# Monitoring and Modulation of the Neutrophil Response

Tjaakje Visser

Monitoring and Modulation of the Neutrophil Response
Bestuderen en bijsturen van de neutrofiel reactie

Thesis, Utrecht University, The Netherlands, with summary in Dutch
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# Monitoring and Modulation of the Neutrophil Response

Bestuderen en bijsturen van de neutrofiel reactie
*(Met een samenvatting in het Nederlands)*

Proefschrift

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**Promotoren** Prof. dr. L.P.H. Leenen
Prof. dr. L. Koenderman

# Contents

# 1

General introduction and outline of the thesis

Neutrophil granulocytes, also referred to as neutrophils or polymorphonuclear leukocytes (PMNs), play a pivotal role in the host first-line defense against invading pathogens. Neutrophils are important effector cells participating in the innate immune response. The human innate immune system consists of genetically programmed defence mechanisms that are meant to kill pathogens and to clear damaged host cells in a rapid non-antigen specific manner. The innate immune system involves recognition mechanisms that identify molecular patterns present in various components of invading pathogens and damaged cells. These patterns are recognized by pattern recognition receptors (PRRs)[1,2]. In contrast to adaptive immune system, the innate immune response does not not confer long-lasting protection against inducing pathogens nor does it amplify its response upon secondary challenge[3].

Under normal conditions neutrophils are abundant in the circulation and account for 60-70% of circulating leukocytes (white blood cells). Upon inflammation this number can rapidly rise up to more than 90% of all leukocytes. Under influence of several humoral factors and cellular associated signals neutrophils migrate to site of injury or infection, where they start to eradicate pathogens and damaged cells.

## Anti-microbial function

One of the mechanisms by which neutrophils can eliminate invading pathogens and infected and/or damaged cells is via phagocytosis. Neutrophils recognize targets that are opsonized by immunoglobins and complement components. Neutrophils use their immunoglobulin receptors (Fc-receptors such as FcγRII(CD32) and FcγRIII(CD16)) to bind to the Fc-part of immunoglobulins attached to their targets[4]. Via the αMβ2 integrin (CD11b/CD18) neutrophils can also bind to particles coated with the complement component C3bi[5]. Once bound to opsonized particels, neutrophils are activated and start t ingest these particles in a process normally referred to as phagocytosis.

Another mechanism by which neutrophils can attack invading pathogens is by release of cytotoxic agents such as proteinases, reactive oxidative and nitric species (ROS and RNS) stored in granules in the neutrophils cytoplasm. These granules may fuse and release their continents to the extracellular space as well as into the phagosome[6].

A third mechanism, more recently discovered, by which neutrophils can kill pathogens is by the release of neutrophil extracellular traps (NETs)[7]. NETs are networks of extracellular fibers, primarily composed of DNA from neutrophils. NETs that can bind pathogens and kill pathogens extrracellularly by several cytotoxic mediators bound to DNA within the traps[8,9].

## Neutrophil priming

In response to local inflammation activated endothelial cells and innate immune cells such as macroghages release pro-inflammatory mediators, such as interleukin (IL)- 1β, IL- 6, tumour necrosis factor (TNF)–α and platelet activation factor (PAF). Interaction with these inflammatory mediators and bacterial components, such as formyl-methionyl-leucyl-phenylalanine (fMLP) or lipopolysaccherides (LPS), results in pre-activation of circulating neutrophils. This pre-activation is also referred to as priming. Priming of cells is not associated with activation of cytotoxic responses but rather enhance these responses

evoked by activating agonists[10]. Priming is associated with altered expression and affinity of several specific surface receptors[11-13].
Neutrophils express a variety of receptors that are involved in cellular priming including specific chemokine receptors, adhesion molecules and immunoglobulinreceptors. Activation of these receptors typically induces a pro-inflammatory change in neutrophil function[14]. Primed neutrophils show *in vitro*, for instance an enhanced oxidative burst and increased migratory response to chemotactic stimulation by IL-8[15-17]. Furthermore, priming of neutrophils has shown to delay neutrophil apoptosis[18].

## PAMPs and DAMPs

As described earlier innate immune cells recognize their targets by PRRs of which Toll like receptors (TLR) are best described in the context of innate immune mechanisms[2]. PRRs are able to identify damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs). DAMPs consist of endogenous cell components released throughout cell damage, whereas PAMPs consist of microbial derived components. Well known DAMPs include mitochondrial components and intracellular proteins such as high mobility group box 1 (HMGB1), heat shock proteins, defensins and annexins[19]. Important PAMPs include bacterial derived endotoxin (LPS), peptites such as fMLP, lipoproteins and nucleic acids[2 20].
Although a distinction can be made between DAMPs and PAMPs based on its origin, there does not seem to be much difference between the two in terms of activating PRRs. Therefore, it has been proposed that PAMPs and DAMPs belong to an ancient subfamily of universal DAMPs and that they activate innate immune cell via the same receptors[21 22].

## Neutrophil homing

Neutrophils are attracted to sides of injury or infection by a variety of chemotactic signals generated at these sites by activated innate immune cells and stromal cells such as epithelial cells. These chemotactic signals include among others complement-derived C5a, leukotriene B4 and the neutrophil chemokine IL-8[3 23]. These chemtactic signals are recognized by chemotaxin receptors CXCR1(CD181), CXCR2(CD182) and C5aR expressed on the neutrophil surface. In addition, certain microbial peptides such as fMLP are chemotaxins for neutrophils and recognized by PRRs such as the fMLP receptor (FPR1)[12].
The transmigration of neutrophils across the vascular endothelium to inflamed tissue is preceded by rolling and firm adhesion of neutrophils to endothelial cells. This multiple step process is mediated via selectins and integrins expressed on the neutrophil surface. The first step of slowing down, or "rolling", of neutrophils is caused by loose adhesion of the neutrophil to the endothelium. Rolling is primarily mediated by reversible binding of L-selectin(CD62L) expressed on the neutrophil surface to its ligands on the endothelium. Next, rolling neutrophils can adhere to the endothelium causing the neutrophil to stop moving. Integrins, like αMβ2 integrin (CD11b/CD18), are involved in the firm adhesion of neutrophils to endothelial wall. Adhered cells can subsequently migrate through the vascular wall to site of injury or infection, a process called homing[14 24].

## Granulopoiesis

Neutrophils belong, together with eosinophils and basophils, to a class of leukocytes referred to as polymorphonuclear leukocytes or granulocytes. They are named for the multilobulated shape of the nucleus (polymorphonuclear) and named according to the staing propereties of their granules in the cytoplasma (neutrophilic, eosinophilic or basophilic granulocytes).

Neutrophils are produced in the bone marrow and  a derived from multipotent hematopoietic stem cells (HSC) that have the capacity to differentiate in to all kinds of leukocytes, erythrocytes and platelets[25]. The granulocyte lineage begins at a mutual precursor cell called myeloblast or CFU-G. Meyloblasts can divide and differentiate into promeyolocytes and myelocytes. All these precursors have the capacity to proliferate and are, therefore, part of the mitotic pool. Myelocytes can also loose their capacity to divide and mature into metameyelocytes, banded neutrophils and eventurally mature neutrophils (Figure 1). These latter cell types forms the post mitotic pool (PMP) in the bone marrow. During the maturation process, the nucleus's shape changes from a round nucleus to a banded nucleus in young neutrophils and to a segmented nucleus after full maturation. The expression of surface receptors also changes during maturation. It is known that for instance FcγR III(CD16) appears with maturation whereas VLA-4(CD49d/CD29) dissapears with neutrophil maturation[26].

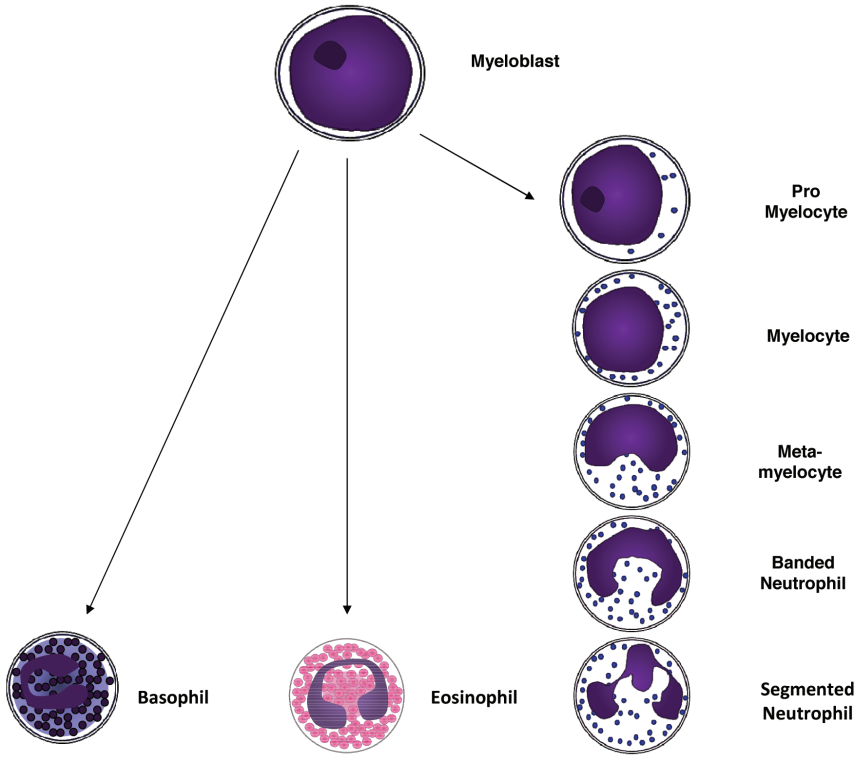


**Figure 1**
Schematic representation of neutrophil granulopoiesis. Adapted from a figure kindly provided by T.Tak.

## Bone marrow release

In steady state 0.3-1.3x10$^9$ neutrophils per kg bodyweight are produced in the bone marrow every day to maintain homeostsis[27]. The retention and maturation-controlled release of neutrophils from the bone marrow is regulated by very late antigen (VLA)-4 (α4β1- integrin) and the CXCR-4(CD184)/stroma derived factor (SDF)-1 axis. VLA-4 and CXCR4 are both highly expressed on neutrophil progenitor cells in the bone marrow[28-31]. Neutrophils progenitors are retained in the bone marrow as they adhere via VLA-4 to vascular adhesion molecule (VCAM)-1, which is expressed on bone marrow endothelium and stroma[30]. Petty at al. showed that blockade of VLA-4/VCAM-1 binding results in a egress of neutrophils from the bone marrow[30]. They furthermore showed that SDF-1 (CXCL12) signaling through neutrophil CXCR4 augments VLA-4 adhesion to VCAM-1 and that inhibition of both CXCR4 and VLA-4 caused a synergistic release of neutrophils from the bone marrow. Neutrophil VLA-4 and CXCR-4 expression decreases during maturation setting the egress from the bone marrow in motion.

In response to inflammation, a variety of signals induce recruitment of neutrophils form the bone marrow into the circulation. Neutrophil recruitment is mainly mediated by granulocyte colony stimulating factor (G-CSF), but also other signals including endotoxin, gluco-corticoids, complement factors and TNF-α can induce neutrophil recruitment[32-34].

During acute inflammation neutrophils are rapidly mobilized from this storage pool, creating a blood neutrophilia. This rapid mobilization is characterized by the appearance of young banded.

## Neutrophil related inflammatory complications

Neutrophils are important for maintaining innate immune surveillance but upon inappropriate activation can also contribute to tissue damage. Within hours after injury, a cascade of humoral and cellular factors results in priming of neutrophils and enhanced mobilization of cells to the tissue that is not necessarily affected by the injury[35-37]. This immune response accompanied with an increased number of circulating leukocytes is generally referred to as systemic inflammatory reaction syndrome (SIRS)[38]. It is thought that an aberrant systemic inflammatory response results in an accumulation of neutrophils in organ capillaries followed by a random migration of neutrophils to organ tissues[36]. An overwhelming sequestration of neutrophils in combination with a massive release of cytotoxic agents may harm the healthy organ parenchyma of the host. Damage of organ tissue can range from mild disease to extreme conditions such as acute respiratory distress syndrome (ARDS) and/or multiple organ failure organ dysfunction syndrome (MODS). MODS and ARDS are life threatening complications that occur frequently in patients after major surgery, trauma and burn injury[39 40]. MODS and ARDS account for 50 to 80% of the late death of all surgical ICU patients[41 42].

The aberrant inflammatory response is not only associated with organ dysfunction early after injury, it is also associated with an increased risk for septic complications and organ dysfunction in a later phase[16 43]. How an excessive early inflammatory response is related to late septic complications is poorly understood. A concept of a compensatory anti-inflammatory reaction syndrome (CARS) following SIRS was developed in the late nineties[44 45]. To date this concept has been more and more abandoned as pro- and anti-inflammatory responses are described to occur simultaneously[43 46].Yet, there is substantial evidence for

a late immunodeficient state during which patients suffer and enhanced susceptibility nosocomial infections[43]. This increased susceptibility for infection and immunodeficiency state is a serious problem as the mortality of septic shock remains high despite appropriate antibiotics and supportive therapy on an intensive care unit (ICU)[47].

## Immunomodulation

Modulation of the inflammatory response has been considered as a possible means to prevent organ dysfunction and septic complications. However, attempts to modulate the inflammatory response have failed to improve survival in human so far. It seems that the innate immune system is hardly sensitive for commonly used immunosuppressive drugs. Therefore, novel agents need to be tested, developed or applied.

Endogenous inhibitors of the innate immune response may be used as potential drugs for this purpose. One of these endogenous inhibitors is acute phase protein C1 esterase inhibitor (C1INH). C1INH is an acute phase protein which is excreted during inflammation by various cells, such as hepatocytes, fibroblasts, monocytes and macrophages[48]. C1INH acts as a potent anti-inflammatory protein because it inhibits all three the pathways of the complement system[49-51]. It additionally inactivates the contact system via inhibition of the formation of kallikrein and FXIIa (Figure 2). Through inhibition of the complement and contact system C1 INH reduces the formation of several neutrophil agonists and restrains vascular leakage and formation of oedema.

However, there is evidence that a part of the effect of C1INH on neutrophils response is independent from complement and contact system activation. In a sepsis model in the mouse substitution of active as well as inactive C1INH (iC1INH) decreased leukocyte adhesion, increased bacterial clearance, and improved survival [51]. Since similar and even better results are obtained with iCINH substitution, it is suggested that C1INH might act directly against neutrophils. This hypothesis is supported by *in vitro* results showing increased bacterial clearance and diminished adhesion molecule expression in presence of C1INH as well as iC1INH and in absence of complement en contact system[52]. These mentioned properties make C1INH a promising drug for antagonizing the pro-inflammatory response.

## Outline of thesis

Aberrant inflammatory response results in high morbidity, mortality and health care costs. So, there is an unmet need for both diagnostics and novel therapeutics for diagnosis/prognosis and treatments for the complications caused by acute and excessive activation of the innate immune response after injury.

The neutrophil response underlies the pathogenesis of inflammatory complications such as MODS[36]. The main topic of this thesis concentrates on the early response of the circulating neutrophil pool upon acute systemic inflammation and on modulation of the early innate immune response *in vivo*.

First, a review of the literature was performed to gain insight of the knowledge on the identification of trauma patients at risk for inflammatory complications (**Chapter 2**).

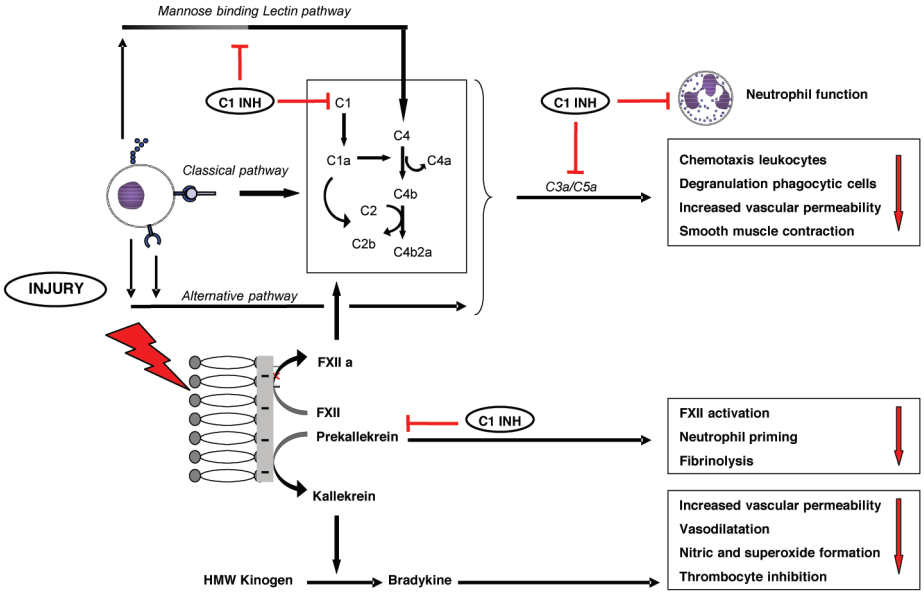


**Figure 2**
C1INH inhibits the classical, (Mannose binging lectin) MBL and alternative pathway of the complement system. In that way, it reduces the production of the anaphylatoxins C4a, C3a and C5a. C1 INH directly inhibits neutrophil function and opsonizes C3b (not shown in figure). C1INH further inactivates prekallikrein and FXII. Inactivation of prekallekrein leads to a reduced bradykine production reduced neutrophil priming and reduced FXII activation. Inactivation of FXII, on the other hand, results in a diminished stimulation of the classical pathway of the complementsystem and the intrinsic pathway of coagulation via FXIIa.

Then, changes in phenotype of circulating neutrophils were studied during the first 24 hours after thoracic injury *in vivo* (**Chapter 3**) and related to inflammatory complications. It appears that systemic inflammation results in a heterogeneous pool of circulating neutrophils that can be divided in different neutrophil subsets[53]. **Chapter 4** focuses on one of these subpopulations. A VLA-4$^{pos}$ neutrophil like population, which emerges in the circulation in severely injured patients, is characterized and phenotype and functional changes are studied *in vitro* in time.

Studying the neutrophil response in trauma patients is hampered by several obstacles and confounders. First of all, the delay between injury and arrival of the patient in hospital makes it almost impossible to investigate the start of inflammatory response. In addition, the neutrophil response in trauma patients is altered by a range of additional inflammatory stimuli (like surgical intervention, need for mechanical ventilation, anemia, wound contamination etc.) that vary greatly among trauma patients. We propose the use a standardized *in vivo* inflammation model to study the neutrophil response. The human endotoxemia model is such a model. In this model intravenous administration of LPS induces a systemic inflammation in response. Yet this model provokes a PAMP induced inflammatory response instead of a mainly DAMP induced inflammation in trauma patients. In **chapter 5** we investigate if neutrophils in the circulation are similarly activated after endotoxemia (PAMP) and trauma (DAMP).

In a systemic review the outcome of applied immunomodulating therapies in trauma patients is evaluated (**Chapter 6**).
The potential immunomodulating effects on the humoral and cellular immune response of intravenous administration of the endogenous acute phase protein C1INH is studied in the human endotoxemia model (**Chapter 7** and **8**).
This thesis concludes with a general discussion (**Chapter 9**) and a summary in Dutch (**Chapter 10**).

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# PART I
# IMMUNOMONITORING

# 2

## *Post injury immune monitoring*
## *Can multiple organ failure be predicted?*

*Curr Opin Crit Care. 2008 Dec;14(6):666-72*

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## Abstract

**Purpose of this review**

Multiple organ failure (MOF) is the main cause of late morbidity and mortality after severe injury. This disease state is driven by a dysfunctional immune system. Prediction of MOF based on clinical parameters appears to be insufficient. A better understanding of immunological pathogenesis underlying MOF may lead to better prediction and innovation in treatment strategy in order to increase survival of trauma-patients.

**Recent findings**

Immune monitoring has increased the knowledge of the pathogenesis of MOF, but many mechanisms underlying the pathogenesis remain to be elucidated. Consequently, adequate predictive markers for diagnosis and monitoring still need to be developed.

**Summary**

General markers of inflammation including cytokines are correlated with posttraumatic complications with a low sensitivity and specificity and are, therefore, of little use as prognostic markers. Current findings regarding the functionality of immune cells are promising and might be of prognostic value in the near future.

# Introduction

Multiple organ failure (MOF) and adult respiratory distress syndrome (ARDS) are life-threatening complications that manifest in 30 % of multi-trauma patients[1**]. Despite improvement of technology and supportive treatment on intensive care units, MOF remains the leading cause of late mortality after trauma[2-5].

There is undisputable evidence that MOF and ARDS results from an unbalanced systemic inflammation[6 7]. The risk of an excessive inflammatory response and subsequent organ failure increases with the severity of initial injury and shock. However, it has become clear that the risk further increases by succeeding activation of the inflammatory response due to a second hit caused by e.g. surgical intervention or inadequate resuscitation therapy. Especially in patients with already a highly activated immune system, adequate treatment strategy is essential to minimize the risk of ARDS and MOF. It is postulated that differences in extent of the initial inflammatory response plays a determining role in the development of MOF and ARDS[8]. Reliable early inflammatory markers that can predict these life threatening conditions are lacking. Development of markers is essential to adjust treatment strategies in those patients who are prone to develop organ failure, because until now it remains unclear why under seemingly similar clinical conditions, some patients endure organ failure and others do not.

Correlations between different serum markers and the incidence of MOF and mortality have been described in many studies. Potential biomarkers include conventional serum markers such as acute phase proteins and coagulation markers, plasma cytokine levels, markers of monocyte or polymorphnuclear leukocyte (PMN) function and many more.

In this review we will summarize the results of several immune monitoring studies performed in trauma-patients during the past decade. We will discuss new aspects and an emerging hypothesis regarding the post injury immune response leading to MOF and what consequences innovative immune-monitoring will have for management of these diseases in due course.

**Conventional serum markers**

Inflammatory markers have been proposed as useful predictors for the occurrence of MOF and ARDS, given that the severity of post injury innate immune response is correlated to the risk of developing organ failure.

The most commonly used inflammatory markers are acute phase proteins, including C-reactive protein (CRP), procalcitonine (PCT) and phospholypase $A_2$ ($PLA_2$). Acute phase proteins are defined as proteins whose plasma concentrations increase or decrease during inflammation. Although interleukin- 6 (IL-6) is considered to be an acute phase protein as well, it will be discussed in the next paragraph regarding cytokines.

CRP as well as $PLA_2$, are significantly elevated at and during admission in patients developing MOF compared to patients who do not[9*]. In addition, $PLA_2$ and CRP levels remain significantly higher (respectively from day 2 and 4 post injury) in multiple injured patients with lethal MOF compared to those who survive MOF[10]. The positive prognostic value for lethality amounts 74% for $PLA_2$ on day 2 whereas that of CRP reaches 86% on day 4 (see Table 1). Yet, most studies show that both these markers are non-specific and therefore have a low predictive value[9* 11-15].

PCT is proposed to be a better and more specific marker for inflammation than CRP, as the kinetics of PCT more closely resembles the kinetics of inflammation[16 17]. Serum levels of PCT more rapidly increase after the onset of inflammation and faster decline as inflammation diminishes. Several studies report that PCT can be useful in discriminating between sepsis and SIRS, but only a few studies have been investigating the predictive value of PCT with regard to development of organ failure after injury[18-20]. Two studies, investigating inflammation-induced complications after trauma, describe significantly increased PCT concentrations with peak levels on day 0 to 3 after trauma[18 20]. The extend of the elevated PCT concentrations showed a correlation to ISS and development of severe sepsis and septic shock. One study found a correlation between the occurrence of MOF[20], whereas the other one did not[18]. Both studies mentioned that the predictive value strongly depends on the cut off value of PCT plasma levels. When the cut off point of plasma PCT levels increases the sensitivity decreases while the specificity increases, changing thereby the positive and negative predictive values.

Other conventional biochemical markers have also been associated with inflammation-induced complications after injury. The innate immune response is triggered by many physiological disturbances occurring as result of trauma. Abnormalities of biochemical laboratory findings can be indicative for the severity of inflammation. Biochemical markers that reflect differences in the patient's physiologic condition include those of the coagulation system (platelet count, (pro)thrombine time) and those associated with shock (base deficit, lactate and haemoglobin). It is known that coagulopathy is associated with lethal outcome[21 22]. Combining markers of the coagulation and inflammation system with injury severity has proven to be a quite accurate method for the prediction of complications[23-25]. Sauaia et al. developed a model in which the Acute Heath Physiology and Chronic Health Evaluation (APACHE) score is combined with shock and coagulopathy indicators. This model could predict MOF as early as 12 hours after injury[2]. Low platelet counts and prolonged protrombine time at admission were determined as most important coagulopathy markers associated with increased risk of MOF. With regard to shock indicators, base deficit and haemoglobin levels seemed to be related to the development of MOF. In contrast to others studies, lactate did not contribute to positive predictive value of the development of MOF[23 26]. This study shows that the predictive value considerably increases when several biochemical markers and demographic data are combined, but unfortunately, the sensitivity and specificity remains low (63 and 84% resp). Interestingly, this study shows that physiological abnormalities during the first 12 hours after trauma are the most predictive for the development of MOF, suggesting that the extent of the initial innate immune response is most important for the development of inflammatory complications.

**Cytokines**

The innate immune response is modulated by cytokines and cytokine levels rapidly change directly after injury. The rate of release of several cytokines depends on the severity of injury. Levels of several important cytokines can easily be measured in the peripheral circulation and, therefore, cytokines have for a long time been proposed as potential markers. Plasma levels of tumor necrosis factor-α (TNF-α) and IL-1β rise within 1 to 2 hours after initial trauma followed by, among others, IL-6, IL-8, IL-12 and the anti-inflammatory cytokine IL-10[12 27 28]. The levels of the latter cytokines are maximal between 6 to 24 hours post-injury and gradually decline during the following days (Figure 1).

**Table 1** Positive predictive value of different markers for organ failure, septic complications and mortality in prospective series with multitrauma patients

| Marker | Result | Positive predictive value |
|---|---|---|
| CRP | Increased from admission until 10 days postinjury in patients developing MOF. | ? |
| | Increased from day 3 postinjury in patients developing MOF. | 73% |
| | Increased preoperative in trauma patients with postoperative MOF. | 75% |
| | Decreased from admission in patients developing MOF. | ? |
| PCT | Increased from day 1 after admission until 21 days postinjury in patients developing sepsis. | 38–67% |
| | Increased at admission in patients developing septic complications. | ? |
| PLA2 | PLA2 Increased from admission until 10 days postinjury in patients developing MOF | ? |
| | Increased from day 2 after admission in patients developing MOF. | 74% |
| Lactate | Increased from admission until day 4 postinjury in patients developing MOF; from day 7 increased in patients with lethal MOF compared with survivors with MOF. | ? |
| | Decreased in trauma patients undergoing late surgery who developed MOF compared with those who did not. | ? |
| | No significant difference between those who developed MOF and those who did not. | - |
| Platelet count | Decreased preoperative in trauma patients with postoperative MOF. | 71% |
| IL-6 | Increased from admission until day 4 postinjury in patients with lethal MOF; patients who survived MOF did not show a significant increase in IL-6 levels compared with those without MOF. | ? |
| | Increased from admission until day 10 postinjury in patients developing MOF. | ? |
| | Increased at admission in all patients and declining to baseline levels in the following day in patients without MOF; patients with early MOF showed a second peak of IL-6 levels day 1postinjury, patients with early and late MOF showed a second peak at day 5 to 6 postinjury and those with late MOF on 7 until day 10 postinjury. | 70% |
| IL-8 | Increased from admission until day 1 postinjury in patients developing MOF; patients with lethal MOF show increased IL-8 levels from admission until 14 days post injury. | ? |
| | Increased at admission in all patients and declining to baseline levels in the following day in patients without MOF; patients with early MOF showed a second peak of IL-8 levels day 1postinjury, patients with early and late MOF and those with only late MOF showed a second peak at day 7 until day 10 postinjury. | 69% |
| IL-10 | Increased from admission until day 10 postinjury in patients developing MOF. | ? |
| | Increased from day 1 postinjury in patients developing sepsis or MOF but not in patients developing ARDS. | ? |
| | Increased at admission and declining to baseline levels in following days in patients developing MOF; patients with late MOF had the highest IL-10 levels followed by patients with early and late MOF; patients developing early MOF had lower IL-10 levels but still increased compared with patients without MOF. | 60% |

Positive predictive value is the proportion of patients with positive test who are correctly diagnosed. ARDS, adult respiratory distress syndrome; CRP, C-reactive protein; MOF, multiple organ failure; PCT, procalcitonin; PLA2, phospholipase A2.

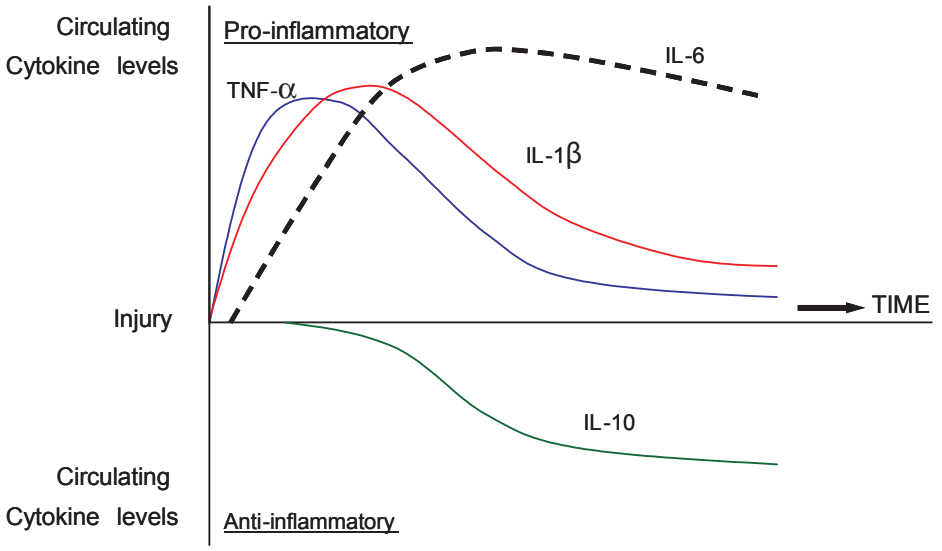


**Figure 1**
Graphic presentation of cytokine release during the first hours after injury (combined data from: 1** 9* 11 13 23 28). TNF-α and IL-β are early mediators, which endorse the release of other cytokines during inflammation. TNF-α and IL-β reach peak concentrations within the first 6 hours after injury. IL-6, IL-8, IL-10 levels rise within hours after injury and reach peak level after 6 and 24 hours. Levels gradually decline after 24 hours post injury. IL-6 is believed to be mainly anti-inflammatory although pro-inflammatory features have been described.

Enhanced IL-6 levels evidently correlate with the severity of injury as well as the incidence on MOF, ARDS, sepsis and mortality[11 23 29 30]. Interestingly, trauma-patients show a second rise of IL-6 in response to secondary hits[31]. Once became clear that surgery during the first after trauma was associated with increased IL-6 levels and poor outcome[32-34] treatment strategy in emergency care considerably changed. Patients in extremis nowadays undergo only damage control surgery (DCS), whereas stable patients receive early total care (ETC). Yet, there is a large group of borderline trauma-patients in which it remains uncertain who benefits from ETC or DCS. To refine treatment strategy in those patients, there is a clear unmet need for biomarkers that can identify patients at risk.
Elevated IL-8 plasma levels also correlates with injury severity and outcome[23 29]. Yet, contrasting results concerning IL-8 have been reported, probably due to the short half-life time of this cytokine in peripheral blood[11 35]. For that same reason, IL-1β and TNF-α have turned out to be poor markers too[11 12]. In addition, the wide variety in value of the cytokine levels among individuals makes it difficult to predict outcome[23]. Yet, positive predictive values of 80 to 90% have been described in studies combining cytokine levels with biochemical markers and clinical signs[9* 19].
Interestingly, cytokine release patterns do not seem to correlate to the biphasic concept in which a severe auto-destructive pro-inflammatory response (also called systemic inflammatory reaction syndrome (SIRS)), causing early-onset ARDS or MOF, is followed by a compensatory anti-inflammatory reaction syndrome (CARS), making the patient susceptible to infection and late-onset MOF (Figure 2). The anti-inflammatory cytokine

IL-10 is considered to be an important mediator in CARS. However, IL-10 levels rise shortly after trauma and do not markedly increase during CARS (Figure 1). These contrasting findings have changed the idea of CARS to a concept of a mixed anti-inflammatory reactions syndrome (MARS). This hypothesis describes that the pro- and anti-inflammatory reaction occur simultaneously. Elevated IL-10 serum levels are known to correlate with ISS and with increased risk of sepsis, MOF and mortality[9* 12 36], but its exact role in the pathogenesis of SIRS and CARS or MARS remains unclear.

Remarkably, only a few studies took the difference in the pathogenesis between early or late-onset MOF in considering when investigating correlation between cytokine release and MOF[1** 23]. These studies showed that cytokine patterns clearly differ in patients with early-onset and late-onset MOF (see Table 1). Maier et al. studied correlation between cytokine levels and the onset of MOF and showed that IL-6 and IL-8 had a positive predictive value of 70 and 69% respectively. Unfortunately, this enfolds that still 30% is false positive.

**Cellular response**

Immune cells play a key role in the inflammatory response and differences in functionality and/or immune phenotype of these cells have been reported to correlate with complications and outcome of trauma-patients. Within hours after injury the number of leukocytes markedly increases, while the number of lymphocytes and monocytes decreases[37]. Post injury leukocytosis is mainly the result of an increased number of PMNs. These PMNs are considered to have an essential role in the final common pathway of the inflammatory response after injury. Several studies have demonstrated that high numbers of PMNs during the first hours after injury are associated with increased risk of organ failure and mortality[38 39]. Migration of PMN to tissues is an important determinant in the pathogenesis of organ failure. Autopsy has shown that large numbers of PMNs are found even in non-injured tissues in patients with ARDS/MOF[7]. In addition, patients developing MOF show a faster decline in leukocyte count, indicating rapid extravasation of PMNs[38 39].

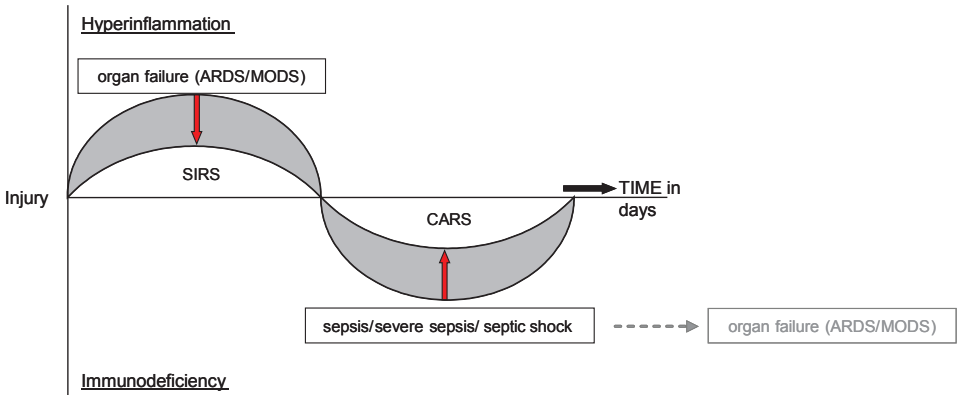


**Figure 2**

Concept op biphasic pattern of SIRS and CARS as introduced by Moore in 19968. During SIRS patients are at risk to develop aseptic organ failure (ARDS/MOF), whereas during CARS they are at risk of developing severe sepsis and septic shock. An ongoing severe sepsis, however can leas to organ failure in the second place. Gray arrows indicate the effect of a reduction of the immune response early after onset.

Not only the number of cells but also the functionality of these cells is associated with clinical outcome. During the first days after trauma circulating PMNs prove to be primed, resulting in not only in an increased migratory capacity but also in enhanced cytotoxic functions[31 40-42]. The levels of neutrophil elastase as indicator of systemic activation of PMNs, rise shortly after injury[23]. In addition, significantly elevated neutrophil elastase levels are seen during the first hours and days in patients with organ failure[7 23]. In surgical patients, neutrophil elastase levels are even suggested to have good positive prognostic value for complications with a sensitivity of 88% and a specificity of 83%[43]. In septic critically ill patients admitted to an ICU, however, non-survivors remarkably show decreased neutrophil elestase levels, suggesting a suppressed functionality of PMN. This reduced PMN function fits the idea of a biphasic pattern of hyperinflammation during SIRS followed by immuneparalysis during CARS (see Figure 2).

Primed PMNs are characterized by an increased expression of the integrin CD11b/CD18 on the cell surface. Expression of this integrin tends to be normal or slightly increased in trauma patients at admission, indicating priming of an amount of peripheral PMNs[44]. Integrin expression declines after several days[44 45]. The migratory capacity of these PMNs seems to decline simultaneously. These findings can lead to the counterintuitive conclusion that systemic neutrophils do not become primed upon injury. However, these findings can also be explained by a more rational hypothesis describing the rapid homing of primed PMNs to the tissues leaving behind non-primed cells in the circulation. This latter hypothesis is supported by several findings. Firstly, integrin expression on PMNs in lung fluid is markedly increased after injury compared to those in the peripheral circulation[46*]. Secondly, other studies show that PMNs of patients, who are prone to develop organ failure, are unresponsive to different stimuli such as the innate immune stimulus fMLP[47 48]. PMN normally show an increased functionality upon interaction with pro-inflammatory cytokines *in vitro* and particularly responses associated with adhesion and chemotaxis are sensitive for these priming signals[49]. It is therefore, reasonable to assume that these functionally primed PMNs have left the circulation in patients with a poor clinical outcome[48]. It indicates that severe inflammation results in the occurrence of functionally refractory immune cells in the peripheral circulation.

**Conclusion**

Despite the numerous studies focussed on the pathogenesis of MOF, the underlying mechanisms are still poorly understood. Consequently no good methods are available to quantify the early host response after injury and to predict outcome have been developed. General inflammatory markers are correlated with posttraumatic complications but with a low sensitivity and even lower specificity and are, therefore, of little use as predictive markers. So far levels of single cytokines have shown the largest potential as a biomakers, however the predictive value of this approach is increasingly under debate. Many studies have shown contrasting results about the correlation between cytokines and complications or outcome. As result, no (single) cytokine turned out to be legible enough to predict outcome. Therefore, determination of the change in functionality of innate immune cells might be more promising as these cells integrate all the pro- and anti-inflammatory signals and change their phenotype accordingly. Detailed analysis of these phenotypes can then help in characterizing and predicting inflammatory complications after injury.

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Papers of particular interest, published within the annual period of review have been highlighted as:
* of special interest
** of outstanding interest

PART I

IMMUNOMONITORING

# 3

# Isolated blunt chest injury leads to transient activation of circulating neutrophils

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## Abstract

The acute respiratory distress syndrome (ARDS) is a severe and frequently seen complication in multi-trauma patients. ARDS is caused by an excessive innate immune response with a clear role for neutrophils. As ARDS is more frequently seen in trauma patients with chest injury, we investigated the influence of chest injury on the systemic neutrophil response and the development of ARDS.

Thirteen patients with isolated blunt chest injury (abbreviated injury score (AIS) 2 to 5) were included. To avoid systemic inflammation caused by tissue damage outside the thorax, injuries in other regions than chest did not exceed an AIS of 2. At 3, 9 and 24 hrs after injury expression of circulating activating molecules on neutrophils and levels of circulating interleukine (IL)-6 were determined. Blood samples from eight healthy volunteers were used as control.

Blunt chest injury resulted in activation of circulating neutrophils, is characterized by decreased expression of L-selectin (CD62L), CXCR2 (CD182b) and C5aR (CD88) compared to control ($p<0.05$). Expression of L-selectin, CXCR2 and C5aR partially restored at 24 hrs after injury. In addition, mean expression of FcγRIII (CD16) dropped ($p<0.001$), indicating recruitment of young neutrophils into the circulation. IL-6 levels increased to maximum mean concentration of 86±31 pg/ml at 24 hrs post injury. None of the patients developed ARDS.

Blunt chest trauma caused a systemic inflammatory reaction with transient activation of neutrophils and mobilization of young neutrophils into the circulation. Isolated chest injury, however, was not abundant enough to cause ARDS and a second-hit seems crucial.

# Introduction

In multi-trauma patients, the acute respiratory distress syndrome (ARDS) is a commonly seen complication. Important risk factors for development of ARDS after trauma are severe injury (Injury Severity Score (ISS) > 25) and pulmonary contusion[1 2]. Although the occurrence of ARDS in multi-trauma patients increases considerably with severity of pulmonary contusion, the incidence is surprisingly low in patients with isolated chest injury[2-4].
An excessive innate immune response to tissue injury is considered to be the cause of ARDS. Due to the heterogeneity of injuries in multi-trauma patients, the specific role of chest injury in the pathogenesis of ARDS is not well known. In this study we investigated the effect of isolated chest injury on the systemic innate immune response to test whether this increased risk of ARDS is caused by priming of the circulating neutrophils or solely due to local damage. We focused on differences in phenotype of circulating neutrophils during the first 24 hrs after chest injury. Neutrophils are important effector cells of the final common pathway of the innate immune response. Activated neutrophils migrate out of the circulation into the alveolar compartment. Massive release of radical oxygen species (ROS) and proteases by neutrophils can cause damage to the parenchyma which can eventually result in organ dysfunction[5].
Systemic inflammation is characterised by activation of circulating neutrophils[6 7] and mobilisation of young neutrophils from the bone marrow. Surface receptors of circulating neutrophils have been used to determine activation of circulating neutrophils in order to discriminate between severity of inflammation and to predict the occurrence of organ failure[6-9]. Systemic neutrophils activation is typically characterized by shedding of L-selectin(CD62L) and upregulation of expression of the αM integrin(CD11b)[10-13]. Decreased L-selectin and increased αM expression have been shown to correlate to ISS and to be associated with the development of posttraumatic complications, such as ARDS[14-20].
A previous study by our group showed a significant decreased responsiveness after *in vitro* stimulation of active FcγRII (CD32), the main IgG-receptor on neutrophils, in multi-trauma patients[20]. Decreased responsiveness of active FcγRII was more pronounced in patients who developed ARDS or acute lung injury (ALI) compared to patients without complications. Expression of active FcγRII is under the control of inside-out signals induced by both chemoattractants and cytokines and seems to be more sensitive to priming stimuli compared with αM/CD11b[21]. Responsiveness of active FcγRII have turned out to correlate better with outcome than other neutrophil receptor expressions in preceding observational studies in trauma patients[11 20].
We investigated whether isolated chest injury leads to a systemic innate immune response quantified by activation of circulating neutrophils. Furthermore, the release of Interleukin (IL)-6 was measured as an additional marker for inflammation.

# Methods

**Patients**

Patients suffering from chest injury with an abbreviated injury score[3] of 2 or more and admitted to the Trauma department of the University Medical Centre Utrecht were included.

Patients with injuries of an AIS >2 in other regions than thorax were excluded to reduce systemic inflammation caused by tissue damage outside the thorax. Other exclusion criteria included age < 18 or > 70 years, death within 24h after admission and patients with an altered immunological status (e.g. corticosteroid use or chemotherapy).
At admission, Injury Severity Score (ISS)[22], New Injury Severity Score (NISS)[23], Apache II Score24 and leukocyte count were determined. All patients were followed until discharge. The presence of ARDS was assessed according to their clinical criteria as determined in the consensus conferences of ARDS[25].
Blood samples ware taken at approximately 3 (2-4) hrs, 9 (8-10) hrs and 24 (22-26) hrs after the accident, to investigate the relationship between chest injury and systemic neutrophil activation. In an *in vivo* human inflammation model we have previously seen that systemic neutrophil activation is most prominent between 2-4 hrs after induction of inflammation[26]. First time point of measurement was therefore set at 3 hrs post injury.
The local ethics committee approved the study and written informed consent was obtained from all patients or their legal representatives in accordance with the Helsinki declaration.

**Expression of activation markers on neutrophils determined by flowcytometry**
For analysis of neutrophil receptor expression by flowcytometry the following commercially available mouse-antihuman monoclonal antibodies were purchased: fluorescein isothiocyanate (FITC) -labelled IgG1 isotype-control (clone MOPC-21, BD Pharmingen, USA), Alexa Fluor® 647- labelled IgG1 isotype-control (clone MOPC-21, BD Pharmingen, USA ) R-phycoerythrin (RPE)- labelled IgG2a isotype-control (clone MRC OX-34, Serotec, Germany), RPE-labelled IgG1 anti-αM (CD11b; clone 2LPM19c, DAKO, Denmark), FITC-labelled IgG1 anti- L-selectin (CD62L; clone Dreg56, BD Pharmingen, USA), Alexa Fluor® 647-labelled IgG1 anti- FCγRIII (CD16; clone 3G8, BD Pharmingen, USA), RPE-labelled IgG2b anti- FcγRII (CD32; clone FLI8.26, BD Pharmingen, USA), FITC-labelled IgG2a anti-CXCR1 (CD181a; clone 42705, R&D Systems Europe, UK), RPE-labelled IgG2a anti-CXCR2 (CD182b; clone 48311, R&D Systems Europe, UK), FITC-labelled IgG2a anti-C5aR (CD88; clone P12/1, Serotec, Germany).
A FITC-labelled monoclonal phage antibody (A27), which recognises the active configuration of FcγRII (CD32), was manufactured at the Department of Respiratory Medicine at the University Medical Centre Utrecht (MoPhab A27, UMC Utrecht, The Netherlands)[21 27]. The functionality and configuration of FcγRII (CD32) on granulocytes is regulated by inside-out control[28]. Visualization of this process by the antibody A27 is a very sensitive means to monitor *in vivo* subtle activation of innate immune cells such as neutrophils.
Blood was collected in a Vacutainer® with sodium heparin as anticoagulant and cooled immediately on melting ice. Blood samples of eight healthy volunteers served as a control values. Red cells were lysed with icecold isotonic $NH_4Cl$[27]. After lyses white blood cells were washed and resuspended in PBS2+ (phosphate buffered saline supplemented with sodium citrate (0.4% wt/vol) and pasteurised plasma protein solution (10% vol/vol)). Resuspended cells were incubated for 45 min on ice with commercial obtained directly labelled antibodies against activation molecules: L-selectin, αM, CXCR1, CXCR2, C5aR, FCγRII and FCγRIII.

After incubation and final wash, expression was measured on FACScalibur Flow cytometer (Becton & Dickenson, Mountain View, CA). The neutrophils were identified according to their specific side-scatter and forward-scatter signal.
For measurement of FcγRII* expression, whole blood was incubated with a FITC-labelled monoclonal phage antibody A27 for 45 min on ice[11]. Active upregulation of FcγRII* expression was measured after 5 min of stimulation of whole blood at 37 °C with N-formyl-methionyl-leucyl-phenylalanine (fMLP $10^{-6}$M) to evaluate the responsiveness of the cells for bacterial derived protein products/peptides. After stimulation, the samples were put on ice again and stained with phage antibody A27. After staining, red cells were lysed and expression was measured on FACScalibur as described above.
Data from individual experiments are depicted as fluorescence intensity as the median fluorescence intesity (MFI) of at least 10,000 neutrophils.

**IL-6**
Plasma samples were obtained at 3, 9 and 24 hrs after injury and stored at -80ºC until further analysis. IL-6 levels were measured by an enzyme-linked immunosorbent assay (ELISA) according to manufactors protocol (Ebioscience, San Diago, USA).

**Statistics**
All data are presented mean ± SE, unless described otherwise. To compare differences of admission variables between patients or control values, Mann Whitney test was used as appropriate. A p-value $< 0.05$ was considered significant.

# Results

**Patient demographics**
From April 2008 until April 2009 seventeen patients were included. Four patients were eventually excluded because of considerable additional injury (AIS >2) diagnosed within 24 hours after admission. All patients, of whom 9 were male and 4 female, had blunt chest injury. Thorax AIS varied from 2 to 5. Injury mechanism and admission characteristics are listed in Table 1. Four patients were under influence of alcohol at admission. One of them was also hypothermic with a body temperature of 35.4°C.
The mean age was 54±4 years, the mean ISS 18±2 and mean NISS 23±3. One patient was diagnosed with a head AIS of more than 2. This patient suffered from diffuse axonal injury (DAI) without signs of bleeding, oedema or compression on CT scan (AIS of 5). Diagnosis was made several days after injury based on clinical presentation. Since the high sore assigned to DAI, is related to an increased risk of mortality caused by direct brain injury rather than to severity of inflammation, this patient was not excluded from further analysis.
The mean Apache II score was 9±2 and mean hospital stay amounted 16±2 days. Six patients needed mechanical ventilation during hospital stay for a mean duration of 6±2 days. None of the patients developed ARDS. Pneumonia was diagnosed in two patients. One patient developed sepsis due to thoracic empyema and underwent thoracotomy for debridement of the pleural cavity.

**Table 1** Patient characteristics. M = male, F = female, MVA = motor vehicle accident, ISS = injury severity score, NISS = new injury severity score

| Pt | Gender | Age | Mechanism of injury | Diagnosis | ISS | NISS | Thorax AIS | Apache II | Leukocyte count * 109/L at admission | Complications |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 25 | MVA | 8 rib fractures unilateral<br>Pneumothorax bilateral | 16 | 25 | 4 | 3 | 12.0 | None |
| 2 | M | 62 | MVA | 3 rib fractures unilateral<br>Clavicula fracture<br>Orbita roof fractur | 17 | 17 | 3 | 15 | 8.0 | Pneumonia |
| 3 | M | 51 | Fall from height | 2 rib fractures unilateral<br>Pneumothorax unilateral | 9 | 9 | 3 | 6 | 9,.0 | None |
| 4 | F | 47 | Fall from horse | 10 rib fractures unilateral<br>Flail thorax | 17 | 17 | 4 | 3 | 19.3 | None |
| 5 | M | 60 | MVA | 5 rib fractures unilateral<br>Pneumothorax unilateral<br>Lungcontusion unilateral<br>Pancreas contusion | 21 | 29 | 4 | 9 | 17.6 | Infected epidural catheter |
| 6 | M | 31 | MVA | 3 rib fractures unilateral<br>Pneumothorax unilateral<br>Lungcontusion unilateral<br>Humerus luxation<br>Clavicula fracture<br>Diffuse Axonal Injury | 38 | 43 | 3 | 18 | 8.0 | None |
| 7 | F | 67 | MVA | 5 rib fractures unilateral<br>Flail thorax<br>Pneumothorax unilateral<br>Lungcontusion bilateral<br>Fracture proc. transverus | 20 | 36 | 4 | 5 | 15.6 | None |
| 8 | M | 59 | Fall from height | 6 rib fractures unilateral<br>Pneumothorax unilateral<br>Lungcontusion uniilateral | 16 | 25 | 4 | 18 | 15.9 | Thorax empyema, sepsis |

*folllow up table 1*

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 9 | M | 69 | MVA | 3 rib fractures unilateral | 5 | 5 | 2 | 5 | 5.0 | Infected epidural catheter |
| 10 | F | 62 | Bicycle accident | 6 rib fractures unilateral<br>Minor laceration kidney<br>Facial heamatoma | 14 | 14 | 3 | 7 | 8.5 | Pleural effusion |
| 11 | F | 53 | Fall from height | 4 ribfractrures unilateral<br>1 rib fracture contra lateral<br>Scapula fracture<br>Fracture cervical vertebral body | 17 | 17 | 3 | 2 | 11.2 | Pleural effusion |
| 12 | M | 62 | MVA | 3 ribfractures<br>Lungcontusion bilateral<br>Minor liver laceration | 20 | 29 | 4 | 15 | 7.8 | Pneumothorax |
| 13 | M | 59 | Attacked by cow | Multiple rib fractures bilateral<br>Flail thorax bilateral<br>Pneumothorax bilateral<br>Lungcontusion bilateral<br>Sternum fracutre<br>Minor liver laceration | 29 | 38 | 5 | 6 | 18.9 | Pneumonia |

**Receptor expression on neutrophil surface**

*L-selectin and CD11b*

L-selectin expression was decreased at 9 hrs post injury (p = 0,002) and remained decreased until 24 hrs ($p$= 0.012)(Figure 1A). αM expression was significantly decreased at 9 hrs post injury compared to control values (Figure 1B). Although, neutrophil activation is typically characterized by αM up-regulation, expression of αM declined at 9 hrs post injury ($p$=0.020) to a minimum in this group of patients. This overall decrease in αM expression is probably due to an increased amount of young neutrophils, which express αM at low levels[29 30].

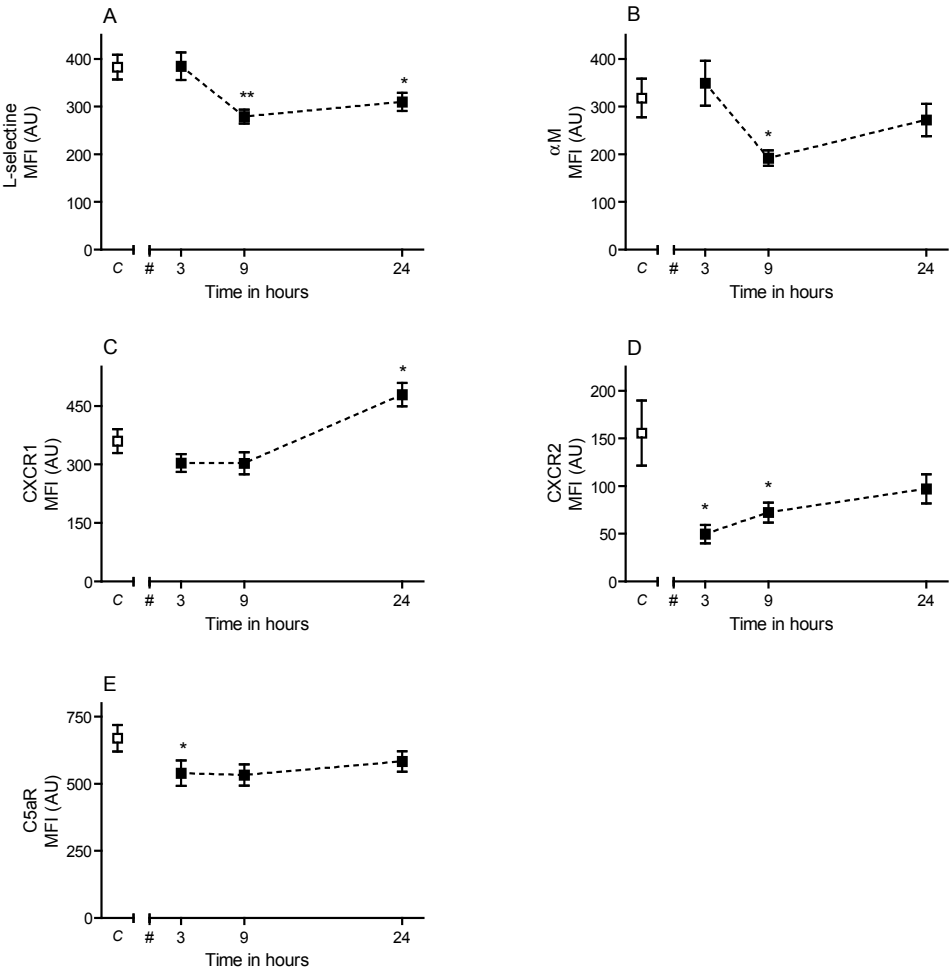


**Figure 1**

Expression of L-selectin (A), αM (CD11b) (B), CXCR1 (C), CXCR2 (D) and C5aR (E) on neutrophil surface during time. Open squares (□) stand for control values from healthy controls (N=8), whereas black squares (■) represent patients (N=13) at 3, 9 and 24 hrs post injury. (C) is control, (#) is time of injury. Data are presented as mean ± SE.(* p<0.05; ** p< 0.01).

*CXCR1, CXCR2 and C5aR*

Neutrophil activation is associated with reduced surface expression of CXCR1, CXCR2 and C5aR[31-33]. After chest injury circulating neutrophils showed a temporary decline in expression of chemokine receptors CXCR1 and CXCR2 and of complement receptor C5aR (Figure 1C-E). This decline was statistically significant for CXCR2 and C5aR at 3 hrs post injury, but not for CXCR1. CXCR2 expression remained low until 9 hrs post injury. It has been demonstrated that upon activation CXCR2 internalizes more rapidly relative to CXCR1[34], which may explain the more pronounced decline of CXCR2 compared to CXCR1 in our results. Expression of CXCR2 and C5aR gradually restored during the first 24 hours after injury, indicating a transient activation of circulating neutrophils. CXCR1 expression increased above control values at 24 hrs after injury ($p$= 0.039)

*FcγRII, active FcγRII and FcγRIII*

Expression of FcγRII was markedly decreased until 24 hrs after injury (p<0.010; Figure 2). Expression of the active form was slightly lower in trauma patients compared to control values, although this decline did not reach statistical significance. Expression of fMLP induced active FCγRII, however, was significantly decreased until 9 hrs after injury ($p$=0,006). Expression of FcγRIII evidently dropped during the first 24 hrs after chest trauma (p<0,001). FcγRIII is normally expressed at lower levels on young (banded) neutrophils compared with more mature forms[35]. This decrease in overall FcγRIII, therefore suggest an influx of young neutrophils.

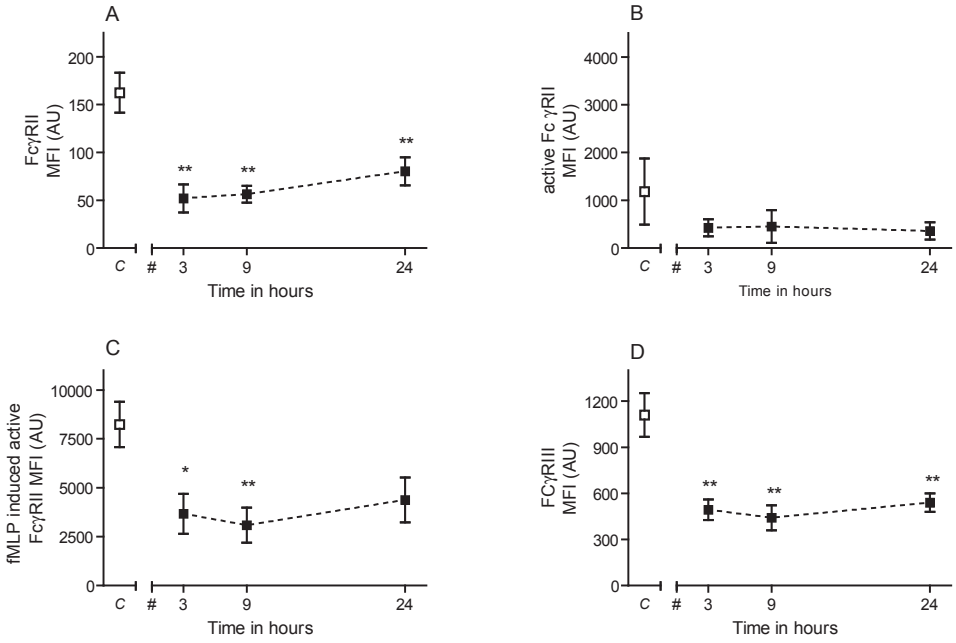


**Figure 2**

Intrinsic expression of FCγRII (A), active FCγRII (B), fMLP induced expression of active FCγRII (C) and expression of FCRγIII (D) on neutrophil surface during time. Open squares (□) stand for control values from healthy controls (N=8), whereas black squares (■) represent patients (N=13) at 3, 9 and 24 hrs post injury. (C) is control, (#) is time of injury. Data are presented as mean ± SE (* p<0.05; ** p< 0.01).

*IL-6 levels*

IL-6 levels were significantly enhanced at 3 hrs post injury compared to control values (mean concentration of 44±15 vs. 0 pg/ml ($p<0.001$)). IL-6 levels further increased to maximum mean concentration of 86±31 pg/ml ($p<0.001$; compared to control values) at 24 hrs after blunt chest injury (Figure 3).

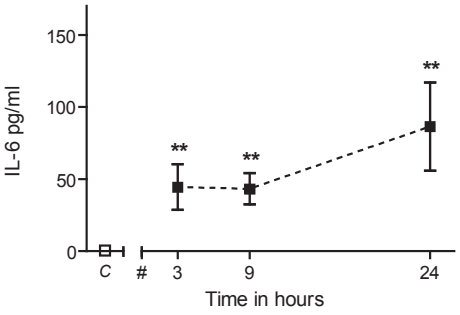


**Figure 3**

Levels of circulating IL-6. Open squares (□) stand for control values from healthy controls (N=8), whereas black squares (■) represent patients (N=13) at 3, 9 and 24 hrs post injury. (C) is control, (#) is time of injury. Data are presented as mean ± SE (** $p< 0.01$).

## Discussion

In this study we show that blunt chest injury leads to a systemic activation of circulating neutrophils, characterized by shedding of L-selectin and down-regulation of CXCR2 and C5aR. It furthermore shows that blunt chest injury is associated with mobilisation of young (FcγRIII-low) neutrophils and with a reduced responsiveness of circulating neutrophils to an inflammatory stimulus.

Although seven patients had a chest AIS ≥ 4, of whom two had a bilateral pulmonary contusion, none of these patients developed ARDS. Lung injury results in endovascular changes, tissue barrier failure and locally increased cytokine levels, facilitating systemic activated neutrophils to infiltrate the parenchyma. Despite vast local damage in the above mentioned cases, the provoked innate immune response was apparently not abundant enough to cause ARDS. Similar findings were found in an earlier study performed by Maier et al.[4]. This study showed that isolated lung contusion resulted in an increase of circulating IL-6, but did not cause ARDS. These findings involved patients with minor as well as major lung contusion (based on CT lung injury score). The same study also described an enhanced inflammatory response, with significantly increased levels of circulating IL-6 and IL-8, plus significant elevated multiple organ failure (MOF) score in multi-trauma patients with major lung contusion compared to multi- trauma patients with minor or no lung contusion. Yet, the exact role of lung contusion on the immune response and the occurrence of organ dysfunction was not completely clear in these severely injured patients. The result was biased by an evidently higher ISS in patients with major lung contusion compared to those with minor or no lung contusion. More severe chest injury is most often accompanied by more severe additional injury in at least one of the other regions, resulting in a higher ISS. The same tendency was noticed in our study. The majority of patients presented at the emergency department with severe chest injury could not be included due to considerable injury in regions other than the thorax. The

challenging in- and exclusion criteria resulted in inclusion of less patients, but also a more homogeneous group.

Earlier studies concerning multi-trauma patients demonstrated that fMLP-induced active-FcγRII expression was decreased in patients compared to controls and that the expression negatively correlated with ISS and adverse outcome[11 20]. A clear difference in median fMLP-induced expression of active FcγRII, measured during the first 24 hrs after injury, was seen between patients with ALI/ARDS (low expression) and patients with an uneventful course (high expression)[20]. The median values fMLP-induced expression of active FcγRII measured in this study are comparable multi-trauma patients with an uneventful clinical course. These findings support the hypothesis that isolated chest injury induces only a restricted activation of the systemic immune response.

The increased number of circulating neutrophils after chest injury is most likely due to mobilisation of young neutrophils, displayed by a decrease of overall FcγRIII expression. However, delayed neutrophil apoptosis has been described after trauma[36 37]. Although this phenomenon remains to be established *in vivo* we can not exclude that the total number of neutrophils are biased by disturbed apoptosis since, apoptotic markers, such as Annexin V, were not measured.

The fact that isolated chest injury rarely leads to ARDS is in contrast to other conditions in which the lung is locally affected and in which this complication is frequently seen, such as during pneumonia or after aspiration[25 38 39]. Presumably the systemic innate immune response evoked by isolated chest injury is less extensive compared to that during infection or after aspiration. In a rodent model, Hoth et al. have demonstrated that lung injury followed by exposure to E.coli lipopolysaccharide (LPS) leads to massive neutrophils infiltration and lung damage, whereas tissue infiltration and damage was far less after LPS or lung injury alone[40]. In addition, serum IL-6 levels were significantly increased compared to LPS exposure or lung injury alone. They concluded that lung injury primes the systemic innate immune response, like was suggested by Maier et al.

In this study we show that the systemic innate immune response caused by isolated chest injury is transient and short. Although IL-6 levels remained elevated until 24 hrs after injury, activation of circulating neutrophils partially restored. Yet, this mild systemic response may be sufficient to enhance the innate immune response caused by a second-hit such as concomitant tissue damage, fat emboli or infection.

We therefore suggest that lung damage alone is not likely to result in an ARDS, but a synergism between inflammation caused by lung injury and an additional stimulus caused by a second-hit results in a markedly increased risk of developing ARDS.

## Conclusion

In this study we demonstrated that isolated blunt chest injury caused transient systemic activation of neutrophils together with mobilization of young neutrophils into the peripheral circulation. In addition, only severe chest injury (AIS >4) results in increased number of circulating neutrophils. However, it seems that chest injury alone is not sufficient to cause ARDS in these cases and a second-hit might be needed.

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PART I

IMMUNOMONITORING

# 4

# Circulating VLA-4 positive neutrophil progenitors differentiate outside the bone-marrow in the absence of cytokines

*Manuscript in preparation*

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## Abstract

A systemic inflammatory response such as seen during infection or after injury is accompanied by changes in the phenotype of circulating neutrophils. One of these phenotypic changes, described to occur under severe inflammatory conditions, is the enhanced expression of very late antigen (VLA)-4 (CD49d/CD29). VLA-4 belongs to the integrin family and is normally expressed on neutrophil progenitor cells in the bone marrow but not on mature neutrophils. This study tested the hypothesis that circulating VLA-4$^{pos}$ neutrophil-like cells are neutrophil progenitors that egress the bone marrow during severe inflammation and that these cells are able to differentiate outside the bone marrow.

Flow cytometry data from 39 trauma patients (with an expected ICU-stay >3 days) were analyzed. The number of VLA-4$^{pos}$ cells varied from 0 to 55% of all circulating neutrophils during the first week after trauma. Morphological examination confirmed that circulating VLA-4$^{pos}$ cells were neutrophil progenitors. No mature VLA-4$^{pos}$ neutrophils were detected. VLA-4$^{pos}$ progenitors were characterized by a marked reduced expression of molecules associated with adhesion and chemotaxis: L-selectin(CD62L), αMβ2 integrin(CD11b/CD18) and C5aR(CD88). Additionally, VLA-4$^{pos}$ progenitors had an impaired anti-microbial function characterized by an impaired respiratory burst.

Maturation of FACS sorted peripheral VLA-4$^{pos}$ neutrophil progenitors obtained from blood from trauma patients and from peripheral blood stem cell (PBSC) donors was studied *in vitro*. During two days of culture progenitor neutrophils differentiated morphologically into neutrophils in the absence of any added cytokines, and lost their VLA-4 expression.

Our study shows that under severe conditions the bone marrow releases neutrophil progenitors that are programmed to become neutrophils by an intrinsic mechanism. This provides the body with an emergency mechanism to direct neutrophils to the tissues, even under conditions when the bone marrow is deficient in producing mature cells.

# Introduction

Systemic inflammation, such as seen during severe sepsis or after trauma, is characterised by activation of circulating neutrophils[1 2] and by mobilisation of young banded neutrophils from the bone marrow. Phenotyping of circulating neutrophils by expression of cell surface markers has been used to determine the activation status of circulating neutrophils in order to discriminate between severity of inflammation and to predict the occurrence of organ failure[1-4].

One of these surface receptors that has gained interest is very late antigen-4 (VLA-4). VLA-4 belongs to the integrin family and has an important function in rolling and adhesion of leukocytes to the endothelium. Leukocytes are able to adhere to the vascular wall via binding of VLA-4 to its ligand vascular cell adhesion molecule-1(VCAM-1), that is expressed on the endothelial cells[5]. VLA-4 is under normal conditions expressed on leukocytes, such as eosinophils and monocytes, but not on neutrophils[6 7]. Yet, some reports have shown that VLA-4 is expressed on cell surface of circulating neutrophils during severe sepsis[8 9]. In septic patients approximately 30-40% of circulating neutrophils express functional VLA-4 compared to 0-5% in healthy controls[8]. It has been suggested that VLA-4 on these neutrophils plays a role in αMβ2 integrin(CD11b/CD18) independent adhesion[10 11]. This type of adhesion would then contribute to lung damage in acute respiratory distress syndrome (ARDS)[8]. Interpretation of this latter study is hampered by the fact surface expression of VLA-4 was not visualized by FACS analysis, nor were these cells examined microscopically in order to verify if these cell were in fact neutrophils.

Although VLA-4 is not expressed on neutrophils under physiological conditions, it is expressed on neutrophil progenitor cells in the bone marrow[6 12 13]. For that reason, we hypothesized that VLA-4 positive neutrophil-like cells that appear in the circulation under extreme inflammatory conditions are neutrophil progenitors which are released from the bone marrow as response to an excessive demand for neutrophils.

Mediators such as granulocyte colony-stimulating factor (G-CSF), interleukins (IL), tumour necrosis factor (TNF) and complement components induce the proliferation and release of mature and immature neutrophils from the bone marrow in acute systemic inflammation[14-16]. This is classically characterised by the appearance of young banded neutrophils in peripheral blood, a so called "left shift". To our knowledge a significant increase in circulating neutrophil progenitor cells during acute inflammation has not been described. Studies on allergic diseases have shown an increased number of circulating progenitor cells (metamyeolocytes and myelocytes) during severe disease state, albeit an increase of only a small percentage (0.12%)[17]. We believe, however, that adequate and profound stimulation can result in the release of a substantial number of progenitor cells from the bone marrow into the circulation. We suppose that under severe inflammatory conditions the number of neutrophil progenitors may rise up to 30 or 40%, explaining the percentage of neutrophils with high VLA-4 expression in septic patients described by Ibbotson et al[8].

Haematopoietic progenitor cells have previously be known to appear in high numbers in the circulation in humans participating in peripheral blood stem cell (PBSC) donation[18 19]. Administration of the growth factor G-CSF induces the release of haematopoietic stem cells into the circulation in PBSC donors[18 19]. G-CSF administration is nowadays commonly used for mobilization of haematopoietic stem cells for autologous as well as allogeneic

PBSC transplantation. PBSC mobilization has proven to be effective and replaced bone marrow as the preferred source of stem cells for patients with haematopoietic malignancies. Successful PBSC mobilization implies that adequate and profound stimulation (possibly also during severe sepsis or trauma) results in the release of progenitor cells from the bone into the circulation.
In this study we tested the hypothesis that VLA-4 is expressed on neutrophil precursors in severely injured patients rather than mature neutrophils, and that these precursors can rapidly differentiate into neutrophils *in vitro*. We furthermore suggest that these neutrophil progenitors differentiate outside to bone marrow to mature neutrophils to perform their designated anti-microbial functions.

# Methods

**Subjects and study design**

*Trauma patients*

Multi trauma patients (age of ≥18 or <80) with an expected ICU-stay of at least three days were included. Patients with an altered immune status (e.g. corticosteroids use or chemotherapy) were excluded. Blood was drawn within 24 hours after admission at the University Medical Centre Utrecht and on consecutive days until maximal 14 days after trauma. Clinical parameters were monitored for all patients during hospital stay. The local ethics committee approved the study and written informed consent was obtained from all patients or their legal representatives in accordance with the protocol.

*Peripheral blood stem cell (PBSC) donors*

Blood from autologous and allogeneic PBSC donors was obtained from the Stem Cell Therapy Department of the University Medical Centre Utrecht. G-CSF was administered twice a day at a dose of 5μg/kg subcutaneously. After 5 consecutive days of administration blood was drawn for prognostic purposes and residual anonymous blood samples were used for study purposes with approval of the institutional ethical review board.

*Reagents and antibodies*

For the analysis of neutrophil receptor expression by flow cytometry (FACScalibur, Becton Dickinson, Mountain View, CA, USA), the following mouse anti-human monoclonal antibodies were commercially purchased: Alexa 647-labelled IgG1 negative control (clone MOPC-21, BD Biosciences, Franklin lakes NJ, USA), FITC-labelled IgG1 negative control (clone MOPC-21, BD Bioscience), FITC-labelled IgG2a negative control (clone MRC OX-34, AbD Serotec, Kidlington UK ), PE-labelled IgG1 negative control (clone DD7, Chemicon, Darmstadt, Germany), RPE-labelled CD11b (clone 2LPM19c, Dako, Heverlee, Belgium), FITC-labelled CD11c (clone BU15, Life technologies (Invitrogen/Gibco), Merelbeke, Belgium), RPE-labelled CD13 (clone SJ1D1, Beckman Coulter, Woerden, The Netherlands), Alexa 647-labelled CD16 (clone 3G8, BD Biosciences), FITC-labelled CD18 (clone L130, BD Biosciences), FITC labeled CD181 (clone 42705, R&D Systems Europe, Oxon, UK), PE-labelled CD32 (clone FLI8.26, BD Biosciences), PE-labelled CD49d (clone 9F10, Bioscience), PE-labelled CD54 (clone

MEM-111, Caltag, Buckingham, UK), FITC-labelled CD62L (DREG-56, BD Biosciences), FITC-labelled CD66b (clone 80H3, Beckman Coulter), FITC-labelled CD88 (clone P12/1, Bio-connect, Huissen, The Netherlands), PE-labelled Annexin-V (Annexin V-PE Apoptosis detection kit, BD Biosciences), PE-labelled CD182 (Clone 48311, R&D systems Europe). The flowing media were used for incubation experiments: Iscove's Modified Dulbecco's Medium (IMDM) (Life technologies) and BRFF-EPM2 (Athena ES, Baltimore, USA). Amplex Red (Molecular Probes, Leiden, The Netherlands), horseradish peroxidase (HPR) (Sigma Chemical Co., St. Louis, MO, USA) and phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co.) were used to measure respiratory burst.

*Flow cytometry*

Analysis was performed on whole blood samples anticoagulated with sodium heparin. Erythrocytes were lysed with ice-cold NH4Cl and washed with phosphate buffered saline (PBS = 0.5% wt/vol) supplemented with sodiumcitrate (0.38% wt/vol) and isotonic pasteurized plasma proteins (10% wt/vol) ($PBS^{2+}$)[20]. Antibodies were added and samples were incubated on ice for 45-60 min. After a final wash with $PBS^{2+}$, the cells were analyzed in a FACScalibur flow cytometer. The neutrophils were identified according to their specific side-scatter and forward-scatter signals. Data from individual experiments are depicted as fluorescence intensity in arbitrary units or summarized as the median channel fluorescence of at least 10,000 events.

*Maturation of VLA-4 positive neutrophil progenitors*

Neutrophils were identified according to their specific side- and forward-scatter signals and sorted based on surface marker expressions with FACSaria (Becton & Dickinson).
VLA-4 positive (VLA-4$^{pos}$) and negative (VLA-4$^{neg}$) neutrophil (progenitor) populations were cultured *in vitro* at 37oC in IMDM supplemented with 8% heat inactivated (HI) fetal calf serum (FCS) as well as in serum-free medium (BRFF-EPM2), to control for serum-induced effects on differentiation.
Immediately after cell sorting, the cells were resuspended in culture medium. After 16hr and after 42hr, differentiation of VLA-4$^{pos}$ cells was analyzed based on morphology, surface marker expression and functionality and compared to VLA-4 negative neutrophils. Apoptosis was determined by flow cytometry using Annexin V-PE staining according the protocol of the manufacturer. In addition, expression of VLA-4 was determined after culture of VLA-4$^{pos}$ cells in IMDM 8% HI FCS in presence of 5 µg/ml fibronectine.

*Morphology*

Morphological neutrophil differentiation was quantified before and after sorting on May-Grunwald Giemsa-stained cytospins, by blind counting of >100 cells per slide by two blinded researchers.

*Respiratory burst*

$H_2O_2$ was determined in a fluoro-luminometer (FluostarOptima, BMGLABTECH, Ottenberg, Germany) by determination of fluorescent resorufin, which was formed from Amplex Red in the presence of $H_2O_2$ and horseradish peroxidase HRP. In short, neutrophils were resuspended in $HEPES^{3+}$ at a concentration of 1.0x106/ml in the presence of Amplex Red

(10mM) and HRP (100U/ml). PMA (0.1ug/ml) was added to obtain maximal burst capacity. $H_2O_2$ release was measured for 40 minutes at 37oC. Maximal slope of $H_2O_2$ production was used to determine maximal respiratory burst capacity.

**Statistics**

Data were analyzed using SPSS version 15.0 software (IBM, Amsterdam, The Netherlands) and Graphpad Prism 4.0 (Graphpad software, San Diego, CA, USA). Results are expressed as means ± SE. Students t-test was used to analyze difference in surface expression between VLA-$4^{pos}$ and VLA-$4^{neg}$ cells. A one-way ANOVA followed by a Bonferroni post hoc was used as appropriate to test differences of expression in time. Correlations were tested by a Spearman's rho test. Statistical significance was defined as $p<0.05$.

# Results

**VLA-4 expression on neutrophils in trauma patients**

Thirty-nine trauma patients were included. Demographics of trauma patients are described in Table 1. FACS analysis of leukocytes from these patients showed a population of cells in the neutrophil gate (identified by forward-sideward scatter signals) that expressed VLA-4 (CD49d/ VLA-4 α-chain; Figure 1). These VLA-$4^{pos}$ cells appeared in the circulation within hours after severe trauma. They were most commonly seen during the first 36 hrs after injury and again around day 5 to 7 after injury (see Figure 2). The number of circulating VLA-4 positive cells differed considerably in patients on consecutive days. The percentage of circulating VLA-$4^{pos}$ cells varied between patients from absent to 55% of the cells in the neutrophil gate during the first 7 days after injury. There was no correlation between injury severity score (ISS)21 and percentage of VLA-$4^{pos}$ cells at day of admission. Nor was there a difference found in percentage of VLA-$4^{pos}$ cells or mean expression of VLA-4 (at any time) between patients developing septic complications and those who did not. Yet, this cross-sectional study was not designed and/or powered to find such correlation.

**Table 1** Patients demographics

| | **Mean (range)** |
|---|---|
| Number of patients | 39 |
| Male:Female | 32:7 |
| Age | 37 (18-78) |
| Injury Severity Score | 29 (10-75) |
| Cause of trauma (N) | |
| - Motor vehicle/bicycle accident | 27 |
| - Fall from height | 7 |
| - crush accident | 2 |
| - Assault | 1 |
| - Stab wound | 2 |

Blood from trauma patients was stained for flow cytometry with anti-bodies against VLA-4 α-chain (CD49d) and FcγRIII (CD16). FcγRIII is highly expressed on neutrophils, whereas it is not expressed on eosinophils[7]. Using double staining, VLA-4$^{pos}$ eosinophils could be easily distinguished from neutrophils. Double staining with anti-bodies against VLA-4 α-chain (CD49d) and FcγRIII showed, however, that FcγRIII expression on VLA-4$^{pos}$ cells in the neutrophil gate was markedly lower than on VLA-4$^{neg}$ neutrophils, but overall not as low as on eosinophils (Figure 1A). Low expression of FcγRIII on VLA-4$^{pos}$ neutrophil-like cells suggested that these cells were immature neutrophils, as FcγRIII is known to be expressed at lower levels on young neutrophils and neutrophil progenitors[22].

Cytospins of cells from trauma patients showed a considerable number of neutrophil progenitor cells, especially in patients with a high number of VLA-4$^{pos}$ cells identified by FACS analysis in the neutrophil gate. Metamyelocytes and myelocytes and sporadic some promyeolcytes were seen in these samples from trauma patients. Counting of neutrophil progenitor cells, on cytospins from 4 trauma patients, revealed that the percentage of these progenitor cells highly correlated to percentage of VLA-4$^{pos}$ neutrophils on FACS plot (Spearman's rho, $p<0.001$; see also Figure 2), indicating that these VLA-4$^{pos}$ neutrophils were likely to be neutrophil progenitor cells rather than neutrophils. This result gave rise to further investigation of these VLA-4$^{pos}$ cells and examination of morphology and function after isolation.

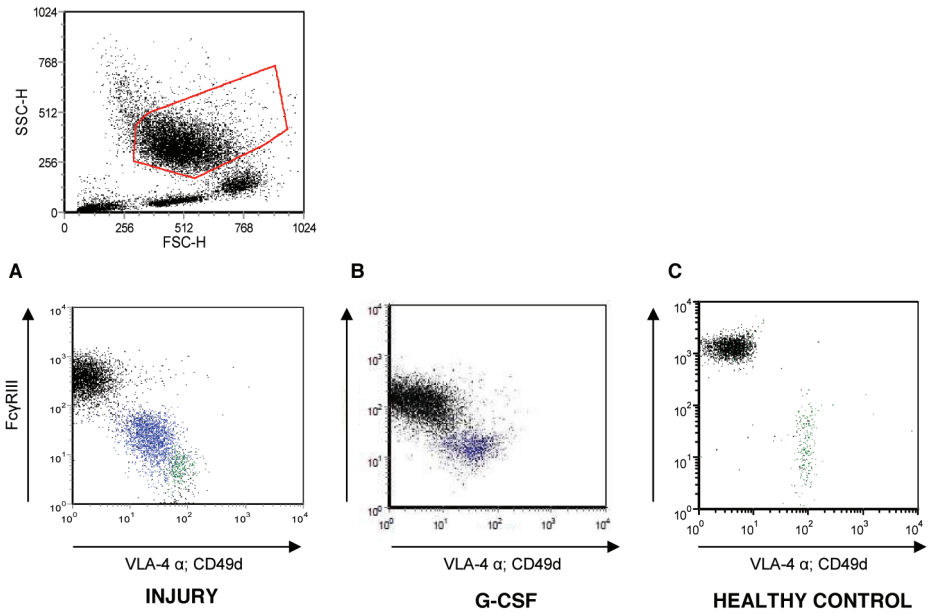


**Figure 1**
Expression of CD49d (VLA-4 α-chain) and FcγRIII (CD16) after selection of neutrophils selected by forward-sideward scatter signals (upper plot) after injury (A) and after G-CSF stimulation (B). VLA-4 positive cells (depicted in blue) are seen after injury and G-CSF stimulation. VLA-4$^{pos}$ cells have a moderate FcγRIII expression, but not such low expression as eosinophils (depicted in green (A)). An example of CD49d and FcγRIII expression of blood of an healthy volunteer who has a substantial number of circulating eosinophils (depcited in green) is shown in C.

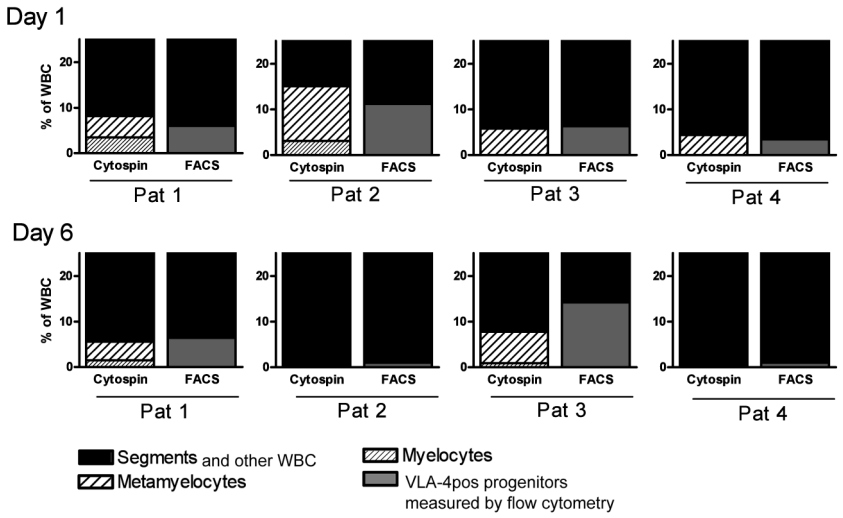


**Figure 2**
Different stages of neutrophil differentiation were determined by microscopic examination of cytospins obtained from 4 trauma patients on day 1 and 6 after injury. After counting of 100 cells, the percentage of neutrophil progenitor cells (promyelocytes, myelocytes and metamyelocytes) was calculated out of all white blood cells (WBC). Bars represent percentage of neutrophil progenitors on a cytospin and are compared to percentage of VLA-4pos neutrophils measured by FACS analysis of the same patient. Percentage of neutrophil progenitor cells counted on cytospins strongly correlated to percentage of VLA-4pos neutrophils measured by FACS analysis (Spearman's rho 0,793 $p < 0,001$, N=4).

### Surface marker expression of VLA-4$^{pos}$ neutrophil progenitor cells

Blood from PBSC donors obtained after 5 days of G-CSF stimulation showed a considerable number of circulating VLA-4$^{pos}$ neutrophil progenitor cells with a low FcγRIII expression (Figure 1B), similarly as seen in trauma patients. Triple staining revealed that VLA-4$^{pos}$ neutrophil progenitor cells from trauma patients and PBCS donors displayed marked differences in expression of several surface markers compared to VLA-4$^{neg}$ neutrophils (Figure 3).

Besides FcγRIII, also FcγRII(CD32) and chemo-attractant receptors C5aR(CD88), CXCR1(CD181) and CXCR2(CD182) were expressed at lower levels on VLA-4$^{pos}$ neutrophil progenitor cells compared to VLA-4$^{neg}$ neutrophils. Also the expressions of L-selectin(CD62L) and integrins αM-(CD11b) and β2(CD18) were significantly lower on VLA-4$^{pos}$ neutrophil progenitor cells compared with VLA-4$^{neg}$ neutrophils. The expression of β1-integrin (CD29), the β unit of the VLA-4 complex, was not significantly different among the two populations.

### Cellsorting

Triple staining revealed that VLA-4$^{pos}$ neutrophil progenitor cells have a low FcγRIII and L-selectin expression (CD16$^{dim}$/CD62L$^{low}$), whereas VLA-4$^{neg}$ neutrophils have a high FcγRIII and L-selectin expression (CD16$^{high}$/CD62L$^{high}$). Due to staining with antibodies against FcγRIII and L-selectin, VLA-4$^{pos}$ neutrophil progenitor cells could be better distinguished from VLA-4$^{neg}$ neutrophils and from eosinophils, which have a low FcγRIII and high L-selectin

expression (CD16$^{low}$/CD62L$^{high}$)(Figure 4A and B). VLA-4$^{pos}$ neutrophil progenitor cells were, therefore, sorted based on their CD16$^{dim}$/CD62L$^{low}$ expression profile, leading to a purer cell sort (Figure 4B). Microscopic examination of VLA-4$^{pos}$ cells isolated by cell sorting confirmed that these cells were indeed neutrophil progenitors (as shown in Figure 4).

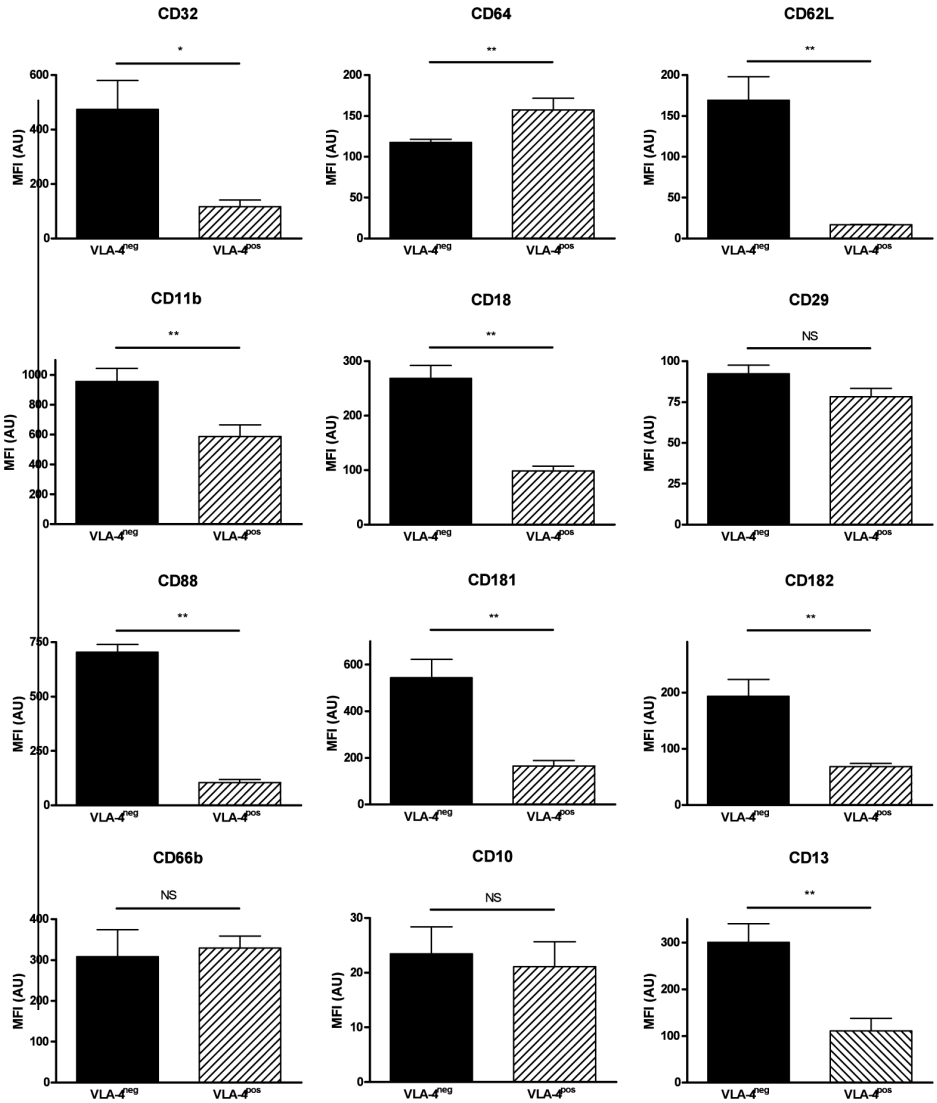


**Figure 3**
Receptor surface expression on neutrophils and VLA-4$^{pos}$ progenitor cells measured by flow cytometry. Black bars stand for segmented VLA-4$^{neg}$ neutrophils, striped bars represents VLA-4$^{pos}$ progenitor cells .Data are presented as mean±SE. MFI (AU) = mean fluorescence intensity (arbitrary units), * = $p<0.05$, ** = $p<0.01$, NS= not significant.

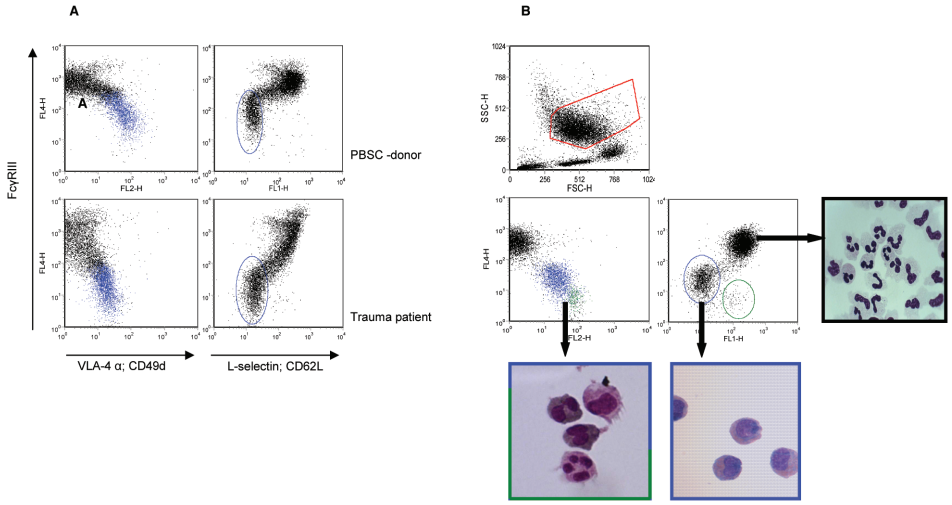


**Figure 4**
Triple staining showed that VLA-4$^{pos}$ neutrophil progenitor cells (blue gate) from trauma patients and PBCS donors have a low FcγRIII and L-selectin expression (CD16$^{dim}$/CD62L$^{low}$; depicted in blue) (A). Using staining with antibodies against FcγRIII and L-selectin, neutrophil progenitor cells (CD16$^{dim}$/CD62L$^{low}$; depicted in blue) and eosinophils (CD16$^{low}$/CD62L$^{high}$; depicted in green) could be better distinguished. Cell sorting based on FcγRIII/L-selecting resulted in a purer cell sort (with absence of eosinophils) compared with cell sorting based on FcγRIII and VLA-4(CD49d) receptor profile (B).

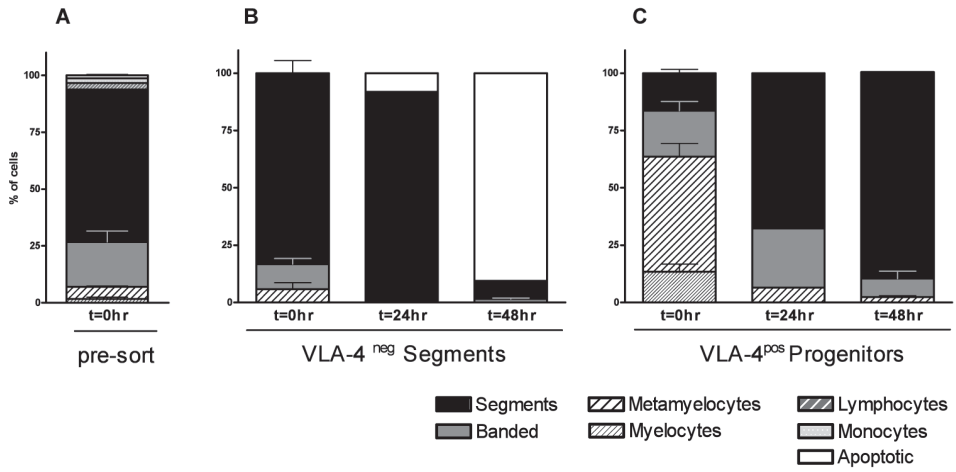


**Figure 5**
Morphological changes of VLA-4$^{pos}$ progenitor cells and segmented VLA-4$^{neg}$ neutrophils during 48hrs of culture at 37ºC in IMDM/8%FCS (N=2) observed by microscopic examination of cytospins. Before cell sorting white blood cell population existed of 70-75% of segmented neutrophils, 15-20% banded cells and between 5 and 10% of neutrophil progenitors (A). Less than 5% of white blood cells consisted of lymphocytes, monocytes and eosinophils. Cell sorting of the CD16 $^{high}$/CD62L$^{high}$ population resulted in a purity of 80% of segmented neutrophils (B). The other 20% consisted of mainly banded neutrophils and a small number of neutrophil progenitors. After 24 hrs of culture no immature neutrophils were seen. A small percentage of the segmented cell population had a small nucleus and these cells were considered as apoptotic cells. After 48hr of culture the majority of cells were in apoptosis. Cell sorting of the CD16$^{dim}$/CD62L$^{low}$ population resulted in 70% purity of neutrophil progenitors (C). The other 30% consisted of banded and mature neutrophils. Neutrophil progenitor cells maturate morphologically into segmented neutrophils, resulting in a percentage of more than 50% after 24hr and more than 90% after 48hr of segmented neutrophils per 100 counted cells on cytospin. All data are presented as mean+SE.

***In vitro* maturation of VLA-4$^{pos}$ neutrophil progenitor cells**

To study maturation of circulating VLA-4 positive neutrophil progenitors, progenitor cells were sorted from blood obtained from PBSC donors and trauma patients. During 2 days of culture, VLA-4$^{pos}$ neutrophil progenitor cells maturate morphologically into mature neutrophils (Figure 5). Maturation is accompanied by a significant loss of VLA-4 expression and a significant increase of FcγRIII(CD16) and L-selectin(CD62L) expression (One way ANOVA, $p$=0.001, $p$=0,039 and $p$=0,021 respectively ; Figure 6A). Maturation of VLA-4$^{pos}$ neutrophil progenitor cells of one trauma patient was compared to that of 5 PBSC donors and appeared to be identical (Figure 6B).

Differentiation of VLA-4$^{pos}$ neutrophil progenitor cells to mature neutrophils occurred *in vitro* in absence of serum in BRFF-EPM2 medium (data not shown). Although no differences in maturation were seen between the culture media, more apoptosis was seen in VLA-4$^{pos}$ neutrophil progenitor cells cultured in serum-free medium compared to those IMDM 8% FCS. Loss of VLA-4 expression was not prevented by adding its natural ligand fibronectine to incubation medium (data not shown).

**Functionality of VLA-4$^{pos}$ neutrophil progenitor cells**

VLA-4$^{pos}$ neutrophil progenitor cells showed a reduced capacity to release $H_2O_2$ in response to PMA stimulation compared to VLA-4$^{neg}$ segmented neutrophils at t=0hr. Although there was no difference in maximum concentration of released $H_2O_2$, the maximal slope was significantly decreased (t-test $p$=0,02) During *in vitro* maturation of neutrophil progenitor cells the capacity to release $H_2O_2$ upon stimulation increased. After one day of incubation there was no difference in respiratory burst produced by neutrophil progenitor cells compared to the burst produced by VLA-4neg segmented neutrophils at t=0hr (Figure 7).

## Discussion

Similarly as found during severe sepsis, a substantial number of circulating cells, identified as neutrophils on FACS scatter plots, expressed VLA-4 in multi trauma patients. The number of VLA-4$^{pos}$ cells could rise up to as high as 55% of all circulating neutrophils. Cell isolation by cell sorting, and morphological examination showed that these circulating VLA-4$^{pos}$ cells were neutrophil progenitor cells. VLA-4$^{pos}$ progenitor cells were characterized by a marked reduction in expression of adhesion molecules and chemo-attractant receptors, as well as by an impaired anti-microbial function.

Our data seem to contradict an earlier study showing VLA-4 expression on mature neutrophils[8] . This can be explained by the fact that this studied focused on the function of VLA-4 by virtue of the binding to its receptor VCAM-1[8]. However, the finding that neutrophils isolated from septic patients or normal neutrophils incubated with septic serum have an increased capacity to bind to VCAM-1 is not sufficient to conclude that the cells express VLA-4. A previous study has clearly shown that the integrin α subunit CD11c is able to bind to VCAM-1 as well[23]. Hence binding of leukocytes to VCAM-1 does not necessarily imply presence of VLA-4 surface expression. In addition, *in vitro* experiments have also shown nonspecific binding of commercially obtained HP 2/1 monoclonal antibody

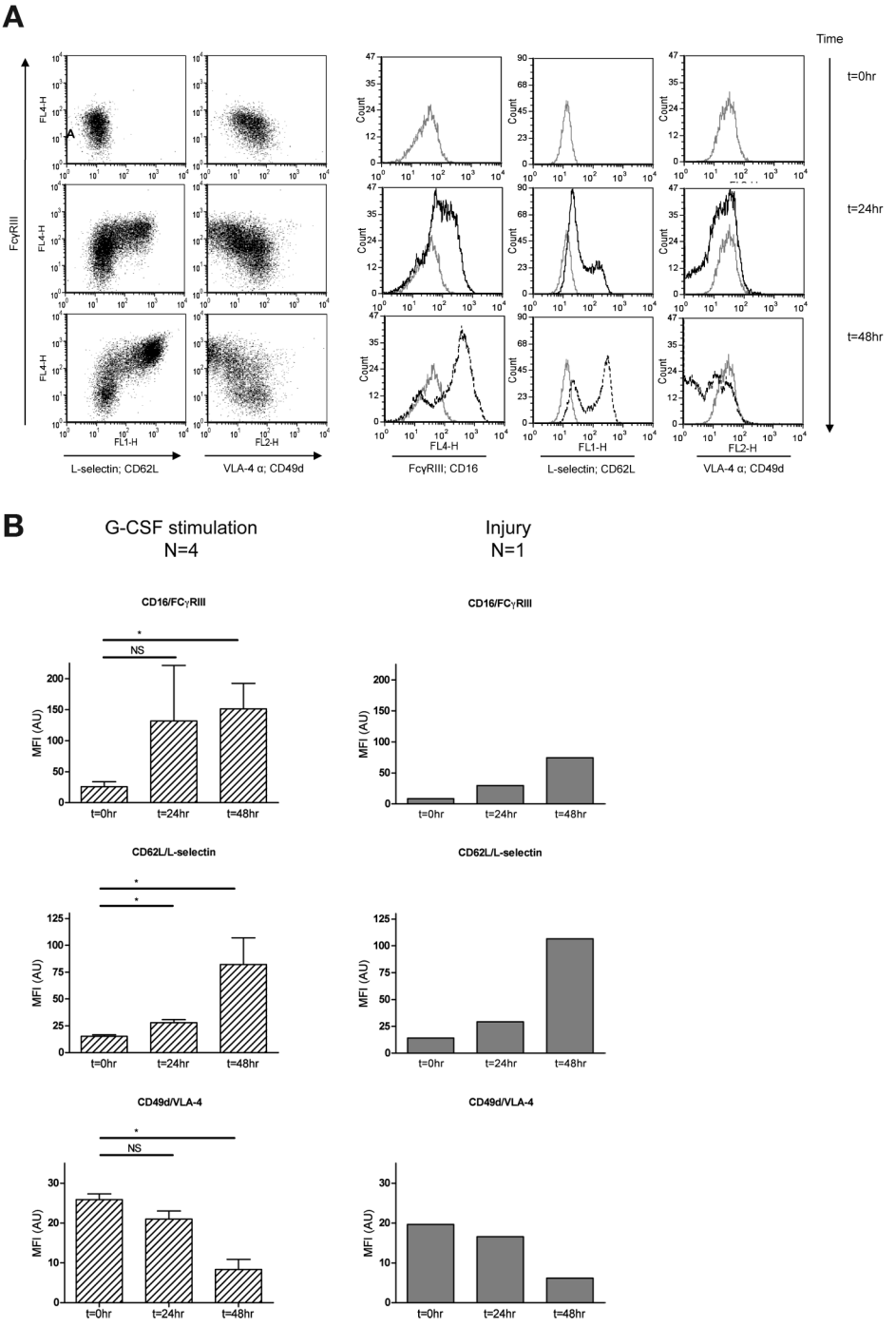


**Figure 6**

Changes in FcγRIII, L-selectin and VLA-4 α expression during culture *in vitro*. During culture expression of FcγRIII and L-selectin increased, whereas the expression of VLA-4 deminished. A representative example of FACS plots and histograms is shown at t=0hr, t=24hr and t=24hr (A). Surface receptor expression on isolated progenitor cells from PSBC donors and from one trauma patient showed similar results (B). * = $p<0.05$, NS= not significant

to activated neutrophils[24]. This non-specific binding of HP 2/1 may explain the increase in VLA-4 expression on mature neutrophils after incubation of neutrophils with plasma from septic patients[8 9].

In our study we showed that circulating VLA-4$^{pos}$ neutrophil progenitors maturated and lost their VLA-4 expression *ex vivo* in the absence of differentiation inducing cytokines. Past research has already shown that haematopoietic progenitor cells obtained from PBSC donors can maturate *ex vivo* in serum free culture medium to which growth factors, like IL-3/GM-CSF fusion protein, were added[25]. The finding that neutrophil progenitor cells maturated in absence of inducing cytokines indicates that maturation occurs via a programmed intrinsic pathway. It seems that no specific conditions are needed for differentiation. It seems, furthermore, that VLA-4$^{pos}$ neutrophil progenitors can maturate outside the bone marrow in order to fulfill their task to eradicate pathogens and remove damaged cells.

A few studies performed in the 70s of past century have shown that neutrophil progenitors are retained in the spleen of mice[26 27]. These progenitor cells are capable to proliferate and maturate outside the bone marrow[26 27]. As far as we know extramedullary proliferation and maturation of granulocyte progenitors have not been described in adult humans.

Maturation of circulating progenitor cells might, to some extent, explain the variation in number of circulating VLA-4$^{pos}$ progenitor cells in consecutive days in severely injured patients. However, the presence of neutrophils and neutrophil precursors in the circulation is very dynamic and influenced by additional factors such as migration and homing of cells to tissue and reversed migration to bone marrow[28 29].

Egress of neutrophil progenitors during severe sepsis and a predetermined maturation process of these cells characterized by a loss of VLA-4 expression argue against the suggestion that circulating VLA-4$^{pos}$ cells seen during severe sepsis are mature neutrophils[8 9]. There is no data to support the hypothesis that VLA-4 will remain on the cell surface during maturation under inflammatory conditions. Nor is it likely that inflammatory factors inhibit the maturation of progenitor cells. Taking these considerations into account, our data indicate that circulating VLA-4$^{pos}$ neutrophil progenitors maturate and lose their VLA-4 expression in the first days after release from the bone marrow.

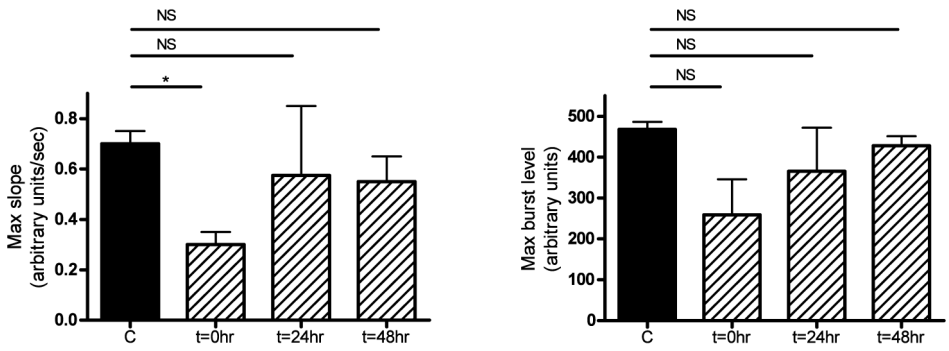


**Figure 7**
$H_2O_2$ release by VLA-4$^{pos}$ progenitors during culture compared to $H_2O_2$ release by segmented VLA-4neg neutrophils at t=0hr (control=c). Respiatory burst depicted as maximum slope (A) and maximum levels during culture (B). * = $p<0.05$, NS= not significant.

It has also been suggested that VLA 4 is re-expressed on cell surface of mature neutrophils[9]. *In vitro* studies have shown that VLA-4 expression on neutrophil cell surface increased after incubation with serum of septic patients[8 9]. It remains uncertain, however, whether this increased expression explains the large number of VLA-4$^{pos}$ cells seen *in vivo* as no VLA-4$^{pos}$ mature neutrophils were seen in any of our experiments. Therefore, VLA-4$^{pos}$ cells found *in vivo* are rather neutrophil progenitor cells than mature neutrophils.

Our data support the hypothesis that VLA-4$^{pos}$ neutrophil progenitors can appear in the circulation during systemic inflammation evoked by both pathogen associated molecular patterns (PAMPs) (e.g. during sepsis) as well as by damage associated molecular patterns (DAMPs) (multi trauma). Which factors induces the release of progenitor cells during inflammation remains to be elucidated. G-CSF and granulocyte macrophage (GM)-CSF are commonly used for stem cell mobilization and are possible candidates, but there might as well be others such as IL-3 and IL-6[30].

The fact that VLA-4$^{pos}$ progenitors are found in critically ill patients and are not found during mild inflammation, implies that liberation of progenitor cells only occurs under extreme conditions. Previous studies performed by our group did not display circulating VLA-4$^{pos}$ neutrophil progenitor cells after *in vivo* endotoxin challenge and mild trauma, although both conditions were accompanied by a release of multiple cytokines and mobilization of banded neutrophils[31 32]. It is likely that the inflammatory stimulus in this model is either too short or too mild to sufficiently deplete the bone marrow from neutrophils. This idea is supported by an early study, performed in 1977 on three healthy male subjects, which demonstrated the appearance of circulating progenitor cells after administration of pseudomonas endotoxin[33]. It is tempting to speculate that the appearance of progenitor cells is a marker for the severity and prolonged time of the inflammatory reaction and might be related to inflammatory complications such as septic shock, organ failure and mortality. The question remains as to why progenitor cells are released from the bone marrow in critically ill patients. Are these cells released because of depletion of mature neutrophils in the bone marrow or do these neutrophil progenitors have a specific function? The first suggestion seems most logical. However, recent work has demonstrated that unique neutrophil subsets appear in the circulation under inflammatory conditions that have specific functions[29 34 35]. Whether VLA-4$^{pos}$ neutrophil progenitors have specific functions remains to be elucidated.

In conclusion, in this study we prove that during severe inflammatory conditions neutrophil progenitors with decreased anti-microbial function are released from the bone marrow into the peripheral circulation. Outside the bone marrow these VLA-4$^{pos}$ neutrophil progenitors are able to differentiate into mature neutrophils in absence of inducing cytokines. Identification of these neutrophil progenitors during severe inflammation provides new insight on the kinetics of the neutrophil response during severe acute inflammation.

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PART I

IMMUNOMONITORING

# 5

# Homology in systemic neutrophil response induced by human experimental endotoxemia and by trauma

*Neutrophil activation in vivo by PAMPs and DAMPs*

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# Abstract

**Introduction**

The investigation of the trauma-induced innate immune responses is hampered by the wide variability in patients, type of trauma en environmental factors. To circumvent this heterogeneity we examined whether the systemic innate immune response towards human experimental endotoxemia is similar to the response during SIRS after trauma. We tested the hypothesis that the innate immune response to pathogen-associated (PAMP, e.g. lipopolysaccharides) and danger-associated (DAMP, as induced by injury) molecular patterns leads to a comparable *in vivo* activation of human neutrophils.

**Methods**

E.coli lipopolysaccharide (LPS, 2 ng/kg) was injected intravenously in 9 healthy volunteers to induce a controlled systemic inflammatory response. Indices of systemic inflammation in this human inflammation model were compared to those of 12 trauma patients with a mean injury severity score of 19. Blood samples were withdrawn at 3 and 24 hours after LPS-challenge or injury. Blood samples of 9 healthy volunteers were used as control. Receptor expression was measured as readout for neutrophil activation by flow cytometry.

**Results**

Endotoxemia and injury resulted in a comparable activation phenotype of circulating neutrophils. This phenotype was characterized by down regulation of chemokine receptors CXCR1 and CXCR2 and of Fcγ receptors II and III. A significant difference between both conditions was seen in CD66b expression, for endotoxin resulted in an increased CD66b expression, whereas injury did not.

Neutrophil activation was present 3 hrs after onset of inflammation, both during experimental endotoxemia as well as in trauma patients.

**Conclusion**

Endotoxin and trauma appear to induce a similar neutrophil activation phenotype.

# Introduction

Tissue injury results in activation of the innate immune system by danger-associated molecular patterns (DAMPs)[1]. DAMPs exist of endogeneous cytosolic components such as high mobility group box 1 (HMGB-1), heat shock proteins, defensins and annexins[2]. After severe injury, activation of the immune system can lead to a systemic inflammatory response syndrome (SIRS) with an increased risk of inflammatory complications such as acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS). A comparable systemic innate immune response is seen during severe infectious diseases such as sepsis and septic shock[3]. Yet, during infection the innate immune response is activated by microbial components in general referred to as pathogen-associated molecular patterns (PAMP) instead of by DAMPs[4 5]. PAMPs are recognized by a limited number of germline-encoded pattern-recognition receptors (PRRs), of which Toll-like receptors are most well known[4].

Recently, there has been much discussion about the distinction between PAMPs and DAMPs. It has been proposed that many micro-organism components and endogenous alarm signals belong to an ancient subfamily of universal DAMP[6, 7]. In addition, several studies have shown that DAMP can also trigger the innate system via toll like receptors[8 9]. We hypothesized that activation by PAMP and DAMP results in a similar neutrophil response as part of the final common pathway of the innate immune response.

Human experimental endotoxemia can be used to investigate cellular innate immune response to PAMP in a standardized manner. Human experimental endotoxemia was developed as model for the host response to infectious diseases and sepsis[10 11]. The model consists of a intravenous challenge of a human volunteer with endotoxin (lipopolysaccharide (LPS)) at low doses (1-4ng/kg)[12 13]. Earlier studies have already shown that the administration of LPS leads to a cytokine release, with increases of, e.g., tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8 and IL-10, comparable to that seen after trauma, albeit in a shorter timeframe[10 12 13].

It is unknown whether inflammation induced by DAMP results in a similar cellular innate immune response as by PAMP-induced inflammation. A recent study has shown similarity in gene expression in circulating leukocytes from trauma patients and from subjects after LPS challenge [14]. Therefore, the present study compared the early neutrophil response after LPS exposure to that of trauma patients, in order to see if the experimental endotoxemia could be used to investigate the acute systemic cellular response after trauma. Since the inflammatory stimulus is short lived after a LPS challenge, the endotoxemia model is in particular useful for investigating the kinetics of the early innate immune response (during the first couple of hours after onset of systemic inflammation). Therefore, we designed a study to compare activation phenotype of circulating neutrophils during this initial phase of the innate immune response, about 3 hrs after onset of inflammation. A second blood sample was drawn after 24 hrs as at this time point the systemic response has normalized in the endotoxemia model[10 12 13].

The endotoxemia model may facilitate the study of the detailed kinetics of the cellular innate immune reaction as it circumvents the heterogeneity seen in trauma patients. In addition, the endotoxemia model is ideal for testing the effect of immunomodulating therapy on the innate immune response during the final common pathway.

# Materials and Methods

**Materials**

U.S. Reference E.coli endotoxin (lot Ec-5, Centre for Biologic Evaluation and Research, Food and Drug Administration, Bethesda, MD); saline 0.9% (Baxter, The Netherlands); 2.5% glucose/0.45% saline (Baxter, The Netherlands); FITC-labelled mouse-antihuman monoclonal antibodies against: L-selectin (CD62L; clone Dreg56, BD Pharmingen, USA), CXCR1 (CD181; clone 42705, R&D Systems Europe, UK), C5aR (CD88; clone P12/1, Serotec, Germany); PE-labelled mouse-antihuman monoclonal antibodies against: αM (CD11b; clone 2LPM19c, DAKO, Denmark), CXCR2 (CD182; clone 48311, R&D Systems Europe, UK), FCγRII (CD32; clone FLI8.26, BD Pharmingen, USA); Alexa 647-labelled monoclonal antibodies against: FCγRIII (CD16; clone 3G8, BD Pharmingen, USA); FITC-labelled IgG1 negative control (clone MOPC-21, BD Biosciences, Belgium), and IgG2a negative control (clone MRC OX-34, Serotec, Germany); PE-labelled and IgG1 negative control (clone DD7, Chemicon, USA); Alexa 647-labelled IgG1 negative control (clone MOPC-21, BD Biosciences, Belgium); FITC-labelled monoclonal phage antibody A27 against active FCγRII (generated and characterized as described previously[15]); N-formyl-methionylleucyl-phenylalanine (fMLP) (Sigma-Aldrich, USA); FACScalibur Flow cytometer (BD Biosciences, USA); SPSS version 15.0 software (The Apache Software Production 2008, USA)

**Trauma patients**

Trauma patients enrolled were part of an observational study performed at University Medical Centre Utrecht, investigating neutrophil activation in patients after chest injury[16]. Written informed consent was obtained from all patients or their legal representatives in accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines.

12 patients suffering from chest injury with an abbreviated injury score (AIS)[17] of 2 or more admitted to the Trauma department of the University Medical Centre Utrecht were enrolled. Exclusion criteria were age < 18 or > 70 years, death within 24h after admission and patients with an altered immunological status (e.g. chronic diseases, corticosteroid use or chemotherapy). Blood samples ware taken at 2-4 hrs and 22-26 hrs after the accident. On average, patients arrived within 1 tot 1.5 hrs after injury at the emergency room (ER). In all trauma patients, a first blood sample was then drawn and analyzed within 1 to 2 hrs after arrival at the ER.

**Human experimental endotoxemia.**

Healthy volunteers undergoing endotoxemia were part of three endotoxin trials (NCT00783068, NCT00916448 and NCT01091571 at www.clinicaltrials.gov) performed at the Radboud Medical Centre Nijmegen. Study protocols were approved by the local Ethical Committees. Written informed consent was obtained from all healthy volunteers in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Human experimental endotoxemia was evoked exactly as described before[12]. In short, 9 male subjects, between 18 and 35 years of age, were enrolled after screening and prehydrated with 1500 ml 2.5% glucose/0.45% saline infusion. E.coli endotoxin, was used in this study. Endotoxin was reconstituted in 5ml saline 0.9% and injected as single

intravenous bolus (2ng/kg) during 1 minute at t=0. Blood samples were taken from the arterial catheter at 3 hrs and 24 hrs after administration of endotoxin.

**FACS analysis**

All blood samples were collected in a vacutainer® with sodium heparin as anticoagulant and cooled immediately on melting ice. Blood samples of 9 healthy lab co-workers served as controls. Red cells were lysed with icecold isotonic NH4Cl. After lysis, white blood cells were washed and resuspended in phosphate buffered saline supplemented with sodium citrate (0.4% wt/vol) and pasteurised plasma protein solution (10% vol/vol) (PBS2+), as previously described[16]. Resuspended cells were incubated on ice with commercial obtained directly labelled mouse-antihuman monoclonal antibodies against L-selectin (CD62L), αM (CD11b), CXCR1 (CD181), CXCR2 (CD182), C5aR (CD88), CD66b, FCγRII (CD32) and FCγRIII (CD16).

After incubation and final wash, labelling was measured on FACScalibur Flow cytometer. The neutrophils were identified according to their specific side-scatter and forward-scatter signal. For measurement of active FCγRII expression, whole blood was incubated a FITC-labelled monoclonal phage antibody A27 for 45 min on ice[16]. Active upregulation of active FCγRII expression was measured after 5 min of stimulation of whole blood at 37°C with fMLP $10^{-6}$M to evaluate the responsiveness of the cells for bacterial derived protein products/peptides. After stimulation, the samples were put on ice again and stained with phage antibody A27. After staining, red cells were lysed and expression was measured on FACScalibur as previously described[16]. Data from individual experiments are depicted as fluorescence intensity as the median fluorescence intensity (MFI) of at least 5,000 neutrophils.

**Leukocyte count and differentiation**

Leukocyte counts were determined by routine laboratory test of the participating hospitals. Percentages of neutrophils and monocytes were calculated out of total amount of white blood cells based on their specific forward-sideward scatter on the FACS plots.

**Statistical analysis**

Data were analyzed using SPSS version 15.0 software. Results are expressed by mean±SE. Normality of variance was confirmed by the Lavene's test. Subsequently, a one-way ANOVA followed by a Bonferroni post hoc was used as appropriate to test differences between the study groups and control at the two different time point. Student's t-test was used to analyze difference in leukocyte count between the two experimental groups. Statistical significance was defined as $p<0.05$.

## Results

**Patient and volunteer demographics**

From April 2008 until April 2009 twelve trauma patients were enrolled, of whom 9 were male and 3 female. The mean age was 53 years (range 25 – 69) and the mean injury severity score (ISS)[17] 19 (range 9 – 56) (Tabel 1). Mean arterial pressure (MAP) at t=3hrs was 97±3

mmHg with a mean heart frequency of 88±5 bpm. None of the patients received blood product during time of study. The mean age of the male endotoxemia volunteers was 22±1 years (range 19-25). Their MAP decreased from 98±2 at baseline to 76±4 mmHg at t=3hrs ($p<0.001$). Heart rate increased from 64±3 to 92±5 bpm ($p<0.001$).

**Table 1** Characteristics of included trauma patients. M = male, F = female, MVA = motor vehicle accident, ISS = injury severity score, NISS = new injury severity score [17]

| Pt | Gender | Age | Mechanism of injury | Diagnosis | ISS | NISS | Apache II |
|---|---|---|---|---|---|---|---|
| 1 | M | 25 | MVA | 8 rib fractures unilateral<br>Pneumothorax bilateral | 16 | 25 | 3 |
| 2 | M | 62 | MVA | 3 rib fractures unilateral<br>Clavicula fracture<br>Orbita roof fracture | 17 | 17 | 15 |
| 3 | M | 51 | Fall from height | 2 rib fractures unilateral<br>Pneumothorax unilateral | 9 | 9 | 6 |
| 4 | F | 47 | Fall from horse | 10 rib fractures unilateral<br>Flail thorax | 17 | 17 | 3 |
| 5 | M | 60 | MVA | 5 rib fractures unilateral<br>Pneumothorax unilateral<br>Lungcontusion unilateral<br>Pancreas contusion | 21 | 29 | 9 |
| 6 | M | 59 | Fall from height | 6 rib fractures unilateral<br>Pneumothorax unilateral<br>Lungcontusion uniilateral | 16 | 25 | 18 |
| 7 | M | 37 | Fall from height | > 20 rib fractures bilateral<br>Hematothorax bilateral<br>Lungcontusion bilateral<br>Pelvic fracture<br>Cerebral hematoma<br>Skul fracture | 56 | 56 | 24 |
| 8 | M | 69 | MVA | 3 rib fractures unilateral | 5 | 5 | 5 |
| 9 | F | 62 | Bicycle accident | 6 rib fractures unilateral<br>Minor laceration kidney<br>Facial heamatoma | 14 | 14 | 7 |
| 10 | F | 53 | Fall from height | 4 ribfractrures unilateral<br>1 rib fracture contra lateral<br>Scapula fracture<br>Fracture cervical vertebral body | 17 | 17 | 2 |
| 11 | M | 62 | MVA | 3 ribfractures<br>Lungcontusion bilateral<br>Minor liver laceration | 20 | 29 | 15 |
| 12 | M | 59 | Attacked by cow | Multiple rib fractures bilateral<br>Flail thorax bilateral<br>Pneumothorax bilateral<br>Lungcontusion bilateral<br>Sternum fracture<br>Minor liver laceration | 29 | 38 | 6 |

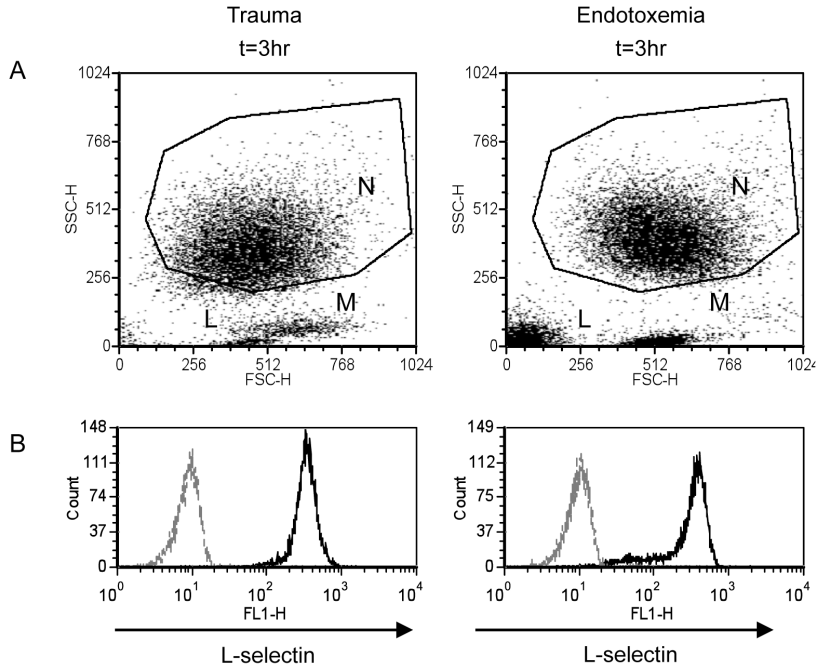


**Figure 1**
Representative example of FACS analysis of a trauma patient and a healthy volunteer undergoing endotoxemia at t=3hr. (A) Neutrophils (N) are gated based on forward-sideward scatter; (M) = monocytes; (L) =Leukocytes. (B) Histogram showing L-selectin (black line) expression and expression of FITC-labelled IgG1 negative control (dotted line) on gated neutrophils.

**Leukocyte count and differentiation**

At 3 hrs after the insult (trauma or LPS administration) leukocyte counts were evidently higher in the trauma group 12.8±1.5x$10^9$ cells/l than the endotoxemia group 6.6±1.8x$10^9$ cells/l ($p$=0.002, unpaired students t-test). Earlier studies have shown that leukocyte counts increase during experimental endotoxemia, but not until 6-8 hrs after infusion of endotoxin[12]. The percentage of neutrophils, however, was equally increased in the endotoxemia and trauma group (87±2% vs. 81± 3%; $p$=0.229) compared to control values 57±2% (p<0.001 both groups) at 3 hrs. The percentage of neutrophils remained high in the trauma group during the first 24hrs (76±2%; p<0.001), whereas it returned to normal levels in the endotoxemia group at time point 24 hrs (64±3%; $p$=0.138 compared to baseline).

A striking difference was seen in the percentage of monocytes between the trauma and the endotoxemia group at 3 hrs. Monocytes almost completely disappeared from the circulation during endotoxemia. The percentage of monocytes was significantly lower in the endotoxemia group (0.7± 0.1%) at 3 hrs compared to the trauma group (5.2±0.7%; p<0.001) and control values (6.7±0.8%; p<0.001). The percentage of circulating monocytes restored to normal at 24 hrs (6.4±0.6% endotoxemia group vs. 7.6±0.7% trauma group; $p$=1.00 both groups compared to control values).

**Receptor expression on the neutrophil surface**

*L-selectin (CD62L) and αM(CD11b)*

It is well known that upon activation neutrophils shed L-selectin and at the same time increase the surface expression of αM(CD11b)[18]. Endotoxin as well as injury-induced inflammation resulted in a tendency towards lower L-selectin expression levels *in vivo*, but this decline did not reach statistical significance (endotoxemia $p$=0.140, trauma $p$=0.066 at t=24hrs; Fig. 2). In contrast, αM(CD11b) expression did not increase as seen during activation *in vitro*, but rather decreased during inflammation. At 24hrs after onset of inflammation αM(CD11b) expression was significantly lower in the endotoxemia group compared to control values ($p$=0.003; Fig. 2). Although αM(CD11b) expression tended to decline in the trauma group at t=24hrs, expression was not significantly lower compared to control values ($p$=0.072). Between the trauma and endotoxemia group, expression of L-selectin and αM(CD11b) did not differ at any time point.

*CXCR1(CD181) and CXCR29CD182)*

Earlier studies have shown that CXCR1 and 2, surface receptors of the chemokine IL-8, are down-regulated upon activation of neutrophils both *in vitro* and *in vivo*[19]. In this study, systemic inflammation resulted in a reduced surface expression of CXCR1 and CXCR2 on circulating neutrophils at t=3hrs in both study groups in comparison to control values

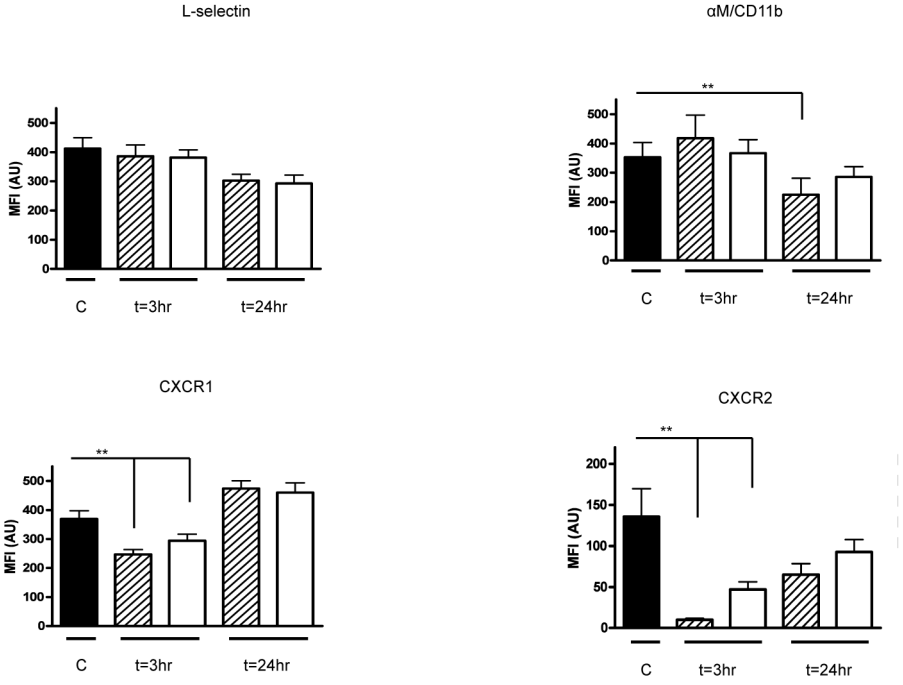


**Figure 2**

Expression of L-selectin, αM/CD11b, CXCR1 and CXCR 2 on circulating neutrophils measured by flow cytometry. Black bars (C) stand for baseline values from healthy controls (n = 9). Striped bars represent healthy volunteers undergoing endotoxemia (n=9) and open bars represent trauma patients (n=12) at 3hrs and 24 hrs after onset of inflammation. Data are presented as mean±SEM. MFI (AU) = mean fluorescence intensity (arbitrary units); *p<0.05; ** p<0.01

(p<0.01; Fig. 2), indicative for neutrophil activation. After 24hrs no significant difference in CXCR1 and CXCR2 expression between the endotoxemia group, trauma group and control values were found.

*FcγRII(CD32) and FcγRIII(CD16)*

Fcγ receptors play in important role in activation of neutrophils. Fcγ receptors bind to immunoglobulins (IgG) either in aggregates or attached to pathogens[20]. Binding of IgG's to Fcγ receptors promotes the oxidative burst and induces phagocytosis[20]. Expression of Fcγ receptors on circulating neutrophils have been shown to decrease during systemic inflammation both in trauma patients as in healthy volunteers during endotoxemia[13 16]. In this study, expression of FcγRII and FcγRIII was significantly lower in both study groups in comparison to control values at 3 hrs after onset of inflammation (FcγRII $p$=0.001 both groups; FcγRIII endotoxemia $p$=0.028, trauma group $p$=0.001; Fig. 3). FcγRIII expression remained low in the trauma group up until 24 hrs ($p$=0.001), whereas FcγRII expression restored ($p$=0.310). Both intrinsic active FcγRII as well as fMLP induced active FcγRII expression showed a tendency to decline during inflammation (Figure 3).Yet, this decline did not reach statistical difference in any of the groups at any time point. A decreased active FcγRII expression on circulating neutrophils, however, has been described in other studies during systemic inflammation after trauma as well as during experimental endotoxemia[13 16 21].

*C5aR(CD88) and CD66b*

C5aR surface expression did not significantly change during inflammation (Figure 4). C5a is a strong chemotaxin for neutrophils and facilitates the oxidative burst and phagocytosis of neutrophils. Decreased C5aR expression has been described in severely injured patients[22] as well as in septic patients[23], but was not seen in this study.

CD66b expression was higher in the endotoxemia group compared to control values (p<0.001) and compared to the trauma group ($p$=0.008), 3hrs after onset of inflammation (Figure 4). CD66b is present in the membrane of specific granules in neutrophils. Upon activation these granules mobilize to the cell surface, resulting in up-regulation of surface CD66b expression[24 25]. CD66b surface expression tended to increase in the trauma group, but did not reach statistical difference ($p$=0.147). At 24hrs no significant differences were seen in CD66b expression between the groups.

## Discussion

Understanding the circulating neutrophil response to injury in severely injured patients has proven to be problematic and has resulted in contradicting data[13 26]. The extend and duration of the innate immune response in trauma patients is influenced by several inevitable confounders such as differences in age, gender, medical history, heterogeneity of injuries, received (blood) products and surgical interventions. In addition, lack of baseline values and an estimated time of the insult and onset of inflammation make the interpretation of data difficult. To circumvent these confounders, we propose the use of the well established human endotoxemia model to accurately study the kinetics of a homogenous early cellular

innate immune response *in vivo* for PAMP- as well as DAMP-associated diseases. The main finding of the present study is that neutrophils, as part of the final common pathway, are similarly activated by endotoxin and trauma. While the human endotoxemia experiments are very standardized and controlled, and the exact time and amount of

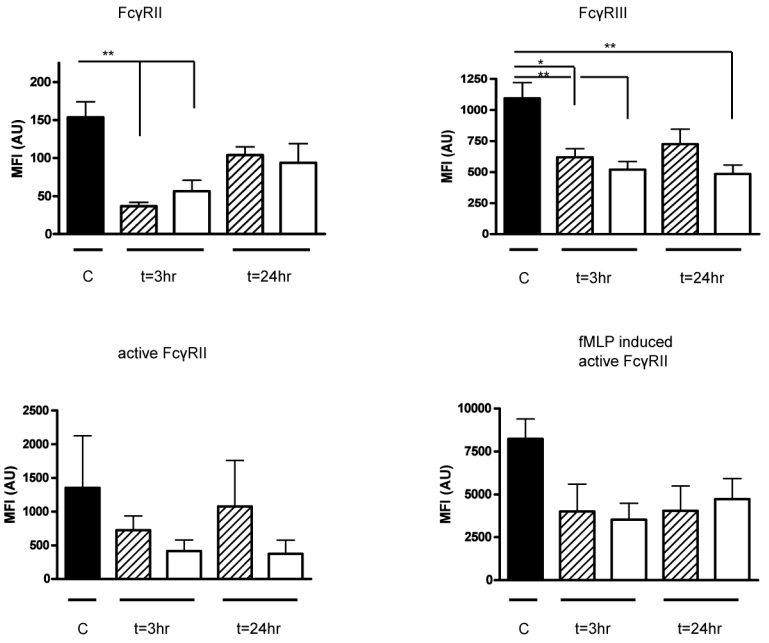


**Figure 3**
Expression of FcγRII, FcγRIII, active FcγRII and fMLP induced active FcγRII on circulating neutrophils measured by flow cytometry. Black bars (C) stand for baseline values from healthy controls (n = 9). Striped bars represent healthy volunteers undergoing endotoxemia (n=9) and open bars represent trauma patients (n=12) at 3hrs and 24 hrs after onset of inflammation. Data are presented as mean±SEM. MFI (AU)= mean fluorescence intensity (arbitrary units); *p<0.05; **p<0.01

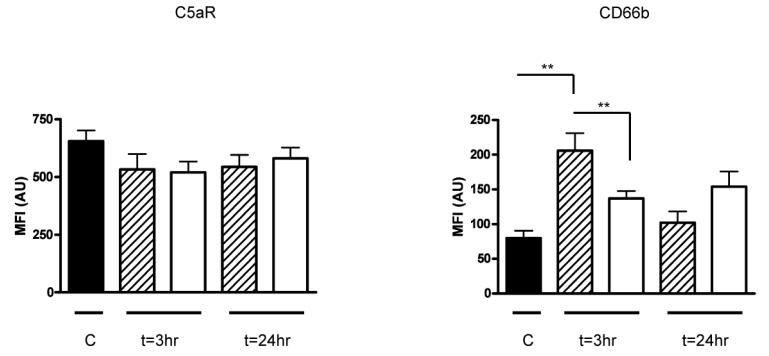


**Figure 4**
Expression of C5aR and CD66b on circulating neutrophils measured by flow cytometry. Black bars (C) stand for baseline values from healthy controls (n = 9). Striped bars represent healthy volunteers undergoing endotoxemia (n=9) and open bars represent trauma patients (n=12) at 3hrs and 24 hrs after onset of inflammation. Data are presented as mean±SEM. MFI (AU)= mean
fluorescence intensity (arbitrary units); *p<0.05; ** p<0.01

endotoxin administration is known, no real measurements for the magnitude of a traumatic stimulus exist. Although also relevant differences were observed that need to be discussed, our study shows that the consequences of the endotoxin and trauma on the activation of neutrophils are comparable. This was most prominently illustrated by the transient down regulation of chemokine receptors CXCR1 and 2.

While circulating neutrophils show in general a similar activation phenotype -indicating an altered expression of surface receptors in comparison to control value due to an inflammatory stimulus - after injury and endotoxemia, also clear differences were observed for example in CD66b expression. Three hours after endotoxin infusion, CD66b expression was significantly increased whereas the expression of CD66b did not change after injury. This disparity may be the direct result of circulating LPS. LPS itself can induce up-regulation of CD66b surface expression *in vitro*[27 28], albeit in much higher concentration than used *in vivo*. It is known that *in vitro* higher concentrations of LPS are needed as *in vivo* LPS binding protein facilitates activation of neutrophils by LPS[29]. CD66b expression is also increased in septic patients[30], which may be induced by circulating LPS during sepsis.

Increased CD66b expression, on the other hand, has to our knowledge not been described in trauma patients. These findings suggest that up-regulation of CD66b might be strongly influenced by the presence of LPS *in vivo* and might, therefore, be potential marker for discriminating between sepsis and SIRS.

Both endotoxin and injury resulted in an increased percentage of circulating neutrophils mounting up to approximately 85% of all leukocytes. These data indicate a prominent role for neutrophils in the early immune response in PAMP and DAMP associated diseases. The absolute number of circulating leukocytes, however, differed among the two groups. Leukocyte count increased more rapidly in trauma patients, reaching increased levels within 3 hrs after onset of inflammation, while during endotoxemia leukocyte counts is known to increase between 6 to 8 hrs after onset of inflammation[12]. A remarkable difference between the endotoxemia and trauma group was also seen in percentage of circulating monocytes, at 3 hrs after the onset of inflammation. During endotoxemia, monocytes almost totally disappeared from the circulation. Interestingly, this phenomenon seems not to occur, or at least to a lesser extent, during trauma. The rapid decline in number of circulating monocytes after LPS challenge is most probably due to homing of monocytes to tissue. Increased apoptosis of monocytes and lymphocytes of septic patients is observed after incubation *in vitro*[31]. However, the implication of this increased apoptosis during sepsis *in vivo* is not clear. To our knowledge, no study has ever reliably identified apoptotic circulating monocytes during inflammation. Therefore, we believe the drop of monocyte count in this study is rather the result of redistribution of monocytes than that of apoptosis.

The above mentioned differences indicate that the responses seen after trauma and after LPS challenge are not identical. This may be the result of PAMP- and DAMP-specific differences, or may be related to the variation in magnitude and duration of the inflammatory stimulus during experimental endotoxemia and trauma patients or other natural courses of inflammatory diseases[10 11]. In addition, differences between the endotoxemia- and trauma group (age, medical history, etc) may also have contributed to dissimilarity between the two responses. Extrapolation of data from the endotoxemia model to clinical conditions should, therefore, be taken with precaution. In our patient group, it appears unlikely that trauma-induced hypotension may have caused intestinal hypoperfusion and transient

endotoxemia. Therefore, we conclude that the observed similarities in neutrophil activation are the result of a PAMP and DAMP driven inflammatory response.

Although neutrophil activation *in vitro* is typically characterized by shedding of L-selectin and up-regulation of αM(CD11b) expression[18], these expected changes in surface expression were not seen on circulating neutrophils *in vivo*. In both study groups L-selectin expression did not significantly decrease. Surprisingly, αM(CD11b) expression *in vivo* showed a reversed activation phenotype compared to *in vitro*. *In vivo* αM(CD11b) surface expression decreased during inflammation whereas expression is known to increase after activation *in vitro*. We can only speculate why surface receptor expression on circulating neutrophils 3 to 24 hrs after onset of inflammation differs form activation phenotype *in vitro*, but it is likely that surface receptor expression *in vivo* is influenced by altered distribution of neutrophils. Activated neutrophils are prone to leave the circulation and home to tissue, whereas young non-activated neutrophils are released from the bone marrow and enter the circulation[3 5]. Changes in circulating neutrophil population can thus explain a different neutrophil receptor phenotype during inflammatory responses *in vivo* compared to neutrophil phenotypes seen after activation *in vitro*.

The overall decrease in αM(CD11b) expression 24 hrs after inflammation *in vivo*, is most likely caused by an increased amount of young neutrophils, expressing αM(CD11b) at lower levels[32 33]. A recruitment of young neutrophils is suggested by an overall decreased expression of FcγRIII, as FcγRIII is known to be expressed at lower levels on banded neutrophils[13 34]. In this study, the appearance of young neutrophil after PAMP and DAMP induced inflammation was not only indicated by low overall FcγRIII expression, but was also confirmed by examination of cytospins showing high numbers of banded neutrophils. Recently, it has been show that neutrophils lifespan is approximately 5 days [35]. This can explain the presence of young neutrophils in the circulation at more than 20 hours after cytokine levels return to normal.

An earlier study from our group has emphasized the importance of identifying neutrophil populations [13]. This study showed that functionality of circulating neutrophils during inflammation varies greatly between young and segmented neutrophils. The complexity of the inflammatory reaction, with appearance of different neutrophil populations, makes it hard to identify the underlying mechanisms by which DAMP and PAMP trigger the early neutrophil response *in vivo*. Isolation of different neutrophil populations by cell sorting at different time points during the initial phase is essential. The controlled neutrophil reaction evoked by the LPS challenge may well serve for this purpose and the endotoxemia model may provide better comprehension of the kinetics and the induction of signalling pathways in the future. However, to accomplish this in trauma patients will prove a difficult task, as blood samples are often not available at the early hours after trauma and yet unknown DAMPs may be involved. So, up until now we can state that DAMP and PAMP induced inflammation results in a similar composition of neutrophil populations, but it remains to be elucidated whether same signalling pathways are involved.

A relatively short lived inflammatory response after LPS challenge limits the extrapolation of the endotoxemia model to more persistent inflammation such as seen in major trauma. Endotoxin infusion leads to a cytokine release that peaks after 2 to 3 hrs resulting in an early and short term leukocytosis [12 13]. Trauma on the other hand can result in elevated cytokine[36] en leukocyte levels for days. Comparison between the LPS challenge and

inflammatory diseases can, in our opinion, thus best be made during the first couple of hours after onset of inflammation. The endotoxemia model has demonstrated to represent a reliable model for examining the kinetics of this early innate immune response in human *in vivo*. Yet, during this initial phase it is very difficult to obtain blood samples from trauma patients at defined time points. We chose to draw blood samples between 2 to 4 hrs.
In conclusion, trauma and experimental endotoxemia result in comparable changes in neutrophil phenotype. Although differences are seen in the innate immune response with respect to absolute neutrophil and monocytes numbers, this study shows clear similarities in endotoxin and trauma induced activation of circulating neutrophils. The endotoxemia model might be a helpful tool not only for investigating the early cellular immune response, but also for testing potential immunomodulating drugs in conditions in which therapy can start shortly after onset of inflammation such as trauma, burn injury and major surgery.

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PART II

IMMUNOMODULATION

# 6

## A systematic review of RCTs exploring the effect of immunomodulative interventions on infection, organ failure, and mortality in trauma patients

*Crit Care. 2010;14(4):R150*

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## Abstract

**Introduction**

Following trauma, patients may suffer an overwhelming pro-inflammatory response and immune paralysis resulting in infection and multiple organ failure (MOF). Various potentially immunomodulative interventions have been tested. The objective of this study is to systematically review the randomized controlled trials (RCTs) that investigate the effect of potentially immunomodulative interventions in comparison to a placebo or standard therapy on infection, MOF, and mortality in trauma patients.

**Methods**

A computerized search of MEDLINE, the Cochrane CENTRAL Register of Controlled Trials, and EMBASE yielded 502 studies, of which 18 unique RCTs were deemed relevant for this study. The methodological quality of these RCTs was assessed using a critical appraisal checklist for therapy articles from the Centre for Evidence Based Medicine. The effects of the test interventions on infection, MOF, and mortality rates and inflammatory parameters relative to the controls were recorded.

**Results**

In most studies, the inflammatory parameters differed significantly between the test and control groups. However, significant changes in infection, MOF, and mortality rates were only measured in studies testing immunoglobulin, IFN-γ, and glucan.

**Conclusion**

Based on level 1b and 2b studies, administration of immunoglobulin, IFN-γ, or glucan have shown the most promising results to improve the outcome of trauma patients.

# Introduction

Trauma remains the leading cause of death in people under the age of 40[1], with multiple organ failure (MOF) accounting for 27.5% of deaths among trauma patients[2]. MOF can be a result of an early over-reaction of the immune system or a late immune paralysis[3]. Several groups have reviewed the changes that occur in the immune system as a result of injury and concluded that pro- and anti-inflammatory reactions play a role in the development of MOF[4-7]. Early MOF, which develops within the first 3 days after injury without signs of infection, is attributed to an overwhelming leukocyte driven pro-inflammatory response clinically defined as a systemic inflammatory response syndrome (SIRS). Late MOF, on the other hand, is most often associated with infection and occurs more than 3 days after injury. Late MOF seems to be the result an inadequate specific immune response with diminished antigen presentation, referred to as compensatory anti-inflammatory response syndrome (CARS). Many argue that SIRS and CARS occur simultaneously as a mixed antagonistic response syndrome (MARS)[4 6] and therefore both reactions contribute to the occurrence of infection, sepsis, and MOF.

This knowledge begs application. Which interventions attenuate both the hyper inflammatory response and immune paralysis and subsequently improve the clinical outcome in trauma patients? Montejo et al.[8] have systematically reviewed the effect of immunonutrition on clinical outcome in trauma patients. Although immunonutrition shortened the time of mechanical ventilation and intensive care unit (ICU) stay, and resulted in a lower incidence of bacteremias and intra-abdominal infections, the incidence of nosocomial pneumonia, wound infection, urinary tract infection, sepsis, and mortality remain unchanged. Other interventions are needed.

The objective of this paper is to systematically review the randomized controlled trials (RCTs) that investigate the effect of non-nutritional potential immunomodulative interventions in comparison to a placebo or standard therapy on infection, MOF, and mortality in trauma patients.

# Methods

### Search

Studies were found via computerized searches of the MEDLINE and EMBASE databases and the Cochrane CENTRAL Register of Controlled Trials. The search syntax included synonyms of trauma (trauma*, injur*), immunomodulation (immun*, inflammat*), and clinical outcome (infectio*, "organ failure", mortality, surviv*) in the titles, abstracts, and keywords. Limits were set to retrieve only studies on humans with high-quality design (meta-analyses, systematic reviews, Cochrane reviews, RCTs, and clinical trials). No limits were imposed on either publication date or language.

### Selection

The search hits were screened for relevance by two authors. Studies were deemed relevant when they investigated the effect of a potentially immunomodulative intervention on clinical

outcome in trauma patients. Therefore, studies including patients other than trauma patients (for example, other ICU patients), patients with specific isolated injury (for example isolated injury to the head or an extremity) or with thermal injuries were excluded. Furthermore, patients needed to be randomly allocated to receive a potentially immunomodulative intervention, standard therapy, or a placebo. Since the effect of immunonutrition has already been systematically reviewed, studies implementing immunonutrition were excluded. To assess the efficacy of the interventions, only studies reporting clinical outcomes were included. References of the relevant studies were checked for other relevant articles that might have been missed in the computerized search.

**Quality assessment**

The methodological quality of each of the studies for which the full text was available was assessed using a checklist for therapy articles from the Centre for Evidence Based Medicine[9 10] One point was accredited for each positive criterion: (1) the study participants were randomized, (2) the study groups had similar characteristics at baseline, (3) the groups were treated equally except for the test intervention, (4) all patients were accounted for, (5) outcome assessors were blinded to the intervention or used well-defined outcome criteria, and (6) outcomes were compared on an intention-to-treat basis.

**Data abstraction**

Data abstraction was completed independently. The studies were gleaned for patient characteristics (number, age, and injury severity score (ISS)), details of the intervention (test, control, delivery route, and duration) and length of follow-up during which outcome variables were measured. Outcome variables included in the analysis were: (a) infections, overall or specified; (b) MOF or mortality; and (c) inflammatory parameters, cellular or humoral. Definitions of infections given by authors were used, including major and minor infections, pneumonia, sepsis, meningitis, surgical site infections, urinary tract infections, and intra-abdominal abscesses. MOF was defined by MOF scores given by the authors. The efficacy of interventions intended to attenuate the hyper-inflammatory response were compared to those intended to reduce the immune paralysis. Interventions that altered the release of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α), active complement factors, leukocyte count or leukocyte-derived cytotoxic mediators were considered modulators of SIRS. Interventions that altered the release of anti-inflammatory cytokines (IL-10, IL-1RA), antigen presenting capacity or bactericidal capacity were considered modulators of CARS.

## Results

**Search and selection**

After filtering out duplicate studies retrieved from the databases, 502 potentially relevant studies were assessed. Studies were excluded that did not include only trauma patients (444), tested interventions that were not intended to immunomodulate (10), studied the effect of immunonutrition (20), did not report clinical outcome (4), or were non-systematic reviews (5) (Figure 1). The full text was not available for two studies[11 12]. By checking

references of the relevant studies, three other relevant studies were found that were missed in the computerized search because the keywords were not included in the titles or abstracts[13-15]. Two articles by Seekamp et al.[16 17] and two articles by Dries et al.[13 18] report on the same study. Therefore, 18 unique RCTs that met the inclusion and exclusion criteria were available for analysis.

### Quality assessment

Using the checklist for therapy articles from the Centre for Evidence Based Medicine[9], all RCTs scored 4 to 6 out of a maximum 6 points (Table 1). Points were lost because the study groups were dissimilar at baseline and/or patients dropped out that were not analysed on an intention-to-treat basis. Studies scoring a full 6 points were deemed high-quality RCTs reporting 1b level of evidence[10]. Studies scoring 4 or 5 points were deemed of lesser quality and thus reporting 2b level of evidence. Data from all studies was used to determine the effect of potential immunomodulative interventions on clinical outcome in trauma patients.

**Table 1** Quality assessment

| Study | Patients randomized | Groups similar at baseline | Groups treated equally | All patients accounted for | Assessor blinded or objective | Intention to treat analysis | TOTAL (max 6) | Level of Evidence |
|---|---|---|---|---|---|---|---|---|
| Browder, 1990[29] | 1 | 1 | 1 | 1 | 1 | 1 | 6 | 1b |
| Bulger, 2008[19] | 1 | 1 | 1 | 1 | 1 | 1 | 6 | 1b |
| Croce, 1998[24] | 1 | 0° | 1 | 1 | 1 | 1 | 5 | 2b |
| de Felippe, 1993[30] | 1 | 1 | 1 | 1 | 1 | 0 | 5 | 2b |
| Douzinas, 2000[32] | 1 | 0* | 1 | 1 | 1 | 0 | 4 | 2b |
| Dries, 1998[18] | 1 | 1 | 1 | 1 | 1 | 0 | 5 | 2b |
| Glinz, 1985[20] | 1 | 1 | 1 | 1 | 1 | 1 | 6 | 1b |
| Livingston, 1994[31] | 1 | 1 | 1 | 1 | 1 | 1 | 6 | 1b |
| Marzi, 1993[25] | 1 | 1 | 1 | 1 | 1 | 1 | 6 | 1b |
| Miller, 1985[14] | 1 | n.r. | 1 | 1 | 1 | 0 | 4 | 2b |
| Nakos, 2002[26] | 1 | 1 | 1 | 1 | 1 | 1 | 6 | 1b |
| Nathens, 2006[21] | 1 | 1 | 1 | 1 | 1 | 1 | 6 | 1b |
| Polk, 1992[22] | 1 | 0° | 1 | 1 | 1 | 1 | 5 | 2b |
| Rhee, 2000[23] | 1 | 0 | 1 | 1 | 1 | 1 | 5 | 2b |
| Rizoli, 2006[27] | 1 | 0 | 1 | 1 | 1 | 0 | 4 | 2b |
| Seekamp, 2004[16] | 1 | 1 | 1 | 1 | 1 | 1 | 6 | 1b |
| Vassar, 1991[15] | 1 | 1 | 1 | 1 | 1 | 1 | 6 | 1b |
| Waydhas, 1998[28] | 1 | 1 | 1 | 1 | 1 | 0 | 5 | 2b |

1: yes, 0: no, n.r.: not reported, ° the test group was older, * the test group had a higher injury severity score, which was corrected for using a multiple regression model.

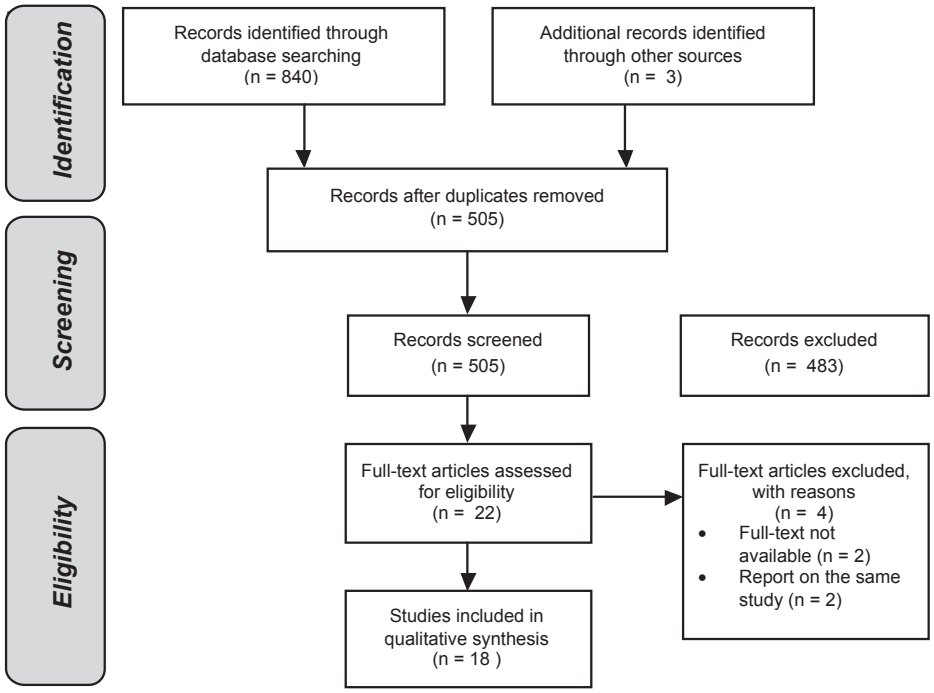


**Figure 1**
Study selection. Computerized search conducted on January 4, 2010.

### Study characteristics

A comparison of the study characteristics of the 18 RCTs reveals marked inter-trial heterogeneity of patients and interventions (Table 2). The number of patients included in the trials ranged from 16 to 268, with five trials studying over 100 patients[19-23]. Of the smaller trials, six were pilot studies[14 24-27]. Three of the trials were phase II trials primarily powered to test dosage and safety, not efficacy[16 23 24]. Patient ages ranged between 13 and 90, with the mean age in the 30s or low 40s for all studies except those of Rizoli et al[27] and Seekamp et al.[16 17] in which the mean age was nearer 50 years. Similarly, the ISS ranged from 0 to 75, with the mean ISS in the 20s or low 30s for most studies. The studies by Nakos et al[26] and Waydhas et al28 averaged more severely injured patients.

Interventions were intended to attenuate the early overwhelming inflammatory response and diminish the immune paralysis. Since many trauma patients are plagued by infections, researchers aimed to augment the host's inflammatory response by stimulating macrophages with glucan[29 30], activating monocytes with dextran[14], upregulating HLA-DR expression with IFN-γ[18 22 26 31], and providing immunoglobulins[20 32]. Since hyper-inflammation causes injury, researchers aimed to taper the host's inflammatory response by infusing leuko-reduced blood[21], prostaglandin E1[15], antioxidants[25], and antithrombin III[28], which, by blocking thrombin, decreases IL-8 production and sequestration of neutrophils. By blocking a neutrophil receptor that binds to endothelium (CD18)[23] or an adhesion molecule (L-selectin)[16] with an antibody, researchers hoped to prevent neutrophils from extravasating

and causing reperfusion injury after hemorrhagic shock. Perflubron is attributed with anti-inflammatory properties since macrophages exposed to it demonstrate significantly less hydrogen peroxide superoxide anion and production[24]. Most of the control groups were given a placebo[15-18 20 22 23 25-32] and four received only standard treatment[14 19 21 24]. The interventions were administered intravenously[14-17 19-21 23 25 27-30 32], subcutaneously[18 22 31], or via inhalation[24 26]. Interventions were initiated as soon as possible after injury by ambulance personnel[19] or as late as 145 hours after hospital admission[30]. The duration of the intervention differed from a single dose to 28 days. The length of follow-up ranged from 10 to 90 days.

**Outcomes**

Among the outcome variables, most of the significant differences between the test and control groups were in inflammatory parameters, suggesting attenuation of SIRS, CARS, or both (Table 3). Only monoclonal antibodies against CD18[23] exacerbated SIRS and hypertonic saline with Dextran had a mixed effect on CARS[27]. Significant changes in infection and mortality rates were only measured in the studies testing IFN-γ[18 26], immunoglobulin[20 32], and glucan[29 30]. These were not the most recently published or largest studies, nor the studies with the longest follow-up, and did not differ from the other studies regarding the ages or ISS of the patients. Besides the test intervention, only the duration of the test intervention distinguished the studies that reported a significant efficacy in preventing adverse clinical outcome from those that did not; none of the single dose interventions proved efficacious[16 17 19 23 27].

## Discussion

While posttraumatic immune deregulation is apparent, the solution is not. In this systematic review we show that administration of immunomodulative interventions often leads to beneficial changes in the inflammatory response. Only administration of immunoglobulin, IFN-γ, or glucan was efficacious in reducing infection and/or mortality rate.

Immunoglobulin and IFN-γ both increase the antigen presenting capacity of the host. After injury circulating IgG levels are decreased[32]. Administration of exogenous immunoglobulins results in normalization of IgG concentrations and thus increases IgG mediated antigen presentation. IgG is a plasma product obtained from healthy donors. IgG was given in the mentioned studies at a dose of 0.25 -1.0 g/kg intravenously and reduced infections in trauma patients, even more clearly in combination with antibiotics[20 32]. IFN-γ increases antigen presentation to lymphocytes via induction of HLA-dr expression on monocytes. Recombinant IFN-γ was given daily at a dose of 100 µg subcutaneously[18 22 26 31], but only had an positive effect on mortality[18] and infection[26] in 2 out 4 studies. Glucan, a component of the inner cell wall of Saccharomycces cerevisiae, reduces the immune paralysis via a different manner. It decreases PGE2 release by macrophages but also stimulate bone marrow proliferation[29]. This bone marrow proliferation may be in favor in the late immune paralysis. Glucan was given at a dose of 50mg/m2 daily[29] or 30mg every 12 hrs[30], resulting in a reduced infection and mortality rate. All these seemingly effective interventions started on day of admission and were continued until at least 3 to 7 days after trauma.

**Table 2** Study characteristics

| | | Patients | | Intervention | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Study** | **N** | **Age (range)** | **ISS (range, ±SD)** | **Test** | **Control** | **Delivery** | **Initiation** | **Duration** | **Length of follow-up** |
| Browder, 1990[29] | 38 | 34 (18-65) | 24 (8-41) | Glucan | placebo (saline) | i.v. | after exploratory laparotomy or thoracotomy | 7 days | 10 days |
| Bulger, 2008[19] | 209 | 38 (13-90) | 28 (0-75) | Hypertonic saline + Dextran | Lactated Ringer solution | i.v. | initial reperfusion fluid | single dose | 28 days |
| Croce, 1998[24] | 16 | 32 (15-75) | 29 | Partial liquid ventilation with perflubron | Conventional mechanical ventilation | inhaled | day of admission | 4 days | hospital discharge |
| de Felippe, 1993[30] | 41 | 35 (16-76) | n.r.* | Glucan | placebo | i.v. | 12-145 hr (mean 46.2 hr) after admission | 3-17 days | hospital discharge |
| Douzinas, 2000[32] | 39 | 32 | 24 (16-50) | Immunoglobulin | placebo (albumin) | i.v. | 12 hr after admission | 6 days | hospital discharge |
| Dries, 1998[18] | 73 | 31 | 34 (21-59) | rhIFN-γ | placebo | s.c. | within 30 hr of injury | 21 days or hospital discharge | 60 days |
| Glinz, 1985[20] | 150 | 39 (15-78) | 30 (9-66) | Immunoglobulin | placebo (albumin) | i.v. | within 24 hr of starting mechanical ventilation | 12 days | 42 days |
| Livingston, 1994[31] | 98 | 30 (>16) | 30 (±8) | rhIFN-γ | placebo | s.c. | day of admission | 10 days | 30 days |
| Marzi, 1993[25] | 24 | 32 (18-57) | 34 (27-57) | superoxide dismutase | placebo (sucrose) | i.v. | within 48 hr of injury | 5 days | 14 days |
| Miller, 1985[14] | 28 | n.r. | >10 | Dextran + standard treatment | standard treatment | i.v. | within 12 hr of admission | 5 days | 4 weeks |
| Nakos, 2002[26] | 21 | 49 (35-67) | 41 (24-62) | rhIFN-γ | placebo | inhaled | 2nd or 3rd day after admission | 7 days | hospital discharge |
| Nathens, 2006[21] | 268 | 42 (>17) | 24 (±11) | Leukoreduced (<5x10^6 WBC) RBC transfusion | Nonleukoreduced (5x10^9 WBC) RBC transfusion | i.v. | within 24 hr of injury | 28 days | 28 days |
| Polk, 1992[22] | 193 | 32 (>15) | 33 (>20) | rhIFN-γ | placebo | s.c. | day of admission | 10 days | 90 days |

*follow up table 2*

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Rhee, 2000[23] | 116 | 40 (>18) | 20 (±11) | rhMAbCD18 | placebo | i.v. | day of admission | single dose | hospital discharge |
| Rizoli, 2006[27] | 24 | 48 (>16) | 26 (±11) | Hypertonic saline + Dextran | placebo (saline) | i.v. | upon arrival in de emergency department | single dose | hospital discharge |
| Seekamp, 2004[16] | 84 | 36 (17-72) | 32 (17-59) | Anti-L-Selectin (Aselizumab) | placebo | i.v. | within 6 hr of injury | single dose | 42 days |
| Vassar, 1991[15] | 48 | 36 | 31 (±3) | Prostaglandin E1 | placebo | i.v. | 24-48 hr after hospital admission | 7 days | hospital discharge |
| Waydhas, 1998[28] | 40 | 33 (18-70) | 41 (±13) | Antithrombin III | placebo (albumin) | i.v. | within 6 hr of injury | 4 days | hospital discharge |

IFN, interferon; ISS, injury severity score; i.v., intravenous; n, number; n.r., not reported; RBC, red blood cell; s.c., subcutaneous; WBC, white blood cell; * Trauma score 10, denoted as "severe multiple trauma"

**Table 3** Study results

| Test intervention | Study | Infection Test group (relative to control) | Effect | MOF, Mortality Test group (relative to control) | Effect | Inflammation Test group (relative to control) | Effect |
|---|---|---|---|---|---|---|---|
| Reduce immune paralysis | | | | | | | |
| Plasma expander | Miller, 1985[14] | | | Mortality 0 vs 0 n.s. | No effect | immune reactive capacity n.s. | No effect |
| | Rizoli, 2006[27] | pneumonia 0.5% vs 0.5% n.s. | No effect | Mortality 0 vs 14.3% n.s., MOF score 1.68 vs 1.9 n.s. | No effect | WBC n.s.; decreased toward normal: CD11b, CD62L, CD16, and TNFα; increased toward normal: CD14, IL-1RA, and IL-10 all p<0.05 | SIRS↓ and CARS↓↑ |
| | Bulger, 2008[19] | nosocomial infections 18.2% vs 15.2% n.s. | No effect | ARDS-free survival, MOF, mortality 29.1% vs 22.2% n.s. | No effect | | |
| Immuno-globulin | Glinz, 1985[20] | any 47% vs 68% $p$=0.02, pneumonia 37% vs 58% $p$=0.01, sepsis 18% vs 26% n.s. | ↓ | Mortality from infection* 12% vs 11% n.s. | No effect | acute phase proteins n.s. | No effect |
| | Douzinas, 2000[32] | pneumonia 10% vs 61% $p$=0.003 | ↓ | Mortality rom infection* 0 vs 0 | No effect | C3 and CH50 n.s., C4 increased $p$=0.04, increased serum bactericidal activity p<0.000001 | CARS↓ |
| IFN-γ | Polk, 1992[22] | major 39% vs 35%, minor 20% vs 28% , pneumonia 27% vs 24% n.s. | No effect | Mortality 9.2% vs 12.5% n.s. | No effect | HLA-DR increased $p$=0.0001 | CARS↓ |
| | Livingston, 1994[31] | major infection 48% vs 31% n.s. | No effect | | | WBC decreased p<0.05, HLA-DR increased p<0.05 | SIRS↓ and CARS↓ |
| | Dries, 1998[18] | major infection 49% vs 58% n.s. | No effect | Mortality 13% vs 42% $p$=0.017 | ↓ | TNFα,IL-1β, IL-2, IL-4, IL-6 n.s. | No effect |
| | Nakos, 2002[26] | ventilator-associated pneumonia 9% vs 50% p<0.05 | ↓ | Mortality 27% vs 40% n.s. | No effect | HLA-DR expression, IL-1β, phospholipase A2 all increased p<0.05; total cells in BAL and IL-10 decreased p<0.01 | SIRS↓ and CARS↓ |
| Glucan | Browder, 1990[29] | sepsis 9.5% vs 49% p<0.05 | ↓ | Mortality from sepsis* 0 vs 18% n.s. | No effect | IL-1β decreased p<0.05, TNFα n.s. | SIRS↓ |
| | de Felippe, 1993[30] | pneumonia 9.5% vs 55% p<0.01, sepsis 9.9% vs 35% p<0.05, either or both 14.3% vs 65% p<0.001 | ↓ | Mortality: general 23.5% vs 42.1%, related to infection 4.8% vs 30% p<0.05 | ↓ | | |

*follow up table 3*

**Reduce hyper inflammation**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Superoxide dismutase | Marzi, 1993[25] | | | Mortality 17% vs 8.3% n.s.<br>MOF score n.s. | No effect | WBC count, CRP, PMN-elastase and IL-6 n.s.; phospholipase A2 and conjugated dienes decreased p<0.05 | SIRS↓ |
| Antithrombin III | Waydhas, 1998[28] | | | Mortality 15% vs 5%,<br>MOF 20% vs 30% n.s | No effect | soluble TNF receptor II, neutrophil elastase, IL-RA, IL-6, and IL-8 n.s. | No effect |
| Anti-CD18 | Rhee, 2000[23] | major and minor 38% vs 40% n.s. | No effect | Mortality 5.8 % vs 6.7%,<br>MOF score n.s. | No effect | WBC increased p-value not reported | SIRS↓ |
| Anti-L-Selectin | Seekamp, 2004[16] | 67% vs 55% n.s. | No effect | MOF n.s., mortality<br>11% vs 25% n.s. | No effect | WBC, IL-6, IL-10, neutrophil elastase, C3a, procalcitonin n.s. | No effect |
| Leukoreduced blood | Nathens, 2006[21] | 30% vs 36% n.s. | No effect | Mortality 19% vs 15% n.s.<br>MOF score 6.6 vs 5.9 n.s. | No effect | | |
| Perflubron | Croce, 1998[24] | pneumonia 50% vs 3 75% n.s. | No effect | Mortality 8.3% vs 25% n.s. | No effect | WBC, neutrophils, IL-6, and IL-10 all decreased p<0.01; capillary leak (BAL protein), TNFα , IL-1β, and IL-8 n.s. | SIRS↓ |
| Prostaglandin E1 | Vassar, 1991[15] | sepsis 28% vs 30%, major wound inf. 65% vs 72%, n.s. | No effect | Mortality 26% vs 28%,<br>ARDS 13% vs 32%,<br>MOF 30% vs 32% n.s. | No effect | PMN superoxide production increased toward normal p<0.02 | CARS↓ |

n.s., not significant; * excluding deaths from cardiac arrhythmias secondary to a pulmonary embolus and myocardial infarction, intracranial pressure, and tracheostomy

As every systematic review this study has its restrictions. A clear limitation of the trials is their relatively small sample size and the heterogeneity of interventions and study populations. Furthermore, we can not completely rule out publication bias. Yet, none of the studies report financial support by a pharmaceutical company and quite some studies show a negative result. Also searching the clinical trial register database[33], no other studies with immunoglobulin, IFN-γ, or glucan in trauma patients were found.

Challenges unique to the trauma population impede designing large RCTs. Polk et al.[22] note that patient homogeneity is difficult to achieve in multicenter trials because different centers tend to receive different patients. In addition, in the rush of the emergency care of severely injured patients, informed consent must wait until a family member is contacted[23] while the initiation of treatment cannot wait. Bulger et al.[19], Nathens et al.[21], and Rizoli et al.[27] solved this problem by gaining permission from their ethics committees to delay informed consent until after the initial treatment, but this approach is not always accepted. Furthermore, assessing patient eligibility for inclusion in the trial is time consuming. Delay to randomize patients can be avoided by using simple inclusion criteria. Nathens et al.[21] used only one criterion, the request of the physician for red blood cells for an expected transfusion, but were then faced with the possible dilution of treatment effect when they performed an intention-to-treat analysis as many randomized patients never received any blood products.

Based on the selected studies, general conclusions regarding the efficacy of potentially immunomodulative interventions cannot be drawn. As explained in the results section, the intended effects of the interventions on the inflammatory response differed. Furthermore, data from pilot studies[14 24-27] and phase II trials[16 23 24] should be used to steer future investigations rather than to draw definitive conclusions. Interventions that did not have a significant effect on clinical outcome may need to be administered earlier[25], continued longer[16 22 25 28], or need sequential specific timing to be effective[22]. Seekamp et al.[16] and Rhee et al.[23] explicitly chose for a single dose of an anti-inflammatory cytokine because they wanted to taper the initial hyper-inflammatory response without compounding the later immune paralysis. Timing is essential in accurate modulation of the immune response after trauma. Lack of a positive effect can be the result of wrong timing rather that to the drug itself. Consequently differences in timing between interventional drugs studied in this systematic review may contribute to disparity in outcome.

Besides changing timing, some authors recommended the use of larger doses[19 28]. Waydhas et al.[28] suggest that concomitant heparinization interfered with the immunomodulative effect of antithrombin III. The use of these drugs is inevitable in severely injured patients. Where theoretically promising approaches did not produce the results hoped for, sufficiently powered phase IV trails are needed.

Another impediment for drawing general conclusions is the fact that study populations differed greatly across the studies. For example, while Croce et al.[24] excluded patients with injuries thought to be lethal within 30 days of injury, others only excluded patients when the injuries were thought to be lethal within only one[28], two[16 20 21 23], or five[30] days. Similarly, while de Felippe et al.[30] only included patients with concomitant head injury, other researchers excluded patients with major head injury[16 19 23 28] or any head injury[14 29]. Mortality by severe head injury or massive bleeding may mask the effect of the interventional drug in an intention-to-treat trial, especially in trials with a small sample size.

Some researchers chose to exclude patients receiving steroids[24 25 31 32], as the efficacy of immunomodulative interventions is likely affected by simultaneous administration of steroids and/or antibiotics during care-as-usual[32]. However, this approach leads to a selection bias including patients that are more likely to have a favorable outcome.

Patient selection is imperative. Where no significant benefit was found for the test group as a whole, study authors postulated more specific inclusion criteria were necessary for future studies. For example, older patients[19 24 26], those with more severe injuries[19 23 26], patients needing >10 units of packed RBCs[24], and those who had a longer time from injury to enrollment in the study[24] were more susceptible to organ dysfunction and thus likely to benefit more from immunomodulative intervention. Selection of patients at risk may favor the outcome where no significant difference was found in a broader group of patients. Researchers suggest future study participants be select based not only the injury severity, but also on sepsis[28] or inflammatory parameters[16] as Nakos et al.[26] did when they only randomized patients after ascertaining immune paralysis by measuring the HLA-DR in bronchoalveolar lavage.

Interpretations of the efficacy of immune modulating therapies in trauma patients remain difficult. More studies with similar study populations will aid comparison of the effect of different interventions in trauma patients.

**Conclusions**

An array of potentially immunomodulative interventions have been tested in a heterogeneous group of trauma patients in level 1b and 2b RCTs. Reported changes in inflammatory parameters could indicate an attenuation of SIRS and/or CARS, however, they were not consistently accompanied by significant changes in infection and mortality rates. Administation of immunoglobulin, IFN-γ, and glucan was efficacious while none of the single dose interventions were. Further trials powered to measure efficacy may reveal which immunomodulative interventions should be routinely implemented to save lives of trauma patients.

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# PART II
# IMMUNOMODULATION

# 7

## C1-esterase inhibitor attenuates the inflammatory response during human endotoxemia

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## Abstract

**Objective**
Besides its role in regulation of the complement and contact system, C1-esterase inhibitor (C1INH) has other immunomodulating effects which could prove beneficial in patients suffering from acute inflammation such as during sepsis or after trauma. We examined the immunomodulating properties of C1INH during human experimental endotoxemia, in which the innate immune system is activated in the absence of activation of the classical complement pathway.

**Design**
Double-blind placebo-controlled study.

**Setting**
Research Intensive Care Unit of the Radboud University Nijmegen Medical Centre.

**Subjects**
Twenty healthy volunteers.

**Interventions**
Intravenous injection of 2 ng/kg of *Escherichia coli* lipopolysaccharide. Thirty minutes thereafter (to prevent binding of lipopolysaccharide), C1INH concentrate (100 U/kg, n=10) or placebo (n=10) was infused.

**Measurements and Main Results**
Pro- and anti-inflammatory mediators, markers of endothelial and complement activation, hemodynamics, body temperature and symptoms were measured. C1-esterase inhibitor reduced the release of pro-inflammatory cytokines as well as CRP (peak levels of: Interleukin-6 1521±209 vs. 932±174 ($p$=0.04), Tumor Necrosis Factor-α 1213±187 vs. 827±167 ($p$=0.10), Monocyte Chemotactic Protein-1 6161±1302 vs. 3373±228 pg/ml ($p$=0.03), Interleukin-1β 34±5 vs. 23±2 ($p$<0.01), C-reactive protein 39±4 vs. 29±2 mg/l ($p$=0.02)). In contrast, release of the anti-inflammatory cytokine Interleukin-10 was increased by C1INH (peak level 73±11 vs. 121±18 pg/ml, $p$=0.03). The increase in Interleukin-1receptor antagonist tended to be smaller in the C1INH group, but this effect did not reach statistical significance ($p$=0.07).
Markers for endothelial activation were increased after LPS infusion but no significant differences between groups were observed. The lipopolysaccharide-induced changes in heart rate ($p$<0.0001 over time), blood pressure ($p$<0.0001 over time), body temperature ($p$<0.0001 over time) and symptoms ($p$<0.0001 over time) were not influenced by C1INH. Complement-fragment C4 was not increased after lipopolysaccharide challenge.

**Conclusions**
This study is the first to demonstrate that C1INH exerts anti-inflammatory effects in the absence of classical complement activation in humans.

# Introduction

Acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS) are the leading causes of death in medical and surgical ICU patients[1-4]. The parenchymal damage and subsequent organ dysfunction are caused by an over-activated inflammatory response[5]. The systemic release of several humoral inflammatory mediators, such as tumour necrosis factor (TNF)-α, Interleukin (IL)-1β and IL-6 activate the vascular endothelium and modulate activation and tissue infiltration of circulating leukocytes[5-7].
A promising intervention to modulate the innate inflammatory response is treatment with a high concentration of C1-esterase inhibitor (C1INH). C1INH is an acute phase protein produced by the liver and important in regulating the activation of the complement and contact system, which play a role in opsonisation and the regulation of coagulation[8-10]. Currently, C1INH administration is applied in patients suffering from a deficiency of the protein causing hereditary angioedema[8].
Interestingly, several animal studies have demonstrated that supraphysiological levels of C1INH during models of acute inflammation (sepsis and (thermal) trauma), improve survival, preserve endothelial function and prevent the occurrence of capillary leak[11-14]. Furthermore, C1INH can inhibit adhesion of leukocytes to the endothelium and reduce tissue infiltration[11 13 15-19]. In septic patients, administration of C1INH reduced leukocyte activation and the release of cytotoxic mediators by degranulation[20]. The mechanisms by which C1INH exerts its actions are only partly understood but appear to be (partly) independent of its effects on the complement and contact system, since its beneficial effects in *in vitro* and animal studies remain intact after cleavage of the reactive centre[12 21].
Up to now, only a few reports exist of the administration of C1INH to patients undergoing major surgery or suffering from severe sepsis or septic shock. Although these studies were only performed in very small patient groups and not always in a placebo-controlled fashion, their results were encouraging demonstrating a significant reduction of renal impairment and small case series suggest that the administration of C1INH is associated with less need for vasopressor therapy and a reduced hospital stay[8 22-25].
However, to elucidate the mechanism by which C1INH exerts its anti-inflammatory effects, patient studies remain difficult to interpret due to heterogenenity of the underlying diseases. In contrast, human experimental endotoxemia provides an *in vivo* model with reproducible systemic inflammation[26]. During human endotoxemia, the release of several humoral mediators, activation of leukocytes and vascular endothelium occurs within a few hours after infusion. Interestingly, this cascade is independent of complement or contact system activation[27-29]. We hypothesized that C1INH can modulate the inflammatory response during human endotoxemia in the absence of contact or complement system activation.

# Materials and methods

**Subjects**

This study was registered at ClinicalTrials.gov as # NCT00785018. After approval from the medical ethical committee, 20 healthy male subjects gave written informed consent to participate in the experiments in accordance with the Declaration of Helsinki. Subjects

using any drugs were excluded. Screening of the subjects within 14 days before the test revealed no abnormalities in medical history and physical examination. Laboratory tests (including serology on HIV and hepatitis B) and ECG were normal. Ten hours before the experiment, subjects refrained from the intake of caffeine, alcohol, and food.

**Study design**

After admission to the research intensive care unit of the Radboud University Nijmegen Medical Centre, purified lipopolysaccharide (LPS) (U.S. Standard Reference Endotoxin *Escherichia coli* O:113) obtained from Pharmaceutical Development Section NIH (Bethesda, MD, USA), was administered at a dose of 2 ng/kg bodyweight at t=0h (hour). Thereafter, subjects were randomized by an independent research nurse to receive C1INH-concentrate (N=10, Cetor®, Sanquin, Amsterdam, The Netherlands, 100 U/kg bodyweight, infused in 30 minutes) or an equivalent volume of placebo (N=10, 0.9% saline, Baxter, Utrecht, The Netherlands) using the sealed envelope method. The dose of 100 U/kg C1INH was chosen based on a study performed in humans with acute myocardial infarction[30]. C1INH solution or placebo was prepared by the independent research nurse and given to the investigator in identical containers ensuring the double-blind fashion of the study. Intravenous infusion of C1INH or placebo was started by the investigators at 30 minutes after LPS to prevent binding to LPS[13 28 31 32].

**Hemodynamic and clinical response**

For continuous monitoring of blood pressure and for blood sampling, the radial artery was cannulated with a 20 gauge arterial catheter. Heart rate monitoring was performed using a 5-lead ECG. A cannula was placed in an antecubital vein to permit infusion of prehydration fluid, endotoxin, C1INH or placebo and the continuous infusion of 150 ml/h 2.5% glucose/0.45% saline. Body temperature (FirstTemp Genius, Tyco Healthcare, Hampshire, UK) and symptoms were scored every 30 minutes. Subjective symptoms were scored using grades varying from 0 (symptoms: absent) to 5 (symptoms: worst ever experienced) in order to define the severity of nausea, headache, shivering, muscle and/or back pain. Thereafter, scores were added leading to an arbitrary 'total symptom score' with a maximum value of 25 points.

**Assays**

Measurements of C1INH antigen and activity complement levels C4, various cytokines, C-reactive protein (CRP), and soluble adhesion molecules were performed before LPS and serially thereafter.

**Objectives and hypothesis**

The primary objective of the present study was to determine whether C1INH can modulate cytokine release during human endotoxemia. Secondary objectives include the effects of C1INH on CRP, hemodynamic and clinical response and the release of soluble adhesion molecules after LPS challenge.

**Data analysis and statistics**

Values are expressed as mean±SEM unless described otherwise. Kolmogorov-Smirnov

tests indicated a normal distribution of almost all the data (a few exceptions of less relevant time points). Hence, a two-way repeated measures ANOVA was used to test variation over time, the variation between interventions, and the interaction between time and intervention (SPSS 16.0 software, SPSS, Chicago, IL, USA). Changes over time alone were analyzed by One-way ANOVA (Graphpad Prism 5, Graphpad Software, La Jolla, CA, USA). To compare differences between groups Student's t-tests were used, as appropriate (SPSS 16.0 software, SPSS, Chicago, IL, USA). As the symptom score is a discontinuous variable, we used non-parametric Friedman ANOVA for changes over time and Mann-Whitney U tests for differences between groups (SPSS 16.0 software, SPSS, Chicago, IL, USA). These data are expressed as median and ranges.

A $p$-value <0.05 was considered to indicate significance. Given the explorative 'proof of concept' nature of this study, no formal sample size calculation was performed. Furthermore, no subgroup analyses were made.

# Results

### Baseline characteristics

After screening 23 healthy volunteers, 20 subjects were enrolled in the study protocol and randomized to receive C1INH or placebo (Figure 1). Besides a difference in body weight (Students t-test $p$=0.03), there were no significant differences in baseline characteristics between both groups. Demographic data are shown in Table 1.

**Table 1** Demographic characteristics

| | Placebo | C1INH | Total group |
|---|---|---|---|
| Age, yrs | 21.6±2.6 | 22.6±3.6 | 22.1±3.1 |
| Height, cm | 187±7 | 181±7 | 184±8 |
| Weight, kg | 80±10 | 71±6 | 75±9 |
| BMI, kg/m$^2$ | 23±2 | 22±1 | 22±2 |
| HR, bpm | 66±4 | 70±12 | 68±9 |
| MAP, mmHg | 103±10 | 98±8 | 101±9 |

Data are presented as mean ± SD. BMI, body mass index; HR, heart rate; MAP, mean arterial blood pressure.

### Safety

No serious adverse events occurred during the experiments. The symptoms observed during the experiments could be related to the administration of LPS and are discussed below.

### Clinical and hematological response

As summarized in Table 2, endotoxin infusion resulted in the expected changes in clinical and hemodynamic parameters in both groups. All endotoxin-induced changes were statistically significant (over time analysis using one-way ANOVA).

Endotoxin-induced symptoms typically started with headache approximately 1h after LPS administration. Symptoms, as scored by the subjects, peaked at t=1.5h at a median value of 5.5 (range 3-10) (out of a maximum of 25), for subjects receiving C1INH versus 6.5 (range

1-11) in subjects receiving placebo (over time $p<0.001$ using Friedman, no significant difference between groups using Mann Whitney U test ($p=0.579$)). Blood pressure dropped by 19±3% in the placebo group compared to 18±2% in the C1INH group (no significant difference between groups ($p=0.392$)). A comparable compensatory rise in heart frequency was observed in both groups. Also, the maximum increase in temperature of 1.7±0.2 °C (C1INH) and 1.9±0.2 °C (placebo) was similar in both groups (Table 2).

**C1INH antigen, activity and levels of complement factor 4**

After LPS infusion, levels of C1INH antigen and activity demonstrated a modest increase in the placebo group (Figure 2, over time: $p<0.01$). After administration of C1INH at 30 minutes after endotoxin infusion, levels of C1INH antigen increased from 0.20 ±0.01 g/l to 0.54 ± 0.03 g/l at t= 1 hr after LPS administration (over time: $p<0.001$, between both groups: $p<0.001$) and remained high throughout the experiment. Also, levels of C1INH activity increased from 0.94 ± 0.03 U/ml to 2.42 ± 0.13 U/ml (over time: $p<0.001$, between groups: $p<0.001$, Figure 2). The levels of C4 remained low in both groups (not significantly different over time ($p=0.585$) in both groups, or between groups ($p=0.735$)).

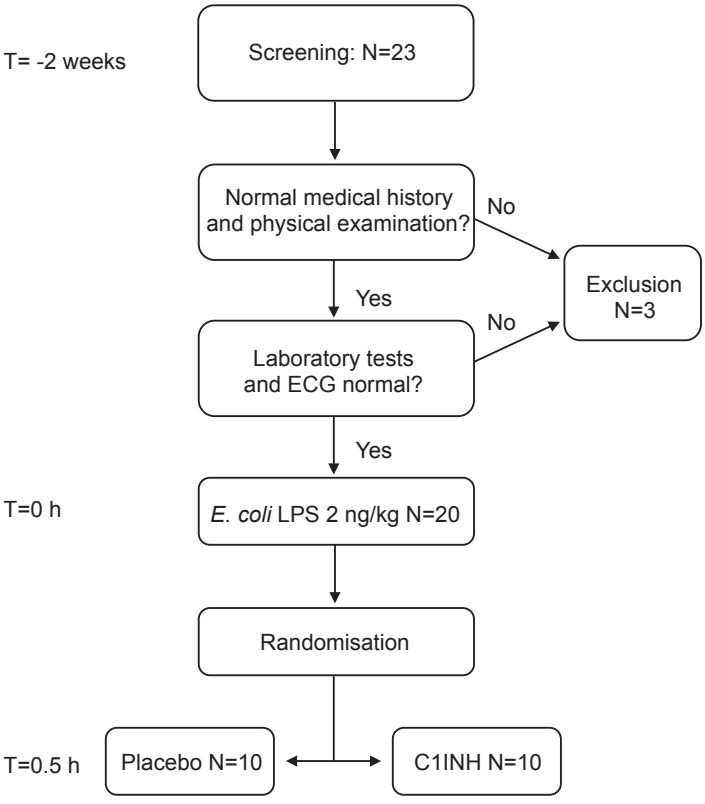


**Figure 1**
Flow diagram of subjects included in the study. After screening of 23 subjects, 20 subjects were included in the experiments. All received 2 ng/kg Escherichia coli lipopolysaccharide. Thereafter subjects were randomized to receive C1-esterase inhibitor (100 U/kg) or placebo.

**Table 2** Hemodynamic parameters, symptoms, hematological and biochemical laboratory data during human endotoxemia in the absence and presence of C1INH.

| | | T=0 | T=1 | T=2 | T=3 | T=4 | T=8 | T=24 | *P-value* |
|---|---|---|---|---|---|---|---|---|---|
| MAP, mmHg | Placebo | 103±4 | 104±2 | 93±2 | 85±4 | 86±3 | 83±6 | ND | 0.392 |
| | C1INH | 98±3 | 97±3 | 86±3 | 83±3 | 83±3 | 83±3 | ND | |
| HR, bpm | Placebo | 66±2 | 73±3 | 81±3 | 90±3 | 91±2 | 81±4 | ND | 0.787 |
| | C1INH | 70±5 | 74±5 | 87±4 | 91±3 | 93±3 | 77±4 | ND | |
| Total symptoms | Placebo | 0±1[a] | 0±2[a] | 3.5±6[a] | 2.5±9[a] | 1±8[a] | 0±1[a] | ND | 0.579[b] |
| | C1INH | 0±3[a] | 0±03[a] | 3±5[a] | 2±6[a] | 1±4[a] | 0±3[a] | ND | |
| ΔTemp., °C | Placebo | NA | 0.6±0.1 | 1.3±0.2 | 1.9±0.2 | 1.8±0.3 | 1.0±0.3 | ND | 0.826 |
| | C1INH | NA | 0.5±0.2 | 1.2±0.2 | 1.7±0.2 | 1.3±0.2 | 0.8±0.2 | ND | |
| Hb, mmol/l | Placebo | 8.4±0.1 | 8.5±0.2 | ND | 8.3±0.1 | ND | 8.3±0.1 | 8.4±0.2 | 0.519 |
| | C1INH | 8.4±0.1 | 8.6±0.1 | ND | 8.5±01 | ND | 8.4±0.1 | 8.5±0.1 | |
| Leuko., $x10^9$/l | Placebo | 5.3±0.6 | 3.1±0.5 | ND | 6.3±0.7 | ND | 11.4±0.7 | 5.5±0.5 | 0.801 |
| | C1INH | 4.6±0.3 | 2.2±0.4 | ND | 7.0±0.5 | ND | 11.0±0.7 | 5.4±0.4 | |
| Thromb., $x10^9$/l | Placebo | 187±10 | 181±9 | ND | 189±13 | ND | 190±10 | 196±12 | 0.426 |
| | C1INH | 167±10 | 159±11 | ND | 179±8 | ND | 181±12 | 184±12 | |
| CRP, mg/l | Placebo | <5 | ND | ND | ND | ND | 9±1 | 39±4 | **0.026** |
| | C1INH | < 5 | ND | ND | ND | ND | 7±1 | 29±2 | |

Time (T) expressed in hours after LPS administration. MAP: mean arterial blood pressure, HR: heart rate, Hb: haemoglobin, Leuko: leukocytes, Thromb.: thrombocytes, CRP: C-reactive protein. ND: not determined, NA: not applicable. Data are expressed as mean±SEM. p values are comparisons between groups over time and were determined by Two-way repeated measures ANOVA. [a] Total symptoms are expressed as median±range. [b] *p* value signifies difference between groups at t=1.5h determined by Mann-Whitney U test

### Inflammatory markers

After the administration of endotoxin, all measured cytokines showed a marked increase as illustrated in Figure 3 (over time: all $p<0.001$). The administration of C1INH attenuated the release of all pro-inflammatory cytokines. Compared to subjects receiving placebo, peak levels of IL-6 were reduced by 39%. TNF-α peak levels were abrogated by 32%. Concentrations of MCP-1 and IL-1β decreased by 45% and 32% compared to placebo respectively. Conversely, the release of the anti-inflammatory IL-10 was increased in subjects receiving C1INH by 66%. The increase in IL-1RA tended to be less in the C1INH group, but this effect did not reach statistical significance ($p=0.07$). CRP was significantly reduced in the C1INH group compared to placebo ($p=0.03$, Table 2).

### Markers for endothelial activation

Endotoxin administration is known to cause a release of soluble adhesion molecules, suggesting activation of endothelial cells[33 34]. As demonstrated in Figure 4, all measured markers for endothelial activation were significantly induced by LPS infusion except for VWF. VWF was not significantly induced after LPS infusion in subjects receiving placebo (over time $p=0.09$). No significant difference in concentrations of circulating endothelial markers was observed between both groups.

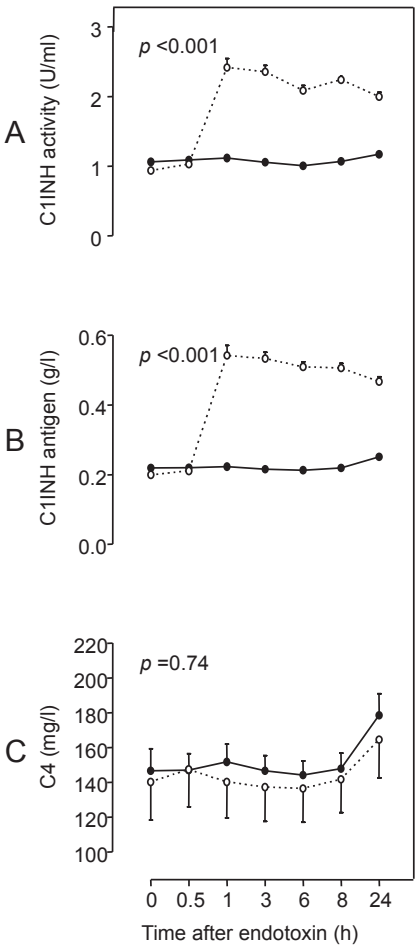


**Figure 2**
Levels of C1-esterase inhibitor activity (panel A), antigen (panel B) and Complement factor 4 (C4) (panel C) after administration of 2ng/kg *Escherichia coli* lipopolysaccharide at t=0h. At t=0.5h C1-esterase inhibitor at a dose of 100U/kg intravenously over 30 minutes(o) or placebo (•) was infused. Data are expressed as mean ± SEM. *p* values in the figure express differences between groups obtained by repeated measures ANOVA over the complete curve.

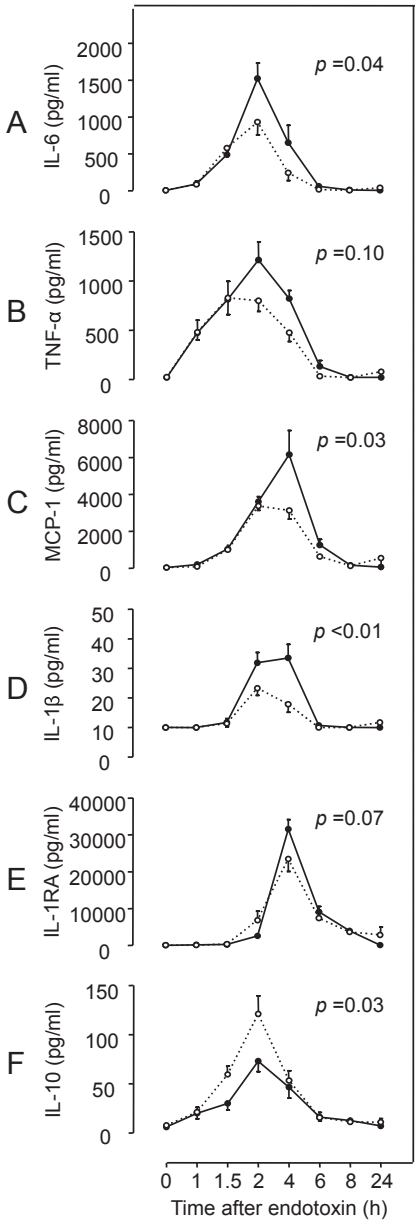


**Figure 3**
Cytokine concentration in the absence (•) and presence (o) of C1-esterase inhibitor after administration of 2ng/kg *Escherichia coli* lipopolysaccharide at t=0h. IL-6: Interleukin-6 (A), TNF-α: Tumor Necrosis Factor-α (B), MCP-1: Monocyte Chemotactic Protein-1 (C), IL-1β: Interleukin-1β (D), IL-1RA: Interleuking-1 receptor antagonist (E), IL-10: Interleukin 10 (F). Data are expressed as mean ± SEM. *p* values in the figure express differences between groups obtained by repeated measures ANOVA over the complete curve.

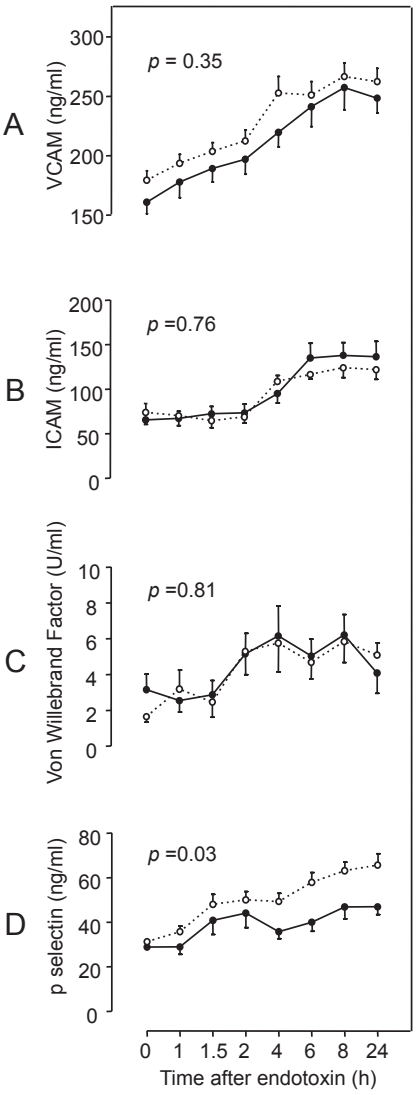


**Figure 4**
Concentrations of soluble adhesion molecules in the absence (•) and presence (o) of C1-esterase inhibitor after administration of 2ng/kg *Escherichia coli* lipopolysaccharide at t=0h. ICAM: Inter-Cellular Adhesion Molecule (A), VCAM: Vascular Cell Adhesion Molecule (B), P-selectin: Platelet selectin (C). Data are expressed as mean ± SEM. p values in the figure express differences between groups obtained by repeated measures ANOVA over the complete curve.

## Discussion

The present study is the first to demonstrate that administration of a high dose of C1INH can modulate a controlled inflammatory response in humans, as elicited by *in vivo* infusion of endotoxin. During experimental endotoxemia, the release of pro-inflammatory cytokines was attenuated by C1INH, whereas the release of the anti-inflammatory cytokine IL-10 was potentiated. This C1INH-mediated shift in the pattern of the inflammatory response occurred in the absence of activation of the complement system and could not be explained by binding to endotoxin.

After administration of C1INH, concentrations of antigen and activity were increased throughout the entire experiment. An increase of C1INH antigen and activity of 340 mg/L and 1.5 U/ml respectively is expected after an average gift of 7500 U. This higher plasma concentration is similar to that found in time during infectious diseases8. It seems that C1INH in acute phase protein concentration has a clear immunoregulating function.

The observed early increase of plasma IL-10 in the C1INH group compared to the placebo group is most remarkable. While the IL-10 levels were significantly increased by C1INH at 1.5h after LPS infusion, the levels of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and MCP-1 did not differ between groups until 2h after LPS infusion. Our findings are in agreement with data published by Storini et al[35], who showed a more pronounced increase of IL-10 mRNA expression after ischemia-reperfusion brain injury in mice treated with C1INH. They also found a concurrent smaller increase of mRNA levels of TNF-α and IL-6 in the C1INH treated group.

Interestingly, in our study the production of the anti-inflammatory cytokine IL-1RA was not potentiated, but even moderately blunted by C1INH in our study. This indicates that C1INH does not induce a general anti-inflammatory response. It is, therefore, tempting to speculate that C1INH acts as an anti-inflammatory mediator in humans by enhancing IL-10 production. To our knowledge, the direct effects of C1INH on IL-10 production have not been studied previously. IL-10 can block the release of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8 *in vitro*[36 37]. In addition, studies *in vivo* have demonstrated that IL-10 can protect mice from lethal endotoxemia[38 39]. Apart from increased levels of inflammatory mediators, IL-10 knockout mice were also characterized by an enhanced cellular inflammatory response in tissue[40]. These results imply a protective role for IL-10 in leukocyte driven inflammation, but the exact mechanism by which C1INH increases IL-10 production remains to be elucidated.

In our study IL-10 levels reached maximum concentrations concomitant with TNF-α at 2 hrs after LPS infusion. In previous experiments IL-10 peaked later than TNF-α, between 2 to 3h after LPS infusion. No measurements were performed at 3h after LPS in these experiments and therefore maximum concentrations of IL-10 on this time point could have been missed. This could explain why it seems that peak values of TNF-α and IL-10 seems to occur at the same time point.

Earlier studies have suggested that the LPS-induced inflammatory reaction can be diminished due to scavenging of LPS by C1INH. This hypothesis was based on *in vitro* studies demonstrating the ability of C1INH to bind LPS and to reduce LPS binding to endothelial cells[13 31]. However, it seems unlikely that the binding of LPS to C1INH explains the anti-inflammatory effects found after administration of C1INH concentrate in our study.

Plasma concentrations of LPS are known to decrease rapidly to undetectable levels within 15 to 20 minutes after administration[28 32]. In our study, C1INH infusion was started 30 minutes after LPS infusion, well after complete plasma clearance of LPS. Furthermore, treatment with C1INH caused enhanced levels of LPS-induced IL-10. Scavenging of LPS due to an interaction with C1INH would have resulted in decreased levels of all cytokines, including IL-10[41]. Therefore, it can be concluded that C1INH has direct immune modulating effects irrespective of a scavenging effect on LPS.

The observation that C1INH has a vast anti-inflammatory effect, even when it is administered after the induction of inflammation, is in agreement with several animal studies demonstrating beneficial effects of C1INH administration well after the onset of inflammation[12 42]. In a murine cecal ligation and puncture (CLP) model, C1INH even increased survival when administered up till 6 h after CLP, but not as much as when administered directly or at 3h after CLP[12].

The anti-inflammatory effects of C1INH seem, at least in part, independent of its function as a serpin, for in our study no signs of activation of the classical pathway of the complement system were found. We only measured serum concentrations of C4, and therefore can not rule out possible activation of the classical pathway via complement components bound to micro particles and a possible effect hereon by C1INH[43]. Although some studies with higher doses of LPS have demonstrated complement activation[44], no studies with a similar low-dose of LPS have shown activation of the final common pathway of the complement system so far [28 29].

The concept that C1INH has immunomodulatory effects independent of its role as a serpin, has been demonstrated by a seminal study performed by Liu et al.[12]. They have showed that serpin-inactive C1INH was at least as effective as active C1INH to prevent mortality in a murine CLP model for sepsis. This indicates that C1INH does not rely on its serpin-dependent properties to evoke its anti-inflammatory effects.

Some animal studies indicate that C1INH can inhibit margination of tissue leukocytes[8 14 18]. However, these studies did not evaluate simultaneous increases in leukocyte counts in the peripheral blood. In our study, we did not find any effect of C1INH on peripheral leukocyte counts (Table 2), which is in agreement with data from an endotoxemia model in rats where similar results were observed[16]. However, CRP was significantly lower at t=24h in the C1INH group, indicating that these subjects have apparently endured less severe inflammation.

As demonstrated in our study, the levels of soluble adhesion molecules ICAM, VCAM and P-selectin as well as VWF increased after induction of systemic inflammation by LPS. Increases in these soluble adhesion molecules are thought to reflect activation of endothelial cells[33].

However, no differences were found between subjects receiving placebo or C1INH. This may point at the inability of C1INH to antagonize activation of endothelial cells after LPS challenge. Apparently, C1INH had no effect on the sheddases which cause the quick release of these molecules from the endothelial surface. This is in contrast to animal studies in which an attenuation of inflammation-induced mRNA synthesis of ICAM-1, VCAM-1 and P-selectin by C1INH was demonstrated[15 39]. However, mRNA synthesis does not always reflect the levels of the circulating adhesion molecules[45].

In our study no difference in clinical response such as hemodynamic changes or symptoms

could be demonstrated. Obviously, human endotoxemia, being a very useful tool to study the innate immune response, can by no means mimic the cascade of events occurring in ICU patients suffering from inflammatory disorders such as septic shock or severe trauma. As an enhanced cytokine release during severe inflammation is associated with development of ARDS and MODS and eventually death[3 46 47], it is tempting to speculate that the ability of C1INH to shift of the inflammatory response to a more anti-inflammatory pattern, especially early in the disease process, could be beneficial in patients suffering from these acute inflammatory syndromes.
A limitation of our study is the fact that only healthy young male subjects were included. As this study should be viewed as a 'proof of concept study', to demonstrate the effects of C1INH on the innate immune response, we aimed to create a homogeneous study population. Therefore, the extrapolation of the effects of C1INH on LPS challenge to putative clinical effects should be taken with caution. Although a few studies have been reported applying C1INH in sepsis or trauma patients, future randomized controlled patient studies are of pivotal importance to determine the effects of this protein in the clinical setting.

**Conclusions**

In the present study, C1INH has potentiated the release of the anti-inflammatory cytokine IL-10 and simultaneously reduced the release of pro-inflammatory cytokines during human experimental endotoxemia. This shift in the pattern of the inflammatory response occurred in the absence of activation of the complement component C4 and could not be explained by binding of C1INH to LPS.

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**Source of drugs**

Sanquin Plasma Products supplied the C1-esterase inhibitor used to conduct the experiments.

**Disclosure of Conflicts of Interest**

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# PART II
# IMMUNOMODULATION

# 8

## Discrepancy between humoral and cellular response after C1-esterase inhibitor administration in a human endotoxemia model

*Manuscript in preparation*

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# Discrepancy between humoral and cellular response after C1-esterase inhibitor administration in a human endotoxemia model

Acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS) are life threatening complications that often complicate the clinical course in patients with infectious (e.g. the sepsis syndrome) or non-infectious (for instance after trauma or major surgery) disorders[1 2]. These severe complications are caused by tissue damage by abundant activation and sequestration of neutrophils, as result of an over-activation of the innate immune system[3].

Early inhibition of neutrophil tissue infiltration in these severe conditions may reduce late inflammatory complications. However, despite numerous attempts to attenuate the innate immune response this has not been accomplished. Current therapies designed to inhibit the immune response have failed to improve survival[4]. This is largely due to the fact that knowledge regarding inhibition of innate immune cell functions *in vivo* is scarce. In marked contrast to adaptive immunity which is very sensitive to immune modulators, such as steroids, the innate immune system cannot be sufficiently targeted by currently available anti-inflammatory drugs.

A promising candidate, with evidence for inhibition of the innate immune system[5], is the endogenously produced C1-esterase inhibitor (C1INH)[5 6]. This protein is an acute phase protein that is found in increased concentrations in inflammatory conditions. CINH is a major inactivator of the contact and complement system, but important additional anti-inflammatory properties have been ascribed[5].

Animal models have shown that C1INH improves outcome both given before and shortly after induction of severe inflammation induced by sepsis or trauma[7-11]. The mechanisms by which C1INH exerts its actions are only partly understood. There is evidence that at least a part of the effect of C1INH on neutrophils is independent from complement and contact system activation[10 12-14]. Hence, C1INH appears to have serpin independent immunomodulatory effects. These serpin independent effects were illustrated by the fact that the protective effects of C1INH remained intact after cleavage of the reactive centre[8]. In a sepsis model in mice, substitution of active as well as inactive C1INH (iC1INH) decreased leukocyte adhesion, increases bacterial clearance, and improved survival[8]. Since same and even better results are obtained with iC1INH substitution, it is suggested that C1INH might act directly against leukocytes. This hypothesis is supported by *in vitro* results showing increased bacterial clearance and diminished adhesion molecule expression in presence of C1INH as well as iC1INH[12 15]. These latter experiments were performed in the absence of complement and contact system.

In a human experimental endotoxemia model we have shown that C1INH significantly reduced the concentration of circulating of pro–inflammatory cytokines interleukin (IL)-6, tumor necrosis factor (TNF)-α and IL-1β16. C1INH treatment led furthermore to an increased level of the anti-inflammatory cytokine IL-10. The attenuation of cytokine release during experimental endotoxemia occurred in the absence of activation of the complement system.

As C1INH is supposed to have also direct inhibitory effects on neutrophil function, the outcome of C1INH substitution on the activation phenotype of circulating neutrophils was

studied as a supplementary part of this latter randomized placebo controlled trial. The endotoxemia experiment was performed as described by Dorresteijn et al[16]. In short, *Escherichia coli* lipopolysaccharide (LPS) was injected intravenously at a concentration of 2 ng/kg in 20 healthy male volunteers. Thirty minutes thereafter C1INH concentrate (Cetor®) (100 U/kg, n=10) or placebo (0.9% saline) (n=10) was infused in a double-blind randomized approach. Blood samples, collected in a vacutainer® with sodium heparin as anticoagulant, were obtained at baseline (before endotoxin injection) and at 3 and 24 hrs after endotoxin administration. Using flow cytometry, receptor surface expression on circulating leukocytes was measured after staining with directly labelled mouse-antihuman monoclonal antibodies directed against L-selectin (CD62L), αM (CD11b), CXCR1 (CD181), CXCR2 (CD182), C5aR (CD88), CD66b, FcγRII (CD32) and FcγRIII (CD16) and after staining with monoclonal phage antibody A27 which recognises the active configuration of FcγRII(CD32)[17 18] (see also *materials and methods*).

Three hours after LPS infusion a clear change in the receptor phenotype of circulating neutrophils was measured. The altered receptor expression was most prominently illustrated by the transient reduction of expression of the chemokine receptors (CXCR1 and CXCR2; see Figure 1) and Fcγ receptors (FcγRII and FcγRIII; see Figure 2). Also, the sensitivity for the innate stimulus N-formylmethionyl-leucyl-phenylalanine (fMLP) was markedly decreased at 3 hrs after endotoxin injection in all subjects (Figure 2).

These results were in line with an earlier study performed by our research group demonstrating a transient reduced expression of the above mentioned surface receptors and a diminished sensitivity of neutrophils for fMLP[19]. Yet, the most outstanding finding of these results was the lack of difference in activation phenotype of circulating neutrophils from subjects treated with C1INH compared to subjects treated with placebo (Figure 1 and 2, repeated measures ANOVA: all p-values >0.05). There was also no difference in sensitivity of neutrophils for fMLP between the two groups (Figure 2, repeated measures ANOVA: p=0.84).These findings were rather unexpected as a significant difference was found between the two treatments in concentration of circulating pro-inflammatory cytokines[16]. In addition, there were also no differences in phenotype of circulating monocytes and eosinophils between the two groups (data not shown). It appears that in this study C1INH did not attenuate the cellular immune response, or did at least not lead to difference in the phenotype of circulating leukocytes.

There is, however, circumstantial evidence that C1INH is a potent antagonist of the cellular innate immune response[5 10-12]. Therefore, we should be careful with the interpretation of our results based only on expression of receptors chosen in this study. The expression of these receptors are very sensitive for activation by cytokines *in vitro*[1820-26], but little is known regarding such signals in the peripheral blood from patients with acute of chronic inflammatory diseases.

Another possible explanation as to why systemic neutrophil response was not affected by C1INH in the experimental endotoxemia model might be the fact that C1INH was given too late after LPS injection to attenuate the neutrophil response provoked by LPS. C1INH was administered 30 minutes after endotoxin injection to mimic a clinical situation and to prevent possible binding of LPS to C1INH[15]. However, since the immune response during experimental endotoxemia is only short lived C1INH substitution 30 minutes after endotoxin injection might not have resulted in a measurable effect on the transient neutrophil response.

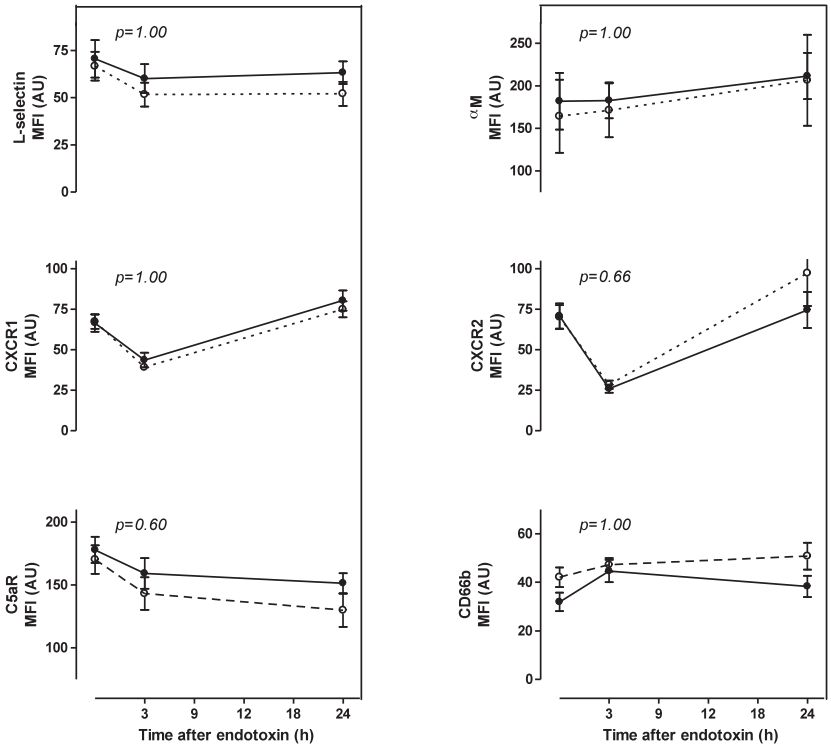


**Figure 1**
Expression of L-selectin, αM(CD11b), CXCR1, CXCR2, C5aR and CD66b on circulating neutrophils measured by flow cytometry, after administration of 2ng/kg Escherichia coli lipopolysaccharide at t=0hr , t=3hr and t=24hr. C1-esterase inhibitor at a dose of 100U/kg intravenously over 30 minutes (o) or placebo (•) was infused. Data are expressed as mean ± SEM. p values in the figure express differences between groups obtained by repeated measures ANOVA over the complete curve.

Interestingly, C1INH did attenuate the humoral response, but not the cellular response in this *in vivo* human inflammatory model. The humoral response did not correlate with the neutrophil response in this situation. Certainly, the cellular response is *in vivo* controlled by a wide variety of mediators and not only by those cytokines measured in this study. Nevertheless, a reduction in concentration of important pro-inflammtory cytokines like IL-6, TNF-α and IL-1β did not affect the neutrophil response at all. It implies that caution must be taken in drawing conclusions regarding changes in circulating cytokine levels as read out of activation of the innate immune system. Apparently, these changes do not necessarily reflect the cellular response in peripheral blood *in vivo*. *In vitro* studies have shown that the effect on leukocytes caused by stimulation of a combination of cytokines does not reflect the sum of the effects caused by individual cytokines[27 28]. Therefore, the interpretation of multiplex analysis of circulating cytokines are difficult to translate to activation of the cellular innate immune system The measurement of the cellular response, as integrator of all pro- and anti-inflammatory signals, might therefore be a more reliable read-out point for inflammation than circulating cytokines levels. At least this should be done in parallel.

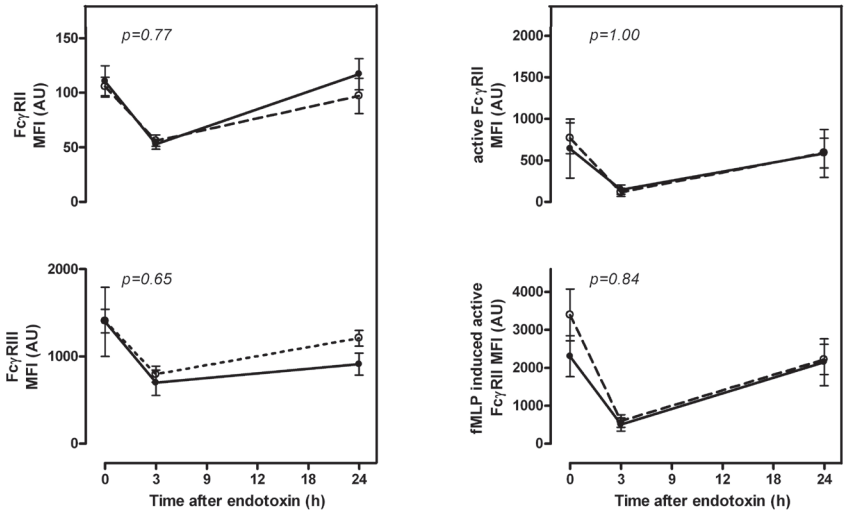


**Figure 2**
Expression of FCγRII, FCRγIII, active FCγRII and fMLP induced expression of active FCγRII on circulating neutrophils measured by flow cytometry after administration of 2ng/kg *Escherichia coli* lipopolysaccharide at t=0hr , t=3hr and t=24hr. C1-esterase inhibitor at a dose of 100U/kg intravenously over 30 minutes (o) or placebo (•) was infused. Data are expressed as mean ± SEM. p values in the figure express differences between groups obtained by repeated measures ANOVA over the complete curve.

In conclusion, modulation of the inflammatory response by C1INH substitution in a human experimental endotoxemia model resulted in attenuation of the humoral, but not of the neutrophil response. This result underlies the frail correlation between the humoral and cellular response *in vivo*.

## Materials

U.S. Reference E.coli endotoxin (lot Ec-5, Centre for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD); C1-esterase inhibitor (Cetor®) (Sanquin Plasma Products, The Netherlands); saline 0.9% (Baxter, The Netherlands); 2.5% glucose/0.45% saline (Baxter, The Netherlands); FITC-labelled mouse-antihuman monoclonal antibodies against: L-selectin (CD62L; clone Dreg56, BD Pharmingen, USA), CXCR1 (CD181; clone 42705, R&D Systems Europe, UK), C5aR (CD88; clone P12/1, Serotec, Germany); PE-labelled mouse-antihuman monoclonal antibodies against: ΜM (CD11b; clone 2LPM19c, DAKO, Denmark), CXCR2 (CD182; clone 48311, R&D Systems Europe, UK), FCΥRII (CD32; clone FLI8.26, BD Pharmingen, USA); Alexa 647-labelled monoclonal antibodies against: FCΥRIII (CD16; clone 3G8, BD Pharmingen, USA); FITC-labelled IgG1 negative control (clone MOPC-21, BD Biosciences, Belgium), and IgG2a negative control (clone MRC OX-34, Serotec, Germany); PE-labelled and IgG1 negative control (clone DD7, Chemicon, USA); Alexa 647-labelled IgG1 negative control (clone MOPC-21, BD Biosciences, Belgium); FITC-labelled monoclonal phage antibody A27 against active FCΥRII (generated and characterized as described previously 29); N-formyl-methionylleucyl-phenylalanine (fMLP) (Sigma-Aldrich, USA); FACScalibur Flow cytometer (BD Biosciences, USA); SPSS version 15.0 software (The Apache Software Production 2008, USA)

## Methods

*FACS analysis*

All blood samples were collected in a vacutainer® with sodium heparin as anticoagulant and cooled immediately on melting ice. Red cells were lysed with icecold isotonic $NH_4Cl$. After lysis, white blood cells were washed and resuspended in phosphate buffered saline supplemented with sodium citrate (0.4% wt/vol) and pasteurised plasma protein solution (10% vol/vol) (PBS2+), as previously described[30]. Resuspended cells were incubated on ice with directly labelled mouse-antihuman antibodies against L-selectin (CD62L), αM (CD11b), CXCR1 (CD181), CXCR2 (CD182), C5aR (CD88), CD66b, FCγRII (CD32) and FCγRIII (CD16). After incubation and final wash, labelling was measured on FACScalibur Flow cytometer. The neutrophils were identified according to their specific side-scatter and forward-scatter signal. For measurement of active FCγRII expression, whole blood was incubated a FITC-labelled monoclonal phage antibody A27 for 45 min on ice[30]. Active upregulation of active FCγRII expression was measured after 5 min of stimulation of whole blood at 37°C with fMLP $10^{-6}$M to evaluate the responsiveness of the cells for bacterial derived protein products/peptides. After stimulation, the samples were put on ice again and stained with phage antibody A27. After staining, red cells were lysed and expression was measured on FACScalibur as previously described[30].

Data from individual experiments are depicted as fluorescence intensity as the median fluorescence intensity (MFI) of at least 5,000 neutrophils.

## Statistics

Values are expressed as mean±SEM. A two-way repeated measures ANOVA was used to test variation over time, the variation between interventions, and the interaction between time and intervention. Statistical significance was defined as $p<0.05$.

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# 9

# General discussion and future perspectives

Acute inflammation, whether it is caused by injury or invading pathogens, results initially in a pro-inflammatory innate immune response. A systemic immune response is defined, according to clinical parameters, as systemic inflammatory reaction syndrome (SIRS). Neutrophil granulocytes play a prominent role in the early innate immune reaction. This first line immune response can in severely injured patients have devastating consequences. Subsequent to disproportional neutrophil sequestration in vital organs, cytotoxic agents released by these neutrophils can cause damage of the parenchyma. Tissue damage of vital organs can consequently lead to multiple organ dysfunction syndrome (MODS). A massive immune response is on the other hand also associated with a late immune deficient state, putting the patient at risk for development of sepsis and septic shock. This condition is clinically defined as compensatory anti-inflammatory reaction syndrome (CARS). Much effort has been made to identify patients at risk for these inflammatory complications. Plasma derived inflammatory markers are analyzed without showing a clear correlation to patient's outcome. Furthermore, the use of immunosuppressive drugs to diminish these complications have been proposed and tested in several studies. However none of these therapies have led to much success. The complexity of the innate immune response and the lack of knowledge regarding the kinetics of the innate immune system probably have contributed to failure of proposed therapy so far.
This thesis has addressed the neutrophil response upon injury and LPS challenge. Furthermore, a new potential drug to antagonize the excessive activation of the innate immune response is tested in a human endotoxemia model.

## Neutrophil response

Within minutes after onset of inflammation altered neutrophil phenotypes are seen[1]. In **Chapter 3** we demonstrated that isolated thorax trauma leads to an altered phenotype of neutrophils during the first hours after injury. An important outcome was that the activation phenotype of neutrophils in trauma patients differs from the activation phenotype of neutrophils after stimulation *in vitro*. This was most clearly demonstrated by a reduced expression of αM(CD11B) and fMLP-induced activated FcγRII on circulating neutrophils *in vivo*, while expression of these markers is known to be increased after priming *in vitro*[2-5]. It is possible that activation phenotype of circulating neutrophils *in vivo* is different to the phenotype *in vitro* because of the attendance of different stimulatory mediators. Priming of neutrophils by a variety of stimuli *in vitro* can impossibly mimic the complexity of mediators present *in vivo*. Nonetheless, dissimilarity in neutrophil phenotype after stimulation *in vitro* and *in vivo* seems also to be the redistribution of primed and non-primed neutrophils *in vivo*. During inflammation, cytokines and altered characteristics of endothelial cells facilitate the extravasation of circulating primed neutrophils to (affected) tissue simultaneously with recruitment of new neutrophils from the bone marrow and marginated pool to the circulation[6-8]. As a result an altered neutrophil population is quickly found in the circulation in response to inflammatory stimuli.
FACS analysis of leukocytes of trauma patients showed that circulating neutrophil granulocytes do not consist of a homogenous population. One of the surface receptors on circulating neutrophils that varied widely during inflammation is FcγRIII (CD16). Normally neutrophils express this FcγR with a narrow expression level showing a homogenous

population of neutrophils. Upon acute inflammation, the expression of FcγRIII broadened from a low to normal expression on circulating neutrophils at the same moment. FcγRIII expression on neutrophils is related to cell maturity[9 10]. Appearance of neutrophils with a low FcγRIII expression indicates recruitment of young banded neutrophils into the circulation, a frequently seen phenomenon in acute inflammation.

Interestingly, mean neutrophil FcγRIII expression was decreased in all trauma patients with thoracic injury, signifying recruitment of young neutrophils in every patient (**Chapter 3**). Yet, the appearance of young neutrophil was not necessarily accompanied by an increased number of circulating neutrophils since half of these trauma patients did not have a leukocytosis (> 12.0 $10^6$/ L). Rapid homing of primed neutrophils to the tissues together with mobilization of neutrophils from the bone marrow may thus lead to an unchanged number of circulating neutrophils with a clear shift in phenotype. A normal leukocyte count therefore does not reflect the absence of a systemic cellular innate immune response per se.

Young neutrophils expressed, besides FcγRIII, several other receptors, such as L-selectin (DC62L), αM(CD11b), FcγRII(CD32), CXCR1(CD181) and CXCR2 (CD182), at low levels[11] (see also **chapter 4**). Recruitment of neutrophils with a reduced expression of activation markers to the circulation makes clear why during inflammation *in vivo* certain receptors are decreased while an increase is expected based on neutrophil priming. Hence, the overall neutrophil phenotype found *in vivo* depends on both *in vivo* priming as well as on the redistribution of neutrophils.

Recent studies have identified circulating neutrophil populations with distinctive surface phenotypes during acute inflammation[1 12]. Heterogeneity of the circulating neutrophil population makes it hard to classify activation status based on the expression of a single surface marker. Mean expression of a neutrophil surface receptor is affected by the size of influx and efflux of neutrophils, a dynamic process that changes in time. This clarifies why single neutrophil surface receptor expression does not correlate well with outcome and why results have been contradictory (**Chapter 2**).

Understanding of the neutrophil kinetics is needed for better interpretation of the innate immune response. Further investigations should be aimed on identification of different neutrophil populations appearing in the circulation in time.

One of the neutrophil populations that was identified in the circulation during severe acute inflammation involved VLA-$4^{pos}$ neutrophil progenitor cells (**Chapter 4**). These VLA-$4^{pos}$ neutrophil progenitors were found in severely injured patients who met the inclusion criteria of multiple injuries and an expected admission to an ICU of more than three days. The fact that these cells were found in these severely injured patients and not in less severe injured patients - e.g. those with isolated thoracic injury- suggest that an excessive inflammatory stimulus is needed for recruitment of these cells. Why and how VLA-$4^{pos}$ neutrophil progenitors appear in the circulation, however, remains to be elucidated.

Under normal conditions, CXCR4(CD184) and stroma derived factor (SDF)-1α signaling pathways play a pivotal role in the retention and maturation controlled release of neutrophils from the bone marrow[13-16]. CXCR4 is one of the key receptors responsible for the retention of granulocyte progenitors in the bone marrow[14 15 17]. During maturation of granulocyte progenitors CXCR4 surface expression decreases, by which neutrophil mobilization is facilitated[18]. The retention of immature neutrophils at the bone marrow is also regulated

via VLA-4[16]. Neutrophil progenitors retain in the bone marrow as they adhere via VLA-4 to vascular adhesion molecule (VCAM)-1, which is expressed on bone marrow endothelium and stroma.

Rapid recruitment of neutrophils from the bone marrow in response to inflammatory stimulus is regulated by a cascade of cellular and molecular signals, in which granulocyte colony stimulation factor (G-CSF) plays a dominant role[6 19 20]. G-CSF is the primary regulator of steady-state and emergency granulopoiesis[6 19]. CXCR4 surface expression is down-regulated by G-CSF, thereby inducing the release of neutrophils from the bone marrow[21 22]. CXCR4 also enhances VLA-4 adhesion to VCAM-1[16]. Inhibition of CXCR4 may thus result in a reduced binding of VLA-4 to VCAM-1 thereby inducing the release of VLA-4$^{pos}$ progenitors during severe inflammation.

Neutrophils are important for first line defense of the host against invading micro organisms, but can be devastating when inappropriately activated and released in high number. Therefore, the number of circulating neutrophils in steady-state is tightly regulated[7]. Under normal conditions neutrophil mobilization is restrained by feedback loops that sense neutrophils homing tot tissue[7]. Also, feedback signals generated throughout neutrophil apoptosis and clearance has been proposed to play a role in the control of bone marrow release[7]. How these feedback systems are regulated is currently unknown. It is clear, however, that in steady-state the number of neutrophils released is securely controlled to maintain homeostasis. How numbers of circulating neutrophils is controlled during inflammation is not completely understood[23]. Acute inflammation is commonly accompanied by neutrophilia. Delayed apoptosis during inflammation is considered to be one of the factors contributing to this neutrophilia [24-26]. Furthermore, the mobilization of neutrophils from the marginated pool, due to stress hormones like gluco-corticoids, is supposed to add to higher number of circulating neutrophils[8]. However, neutrophilia is most importantly the result of an increased proliferation and egress of neutrophils from the bone marrow induced by cytokines, of which G-CSF is most prominent[19].

Because of the potential destructive character of neutrophils, it seems highly unlikely that the extent of neutrophil mobilization is not controlled during inflammation. Hence, the recruitment of neutrophils in response to inflammatory stimuli must be a carefully controlled process. Neutrophils must be released during inflammation in a manner which is securely regulated by feedback signals regarding neutrophil homing and neutrophils present in the peripheral circulation. If we assume that neutrophil trafficking is conducted by several feedback signals from tissue, endothelium and immune cells, it is tempting to hypothesize that those cells appearing in the circulation represent the extent and/or duration of inflammation. This concept denotes that for instance VLA-4$^{pos}$ neutrophil progenitors enter the circulation with a particular reason.

It seems logical that VLA-4$^{pos}$ neutrophil progenitors are recruited during inflammation due to depletion of more mature neutrophils in the bone marrow. Appearance of VLA-4$^{pos}$ neutrophil progenitors consequently implies a recruitment of the majority of neutrophils stored in the bone marrow. Massive recruitment can by caused by a reduction in circulating neutrophils as a result of substantial homing of neutrophils to tissue or by excessive blood loss.

It cannot be excluded, however, that VLA-4$^{pos}$ neutrophil progenitors are released because of a specific function. Previous work has shown that distinctive neutrophil subsets can

have particular functions[1]. A neutrophil subpopulation with a unique phenotype (CD62L$^{dim}$/ CD16$^{bright}$/CD11b$^{bright}$/ CD54$^{bright}$), found in the peripheral circulation after LPS-challenge, was shown to have an immunosuppressive capacity as it can inhibit T-cell response. For a long time, neutrophils were considered to belong to a homogenous population of cells with a same function and their phenotypes were related to different stage of priming and maturation. This later study shows that different neutrophil subsets can have different function and, therefore, we must keep in mind that these that VLA-4$^{pos}$ neutrophil progenitors may have a specific function as well.

An inevitable shortcoming of studying the innate immune response in human is that we can rather easily obtain neutrophils from the circulation but not from tissue. What happens in organ parenchyma remains uncertain. It also remains uncertain which cells and what part of these cells home. Until now research has given us a slightly better insight of the complexity of the neutrophil response based on changed neutrophil populations appearing in the circulation. Yet, the innate immune response in human in tissue is still hardly understood. It remains unsure if the neutrophil populations in the circulation indeed reflect the immune reaction in tissue.

Intravital microscopy may provide more insight in neutrophil migration during inflammation, although this method is hard to apply in patients. *In vivo* imaging studies in transparent zebrafisch performed by Huttelocher et al. has provided new insight concerning neutrophil trafficing. They have elucidated several singalling mechanisms involved in neutrophil attraction to site of inflammation[27 28]. They were also able to make the phenomonon of reversed migration visible, as they were abel to show neutrophils leaving the site of inflammation[29]. These results illustrate the potential of *in vivo* imaging studies.

## DAMPs and PAMPs

**Chapter 5** shows that trauma and endotoxemia result in a similar neutrophil response in the peripheral blood during the first hours after onset of inflammation. The inflammatory reaction after trauma is induced mostly by damage associated molecular patterns (DAMP), whereas during endotoxemia inflammation is induced by pathogen associated molecular patterns (PAMP). DAMPs and PAMP are recognized by innate immune cells via pattern recognition receptors (PRRs) of which Toll like receptors (TLR) are most well known[30]. Both DAMPs and PAMPs induced inflammation resulted in recruitment of young neutrophils in the peripheral blood with similar changes in surface marker expression. This similarity indicates that the neutrophil response forms the final common pathway of inflammatory cascades. The neutrophil reaction seems independent of its trigger (damage or pathogen associated). This may be because same or even similar PRRs recognize DAMPS and PAMPs[31-34].

Yet, similarity in neutrophil response after trauma and LPS-challenge at onset, does not mean that the response remains identical in time. One of the limitations of the human endotoxemia model is that only a short ($t_{1/2}$ of plasma LPS is 15 min) and moderate inflammatory stimulus is supplied and the innate immune response is restored within 24 hours. *In vivo* LPS-challenge is, therefore, an excellent model to study the onset of the inflammatory response in a controlled manner *in vivo* in human[35-37]. Yet, the endotoxemia model does by no means resemble the ongoing inflammatory response provoked by signals

from e.g. damaged tissue, surgical intervention or form hypoperfusion due to anemia and hemodynamic instability in severely injured patients. Then again, the human endotoxemia model has also advantages above animal models that yield more severe and long-term inflammation models. *In vivo* immunological experimentation are most often performed in mice models. However, there are significant differences between mice and humans in activation and response to challenge, in both the innate and adaptive immune systems[38]. Results from these models are often difficult to translate to the human situation.

Although DAMPs and PAMPs result in a similar early immune response, the innate immune response of a trauma patient should not be compared with that of a septic patient. One of the most important differences between injury and sepsis is that the beginning of inflammation in a septic patient is unknown. Even when there seems to be a clear clinical onset of infection, it remains unclear how long the inflammatory reaction has been going on without clinical symptoms. The process of cell homing and recruitment might thus have been initiated for days in a septic patient and the innate immune response might therefore appear different to that of a trauma patient.

As the innate immune response is a dynamic process influenced by persistent or new inflammatory stimuli *in vivo* investigation of the immune response is difficult. A standardized inflammatory representation such as the human endotoxemia model may therefore be useful to gain better understanding of the multifaceted innate immune response.

## Organ failure and sepsis

Acute respiratory distress syndrome (ARDS) and MODS are severe complications that are frequently seen after severe injury and are caused by an overwhelming neutrophil response[39-42]. An interesting finding, described in **chapter 3**, was that isolated lung injury rarely results in ARDS, even in cases with major bilateral thoracic trauma. Under these circumstances local damage and a transient systemic inflammatory response did not result in a disproportional neutrophil reaction in the lung parenchyma. This outcome was unexpected thoracic injury is associated with and increased risk of developing ARDS[43 44]. This result underlines the importance of a more pronounced and/or prolonged systemic response in the development of organ dysfunction. Several reports have proposed that neutrophils, released form bone marrow, are stiffer and therefore more easily sequester in the lung microvasculature[45]. It appears that during systemic inflammation certain circulating neutrophils are prone to enter organ tissue due to altered properties. This may explain why neutrophils are found even in non-injured tissues in patients with ARDS and MODS[39 40]. Characterization of these seeming randomly "homing" neutrophils may be useful, but it remains to be seen whether specific neutrophils subpopulations exist that enhance the sequestration of neutrophils in organs and in that way increase the risk of organ failure.

Besides the risk of developing early organ failure, trauma patients are at risk for septic complications. Trauma patients typically attain septic complications around day 5 to 10 after injury[46 47]. Hypotheses that explain as to why trauma patients are susceptible for sepsis and septic shock days after trauma have been put forward. The concept of a 'Compensatory anti-inflammatory reaction syndrome' (CARS) was proposed in 1996 by R. Bone[48] (see also above). Yet, this concept has been under debate for a long time. Plasma concentrations of

pro-inflammatory ( e.g IL-6, TNF-α, IL-1β) and anti-inflammatory (IL-10, TGF-β, IL-1β receptor antagonist) cytokines are simultaneously increased in the first days after injury[49 50]. There is no direct evicence supporting the concept of a direct overwhelming anti-inflammatory response.

A completely different theory is that the immune deficient state is caused by failure of the pro- inflammatory response due to exhaustion of the immune system in a late phase. The appearance of circulating VLA-4$^{pos}$ neutrophil progenitors favors this latter hypothesis. Mobilization of high numbers of VLA-4$^{pos}$ neutrophil progenitors implicates an enormous demand for innate immune cells shortly after onset of inflammation. Although VLA-4$^{pos}$ neutrophil progenitors maturate outside the bone marrow -and probably can in time adequately eradicate invading pathogens- the massive recruitment may lead to a relative shortness of neutrophils stored in the bone marrow. The myelocyte pool in the bone marrow is considered to consist of two distinct pools[6 51 52]. One pool with a high turn-over contributes to steady-state granulopoiesis and one is reserved for emergency granulopoiesis. The slowly dividing pool of myelocytes for emergency granulopoiesis has a generation time of 70 days[51]. Although proliferation is increased during infection it remains to be elucidated how long it will take for the bone marrow to be restored and to be able to cope new inflammatory events.

Recently, it was shown that neutrophils have a life-span of 5 to 6 days after release form the bone marrow. As a result, sufficient numbers of neutrophils may be at hand during the first week after massive recruitment but a shortage of adequate functioning neutrophils may evolve in the period thereafter. This hypothesis is in line with results from trauma patients that are at risk for septic complications typically days 5 to 10 after trauma. A relative shortness and/or presence of young neutrophils with an impaired anti-microbial function may explain an increased susceptibility for patients to invading pathogens in late phase after injury.

Future treatment should, therefore, be aimed against overwhelming recruitment of neutrophils during the first days after trauma. In that way, accumulation of great numbers of neutrophils in tissue as well as bone marrow depletion can be prevented. Additional therapy may include anti-biotic prophylaxes or leukocyte transfusion in a later phase for instance in patients with excessive blood loss at admission.

## Modulation of neutrophil response

It has become clear that there is a vast inflammatory reaction in the first hours after trauma. Several previous studies have shown that avoidance of second inflammatory hits shortly after injury reduces the risk of organ failure and sepsis later on[53-55]. The outcome of these studies underlines the role of the early inflammatory response in the development of these life-threatening complications. For that reason we believe that adequate suppression of the innate immune response as early as possible in patients at risk for these inflammatory complications is crucial for success.

In marked contrast to adaptive immunity that is very sensitive to immune modulators such as gluco-cortico-steroids, the innate immune system cannot be sufficiently targeted with currently available anti-inflammatory drugs (**Chapter 6**). This is largely due to the fact that knowledge regarding antagonism of innate immune cell function is scarce.

C1 esterase inhibitor (C1INH) is an endogenous inhibitor of the innate immune response. C1INH is an acute phase protein that has potent anti-inflammatory properties. It inhibits the complement system but is also directly acts against neutrophils[56-58]. Animal studies have shown that C1INH reduces the influx of neutrophils in tissue in inflammatory models[59 60]. C1INH may therefore be a potential drug for limiting the devastating neutrophil response such as seen after major trauma or sepsis.

In **chapter 7** C1INH was given 30 minutes after LPS-challenge in an attempt to block the innate immune response. Several animal studies have shown C1INH to preserve endothelial function and prevent capillary leakage during sepsis and after (thermal) trauma[61-67]. Furthermore C1INH inhibits adhesion of neutrophils to the endothelium in LPS induced inflammation[59 68 69]. Also, histology shows less infiltration of neutrophils to the tissues as well as less neutrophil mediated tissue damage in animals treated with C1INH[59 62 68 70].

In the endotoxemia model C1INH administration resulted in a reduced concentration of circulating pro-inflammatory cytokines IL-6, IL-1β and TNF-α. A deminished rise of pro-inflammatory cytokine levels was preceded by an increase of the ant-inflammatory cytokine IL-10 levels in humans treated with C1INH. Increasing IL-10 levels seems a new potential mechanism by which C1INH inhibits the pro-inflammatory reaction.

In contrast to the humoral response, C1INH administration did not seem to affect the cellular response (**chapter 8**). One could, therefore, argue that C1INH is not efficient as therapy for prevention of inflammatory complications, but there are arguments that need to be taken into account. C1INH was given shortly after LPS challenge to mimic a clinical situation. Perhaps the timing of C1INH administration was to too late in this study because of the short inflammatory response provoked by LPS in the endotoxemia model. A more pronounced effect on the humoral response and a considerable effect on cellular response and clinical signs might be found when C1INH is given beforehand.

Interestingly and counter-intuitively, no correlation was found between cytokine levels and neutrophil response in the endotoxemia experiment. The effect of cytokines depends on several factors. Their effect depends on the ratio between agonistic and antagonistic mediators and the responsiveness of cells for the cytokine. Cytokines by itself do, therefore, *in vivo* not correlate well with cellular responses. *In vitro* studies also showed that the effect on neutrophils caused by stimulation of a combination of cytokines does not reflect the sum of the effects caused by individual cytokines[71 72]. Stimulation of neutrophils *in vitro* by TNF-α or GM-CSF resulted in different regulated protein spots measured by two-dimensional gel electrophoresis[72]. Yet, regulated protein spot profile after stimulation of both TNF-α and GM-CSF did not match the individual profiles. Since there is always a combination of cytokines *in vivo*, the effect of a single cytokine on neutrophils remains unknown. For that reason, the cellular response seems more reliable to measure.

**Conclusion**

Research in this thesis describes the complexity of the neutrophil response as part of a systemic innate immune reaction. Due to redistribution, neutrophils subsets with unique phenotypes appear in the circulation at certain time. Future perspectives should be focused on role and function of these neutrophil subpopulations. To gain more insight on neutrophil distribution further research may involve investigation of neutrophils other sides that peripheral circulation including bone marrow and organ parenchyma.

Immunomodulation in trauma patients should be aimed on the early inhibition of neutrophil recruitment from the bone marrow and sequestration neutrophils in tissue in order to improve survival. C1INH may have the potential to do so, but its therapeutic use in human must still be explored. At this moment, our study group investigates the effect of C1INH on the systemic inflammatory reaction in a randomized placebo control trial in trauma patients with femur fractures[73]

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# 10

Summary in Dutch

Nederlandse Samenvatting

# Samenvatting

Het immuunsysteem beschermt het lichaam tegen binnendringende ziektekiemen, zoals bacteriën, virussen en parasieten. Daarnaast is het betrokken bij het opruimen van zieke of beschadigde cellen. Het immuunsysteem is onder te verdelen in een aangeboren (aspecifieke) en een verworven (specifieke) afweersysteem. De aangeboren afweer reactie is snel werkzaam, maar minder specifiek voor de ziekteverwekker. De aangeboren immuun reactie is verantwoordelijk voor de eerstelijns afweer bij acute ontstekingen (inflammatie), zoals na een verwonding.
De neutrofiele granulocyt, ook wel neutrofiel genoemd, behoort tot de groep van leukocyten (witte bloedcellen). De neutrofiel speelt een zeer belangrijke rol in de eerstelijns afweer. Neutrofielen zijn in staat om schadelijke stoffen op te nemen en te verteren, een proces genaamd fagocytose. Daarnaast kunnen neutrofielen cytotoxische stoffen uitscheiden. Door verschillende signaleringstoffen die vrijkomen tijdens inflammatie, bijvoorbeeld cytokinen, worden neutrofielen geactiveerd. Activatie van neutrofielen leidt onder ander tot een veranderde expressie van receptoren op de celmembraan. Geactiveerde neutrofielen zijn beter in staat om naar de plaats van inflammatie migreren en hebben een verhoogde antimicrobiële capaciteit.
Gebleken is dat bij ernstige gewonde traumapatienten de neutrofiel reactie dusdanig hevig kan zijn dat het zich ook tegen het eigen lichaam keert. Een ongecontroleerde afweer reactie kan aanleiding geven tot het ontstaan ernstige complicaties zoals orgaanfalen met dood tot gevolg. Weefselonderzoek heeft aangetoond bij patiënten met orgaanfalen grote hoeveelheden neutrofielen zich in het parenchym van organen bevinden. Cytotoxische mediatoren die massaal uitgescheiden worden door geactiveerde neutrofielen, beschadigen in deze gevallen niet alleen pathogenen maar ook gezonde eigen lichaamscellen. Om beter inzicht te verkrijgen in de ongecontroleerde neutrofiel reactie, hebben wij de neutrofiel reactie onderzocht bij ongevalpatiënten. Daarnaast hebben wij gekeken of de we neutrofiel reactie in een vroeg stadium kunnen remmen.

**Hoofdstuk 2** beschrijft een literatuurstudie waarbij gekeken is naar voorspellende waarden van circulerende inflammatoire signaleringmarkers voor het ontstaan van complicaties zoals orgaanfalen bij ongevalpatiënten. Daaruit blijkt dat vrijwel alle signaleringstoffen gemeten in de circulatie een slechte voorspeller zijn voor het wel of niet ontwikkelen van ernstige complicaties.

Indien traumapatiënten orgaanfalen ontwikkelen is de long het orgaan dat het meest frequent is aangedaan. Enkele studies hebben beschreven dat longfalen (ARDS) vaker voorkomt bij patiënten met thoraxletsel (letsel van de borstkast) dan bij patiënten zonder thoraxletsel. In **hoofdstuk 3** hebben we de invloed van geïsoleerd thoraxletsel op de neutrofiel reactie in het bloed gemeten. Gedurende de eerste 24 uur na ongeval hebben we op drie momenten bij patiënten met thorax letsel bloed afgenomen en met behulp van flowcytometrie de activatie status van circulerende neutrofielen bepaald. Daarnaast hebben we de concentratie van pro-inflammatoire cytokine IL-6 in het bloed gemeten. Deze waarden hebben we vergeleken met waarden die gemeten zijn bij gezonde vrijwilligers.

Binnen drie uur na ongeval hadden circulerende neutrofielen een veranderde expressie van oppervlakte receptoren in vergelijking met circulerende neutrofielen van gezonde vrijwilligers. Daarbij zagen we met name een verminderde expressie van L-selectin(CD62L), CXCR2(CD182b) and C5aR (CD88). Dit veranderde receptor fenotype was 24 uur na verwonding vrijwel volledig genormaliseerd. De concentratie van de pro-inflammatoire cytokine IL-6 was na thoraxletsel verhoogd aanwezig in de circulatie. De concentratie van IL-6 nam gedurende de 24 uur na ongeval toe. De gepresenteerde resultaten uit deze studie geven weer dat een geïsoleerd thoraxletsel niet alleen leidde tot een lokale afweerreactie maar ook tot een systemische reactie die in de circulatie te meten was.
Opvallend genoeg ontwikkelde geen van de 14 patiënten met een geïsoleerd thoraxletsel ARDS. Tot de patiëntgroep behoorden ook patiënten met een ernstig thoraxletsel met onder andere longcontusies beiderzijds. Een kortdurende systemische neutrofiel reactie in combinatie met schade aan het longparenchym leidde in deze gevallen dus niet tot orgaanfalen. Blijkbaar leidt een kortdurend systemische inflammatoire reactie, zelfs in combinatie met ernstige schade aan het orgaan, zelden tot orgaanfalen. Een hevigere systemische inflammatoire reactie lijkt nodig te zijn voor aspecifieke uittreding van neutrofielen met destructie van het parenchym van vitale organen als gevolg.

Op basis van een veranderd receptor profiel konden we concluderen dat er na thoraxletsel jonge staafkernige neutrofielen in de circulatie verschenen die hoogst waarschijnlijk uit het beenmerg zijn gerekruteerd. Mobilisatie van jonge neutrofielen vanuit het beenmerg naar de circulatie ging niet noodzakelijkerwijs gepaard met optreden van een leukocytose (verhoogd leukocyten aantal (>12*$10^6$ /ml)). Een mogelijke oorzaak hiervan is dat jonge cellen vanuit het beenmerg worden aangetrokken terwijl tegelijkertijd geactiveerde cellen de bloedbaan verlaten.
Onderzoek naar receptor expressie toonde tevens aan dat neutrofiel populatie in de circulatie na inflammatie bestaat uit een heterogene populatie. Verschillende neutrofiel populaties konden worden onderscheiden door hun unieke receptor fenotype. In **hoofdstuk 4** hebben we één van deze subpopulaties geïsoleerd en geïdentificeerd. Het betrof hier circulerende neutrofielen die de receptor very late antigen (VLA)-4 op de celmembraan tot expressie brachten. VLA-4 komt normaliter voor op de membraan van neutrofiel voorlopercellen in het beenmerg, maar wordt niet tot expressie gebracht bij staafkernige of segmentvormige neutrofielen. Microscopisch onderzoek liet zien dat deze in de circulatie aangetroffen populatie geen volwassen neutrofielen betrof maar dat deze populatie bestond uit neutrofiele voorlooper cellen. We hebben aangetoond dat neutrofiele voorloper cellen een verminderde antibacteriële functie hadden. Zij konden echter buiten het beenmerg wel uitrijpen tot volwassen neutrofielen, zelfs in afwezigheid van stimulerende mediatoren.
Circulerende neutrofiele voorloper cellen werden alleen aangetroffen bij zeer ernstig verwonde traumapatiënten. Eerdere studies hebben VLA-4 positieve neutrofielen gevonden bij patiënten met ernstig sepsis. Het lijkt er dus op dat deze cellen vanuit het beenmerg gemobiliseerd worden bij zeer ernstige aandoeningen. Het verschijnen van VLA-4 positieve neutrofiele voorloper cellen in de circulatie is dus mogelijk een maat voor ernst van inflammatie.

Onderzoek naar de aangeboren immuunreactie in traumapatiënten is een lastige opgave en wordt bemoeilijkt door allerlei factoren die de afweer beïnvloeden. Factoren die de afweer reactie beïnvloeden zij onder anderen de aard van de letsels, behandeling (zoals operatieve ingrepen en bloedtransfusies) en de medische voorgeschiedenis van de patiënt.
Om het onderzoek naar de neutrofiel reactie te vereenvoudigen zou in een experimenteel model waarin een gecontroleerde systemische afweer reactie wordt opgewekt uitkomst kunnen bieden.
Een bekend inflammatie model, gebruikt voor onderzoek naar onder andere sepsis, is het humane endotoxemie model. In dit model krijgen gezonde vrijwilligers lipopolysaccharide (LPS), een endotoxine, intraveneus geïnjecteerd. Endotoxinen zijn celwandbestanddelen van gramnegatieve bacteriën die in het lichaam een afweerreactie induceren.
De vraag is echter of de immuunreactie geïnduceerd door pathogenen vergelijkbaar is aan die geïnduceerd door verwonding. Het is bekend dat cellen van het aangeboren immuunstysteem hun op te ruimen doelwitten herkennen middels pattern recognition receptoren (PRR). PPRs kunnen specifieke bestanddelen van pathogenen (pathogen associated molecular patterns (PAMPs) genoemd) herkennen. Daarnaast kunnen PRRs stoffen die vrijkomen bij beschadiging van lichaamseigen cellen herkennen. Deze lichaamseigen partikels worden damage associatied patterns (DAMPs) genoemd. In **hoofdstuk 5** hebben we gekeken of PAMPs and DAMPs een zelfde afweerreactie induceren. De neutrofielreactie van traumapatiënten werd hiebij vergeleken met die van gezonde personen die LPS van Escheria Coli bacterie (*E. Coli*) kregen toegediend. Voor dit doeleinde werden receptor expressies op circulerende neutrofielen bepaald op 3 uur en 24 uur na LPS toediening en na ongeval. Receptor expressie van gezonde vrijwilligers die geen LPS toegediend hadden gekregen werden gebruikt als controle waarden.
Endotoxemie en trauma resulteerden in een vergelijkbaar activatie fenotype van circulerende neutrofielen. Dit fenotype werd gekenmerkt door verminderde expressie van chemokine-receptoren CXCR1(CD181) en CXCR2(CD182) en van FcγR II (CD32) en III(CD16). Zowel tijdens experimentele endotoxemie als na trauma was er sprake van systemische neutrofiel activatie 3 uur na begin van inflammatie die normaliseerde in de daarop volgende 24 uur. Hoewel, de inflammatoire reactie in beginsel door verschillende factoren wordt geactiveerd lijkt de neutrofiel reactie dus vergelijkbaar. Het humane endotoxemie zou daarom kunnen worden gebruikt als model voor verder onderzoek naar neutrofiel reactie gedurende eerste uren van inflammatie. Tevens zou het model uitkomst kunnen bieden bij een eerste testfase voor immunomodulerende therapieën.

**Hoofdstuk 6** is een systematische beschouwing van de literatuur naar het effect van immunomodulerende therapieën. Gerandomiseerde studies die het effect van potentiële immunomodulerende therapieën op infectie, meervoudig orgaanfalen en mortaliteit bij traumapatiënten vergeleken met een placebo of een standaard therapie werden voor deze analyse geïncludeerd. In de meeste studies werd een significant verschil gezien tussen inflammatoire parameters. Er werden echter geen significante verschillen gemeten in infectie, orgaanfalen of sterfte.
Een verklaring waarom de geteste therapieën tot op heden geen verbetering in klinische uitkomst lieten zien is mogelijk gelegen in het feit dat deze therapieën voornamelijk de verworven afweerreactie remmen en niet de aangeboren afweerreactie. We zouden dus

op zoek moeten naar een remmer van de aangeboren afweerreactie. Een lichaamseigen remmer van de aangeboren afweerreactie is het acute fase eiwit C1-esterase inhibitor (C1INH). In **hoofdstuk 7** testten wij het effect van toediening C1INH op de vroege afweerreactie. Toediening van C1INH 30 minuten na LPS injectie resulteerde in een afname van de pro-inflammatoire cytokines IL-6, IL-1β and TNF-α in vergelijking met placebo. Een opvallende bevinding was dat tevens een verhoogde concentratie van de ant-inflammatoire (remmende) cytokine IL-10 werd gevonden in personen die C1INH toegediend hadden gekregen. Mogelijk speelt IL-10 een rol in de afremming van de pro-inflammatoire reactie wanneer C1INH in hoge dosis wordt toegediend.
Hoewel er een significant verschil werd gevonden in circulerende signaleringstoffen, zorgde toediening van C1INH niet voor een remming van de neutrofielreactie (**hoofdstuk 8**). Dit zou kunnen komen doordat in het endotoxemie model C1INH relatief laat wordt toegediend waardoor het geen meetbaar effect op de kortdurende neutrofiel reactie heeft. Deze bevinding belicht echter wel een belangrijk punt. Er lijkt geen correlatie lijkt te zijn tussen concentratie circulerende signaleringsstoffen en de neutrofiel reactie.

**Conclusie**

Onderzoek in dit proefschrift beschrijft de complexiteit van de neutrofiel reactie als onderdeel van een systemische aangeboren immuun reactie. Als gevolg van herverdeling van neutrofielen vanuit beenmerg naar de weefsels verschijnen verschillende neutrofiele subpopulaties met unieke fenotypen tijdens de afweer reactie in de bloedbaan. Toekomstig onderzoek zal zich moeten richten op de rol en functie van deze neutrofiele subpopulaties.
Het bijsturen van de afweer reactie in trauma patiënten moet gericht zijn op de vroege remming van mobilisatie van neutrofiel uit het beenmerg en voorkomen van massaal uittreden van neutrofielen naar de weefsels ten einde de kans op complicaties zoals orgaanfalen en mortaliteit te doen afnemen. C1INH heeft de potentie om dit te doen, maar het therapeutische effect moet nog bij de mens nog worden onderzocht. Op dit moment onderzoekt onze studiegroep het effect van C1INH op de systemische afweer reactie in traumapatiënten met dijbeenfracturen in gerandomiseerde placebogecontroleerde studie.

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List of publications

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Cirruculum vitae

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# Curriculum vitae

Tjaakje Visser was born on 29th of April 1979 in Oosterhout (NB), The Netherlands. After graduating secondary school in 1997 at the Sint Oelbert gymnasium in Oosterhout, she started studying Biomedical Sciences at the Utrecht University. Two years later she was admitted at medical school at the Utrecht University. During Medical school she followed several internships abroad: Gynecology & Obstetrics at the King Edward VII Hospital in Durban, South Africa, Dermatology at the Lago Maggiore in Mendoza, Argentina, and Ophthalmology at the Hospital Central also in Mendoza, Argentina. In 2005 she graduated from Medical school. That same year she got her bachelor degree in Biomedical Sciences. She wrote a bachelor thesis entitled "*Timing of intramedullary nailing of femur fracture in multi-trauma patients. Posttraumatic inflammatory reactions and their influence on the orthopaedic treatment*" under the supervision of Prof. dr. L.P.H. Leenen. She then started working at the surgical department of the Haga Hospital, The Hague. In 2007 she commenced working at de surgical department of the University Medical Center Utrecht, where she initially was dedicated to the education and supervision of medical students before starting her PhD project. During her PhD project that is described in this thesis, she was supervised by her promoters Prof. dr. L.P.H. Leenen of the department of Trauma and Prof. dr. L. Koenderman of the department of Respiratory Medicine. The project concerned the neutrophil response in severe acute inflammation and its role in development of inflammatory complications.

From June 2010 she worked at the surgical department of the Jeroen Bosch Hospital in Den Bosch under supervision of Dr. K. Bosscha. In January 2012 she started the first two years of her residency in general surgery under the supervision of Dr. F.M. van Lammeren at the Slingeland Hospital in Doetinchem. In 2014 she will continue her surgical residency at the Radboud University Nijmegen Medical Centre under supervision of Prof. Dr. C.J.H.M. van Laarhoven.