) was used according to protocol. Again, reamer heads of 12 mm were utilized. Reamer heads were replaced after every 5 experiments in all study groups. Sequential reaming at intervals of 0.5 mm was performed. Final reaming with the 12 mm drill head was repeated twice. In order to optimize standardization of our study conditions, all fractures treated by a treatment modality including intramedullary nailing, have been reamed 10 times in total. The experimental design is presented as a flowchart in Figure 1.

Figure 1. Experimental design



Systemic body temperature regulation

Core temperature (T_{core}) was monitored by using an oesophageal temperature probe connected to a Datascope Passport2 Patient Monitoring System (Pacific Medical, Tracy, USA). Core temperature was corrected by: (1) controlling room temperature, (2) administering pre-heated fluid infusions, and (3) warming blanket adjustment (Bair Hugger, 3M, Saint Paul, Minnesota, USA). A local temperature probe (Electronic Precision Thermometer ama-digit ad 15th, Amarell GmbH & Co.KG, Kreuzwertheim, Germany) was placed near the fracture site (T_{tissue}). Standardized probe positioning was achieved by fluoroscopically guiding the probe one shaft diameter lateral to the fracture, a representative example is shown in supplement 3. During

surgery, temperature was measured continuously, and peak values (lowest and highest intra-operative temperatures) were compared between groups.

Termination and tissue collection

Animals were terminated after an observation period of 6 hours with a Na-pentobarbital (Esconarkon ad us. Vet., Streuli Pharma AG, Uznach, Switzerland) infusion. Prior to termination, samples of FH were collected.

Laboratory analysis and flow cytometry

FH-single cell solutions were made by flushing/crushing collected FH-material. Thereafter, FH samples were continuously kept on ice. The solution was centrifuged for 4 minutes at 1500 rpm at 4°C, and supernatants were taken up in RBC-lysing buffer (Biolegend, San Diego, USA). After lysis, FACS-buffer (phosphate buffered saline (PBS) enriched with 0.5% bovine serum albumin and 0.5mM EDTA) was added to the samples and two washing steps (4 minutes, 1500 rpm at 4°C) were performed. Thereafter, conjugated antibody mixes were added and allowed to incubate for 45 minutes. Of note, in the case of Annexin-V analysis, solutions were resuspended in Annexin-V-Binding Buffer (Abcam, Cambridge, UK) prior to antibody incubation. The following antibodies were utilized: CD11b (Clone 2F/11, AbDSerotec, Kidlington, UK), CD16 (Clone G7, AbDSerotec, Kidlington, UK), and CD45 (Clone K252.1E4, AbDSerotec, Kidlington, UK). After incubation, two additional washing steps were performed and solutions were directly analyzed by flow cytometry. A standardized Annexin-V staining protocol was utilized to test for cell apoptosis (Abcam, Cambridge, UK). A Canto II-device (Becton & Dickinson, Mountain View, CA, USA) and FACS Diva Software (Becton & Dickinson, Mountain View, CA, USA) were used.

CD11b (Mac-1) is an integrin family member. Functionally CD11b regulates neutrophil adhesion and migration. Moreover, CD11b is a traditional neutrophil-activation marker and cell activation is characterized by upregulation of this integrin in both *in vitro* and *in vivo* studies [22-24].

CD16 (FcγRIII) is an important and well-described Fcγ-receptor. This receptor binds to immunoglobulins (IgG) either in aggregates or attached to pathogens. Binding of IgG to Fcγ receptors promotes the oxidative burst and induces phagocytosis. [25] Short term *in vitro* activation of neutrophils by lipopolysaccharide is associated with an initial increase in neutrophil CD16-expression [26]. Neutrophil FcγRII-receptor expression increases during cell maturation, with young neutrophils typically having relatively low CD16-cell surface

expression compared to older counterparts [27,28].

CD45 is a pan-leukocyte marker and is utilized to identify immune cells in blood and the tissue compartment [29].

FlowJo (Becton & Dickinson, Mountain View, CA, USA) was utilized for the evaluation of flow cytometry data and the gating strategy utilized is summarized in Supplement 1. Leukocyte subtype identification is based on forward-sideward scatter gating and CD45-expression levels. This protocol has previously been validated in pilot experiments using both porcine blood and hematoma samples. Differential cell counts were obtained with cytopspin-prepared slides stained with May-Grünwald-Giemsa.

Sample size calculation

Sample size calculations were based on tissue neutrophil presence after intervention. In a previous study on pulmonary neutrophil migration after polytrauma performed by this research group, 12 animals were exposed to polytrauma while 6 animals were included in a sham control group. Neutrophil presence in lung tissue in the intervention group ($n=12$) was determined as 2.38 (SD: 0.76) cells per high power field, whereas 0.98 (SD 0.2) cells/high power field were counted in the sham group ($n=6$). This results in power of 98% if the sample size in two groups is 4 at a 5% significance level [30]. However, the number of animals and the allocation ratio of 2:1:1 in the current study was based primarily on logistic and ethical considerations instead of a formal a-priori sample size calculation. Sample sizes of 12 and 6 in the groups were used to avoid an underpowered study protocol.

Statistical analysis

All statistical analysis were performed using GraphPad Prism Version 7 (San Diego, United States). Unpaired T-tests (normally distributed datasets) or Mann-Whitney U tests (non-parametrical analysis) were performed. Correlation analysis and plotting was performed with Excel (Microsoft, Redmond, USA) and a web-based Pearson Correlation Coefficient Calculator, retrieved from Social Science Statistics at www.socscistatistics.com. A P -value < 0.05 was considered statistically significant. All data are presented as standard error of the mean (SEM).

Results

All 24 animals survived the observation period. No intra- or postoperative complications were observed.

Fracture hematoma immune cell subpopulation composition

Flow analysis demonstrated no differences in FH-white blood cell subpopulation composition between the different study groups (Supplement 2). Overall, the majority (63.12 ± 2.72 %) of isolated fracture hematoma leukocytes (defined as all nucleated/CD45⁺-cells) were identified as granulocytes. Additional morphological analysis demonstrated that among the granulocytes, polymorphonuclear neutrophils (PMNs) were the most prevalent FH immune cell type. More specifically, 61.23 ± 2.18 percent of FH-white blood cells were identified as PMNs. The second most predominating type of leukocyte in FH were lymphocytes (32.01 ± 2.75 percent of all nucleated CD45⁺-cells). Finally, 1.85 ± 0.37 percent of FH-immune cells showed morphology characteristic of eosinophils, while 4.21 ± 0.98 percent of cells were monocytes/macrophages and less than 1 percent of FH-immune cells were basophils.

Fracture hematoma immune cell apoptosis rates

FH-immune cell apoptosis rates were significantly lower in the RIA group (3.50 ± 0.53 %/ FH-CD45⁺ Annexin-V⁺ cells) than in the UFN and RFN groups (12.50 ± 5.22 % Annexin-V⁺ / FH-CD45⁺ cells, $P=0.028$ and 13.30 ± 3.18 % Annexin-V⁺ / FH-CD45⁺ cells, $P<0.001$, respectively). Of note, immune cell apoptosis rates did not differ between the UFN and RFN groups ($p=0.90$). Apoptosis rates are displayed in Figure 2.

Figure 2. The impact of reaming strategy on early immune cell apoptosis in fracture hematoma

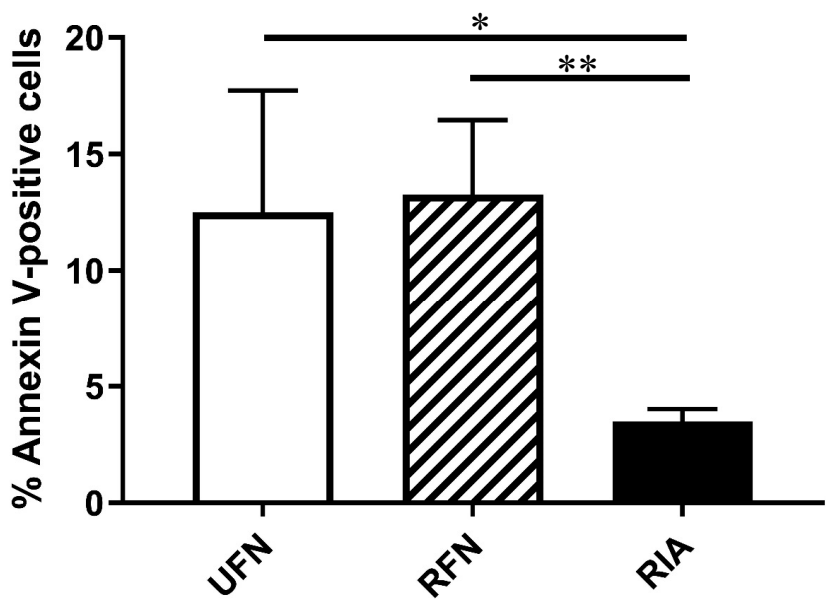


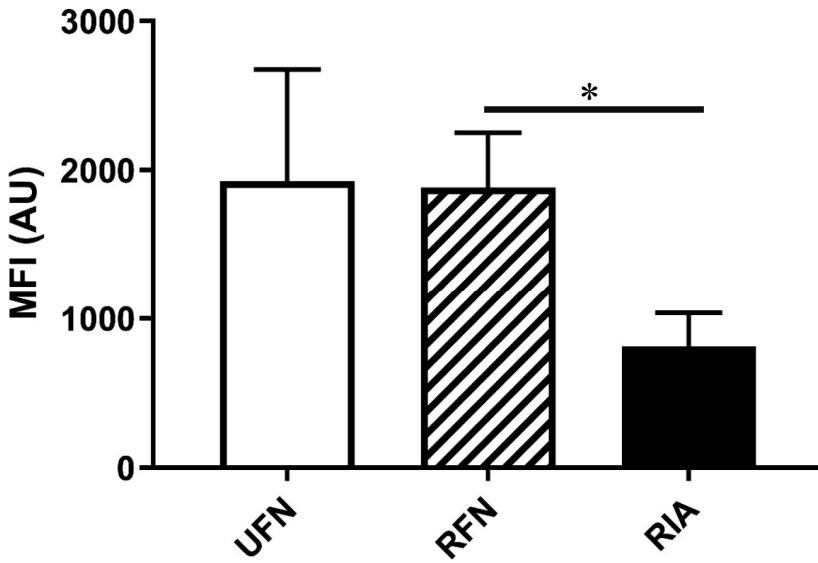
Fig. 2. This figure displays the impact of reaming strategy on early immune cell apoptosis in fracture hematoma. Immune cell hypothesis was determined 6-hrs after insult. Bars represent the percentage (mean ± SEM) of Annexin-V-positive immune cells following different reaming protocols. *, $P < 0.05$; **, $P < 0.01$.

Neutrophil Mac-1 (CD11b) and FcγRIII-receptor (CD16) expression profiles of FH-PMNs

Mac-1 cell surface expression levels on FH-PMNs were significantly lower in the RIA group compared with the RFN group ($p=0.02$). In addition, FcγRIII-expression in FH-PMNs was also significantly lower in the RIA group compared to the RFN group ($p=0.04$). FH-PMN cell surface receptor expression levels observed in the different study groups are summarized in Figure 3.

Figure 3. Neutrophil cell surface expression patterns of Mac-1 and FcγRIII in early fracture hematoma following different reaming protocols

(a) Mac-1 (CD11b) cell surface expression on fracture hematoma neutrophils



(b) FcγRIII (CD16) cell surface expression on fracture hematoma neutrophils

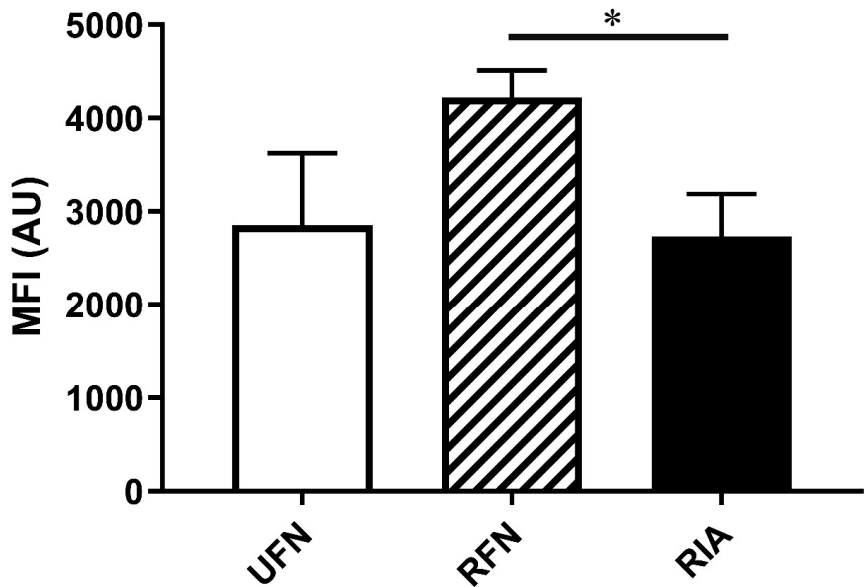


Fig. 3.
Fig 3a. This figure displays differences in neutrophil Mac-1 cell/CD11b surface expression on fracture hematoma neutrophils following different reaming protocols. Bars represent the median fluorescent intensity (MFI) in arbitrary units (AU) of Mac-1 on neutrophils in FH determined by flow analysis per study conditions. Study groups have been compared. *, P < 0.05.

Fig 3b. This figure displays differences in neutrophil FcγRIII cell/CD16 surface expression on fracture hematoma neutrophils following different reaming protocols. Bars represent the median fluorescent intensity (MFI) in arbitrary units (AU) of Mac-1 on neutrophils in FH determined by flow analysis per study conditions. Study groups have been compared. *, P < 0.05.

In vivo fracture site temperatures

With comparable baseline temperature levels, fracture site temperatures during surgery were lower in the RIA-group (mean $T_{\text{tissue/min}}$: $34.5 \pm 0.3^{\circ}\text{C}$) versus the UFN group ($35.7 \pm 0.2^{\circ}\text{C}$, $P=0.0194$) and the RFN group ($36.1 \pm 0.3^{\circ}\text{C}$, $P=0.0026$).

Higher intra-operative mean peak temperatures were encountered in the RFN group ($37.2 \pm 0.4^{\circ}\text{C}$) vs. the UFN group ($35.8 \pm 0.2^{\circ}\text{C}$, $P=0.0194$) and RIA-reamed group ($35.3 \pm 0.397^{\circ}\text{C}$, $P=0.0079$). Temperature changes are shown in Table 1.

Table 1. Differences in local temperature alterations during various fracture fixation strategies

<i>Condition:</i>	T_{core} :baseline	T_{tissue} :baseline	T_{tissue} :intra- operative min	T_{tissue} :intra- operative max	$T_{\text{coefficient}}$:intra- operative
<i>UFN</i>	37.4 (SEM 0.230)	35.8 (SEM 0.181)	35.7 (SEM 0.227) [§]	35.8 (SEM 0.150) ^{**}	+0.020 (SEM 0.073) ^{§,**}
<i>RFN</i>	37.0 (SEM 0.193)	36.1 (SEM 0.270)	36.1 (SEM 0.262) [*]	37.2 (SEM 0.428) ^{***}	+1.15 (SEM 0.339) ^{*,**}
<i>RIA-nailing</i>	37.4 (SEM 0.165)	35.2 (SEM 0.083)	34.5 (SEM 0.282) ^{§,*}	35.3 (SEM 0.397) [*]	-0.890 (SEM 0.179) ^{§,*}

Differences in core temperature at baseline (T_{core}), local tissue temperature at the fracture site prior to surgery (T_{tissue}), lowest tissue temperature during fracture fixation ($T_{\text{tissue/min}}$), peak tissue temperature during fracture fixation ($T_{\text{tissue/max}}$) and the temperature coefficient ($T_{\text{coefficient}}$).

[§] $P < 0.05$ RIA-enhanced reaming vs. UFN

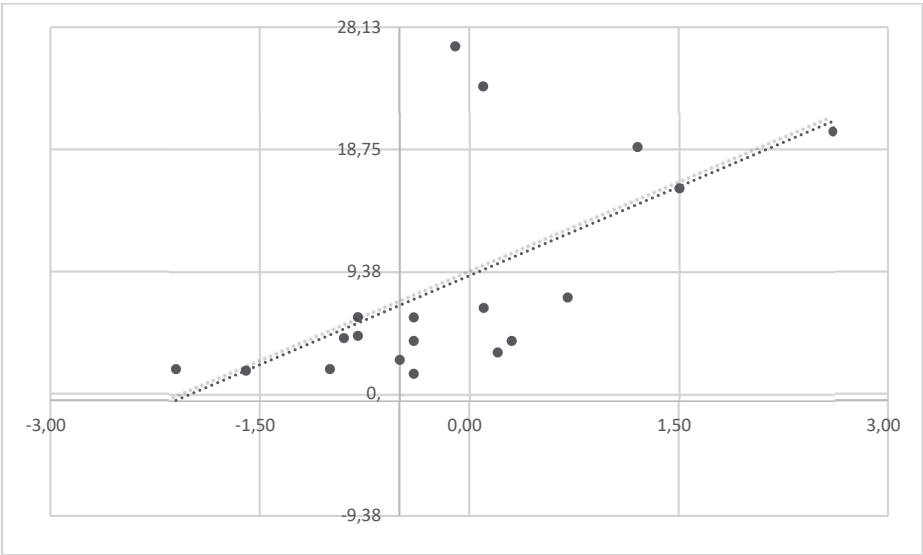
^{*} $P < 0.05$ RIA-enhanced reaming vs. RFN

^{**} $P < 0.05$ UFN vs. RFN

As shown in Figure 4, intra-operative temperature and early FH immune cell apoptosis were strongly correlated ($r(17) = .61$, $p = .006$), while neutrophil activation in early FH moderately correlated with local tissue temperature ($r(17) = .53$, $p = .02$).

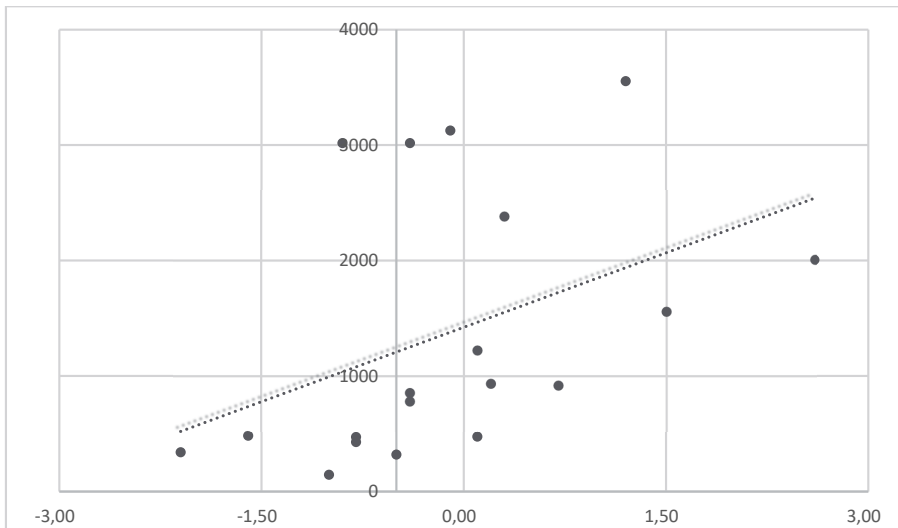
Figure 4.

(a) Correlation between local thermal changes during fracture fixation surgery and fracture hematoma immune cell apoptosis



R-score = 0.61
R² = 0.38
N = 19
P-value = 0.006

(b) Correlation between local thermal changes during fracture fixation surgery and FH-neutrophil activation (reflected by Mac-1 expression)



R-score = 0.53

R² = 0.28

N = 19

P-value = 0.02

Fig. 4.

Fig 4a. This figure demonstrates the correlation between local thermal changes during fracture fixation surgery and fracture hematoma immune cell apoptosis. *y-axis*: percentage of early fracture hematoma immune cell apoptosis in FH as measured by percentage of Annexin-V positive immune cells; *x-axis*: temperature coefficient (in °C). All dots represent individual samples.

Fig 4b. This figure demonstrates the correlation between local thermal changes during fracture fixation surgery and fracture hematoma neutrophil activation. FH-neutrophil activation is determined by neutrophil CD11b cell surface expression levels at termination. *y-axis*: neutrophil CD11b/Mac-1 expression in early fracture hematoma; *x-axis*: temperature coefficient (in °C). All dots represent individual samples.

Discussion

Studies have shown that fracture-healing success is driven largely by the early inflammatory stages after injury. This inflammatory phase sets the stage for the second phase of fracture healing, the repair process [1,2,31]. Hauser et al. have demonstrated that 4-hour FH is rich in activated monocytes, neutrophils and soluble cytokines such as interleukin 6/8 and 10 [7]. In contrast to the local humoral immune response at the fracture site [7,8,10], the regional cellular immune response is still poorly understood. The current study is the first to describe fundamental characteristics of FH immune cell content. Furthermore, this study describes the impact of different reaming strategies on FH immune cell homeostasis and their potential interplay with local temperature changes during surgery.

Excessive systemic immune activation precedes inadequate bone healing by altering the immunological composition of early FH [32-35]. Specifically, studies suggest that excessive influx of activated neutrophils into FH is associated with inadequate fracture healing [11]. Despite varying reaming strategies, PMN levels in FH were consistent across all groups in the current study. Interestingly, however, while no quantitative differences in immune cell composition were found, prominent variations in FH neutrophil activation status was encountered between groups.

Based on this revelation, the key findings in this study may provide relevant new information:

1. Neutrophils were the most prevalent FH immune cells and the composition of immune cell subtypes was unaffected by reaming strategy in nail fixation.
2. RIA was associated with diminished FH immune cell apoptosis compared with unreamed and reamed femoral nailing. Further, RIA was associated with lower FH-neutrophil activation compared with reamed femoral nailing.
3. RIA induced local hypothermia at the fracture site, and negatively correlated with both FH immune cell apoptosis and neutrophil pool activation.

Sheep experiments have shown that leukocyte counts in early FH start to rise immediately after fracture induction. The percentage of FH-myeloid cells also increases immediately after injury, with FH concentrations equaling circulatory levels within 4 hours. During this time period,

however, the proportion of non-viable leukocytes in FH also increases [8,9,10]. The current investigation is the first to demonstrate that *fracture fixation strategy*, or more specifically, reaming strategy, influences white blood cell apoptosis in FH. Specifically, reamed-irrigation augmented nailing is associated with less early cell apoptosis compared with unreamed and stepwise reaming protocols.

The current study demonstrated, in line with other animal [7] and human trauma studies [36], that neutrophils are initially the most prevalent immune cells in early FH. This study, further reveals that the percentage of myeloid cells (including neutrophils) in 6-hour fracture hematoma was not affected by reaming strategy. Interestingly, however, RIA-enhanced reaming was associated with diminished FH neutrophil activation, demonstrated by lower levels of PMN-CD11b surface expression [37,38], compared with the other study groups. It is tempting to hypothesize that irrigation/aspiration induces less profound regional immune cell activation than alternative reaming methods. Knowledge about which specific characteristics of the RIA-technique modulate early FH immune cell response is important as it could guide development of future reaming strategies.

Since this study demonstrated a correlation between *in vivo* local temperature and local neutrophil activation, it is tempting to speculate that observed differences in PMN-activation levels between the study groups may have primarily been a function of thermal differences between the reaming strategies. These findings are in line with *in vitro* studies that assessed neutrophil activation in the field of pediatric extracorporeal circuits and cardiac bypass surgery in which cooling procedures decreased neutrophil CD11b expression [39]. Furthermore, *in vitro* experiments evaluating different neutrophil preservation strategies, showed that PMNs at 37°C vs. 4°C exhibited a 2.5-fold increase in formyl-methionyl-leucyl-phenylalanine (FMLP)-receptor expression. In addition, increased cellular functional activity, demonstrated by increased levels of FMLP-induced superoxide generation, was seen as well [40]. Human PMNs heated to temperatures above 37°C exhibited higher levels of reactive oxygen intermediates release upon *in vitro* lipopolysaccharide stimulation compared with PMNs stored at lower temperatures [41].

The importance of thermal regulation on local immune cell homeostasis has previously been posited by our group based on data from a standardized hypothermia model in the setting of porcine polytrauma that was associated with reduced hepatic granulocyte infiltration compared with normothermia [13]. However, experimentally induced hypothermia in a long-term porcine

model of combined trauma led to prolonged (48 hours) enhancement of systemic and remote humoral parameters (including high mobility group box 1 and interleukin-6) compared with normothermic controls [12].

A correlation between thermal changes and FH-immune cell apoptosis was also observed. It has previously been demonstrated that relatively moderate temperature changes within febrile temperature ranges trigger neutrophilic cell apoptosis. Within just fifteen minutes of culturing neutrophils at 39.5°C, Caspase-8 reaches peak levels. Interestingly, Caspase-8 inhibition protects cells from heat-induced apoptosis [42]. Further, *in vitro* studies have demonstrated that mild hypothermia protects mammalian cells from apoptosis induced by various stimuli [43].

Reamer head-design is an important factor in local heat production during reaming. Utilization of a large and blunt reamer has been shown to exacerbate local temperature elevation [44,45]. Given the increased local temperatures observed during conventional reaming in this study, temperature likely contributed to increased FH immune cell apoptosis in the RFN conventional reaming group, while RIA-induced transient local hypothermia induced immune cell preservation.

Further, conventional RFN was associated with enhanced FH-neutrophil CD16-receptor expression, which is considered an essential receptor in the phagocytic process [46]. Decreased PMN-CD16-expression after RIA may be due to the influx of immature band neutrophils from the bone marrow [47,48]. Further deciphering CD16 expression in long bone reaming is certainly an area of future study. Additionally, future studies should more specifically investigate the impact of local hypothermia on FH neutrophil homeostasis, e.g. through varying cooling protocols during reaming.

These study results must, however, be qualified considering several possible limitations. First, this experiment focused on the early cellular immune reaction at the fracture site, which is dominated by neutrophils. Long-term effects of different reaming protocols were therefore not investigated. Furthermore, to avoid artificial manipulation at the fracture site, local temperature probes were not inserted directly into the fracture but were fluoroscopically guided to a standardized position near the fracture site. As a result, FH temperature measurements in this study were indirect, but consistent approximations across all study groups. Further, as humoral immune characteristics of FH have been studied in detail before [1,7,10,12], we decided to focus on immune effector cells only. Therefore, we were unable to determine the interplay

between early humoral and cellular immune response in FH. For the current large-animal study, 4-month-old male animals were used. Four-month-old pigs are considered young adults. These animals at this age therefore represent the most common patient group with transverse mid-shaft femur fractures. To avoid the potential confounding effect of hormonal factors, male animals were chosen. Therefore, caution should be taken in extrapolating our experimental findings to other trauma subgroups (e.g. women/geriatric patients).

In conclusion, this standardized porcine study appears to suggest that RIA induced transient local hypothermia in bone and soft tissues may reduce early immune cell apoptosis and decrease neutrophil activation in FH. The current study further revealed that FH immune cell composition is not affected by reaming strategy. These findings inspire further investigation and should serve as a foundation for designing new, enhanced reaming protocols which may ultimately help optimize fracture healing.

References

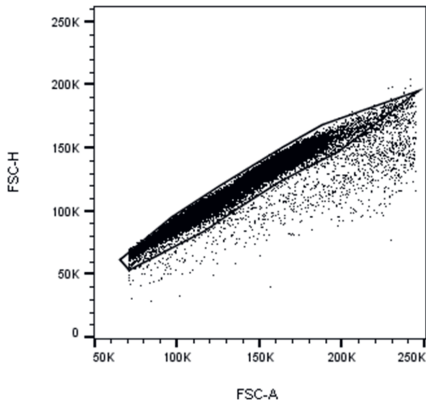
1. Kolar P, Schmidt-Bleek K, Schell H, et al. 2010. The early fracture hematoma and its potential role in fracture healing. *Tissue Eng Part B Rev* 16(4):427-434.
2. Grundnes O, Reikeras O. 2010. The importance of the hematoma for fracture healing in rats. *Acta Orthop Scand*. 64(3):340-342.
3. Pfeifer R, Sellei R, Pape HC. 2010. The biology of intramedullary reaming. *Injury* 41S2:S4-8.
4. Giannoudis PV, Snowden S, Matthews SJ, et al. 2002. Temperature rise during reamed tibial nailing. *CORR* 395:255-261.
5. Mueller CA, Rahn BA. 2003. Intramedullary pressure increase and increase in cortical temperature during reaming of the femoral medullary cavity: the effect of draining the medullary contents before reaming. *J Trauma* 55(3):495-503.
6. Hartsock LA, Barfield WR, Kokko KP. 2010. Randomized prospective clinical trial comparing reamer irrigator aspirator (RIA) to standard reaming (SR) in both minimally injured and multiply injured patients with closed femoral shaft fractures treated with reamed intramedullary nailing (IMN). *Injury* 52:S94-98.
7. Hauser CJ, Zhou X, Joshi P, et al. 1997. The immune microenvironment of human fracture/soft-tissue hematomas and its relationship to systemic immunity. *J Trauma* 42(5):895-903.
8. Schmidt-Bleek K, Schenll H, Kolar P, et al. 2009. Cellular composition of the initial fracture hematoma compared to a muscle hematoma: a study in sheep. *J Orthop Res* 27(9):1147-1151.
9. Chung R, Cool JC, Scherer MA, et al. 2006. Roles of neutrophil-mediated inflammatory response in the bony repair of injured growth plate cartilage in young rats. *J Leukoc Biol*. 80(6):1272-1280.
10. Horst K, Greven J, Lüken H, et al. 2019. Trauma severity and its impact on local inflammation in extremity injury- insights from a combined trauma model in pigs. *Front Immun*. 10:3028.
11. Bastian O, Pillay J, Alblas J, et al. 2011. Systemic inflammation and fracture healing. *J Leukoc Biol*. 89(5):669–673.
12. Horst K, Eschbach D, Pfeifer R, et al. 2016. Long-term effects of induced hypothermia on local and systemic inflammation – results from a porcine long-term trauma model. *PLoS One* 11(5):e0154788.
13. Fröhlich M, Hildebrand F, Weuster M, et al. 2014. Induced hypothermia reduces the hepatic inflammatory response in a swine multiple trauma model. *J Trauma* 76(6):1425-1432.
14. Pfeifer R, Kobbe P, Knobe M, Pape HC. 2011. The reamer-irrigator-aspirator (RIA) System. *Open Orthop Traumatol*. 23(5):446-452.
15. Pape HP, Zelle BA, Hildebrand F, et al. 2005. Reamed Femoral Nailing in Sheep: Does Irrigation and Aspiration of Intramedullary Contents Alter the Systemic Response?, *JBJS*. 87:2515-2522.
16. Halvachizadeh S, Teuben M, Lempert M, et al. (2021) Protective effects of new femoral reaming techniques (Reamer irrigator aspirator, RIA I and II) on pulmonary function and posttraumatic contusion (CT morphology) - results from a standardized large animal model. *Injury* 52(1):26-31.

17. Horst K, Eschbach D, Pfeifer R, et al. 2016. Long-Term Effects of Induced Hypothermia on Local and Systemic Inflammation - Results from a Porcine Long-Term Trauma Model. *PLoS One* 11(5):e0154788.
18. Horst K, Simon TP, Pfeifer R, et al. 2016. Characterization of blunt chest trauma in a long-term porcine model of severe multiple trauma. *Sci Rep.* 6:39659.
19. Kalbas Y, Qiao Z, Horst K, et al. 2018. Early local microcirculation is improved after intramedullary nailing in comparison to external fixation in a porcine model with a femur fracture. *Eur J Trauma Emerg Surg.* 44(5):689-696.
20. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. *Guide for the Care and Use of Laboratory Animals.* 8th ed. Washington, DC: National Academ. Press. 2011.
21. Kilkenney C, Browne WJ, Cuthill IC, et al. 2010. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* 8:e1000412.
22. Neeley SP, Hamann KJ, White SR, et al. 1993. Selective regulation of expression of surface adhesion molecules Mac-1, L-selectin, and VLA-4 on human eosinophils and neutrophils. *Am J Respir Cell Mol Biol*;8(6):633-9
23. Ley K. 2002. Integration of inflammatory signals by rolling neutrophils. *Immunol Rev*; 186:8-18.
24. Griensven van M, Krettek C, Pape HC. 2003. Immune reactions after trauma. *Eur J Trauma*;29:181-92.
25. Huizinga TW, Roos D, von dem Borne AE. 1990. Neutrophil Fc-gamma receptors: a two-way bridge in the immune system. *Blood*;75(6):1211-4
26. Wagner C, Deppisch R, Deneffle B, et al. 2003. Expression patterns of the lipopolysaccharide receptor CD14, and the Fc-gamma receptors CD16 and CD64 on polymorphonuclear neutrophils: data from patients with severe bacterial infections and lipopolysaccharide-exposed cells. *Shock*; 19(1):5-12.
27. Orr Y, Taylor JM, Bannon PG, et al. 2005. CD10-/CD16low neutrophils provide a quantitative index of active bone marrow neutrophil release. *Br J Haematol*;131(4):508-19.
28. Drifte G, Dunn-Siegrist I, Tissières P, et al. 2013. Innate immune functions of immature neutrophils in patients with sepsis and severe systemic inflammatory response syndrome. *Crit Care Med.*;41(3):820-32.
29. Lacombe F, Durrieu F, Briaux A, et al. 1997. Flow cytometry CD45 gating for immunophenotyping of acute myeloid leukemia. *Leukemia.* 1997;11(11):1878-86.
30. Dhand NK, Khatkar MS. 2014. Statulator: An online statistical calculator. Sample Size Calculator for Comparing Two Independent Means. <http://statulator.com/SampleSize/ss2M.html>
31. Claes L, Recknagel S, Ignatius A. 2012. Fracture healing under healthy and inflammatory conditions. *Nat Rev Rheumatol.* 8(3):133-143.
32. Bastian OW, Kuijer A, Koenderman L, et al. 2016. Impaired bone healing in multitrauma patients is associated with altered leukocyte kinetics after major trauma. *J Inflamm Res.* 9:69–78.
33. Bastian OW, Croes M, Alblas J, et al. 2018. Neutrophils inhibit synthesis of mineralized extracellular matrix by human bone-marrow derived stromal cells in vitro. *Front Immunol.* 9: 945.

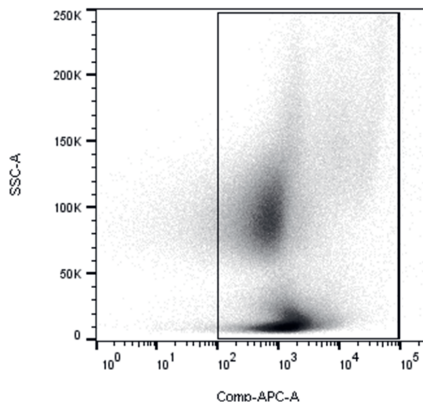
34. Reikerås O, Shegarfi H, Wang JE, et al. 2005. Lipopolysaccharide impairs fracture healing: an experimental study in rats. *Acta Orth.* 76:749–753.
35. Recknagel S, Bindl R, Brochhausen C, et al. 2013. Systemic inflammation induced by a thoracic trauma alters the cellular composition of the early fracture callus. *J Trauma* 74(2):531–537.
36. Bastian OW, Koenderman L, Alblas J, et al. 2016. Neutrophils contribute to fracture healing by synthesizing fibronectin+ extracellular matrix rapidly after injury. *Clin Immun.* 164:78-84.
37. Neeley SP, Hamaan KJ, White SR, et al. 1993. Selective regulation of expression of surface adhesion molecules Mac-1, L-selectin, and VLA-4 on human eosinophils and neutrophils. *Am J Respir Cell Mol Biol.* 8:633-639.
38. Wagner JG, Roth RA. 1999. Neutrophil migration during endotoxemia. *J Leukoc Biol.* 66:10-24.
39. El Habbal MH, Carter H, Smith LJ, et al. 1995. Neutrophil activation in paediatric extracorporeal circuits: effect of circulation and temperature variation. *Cardiovasc Res.* 29:102-107.
40. Tennenberg D, Zemlan FP, Solomkin JS. 1988. Characterization of N-formyl-methionyl-leucyl-phenylalanine receptors on human neutrophils. Effects of isolation and temperature on receptor expression and functional activity. *J Immunol.* 141(11):3937-3944.
41. Rosenspire AJ, Kindzelskii AL, Petty HR. 2002. Cutting edge: Fever-associated temperatures enhance neutrophil responses to lipopolysaccharide: A potential mechanism involving cell metabolism. *J Immunol.* 169:5396–5400.
42. Nagarsekar A, Greenberg RS, Shah NG, et al. 2008. Febrile-range hyperthermia accelerates Caspase-Dependent Apoptosis in Human Neutrophils. *J Immunol.* 181(4):2636-2643.
43. Sakurai T, Itoh K, Liu Y, et al. 2005. Low temperature protects mammalian cells from apoptosis initiated by various stimuli in vitro. *Exp Cell Res.* 309(2):264-272.
44. Hupel TM, Aksenov SA, Schemitsch EH. 1998. Muscle perfusion after intramedullary nailing of the canine tibia. *J Trauma* 45(2):256-262.
45. Wozasek GE, Simon P, Redl H, et al. 1994. Intramedullary pressure changes and fat intravasation during intramedullary nailing: an experimental study in sheep. *J Trauma.* 36(2):202-207.
46. Buckley CD, Ross EA, McGettrick HM, et al. 2006. Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. *J Leuk Biol.* 79:303–311.
47. Drifte G, Dunn-Siegrist I, Tissièrès P, et al. 2013. Innate immune functions of immature neutrophils in patients with sepsis and severe systemic inflammatory response syndrome. *Critical Care Med.* 41:820–832.
48. Leliefeld PH, Wessels CM, Leenen LP, et al. 2016. The role of neutrophils in immune dysfunction during severe inflammation. *Crit Care.* 20:73.

Supplement 1. Multistep gating strategy of porcine fracture hematoma leukocyte subtypes

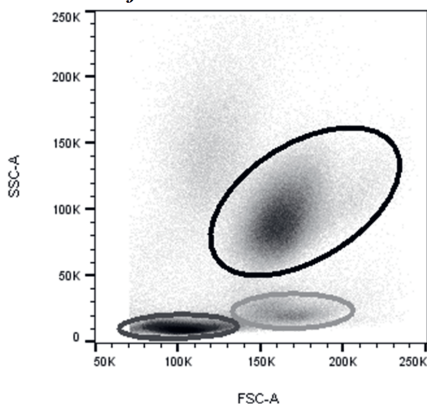
1. Singlet gating: *exclusion of doublets*



2. CD45+/SSC-gating of FH-immune cells: *purification of immune cells*

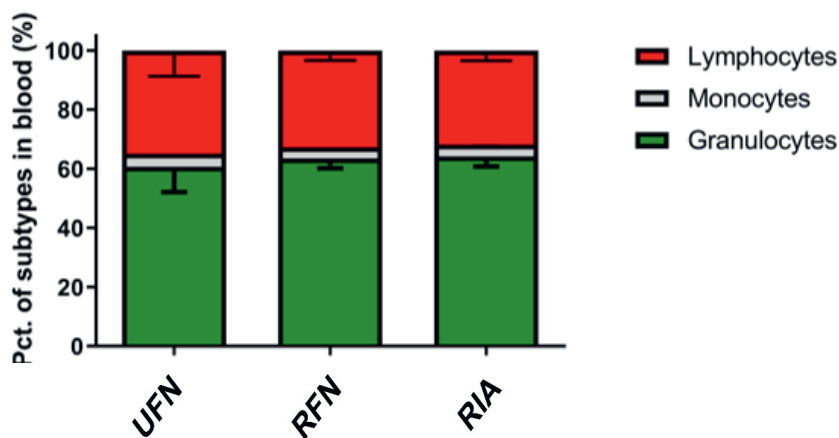


3. FSC/SSC-gating of specific leukocyte subpopulations: *leukocyte subtype identification*



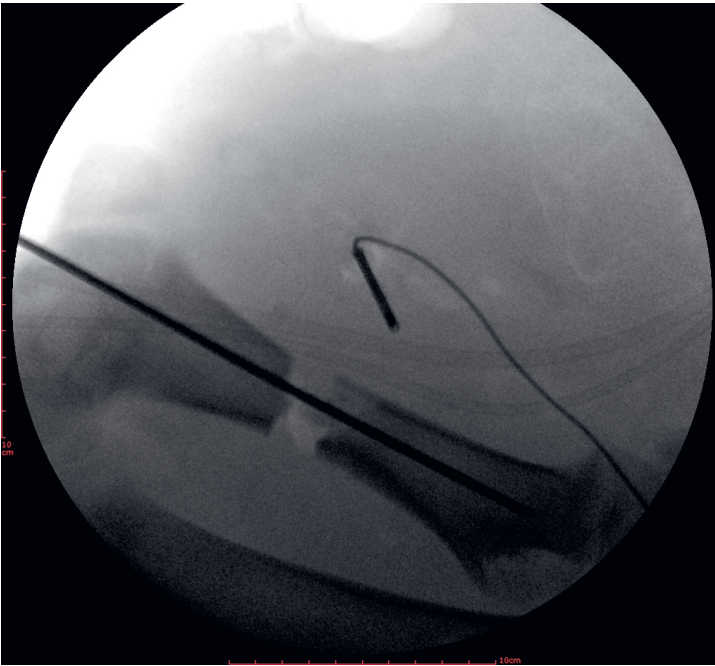
Granulocytes
Monocytes/macrophages
Lymphocytes

Supplement 2: Early fracture hematoma white blood cell content determined by morphological analysis of cytological samples



Supplement 2. Granulocytes were the most prominent immune cell type in early fracture hematoma. Furthermore, the immune cell composition of fracture hematoma was not affected by reaming protocol.

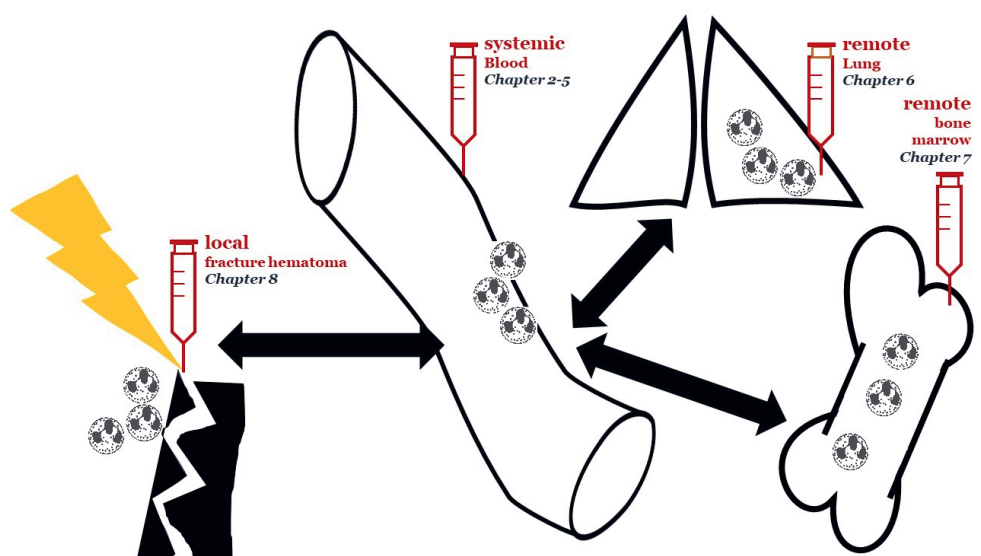
Supplement 3. Representative example of fluorescence-guided standardized temperature probe positioning



Supplement 3. A representative intra-operative imaging with temperature positioning and reamer introduction in a midshaft femur fracture.

Chapter 9

General discussion



GENERAL DISCUSSION

Tissue damage activates the immune system and leads to local immune activation. Clinically this is characterized by *rubor* (redness), *calor* (increased heat), *tumor* (swelling), *dolor* (pain) and *functio leasa* [1]. In the case of polytrauma, multiple triggers, such as fractures, organ injury, burns, hypovolemic shock and infection may cause tissue damage and subsequent inflammation. Furthermore, medical treatment, paradoxically, may cause additional tissue damage and immune activation as well. Procedures such as intubation/mechanical ventilation, blood transfusion, drugs and surgical intervention have the potency to boost immune responses and thereby unintentionally lead to iatrogenic collateral damage to parenchymal organ cells [2]. Inadequate orchestration of post-traumatic immune responses, play an important role in the development of major complications after injuries such as infections, inadequate wound/fracture healing and single or multiple organ dysfunction syndrome (MODS) [3-5].

In general, the development of MODS follows a specific sequence of events with consecutive failure of these organs: lungs, liver, intestines, and kidneys [6]. The lungs tend to fail first and are involved most frequently in the critically ill. Pulmonary failure is described as acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). Clinical features include bilateral infiltrates on imaging without signs of hypertension or cardiac failure with acute onset. ALI and ARDS are discriminated by differences in respiratory parameters. More specifically, ALI is defined as a $\text{PaO}_2/\text{FiO}_2 < 300$ mmHg with $\text{PEEP} > 6$ cm H₂O and ARDS is a subset of ALI more profound hypoxemia reflected by a $\text{PaO}_2/\text{FiO}_2 < 200$ mmHg with $\text{PEEP} > 6$ cm H₂O [7]. Specific ventilation protocols aimed to preserve respiratory function in the critically ill have been developed and rolled out over time. Unfortunately, the mortality rate of pulmonary failure after trauma did not improve over the last four decades and is approximately 20% [8].

The immune barrier and local immune responses in injury

The body's immune response forms an essential protection mechanism against harmful agents. Different levels of defence have been described. Mechanical barriers, such as in the skin and gut. Here, well-organized layers with specific protective properties prevent pathogens to enter the body. In addition, there are (dynamic) biological immune components. These are complementary to (static) mechanic immune defence layers and can be divided into humoral and cellular elements. Humoral factors are involved in the orchestration of immune responses by influencing cellular signalling processes and the modification of biological pathways. Immune effector cells, however, eventually fight pathogens and release chemical immune factors. Thereby immune cells form the endpoint of immune cascades. Immune cells are divided into innate and adaptive cells. Innate immune cells highly outnumber adaptive cells. These cells are the first line of defence against invaders and also play a role in the activation of adaptive immune reactions [9-11]. Hence, the innate immune system, rather than the adaptive system, determines the overall success of the body's attempt to prevent the invasion of potentially harmful pathogens and to rapidly wipe out pathogens after tissue damage in trauma [10,11]. The interplay between static and dynamic immune elements determines the course of the local immune response and the success of systemic immune surveillance. The immune system also affects other relevant biological processes upon trauma such as wound and fracture healing. Moreover, the local immune response to tissue damage, modifies distant immune reactions and regulation [9-11].

Transition from local to systemic inflammation

Following minor injuries, such as a cut in the skin, local immune resources are sufficient to achieve uncomplicated healing. In the case of multiple or more extensive injuries, a transition from local into systemic inflammation occurs. This transition is initiated by endogenous local mediators such as high-mobility group box 1 protein (HMGB1) [12], Heat Shock Protein, complement factors [13], cytokines and local innate immune cells. Interleukin (IL)-1 β , Tumor Necrosis Factor (TNF)- α , IL-6/8 and Interferon (IFN)- γ are among the most relevant reactive pro-inflammatory cytokines and these components originate mainly from local immune cells and endothelium [14]. TNF- α and IL-1 are the first (short living) cytokines to react to trauma and stimulate the secretion of pro-inflammatory cytokines IL-6 /8 as well as of the anti-inflammatory IL-10 [15-17]. These clinical patterns have been verified in

controlled porcine trauma experiments as well [18-20]. Interestingly, genomic studies in trauma patients have shown that both pro- and anti-inflammatory mechanisms of the innate immune system get instantly activated after insult [21]. Post-traumatic circulatory cytokine kinetics over time, however, do not fully align with these genomic findings as traditionally, a biphasic pattern of systemic interleukin levels has been described. In this biphasic model an initial pro-inflammatory response (SIRS) precedes, an anti-inflammatory phase. The anti-inflammatory response is known as the compensatory anti-inflammatory reaction syndrome (CARS) [22]. The incompatibility between genomic and humoral studies models may be partly due to the role of tissue compartments' immune reaction. In fact, essential immune processes may take place outside the circulatory system, and not intravascular. Up to now, trauma studies have mainly focussed on circulatory processes and immune responses in the tissue compartment are probably a frequently overlooked factor in studies on inflammatory responses upon trauma.

The role of neutrophils in (vital) organ inflammation after trauma

End-organ dysfunction is the direct cause of inflammation-related mortality after trauma. Therefore, it is essential to focus on tissue compartments of vital organs when studying inflammatory complications after trauma. Peripheral blood should be considered as a readout of the body's immune homeostasis, rather than being considered as the key dictating compartment of immune regulation after trauma. Currently, the exact interplay between local, systemic and remote/organ inflammation is relatively unclear. Although, it has been demonstrated before that trauma-severity is associated with the magnitude of the inflammatory response [23,24]. Moreover, the degree of systemic inflammation is linked with the intensity of local and remote end-organ inflammation. Experimental studies have pointed out that more extensive tissue damage is associated with boosted transition from local to systemic and/or remote inflammation as well [18,20,23,24]. In remote organ inflammation, by definition, non-directly injured organs become inflamed, even in the absence of direct local trauma. Remote inflammation of vital organs may lead to organ failure (such as ARDS), even in the absence of direct trauma involvement of the organ. Of note, combined local and remote pulmonary inflammation may occur in polytrauma. Namely in the case of concurrent pulmonary trauma, thoracotomy or pneumonia. These three factors have been identified as independent risk factors for post-traumatic ARDS in the clinical setting [25].

A landmark post-mortem trauma study demonstrates that ARDS-related mortality is characterized by massive pulmonary neutrophil occurrence [26]. This underlines the relevance of neutrophil tissue homeostasis after trauma. Based on current understanding of the pathophysiology of severe trauma, post-traumatic inflammation is characterized by enhanced mobilization and activation of neutrophils. Activated circulatory neutrophils migrate into the tissue compartment, including highly vascularized lung-tissue. Upon activation, tissue residing neutrophils may release radical oxygen species (ROS) and proteases, which forms the basis for collateral damage to parenchymal cells and the development of organ dysfunction [27,28]. Neutrophils further modify humoral and adaptive cellular pathways. The current thesis aims to gain more insights on neutrophil homeostasis and its role in the development of inflammatory complications after trauma.

Systemic neutrophil homeostasis: quantitative and qualitative parameters

Neutrophils are considered as the key effector cells in trauma induced inflammation and these cells form the endpoint of essential immunological cascades [29,30]. Under homeostatic conditions, neutrophils, are the most prevalent subtype of circulatory leukocytes. Neutrophils outnumber other granulocytes in blood: basophils, eosinophils and mast cells, as well as mononuclear cells (lymphocytes and monocytes) [31].

Systemic inflammation further boosts circulatory neutrophil presence. During inflammation, shifts in neutrophil tissue distribution are seen as well. These effects are the result of (i) homing of specific cell (populations) in the tissue compartment and (ii) altered intra-organ compartmentalization [32]. The demarcation between physiological and pathological neutrophil tissue migration, however, is unclear. And this issue is a topic of high debate. In short, excessive circulatory neutrophil presence is not considered to be harmful by itself, whereas *excessive neutrophil tissue residence plus uncontrolled activation* in the tissue compartments of viable organs is a prerequisite for pathological inflammatory responses in trauma patients. To gain more insight in the exact interplay between local-systemic-remote neutrophil homeostasis after trauma, controlled studies are required. Animal models allow for these experiments. In contrast to human studies, animal experiments enable protocolized sampling in standardized trauma settings subjects that are highly similar to each other genetically. Furthermore, animal projects allow for the collection of baseline and tissue samples and control groups can be added. Therefore, the author and colleagues aimed to utilize

animal models to test several hypotheses. Different trauma models with varying trauma loads have been used and these studies resulted in identification of essential neutrophil changes over time in different compartments (e.g. fracture hematoma, circulation, bone marrow and lungs).

Increasing availability of novel anti-pig monoclonal antibodies (mAbs) allowed for multichannel flowcytometry studies on tissue neutrophils and neutrophil subsets in pigs. The author of this thesis further contributed to the development of novel anti-pig mAbs and the validation of unique flowcytometry gating protocols for neutrophils. Findings from hypothesis-driven experimental studies as described in this thesis comprise an important adjunct to available data obtained from non-standardized clinical studies on trauma neutrophil homeostasis. Moreover, these data, which will be discussed in more detail later, may form the impetus for upcoming immune modulatory interventions for trauma.

Systemic neutrophil homeostasis: monitoring of quantitative parameters in a controlled setting

In contrast to trauma, in the field of cardiovascular research, there are several well-validated animal models and hypothesis-driven studies are frequently performed. Given the similarities in anatomy, physiology and immunology between humans and pigs, porcine models are considered as highly relevant to study inflammatory responses following myocardial infarction (MI) [33]. MI is a key cardiovascular killer, however, due to novel treatment strategies, survival after MI improved markedly over time [34]. Consequently, incidences of the most relevant long-term complication after MI: heart failure, rose as well [35]. Hence, this process became an important research focus and neutrophils are believed to play an important role in the initial inflammatory response to MI-related tissue damage.

In *Chapter 2* of this thesis, immune responses between two different conditions (closed chest model vs. open chest surgery model) with ischemia-reperfusion injury following left coronary artery occlusion in pigs have been compared. Initial 2hr-systemic leukocyte numbers did not differ between conditions, however after 8 hours of observation, leukocyte counts were significantly higher in animals exposed to open chest interventions, than in the closed chest group. This was mainly the result of selective expansion of the circulatory neutrophil population. These findings indicate that more severe insult (tissue damage) is associated with more prominent innate immune cell responses. A similar observation was made in polytrauma

patients as the intensity of innate immune responses has been linked with the severity of trauma (Injury Severity Scoring) [24,36,37]. Besides, circulatory leukocyte numbers over time after porcine-MI in our study mimic kinetics after experimental trauma. This suggests the existence of integral pathways and overlap in the (patho)physiology of acute inflammation.

Chapter 2 further describes that a medial sternotomy is associated with reduced infarct size, enhanced cardiac function and higher Troponin levels after MI than the closed MI-condition. This finding implies that surgical intervention prior to standardized ischemia-reperfusion is associated with improved outcome. Several explanations for these observations have been postulated, including the cardioprotective effect of preconditioning [38]. *Ischemic preconditioning* is a well-described experimental concept in the field of cardiovascular research. In short, increased resistance to hypoperfusion in tissue is generated by prior application of multiple minor insults to end-organs (heart). This leads to improved coping of the organ to more substantial insults later on [39].

Additionally, *surgical and bacterial preconditioning* have also been described in literature [38,40,41]. The principle of surgical preconditioning entails the artificial induction of remote tissue damage, prior to the actual core insult. This modifies essential and potentially harmful processes and results in enhanced outcome. The concept of surgical preconditioning has not been explored in the field of trauma yet and may be an interesting topic to focus on in the future [38,40,41]. A prerequisite for preconditioning techniques is that the intervention should be made prior to insult. Trauma cannot be planned and therefore an alternative concept, called *post-conditioning* seems more promising to focus on. In post-conditioning, impaired outcome is mitigated by applying previously mentioned preconditional principles not before but after insult [42]. As polytrauma -by definition- is characterized by various insult conditions, some form of preconditioning naturally applies to trauma cases. Hence, more research on the interplay between different insults may contribute to the development of more differentiated trauma care in the future. Moreover, pre- or postconditioning interventions are potentially interesting therapeutic concepts. In fact, some pre-/post-conditioning has already been implemented in daily trauma care. The principle of dosing insults according to specific patient and trauma characteristics forms the basis for modern treatment protocols in polytrauma such as early appropriate care (EAC), damage control orthopaedics (DCO), and safe definitive surgery (SDS) [43-45]. The goal of modern trauma concepts is to apply calculated additional surgical loads to patients by spreading interventions over time. It is believed that major surgical

interventions such as definitive care of complex fractures should be postponed until the patient is stabilized. Timing of surgery is not only determined by the number of injuries solely, but also by the (immune) reserves of individuals. This may lead to the performance of additional temporary interventions, including placement of external fixators in the acute setting and delayed definitive surgery. DCO-practices have been introduced two decades ago and have been rolled out increasingly in European trauma care [46]. The success of these protocols has been demonstrated in large clinical studies [47]. Nevertheless, according to the author of this thesis, future trauma protocols should entail more differentiated (artificial) immune modulation. DCO and SDS-algorithms are limited by the fact that they fully rely on the modification of *patient-dependent* factors. More specifically, physicians' treatment options depend on the patients' injuries spectrum. In the authors' view, sophisticated trauma care in the future should include differentiated modification of *patient-independent* factors. This includes the providence of immune modulatory substances aimed to alter the immunological response. Adding calculated artificial immune triggers to natural serial insults in polytrauma may improve outcome of trauma patients in the future. Promising interventions include *pre-/postconditioning techniques* and *intravenous/intraosseous/inhalation application of immunomodulatory substances* [48]. Besides, pre-/postconditioning interventions may also be interesting for monotrauma as treatment in these cases is less dictated by urgency and allows for more planning of care.

Kinetics of circulatory neutrophil numbers upon different insult conditions

Peak systemic leukocyte levels are seen within the first hours after trauma. Clinical trauma studies have linked increased circulatory innate immune cell presence with impaired outcome [29,49]. This instant reactive leucocytosis is mainly caused by enhanced circulatory neutrophil influx [50-52].

Despite heterogenous insult conditions, patterns of systemic innate immune cell kinetics over time are very uniform. Aberrant situations of either deprived or excessive circulatory innate immune cell presence are associated with impaired outcome [29,53,54]. The demarcation between physiological and pathological circulatory neutrophils responses is a topic of high debate. For this thesis, several experiments with different insult conditions and observation periods have been performed. The findings from different experiments have been summarized in figure 1.

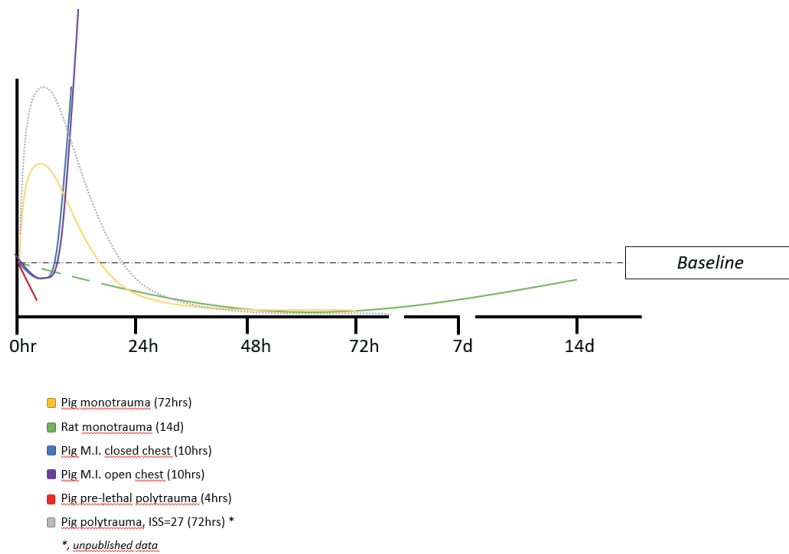


Fig. 1. demonstrates that patterns of systemic leukocyte kinetics are very similar, regardless of the specific type of insult. This further underlines the feasibility of all utilized models in this thesis (e.g. orthopedic monotrauma (rat/pig), polytrauma with HS (pig), pre-lethal thoracoabdominal surgery) to execute future studies on immunomodulation or proof-of-principle studies for novel innovative interventions. Based on the personal experiences from the author, we suggest to utilize pig models for short-term trauma observation models (up to 72 hrs), whereas rat models are most suitable for long-term (>72hrs) trauma investigations.

In order to substantially improve mortality in polytrauma patients, it is essential to study potentially lethal trauma, rather than likely survivable trauma. To do so, a pre-lethal model of ongoing thoraco-abdominal trauma surgery was developed in collaboration with the international DSTCTM-course. By using this model it was shown in **Chapter 4** of this thesis that in contrast to what is generally believed, systemic innate immune cell levels do not increase during extensive pre-lethal trauma surgery, but in fact gradually drop over time. Moreover, the percentage of mature neutrophils decreases, whereas novel subtypes with unknown functionality appear in circulation. This phenomenon may form a pathophysiological basis for the concept of damage control surgery/DCO as circulatory withdrawal of mature neutrophils became significantly more intense after 60 minutes of ongoing surgery. Especially as leukocyte depletion has been associated with altered outcome in clinical studies on critically ill patients [53].

Explanations for circulatory neutrophil depletion upon extensive polytrauma include: (i)

increased neutrophil extravasation, (ii) decreased neutrophil release from the bone marrow, (iii) impaired neutrophil re-migration into circulation from the tissue compartment, (iv) altered neutrophil apoptosis. Most likely, however, a combination of these processes takes place. The selective withdrawal of mature neutrophils and increased circulatory influx of novel neutrophil subsets may also be explained by the previously mentioned effects.

Chapter 5 of this thesis reports on a long-term observation model after a unilateral femur shaft fracture and fixation in rats. Reduced absolute neutrophil numbers in blood were found 3 days after trauma. Thereafter, circulatory neutrophil numbers gradually returned to baseline values. Decreased circulatory neutrophil numbers were also seen 3 days after intramedullary nailing (IMN) of a unilateral femur shaft fracture in a pig model (**Chapter 3**). In the pig-model, the drop of circulatory neutrophil numbers was preceded by a phase of increased systemic neutrophil numbers. Blood neutrophil kinetics as demonstrated in experimental rat (**Chapter 5**) and pig (**Chapter 3**) studies are in line with clinical trauma studies [54,55]. This underlines the feasibility of these animal models to perform future proof-of-principle studies to test novel interventions for trauma.

Characteristics of blood neutrophils: changes in qualitative parameters after trauma

In addition to altered circulatory white blood cell counts (quantitative parameters) after trauma, characteristics of neutrophil populations (qualitative parameters) change as well. Properties of circulatory neutrophils vary, and this is reflected by (i) cell surface receptor expression alterations and (ii) modified responses upon *in vitro* functional testing.

Changes of qualitative parameters of the blood neutrophil pool upon insult have not been studied in detail in trauma yet. Clinical relevance of immune cell characteristics in predicting infectious complications, however, is growing [56-59]. The flow experiments as described in this thesis provide new insights in porcine neutrophil characteristics both under homeostatic conditions and during acute systemic inflammation. Neutrophil receptor expression dynamics of essential cell-surface receptors upon trauma have been determined in a controlled context. Given the important role of neutrophils in the pathogenesis of inflammatory complications after trauma, better understanding of cellular responses and identification of key cellular processes is key. The findings as described in this thesis may form the basis for new diagnostic tools and contribute to the potential identification of novel targets for future immunomodulatory interventions.

PMN-receptor expression dynamics after trauma: the role of integrin-signaling in inflammatory complications upon IMN

In **Chapter 3**, a porcine model of standardized IMN of a femur fracture was utilized to determine polymorphonuclear neutrophil (PMN) receptor expression dynamics within the first 72 hours after trauma. It shows that IMN, rather than general anaesthesia and mechanical ventilation (GA+MV, control condition) evokes prompt and profound activation of circulatory PMNs. Blood PMN-counts are not largely affected by IMN. Interestingly, this phenomenon precedes later pulmonary PMN invasion, which has been demonstrated in a different study with our monotrauma pigmodel [60].

The experiment focuses on IMN as literature has shown that IMN is associated with increased occurrence of ARDS [61]. Interestingly, **Chapter 3** further reveals that GA+MV in pigs also affects circulatory PMN numbers and cell surface receptor expression. This is in line with a rodent trauma study by van Wessem et al. and implies that GA+MV should also be considered as a relevant trigger for the immune system [62]. Therefore, it may be interesting to investigate the impact of different GA+MV-strategies on immunological responses after trauma in future projects.

A femur fracture and subsequent instrumentation, however, intensifies the systemic neutrophil response. This is reflected by more prominent alterations of cell surface receptor expression of CD11b on circulatory cells after IMN, compared with the GA+MV-condition. This supports the belief that IMN of a femur fracture is a substantial and specific stimulus for the innate cellular immune response. CD11b-signaling may even link IMN and boosted immune activation in trauma. CD11b is a traditional activation marker for neutrophils. And *in vitro* neutrophil activation is associated with increased neutrophil-CD11b membrane expression [63]. Moreover, *in vivo* studies with systemic inflammation models showed that circulatory neutrophil-CD11b expression rises upon insult [24,64,65]. Findings as described in **Chapter 3** also exhibit that changes in PMN-CD11b expression after porcine-IMN are in line with human trauma studies. Interestingly, peak PMN-CD11b levels have been found 24 hours post-insult in the current pig project, whereas peak levels in clinical trauma studies were encountered slightly earlier after insult [24,64,65]. The minor differences between the current pig model and human trauma studies may be due to variations in (i) timing of sampling: in human studies there is an unavoidable delay between trauma and the first sampling time point, (ii) in trauma severity, which was higher in clinical studies than in the porcine monotrauma model. Thereby the current study also indicates that higher trauma load not only intensifies [24,60] the

magnitude of the inflammatory response, but also accelerates the early innate immune response.

After 72 hours of observation following trauma, CD11b expression levels on porcine blood PMNs dropped again, as neutrophil CD11b-expression levels did in a clinical trauma study from Bathia et al. [66]. Integrins have an important biological function in innate immune homeostasis. Changes in circulatory PMN-CD11b expression may therefore be an interesting marker to monitor the circulatory immune response. It may even be utilized to guide timing of intervention and more specifically fracture fixation. Based on our experimental findings and clinical data from Bathia et al. [66] it is worth considering postponing IMN for the first 72 hours and allow integrin levels to return to homeostatic levels in patients prone to develop inflammatory complications. Alternatively, clearing patients for surgery based on routine PMN-CD11b-measurements may be an element of more-differentiated trauma protocols in the future. It is tempting to hypothesize that the optimal timepoint for definitive fracture fixation differs between individuals. Measuring PMN-CD11b over time may be used to define the optimal timepoint for definitive surgery in severe trauma cases. Besides, additional *in vitro* analyses may provide even more specific laboratory markers to time surgery in severe trauma. Additional *in vitro* stimulation of blood samples enables physicians to investigate PMN-responsiveness over time and this parameter likely mimics a potential response to IMN as well. In the view of the author, both PMN-CD11b-expression and PMN-responsiveness are promising markers to guide timing of definitive surgery (including IMN) in polytrauma cases. Prospective multicentre studies are needed to investigate the potential clinical relevance of these parameters in trauma patients. The recent introduction and ongoing validation of automatized flow devices is a very promising development that likely stimulates advancement in flow cytometry diagnostics for trauma [67]. Furthermore, application of agents to block or modify CD11b/integrin-signalling after insult may dampen the cellular immune response after trauma and this is an interesting target to focus future experimental research projects on as well.

PMN-expression of Fc-gamma and selectin receptors on blood PMNs after monotrauma

Chapter 3 also describes receptor dynamics of three other essential PMN-receptors: CD16 (FcγRIII) and CD32 (FcγRII), which are members of the Fcγ-receptor family and CD62L (L-selectin). Fcγ-receptors bind to immunoglobins (IgG), either in aggregates or attached to pathogens and thereby contribute to neutrophil activation. More specifically, binding induces

phagocytosis or promotes oxidative burst [68]. L-selectin binds to its ligands on vascular endothelium and thereby facilitates slowing down, or 'rolling' of neutrophils and this receptor plays a critical role in neutrophil transmigration [69]. As PMN-activation leads to shedding of L-selectin from the cell surface, *in vitro* cell activation induces a decrease of L-selectin expression [63]. In **Chapter 3**, it was demonstrated that PMN-expression of CD16 rose over time after IMN and a femur fracture. This is in contrast with data from other studies on polytrauma and severe burn injuries [65,70].

L-selectin expression on PMNs did not differ significantly between conditions nor over time. A similar effect was seen in patients undergoing elective orthopaedic surgery [71], whereas striking alterations of neutrophil CD62L-expression levels were seen after severe trauma [72]. More severe injury than monotrauma and surgical fracture fixation may be required to evoke systemically relevant L-selectin receptor responses.

Of note, CD32 expression levels did not differ significantly between conditions and over time in our study and, which is in accordance with a clinical trauma study from Visser et al. [73]. The differences in PMN-receptor expression changes between the current porcine monotrauma study and clinical studies most likely relate to discrepancies in the severity of trauma between the studies. The porcine monotrauma model was probably not intense enough to evoke massive shifts in neutrophil activation and compartmentalization. In contrast to the polytrauma study described in **Chapter 4**, no CD16/CD62L-heterogeneity of circulatory neutrophils was found upon monotrauma. This further underlines the authors view that IMN for orthopedic monotrauma does not trigger the immune response enough to evoke massive mobilization of novel subsets, and more specifically CD16^{low}-PMNs [74,75]. This indicates that trauma severity does not only influences the *speed* and *intensity* of early cellular immune responses but also *the occurrence of specific receptor expression profiles on circulatory neutrophils* and *the appearance of trauma-associated PMN-subsets in circulation*. There may even be a, currently undefined, tipping point/threshold for generating specific neutrophil receptor expression profiles or for the release of aberrant neutrophil phenotypes in circulation. Based on the experiments in this thesis, it seems that the combination of GA+MV plus monotrauma and fracture fixation (ISS = 9) does not exceed this threshold. Prerequisites for passing this threshold and initiation of marked heterogenization of the blood neutrophil population following (poly)trauma are to be defined in future experimental projects. Besides, the role and potential pathological relevance of this phenomenon are currently unclear and requires further investigation.

Neutrophil diversity and heterogeneity after trauma

Neutrophils do not belong to a homogenous population of cells. During systemic inflammation, three different subtypes can be identified by differences in nuclear morphology and receptor membrane expression of FcγRIII (CD16) and L-selectin (CD62L). During homeostatic conditions, different subsets are virtually absent in blood and the blood neutrophil population consists almost exclusively of CD16^{high}/CD62L^{high}-cells. However, two additional subsets however appear in circulation during systemic inflammation and are believed to be of great relevance because of their varying functional capacities [75-78]. Given their unique pattern of mobilization and their specific morphological and functional capacities these neutrophil subtypes represent different phenotypes as well [75-78]. In short, hypersegmented neutrophils (CD16^{high}/CD62L^{dim}) exhibit T-cell suppressive capacities and impaired execution of bactericidal tasks compared with other subsets [75-78]. CD16^{dim}/CD62L^{high}-neutrophils have a band-shaped nucleus, are less mature neutrophils [75] and are superior in containing bacteria [75-78]. As described in **Chapter 4** of this thesis, porcine neutrophil subsets have similar morphological characteristics, pattern of mobilization and cell-membrane receptor co-expression profiles as human subsets, which were described first by Pillay et al. [84].

In line with human *in vivo* studies with bacteremia-induced systemic inflammation, it was demonstrated in **Chapter 4** that the proportion of banded (CD16^{dim}/CD62L^{high}) cells in blood increases rapidly in trauma-induced inflammation as well [75]. In addition, these systemic subsets appeared almost immediately after porcine trauma induction and their fractions rose gradually during ongoing surgery.

The role and function of these subsets in trauma and their potential to contribute to pathological immune states in pre-lethal trauma are unclear but require in depth further analysis.

A distinct circulatory neutrophil subset which was identified after rodent monotrauma (CD11a^{high}/CD11b^{high}), has been described in **Chapter 5** of this thesis. This subtype appears several days after trauma in circulation and this subset may be relevant for later (immune) repair processes after trauma. Their specific function should be determined in later experimental studies as well.

Concurrent systemic neutrophil deprivation despite the emergence of novel subsets: the road to disaster?

As shown in **Chapter 4**, the appearance of neutrophil subtypes in blood and subsequently

increased heterogeneity of the systemic neutrophil population occurred in a setting of ongoing circulatory neutrophil depletion. A unique phenomenon that has not been described before and which may be of great relevance for the understanding of the pathophysiology of severe trauma. Especially, as this specific subtype of neutrophils is less mature and demonstrates superior containment of bacteria such as *S. Aureus*/MRSA but impaired interaction with *S. epidermis* [75,76]. The mobilization of banded ($CD16^{\text{dim}}/CD62L^{\text{high}}$) cells in pigs were validated by PMN cell sorting experiments and subsequent morphological analysis. In the case of massive influx of $CD16^{\text{dim}}$ -cells, the low cell surface expression levels of CD16 (Fc γ RIII) on this specific subset may also contribute to an overall reduction of CD16 on circulatory neutrophils following severe trauma. Interestingly, however, a rise of overall CD16-expression on neutrophils was seen in standardized porcine monotrauma + IMN (*Chapter 3*). Increased CD16 on PMNs can be either be the result of altered activation status of the circulatory neutrophil pool or increased cell dead/maturity. It seems that in minor trauma situations, these processes determine blood PMN-CD16 expression profiles, whereas *major trauma* evokes prominent shifts in the composition of the blood neutrophil pool. Hence, the overall impact of newly released $CD16^{\text{dim}}$ -cells likely outweighs the previously mentioned processes affecting circulatory neutrophil-CD16-expression in minor trauma. The impact of the invasion of aberrant neutrophil subtypes on restoration of homeostasis in major trauma is unclear. Although, pre-lethal trauma as described in *Chapter 4/7* is characterized by progressive circulatory neutrophil depletion and prominent circulatory occurrence of aberrant neutrophil subsets. A phenomenon that may segregate likely *non-survivable* polytrauma from likely *survivable* inflammatory major trauma conditions. Statistically significant shifts in the composition of the blood neutrophil pool are observed first after 60 minutes of ongoing trauma surgery. This observation may be considered an important rationale behind current treatment strategies in severely injured trauma patients which are aimed to minimize surgical intervention (damage control surgery) and respect the 'golden hour of trauma' [43-47].

Long-term circulatory neutrophil homeostasis after monotrauma and IMN

Late inflammatory complications such as wound and fracture healing issues or prolonged MODS affect morbidity and mortality after trauma as well [79-81]. There is increasing evidence that neutrophils shape adaptive immune responses and play an important role in the pathogenesis of late post-traumatic complications [75,80,82]. Multiple organ dysfunction

syndrome is diagnosed first after several days of ICU-stay and this makes it very interesting to study longer intervals than the first 72-hours as well [2,5,8,22,83]. **Chapter 5** of this thesis, is the first experimental study to describe long-term circulatory neutrophil responses after IMN and a femur fracture. As described by Hildebrand et al. large-animal models are not feasible to study trauma/hypovolemic shock for a longer period than 72 hours. In these cases, rodent modelling is preferable [84,85]. A rodent model was developed to study the systemic neutrophil response to standardized intramedullary nailing and a femur fracture over a time period of 14 days. As anticipated, both the number and activation status of circulatory neutrophils dropped at 72 hours after femoral fracturing and IMN in rats. These findings are in line with other orthopaedic trauma studies [86]. Thereafter, neutrophil receptor expression of CD11b as well as circulatory cell counts returned to baseline values. Most likely, two processes are involved in the encountered temporary systemic neutrophil withdrawal. Firstly, instantly after fracture, massive invasion of neutrophils in fracture hematoma as well as surrounding tissues occurs. [80, 87,88]. Early enhanced neutrophil tissue homing is driven by the local release of mitochondrial DAMPs (damage-associated molecular patterns) and different chemo-attractants in the surrounding tissue. Subsequently increased fracture site neutrophil recruitment may lead to a second drop of circulatory neutrophils [80,87]. Secondly, increased neutrophil homing of PMNs into other organ systems after IMN occurs. The lungs have been identified as the primary target organ. And there, neutrophils potentially damage parenchymal lung tissue. This process forms the basis for post-traumatic inflammatory complications [89,90]. Both clinical and experimental trauma studies from the author (**Chapter 6**) and others have demonstrated that pulmonary neutrophil infiltration peaks at 72 hours after fracture fixation [23,56].

In addition to systemic neutrophil presence, neutrophil functionality is also key to assessing the long-term competence of the innate immune system as well as its potential to harm the host. Therefore, relevant receptor expression dynamics over time have been investigated in our long-term observation study as described in **Chapter 5** as well. Cell surface expression of L-selectin (CD62L), MAC-1 (CD11b) and LFA1 (CD11a) have been studied. Unfortunately, it is not possible to study markers from the Fc γ -receptor family in rats as these receptors markedly differ between species and in fact there is consensus that rodents do not allow for these investigations [91]. The experiments from **Chapter 5** did not show differences in L-selectin (CD62L) surface expression levels between different time points nor as compared with control conditions. This is in line with our 72-hour observation study on porcine monotrauma (**Chapter 3**) and with clinical studies on cardiac surgery and elective orthopaedic lower limb surgery

[71,92,93].

Neutrophil CD11b-expression on circulatory neutrophils did significantly change over time after monotrauma and IMN. More specifically, 3 days after trauma, there was a drop of systemic neutrophil-CD11b expression. A similar temporary drop of systemic neutrophil CD11b expression in the initial phase after trauma has also been encountered in clinical studies [64,92]. Thereafter restoration of CD11b-receptor presence on circulatory neutrophils was seen. Eventually, neutrophils gradually reach homeostatic CD11b-expression levels within 14 days. This indicates that restoration of integrin signalling upon orthopaedic trauma and surgery takes about 14 days. Decreased neutrophil CD11b-expression is suggestive for impaired immunocompetence. During this phase, patients may be at increased risk of developing inflammatory (ARDS/MODS) or infectious complications (pneumonia, wound infections) and fracture healing issues. Our results are supported by a clinical study on isolated fractures by Baehl et al. as in this cohort an initial decrease of CD11b-expression on blood neutrophils was followed by a gradual increase over 6 months of follow-up observation [95]. In addition, multichannel flow analysis of different integrins (CD11b and CD11a) was performed for the study described in *Chapter 5*. This led to the identification of a novel CD11b^{high}/CD11a^{high}-neutrophil subset, which appeared first in circulation at day 7, during the restoration phase. Additional co-receptor studies have demonstrated a significantly higher co-expression of very late antigen-4 (VLA-4/CD49d) on CD11b^{high}/CD11a^{high}-neutrophils than on regular neutrophils. The origin, role and functionality of these cells are currently unclear. Given the timing of appearance in blood, these cells may play a role in the regulation of the anti-inflammatory phase following trauma. Moreover, increased CD49d expression suggests that these cells may originate from the bone (marrow) [96,97].

Remote pulmonary neutrophil homeostasis after trauma

Trauma without thoracic involvement may also affect pulmonary neutrophil homeostasis. Remote immune responses may even cause inflammatory complications such as ALI or ARDS [2,11,25,98]. IMN for femur fractures is associated with increased incidences of pulmonary complications and neutrophils seem to play a role [26,90,98,99]. Histological studies on post-trauma ARDS-fatalities have demonstrated substantial pulmonary neutrophil occurrence [26,100]. Key characteristics of the pulmonary neutrophil pool after femoral IMN have been investigated in *Chapter 6*. A rat trauma model was utilized to monitor pulmonary neutrophil

characteristics over 14 days. In line with literature, IMN and a unilateral femur fracture in rats are associated with increased pulmonary neutrophil deposition [23]. Even more interestingly, the rodent study as described in **Chapter 6** revealed that concurrent activation of the pulmonary PMN-pool occurs. After the peak of both pulmonary neutrophil presence and activation at 72 hours after insult, both variables gradually returned to baseline values.

As no complications were seen in this study, the model most likely reflects the physiological pulmonary neutrophil response to IMN and a femur fracture. However, it is tempting to hypothesize that a boosted response (for instance in the case of polytrauma or in fragile patients) may lead to intensified pulmonary neutrophil invasion and activation and forms the basis for inflammatory complications.

Increased overall CD62L-expression on pulmonary neutrophils (after 72 hrs) may partly be the result of the appearance of novel (CD16^{low}/CD62L^{high}) neutrophils in circulation and (L-selectin dependent) tissue homing of cells. The observed shifts of integrin (LFA-1 and Mac-1) cell surface receptor expression, underline the biological function of these receptors in post-traumatic pulmonary neutrophil compartmentalization. In other studies, specific blockage of single receptors did show minor effect on post-insult neutrophil kinetics [101]. This may be explained by the existence of multiple independent pathways for integrin-mediated trans endothelial migration with compensatory capacities [102]. This concept has been described in detail by Young-Min et al. and entails overlapping roles of integrins LFA-1 and Mac-1 in neutrophil extravasation through hotspots I and II. Selective blocking of single receptors may therefore result in aberrant outcome as the harmonized regulation of integrin signalling pathways becomes disturbed [103]. Compensatory action of the non-blocked integrin receptor may also occur. To overcome this issue, an integral (pan)-integrin blockage may be an interesting approach to dampen neutrophil pulmonary tissue homing in trauma. Inhalation application of pan-integrin blocking agents may further lead to optimized local effects in the lungs and minimal systemic impact and this should be studied in a controlled setting in the future [104].

Early local immune homeostasis: fracture hematoma neutrophils

Local tissue damage after trauma triggers cytokine production. This initial increased production of cytokines primarily consists of pro-inflammatory cytokines such as TNF- α , interleukin (IL)-1 β , IL-6 as well as IL-8 [2]. TNF- α and IL-1 β stimulate hepatic cells to

produce IL-6 and thereby stimulate the rise of systemic IL-8 [105]. IL-8 is a key cytokine involved in recruitment, tissue migration and enhanced oxidative burst of neutrophils (PMNs) [106]. Next to pro-inflammatory cytokines, anti-inflammatory cytokines are released as well. The most prominent anti-inflammatory protein is IL-10, which provides negative feedback on the production of TNF- α , IL-6 and IL-8 [107]. These cascades eventually modify the influx of immune effector cells such as neutrophils. Neutrophils are the first cells to infiltrate in inflamed regions and also the most prominent white blood cell type in early healing processes, including fracture healing (*Chapter 8*).

Fracture healing is divided into three consecutive but overlapping phases: (1) an early inflammatory phase, (2) a repair and (3) a remodeling phase [108]. The composition of early fracture hematoma (FH), dictates the overall success of the fracture healing process [109]. The importance of the early FH composition is underlined by the observation that removal of early fracture hematoma during the first days results in delayed or non-union [110]. The quality of early fracture hematoma content is the result of the skeletal, immune and vascular systems [108,111]. Thus, in the case of adequate fracture fixation, the success of fracture healing is mainly determined by adequate blood supply to the fracture site and local inflammation.

Studies from Hauser et al. demonstrated that early (4 hours) fracture hematoma is rich in activated monocytes, neutrophils and a variety of soluble cytokines (IL-6, IL-8, IL-1). IL-1 and TNF- α , are increased during the initial inflammatory phase and are known to upregulate endothelial adhesion molecules and to induce recruitment of inflammatory cells [109, 112]. IL-6 is mostly known as a pro-inflammatory bone resorbing cytokine that stimulates the differentiation and proliferation of osteoclasts. Furthermore, IL-6-/-mice had delayed callus maturity, mineralization, and remodeling compared with wild type mice. These effects were transient, indicating that the role of IL-6 appears to be most important in the early stages of fracture healing [113].

According to previous large animal studies, leukocyte numbers in fracture hematoma start to rise directly following fracture. And the percentages of PMNs and monocytes/macrophages in FH increase instantly after insult and reach concentrations similar as blood within 4 hours. During this time period, the proportion of non-vital leukocytes in FH also increases. The composition of the fracture hematoma is established predominantly by factors that are produced by infiltrated inflammatory cells, illustrating the importance of local controlled inflammation for adequate bone repair [114].

The early cellular local immune response is characterized by two subsequent phases: during

the first day there is a massive influx of neutrophils into the fracture side and neutrophils outnumber all other white blood cells. Thereafter, neutrophils become gradually replaced by macrophages [115]. Adequate regulation of FH-neutrophil homeostasis is essential and aberrant neutrophil influx is detrimental [86]. Furthermore, systemic neutrophil reduction in rats with growth plate trauma results in improved bone repair [80]. Neutrophils also contribute significantly to the later influx of macrophages in FH, by release of the chemoattractants MCP-1/IL-6 [116]. Thereby neutrophils also modify later immune stages. The study described in **Chapter 8** has confirmed the previous findings from human studies in a controlled animal experiment. It was shown that neutrophils are the most prevalent immune cell in porcine early fracture hematoma. The applied fracture fixation method did not affect the fractions of immune cell subtypes in FH after monotrauma. Interestingly, no differences in quantitative parameters of early fracture hematoma immune cell composition were seen after different fracture fixation techniques (e.g. unreamed femoral nailing (UFN), reamed femoral nailing (RFN) and reamed-irrigation and aspiration enhanced intramedullary nailing (RIA-group)). Although, obvious differences in qualitative characteristics of the FH immune cell content were found. First, RIA was associated with relatively more viable immune cells in fracture hematoma, than alternative treatment strategies. Secondly, lower FH-neutrophil activation and maturation status were reported upon RIA than after RFN or UFN. Activation status and maturation status were measured by flow studies measuring relevant cell surface receptor expression levels on isolated FH-neutrophils. Mac-1/CD11b was utilized to determine cell activation, whereas Fc γ RIII/CD16 was measured as a readout for cell maturation. Of note, an association between local temperature alterations and fracture hematoma immune cell viability as well as neutrophil activation was found as well. This suggests that local cooling (by RIA) may be beneficial for the quality of fracture hematoma immune cells. These insights require clinical validation and prospective studies are needed to optimize IMN-protocols.

Bone marrow neutrophil homeostasis during extensive ongoing trauma surgery

In **Chapter 4** a decline in circulatory neutrophil numbers during extensive trauma surgery was found. This observation formed the impetus for the study on the reactive bone marrow response which is described in **Chapter 7**. It was hypothesized that trauma-induced circulatory neutrophil depletion is mainly the result of inadequate medullary compensation for enhanced neutrophil extravasation. This suboptimal compensation eventually leads to a mismatch of

circulatory neutrophils supply and demands in pre-lethal polytrauma. The rationale for this hypothesis is described hereafter.

Besides being the most abundant circulatory cells, neutrophils are also the most prevalent leukocyte population in bone marrow. More specifically, about 60 percent of bone marrow leukocytes comprise granulocyte (precursors) [117]. Under homeostatic conditions, the bone marrow produces approximately $0.5-1.0 \times 10^{11}$ neutrophils within 24 hours [117]. Compensatory mobilization of bone marrow (BM) immune cells is an essential mechanism in the maintenance of circulatory neutrophil homeostasis during states of increased circulatory immune cell demands, for instance after trauma. Trauma-evoked systemic inflammation and increased innate immune cell mobilization from the bone marrow comes along with instant shifts in the composition of the blood neutrophil pool [118,119]. Hematopoiesis, however, is a time-consuming process [118,119]. Adequate medullary compensation to inflammation-associated circulatory cellular depletion entails both enhanced differentiation and maturation of hematopoietic cells into mature/post-mitotic neutrophils [119]. Given the observations as described in **Chapter 4**, it was hypothesized that in the case of extensive trauma-induced systemic inflammation, the bone marrow fails to compensate for this extreme immune state. Especially as literature links extreme inflammatory conditions with an '*empty bone-marrow*'-phenomenon [120-123]. Moreover, a distinct process, known as '*emergency hematopoiesis*', has been described both in trauma situations and after severe infection. In short, this process describes the compensatory bone marrow response aimed to cope with instant increased circulatory effector cell demands by stimulating BM-hematopoiesis [120,124]. This response merely describes the expansion of hematopoietic progenitor cells (early stages of hematopoiesis), rather than specific alterations in more differentiated BM-cell populations (end-stage hematopoiesis/granulocytopoiesis) [125-126]. In **Chapter 7** we have utilized our large animal model of pre-lethal trauma surgery to study the medullary neutrophil response in a controlled setting. By doing so, it was demonstrated again (**Chapter 4**) that extensive porcine trauma surgery is associated instant circulatory neutropenia. Interestingly however, medullary neutrophil depletion was not detected. Instead, concurrent expansion of the BM-neutrophil fraction was seen. It was further revealed that reactive expansion of the BM-neutrophil fraction pool was associated with concurrent increased overall cell senescence. Moreover, a novel subset of bone marrow PMNs with an attenuated maturity profile was identified and characterized.

These interesting novel observations during the early immune response to extensive trauma are

in contrast with well-accepted theories. First, it contradicts the belief that the neutrophil pool shrinks during acute systemic inflammation due to increased circularization of cells. Secondly, the current experimental study also contradicts the concept of the empty-bone marrow syndrome which is characterized by medullary hypocellularity upon insult [120-122]. According to the author, upsizing of the BM-neutrophil pool may reflect emergency hematopoiesis after trauma, which was recently also described by Fuchs et al [124]. However, increased senescence of the BM-neutrophil population makes this unlikely. Alternatively, the post-mitotic neutrophil pool after trauma may also increase due to re-migration of circulatory neutrophils. The concept of medullary re-migration of (old) CXCR4^{high} neutrophils was described by Martin et al. [127]. In line with the results from these studies, an overrepresentation of BM-CXCR4^{high} neutrophils after trauma was identified after trauma. The observations from the study as described in **Chapter 7** may be unique for cases of pre-lethal trauma, rather than less severe conditions of trauma.

In our view, the discrepancy between the results of the current study with studies on the empty-bone marrow syndrome may be caused by differences in trauma severity and timing of sampling and subsequent observation periods. In short, our pig model of pre-lethal trauma focuses on the instant response to extensive surgery and describes alterations that occurred within the first 4 hours after trauma induction.

Translational considerations of pig experiments in trauma

Historically, rodents have been the primary model system in most fields of medical research. However, the shortcomings of rodent's preclinical models are widely recognised, and these models are considered suboptimal regarding translating basic knowledge into clinical application as they do not always accurately mimic human pathologies. Consequently, many new clinical interventions based on preclinical rodent study fail after translation into human patients [128,129].

The pig as model for human trauma research is becoming more popular, especially as pigs share valuable similarities of key anatomical and physiological features with humans. Similarities between human and pig regarding hemodynamic responses, metabolic responses to drugs and wound healing have been well described [130,131]. Sequencing studies of the porcine genome has widened support for using this species as a model for humans by demonstrating large similarities at the gene and chromosomal levels with humans [132].

Alternative large animal models include sheep, goat and primate models. However, validation studies for key inflammation markers in these species are lacking and this largely limits immunological analysis. Furthermore, due to ethical considerations it is unlikely that essential knowledge regarding primate inflammatory processes will become available soon. Regarding pigs however, a large number of validation studies on porcine leukocyte antibodies have been performed and international consensus meetings have been held to validate antibodies for flow studies [133-135].

The results of these international consensus meetings, related papers and later validation studies paved the way for the experiments as described in this thesis [136-138]. Moreover, a novel monoclonal antibody against porcine-CD62L has been developed and validated by the author and his supervisors. This gave us the unique opportunity to identify different immune cell subsets in pigs in a similar setting as others did before in human. Nevertheless, to prevent overinterpretation of findings it is important to highlight shortcomings of porcine modelling as well. Overall shortcomings include interspecies differences of specific biological characteristics such as bone-muscle relations, pulmonary anatomy regarding non-upright positioning of pig.

Specific inflammatory differences between human and pigs have been encountered by our experiments as well. Baseline leukocyte and neutrophil levels in pigs are higher than in healthy human beings. This relative leucocytosis in pigs may have several causes. First, as initial blood sampling in pigs always involves some degree of sedation and intervention a stress-leucocytosis may be involved. Secondly, transportation and altered housing circumstances as well as nutrition prior to the experiments may induce additional stress to experimental animals. In addition, the porcine coagulation system largely differs from the human system and it has been described that experimental posttraumatic coagulation responses in pigs do not adequately mimic the human situation. This is of great importance as coagulation disorders in polytrauma patients are an important outcome-determining factor [136-139]. Some groups opt to implement controlled haemodilution in their trauma models to induce coagulopathy, however this is not considered as an adequate way to simulate the specific and complex condition of trauma-associated coagulopathy [84,140,141].

Furthermore, the experimental setup of large animal experiments has some shortcomings. Due to ethical reasons, insult induction is always performed under anaesthesia and mechanical ventilation (MV). In human trauma insult occurs in the absence of anaesthesia and MV. Anaesthesia and MV likely affect the stress responses to insult as well and may either mask or

boost essential responses to insult [62]. Sequential sampling during experimental research (e.g. broncho-alveolar lavage or fracture hematoma puncture) may also affect outcome, should be considered as a potential confounder as it exhibits additional triggers for immune activation, and may alter the local immune environment significantly as well. Studies for this thesis were designed in a way repetitive invasive sampling was avoided.

FUTURE PERSPECTIVES

The different studies as described in this thesis resulted in novel insights in post-traumatic neutrophil homeostasis. This thesis describes novel local, systemic and organ neutrophil changes after trauma in a controlled environment. Not only changes in neutrophil numbers, but also essential cell surface receptor expression dynamics have been documented and novel porcine neutrophil subsets have been identified. Both in mono trauma and in extreme polytrauma scenarios. It was demonstrated that both the number of neutrophils in circulation, as well as their activation status change over time according to a universal pattern. A comparison of the different insult conditions, with varying trauma severity, indicate that more severe trauma does not only result in more intensified systemic neutrophil changes but also in faster occurrence of these changes. Moreover, trauma resulted in increasing heterogeneity of the circulatory neutrophil pool, characterized by the appearance of novel neutrophil subsets in circulation among intense trauma. As these subsets were not identified in minor trauma models of single fractures it is tempting to hypothesize that severe injury is a prerequisite for this phenomenon. In addition to the prominent changes of circulatory neutrophil numbers and selective expansion of specific neutrophil populations in blood, the activation status of the systemic neutrophil pool also fluctuated over time. Overall, more prominent, and faster fluctuations were seen in more severe trauma than in less intense trauma.

All these novel insights may form the basis for future clinical diagnostic and therapeutic studies. Regarding diagnostics, specific effects (e.g. more prominent alterations of qualitative/quantitative characteristics of the systemic neutrophil pool) may be predictive for inflammatory complications. Due to the recent development of automatized flow systems, cellular immune monitoring in the clinical setting became easier. This potentially allows for

fast analysis of cellular innate immune parameters in clinical cases. Augmented laboratory protocols including *in vitro* stimulation of blood/tissue samples from trauma patients may even improve the predictive value of immunomonitoring for adverse outcome and thereby guide timing of interventions in polytrauma. This may be utilized to guide surgical treatment strategy (damage control orthopedics/early total care and safe definitive surgery). However, it is of utmost relevance that our findings will be validated by clinical studies first. Thereafter, big-data analysis may help to identify those patients at risk for the development of inflammatory complications.

As blood is just a readout for the whole-body immune response, it is essential to monitor alternative tissue compartments as well. In my view, several specific, easily available tissue samples may entail important information about the tissue response to trauma: urine, stool, saliva, bronchial secretions, cerebro-spinal fluid.

This thesis may also form the basis for novel therapeutic concepts. Patients prone to develop inflammatory complications after trauma may be considered as adequate candidates for immune modulatory interventions. The pig model for polytrauma, as utilized in this thesis, is suitable to test potential future immune modulatory interventions in a controlled setting. Potential interventions include pre- or postconditioning interventions (integrated in novel protocols with recommendations on timing and clearance for surgery) or the application of immune modulatory substances. As potential immune modulatory interventions tend to become more differentiated, the development of such interventions should be a key focus of future experimental investigations. This thesis also provided novel insights in specific neutrophil processes after trauma which may be utilized to develop novel interventions. Local inflammation, e.g. early fracture hematoma neutrophil population, was affected by intramedullary nailing strategy. More specifically, reamed-irrigation and aspiration enhanced nailing was associated with more viable and less activated FH-immune cells than alternative fracture fixation protocols. Interestingly, a correlation between intra-operative temperature changes and quality of FH-immune cells was found. This implies that irrigation, and thereby cooling, of the intramedullary channel, dampens the local immune response. This observation should be focus of upcoming experiments in which the impact of RIA-enhanced nailing on systemic immune homeostasis should be investigated. Not only in mono trauma but given the high rates of severe inflammatory complications after polytrauma, also in more severe trauma conditions. Furthermore, more differentiated reaming protocols should be compared to identify the optimal reaming protocol for both mono- and polytrauma in future projects. The following

parameters are of special interest: drill speed, duration of drilling, reamer head properties, irrigation solution content, flushing solution temperature, irrigation and aspiration pressures, intermittent/continuous irrigation-aspiration settings. Moreover, addition of specific substances to affect inflammation, coagulation, pH-alterations (buffers) may be tested. The porcine trauma models as developed by the TREAT-Research consortium and which have been utilized for our studies on intramedullary nailing are optimal to execute the previously mentioned investigations.

In addition to local inflammation and systemic inflammation, the studies of this thesis are unique as remote immune responses were studied as well. As life-threatening inflammatory complications mainly involve lung dysfunction, the pulmonary neutrophil response was investigated in detail as well. Both parenchymal and alveolar neutrophil populations were studied in a 2-week observation model after singular femoral nailing and a 3-day pig model. It was demonstrated that pulmonary neutrophil numbers and subsequent activation status of these cells increased within the first 3 days after trauma. After 3 days, cell numbers and activation status of neutrophils both systemic and pulmonary populations returned to baseline values. As pulmonary complications were not observed in both studies it is tempting to speculate that these models in fact reflect physiological alterations after fracture and trauma care. Future studies should focus on the demarcation between physiological and pathological neutrophil tissue responses. This can be done by standardized titration of trauma intensity.

Moreover, not only systemic neutrophil changes should be studied but as the interplay between blood and end-organ homeostasis is unclear, tissue (including lung) neutrophil populations should be analysed in more detail as well. And selective blocking of key pulmonary neutrophil homing receptors are interesting therapy options to investigate in future experimental projects. Administration of such agents by inhalation may optimize local tissue concentrations and the eventual impact. Furthermore, simultaneous blocking of several receptors (pan-integrin/selectin) may help to overcome potential bypassing by alternative pathways for neutrophil migration when blocking a single receptor.

In addition, extensive polytrauma was associated with instant circulatory neutrophil depletion, the concurrent bone marrow response was studied as well. This standardized study revealed that no instant depletion of the bone marrow neutrophil pool occurred upon extensive trauma. On the contrary, increased neutrophil presence in the bone marrow was seen and the BM-neutrophil pool became more heterogeneous.

Immune modulation by targeting the bone marrow in trauma is a relatively unexplored field.

However, given the essential role of bone marrow in immune homeostasis, potential interventions are promising. And the different porcine models as described in this thesis may be used to perform preclinical studies in this field. Prior to intervention studies, more insights in medullary immune responses may be obtained by either porcine modelling or repetitive sampling in human studies by intraosseous sampling. Novel studies on this issue have been initiated by the author prior to the completion of this thesis and these results may form the basis for bone-marrow aimed immune modulatory interventions in severe trauma.

References

1. Rather LJ. Disturbance of function (functio laesa): the legendary fifth cardinal sign of inflammation, added by Galen to the four cardinal signs of Celsus. *Bull N Y Acad Med*. 1971 Mar;47(3):303-22.
2. Keel M, Trentz O. Pathophysiology of polytrauma. *Injury*. 2005 Jun;36(6):691-709.
3. Metsemakers WJ, Handojo K, Reyniers P, Sermon A, Vanderschot P, Nijs S. Individual risk factors for deep infection and compromised fracture healing after intramedullary nailing of tibial shaft fractures: a single centre experience of 480 patients. *Injury*. 2015 Apr;46(4):740-5.
4. Hildebrand F, van Griensven M, Huber-Lang M, Flohe SB, Andruszkow H, Marzi I, Pape HC; Trauma Research Network of the German Society of Trauma, DGU. Is There an Impact of Concomitant Injuries and Timing of Fixation of Major Fractures on Fracture Healing? A Focused Review of Clinical and Experimental Evidence. *J Orthop Trauma*. 2016 Mar;30(3):104-12.
5. Goris RJ, te Boekhorst TP, Nuytinck JK, Gimbrère JS. Multiple-organ failure. Generalized autodestructive inflammation? *Arch Surg*. 1985 Oct;120(10):1109-15.
6. Fry DE, Pearlstein L, Fulton RL, Polk HC Jr. Multiple system organ failure. The role of uncontrolled infection. *Arch Surg*. 1980 Feb;115(2):136-40.
7. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, Legall JR, Morris A, Spragg R. The American-European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. *Am J Respir Crit Care Med*. 1994 Mar;149(3 Pt 1):818-24.
8. Birkner DR, Halvachizadeh S, Pape HC, Pfeifer R. Mortality of Adult Respiratory Distress Syndrome in Trauma Patients: A Systematic Review over a Period of Four Decades. *World J Surg*. 2020 Jul;44(7):2243-2254.
9. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell*. 2006 Feb 24;124(4):783-801.
10. Murphy K, Travers P, Walport M, Janeway C (2008). *Janeway's immunobiology*. New York: Garland Science.
11. Huber-Lang M, Lambris JD, Ward PA. Innate immune responses to trauma. *Nat Immunol*. 2018 Apr;19(4):327-341.
12. Harris HE, Raucci A. Alarmin(g) news about danger: workshop on innate danger signals and HMGB1. *EMBO Rep*. 2006 Aug;7(8):774-8.
13. Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, Lambris JD, Warner RL, Flierl MA, Hoesel LM, Gebhard F, Younger JG, Drouin SM, Wetsel RA, Ward PA. Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med*. 2006 Jun;12(6):682-7.
14. Svoboda P, Kantorová I, Ochmann J. Dynamics of interleukin 1, 2, and 6 and tumor necrosis factor alpha in multiple trauma patients. *J Trauma*. 1994 Mar;36(3):336-40.
15. Ferguson KL, Taheri P, Rodriguez J, Tonapi V, Cardellio A, Dechert R. Tumor necrosis factor activity increases in the early response to trauma. *Acad Emerg Med*. 1997 Nov;4(11):1035-40.
16. Dinarello CA. Biology of interleukin 1. *FASEB J*. 1988 Feb;2(2):108-15.

17. Keel M, Ecknauer E, Stocker R, Ungethüm U, Steckholzer U, Kenney J, Gallati H, Trentz O, Ertel W. Different pattern of local and systemic release of proinflammatory and anti-inflammatory mediators in severely injured patients with chest trauma. *J Trauma*. 1996 Jun;40(6):907-12; discussion 912-4.
18. Horst K, Eschbach D, Pfeifer R, Hübenthal S, Sassen M, Steinfeldt T, Wulf H, Ruchholtz S, Pape HC, Hildebrand F. Local inflammation in fracture hematoma: results from a combined trauma model in pigs. *Mediators Inflamm*. 2015;2015:126060.
19. Horst K, Eschbach D, Pfeifer R, Relja B, Sassen M, Steinfeldt T, Wulf H, Vogt N, Frink M, Ruchholtz S, Pape HC, Hildebrand F. Long-Term Effects of Induced Hypothermia on Local and Systemic Inflammation - Results from a Porcine Long-Term Trauma Model. *PLoS One*. 2016 May 4;11(5):e0154788.
20. Horst K, Greven J, Lüken H, Zhi Q, Pfeifer R, Simon TP, Relja B, Marzi I, Pape HC, Hildebrand F. Trauma Severity and Its Impact on Local Inflammation in Extremity Injury-Insights From a Combined Trauma Model in Pigs. *Front Immunol*. 2020 Jan 9;10:3028.
21. Xiao W, Mindrinos MN, Seok J, Cuschieri J, Cuenca AG, Gao H, Hayden DL, Hennessy L, Moore EE, Minei JP, Bankey PE, Johnson JL, Sperry J, Nathens AB, Billiar TR, West MA, Brownstein BH, Mason PH, Baker HV, Finnerty CC, Jeschke MG, López MC, Klein MB, Gamelli RL, Gibran NS, Arnoldo B, Xu W, Zhang Y, Calvano SE, McDonald-Smith GP, Schoenfeld DA, Storey JD, Cobb JP, Warren HS, Moldawer LL, Herndon DN, Lowry SF, Maier RV, Davis RW, Tompkins RG. Inflammation and Host Response to Injury Large-Scale Collaborative Research Program. A genomic storm in critically injured humans. *J Exp Med*. 2011 Dec 19;208(13):2581-90.
22. Hietbrink F, Koenderman L, Rijkers G, Leenen L. Trauma: the role of the innate immune system. *World J Emerg Surg*. 2006 May 20;1:15.
23. Störmann P, Wagner N, Köhler K, Auner B, Simon TP, Pfeifer R, Horst K, Pape HC, Hildebrand F, Wutzler S, Marzi I, Relja B. Monotrauma is associated with enhanced remote inflammatory response and organ damage, while polytrauma intensifies both in porcine trauma model. *Eur J Trauma Emerg Surg*. 2020 Feb;46(1):31-42.
24. Hietbrink F, Koenderman L, Leenen LP. Intramedullary nailing of the femur and the systemic activation of monocytes and neutrophils. *World J Emerg Surg*. 2011 Oct 31;6:34.
25. Bakowitz M, Bruns B, McCunn M. Acute lung injury and the acute respiratory distress syndrome in the injured patient. *Scand J Trauma Resusc Emerg Med*. 2012 Aug 10;20:54.
26. Nuytink HK, Offermans XJ, Kubat K, Goris JA. Whole-body inflammation in trauma patients. An autopsy study. *Arch Surg*. 1988 Dec;123(12):1519-24.
27. Abraham E. Neutrophils and acute lung injury. *Crit Care Med*. 2003 Apr;31(4 Suppl):S195-9.
28. Lenz A, Franklin GA, Cheadle WG. Systemic inflammation after trauma. *Injury*. 2007 Dec;38(12):1336-45.
29. Pallister I, Dent C, Topley N. Increased neutrophil migratory activity after major trauma: a factor in the etiology of acute respiratory distress syndrome? *Crit Care Med*. 2002 Aug;30(8):1717-21.
30. Wickel DJ, Mercer-Jones M, Peyton JC, Shrotri MS, Cheadle WG. Neutrophil migration into the peritoneum is P-selectin dependent, but sequestration in lungs is selectin independent during peritonitis. *Shock*. 1998 Oct;10(4):265-9..
31. Rosales C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types?. *Front Physiol*. 2018;9:113. Published 2018 Feb 20.

32. Fortunati E, Kazemier KM, Grutters JC, Koenderman L, Van den Bosch vJ. Human neutrophils switch to an activated phenotype after homing to the lung irrespective of inflammatory disease. *Clin Exp Immunol*. 2009 Mar;155(3):559-66.
33. Munz MR, Faria MA, Monteiro JR, Aguas AP, Amorim MJ. Surgical porcine myocardial infarction model through permanent coronary occlusion. *Comp Med*. 2011 Oct;61(5):445-52.
34. Mendis S, Abegunde D, Yusuf S, Ebrahim S, Shaper G, Ghannem H, Shengelia B. WHO study on Prevention of REcurrences of Myocardial Infarction and Stroke (WHO-PREMISE). *Bull World Health Organ*. 2005 Nov;83(11):820-9. Epub 2005 Nov 10.
35. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER 3rd, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Wong ND, Woo D, Turner MB; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation*. 2014 Jan 21;129(3):e28-e292.
36. Okeny PK, Ongom P, Kituuka O. Serum interleukin-6 level as an early marker of injury severity in trauma patients in an urban low-income setting: a cross-sectional study. *BMC Emerg Med* 15, 22 (2015).
37. Almahmoud K, Namas RA, Abdul-Malak O, Zaaqoq AM, Zamora R, Zuckerbraun BS, Sperry J, Peitzman AB, Billiar TR, Vodovotz Y. Impact of Injury Severity on Dynamic Inflammation Networks Following Blunt Trauma. *Shock*. 2015 Aug;44(2):101-9.
38. Gross GJ, Hsu A, Gross ER, Falck JR, Nithipatikom K. Factors mediating remote preconditioning of trauma in the rat heart: central role of the cytochrome p450 epoxigenase pathway in mediating infarct size reduction. *J Cardiovasc Pharmacol Ther*. 2013 Jan;18(1):38-45.
39. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986 Nov;74(5):1124-36.
40. Lin HY, Huang CC, Chang KF. Lipopolysaccharide preconditioning reduces neuroinflammation against hypoxic ischemia and provides long-term outcome of neuroprotection in neonatal rat. *Pediatr Res*. 2009 Sep;66(3):254-9.
41. Gross GJ, Baker JE, Moore J, Falck JR, Nithipatikom K. Abdominal surgical incision induces remote preconditioning of trauma (RPCT) via activation of bradykinin receptors (BK2R) and the cytochrome P450 epoxigenase pathway in canine hearts. *Cardiovasc Drugs Ther*. 2011 Dec;25(6):517-22.
42. Kloner RA, Rezkalla SH. Preconditioning, postconditioning and their application to clinical cardiology. *Cardiovasc Res*. 2006 May 1;70(2):297-307.
43. Vallier HA, Wang X, Moore TA, Wilber JH, Como JJ. Timing of orthopaedic surgery in multiple trauma patients: development of a protocol for early appropriate care. *J Orthop Trauma*. 2013 Oct;27(10):543-51.
44. Pape HC, Giannoudis P, Krettek C. The timing of fracture treatment in polytrauma patients: relevance of damage control orthopedic surgery. *Am J Surg*. 2002 Jun;183(6):622-9.
45. Pape HC, Pfeifer R. Safe definitive orthopaedic surgery (SDS): repeated assessment for tapered application of Early Definitive Care and Damage Control?: an inclusive view of recent advances in polytrauma management. *Injury*. 2015 Jan;46(1):1-3.

46. Bläsius FM, Laubach M, Andruszkow H, Lichte P, Pape HC, Lefering R, Horst K, Hildebrand F; Trauma Register DGU®. Strategies for the treatment of femoral fractures in severely injured patients: trends in over two decades from the TraumaRegister DGU®. *Eur J Trauma Emerg Surg.* 2021 Feb 15:1–10.
47. Nahm NJ, Como JJ, Wilber JH, Vallier HA. Early appropriate care: definitive stabilization of femoral fractures within 24 hours of injury is safe in most patients with multiple injuries. *J Trauma.* 2011 Jul;71(1):175-85.
48. Szabó A, Varga R, Keresztes M, Vízler C, Németh I, Rázga Z, Boros M. Ischemic limb preconditioning downregulates systemic inflammatory activation. *J Orthop Res.* 2009 Jul;27(7):897-902.
49. Botha AJ, Moore FA, Moore EE, Sauaia A, Banerjee A, Peterson VM. Early neutrophil sequestration after injury: a pathogenic mechanism for multiple organ failure. *J Trauma.* 1995 Sep;39(3):411-7.
50. Ghebrehiwet B, Müller-Eberhard HJ. C3e: an acidic fragment of human C3 with leukocytosis-inducing activity. *J Immunol.* 1979 Aug;123(2):616-21.
51. Hernandez LA, Grisham MB, Twohig B, Arfors KE, Harlan JM, Granger DN. Role of neutrophils in ischemia-reperfusion-induced microvascular injury. *Am J Physiol.* 1987 Sep;253(3 Pt 2):H699-703.
52. Suratt BT, Petty JM, Young SK, Malcolm KC, Lieber JG, Nick JA, Gonzalo JA, Henson PM, Worthen GS. Role of the CXCR4/SDF-1 chemokine axis in circulating neutrophil homeostasis. *Blood.* 2004 Jul 15;104(2):565-71.
53. Gulack BC, Englum BR, Lo DD, Nussbaum DP, Keenan JE, Scarborough JE, Shapiro ML. Leukopenia is associated with worse but not prohibitive outcomes following emergent abdominal surgery. *J Trauma Acute Care Surg.* 2015 Sep;79(3):437-43.
54. Bastian OW, Kuijper A, Koenderman L, Stellato RK, van Solinge WW, Leenen LP, Blokhuis TJ. Impaired bone healing in multitrauma patients is associated with altered leukocyte kinetics after major trauma. *J Inflamm Res.* 2016 May 18;9:69-78.
55. Visser T, Pillay J, Pickkers P, Leenen LP, Koenderman L. Homology in systemic neutrophil response induced by human experimental endotoxemia and by trauma. *Shock.* 2012 Feb;37(2):145-51.
56. Relja B, Taraki R, Teuben MP, Mörs K, Wagner N, Wutzler S, Hildebrand F, Perl M, Marzi I. Sera from severe trauma patients with pneumonia and without infectious complications have differential effects on neutrophil biology. *BMC Pulm Med.* 2016 Dec 1;16(1):171.
57. Janicova A, Relja B. Neutrophil Phenotypes and Functions in Trauma and Trauma-Related Sepsis. *Shock.* 2021 Jul 1;56(1):16-29.
58. Leonard JM, Zhang CX, Lu L, Hoofnagle MH, Fuchs A, Clemens RA, Ghosh S, Hughes SW, Bochicchio GV, Hotchkiss R, Turnbull IR. Extrathoracic multiple trauma dysregulates neutrophil function and exacerbates pneumonia-induced lung injury. *J Trauma Acute Care Surg.* 2021 Jun 1;90(6):924-934
59. Gelbard RB, Hensman H, Schobel S, Stempora LL, Moris D, Dente CJ, Buchman TG, Kirk AD, Elster E. An integrative model using flow cytometry identifies nosocomial infection after trauma. *J Trauma Acute Care Surg.* 2021 Jul 1;91(1):47-53.
60. Störmann P, Auner B, Schimunek L, Serve R, Horst K, Simon TP, Pfeifer R, Köhler K, Hildebrand F, Wutzler S, Pape HC, Marzi I, Relja B; This study was performed within the study consortium of the TREAT Research Group. Leukotriene B4 indicates lung injury and on-going inflammatory changes after severe trauma in a porcine long-term model. *Prostaglandins Leukot Essent Fatty Acids.* 2017 Dec;127:25-31.

61. Hietbrink F, Koenderman L, van Wessem KJ, Leenen LP. The Impact of Intramedullary Nailing of Tibia Fractures on the Innate Immune System. *Shock*. 2015 Sep;44(3):209-14.
62. van Wessem KJ, Hennus MP, van Wagenberg L, Koenderman L, Leenen LP. Mechanical ventilation increases the inflammatory response induced by lung contusion. *J Surg Res*. 2013 Jul;183(1):377-84.
63. Neeley SP, Hamann KJ, White SR, Baranowski SL, Burch RA, Leff AR. Selective regulation of expression of surface adhesion molecules Mac-1, L-selectin, and VLA-4 on human eosinophils and neutrophils. *Am J Respir Cell Mol Biol*. 1993 Jun;8(6):633-9.
64. Botha AJ, Moore FA, Moore EE, Peterson VM, Goode AW. Base deficit after major trauma directly relates to neutrophil CD11b expression: a proposed mechanism of shock-induced organ injury. *Intensive Care Med*. 1997 May;23(5):504-9.
65. Johansson J, Sjögren F, Bodelsson M, Sjöberg F. Dynamics of leukocyte receptors after severe burns: an exploratory study. *Burns*. 2011 Mar;37(2):227-33.
66. Bhatia RK, Pallister I, Dent C, Jones SA, Topley N. Enhanced neutrophil migratory activity following major blunt trauma. *Injury*. 2005 Aug;36(8):956-62.
67. Spijkerman R, Hesselink L, Bongers S, van Wessem KJP, Vrisekoop N, Hietbrink F, Koenderman L, Leenen LPH. Point-of-Care Analysis of Neutrophil Phenotypes: A First Step Toward Immuno-Based Precision Medicine in the Trauma ICU. *Crit Care Explor*. 2020 Jul 17;2(7):e0158.
68. Huizinga TW, Roos D, von dem Borne AE. Neutrophil Fc-gamma receptors: a two-way bridge in the immune system. *Blood*. 1990 Mar 15;75(6):1211-4.
69. Seely AJ, Pascual JL, Christou NV. Science review: Cell membrane expression (connectivity) regulates neutrophil delivery, function and clearance. *Crit Care*. 2003 Aug;7(4):291-307.
70. White-Owen C, Alexander JW, Babcock GF. Reduced expression of neutrophil CD11b and CD16 after severe traumatic injury. *J Surg Res*. 1992 Jan;52(1):22-6.
71. Mommsen P, Barkhausen T, Hildebrand F, Zeckey C, Krettek C, van Griensven M. Regulation of L-selectin expression by trauma-relevant cytokines. *Pathol Res Pract*. 2011 Mar 15;207(3):142-7.
72. Maekawa K, Futami S, Nishida M, Terada T, Inagawa H, Suzuki S, Ono K. Effects of trauma and sepsis on soluble L-selectin and cell surface expression of L-selectin and CD11b. *J Trauma*. 1998 Mar;44(3):460-8.
73. Visser T, Hietbrink F, Groeneveld KM, Koenderman L, Leenen LP. Isolated blunt chest injury leads to transient activation of circulating neutrophils. *Eur J Trauma Emerg Surg*. 2011 Apr;37(2):177-84.
74. Orr Y, Taylor JM, Bannon PG, Geczy C, Kritharides L. Circulating CD10-/CD16low neutrophils provide a quantitative index of active bone marrow neutrophil release. *Br J Haematol*. 2005 Nov;131(4):508-19.
75. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers JW, Ulfman LH, Leenen LP, Pickkers P, Koenderman L. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest*. 2012 Jan;122(1):327-36.
76. Leliefeld PHC, Pillay J, Vrisekoop N, Heeres M, Tak T, Kox M, Rooijackers SHM, Kuijpers TW, Pickkers P, Leenen LPH, Koenderman L. Differential antibacterial control by neutrophil subsets. *Blood Adv*. 2018 Jun 12;2(11):1344-1355.
77. Kamp VM, Pillay J, Lammers JW, Pickkers P, Ulfman LH, Koenderman L. Human suppressive neutrophils CD16bright/CD62Ldim exhibit decreased adhesion. *J Leukoc Biol*. 2012 Nov;92(5):1011-20.

78. Sauce D, Dong Y, Campillo-Gimenez L, Casulli S, Bayard C, Autran B, Boddaert J, Appay V, Elbim C. Reduced Oxidative Burst by Primed Neutrophils in the Elderly Individuals Is Associated With Increased Levels of the CD16bright/CD62Ldim Immunosuppressive Subset. *J Gerontol A Biol Sci Med Sci*. 2017 Feb;72(2):163-172.
79. Wang J. Neutrophils in tissue injury and repair. *Cell Tissue Res*. 2018;371(3):531-539.
80. Chung R, Cool JC, Scherer MA, Foster BK, Xian CJ. Roles of neutrophil-mediated inflammatory response in the bony repair of injured growth plate cartilage in young rats. *J Leukoc Biol*. 2006 Dec;80(6):1272-80.
81. Shepherd JM, Cole E, Brohi K. Contemporary Patterns of Multiple Organ Dysfunction in Trauma. *Shock*. 2017 Apr;47(4):429-435.
82. Mortaz E, Alipoor SD, Adcock IM, Mumby S, Koenderman L. Update on Neutrophil Function in Severe Inflammation. *Front Immunol*. 2018 Oct 2;9:2171.
83. Visser T, Pillay J, Koenderman L, Leenen LP. Postinjury immune monitoring: can multiple organ failure be predicted? *Curr Opin Crit Care*. 2008 Dec;14(6):666-72.
84. Hildebrand F, Andruszkow H, Huber-Lang M, Pape HC, van Griensven M. Combined hemorrhage/trauma models in pigs-current state and future perspectives. *Shock*. 2013 Oct;40(4):247-73.
85. Hildebrand F, Weuster M, Mommsen P, Mohr J, Fröhlich M, Witte I, Keibl C, Ruchholtz S, Seekamp A, Pape HC, Flohe S, van Griensven M. A combined trauma model of chest and abdominal trauma with hemorrhagic shock--description of a new porcine model. *Shock*. 2012 Dec;38(6):664-70.
86. Grøgaard B, Gerdin B, Reikerås O. The polymorphonuclear leukocyte: has it a role in fracture healing? *Arch Orthop Trauma Surg*. 1990;109(5):268-71.
87. Andrew JG, Andrew SM, Freemont AJ, Marsh DR. Inflammatory cells in normal human fracture healing. *Acta Orthop Scand*. 1994 Aug;65(4):462-6.
88. Greven J, Horst K, Qiao Z, Bläsius FM, Mert Ü, Teuben MPJ, Becker NH, Pfeifer R, Pape HC, Hildebrand F. Fracture fixation strategy and specific muscle tissue availability of neutrophilic granulocytes following mono- and polytrauma: intramedullary nailing vs. external fixation of femoral fractures. *Eur J Med Res*. 2020 Nov 26;25(1):62.
89. Pape HC, Grimme K, Van Griensven M, Sott AH, Giannoudis P, Morley J, Roise O, Ellingsen E, Hildebrand F, Wiese B, Krettek C; EPOFF Study Group. Impact of intramedullary instrumentation versus damage control for femoral fractures on immunoinflammatory parameters: prospective randomized analysis by the EPOFF Study Group. *J Trauma*. 2003 Jul;55(1):7-13.
90. Griensven M, Krettek, Pape H. Immune reactions after trauma. *Eur J Trauma*. 2003.
91. Lux A, Nimmerjahn F. Of mice and men: the need for humanized mouse models to study human IgG activity in vivo. *J Clin Immunol*. 2013 Jan;33 Suppl 1:S4-8.
92. Fung YL, Silliman CC, Minchinton RM, Wood P, Fraser JF. Cardiopulmonary bypass induces enduring alterations to host neutrophil physiology: a single-center longitudinal observational study. *Shock*. 2008 Dec;30(6):642-8.
93. Ilton MK, Langton PE, Taylor ML, Misso NL, Newman M, Thompson PJ, Hung J. Differential expression of neutrophil adhesion molecules during coronary artery surgery with cardiopulmonary bypass. *J Thorac Cardiovasc Surg*. 1999 Nov;118(5):930-7.

94. Scannell G, Waxman K, Vaziri ND, Zhang J, Kaupke CJ, Jalali M, Hect C. Effects of trauma on leukocyte intercellular adhesion molecule-1, CD11b, and CD18 expressions. *J Trauma*. 1995 Oct;39(4):641-4.
95. Baëhl S, Garneau H, Le Page A, Lorrain D, Viens I, Svotelis A, Lord JM, Phillips AC, Cabana F, Larbi A, Dupuis G, Fülöp T. Altered neutrophil functions in elderly patients during a 6-month follow-up period after a hip fracture. *Exp Gerontol*. 2015 May;65:58-68.
96. Lund-Johansen F, Terstappen LW. Differential surface expression of cell adhesion molecules during granulocyte maturation. *J Leukoc Biol*. 1993 Jul;54(1):47-55.
97. Soligo D, Schiró R, Luksch R, Manara G, Quirici N, Parravicini C, Lambertenghi Delilieri G. Expression of integrins in human bone marrow. *Br J Haematol*. 1990 Nov;76(3):323-32.
98. Pape HC, Auf'm Kolk M, Paffrath T, Regel G, Sturm JA, Tscherne H. Primary intramedullary femur fixation in multiple trauma patients with associated lung contusion--a cause of posttraumatic ARDS? *J Trauma*. 1993 Apr;34(4):540-7; discussion 547-8.
99. Dunn RH, Jackson T, Burlew CC, Pieracci FM, Fox C, Cohen M, Campion EM, Lawless R, Mauffrey C. Fat emboli syndrome and the orthopaedic trauma surgeon: lessons learned and clinical recommendations. *Int Orthop*. 2017 Sep;41(9):1729-1734.
100. Pape HC, Remmers D, Kleemann W, Goris JA, Regel G, Tscherne H. Posttraumatic multiple organ failure--a report on clinical and autopsy findings. *Shock*. 1994 Sep;2(3):228-34.
101. Redl HR, Martin U, Khadem A, Pelinka LE, van Griensven M. Anti-L-selectin antibody therapy does not worsen the postseptic course in a baboon model. *Crit Care*. 2005;9(6):R735-44.
102. Smith CW, Marlin SD, Rothlein R, Toman C, Anderson DC. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. *J Clin Invest*. 1989 Jun;83(6):2008-17.
103. Hyun YM, Choe YH, Park SA, Kim M. LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) distinctly regulate neutrophil extravasation through hotspots I and II. *Exp Mol Med*. 2019 Apr 9;51(4):1-13.
104. Lichte P, Pfeifer R, Kobbe P, Tohidnezhad M, Pufe T, Almahmoud K, Hildebrand F, Pape HC. Inhalative IL-10 treatment after bilateral femoral fractures affect pulmonary inflammation in mice. *Ann Anat*. 2015 Jul;200:73-8.
105. Muehlstedt SG, Richardson CJ, Lyte M, Rodriguez JL. Systemic and pulmonary effector cell function after injury. *Crit Care Med*. 2002 Jun;30(6):1322-6.
106. Edderkaoui B. Potential Role of Chemokines in Fracture Repair. *Front Endocrinol (Lausanne)*. 2017 Mar 2;8:39.
107. Walsh DS, Thavichaigarn P, Pattanapanyasat K, Siritongtaworn P, Kongcharoen P, Tongtawe P, Yongvanitchit K, Jiarakul N, Dheeradhada C, Pearce FJ, Wiesmann WP, Webster HK. Characterization of circulating monocytes expressing HLA-DR or CD71 and related soluble factors for 2 weeks after severe, non-thermal injury. *J Surg Res*. 2005 Dec;129(2):221-30.
108. Claes L, Recknagel S, Ignatius A. Fracture healing under healthy and inflammatory conditions. *Nat Rev Rheumatol*. 2012 Jan 31;8(3):133-43.
109. Kolar P, Schmidt-Bleek K, Schell H, Gaber T, Toben D, Schmidmaier G, Perka C, Buttgeriet F, Duda GN. The early fracture hematoma and its potential role in fracture healing. *Tissue Eng Part B Rev*. 2010 Aug;16(4):427-34.

110. Grundnes O, Reikerås O. The importance of the hematoma for fracture healing in rats. *Acta Orthop Scand*. 1993 Jun;64(3):340-2.
111. Hoff P, Maschmeyer P, Gaber T, Schuetze T, Raue T, Schmidt-Bleek K, Dziurla R, Schellmann S, Lohanatha fL, Roehner E, Ode A, Burmester GD, Duda GN, Perka C, Buttgerit F. Human immune cells' behavior and survival under bioenergetically restricted conditions in an in vitro fracture hematoma model. *Cell Mol Immunol* 10, 151–158;2013.
112. Hauser CJ, Zhou X, Joshi P, Cuchens MA, Kregor P, Devidas M, Kennedy RJ, Poole GV, Hughes JL. The immune microenvironment of human fracture/soft-tissue hematomas and its relationship to systemic immunity. *J Trauma*. 1997 May;42(5):895-903.
113. Yang X, Ricciardi BF, Hernandez-Soria A, Shi Y, Pleshko Camacho N, Bostrom MP. Callus mineralization and maturation are delayed during fracture healing in interleukin-6 knockout mice. *Bone*. 2007 Dec;41(6):928-36.
114. Schmidt-Bleek K, Schell H, Kolar P, Pfaff M, Perka C, Buttgerit F, Duda G, Lienau J. Cellular composition of the initial fracture hematoma compared to a muscle hematoma: a study in sheep. *J Orthop Res*. 2009 Sep;27(9):1147-51.
115. Bastian O, Pillay J, Alblas J, Leenen L, Koenderman L, Blokhuis T. Systemic inflammation and fracture healing. *J Leukoc Biol*. 2011 May;89(5):669-73.
116. Hurst SM, Wilkinson TS, McLoughlin RM, Jones S, Horiuchi S, Yamamoto N, Rose-John S, Fuller GM, Topley N, Jones SA. IL-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. *Immunity*. 2001 Jun;14(6):705-14.
117. Dancey JT, Deubelbeiss KA, Harker LA, Finch CA. Neutrophil kinetics in man. *J Clin Invest*. 1976 Sep;58(3):705-15.
118. Eash KJ, Means JM, White DW, Link DC. CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. *Blood*. 2009 May 7;113(19):4711-9.
119. Furze RC, Rankin SM. Neutrophil mobilization and clearance in the bone marrow. *Immunology*. 2008 Nov;125(3):281-8.
120. Livingston DH, Anjaria D, Wu J, Hauser CJ, Chang V, Deitch EA, Rameshwar P. Bone marrow failure following severe injury in humans. *Ann Surg*. 2003 Nov;238(5):748-53.
121. Zhang H, Rodriguez S, Wang L, Wang S, Serezani H, Kapur R, Cardoso AA, Carlesso N. Sepsis Induces Hematopoietic Stem Cell Exhaustion and Myelosuppression through Distinct Contributions of TRIF and MYD88. *Stem Cell Reports*. 2016 Jun 14;6(6):940-956.
122. Marsh JC, Boggs DR, Cartwright GE, Wintrobe MM. Neutrophil kinetics in acute infection. *J Clin Invest*. 1967 Dec;46(12):1943-53.
123. Amos RJ, Deane M, Ferguson C, Jeffries G, Hinds CJ, Amess JA. Observations on the haemopoietic response to critical illness. *J Clin Pathol*. 1990 Oct;43(10):850-6.
124. Fuchs A, Monlish DA, Ghosh S, Chang SW, Bochicchio GV, Schuettpeiz LG, Turnbull IR. Trauma Induces Emergency Hematopoiesis through IL-1/MyD88-Dependent Production of G-CSF. *J Immunol*. 2019 May 15;202(10):3020-3032.
125. Scumpia PO, Kelly-Scumpia KM, Delano MJ, Weinstein JS, Cuenca AG, Al-Quran S, Bovio I, Akira S, Kumagai Y, Moldawer LL. Cutting edge: bacterial infection induces hematopoietic stem and progenitor cell expansion in the absence of TLR signaling. *J Immunol*. 2010 Mar 1;184(5):2247-51.

126. Boettcher S, Manz MG. Regulation of Inflammation- and Infection-Driven Hematopoiesis. *Trends Immunol.* 2017 May;38(5):345-357.
127. Martin C, Burdon PC, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity.* 2003 Oct;19(4):583-93.
128. Mak IW, Evaniew N, Ghert M. Lost in translation: animal models and clinical trials in cancer treatment. *Am J Transl Res.* 2014 Jan 15;6(2):114-8.
129. Justice MJ, Dhillon P. Using the mouse to model human disease: increasing validity and reproducibility. *Dis Model Mech.* 2016 Feb;9(2):101-3.
130. Tsukamoto T, Pape HC. Animal models for trauma research: what are the options? *Shock.* 2009 Jan;31(1):3-10.
131. Hauser CJ. Preclinical models of traumatic, hemorrhagic shock. *Shock.* 2005 Dec;24 Suppl 1:24-32.
132. Lunney JK. Advances in swine biomedical model genomics. *Int J Biol Sci.* 2007 Feb 10;3(3):179-84.
133. Lunney JK, Walker K, Goldman T, Aasted B, Bianchi A, Binns R, Licence S, Bischof R, Brandon M, Blecha F, et al. Overview of the First International Workshop to Define Swine Leukocyte Cluster of Differentiation (CD) Antigens. *Vet Immunol Immunopathol.* 1994 Oct;43(1-3):193-206.
134. Saalmüller A, Pauly T, Lunney JK, Boyd P, Aasted B, Sachs DH, Arn S, Bianchi A, Binns RM, Licence S, Whyte A, Blecha F, Chen Z, Chu RM, Davis WC, Denham S, Yang H, Whittall T, Parkhouse RM, Dominguez J, Ezquerro A, Alonso F, Horstik G, Howard C, Zuckermann F, et al. Overview of the Second International Workshop to define swine cluster of differentiation (CD) antigens. *Vet Immunol Immunopathol.* 1998 Jan 30;60(3-4):207-28.
135. Haverson K, Saalmüller A, Alvarez B, Alonso F, Bailey M, Bianchi AT, Boersma WJ, Chen Z, Davis WC, Dominguez J, Engelhardt H, Ezquerro A, Grosmaire LS, Hamilton MJ, Hollemweguer E, Huang CA, Khanna KV, Kuebart G, Lackovic G, Ledbetter JA, Lee R, Llanes D, Lunney JK, McCullough KC, Molitor T, Nielsen J, Niewold TA, Pescovitz MD, de la Lastra JM, Rehakova Z, Salmon H, Schnitzlein WM, Seebach J, Simon A, Sinkora J, Sinkora M, Stokes CR, Summerfield A, Sver L, Thacker E, Valpotic I, Yang H, Zuckermann FA, Zwart R. Overview of the Third International Workshop on Swine Leukocyte Differentiation Antigens. *Vet Immunol Immunopathol.* 2001 Jul 20;80(1-2):5-23.
136. Deloizy C, Bouguyon E, Fossum E, Sebo P, Osicka R, Bole A, Pierres M, Biacchesi S, Dalod M, Bogen B, Bertho N, Schwartz-Cornil I. Expanding the tools for identifying mononuclear phagocyte subsets in swine: Reagents to porcine CD11c and XCR1. *Dev Comp Immunol.* 2016 Dec;65:31-40.
137. Gerner W, Talker SC, Koinig HC, Sedlak C, Mair KH, Saalmüller A. Phenotypic and functional differentiation of porcine $\alpha\beta$ T cells: current knowledge and available tools. *Mol Immunol.* 2015 Jul;66(1):3-13.
138. Piriou-Guzylack L, Salmon H. Membrane markers of the immune cells in swine: an update. *Vet Res.* 2008 Nov-Dec;39(6):54.
139. Dawson HD, Lunney JK. Porcine cluster of differentiation (CD) markers 2018 update. *Res Vet Sci.* 2018 Jun;118:199-246.
140. Grottke O, Braunschweig T, Philippen B, Gatzweiler KH, Gronloh N, Staat M, Rossaint R, Tolba R. A new model for blunt liver injuries in the swine. *Eur Surg Res.* 2010;44(2):65-73.

141. Grottke O, Braunschweig T, Henzler D, Coburn M, Tolba R, Rossaint R. Effects of different fibrinogen concentrations on blood loss and coagulation parameters in a pig model of coagulopathy with blunt liver injury. *Crit Care*. 2010;14(2):R62

SAMENVATTING IN HET NEDERLANDS

Neutrofiele granulocyten en systemische inflammatie

Neutrofiele granulocyten zijn de meest voorkomende immuuncellen van het aangeboren afweersysteem in perifeer bloed. Deze cellen zijn essentieel voor het verloop van de immuunreactie tegen binnendringende pathogenen zoals bacteriën. Neutrofielen beschikken over een breed scala aan mogelijkheden om deze pathogenen te bestrijden.

Naast de mogelijkheden die direct gericht zijn op het uitschakelen van pathogenen, zijn neutrofielen ook belangrijke cellen voor het samenspel tussen andere onderdelen van het immuunsysteem. Door het uitscheiden van signaalstoffen (zoals cytokines/interleukines) sturen neutrofielen de lokale immuunreactie tegen binnendringers aan. Klinische kenmerken van deze lokale inflammatoire response zijn *calor* (warmte), *dolor* (pijn), *rubor* (roodheid), *tumor* (zwellings) en *funtio laesa* (verstoorde functie). Dit zijn typische kenmerken van een lokale infectie.

Bovendien kan bij een uitgebreidere invasie door pathogenen ook een systemische immuunreactie geactiveerd worden. Hierbij beperkt de ontstekingsreactie zich niet meer uitsluitend tot de locatie van binnendringen van de pathogenen, maar is het gehele organisme betrokken. Klinisch wordt een systemische immuunreactie gedefinieerd als een Systemic Inflammatory Response Syndrome (SIRS)-reactie. We spreken van een SIRS-response indien individuen voldoen aan 2 of meer van de volgende criteria:

- i. afwijkende lichaamstemperatuur ($<36^{\circ}\text{C}$ of $>38^{\circ}\text{C}$),
- ii. tachycardie (hartfrequentie $> 90/\text{min}$),
- iii. tachypnoe (> 20 ademhalingen/min) of hypocapnie (arteriële koolzuurspanning $< 4,3$ kPa),
- iv. afwijkend aantal leukocyten in het bloed ($<4 \times 10^9$ of $>12 \times 10^9$ of $>10\%$ staafvormige cellen).

Behalve door het binnendringen van bacteriën (d.w.z. infectie) kan het immuunsysteem ook middels andere triggers geactiveerd worden. Infarcten, obstructie en reperfusie van vaten, brandwonden maar ook fracturen en orgaanletsels resulteren in een immuun-/ontstekingsreactie uit. Bacteriële infecties variëren van een kleine infecties tot een zware

(long)ontsteking, zo variëren letsels ook in intensiteit, alsmede de daaropvolgende immuunreactie. Een kleine snijwond in een vinger vereist een beperkte lokale immuunreactie, die primair het binnendringen van bacteriën dient te voorkomen en bovendien de wondgenezing stimuleert. Aan de andere kant van het spectrum is er de multitraumapatiënt met een groot aantal wonden, breuken en letsels van inwendige organen. Bij de laatstgenoemde patiënt zal de immuunreactie zich niet beperken tot de lokale omgeving van het letsel, maar wordt een systemische immuunreactie gezien.

Hierbij bestaat bovendien het risico dat de immuunreactie ongecontroleerd verloopt en in plaats van de genezing bevordert, resulteert in levensbedreigende inflammatoire complicaties zoals orgaanfalen en Acute Respiratory Distress Syndrome (ARDS). ARDS is een frequente doodsoorzaak bij trauma patiënten en het overlijdenspercentage van dit ziektebeeld is in de afgelopen decennia helaas niet verbeterd. ARDS is een multifactorieel ziektebeeld waarbij een ontspoorde immuunreactie essentieel is.

Neutrofielen en de ontspoorde systemische immuunreacties na trauma

Neutrofielen spelen een grote rol bij het ontstaan van een ontspoorde immuunreactie. Dit is onder andere aangetoond door studies bij trauma patiënten die door long falen zijn overleden. De histologische analyses van longweefsel (na overlijden) bij deze individuen, worden gekenmerkt door een overmatige aanwezigheid van neutrofielen in de longen. Dit gebeurde zelfs in patiënten die geen directe letsels van de longen hadden opgelopen.

Bovendien zijn bij traumapatiënten zowel het voorkomen van verhoogde, als van verlaagde, waarden van leukocyten (lees: neutrofielen) in het bloed geassocieerd met een gecompliceerd beloop. Het onderscheid tussen een normale en een abnormale hoeveelheid neutrofielen die in na trauma in het bloed voorkomen, is echter op dit moment niet duidelijk. Deze thesis heeft middels experimentele trauma studies, de veranderingen van het aantal neutrofielen en karakteristieken van deze cellen in bloed en weefsel, onderzocht. Door gebruik te maken van een gevalideerd varkensmodel zijn vroege (<72 uur) neutrofiel reacties onderzocht. Terwijl een rattenmodel ontwikkeld is om de late (>72 uur) neutrofiel reacties op een gecontroleerde wijze te onderzoeken. Aan de basis van deze projecten staat een samenwerking met verschillende onderzoeksgroepen, zowel in nationaal als in internationaal verband. Validatie van laboratoriumprotocollen voor varkensstudies zijn in samenwerking met cardiologische experimenten gerealiseerd.

Behalve het doorvoeren van validatie experimenten voor trauma studies hebben deze eerste (cardiologische) varkensstudies van de auteur van dit proefschrift ook andere relevante bevindingen opgeleverd. Deze experimenten (**Hoofdstuk 2**) hebben aangetoond dat myocard ischemie geassocieerd is met een toename van neutrofielen in bloed. Bovendien is een intensiever model, namelijk ischemie inductie na een sternotomie (chirurgische insnijding van de borstkas door het sternum), geassocieerd met een nog sterkere toename van circulerende aantallen neutrofielen. Een sternotomie resulteert tevens in een sterkere systemische troponine response, dan minimaal-invasieve geïnduceerde ischemie. Microscopisch onderzoek van hartweefsel liet echter zien dat de omvang van het uiteindelijke infarct kleiner was na sternotomie dan na een minimaal-invasief geïnduceerd myocardinfaarct. Dit effect wordt toegewezen aan het zogenoemde *preconditioneren* van eindorgaan weefsel (myocard). Een fenomeen dat ook voor de traumatologie een interessante therapeutische techniek zou kunnen vertegenwoordigen.

In aanvulling op deze bevindingen werd in dit cardiologische model ook de mobilisatie van neutrofiel-subtypen waargenomen. In tegenstelling tot hetgeen over het algemeen wordt aangenomen, behoren neutrofielen niet tot een homogene populatie van cellen. Integendeel, tijdens acute inflammatie kunnen verschillende subtypen van neutrofielen onderscheiden worden. Deze subtypen zijn in het verleden tijdens experimentele bacteriemie in gezonde vrijwilligers door Pillay et al. geïdentificeerd en getypeerd. De verschillende subtypen zijn tijdens normale (homeostatische) omstandigheden vrijwel afwezig in bloed en verschijnen plotseling tijdens inflammatoire situaties. De subtypen laten zich onderscheiden door specifieke cel oppervlakte expressie van CD16 (FcγRIII) en CD62L (L-selectin). Bovendien hebben de, in totaal drie, verschillende populaties specifieke morfologische kenmerken, een specifiek patroon van mobilisatie en functionele eigenschappen. Hierdoor mag ook gesproken worden van drie verschillende fenotypen. In afwezigheid van inflammatie zijn hoofdzakelijk volwassen/mature neutrofielen (CD16^{hoog}/CD62L^{hoog}) in het bloed aanwezig. Na ernstig letsel (**Hoofdstuk 4**) verschijnen ondanks afnemende circulerende neutrofielen aantallen, twee andere subtypen in toenemende mate in de circulatie. De meest prominent toenemende neutrofiel populatie betreft bandvormige/jonge neutrofielen (CD16^{laag}/CD62L^{hoog}). De waargenomen verschuivingen en depletie van regulaire/mature neutrofielen worden over de tijd prominenter. Na drie uur opereren is er sprake van een uitgebreide depletie van de hoeveelheid beschikbare mature neutrofielen. Statistisch significante verschillen worden al na meer dan één uur opereren waargenomen. De observaties uit deze studie vormen een basis voor

damage control chirurgie in ernstig meervoudig letsel waarbij het doel is om de operatietijd tot maximaal een uur te beperken (tot 60 minuten) in acute leven bedreigde traumapatiënten.

In **Hoofdstuk 3** van dit proefschrift werd in een experimentele varkensstudie na monotrauma en operatieve interventie, echter geen toenemende heterogeniteit van de neutrofielen populatie in bloed gevonden. Maar uit deze monotrauma-studie bleek dat anesthesie en mechanische beademing in varkens resulteert in een snelle toename van neutrofielen-aantallen in bloed (binnen 24 uur). Deze initiële toename ging vooraf aan in een latere daling dan circulerende neutrofielen. Drie dagen na interventie is het aantal neutrofielen in bloed duidelijk lager dan tijdens homeostatische condities. Bovendien veranderde de expressie van relevante cel-receptoren op deze cellen (CD11b/Mac-1, CD16/FcγRIII/CD62L) niet statistisch significant na anesthesie en mechanische beademing. In een andere onderzoeksgroep werd na aanvullende fractuurinductie en mergpenfixatie een vergelijkbaar patroon veranderende neutrofielen aantallen in het bloed waargenomen. Daarentegen waren de karakteristieken van de circulerende neutrofielen in het bloed duidelijk veranderd ten opzichte van de uitgangsmetingen en de interventie conditie met alleen anesthesie en mechanische ventilatie. Een dag na interventie was er namelijk een duidelijke toename van de oppervlakte expressie van CD11b en CD16-receptoren op circulerende neutrofielen.

Zodoende suggereren deze studies dat het verschijnen van verschillende neutrofiel-subtypen geassocieerd is met ernstig trauma/polytrauma, in plaats van met monotrauma. Monotrauma in een experimentele setting resulteert echter in veranderende expressiepatronen van relevante oppervlaktereceptoren op neutrofielen. De rol, herkomst en eigenschappen van de verschillende neutrofiel-subtypen is nog onduidelijk, hetgeen in toekomstige studies verder onderzocht dient te worden.

Hoofdstuk 5 beschrijft karakteristieken van neutrofielen na monotrauma in ratten tijdens een observatieperiode van 14 dagen. Deze studie bevestigt eerdere observaties betreffende neutrofielen kinetiek in het bloed van andere dierexperimentele studies na myocardinfarct (**Hoofdstuk 2**) en monotrauma (**Hoofdstuk 3**). In de rattenstudie van **Hoofdstuk 5** werd namelijk ook, na 72 uur, een relatieve reductie waargenomen van neutrofielenaantallen in het bloed. Daarna normaliseerden de neutrofielenaantallen zich tijdens de eerste 14 dagen. Eenzelfde patroon is ook meermaals door anderen beschreven in humane studies en dit benadrukt de translationele waarde van de door ons ontwikkelde ratten- en varkensmodellen

voor studies naar inflammatie bij trauma. Bovendien is in **Hoofdstuk 5** voor het eerst de simultane veranderingen van oppervlaktereceptoren op circulerende neutrofielen beschreven in een experimentele studie. Simultaan met de neutrofiel depletie fase (dag 3 na trauma) bestond ook een duidelijke afname van CD11b-expressie op circulerende neutrofielen. Dit suggereert een verminderde activatie van neutrofielen. Een week na trauma werd er bovendien een nieuw subtype van neutrofielen in het bloed van ratten waargenomen. Deze neutrofielen kenmerken zich door hoge expressie-niveaus van CD11b en CD11a op het membraan van circulerende cellen. Verdere karakterisering van deze cellen en de eventuele immuun modulerende rol na trauma dient focus van nieuwe onderzoeksprojecten te zijn.

Langetermijn experimentele observatiestudies in een rattenmodel met monotrauma tonen overeenkomstige resultaten met humane studies omtrent kinetiek van neutrofielen-aantallen in het bloed. Bovendien blijkt de fase waarin neutrofielen aantallen gereduceerd zijn, samen te gaan met verminderde activatiestatus van de desbetreffende cellen. Tijdens de herstelfase, die daarna optreedt, wordt (na 7 dagen) een nieuw subtype van circulerende neutrofielen in het bloed waargenomen. De rol en herkomst van deze cellen is op dit moment nog niet duidelijk.

Lokale inflammatie

Neutrofielen spelen ook een belangrijke rol in de fractuurgenezing. Fractuurgenezing verloopt volgens een vast patroon waarbij verschillende fases elkaar opvolgen. Experimenteel onderzoek heeft aangetoond dat de eerste fases essentieel zijn voor adequate fractuurgenezing. Tijdens deze fase zijn neutrofielen de meest voorkomende immuuncellen in vers fractuurhematoom. De hoeveelheid en specifieke eigenschappen van deze neutrofielen hebben een belangrijke invloed op de verdere fractuurconsolidatie. In polytrauma patiënten is een afwijkende hoeveelheid neutrofielen geassocieerd met het optreden van suboptimale fractuurgenezing. De rol van fractuurbehandelingsstrategieën op de hoeveelheid en kwaliteit van immuun cellen in het vroege fractuurhematoom was onderwerp van studie in **Hoofdstuk 7**. Door gebruik te maken van een gevalideerd monotrauma model in varkens werd aangetoond dat de toegepaste fractuurbehandelingsmethode invloed heeft op de samenstelling van de immuuncelpopulatie in vers fractuurhematoom. De samenstelling van vers fractuurhematoom in een varkensmodel komt overeen met de humane situatie. Eveneens bleek uit de

varkensstudie dat ook de lokale temperatuur, hetgeen afhankelijk is van de lokaal gegenereerde energie door de toegepaste behandelingsmethode, samenhangt met de cellulaire immunologische samenstelling van fractuurhematoom. Het toepassen van een reamed-irrigation en aspiratie (RIA) techniek, vóór het invoeren van de uiteindelijke intraossale stabilisatie is geassocieerd met een meer vitale immuuncelpopulatie, alsmede een minder geactiveerde status van neutrofielen in fractuurhematoom, dan alternatieve operatietechnieken. Deze alternatieve, doch gangbare technieken betreffen de niet geboorde intramedullaire osteosynthese en de opgeboorde intramedullaire osteosynthese.

De toegepaste fractuurbehandeltechniek beïnvloedt de receptor-expressie van oppervlakte-receptoren op neutrofielen in vers fractuurhematoom. Het toepassen van een aanvullende irrigatie-aspiratie techniek (RIA) is geassocieerd met een geoptimaliseerde vitaliteit van immuuncellen in fractuurhematoom alsmede een verminderde activatiestatus van neutrofielen. De nieuwe inzichten verkregen uit een monotrauma varkensmodel onderschrijven de meerwaarde van het routinematig toepassen van RIA-ondersteunde intramedullaire osteosynthesen bij patiënten met een verhoogd risico op een gecompliceerde fractuurgenezing.

Remote inflammatie

Behalve lokale en systemische inflammatie bestaat ook nog een zogeheten remote inflammatie, ofwel inflammatie 'op afstand'. Remote inflammatie wordt gekenmerkt door het voorkomen van ontstekingsprocessen op locaties in het lichaam die niet direct uitgelokt worden door lokale triggers (letsels). Een relevant voorbeeld van remote inflammatie is een ontstekingsreactie in de longen van multitrauma patiënten, zonder dat de longen direct letsel hebben opgelopen door het ongeval. Het exacte verband tussen polytrauma en het latere ontstaan van longfalen, in afwezigheid van directe thoraxletsels, is op dit moment nog onduidelijk. Neutrofielen lijken echter een belangrijke rol te spelen. Opvallend is bovendien dat trauma patiënten met fracturen van lange botten (zoals dijbeenbreuken) een duidelijk verhoogde kans hebben op het ontwikkelen van longfalen. Tevens is een mergpinfixatie (als operatieve verzorging) geassocieerd met het meer voorkomen van inflammatoire longcomplicaties. Om meer inzicht te verkrijgen in het verband tussen fracturen van grote botten, mergpinfixatie van de

eerdergenoemde fracturen en pulmonale complicaties, is in **Hoofdstuk 6** van deze thesis een experimentele rattenstudie doorgevoerd. De resultaten suggereren dat een mergpinfixatie en een unilaterale dijbeenfractuur geassocieerd is met een toegenomen influx van neutrofielen in de longen, alsmede een specifiek patroon van activatie van pulmonale neutrofielen. Studie van de celmembraanexpressie van CD11b/Mac-1 op pulmonale neutrofielen middels flowcytometrie, van verschillende longcompartimenten, heeft een aantal relevante bevindingen opgeleverd. Ten eerste, werd drie dagen na trauma, net als reeds in de literatuur beschreven, een piek van pulmonale neutrofielen aantallen gezien. Ten tweede, werd aangetoond dat de neutrofielenpopulatie in de longen, drie dagen na fractuur-inductie een duidelijk verhoogde activatiestatus van deze cellen kent. Ten derde, bleek dat na de piek (op het drie dagen tijdpunt na trauma), zowel het aantal neutrofielen als de activatiestatus van neutrofielen in rattenlongen weer afneemt. Deze afname zet zich voort. Na twee weken zijn beide eerdergenoemde factoren (neutrofielen aantal en activatiestatus) bijna op het niveau van homeostatische condities. Tevens zijn in deze studie de neutrofielenkarakteristieken in verschillende compartimenten van de long met elkaar vergeleken. Hieruit bleek dat de oppervlaktereceptoren expressieniveaus van belangrijke receptoren sterk verschillen tussen neutrofielen in longparenchym en neutrofielen uit de broncho-alveolaire ruimte. Dit is mogelijk het gevolg van een biologisch functie van de onderzochte receptoren. Hetgeen in de toekomst een mogelijk aangrijpingspunt voor interventies vertegenwoordigt. Cellulaire studies van de longen zijn over het algemeen gebaseerd op weefsel dat verkregen wordt door een broncho-alveolaire lavage (BAL). Aangezien aangenomen wordt dat neutrofielen die zich daadwerkelijk in de long (parenchym) bevinden ook een belangrijk rol spelen in de pulmonale inflammatie, wordt door het enkel en alleen onderzoeken van BAL, mogelijk veel relevante informatie over long-neutrofielen gemist. Het is daarom het overwegen waard om de toepassing van alternatieve (en meer inclusieve) spoeling methoden (bijv. met anticoagulantia) of andere samplingtechnieken (biopsie) te testen bij toekomstige experimentele studies.

In een rattenstudie naar de pulmonaire neutrofielenresponse werd drie dagen na trauma een relatieve toename van pulmonale neutrofielen in longparenchym waargenomen. Simultaan bestond een toegenomen activatiestatus van parenchymale neutrofielen. Het verdere verloop werd gekenmerkt door een graduele afname van zowel het aantal neutrofielen in de long, alsmede de activatiestatus. Deze nieuwe inzichten kunnen de basis vormen voor nieuwe differentieerde therapieconcepten.

Systemische inflammatie na trauma en de beenmerg-response

In eerdergenoemde experimentele studies is duidelijk geworden dat neutrofielen-aantallen in het bloed veranderen na trauma. Enerzijds wordt een afname direct na trauma waargenomen, anderzijds wordt een toename van circulerende neutrofielen gezien na trauma in andere studies. De balans tussen de aanvoer van circulerende neutrofielen en het verlaten van de bloedbaan van neutrofielen bepaalt het uiteindelijke aantal van circulerende cellen. De aanvoer van neutrofielen is grotendeels afhankelijk van de productie en het vrijkomen van cellen uit het beenmerg. Naar aanleiding van de bevindingen uit **Hoofdstuk 2** (massale depletie van circulerende neutrofielen na trauma) heeft de auteur van dit proefschrift een nieuwe studie naar de parallelle beenmerg reactie op trauma uitgevoerd. Eenzelfde traumamodel als in **Hoofdstuk 2** gebruikt is, werd ditmaal aangewend om het beenmergcompartiment te onderzoeken. Beenmergpuncties voor **Hoofdstuk 8** werden verricht op twee tijdstippen: namelijk vóór, en na drie uur traumachirurgie. Gebaseerd op de literatuur werd verondersteld dat de samenstelling van de neutrofielenpopulatie in beenmerg na drie uur zeer intensieve traumachirurgie veranderd. De resultaten van deze studie waren dat het relatieve aantal neutrofielen na trauma (t.o.v. andere immuun cellen in het beenmerg) na trauma toegenomen was. Bovendien werd aangetoond dat de neutrofielen-populatie in beenmerg andere karakteristieken had dan onder homeostatische condities. Zo was het receptor-expressie patroon van oppervlaktereceptoren op de neutrofielenpopulatie in het beenmerg na trauma, suggestief voor een relatief oudere populatie van cellen dan voor trauma-inductie het geval was. Opvallend was bovendien dat er na drie uur traumachirurgie sprake was van een relatieve toename van met name een specifieke neutrofielen populatie: namelijk cellen met een verhoogde expressie van CD184/CXCR4. Neutrofielen met een hoge expressie van CXCR4 zijn door andere ondergroepen beschreven als remigrerende (terugkerende) neutrofielen. Daarmee is de studie van Hoofdstuk 8 suggestief voor verhoogde terugkeer van neutrofielen uit bloed naar het beenmerg ten tijde van extreme inflammatie door traumachirurgie.

De beenmergresponse op zeer intensief trauma wordt gekenmerkt door een veranderende samenstelling van de neutrofielen populatie in het beenmerg. Concreet neemt de heterogeniteit van de medullaire neutrofielen populatie toe en neemt het aantal neutrofielen toe ten opzichte van andere immuun cellen. Bovendien zijn aanvullende analyses suggestief voor het meer volwassen worden van de medullaire neutrofielen populatie na trauma. Tevens bleek er sprake van een toegenomen aanwezigheid van een specifiek type neutrofielen in het

beenmerg. Deze cellen zijn vermoedelijk uit het bloed teruggekeerde neutrofielen. Hiermee beschrijft deze experimentele varkensstudie als eerste in detail de simultane beenmerg response op extreem trauma, hetgeen de basis kan vormen voor nieuwe diagnostische en therapeutische innovaties voor trauma.

Translationele implicaties van ratten en varkens experimenten voor de humane situatie

De bovengenoemde bevindingen dienen enigszins met voorzichtigheid geïnterpreteerd te worden aangezien alle experimenten in niet-primaten zijn doorgevoerd. Door gebruik te maken van varkens- en rattenmodellen bestond de mogelijkheid om experimenten te standaardiseren, hetgeen in humane traumastudies een groot obstakel is. Toetsing van de translationele waarde van onze modellen was niet het hoofdthema van deze thesis. Alleszins zijn de implicaties van de gebruikte modellen voor de humane situaties steeds gereflecteerd en benoemd in de discussiesectie van de verschillende manuscripten. Op basis van de literatuur wordt het varken gezien als het meest optimale model (primaten-modellen daargelaten) om trauma-studies te verrichten. Deze aanname is gebaseerd op overeenkomsten op het vlak van anatomie, fysiologie, dimensions, farmacotherapeutische effecten, alsmede immunologie. De bevindingen uit onze studies sluiten hierbij aan. Concreet zijn vele gelijkenissen te zien betreffende neutrofielen kinetiek in humane studies en experimentele varkensstudies van de auteur. Aangezien een langere observatietijd als 72 uur op dit moment voor multitrauma modellen voor het varken nog niet mogelijk waren, werd soms uitgeweken naar rattenmodellen. Ook bij rattenstudies zijn veel parallellen gevonden tussen humane en ratten-data. Dit onderschrijft de geschiktheid van beide experimentele modellen om in de toekomst interventies studies uit te voeren waarbij cellulaire facetten van de immuunreactie onderwerp van studies zijn.

REVIEW COMMITTEE

Prof. dr. H.P. Haagsman

Prof. dr. J.A.G. van Strijp

Prof. dr. F.C. Oner

Prof. dr. F. van Wijk

Prof. dr. I. Marzi

ACKNOWLEDGMENTS (Dankwoord)

Trauma is teamwork, net zoals onderzoek. Dit proefschrift, alsmede de vele projecten, publicaties en presentaties die dit proefschrift niet gehaald hebben zijn het resultaat van teamwork. Veel personen hebben in meer of mindere mate een belangrijke rol gespeeld in de totstandkoming van dit boekje. Graag wil ik een aantal personen uitlichten. Ook wil ik mij verontschuldigen als ik daarbij iemand vergeten ben.

Professor Dr. Leenen,

Als tweedejaars geneeskundestudent heb ik u leren kennen en vanaf de eerste dag was ik onder de indruk van uw bevoegdheid. Dit is altijd zo gebleven. De wekelijkse meetings om 6.30 gaven mij als student soms stress, maar ook structuur. De lat lag altijd hoog, niet alleen bij het onderzoek maar ook in de patiëntenzorg, het onderwijs en de organisatie van de zorg voor traumapatiënten - regionaal, nationaal en internationaal. Ik ben dankbaar dat ik mee heb mogen kijken naar hoe u al die ballen succesvol in de lucht weet te houden.

Professor Dr. Koenderman,

Ik ben blij dat ik de kans heb gekregen om experimenteel, oftewel 'echt' onderzoek, te mogen doen. Bedankt voor alle uitleg over laboratoriumonderzoek, celbiologie en alle achtergronden die mij als niet-bioloog in den beginne allemaal vreemd waren. Nog meer wil ik je bedanken voor de ondersteuning aan het einde van het promotietraject, bij de interpretatie van de onderzoeksresultaten en het corrigeren van de manuscripten.

Sehr geehrter Herr. Prof. Dr. Pape,

seit ich Sie in Aachen kennengelernt habe, bin ich beeindruckt von ihrer Arbeitsweise. Ich möchte mich für Ihre Unterstützung in Aachen bedanken sowie für die Möglichkeit, mit Ihnen nach Zürich zu kommen. Auch bin ich sehr dankbar für Ihr Vertrauen in mich - als Forscher und als Arzt. Sie haben mir ermöglicht meine Ziele zu erreichen; sowohl auf der Arbeit wie auch privat. Ich freue mich auf die weitere Zusammenarbeit am USZ.

Dear members of the thesis assessment committee, Prof. Haagsman, Prof. Van Strijp, Prof. Oner, Prof. Van Wijk, Prof. Marzi, thank you for your time and for assessing the quality of this thesis.

Sehr geehrter Prof. Dr. Pfeifer, lieber Roman,
ich freue mich sehr, Dich zum richtigen Zeitpunkt kennengelernt zu haben. Du hast mir weitergeholfen, viele angefangene Projekte in Manuskripte umzuwandeln. Deine Effizienz ist einzigartig und ich freue mich, täglich mit dir als Kollege zusammenarbeiten zu dürfen. Du bist ein super Kollege und noch besserer Freund. Ich freue mich auf weitere gemeinsame Projekte, Reisen oder Thanksgiving mit deiner Familie.

Dr. Blokhuis, beste Taco,

Jij was vanaf het begin van mijn PhD-project betrokken bij de opbouw en organisatie van de experimentele studies. Ik wil je bedanken voor de hulp bij het schrijven en corrigeren van de eerste artikelen en met name de stukken over de milt. Bovendien ben ik je erg dankbaar voor de mogelijkheden die je hebt geboden om onderzoek te doen bij de DSTC-Cursussen in Nijmegen. Gelukkig zien we elkaar nog regelmatig op verschillende plekken in Europa.

Het trauma en longenlab in Utrecht: Marjolein Heeres, Pieter Leliefeld, Okan Bastian, Janesh Pillay, Falco Hietbrink, Nienke Vrisekoop, Jan van der Linden, Leo Houben, Corneli van Aalst, Deon Kanters, Karin Kazemir, Tamar Tak, Erinke van Grinsven, Bart Hilvering, Susanne Vijverberg en Vera Kamp. Allemaal hebben we de neutrofiel weten te omarmen en een aantal van ons hebben de neutrofiel noodgedwongen ook weer een beetje los moeten laten. Ik ben mij ervan bewust dat de complexiteit en met name het internationale karakter van mijn studies regelmatig voor een volksverhuizing hebben gezorgd. Mijn excuses voor de *collateral damage* en bedankt voor de ondersteuning bij het tot een goed einde brengen van mijn gevecht met de neutrofiel.

Collegae promovendi uit de verschillende laboratoria in het UMC Utrecht, dank voor de gezelligheid en het delen van jullie kennis tijdens de officiële meetings en meer nog tijdens de vele koffiemomenten en lunches. Ook het sociale programma van de congressen met jullie zal ik nooit vergeten.

De medewerkers van de proefdierlaboratoria in Utrecht en Nijmegen ben ik dank verschuldigd voor hun assistentie bij onze experimentele studies. Bovendien wil ik Dr. Edward Tan en het mobiele laboratorium van Defensie bijzonder bedanken voor de hulp tijdens het opzetten en uitvoeren van de experimenten tijdens de verschillende DSTC-cursussen.

Studenten in Utrecht: Marco, Arne, Eric, Roy en Leon. Inmiddels zijn jullie al lang geen student meer, maar toch wil ik jullie graag bedanken voor alle hulp, reisjes naar verre oorden en vooral leuke tijd. Gemotiveerde studenten zijn onmisbaar bij wetenschappelijk onderzoek. Ook dit resulteerde soms in *collateral damage*. Bijvoorbeeld als de huiscavia te koop gezet moest worden om aan de laboratorium experimenten deel te kunnen nemen.

Die (Forschungs)kollegen aus Aachen und Zürich, insbesondere Prof. Hildebrand, Klemens Horst, Martijn Hofman, Miguel Pischnamaz, Bergita Ganse, Johannes Greven und George Zhi aus Aachen, sowie PD Paolo Cinelli, Henrik Teuber, Max Lempert, Nikola Cesarovic, Sonja Märsmann, Till Berk, Prof. Gerrolt Jukema, Prof. Christian Hierholzer, PD Florin Alleman, Prof. Valentin Neuhaus, PD Ladislav Mica, PD Kai Sprengel, Thomas Rauer, Frank Schäfer, Sandro Heining, Rosmarie Widmer, Yannik Kalbas, Felix Karl-Ludwig Klingebiel, Mirian Weisskopf, Yohei Kumabe, Florencia Vonarburg, Kevin Arnke und Georg Osterhoff aus Zürich. Wir haben in der Vergangenheit viele Projekten aufgebaut, fertig gestellt und Publikationen veröffentlicht. Ich freue mich auf neue internationale Kollaborationen.

Insbesondere möchte ich mich bei Sascha Halva(chizadeh) bedanken für die unzählbare Stunden die wir zusammen im Labor, in der Klinik, im Auto oder bei der Burger King verbracht haben. Es passiert immer etwas mit dir und es ist nie langweilig. Die gemeinsame Zeit hat bereits zu vielen erfolgreichen Publikationen und Projekten geführt. Ich freue mich auf die weitere Zusammenarbeit in der Klinik, im Labor und im Privaten.

I would further like to thank the members of the TREAT Research Group for their support during the experimental studies of this thesis.

Professor B.L. Warren and Dr. T. Hardcastle, I would like to thank you for your support during the studies in South-Africa. I had a very enjoyable time and I am glad that our work resulted in some nice publications.

De stafleden, fellows, arts-assistenten en wetenschappers uit het UMC Utrecht en het Elisabeth Ziekenhuis in Tilburg, wil ik bedanken. Helaas kan ik niet iedereen bij naam en toenaam noemen. Bedankt voor de hulp bij alle klinische en experimentele studies. En de collega's uit Tilburg voor de flexibiliteit bij het wisselen van diensten ten behoeve van de wetenschap.

Ingrid Bouwman en Gioya Bouwman, ik wil jullie bedanken voor het organiseren van zowat alles. Ik heb in een vroeg stadium van mijn opleiding ingezien dat het secretariaat de belangrijkste schakel in een organisatie is. En dankzij jullie flexibiliteit en hulp heb ik een aantal allesbeslissende deadlines gehaald.

Prof. Paul Coffe, Lucette Teurling, selectiecommissie en lichtingsgenoten van het Alexandre Suermanprogramma,

Ik ben erg dankbaar voor de selectie voor het MD/PhD-Programma. Dit was een absolute *gamechanger* en lag aan de basis van alles wat ik de afgelopen jaren aan wetenschappelijk onderzoek heb kunnen doen. De masterclasses waren erg verrijkend en de begeleiding was ook zeer professioneel.

Kai-Oliver Jensen,

thank you for being a great teacher and friend. You are a great mentor, not only for me but also for your other Mentee's. It was a great experience to work on the S3-Guidelines with you and in the meantime we even managed to publish a bunch of papers.

Ehemalige Arbeitskollegen aus dem Rheinmaasklinikum (Prof. Dr. med. R. Sobottke): Es hat immer viel Spaß gemacht, mit Euch zusammen zu arbeiten, auch wenn die Reise dann leider weitergegangen ist. Ich freue mich, unsere gemeinsamen Projekten in der Zukunft fortzusetzen und ich möchte mich bedanken für die tolle und die lehrreiche Zeit. Euer Standard ist extrem hoch und ich bin froh, die Chance gehabt zu haben die endoskopische und navigationsgesteuerte Wirbelsäulenchirurgie bei euch kennenlernen zu dürfen. Ich freue mich auf weitere Projekten und möchte mich bei Mohamed Agha Mahmoud und Anas Afifi bedanken für die Kollaboration. Die Forschung macht noch mehr Spass, wenn man sie mit Freunden betreibt.

Ehemalige Arbeitskollegen der operativen Intensivmedizin im UK Aachen (Prof. Dr. G. Marx): Es war eine intensive und lehrreiche Zeit auf der Intensivstation. Vielen Dank für die Möglichkeit, meine Kenntnissen und Fähigkeiten in der chirurgischen Intensivmedizin auszubauen. Sowohl die Quantität der Patienten die Ihr betreut als auch die Qualität der intensivmedizinischen Behandlung ist außergewöhnlich. Wenn alle ICUs wie die eure arbeiten, wird es in Europa nie Bettennotstände geben! Sowohl mit wie auch ohne COVID.

Ehemaligen Kollegen vom Kantonsspital Thurgau (Prof. Dr. M. Müller): Ich möchte mich ganz herzlich für die Zusammenzeit bei euch bedanken. Die lückenlose Organisation der Abteilung führt dazu, dass man außergewöhnlich viel Zeit im Operationssaal verbringen kann. Es war eine tolle Zeit mit super Kollegen wobei sich ernsthafte Arbeit abwechselte mit Witzen und Spass. Der Abschied wie auch die Abschiedsparty fiel mir schwer.

Arbeitskollegen am Universitätsspital Zürich: Seit ich vor etwa 6 Jahren in Zürich angefangen habe, ist es für mich wie zu Hause. Es macht jeden Tag Spass, mit euch zusammen zu arbeiten, sowohl in der Klinik, in der Forschung oder in Lehre. Ich bin der Meinung, wir machen Spitzenmedizin und treiben die traumatologische Forschung weiter voran. Ich freue mich auf die weitere Zusammenarbeit.

Huisgenoten en jaarclubgenoten, het was voor mij een voorrecht tijd te mogen spenderen met vrienden die niets van geneeskunde willen weten. Verhalen genoeg, maar niets voor in dit boekje. Vriendschappen zijn helaas niet immuun voor afstand en tijd, maar het doet me goed te zien dat het iedereen goed gaat. Bedankt voor de mooie tijd en hopelijk tot snel.

Maarten Smits en Dino Colo, ieder is zijn eigen weg gegaan en heeft zijn weg gevonden. Ondanks dat wij allemaal in andere landen wonen en elkaar te weinig hebben gezien is het altijd als de dag van gisteren. Ook hier, verhalen genoeg. Maar niets voor dit boekje.

IC, het was een super tijd en ondanks de evacuatie en de (over)digitalisering is het allemaal goed gekomen.

Freunden aus Züri und Aachen: 4-mal auswandern in einem Zeitraum von 5 Jahren ist recht hochfrequent. Allerdings freue ich mich immer auf das Wiedersehen, sowohl mit den Freunden in Aachen wie auch den Freunden in Zürich. Zum Glück gibt es in beiden Orten recht gute Weihnachtsmärkte und wir verlieren wir uns nicht aus den Augen.

Maurijn en Roy, ik ben bij dat jullie mij als paranimfen tijdens mijn promotie begeleiden. Ik heb geleerd dat het belangrijk is om positieve mensen om je heen te verzamelen. Voor deze mijlpaal kwam ik dan ook snel bij jullie uit.

Spijkermanroy, tijdens de anatomielessen zijn wij elkaar tegen het lijk gelopen. Vanaf de eerste dag gemotiveerd en altijd vrolijk. Na de vragenlijst studie kwamen nog vele projecten, reizen en vooral avonturen. De webcamsessies met het lab onder de palmbomen in Cape Town (incl. Lesotho en de 50/50 bar) Nijmegen, Aachen, Homburg, Bangkok, Sarajevo. Jouw credo is 'consider it done'. Steeds als ik je vroeg me met iets te helpen, was jouw antwoord: 'heb ik al geregeld'. Ik ken niemand die zo goed dingen kan regelen als jij. Zonder jouw hulp was dit proefschrift een erg dun boekje geworden.

Maurijn Gadellaa, ondanks dat je geen geneeskunde gestudeerd hebt, denk ik dat ik jou toch een beetje voor de geneeskunde en neutrofielen heb kunnen enthousiasmeren. Sterker nog, ik denk dat jij zelfs bij de Utrechtse Voortgangstoets Geneeskunde best een eind zou komen. Het waren mooie tijden als vriend, huisgenoot, en met als kers op de taart natuurlijk de jaarlijkse groepsvakanties in augustus. Behalve de eindeloze gesprekken over politiek, filosofie, reizen en eigenlijk alles, heb jij ook een grote rol gespeeld in mijn promotietraject. Je was de perfecte sparringspartner en jouw creatieve ideeën en andere invalshoek hebben een belangrijke bijdrage geleverd aan mijn promotie. De ontelbare uren die wij gezamenlijk in de bibliotheek, en nog meer uren in het WKZ, hebben doorgebracht lagen aan de basis van dit proefschrift. Alsmede de tantelets, elixers, het binnen barbequen en het inwijden van de studentenkamers met goede sigaren.

~~Vrienden van de sportschool.~~

Familie Janssen en Familie Teuben, ooms, tantes, neven en nichten, jullie zijn een super familie en ondanks dat wij elkaar niet meer zoveel zien door de drukke levens en afstand kijk ik enorm uit naar volgende familieweekends of kerstdiners. Laten we deze tradities in stand houden, ondanks dat de families steeds groter worden.

Papa, mama en opa's en oma's, van alle mensen die op de een of andere manier bijgedragen hebben aan mijn ontwikkeling en dit boekje zijn jullie de belangrijkste. Bedankt voor de steun de goed(b)e(doelde) raad. Jullie hebben mij altijd ondersteund, ook als ik mijn eigen weg koos. Dit vertrouwen, jullie liefde en vrijheid die ik van kinds af aan heb gekregen hebben mij gemaakt tot wie ik ben. Daarvoor ben ik jullie erg dankbaar. Papa bedankt voor de correcties van mijn boekje en mama bedankt voor de vele leuke reisjes die we hebben kunnen combineren

met de congressen.

Marc, Mick, Mats en Charlotte. Ik heb erg genoten van de afgelopen jaren in Aachen omdat we elkaar vaker hebben kunnen zien. De komende tijd zullen we het weer moeten doen met tussenstops op doorreis naar het Gardameer. Helaas is de keerzijde van onze carrières dat wij elkaar niet veel zien. Maar ik ben ontzettend trots op wat jullie allemaal bereikt hebben.

Lieve Alba, met jou is alles leuker en gemakkelijker. Ik ben trots op wat jij allemaal bereikt hebt. Maar ik weet hoe goed je bent, niet alleen als mens maar ook als chirurg dus ik sta er niet van te kijken. Ik wil je bedanken voor jouw liefde en hulp. Iedere dag samen werken is de uitkomst gebleken voor onze drukke schema's. Maar voor mij is niets leuker dan samen niet-werken. En vooral kijk ik uit naar de volgende tripjes met jou of familie in Bologna of Albanië. Of als het moet, naar een congres ergens anders.

LIST OF PUBLICATIONS

63. Mahmoud MA, Teuben M, Sobottke R. Minimalinvasive Hybridstabilisierung – Überlebensrate und Mortalität bei osteoporotischen Patienten mit instabilen Wirbelkörperfrakturen des thorakolumbalen Übergangs. *Orth Unf Nachr* 2022;Dec11:17.
62. Halvachizadeh S, Goezmen S, Schuster S, Teuben M, Baechtold M, Probst P, Hauswirth F, Muller MK. The implementation of physicians assistant in a surgical ward improves continuity in daily clinical work and increases comprehensibility of nurses and physicians. *Pat Saf Surg* 2022 Nov;716(1):34.
61. Afifi A, Ringe M, Sobottke R, Oikonomidis S, Michel Paul Johan Teuben. Lumbar Facet Joint Radiofrequency Denervation Therapy for Chronic Low Back Pain: Enhanced Outcome Compared With Chemical Neurolysis (Ethyl Alcohol 95% or Glycerol 20%). *Int J Spine Surg.* 2022 Feb;16(1):33-41.
60. Cobianchi L, Dal Mas F, Massaro M, Biffl W, Catena F, Coccolini F, Dionigi B, Dionigi P, Di Saverio S, Fugazzola P, Kluger Y, Leppäniemi A, Moore EE, Sartelli M, Velmahos G, Woltz S, Angelos P, Ansaloni L; Team Dynamics Study Group. Diversity and ethics in trauma and acute care surgery teams: results from an international survey. *World J Emerg Surg.* 2022 Aug 10;17(1):44.
59. Teixeira H, Halvachizadeh S, Teuben MPJ, Probst P, Muller MK. Transition from a circular to a linear stapling protocol in laparoscopic Roux-en-Y gastric bypass surgery and its impact on quality of life: a 5-year outcome study. *Langenbecks Arch Surg.* 2022 Aug 10.
58. Pfister M, Teuben MPJ, Teuber H, Nocito A, Probst P, Muller MK. Mid-term quality of life after gastric band removal and single-stage conversion to gastric bypass: a single-center cohort study. *Langenbecks Arch Surg.* 2022 Jul 27.
57. Kumabe Y, Kalbas Y, Halvachizadeh S, Teuben M, Cesarovic N, Weisskopf M, Hülsmeier A, Hornemann T, Cinelli P, Pape HC, Pfeifer R. Occult hypoperfusion and changes of systemic lipid levels after severe trauma: an analysis in a standardized porcine polytrauma model. *Eur J Trauma Emerg Surg.* 2022 Jul 12.
56. Reichert M, Sartelli M, Weigand MA, Hecker M, Oppelt PU, Noll J, Askevold IH, Liese J, Padberg W, Coccolini F, Catena F, Hecker A; WSES COVID-19 emergency surgery survey collaboration group. Correction to: Two years later: Is the SARS-CoV-2 pandemic still having an impact on emergency surgery? An international cross-sectional survey among WSES members. *World J Emerg Surg.* 2022 Jul 8;17(1):39.
55. Halvachizadeh S, Kalbas Y, Teuben MPJ, Teuber H, Cesarovic N, Weisskopf M, Cinelli P, Pape HC, Pfeifer R. Effects of Occult Hypoperfusion on Local Circulation and

Inflammation - An Analysis in a Standardized Polytrauma Model. *Front Immunol.* 2022 Jun 21;13:894270.

54. Reichert M, Sartelli M, Weigand MA, Hecker M, Oppelt PU, Noll J, Askevold IH, Liese J, Padberg W, Coccolini F, Catena F, Hecker A; WSES COVID-19 emergency surgery survey collaboration group. Two years later: Is the SARS-CoV-2 pandemic still having an impact on emergency surgery? An international cross-sectional survey among WSES members. *World J Emerg Surg.* 2022 Jun 16;17(1):34.

53. Teuben MPJ, Heeres M, Blokhuis T, Spijkerman R, Knot E, Vrisekoop N, Pfeifer R, Pape HC, Koenderman L, Leenen LPH. Shift of Neutrophils From Blood to Bone Marrow Upon Extensive Experimental Trauma Surgery. *Front Immunol.* 2022 May 17;13:883863.

52. Halvachizadeh S, Störmann PJ, Özkurtul O, Berk T, Teuben M, Sprengel K, Pape HC, Lefering R, Jensen KO; TraumaRegister DGU. Discrimination and calibration of a prediction model for mortality is decreased in secondary transferred patients: a validation in the TraumaRegister DGU. *BMJ Open.* 2022 Apr 13;12(4):e056381.

51. Pfeifer R, Teuben M, Halvachizadeh S, Pape HC. Education in trauma surgery. *Swiss Knife* 2021;1:14-5.

50. Teuben MPJ, Halvachizadeh S, Kalbas Y, Qiao Z, Cesarovic N, Weisskopf M., Teuber H, Kalbitz M, Cinelli P, Pfeifer R, Pape HC; TREAT Research Group. Cellular activation status in femoral shaft fracture hematoma following different reaming techniques - A large animal model. *J Orthop Res.* 2022 Mar 17.

49. Roemer N, Hauswirth F, Teuber H, Teuben M, Neff TA, Muller MK. Improved Clinical and Financial Outcomes in Proximal Gastric Bypass Surgery Following the Transition from a Conventional Circular Stapling to an Augmented Linear Stapling Protocol. *Obes Surg.* 2022 May;32(5):1601-9.

48. Cobianchi L, Dal Mas F, Massaro M, Fugazzola P, Coccolini F, Kluger Y, Leppäniemi A, Moore EE, Sartelli M, Angelos P, Catena F, Ansaloni L; Team Dynamics Study Group. Team dynamics in emergency surgery teams: results from a first international survey. *World J Emerg Surg.* 2021 Sep 16;16(1):47.

47. Kalbas Y, Lempert M, Ziegenhain F, Scherer J, Neuhaus V, Lefering R, Teuben M, Sprengel K, Pape HC, Jensen KO; TraumaRegister DGU. A retrospective cohort study of 27,049 polytraumatized patients age 60 and above: identifying changes over 16 years. *Eur Geriatr Med.* 2022 Feb;13(1):233-41.

46. Afifi A, Mahmoud MA, Teuben M. Lumbale Facettengelenksarthropathie:

Mutisegmental perkutane Radiofrequenz-Neurotomie liefert besseres Ergebniss als endoskopische Neurotomie. *Orth Unf Nachr* 2021;Dec11:15.

45. Teuben MPJ, Pfeifer R, Horst K, Simon TP, Heeres M, Kalbas Y, Blokhuis T, Hildebrand F, Koenderman L, Pape HC, Leenen L; TREAT-Research Collaboration. Standardized porcine unilateral femoral nailing is associated with changes in PMN activation status, rather than aberrant systemic PMN prevalence. *Eur J Trauma Emerg Surg.* 2022 Jun;48(3):1601-11.

44. Ziegenhain F, Scherer J, Kalbas Y, Neuhaus V, Lefering R, Teuben M, Sprengel K, Pape HC, Jensen KO, The TraumaRegister Dgu. Age-Dependent Patient and Trauma Characteristics and Hospital Resource Requirements-Can Improvement Be Made? An Analysis from the German Trauma Registry. *Medicina (Kaunas).* 2021 Apr 1;57(4):330.

43. Weber B, Lackner I, Miclau T, Stulz J, Gebhard F, Pfeifer R, Cinelli P, Halvachizadeh S, Teuben M, Pape HC, Lipiski M, Cesarovic N, Kalbitz M. Early myocardial damage (EMD) and valvular dysfunction after femur fracture in pigs. *Sci Rep.* 2021 Apr 19;11(1):8503.

42. Scherer J, Kalbas Y, Ziegenhain F, Neuhaus V, Lefering R, Teuben M, Sprengel K, Pape HC, Jensen KO. The GERTality Score: The Development of a Simple Tool to Help Predict in-Hospital Mortality in Geriatric Trauma Patients. *J Clin Med.* 2021 Mar 25;10(7):1362.

41. Teuben MPJ, Mand C, Moosdorf L, Sprengel K, Shehu A, Pfeifer R, Ruchholtz S, Lefering R, Pape HC, Jensen KO. Simultaneous Casualty Admissions-Do they Affect Treatment in the Receiving Trauma Center? *World J Surg.* 2021 Jul;45(7):2037-45.

40. Teuben MPJ, Hollman A, Blokhuis T, Pfeifer R, Spijkerman R, Teuber H, Pape HC, Leenen LPH. Splenectomy is associated with altered leukocyte kinetics after severe trauma. *Eur J Med Res.* 2021 Mar 15;26(1):26.

39. Halvachizadeh S, Mica L, Kalbas Y, Lipiski M, Canic M, Teuben M, Cesarovic N, Rancic Z, Cinelli P, Neuhaus V, Pape HC, Pfeifer R. Zone-dependent acute circulatory changes in abdominal organs and extremities after resuscitative balloon occlusion of the aorta (REBOA): an experimental model. *Eur J Med Res.* 2021 Jan 21;26(1):10.

38. Weber B, Lackner I, Baur M, Gebhard F, Pfeifer R, Cinelli P, Halvachizadeh S, Teuben M, Pape HC, Imhof A, Lipiski M, Cesarovic N, Kalbitz M. Early myocardial damage (EMD) and valvular insufficiency result in impaired cardiac function after multiple trauma in pigs. *Sci Rep.* 2021 Jan 13;11(1):1151.

37. Greven J, Horst K, Qiao Z, Bläsius FM, Mert Ü, Teuben MPJ, Becker NH, Pfeifer R, Pape HC, Hildebrand F. Fracture fixation strategy and specific muscle tissue availability of neutrophilic granulocytes following mono- and polytrauma: intramedullary nailing vs. external fixation of femoral fractures. *Eur J Med Res.* 2020 Nov 26;25(1):62.

36. Teuben M, Heeres M, Blokhuis T, Hollman A, Vrisekoop N, Tan E, Pfeifer R, Pape HC, Koenderman L, Leenen LPH. Instant intra-operative neutropenia despite the emergence of banded (CD16^{dim}/CD62L^{bright}) neutrophils in peripheral blood - An observational study during extensive trauma-surgery in pigs. *Injury*. 2021 Mar;52(3):426-33.
35. Halvachizadeh S, Teuben M, Lempert M, Kalbas Y, Cesarovic N, Lipiski M, Benninger E, Cinelli P, Pfeifer R, Pape HC. Protective effects of new femoral reaming techniques (Reamer irrigator aspirator, RIA I and II) on pulmonary function and posttraumatic contusion (CT morphology) - results from a standardized large animal model. *Injury*. 2021 Jan;52(1):26-31.
34. Teuben MPJ, Hofman M, Greven J, Shehu A, Teuber H, Pfeifer R, Pape HC, Hildebrand F. Altered cell surface receptor dynamics and circulatory occurrence of neutrophils in a small animal fracture model. *Pathol Res Pract*. 2020 Oct;216(10):153108.
33. Lackner I, Weber B, Miclau T, Holzwarth N, Baur M, Gebhard F, Teuben M, Halvachizadeh S, Cinelli P, Pfeifer R, Lipiski M, Cesarovic N, Haffner-Luntzer M, Pape HC, Kalbitz M; TREAT Research Group. Reaming of femoral fractures with different reaming irrigator aspirator systems shows distinct effects on cardiac function after experimental polytrauma. *J Orthop Res*. 2020 Dec;38(12):2608-18.
32. Halvachizadeh S, Teuben M, Berk T, Neuhaus V, Pape HC, Pfeifer R. The impact of SARS-CoV-2 (COVID-19) pandemic on trauma bay management and guideline adherence in a European level-one-trauma centre. *Int Orthop*. 2020 Sep;44(9):1621-1627.
31. Teuben MPJ, Pfeifer R, Teuber H, De Boer LL, Halvachizadeh S, Shehu A, Pape HC. Lessons learned from the mechanisms of posttraumatic inflammation extrapolated to the inflammatory response in COVID-19: a review. *Patient Saf Surg*. 2020 Jul 9;14:28.
30. Baur M, Weber B, Lackner I, Gebhard F, Pfeifer R, Cinelli P, Halvachizadeh S, Teuben M, Lipiski M, Cesarovic N, Pape HC, Kalbitz M. Structural alterations and inflammation in the heart after multiple trauma followed by reamed versus non-reamed femoral nailing. *PLoS One*. 2020 Jun 25;15(6):e0235220.
29. Jensen KO, Teuben MPJ, Lefering R, Halvachizadeh S, Mica L, Simmen HP, Pfeifer R, Pape HC, Sprengel K; TraumaRegister DGU. Pre-hospital trauma care in Switzerland and Germany: do they speak the same language? *Eur J Trauma Emerg Surg*. 2021 Aug;47(4):1273-80.
28. Teuben M, Spijkerman R, Teuber H, Pfeifer R, Pape HC, Kramer W, Leenen L. Splenic

injury severity, not admission hemodynamics, predicts need for surgery in pediatric blunt splenic trauma. *Patient Saf Surg.* 2020 Jan 3;14:1.

27. Teuben MPJ, Hofman M, Shehu A, Greven J, Qiao Z, Jensen KO, Hildebrand F, Pfeifer R, Pape HC. The impact of intramedullary nailing on the characteristics of the pulmonary neutrophil pool in rodents. *Int Orthop.* 2020 Mar;44(3):595-602.

26. Teuben M, Spijkerman R, Blokhuis T, Pfeifer R, Teuber H, Pape HC, Leenen L. Nonoperative management of splenic injury in closely monitored patients with reduced consciousness is safe and feasible. *Scand J Trauma Resusc Emerg Med.* 2019 Dec 5;27(1):108.

25. Hofman M, Rabenschlag F, Andruszkow H, Andruszkow J, Möckel D, Lammers T, Kolejewska A, Kobbe P, Greven J, Teuben MPJ, Poeze M, Hildebrand F. Effect of neurokinin-1-receptor blockage on fracture healing in rats. *Sci Rep.* 2019 Jul 5;9(1):9744.

24. Pfeifer R, Halvachizadeh S, Schick S, Sprengel K, Jensen KO, Teuben M, Mica L, Neuhaus V, Pape HC. Are Pre-hospital Trauma Deaths Preventable? A Systematic Literature Review. *World J Surg.* 2019 Oct;43(10):2438-46.

23. Teuben M, Spijkerman R, Pfeifer R, Blokhuis T, Huige J, Pape HC, Leenen L. Correction to: Selective non-operative management for penetrating splenic trauma: a systematic review. *Eur J Trauma Emerg Surg.* 2019 Dec;45(6):987.

22. Teuben M, Löhr N, Jensen KO, Brüesch M, Müller S, Pfeifer R, Mica L, Pape HC, Sprengel K. Improved pre-hospital care efficiency due to the implementation of pre-hospital trauma life support (PHTLS[®]) algorithms. *Eur J Trauma Emerg Surg.* 2020 Dec;46(6):1321-5.

21. Teuben M, Spijkerman R, Pfeifer R, Blokhuis T, Huige J, Pape HC, Leenen L. Selective non-operative management for penetrating splenic trauma: a systematic review. *Eur J Trauma Emerg Surg.* 2019 Dec;45(6):979-85.

20. Busuttil T, Teuben M, Pfeifer R, Cinelli P, Pape HC, Osterhoff G. Screw fixation of ACPHT acetabular fractures offers sufficient biomechanical stability when compared to standard buttress plate fixation. *BMC Musculoskelet Disord.* 2019 Jan 24;20(1):39.

19. Teuben MPJ, Spijkerman R, Blokhuis TJ, Pfeifer R, Teuber H, Pape HC, Leenen LPH. Safety of selective nonoperative management for blunt splenic trauma: the impact of concomitant injuries. *Patient Saf Surg.* 2018 Nov 27;12:32.

18. Spijkerman R, Teuben MP, Hietbrink F, Kramer WL, Leenen LP. A cohort study to

evaluate infection prevention protocol in pediatric trauma patients with blunt splenic injury in a Dutch level 1 trauma center. *Patient Prefer Adherence*. 2018 Aug 28;12:1607-17.

17. Tiziani S, Dienstknecht T, Osterhoff G, Hand TL, Teuben M, Werner CML, Pape HC. Standards for external fixation application: national survey under the auspices of the German Trauma Society. *Int Orthop*. 2019 Aug;43(8):1779-85.

16. Kalbas Y, Qiao Z, Horst K, Teuben M, Tolba RH, Hildebrand F, Pape HC, Pfeifer R; TREAT Research Group. Early local microcirculation is improved after intramedullary nailing in comparison to external fixation in a porcine model with a femur fracture. *Eur J Trauma Emerg Surg*. 2018 Oct;44(5):689-96.

15. Serve R, Sturm R, Schimunek L, Störmann P, Heftrig D, Teuben MPJ, Oppermann E, Horst K, Pfeifer R, Simon TP, Kalbas Y, Pape HC, Hildebrand F, Marzi I, Relja B. Comparative Analysis of the Regulatory T Cells Dynamics in Peripheral Blood in Human and Porcine Polytrauma. *Front Immunol*. 2018 Mar 13;9:435.

14. Kalbitz M, Schwarz S, Weber B, Bosch B, Pressmar J, Hoenes FM, Braun CK, Horst K, Simon TP, Pfeifer R, Störmann P, Hummler H, Gebhard F, Pape HC, Huber-Lang M, Hildebrand F; TREAT Research Group. Cardiac Depression in Pigs after Multiple Trauma - Characterization of Posttraumatic Structural and Functional Alterations. *Sci Rep*. 2017 Dec 19;7(1):17861.

13. Schimunek L, Serve R, Teuben MPJ, Störmann P, Auner B, Woschek M, Pfeifer R, Horst K, Simon TP, Kalbitz M, Sturm R, Pape HC, Hildebrand F, Marzi I, Relja B. Early decreased TLR2 expression on monocytes is associated with their reduced phagocytic activity and impaired maturation in a porcine polytrauma model. *PLoS One*. 2017 Nov 10;12(11):e0187404.

12. Qiao Z, Horst K, Teuben M, Greven J, Yin L, Kalbas Y, Tolba RH, Pape HC, Hildebrand F, Pfeifer R; TREAT group. Analysis of skeletal muscle microcirculation in a porcine polytrauma model with haemorrhagic shock. *J Orthop Res*. 2018 May;36(5):1377-82.

11. Yin L, Busch D, Qiao Z, van Griensven M, Teuben M, Hildebrand F, Pape HC, Pfeifer R. Dose-dependent effects of peroxisome proliferator-activated receptors β/δ agonist on systemic inflammation after haemorrhagic shock. *Cytokine*. 2018 Mar;103:127-32.

10. Spijkerman R, Teuben MPJ, Hoosain F, Taylor LP, Hardcastle TC, Blokhuis TJ, Warren BL, Leenen LPH. Non-operative management for penetrating splenic trauma: how far can we go to save splenic function? *World J Emerg Surg*. 2017 Jul 25;12:33.

9. Pfeifer R, Schick S, Holzmann C, Graw M, Teuben M, Pape HC. Analysis of Injury and Mortality Patterns in Deceased Patients with Road Traffic Injuries: An Autopsy Study. *World J Surg.* 2017 Dec;41(12):3111-19.
8. Horst K, Simon TP, Pfeifer R, Teuben M, Almahmoud K, Zhi Q, Santos SA, Wemmers CC, Leonhardt S, Heussen N, Störmann P, Auner B, Relja B, Marzi I, Haug AT, van Griensven M, Kalbitz M, Huber-Lang M, Tolba R, Reiss LK, Uhlig S, Marx G, Pape HC, Hildebrand F. Characterization of blunt chest trauma in a long-term porcine model of severe multiple trauma. *Sci Rep.* 2016 Dec 21;6:39659.
7. Relja B, Taraki R, Teuben MP, Mörs K, Wagner N, Wutzler S, Hildebrand F, Perl M, Marzi I. Sera from severe trauma patients with pneumonia and without infectious complications have differential effects on neutrophil biology. *BMC Pulm Med.* 2016 Dec 1;16(1):171.
6. Almahmoud K, Teuben M, Andruszkow H, Horst K, Lefering R, Hildebrand F, Pape HC, Pfeifer R. Trends in intubation rates and durations in ventilated severely injured trauma patients: an analysis from the TraumaRegister DGU®. *Patient Saf Surg.* 2016 Nov 3;10:24.
5. Pfeifer R, Teuben M, Andruszkow H, Barkatali BM, Pape HC. Mortality Patterns in Patients with Multiple Trauma: A Systematic Review of Autopsy Studies. *PLoS One.* 2016 Feb 12;11(2):e0148844.
4. Kaltbeitzel D, Hopmann C, Eggert F, Pishnamaz MA, Teuben MP. Faserverstärkte Flüssigsiliconautschuke – Kreuzbandersatz der Zukunft. Gummi, Fasern, Kunststoffe. 2016;69(4):227-231.
3. Teuben M, Pfeifer R, Pape HC. Treatment of severely injured patients. *Notfall + Rettungsmedizin* 2016; 1.
2. van Hout GP, van Solinge WW, Gijsberts CM, Teuben MP, Leliefeld PH, Heeres M, Nijhoff F, de Jong S, Bosch L, de Jager SC, Huisman A, Stella PR, Pasterkamp G, Koenderman LJ, Hoefer IE. Elevated mean neutrophil volume represents altered neutrophil composition and reflects damage after myocardial infarction. *Basic Res Cardiol.* 2015 Nov;110(6):58.
1. van Hout GP, Teuben MP, Heeres M, de Maat S, de Jong R, Maas C, Kouwenberg LH, Koenderman L, van Solinge WW, de Jager SC, Pasterkamp G, Hoefer IE. Invasive surgery reduces infarct size and preserves cardiac function in a porcine model of myocardial infarction. *J Cell Mol Med.* 2015 Nov;19(11):2655-63.

LIST OF PRESENTATIONS AT SCIENTIFIC MEETINGS

116. **Teuben M**, Loehr N, Shehu A, Berk T, Jensen, KO, Brueesch M, Mueller S, Pfeifer R, Mica L, Pape HC, Sprengel K - The benefit of pre-hospital trauma life support courses for experienced medical personnel in a metropolitan area in Europe. *42nd SICOT Orthopedic World Congress 2022*; Kuala Lumpur, Malaysia.
115. **Teuben M**, Halvachizadeh S, Kalbas Z, Zhi Q, Cesarovic N, Cinelli P, Pfeifer R, Pape HC - Initial trauma and not nailing strategy dictates the early systemic neutrophil response in cardiopulmonary compensated polytrauma: a large animal experiment. *42nd SICOT Orthopedic World Congress 2022*; Kuala Lumpur, Malaysia.
114. **Teuben M**, Halvachizadeh S, Shehu A, Pfeifer R, Pape HC - The 24hr leukocyte-gap: a novel early predictor for sepsis in polytrauma patients - *42nd SICOT Orthopedic World Congress 2022*; Kuala Lumpur, Malaysia.
113. **Teuben M**, Halvachizadeh S, Pfeifer R, Pape HC - Which pathophysiologic change is most relevant for delaying definitive surgery in polytrauma patients? *42nd SICOT Orthopedic World Congress 2022*; Kuala Lumpur, Malaysia.
112. **Teuben M** - Case discussion: Spinal trauma. *International Polytraumacourse 2022*; Kuala Lumpur, Malaysia.
111. **Teuben M** - Case discussion: Vascular injury. *International Polytraumacourse 2022*; Kuala Lumpur, Malaysia.
110. **Teuben M** - Polytrauma is a disease. *OTTSCON 2022*; Coimbatore, India.
109. **Teuben M** - Long-term outcome of polytrauma. *OTTSCON 2022*; Coimbatore, India.
108. **Teuben M** - Case discussion (Safe definitive Surgery). *International Polytraumacourse 2022*; Nur-Sultan, Kazakhstan.
107. **Teuben M** - Case discussion (polytrauma and unstable spinal injuries). *International Polytraumacourse 2022*; Nur-Sultan, Kazakhstan.
106. **Teuben M**, Halvachizadeh S, Kalbas Z, Zhi Q, Cesarovic N, Cinelli P, Pfeifer R, Pape HC - Immune cell characteristics of femoral shaft fracture hematoma following different reaming techniques – a large animal model. *21st European Congress of Trauma and Emergency Care 2022*; Oslo, Norway.
105. **Teuben M**, Loehr N, Shehu A, Berk T, Jensen, KO, Brueesch M, Mueller S, Pfeifer R, Mica L, Pape HC, Sprengel K - The value of pre-hospital trauma life support (PHTLS®)

courses for experienced medical personnel involved in pre-hospital trauma care. *21st European Congress of Trauma and Emergency Care 2022*; Oslo, Norway.

104. **Teuben M** - COVID-19 and its impact on polytrauma treatment. *21st European Congress of Trauma and Emergency Care 2022*; Oslo, Norway.

103. **Teuben M** - Case discussion: Surgical priorities. *21st European Congress of Trauma and Emergency Care 2022*; Oslo, Norway.

102. **Teuben M**, Römer N, Hauswirth F, Teuber H, Neff TA, Müller MK. Improved outcomes in proximal gastric bypass surgery following the transition from a conventional circular stapling to an augmented linear stapling protocol. *Jahrestagung der Schweizer Gesellschaft für Chirurgie 2022*; Bern, Switzerland.

101. **Teuben M**, Afifi A, Mahmoud M, Sobottke R - Superior long-term pain reduction and lower complication rates upon radiofrequency vs. endoscopic neurotomy for facet joint arthropathy. *72nd Jahrestagung der Deutschen Gesellschaft für Neurochirurgie*. Germany

100. **Teuben M** - Chairmen Session Trauma Papers 1, *41st SICOT Orthopedic World Congress 2021*; Budapest, Hungary.

99. **Teuben M** - Chairmen Session Trauma Papers 2, *41st SICOT Orthopedic World Congress 2021*; Budapest, Hungary.

98. **Teuben M** - Percutaneous treatment of pelvic injuries. *41st SICOT Orthopedic World Congress 2021*; Budapest, Hungary.

97. **Teuben M** - Case Discussions: Safe Definitive Surgery. *Polytraumacourse: 41st SICOT Orthopedic World Congress 2021*; Budapest, Hungary.

96. **Teuben M** - Case Discussion (Safe Definitive Surgery). *International Polytraumacourse 2021*; Nur-Sultan, Kazakhstan.

95. **Teuben M** - Case Discussion (Pelvic and Extremity injuries). *International Polytraumacourse 2021*; Nur-Sultan, Kazakhstan.

94. **Teuben M** - Case discussion (Modern treatment of pelvic injuries). *International Polytraumacourse 2021*; San Antonio, USA.

93. **Teuben M** - Spinal injury in polytrauma. *International Polytraumacourse 2021*; Zurich, Switzerland.

92. **Teuben M** - Case discussion: spinal injury. *TRAUMA-Conference 2020*; Moscow, Russian Federation.

91. **Teuben M**, Mand C, Moosdorf L, Sprengel K, Shehu A, Pfeifer R, Ruchholtz S, Lefering R, Pape HC, Jensen KO. - Multiple simultaneous casualty admissions: do they affect treatment in the receiving trauma center? *American College of Surgeons COT, Annual Clinical Congress 2020*.

90. **Teuben M**, Halvachizadeh S, Kalbas Z, Zhi Q, Cesarovic N, Cinelli P, Pfeifer R, Pape HC - Systemic Neutrophil Homeostasis Is Not Affected by Reaming Techniques for Intramedullary Nailing in a Standardized Porcine Polytrauma Model. *Orthopedic Trauma Association Annual Meeting 2020*.

89. **Teuben**, Mand C, Moosdorf L, Sprengel K, Shehu A, Pfeifer R, Ruchholtz S, Lefering R, Pape HC, Jensen KO - Multiple simultaneous casualty admissions: do they affect treatment in the receiving trauma center? *ATLS Europe Association Annual Meeting 2020*.

88. **Teuben M** - Management of SIRS and MODS after polytrauma. *40th SICOT Orthopedic World Congress 2019*; Muscat, Oman.

87. **Teuben M** - Timing of surgery: How early is early? *40th SICOT Orthopedic World Congress 2019*; Muscat, Oman.

86. **Teuben M** - My approach to soft-tissue damage and soft-tissue loss in open fractures. *40th SICOT Orthopedic World Congress 2019*; Muscat, Oman.

85. **Teuben M**, Loehr N, Jensen K, Brueesch M, Pfeifer R, Müller S, Pape HC, Sprengel K - The impact of introduction of pre-hospital trauma life support (PHTLS) algorithms on pre-hospital care in a European metropolitan area. *40th SICOT Orthopedic World Congress 2019*; Muscat, Oman.

84. **Teuben M**, Mand C, Shehu A, Pfeifer R Ruchholtz S, Lefering R, Pape HC, Jensen K - Outcome of simultaneous treatment of multiple severely injured trauma patients - an analysis of the german trauma registry. *40th SICOT Orthopedic World Congress 2019*; Muscat, Oman.

83. **Teuben M**, Hofman M, Shehu A, Greven J, Zhi Q, Jensen K, Hildebrand F, Pfeifer R, Pape HC - Transient impaired neutrophil activation after intramedullary nailing and a femur fracture: indicative of selective immunodepletion? *23. Chirurgische Forschungstage der Deutschen Gesellschaft für Chirurgie 2019*; Aachen, Germany.

82. **Teuben M**, Loehr N, Jensen K, Brueesch M, Pfeifer R, Müller S, Pape HC, Sprengel K - The implementation of pre-hospital trauma life support (PHTLS) algorithms is associated

with on pre- hospital care in a European metropolitan area. *20th European Congress of Trauma and Emergency Care 2019*; Prague, Czech Republic.

81. **Teuben M**, Jensen K, Dienstknecht T, Hildebrand F, Pfeifer R, Pape HC, Sprengel K - Epidemiology and outcome of pelvic injury in the elderly: what is the impact of concurrent injuries? *20th European Congress of Trauma and Emergency Care 2019*; Prague, Czech Republic.

80. **Teuben M**, Mand C, Shehu A, Pfeifer R Ruchholtz S, Lefering R, Pape HC, Jensen K - Simultaneous treatment of multiple severely injured trauma patients and impact on outcome - an analysis of the german trauma registry. *20th European Congress of Trauma and Emergency Care 2019*; Prague, Czech Republic.

79. **Teuben M**, Halvachizadeh S, Qiao Z, Cinelli P, Cesarovic N, Pape HC, Pfeifer - Intramedullary nailing does not deprive physiology nor inflammatory status in porcine polytrauma with rapid cardiopulmonary improvement. *20th European Congress of Trauma and Emergency Care 2019*; Prague, Czech Republic.

78. **Teuben M** - Pelvic trauma in polytrauma patients (Case discussion). *Polytraumacourse 2019, 20th European Congress of Trauma and Emergency Care 2019*; Prague, Czech Republic.

77. **Teuben M** - The pathophysiology of ARDS and multiple organ dysfunction syndrome. *International Conference on Trauma 2018*; Moscow, Russian Federation.

76. **Teuben M** - Aortic dissection in trauma (Case discussion). *Deutscher Kongress für Orthopädie und Unfallchirurgie/Polytraumacourse 2018*; Berlin, Germany.

75. **Teuben M**, Hofman M, Greven J, Zhi Q, Hildebrand F, Pfeifer R, Pape HC - The systematic neutrophil response to intramedullary nailing in rats. *39th SICOT Orthopedic World Congress 2018*; Montreal, Canada.

74. **Teuben M** - Management of SIRS and MODS After Trauma. *39th SICOT Orthopedic World Congress 2018*; Montreal, Canada.

73. **Teuben MP**, Hofman M, Shehu A, Greven J, Zhi Q, Jensen K, Hildebrand F, Pfeifer R, Pape HC – The impact of intramedullary nailing on characteristics of the pulmonary neutrophil pool. *39th SICOT Orthopedic World Congress 2018*; Montreal, Canada.

72. **Teuben MP**, Blokhuis T, Heeres M, Pfeifer R, Koenderman L, Leenen L, Pape HC – Extensive thoraco-abdominal surgery causes intra-operative neutrophenia despite the emerge of banded neutrophils in peripheral blood. *19th European Congress of Trauma and Emergency Care 2018*; Valencia, Spain.

71. **Teuben MP**, Hofman M, Pfeifer R, Greven J, Hildebrand F, Pape HC - The pulmonary neutrophil response to long bone fractures and intramedullary nailing in rats. *19th European Congress of Trauma and Emergency Care 2018*; Valencia, Spain.
70. **Teuben M** - Treatment strategies in polytrauma. *38th SICOT Orthopedic World Congress 2017*; Cape Town, South-Africa.
69. **Teuben M** - Pathophysiology of ARDS and organ failure in severe trauma. *38th SICOT Orthopedic World Congress 2017*; Cape Town, South-Africa.
68. **Teuben M**, Pfeifer R, Horst K, Zhi Q, Hildebrand F, Pape HC - Hemorrhagic shock and surgical strategy (nailing vs. external fixation) affect microcirculation in soft tissues. *33rd Orthopedic Trauma Association Annual Meeting 2017*; Vancouver, Canada
67. **Teuben M** - Standardized treatment of polytraumapatient and outcome. *Pirogov Forum 2017*; Moscow, Russian Federation.
66. **Teuben M** - Early Total Care vs. Damage Control Orthopedics. *Pirogov Forum 2017*; Moscow, Russian Federation.
65. **Teuben M** - Pathophysiology of Polytrauma. *Pirogov Russian National Research Medical University*. 2017; Moscow, Russian Federation.
64. **Teuben M** - Clearing traumapatient for Surgery. *Pirogov Russian National Research Medical University*. 2017; Moscow, Russian Federation.
63. **Teuben M** - Principles of ATLS and influence on the quality of trauma care. *18th European Congress of Trauma and Emergency Care 2017*; Bucharest, Romania.
62. **Teuben M** - Objective outcome after trauma. *18th European Congress of Trauma and Emergency Care 2017*; Bucharest, Romania.
61. **Teuben M** - Coagulopathy in polytrauma: case discussion. *18th European Congress of Trauma and Emergency Care 2017*; Bucharest, Romania.
60. **Teuben M** - The systemic immune response in polytrauma. *134. Kongress der Deutschen Gesellschaft für Chirurgie 2017*; München, Germany
59. **Teuben M** - Clearing trauma patients for surgery: Which parameters are the best? *134. Kongress der Deutschen Gesellschaft für Chirurgie 2017*; München, Germany

58. **Teuben M** - Polytraumapatienten with complex Pelvic Injury. *International Conference on Trauma 2017*; Moscow, Russian Federation.
57. **Teuben M** - Long Term outcome in Polytrauma. *International Conference on Trauma 2017*; Moscow, Russian Federation.
56. **Teuben M** - Early total Care vs. Damage Control Surgery. *International Conference on Trauma 2017*; Moscow, Russian Federation.
55. **Teuben M** - Initial assessment of the polytraumapatient. *International Conference on Trauma 2017*; Moscow, Russian Federation.
54. **Teuben M** - Objective outcome after polytrauma. *Deutscher Kongress für Orthopädie und Unfallchirurgie/Polytraumacourse 2016*; Berlin, Germany.
53. **Teuben M**, Pfeifer R, Greven J, Hofman M, Hildebrand F, Pape HC - The porcine neutrophil response in acute systemic inflammation. *Forschungskolloquium Zürich 2016*; Zürich, Switzerland
52. **Teuben M**, Pfeifer R, Greven J, Hofman M, Hildebrand F, Pape HC - The impact of intramedullary nailing and a femur fracture on neutrophil activation in rats. *32nd Orthopedic Trauma Association Annual Meeting 2017*; Maryland, USA.
51. **Teuben M**, Pfeifer R, Qiao Z, Greven J, Hildebrand F, Pape HC - The systemic neutrophil response to long bone fractures and intramedullary nailing in rats. *37th SICOT Orthopedic World Congress 2016*; Rome, Italy.
50. **Teuben M** - Compartment Syndrome in polytrauma. *37th SICOT Orthopedic World Congress 2016*; Rome, Italy.
49. Chairmen **Teuben M** - Session: Miscellaneous. *37th SICOT Orthopedic World Congress 2016*; Rome, Italy.
48. **Teuben M**, Rachid T, Pfeifer R, Kaltbeitzel D, Effert F, Pishnamaz MA, Pape HC - In vitro biomechanical characteristics of the porcine anterior cruciate ligament. *17th European Congress of Trauma and Emergency Care 2016*; Vienna, Austria.
47. **Teuben M**, Heeres M, Blokhuis TJ, Pfeifer R, Hildebrand F, Koenderman L, Leenen LP, Pape HC - Splenic homing of neutrophil subtypes in a mice model of hypovolemic shock and trauma. *17th European Congress of Trauma and Emergency Care 2016*; Vienna, Austria.

46. **Teuben M**, Pfeifer R, Greven J, Hofman M, Hildebrand F, Pape HC - Neutrophilen Aktivierung und Mobilisation im Rahmen der akuten post- traumatischen systemischen Reaktion. *Aachener Unfallchirurgisches Forschungstreffen*. 2016; Dalhem, Germany.
45. Teuben M, Pfeifer R, Greven J, Hildebrand F, Pape HC, Hofman M - Impact of Substance P inhibition on fracture healing in rats. *Aachener Unfallchirurgisches Forschungstreffen*. 2016; Dalheim, Germany.
44. **Teuben M** - Neutrophilen Aktivierung und Mobilisation im Rahmen der akuten post-traumatischen systemischen Reaktion. 6. *Treffen Netzwerk Traumaforschung 2016*: Frankfurt am Main, Germany.
43. **Teuben M**, Horst K, Tan E, Blokhuis TJ, Hildebrand F, Koenderman L, Pape HC, Leenen LP, Pfeifer R - Porcine neutrophil activation and phenotypes in blood and bone marrow in a model of trauma surgery. *Deutscher Kongress für Orthopädie und Unfallchirurgie 2015*; Berlin, Germany.
42. **Teuben M**, Pfeifer R, Horst K, Blokhuis TJ, Hildebrand F, Koenderman L, Pape HC, Leenen LP - Splenic neutrophil migration in a traumamodel with hemorrhagic shock in mice. *Deutscher Kongress für Orthopädie und Unfallchirurgie 2015*; Berlin, Germany.
41. **Teuben M** - Thoracic trauma in polytraumapaticients. *Deutscher Kongress für Orthopädie und Unfallchirurgie/Polytraumacourse 2015*; Berlin, Germany.
40. **Teuben M**, Pfeifer R, Horst K, Blokhuis TJ, Hildebrand F, Koenderman L, Pape HC, Leenen LP – The impact of intramedullary nailing of the femur on systemic activation and tissue migration of neutrophils. *Annual Meeting Orthopedic Trauma Association 2015*; San Diego, USA.
39. **Teuben M**, Pfeifer R, Andruskow H, Horst K, Hildebrand F, Pape HC - Long-Term Analysis (10 versus 20 Years) After Severe Trauma: What are the Effects on Quality of Life? *Annual Meeting Orthopedic Trauma Association International Forum 2015*; San Diego, USA.
38. **Teuben M**, Heeres , Pfeifer R, Horst K, Blokhuis TJ, Hildebrand F, Koenderman L, Leenen LP, Pape HC - The impact of a unilateral femur fracture on neutrophil activation in pigs. *International Conference on Complexity in Acute Illness/European Shock Society 2015*; Köln, Germany.
37. **Teuben M**, Heeres M, Pfeifer R, Tan E, Blokhuis TJ, Pape HC, Koenderman L, Leenen LP - The impact of traumasurgery on the activation status of blood and bone marrow neutrophils in pigs. *World Congress of Surgery 2015*; Bangkok, Thailand.

36. **Teuben M**, Spijkerman R, Hoosain F, Blokhuis TJ, Warren BL, Hardcastle T, Leenen LP - Penetrating splenic injuries and the safety of selective nonoperative management. *World Congress of Surgery 2015*; Bangkok, Thailand.
35. **Teuben M**, Kramer WL, Spijkerman R, Blokhuis TJ, Leenen LP - Nonoperative management, and not spleen preserving surgery for blunt splenic injury results in lower in-hospital infection rates. 16th European, Congress of Trauma and Emergency Surgery 2015; Amsterdam, The Netherlands.
34. **Teuben M**, Heeres M, Pfeifer R, Tan E, Blokhuis TJ, Pape HC, Koenderman L, Leenen LP - The impact of trauma surgery on the activation status of blood and bone marrow neutrophils subsets in pigs. 16th European, Congress of Trauma and Emergency Surgery 2015; Amsterdam, The Netherlands.
33. **Teuben M**, Heeres M, Pfeifer R, Blokhuis TJ, Pape HC, Koenderman L, Leenen LP - Splenic neutrophil compartmentalization in a mouse model with trauma and hemorrhagic shock. 16th European, Congress of Trauma and Emergency Surgery 2015; Amsterdam, The Netherlands.
32. **Teuben M**, Spijkerman R, Hoosain F, Blokhuis TJ, Warren BL, Hardcastle T, Leenen LP - Nonoperative management for the treatment of penetrating splenic injury. 16th European, Congress of Trauma and Emergency Surgery 2015; Amsterdam, The Netherlands.
31. **Teuben M**, Koenderman L, Leenen LP - Neutrophil kinetics in a porcine trauma model. *DSATC Course Faculty Meeting 2015*; Nijmegen, The Netherlands.
30. **Teuben M**, Heeres M, Blokhuis TJ, Leenen LP - The cellular immune response to trauma in pigs. *Annual Dutch Trauma Congress 2014*; Amsterdam, The Netherlands.
29. **Teuben M**, Spijkerman R, Blokhuis TJ, Kramer WL, Leenen LP - Selective nonoperative management for blunt splenic trauma: adults are not large children. *Annual Scientific Meeting on Intensive Care 2014*; Kuala Lumpur, Malaysia.
28. **Teuben M**, Heeres M, Blokhuis TJ, Koenderman L, Leenen LP - The impact of damage control surgery on the mobilization of neutrophils in pigs. *Annual Scientific Meeting on Intensive Care 2014*; Kuala Lumpur, Malaysia.
27. **Teuben M**, Blokhuis TJ, Leenen LP, Koenderman L - Systemic neutrophil activation in a porcine model of tissue damage. *International Neutrophil Symposium 2014*, Montreal, Canada.
26. **Teuben M**, Blokhuis TJ, Kramer WL, Leenen LP - Selective nonoperative management for blunt splenic injury in polytrauma patients. *15th European Congress of Trauma and*

Emergency Surgery 2014 & 2nd World Trauma Congress 2014; Frankfurt am Main, Germany.

25. **Teuben M**, Blokhuis TJ, Kramer WL, Leenen LP - Prevention of infectious complications in pediatric patients with blunt splenic injury. *15th European Congress of Trauma and Emergency Surgery 2014 & 2nd World Trauma Congress 2014*; Frankfurt am Main, Germany.

24. **Teuben M**, Blokhuis TJ, Koenderman L, Leenen LP - Homology in systemic neutrophil response induced by a polytraumamodel in pigs and by human trauma. *15th European Congress of Trauma and Emergency Surgery 2014 & 2nd World Trauma Congress 2014*; Frankfurt am Main, Germany.

23. **Teuben M**, Blokhuis TJ, Leenen LP, Koenderman L - Acute systemic inflammation is associated with occurrence of neutrophil phenotypes in humans and pigs. *48th Annual Scientific Meeting of the European Society for Clinical Investigation*; Utrecht, The Netherlands.

22. **Teuben M**, Blokhuis TJ, Tan E, Koenderman L, Leenen LP - *New Insights into the Pathophysiological Background for Damage Control Surgery*. Singapore Trauma Conference 2014; Singapore, Singapore.

21. **Teuben M**, Blokhuis TJ, Koenderman L, Leenen LP - Nonoperative Management for Blunt Splenic Injury: The Impact of Concomitant Injury on Outcome. *Singapore Trauma Conference 2014*; Singapore, Singapore.

20. **Teuben M**, Blokhuis TJ, Leenen LP, Koenderman L - The pig: a model for the systemic neutrophil response to trauma. *Trauma and Blast Inflammation Symposium 2014*; Birmingham, United Kingdom.

19. **Teuben M**, Blokhuis TJ, Tan E, Koenderman L, Leenen LH - Pathophysiological basis for Damage Control Surgery. *Dutch Trauma Associations' Resident Symposium 2014*; Soest, The Netherlands.

18. **Teuben M** - Physiology of the spleen. *Dutch Conference for Pediatric ICU Specialists 2014*; Utrecht, The Netherlands.

17. **Teuben M**, Blokhuis TJ, Koenderman L, Leenen LP - The systemic neutrophil response to extensive traumasurgery in pigs. *DSATC Faculty Meeting 2013*; Nijmegen, The Netherlands.

16. **Teuben M**, Kramer WL, Leenen LP - Differences in therapy for blunt splenic injury between adults and pediatric patients. *14th European Congress of Trauma and Emergency Surgery 2013*; Lyon, France.
15. **Teuben M**, Leenen LP - Blunt splenic injury: how safe is our strive for the preservation of splenic function? *14th European Congress of Trauma and Emergency Surgery 2013*; Lyon, France.
14. **Teuben M**, Leenen LP, Kramer WL- The treatment of blunt splenic injury in pediatric patients. *Dutch National Pediatric Congress 2012*; Veldhoven, The Netherlands.
13. **Teuben M**, Leenen LP - Early infection rate in patients sustaining blunt splenic injury. *13th European Congress of Trauma and Emergency Surgery 2012*; Basel, Switzerland.
12. **Teuben M**, Kramer WL, Leenen LP - Treatment of pediatric splenic injury in a Dutch level onetrauma center: a twelve year experience. *13th European Congress of Trauma and Emergency Surgery 2012*; Basel, Switzerland.
11. **Teuben M**, Kramer WL, Leenen LP - Treatment of pediatric patients sustaining blunt splenic injury. *Surgical Research Conference Utrecht 2012*; Utrecht, The Netherlands.
10. **Teuben M**, Kramer WL, Leenen LP - The impact of concurrent injuries on outcome of patients treated by nonoperative management for splenic trauma. *Annual Research Conference Utrecht 2012*; Utrecht, The Netherlands.
9. **Teuben M**, Kramer WL, Leenen LP - Blunt splenic injury: How safe is nonoperative management? *Annual Dutch Surgical Congress 2012*; Veldhoven, The Netherlands.
8. **Teuben M**, Leenen LP - The safety of nonoperative management for penetrating splenic injury. *ASGBI International Surgical Congress 2012*; Liverpool, United Kingdom.
7. **Teuben M**, Leenen LP - Nonoperative management of penetrating splenic injury: a systematic review. *22nd International Congress of the Israel Society of Anesthesiologists 2011*; Tel Aviv, Israel.
6. **Teuben M**, Leenen LP - The impact of neurological impairment on outcome of observational treatment for blunt splenic injury. *12th European Congress of Trauma and Emergency Surgery 2011*; Milan, Italy.
5. **Teuben M**, Kramer WL, Leenen LP - Factors affecting management in pediatric patients sustaining blunt splenic trauma. *12th European Congress of Trauma and Emergency Surgery 2011*; Milan, Italy.

4. **Teuben M**, Leenen LP - Nonoperative versus operative management in patients with blunt splenic injury. *12th European Congress of Trauma and Emergency Surgery 2011*; Milan, Italy.
3. **Teuben M**, Leenen LP - The impact of concomitant trauma on failure of nonoperative management for blunt splenic injury. *Annual Dutch Trauma Congress 2010*; Amsterdam, The Netherlands.
2. **Teuben M**, Kramer WL, Leenen LP - Nonoperative management for blunt splenic injury. *Annual Dutch Surgical Congress 2010*; Veldhoven, The Netherlands.
1. **Teuben M**, Leenen LP - The impact of concomitant injury on outcome of nonoperative management of blunt splenic trauma. *11th European Congress of Trauma and Emergency Surgery 2010*; Brussels, Belgium.

CURRICULUM VITAE AUCTORIS

Michel Paul Johan Teuben was born on the 21st of August 1986 in 's-Hertogenbosch, The Netherlands. He graduated from secondary school (Ds. Pierson College, 's-Hertogenbosch) in the year 2005 and the same year he enrolled in the medical program of the University of Utrecht.



During medical school, he was an instructor at the anatomy teaching lab and he served as a student representative/member of the Programme Committee of Medicine for 2 years. And in order to focus on extra-curricular activities (supported by a bursary-salary from Utrecht University), he decided to temporarily discontinue his study in the year 2011. He was awarded with the Alexander Suermanstipend and selected for the M.D./Ph.D.-Talent Program from the University Medical Center Utrecht. After trauma internships at the University Hospital Sarajevo and Groote Schuur Hospital in Cape Town, he finished medical school in 2012. Afterwards he was appointed as surgical resident at the St. Elisabeth Hospital Tilburg, The Netherlands (Dr. P. Vriens/Dr. J. Heisterkamp). In 2013 he started his M.D./Ph.D.-Trajectory at the University Medical Center Utrechts' Dept. of Trauma (Prof. L. Leenen) and the Laboratory for Translational Immunology (Prof. L. Koenderman). Fellowships were executed at the Trauma Unit of the Tygerberg Hospital in Stellenbosch and at the Inkosi Albert Luthuli Central Hospital in Durban (South-Africa). In 2014, a research fellowship, which was kindly sponsored by the Deutsche Akademischer Austausch Dienst, Michael van Vlotenfund and Annafund for Orthopedic Research was executed at the Harald Tscherne Laboratory for Orthopedic Research (Prof. Pape, University Hospital RWTH Aachen, Germany). Thereafter, he continued his residency training under the supervision of Prof. H.C. Pape at the Dept. of Traumatology at the University Hospital RWTH Aachen (Germany), and later at the University Hospital Zurich (Switzerland). Clinical rotations were performed at the University Hospital RWTH Aachen (Prof. G. Marx), Rhein-Maasklinikum Würselen/Aachen (Prof. R. Sobottke) and at the Cantonal Hospital Thurgau (Prof. M. Müller). After finishing residency training for trauma and orthopedic surgery, he completed the EUROSPINE Diploma Curriculum. Obtaining additional board certification for general surgery is scheduled for 2023. During his academic career, he obtained several travel grants and was further awarded an AO-Start-Up grant, the University Aachen Startup-Grant, and an OTC-Grant. He also received several awards including an Award for the Best Oral Presentation at the Annual Meeting on Intensive Care in Kuala Lumpur (Malaysia/2014), the Young Investigator Award at the SICOT World Congress in Montreal (Canada/2018), and the Best European Resident Trauma Paper from the Committee on Trauma/American Colleges of Surgeons (2020). He is further involved in the organisation of the certified 'Polytraumamangement-Beyond ATLS'-courses and he is a co-author of the upcoming German S3-Guidelines 'Polytrauma'. He currently works as a certified trauma and orthopedic surgeon at the University Hospital Zürich and lives with his wife in Zürich.

