KINETICS OF THE EXCESSIVE CELLULAR INNATE IMMUNE RESPONSE AFTER INJURY

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Kinetics of the excessive cellular innate immune response after injury
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The wildebeest migration on the plains of Africa is voted one of the New Seven Wonders of the World. Every January 1.5 million animals travel to the NgoroNgoro crater in the Serengeti to banquet on its young grass and fresh waters. It was not until 2006 that the kinetics of this massive migration was thoroughly analyzed. It was demonstrated that many wildebeests do not survive the journey, while others are distributed over the planes between Kenya and Tanzania. As a result, equilibrium is reached: the reduced number of animals prevents exhaustion of the grasslands, while sufficient wildebeests reach the Serengeti to assure the survival of the large predators that populate the crater.
Nevertheless, this every year phenomenon is inferior to the massive migration of billions of neutrophils to the tissues after severe injury. Like the Serengeti, migration and clearance of neutrophils and macrophages has to be in balance for proper functioning. Devastating migration of neutrophils or failure in clearance of these cells by macrophages would lead to the destruction of the tissues.

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Kinetiek van de buitensporige cellulaire immunologische reactie na trauma

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad doctor aan de Universiteit Utrecht op gezag van rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op

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Prof. dr. L. Koenderman

Aan mijn ouders

Voor Jessica

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Chapter 1

General introduction and outline of the thesis

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TRAUMA AND ORGAN FAILURE

Trauma or injury is the leading cause of death worldwide (1). In the Western world injury is an important cause of mortality, disabilities and loss of working years (2,3). The Road Safety Organisation (VVN) in the Netherlands has estimated that 850–900 traffic accident deaths occur every year and an additional 17.000 injured patients. The mortality is either caused immediately due to the extent of their injuries (e.g. hemorrhagic shock or severe brain injury), or by the development of organ failure in the phase following resuscitation (4). These causes are equally distributed for patients with blunt trauma, which form the majority in the Netherlands (5). Mortality caused by organ failure is in part mediated by pathology in organs that were not injured at the time of impact. The result of this process is a syndrome known as multiple organ failure (MOF). Up to 15% of all patients admitted with severe trauma develop single or multiple organ failure (6). The overall mortality rate in patients with MOF has decreased during the last decade from 80% in the early nineties to 20-40% in the beginning of the 21st century (7-10). However, the incidence of MOF after trauma as such only decreased moderately (10-12). As a result, the use of intensive care support has increased over the this period. Severely injured patients that develop organ failure require on average longer intensive care support (21 – 35 days) and mechanical ventilation (20 – 26 days) as compared to patients without organ failure (10 – 17 days and 3 – 13 days respectively) (9,13). This need for high level of care during an extended period of time makes that organ failure accounts for 1.7% of the annual healthcare budget in the Netherlands (14). In addition, morbidity rates after MOF remain high. Up to 80% of the patients who recovered from MOF suffer severe disabilities (14,15).

ORGAN FAILURE AND SURGERY

At present, no adequate treatment exists once organ failure has manifested: current treatment is supportive and symptom directed (8). Early identification and prevention of organ failure is therefore essential to establish a reduction in both mortality and morbidity. However, the largely unexplained pathophysiology limits the possibilities for early adequate prediction, identification and prevention of multiple organ failure.

Until the mid seventies severely injured patients were deemed “to sick to operate on”, thus extensive surgical procedures were postponed. Frequently, this resulted in systemic complications such as pulmonary embolism or severe infections. With improvement of resuscitation during the Vietnam war, a phenomenon called the “Da Nang” lung was frequently seen, as a syndrome later known as the Adult Respiratory Distress Syndrome. With further progress in anaesthesia came the possibility for surgeons to perform extensive interventions on severely injured patients (16). In this elaborate strategy of “early total care”, patients undergo all necessary surgical interventions in one procedure (17-21). On average, this strategy reduced complications and improved recovery. However, with this policy part of the patients still developed organ failure (Figure 1).

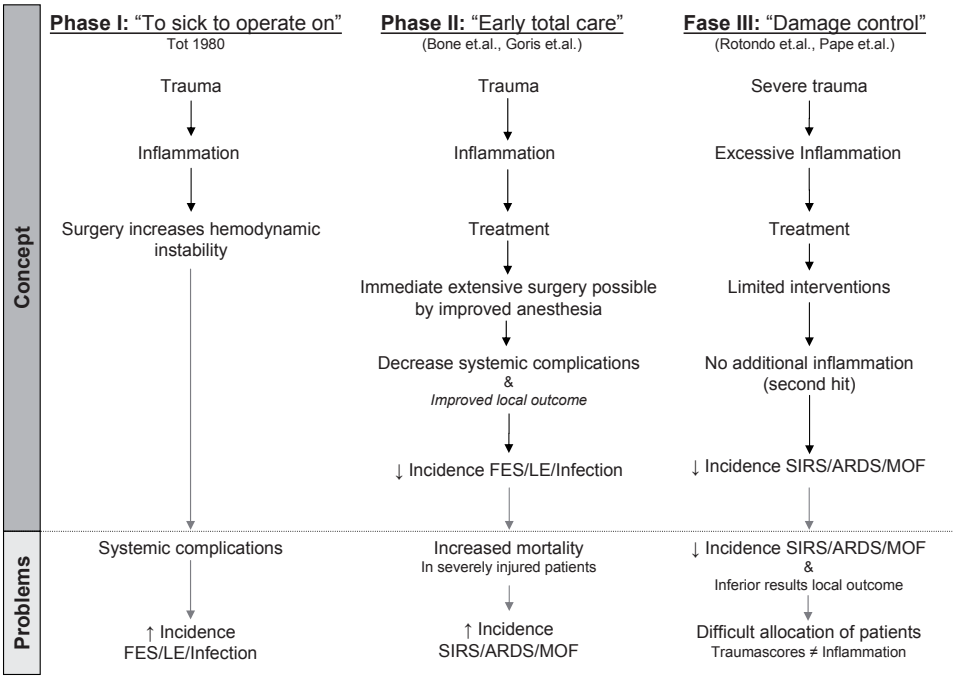

Figure 1. Treatment strategies over the last decades.
The treatment concept for severely injured patients has changed over the last decades. Initially, patients were deemed to sick to operate on, but with improvement of anaesthesia elaborate surgery became possible. Although the early total care regime resulted in a reduced incidence of complications, the most severely injured patients still developed multiple organ failure. Damage control surgery and orthopaedics was invented, which appears to result in a lower incidence of organ failure in the severely injured patients, but has an adverse effect on tissue and fracture healing. Correct allocation of patients to the diverse treatment strategies is therefore essential.

CLINICAL COURSE OF ORGAN FAILURE

In trauma patients the pathophysiology which leads to MOF is thought to be similar in the majority of cases. This is in contrast to the development of MOF during sepsis in patients with an impaired immune system, such as with chronic immunological disorders or haematological malignancies. Investigation of organ failure in injured patients enables the analysis of this life threatening disease throughout its' course with a known point of onset.

Based on it's clinical presentation, organ failure after injury can be divided in two types (22,23). Epidemiological studies have shown that organ failure can occur early between 2 – 3 days, or late between 7 – 14 days after the injury is sustained. Post-mortem analysis of patients who died of multiple organ failure revealed leukocytosis in many organs, even in those without an infection present (24). It was hypothesized that an auto-destructive inflammatory response caused the clinical syndrome of multiple organ failure. However, further analysis demonstrated that early phase organ failure is not associated with infections. In contrast, late phase organ failure is virtually always preceded by an infection. The infection and subsequent sepsis are often the cause of late phase organ failure (25,26).

During the development of multiple organ failure, organs regularly become dysfunctional in a predetermined order; lungs, liver, gastric mucosa (intestines) and kidneys (23). The lung is the organ that fails the most. The clinical presentation of pulmonary failure was formerly known as the Adult Respiratory Distress Syndrome, but is since 1994 defined as acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). ALI and ARDS are both characterised by the acute onset, bilateral infiltrates on the AP-thorax photo and no signs of hypertension or cardiac failure (Wedge pressure < 18 mmHg) and for ALI a PaO_2/FiO_2 < 300 mmHg with PEEP > 6 cm H_2O and for ARDS a PaO_2/FiO_2 < 200 mmHg with PEEP > 6 cm H_2O (27). Solitary pulmonary failure is a complication that is mostly seen in the early form of organ failure (28,29).

PATHOPHYSIOLOGY OF ORGAN FAILURE

Autopsy studies demonstrated large amounts of leukocytes in the dysfunctional organs, however these organs were unaffected by an infection (24). It was concluded that organ failure is the result of an excessive inflammatory response. Although the elaborated initial surgery resulted in a decrease in the incidence of organ failure after trauma, part of the patients still developed organ failure. In an extensive review of their results, Pape *et al.* demonstrated that extensive surgical procedures in severely injured patients had detrimental effects on outcome (30). They argued that the surgery further amplified the inflammatory response, resulting in a further aggravation of this inflammatory response and subsequently worsened the depth of ARDS and MOF.

Therefore, it was thought that patients with a severe inflammatory reaction after trauma have an adverse outcome in the early total care strategy. The major surgical procedures function as a “second hit” and attribute to the development of organ failure (25,31). Minimizing the surgical burden attenuates the inflammatory reaction and reduces the incidence of inflammatory complications such as organ failure (32-34). This led to a change in approach of the severely injured patient, with high chances of ARDS and MOF. In an attempt to attenuate the inflammatory response, only limited surgical procedures were initiated in the early phase just to control the damage (32). This was achieved by limiting contamination, bleeding and minimizing the extent of orthopaedic procedures with the use of external fixators (35). However, there is evidence that this limited initial surgical intervention, i.e. the “damage control” strategy, impairs local outcome on the site of injury (36).

Determining which patient requires which of the different treatment strategies at hand demands more insight in the pathophysiology of organ failure after injury. It has been well established that the inflammatory reaction after injury has a multi-factorial aetiology. Several inflammatory factors, as components of diverse immunological cascades, have been subject of study (37-43). However, these soluble proteins did not possess sufficient discriminative power to differentiate high and low risk patients. Therefore they have not been widely implicated in daily clinical practise. Current risk assessment and treatment allocation is still based on the clinical expertise of the attending physician. A more objective stratification for the identification of patients at risk for early or late organ failure is needed for a reduction in the incidence of MOF.

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1

INNATE IMMUNE SYSTEM AFTER TRAUMA

Cells of the innate immune system, polymorphonuclear granulocytes (neutrophils or PMNs) and monocytes play an essential role in the development of organ failure after trauma. Excessive activation of PMNs after injury is thought to be responsible for early organ failure. The physiological function of PMNs is to neutralize bacterial pathogens with mechanisms dependent on reactive oxygen species (ROS) and cytotoxic proteins such as proteases (44-47). However, when excessively activated, PMNs can target the tissue of the host with these perilous products, which can lead to organ failure (37,48-51). In contrast, inactivation or exhaustion of PMNs and monocytes would facilitate late phase sepsis. Decreased clearance of bacterial pathogens by PMNs and reduced interaction between monocytes and the adaptive immune system have been suggested as cause of sepsis (52,53). Elucidation of the pathophysiological process of inflammation after severe injury could provide tools for early identification allocation to treatment protocol of injured patients. Patients undergoing major surgical procedures behave similarly as patients after trauma. These patients also could benefit from the knowledge obtained from trauma patients (54).

There is some evidence that a relation exists between the amount of injury and the extent of the inflammatory reaction (55). In an experimental model we found that the MPO (myeloperoxidase; an enzyme used by PMNs) content in the lungs increased in a dose dependent fashion when the ischemia reperfusion time increased (56). If the inflammatory state of the cellular innate immune system can be quantified patients at risk for the development of organ failure can be adequately identified. However, current knowledge on the pathophysiological processes limits this possibility. The development of organ failure needs to be further detailed. Although the cellular innate immune system plays a key role, additional factors beside the cellular innate immune system appear involved.

A higher incidence of dysfunctional organs was found in organ systems already injured, which is particularly the case in pulmonary failure (7,33). This suggested that local tissue factors play an important role in the development of organ failure (Figure 2). This hypothesis is supported by the absence of PMNs in the alveolar space after ischemia reperfusion injury, while the MPO content was increased and

alveolar oedema was present (50,56). Thus, although PMNs accumulate in the pulmonary vasculature during inflammation, these cells can not enter the interstitial space without an additional factor.

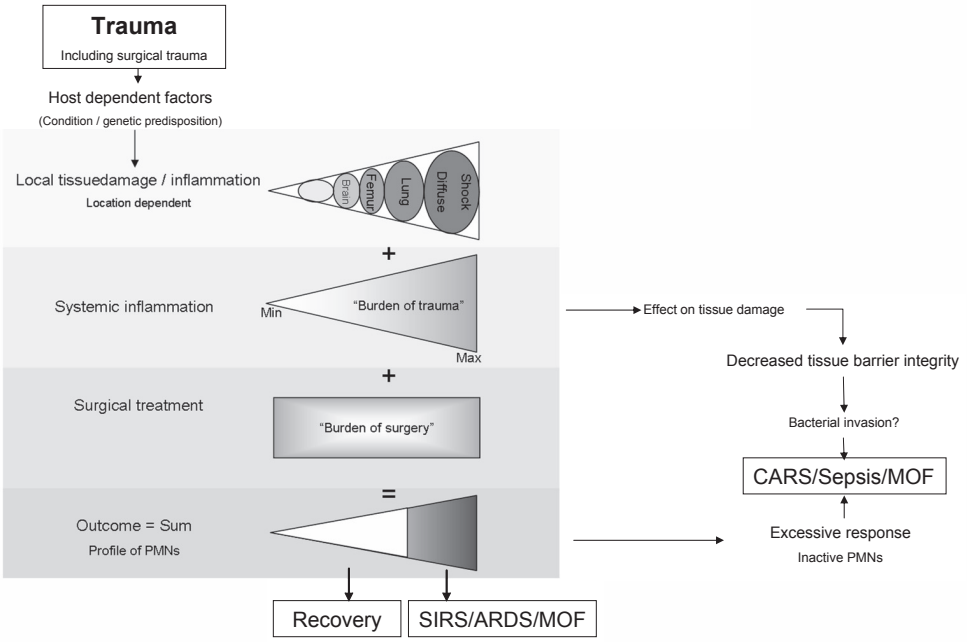

Figure 2. Impact of trauma and surgery on the development of organ failure.
During injury, organs can be damaged and endothelium can be activated. In addition, injury induces an inflammatory response. It is thought that surgery enhances this inflammatory response, thereby inducing organ failure in the severely injured patients with a pronounced inflammatory response. The initial trauma, the subsequent surgery and the inflammatory response cause damage to the tissues. This leads to a decreased barrier integrity, which might facilitate bacterial invasion.

BIOLOGY OF INNATE IMMUNE CELL PHENOTYPE

Quantification of the inflammatory response has been attempted by the measurement of both humeral proteins in body fluids such as serum and plasma and cell associated features such as expression of surface proteins as single markers (57). However, analysis of these single mediators did not provide satisfactory results, as the situation after trauma is often complex (58). Especially studies performed on humeral factors demonstrated a large interpersonal variation and are for that reason less suitable

for clinical implementation (43). Immune cells express receptors for many of these inflammatory mediators and, therefore, integrate all these different signals. The resulting phenotype can be used as read-out for the complex inflammatory signals in both chronic and acute inflammation.. The pro- and anti-inflammatory cytokines, complement fragments and mediators liberated from coagulation pathway all have impact on the phenotype of immune cells. Particularly, effector cells of the innate immune system, such as PMNs and monocytes change their phenotype under these conditions (57,59).

The study of cellular phenotypes of cells of the innate immune system is complicated by the absence of clear definitions of phenotypes, subpopulations, activation and differentiation stages and priming status. Although we realize that there is an overlap in the definitions of phenotype, subpopulation and differentiation stage, the following definitions are used in this thesis:

- *A phenotype* of innate immune cells is defined as cells that are functionally distinct from other cells. Very typical is the situation that inflammation affects the whole population of blood neutrophils or monocytes and leads to marked change in all cells at a certain time of sampling. Cells change rapidly in response to inflammatory mediators and this change can also be induced in vitro. A clear example is the priming phenotype which is seen in granulocytes *in vivo* in chronic inflammatory diseases such as asthma and COPD (60,61). Under these conditions granulocytes acquire an uniform priming phenotype at the time of sampling. A similar phenotype can be induced *in vitro* by treatment of the cells with cytokines such as TNF, GM-CSF and IL-5 (62). In addition, this priming phenotype can be reversed (63).

- *A subpopulation* of innate immune cells is defined as a cell population that can be identified on the basis of expression of distinct markers and/or unique functionality. Subpopulations exist next to each other at the time of sampling. A clear example are the cells characterized by distinct expression of certain cell surface markers such as VLA-4, HLA-DR, L-selectin and/or FcγRIII (see **chapters 5-9**). The induction of subpopulation *in vitro* is difficult and when possible requires prolonged incubation with cytokines such as IFNγ.

- *A differentiation stage* is defined as cells at a certain stage of their normal differentiation in the bone marrow. Cells with different maturation stages can be liberated from the bone marrow.

There are several techniques to investigate the cellular phenotype: 1) by measurement of the release of granule products, 2) by measurement of de novo protein synthesis and 3) by measurement of functionality:

1. Cell surface markers: A well known example of the first method in trauma patients is the measurement of CD11b (MAC-1) on PMNs. Although this marker is considered the most precise surface protein for the detection of activated cells, it is rather insensitive (38). In order to provide a more sensitive marker, alternative proteins have been investigated. Some of these proteins are shed from the cellular surface after activation, such as L-selectin (CD62L). The soluble form of this protein (sL-selectin) has been intensively analyzed to quantify the inflammatory response. However, an extensive literature review provided no evidence for a relation between the amount of soluble L-selectin and the development of organ failure (64).

2. Proteomics/Genomics: Analysis of gene transcription and protein synthesis by for instance proteomics. can be performed with techniques ranging from western/northern blots to genomics/proteomics. Although these methods provide large amounts of information it is time consuming and requires isolation of many cells, which might induce artificial activation (65).

3. Functionality: A multitude of assays is available to measure the functionality of innate immune cells. Again many of these techniques are not very practical to apply in clinical studies, because they are laborious, need many isolated cells and biases by isolation artefacts. However, analysis of inside-out control, of immune receptor function is very sensitive and can be performed in whole blood assays. . The functionality of several immune receptors, such as integrins and Fc-receptors are tightly regulated by cellular signals initiated from activated cytokine and chemokines receptors (66,67). This mechanism determines whether an innate immune cell can respond to adhesion ligands or immunoglobulins. This inside-out control typically

occurs in the peripheral blood and is, therefore, an excellent target for the determination of preactivation of these cells. Excellent antibodies became available that recognize only the activated forms of both integrins (68) and FcγRII (66,69). The downside of measuring inside out control by these antibodies is that non-specific activation by *ex vivo* manipulation can result false interpretation. Therefore, these measurements should be performed on whole blood chilled immediately upon collection.

Analysis of the modulation of innate immune cells in the context of trauma has been focussed on activation markers with low sensitivity (e.g. CD11b expression) mainly on single time points. These studies had limited success in defining and prognosing pathology following multi trauma. This thesis focussed on new more sensitive markers and was designed to obtain more insight into the mechanisms causing inflammatory complications after trauma.

OUTLINE AND RESEARCH QUESTIONS IN THIS THESIS

The central theme in this thesis is the pathophysiology of injury-induced inflammation and its clinical consequences. In **chapter 2** the most important components of the innate immune system are reviewed. While damage to organs reduces the threshold for the development of clinical symptoms, organ failure is caused by excessive inflammation (Figure 3). Neutrophils (or PMNs) play an essential role in this excessive inflammation and consequently the development of organ failure after trauma. Hence, most of the research performed in this thesis focused on these cells (**chapter 3–6, 9)**.

Monocytes are essential in the clearance of inflammation. In addition, monocytes are important in the phagocytosis of bacteria and antigen-presentation to the adaptive immune system. It is thought that the immune system is actively down-regulated following injury. During this state patients are prone for the development of sepsis with subsequent organ failure. Therefore, monocyte changes during injury induced inflammation were analyzed in relation to organ failure (**chapter 7, 8**).

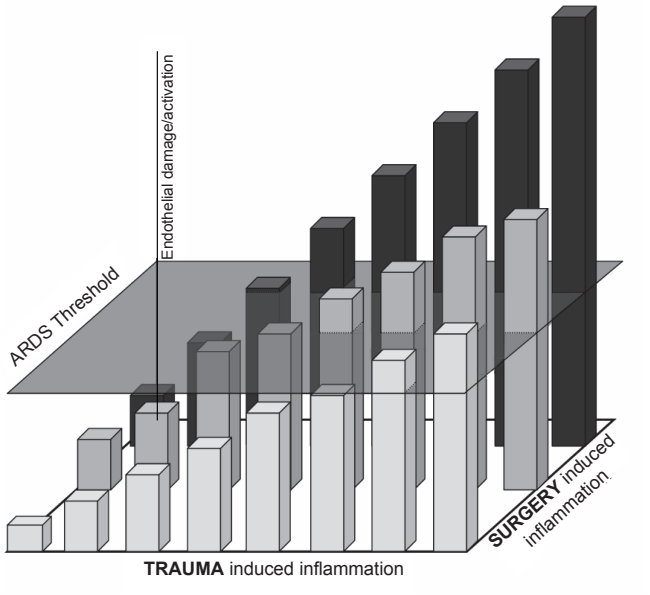

Figure 3. Hypothesis for the development of ARDS after trauma and subsequent surgery.
Injury induces a certain amount of inflammation. In addition, surgery is thought to induce or enhance the inflammatory response. Activation or damage of the endothelium is involved in the extravasation of innate immune cells. These three factors determine the onset of clinical symptoms of organ failure after trauma.

The studies presented in this thesis were guided by the following research questions:

- Can cellular parameters in the circulation be used as a read-out for the severity of innate immune activation (i.e. inflammation) after trauma and do these parameters represent processes in the interstitial space? (**chapters 3, 4, 7**)
- Is the magnitude of the cellular innate immune response related to the development of early phase organ failure? (**chapters 4, 7**)
- What impact does surgery (i.e. primary or secondary intramedullary nailing) have on the severity of inflammation or endothelial damage/activation in relation to the development of ARDS? (**chapter 5**)
- Is the magnitude of the initial inflammatory response related to the subsequent state of immune paralysis and development of late phase organ failure? (**chapter 6**)
- Is the late phase sepsis related state of innate immune, caused by active immunological down-regulation or by exhaustion or redistribution of the innate immune system? (**chapters 6, 8, 9**)

1

In order to study the innate immune response in relation to the development of organ failure after injury, several prospective cohort studies were developed. In a first cohort, it was tested if PMN characteristics in the circulation can be used to analyze the inflammatory response and cellular processes in the tissues (**chapter 3**). In a second cohort, the effect of trauma and surgery on cellular innate immune activation and the development of organ failure was investigated (**chapters 4, 5, 7**). In a third cohort, longitudinal measurements were performed in severely injured patients prone for the development of sepsis, to analyze the kinetics of the innate immune response and the presence of neutrophil and monocyte subpopulations for the development of immune paralysis (**chapters 6, 8**). Finally, several small cohorts were used to analyze innate immune cells in different tissues during severe inflammation, in order to investigate mechanisms leading to immune paralysis (**chapter 9**).

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CHAPTER 2

TRAUMA: THE ROLE OF THE INNATE IMMUNE SYSTEM

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ABSTRACT

Immune dysfunction can provoke (multiple) organ failure in severely injured patients. This dysfunction manifests in two forms, which follow a biphasic pattern. During the first phase, in addition to the injury by trauma, organ damage is caused by the immune system during a systemic inflammatory response. During the second phase the patient is more susceptible for sepsis due to host defence failure (immune paralysis). The pathophysiological model outlined in this review encompasses etiological factors and the contribution of the innate immune system in the end organ damage. The etiological factors can be divided into intrinsic (genetic predisposition and physiological status) and extrinsic components (type of injury or “traumaload” and surgery or “intervention load”). Of all the factors, the intervention load is the only one which can be altered by the attending emergency physician. Adjustment of the therapeutic approach and choice of the most appropriate treatment strategy, can minimize the damage caused by the immune response and prevent the development of immunological paralysis. This review provides a pathophysiological basis for the damage control concept, in which a staged approach of surgery and post-traumatic immunomonitoring has become important aspects of the treatment protocol. The innate immune system is the main objective of immunomonitoring as it has the most prominent role in organ failure after trauma. Polymorphonuclear phagocytes and monocytes are the main effector-cells of the innate immune system in the processes that lead to organ failure. These cells are controlled by cytokines, chemokines, complement factors and specific tissue signals. The contribution of tissue barrier integrity and its interaction with the innate immune system is further evaluated.

Trauma is one of the major causes of mortality in people under the age of 50 in the Western world. Patients die as a direct consequence of their sustained injuries, or by the additional damage caused by subsequent immune reactions (1). About 5% of the patients admitted after severe trauma develops (multiple) organ failure (MOF). Multiple organ failure is a clinical syndrome in which the functionality of several organs fail subsequently or simultaneously (i.e. liver, lungs, kidneys, heart). This review outlines the initiating factors and underlying mechanisms for the development of post-traumatic organ failure. It provides a pathophysiological basis for the so called damage control concept. This concept involves a treatment strategy in which a staged approach of surgery in severely injured patients and post-traumatic immuno-monitoring have become important aspects, to minimize the negative effects of a dysfunctional innate immune system.

MULTIPLE ORGAN FAILURE

Multiple organ failure after trauma has a multifactorial etiology, which can be divided in endogenous and exogenous factors. Endogenous factors, such as genetic predisposition and physical condition form the basis of the patients susceptibility for the development of organ failure. Recent studies have shown that genetic variations (e.g. TNF-α polymorphisms) are strongly associated with the development of organ failure (2). Exogenous factors, like the injury itself (the “first hit” or “trauma-load”) and the resuscitation or surgical intervention (the “second hit” or “intervention load”) play a key role in the development and clinical presentation of organ failure. Organ damage and subsequent organ failure is the result of a dysfunctional immune system. A localized inflammatory reaction after injury is physiological, which can be explained by the “danger model”, an immunological theory coined by Matzinger. The “danger model” explains that alarm signals can provoke an inflammatory reaction (3). These alarm signals can be secreted by healthy cells or released by necrotic cells, which are present after injury is sustained. The combination of type of tissue and type of alarm signal decides what kind of response follows. Neutrophils and macrophages (effectors) are involved in immune surveillance and injury control and after trauma are activated through mediators (cytokines, chemokines and complement). This local inflammatory response can exacerbate and a systemic inflammatory response (SIRS) develops. When SIRS leads to a multiple organ dysfunction syndrome (MODS) mortality can increase up to 50-80% (Figure 1) (2,4,5).

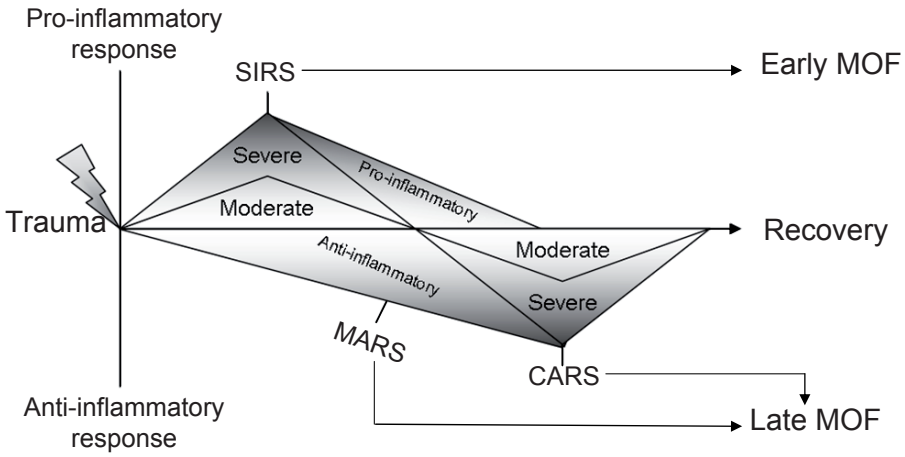

Figure 1. Biphasic model of organ failure.
Depiction of the biphasic model of organ failure (MOF), originally coined by Moore (8). The relative degree of immune activation is displayed on an arbitrary scale on the vertical axis. The horizontal axis indicates the time following trauma. When injury is sustained, a pro-inflammatory response is evoked which can lead to the early version of MOF. At a later stage CARS and MARS can lead to immune paralysis and subsequently, the late form of organ failure.

To restore the equilibrium of the excessive pro-inflammatory reaction, an anti-inflammatory response is evoked. In a propitious case, homeostasis is achieved. However, an overreaction of the anti-inflammatory response can lead to either a compensatory anti-inflammatory response (CARS), or a mixed antagonist response (MARS) (6). In the latter syndrome the pro-inflammatory and anti-inflammatory responses counterbalance each other. In both situations (CARS and MARS), the body is in a state of immune paralysis and is unable to produce an adequate reaction to a new threat (i.e. infection). In this state the patient is extremely prone to micro-organisms as there is a defect in an important defense mechanism formed by the cells of the innate immune system (7). Resulting infections can cause serious complications like sepsis and septic shock with subsequent organ failure (8). In conclusion, SIRS and sepsis (predisposed by CARS or MARS), despite different pathophysiological processes, can all result in multiple organ failure (Figure 2).

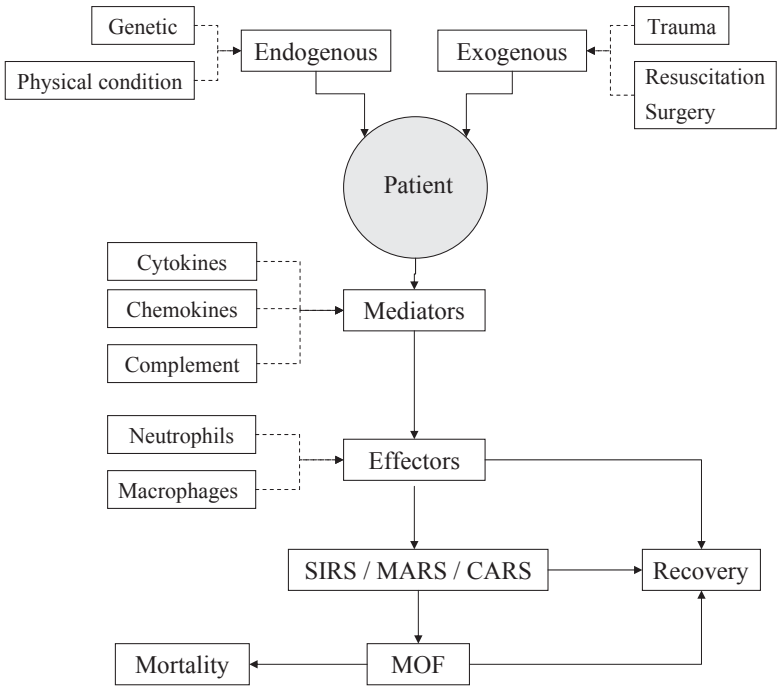

Figure 2. Factors involved in the etiology of post-traumatic organ failure.
Shows the complex of factors, mediators and effectors involved in the development of organ failure. The endogenic factors (genetic predisposition and physical condition) form the basis for the susceptibility of a patient to post-traumatic organ failure. The sustained injury is seen as the first hit on the immune response and the "burden of surgery" is seen as the second hit, which can excacerbate the inflammatory reaction. The mediators stimulate the effectors which cause end-organ damage.

CELLULAR RESPONSE: NEUTROPHILS

Tissue damage leads to the activation of neutrophils and macrophages (9). Hemorrhagic shock induces ischemia and this causes the tissue to change its metabolism to anaerobic. During resuscitation, thus reperfusion, oxygen is transported to the ischemic area in the tissue and radical oxygen species (ROS) are formed. These ROS are chemo-attractors and activators of neutrophils (Figure 3) (10,11). Polymorphonuclear granulocytes have an important role in the defense and debridement of the injured tissue from the first 10 minutes until 3 days after injury (12). Priming, or pre-activation, is an essential step for neutrophils which enhances functional responses of these cells (13,14).

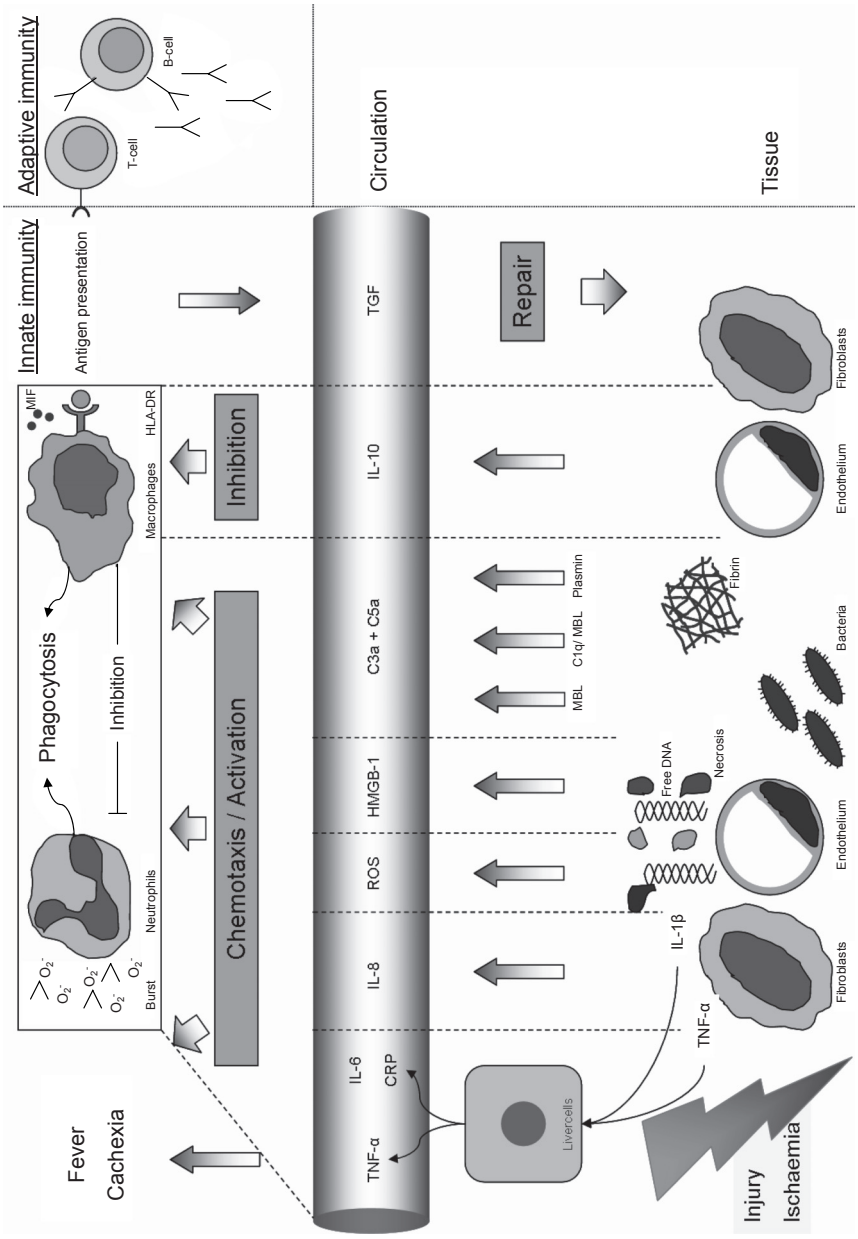

Figure 3. Innate immunity in tissue damage.
Shows the relation between several important factors involved in the pathophysiology of organ failure after tissue injury. The figure is explained in detail in the article. C3a = Complement factor 3a; C5a = Complement factor 5a; O_2^- = Radical oxygen; MBL = Mannose binding lectin; C1q = Complement factor 1q

Priming

Priming is the result of pre-exposure to priming agents, like granulocyte macrophage colony stimulating factor (GM-CSF) or tumor necrosis factor (TNF-α) (15,16). These priming agents are found in increased concentrations in the peripheral blood of severely injured patients and several priming enhanced functions of neutrophils have been demonstrated in traumapatients and patients undergoing major abdominal surgery (17,18). The enhanced functional response after priming encompasses chemotaxis, adhesion, rolling, diapedesis and the oxidative burst.

Oxidative burst

The increased oxidative burst (a cytotoxicity associated response) is necessary to prepare the neutrophils for invading micro-organisms. This increased functional response in the form of oxidative radical production correlates with the incidence of SIRS and MOF (19). It is thought that the increased cytotoxic potential of neutrophils is a sign of an uncontrolled inflammatory reaction which causes damage to tissues and leads to early MOF. Maximum increased priming for cytotixicity (after *in vitro* stimulation) was found between 3 and 24 hours after trauma (20). An elevated priming index (elevation of the spontaneous oxidative burst from normal values) was found between day 2 and 5 after trauma and remained above normal until day 13 after trauma (21). This increased oxidative burst is thought to cause additional damage to the tissue. Furthermore, the newly formed ROS contribute to the attraction and subsequent activation of neutrophils, which attributes to the accumulation of activated neutrophils in the tissue (11). The harmful effects of neutrophil activity can only occur when these cells enter the tissue, therefore, an interaction between the neutrophil and endothelium has to occur. Interactive processes with the endothelium, like rolling, adhesion and diapedesis, are necessary for leukocytes to exert their function in the target tissue. These leukocyte functions are altered after trauma and during early organ failure.

Rolling

Rolling is regulated and controlled by selectins. These proteins undergo interactions which slow down the leukocytes at the endothelial cell surface (22). E-selectin, which can bind carbohydrate molecules, is presented on endothelial cells and are involved in the initial contact between endothelial cells and leukocytes. Leukocytes express L-selectin on their surface and is important in secondary tattering, a process

in which attached leukocytes provide adhesion for other leukocytes. As a result, leukocytes bind directly to each other and thus enhance the effect of the homing process (23). L-selectin is shed after interaction with the endothelium and integrins take over to regulate the next step in the transmigration process. Some authors have reported a correlation between decreased L-selectin expression on leukocytes and the incidence of SIRS or early MOF, indicating to a relation between the degree of neutrophil activation and the development of complications occurring during the pro-inflammatory phase (24,25). The shedded molecules can be found as soluble factors in serum (sL-selectin). Consequently, the activation level of the neutrophil population is associated with the level of sL-selectin in the blood. Maximum sL-selectin levels in serum are found 6 hours after trauma, giving an indication on the time when the highest amount of neutrophils have lost their L-selectin to migrate to the tissue (26).

Adhesion

Integrins are involved in the adhesion of leukocytes to the endothelium. The integrin αmβ2, or MAC-1 (CD11b/CD18) and the ligand ICAM-1 (intercellular adhesion molecule 1) form a high affinity stationary connection between leukocyte and endothelium. This is in contrast to the low affinity, reversible binding of selectins. Functional integrins are only expressed upon activation of the neutrophil and are necessary for an adequate transmigration process (27). An increased expression of MAC-1 is found on neutrophils from patients who were admitted with an ISS > 16 as compared to traumapatients with an ISS < 16, indicating to activated neutrophils after injury (26). Increased expression of MAC-1 is also found in experimental models and patients who received large amounts of blood products for resuscitation (28). In contrast, during late organ failure a decreased expression of MAC-1 is found on neutrophils from patients who died from the consequences of sepsis as compared to patients who survived (29). These results are congruent with the decreased percentage of MAC-1 positive neutrophils of critically ill surgical patients with severe disease as compared with surgical intensive care patients with less severe disease (30).

ICAM-1, normally expressed by activated entothelium, also exists as a soluble factor in serum (sICAM-1) and increased concentrations in septic patients correlate with the incidence of organ failure and mortality (26,29). Expression of MAC-1 or sICAM

give an indication on the activation of neutrophils or tissue and are both related with the development of organ failure. A high activation state of neutrophils is associated with SIRS, whereas a low activation state is related with sepsis. The activation state of neutrophils changes over time and could provide a partial explanation for the biphasic pattern of MOF (8).

Apoptosis

Billions of neutrophils are produced by the bone marrow on a daily basis (31). Neutrophils which have completed their function in the tissue go into apoptosis. Apoptosis is necessary to limit the absolute number of neutrophils present in the tissues. After trauma a delayed programmed cell death (delayed apoptosis), has been demonstrated (21). This delay is seen directly after trauma and can last up to 3 weeks (32). Delayed apoptosis causes accumulation of neutrophils in the tissue, where they can produce more cytotoxic products (oxygen radicals and proteases) and promote tissue damage. This delayed apoptosis is found in patients with sepsis as well (33). Bacterial products can inhibit apoptosis. In contrast to the large population of neutrophils which show decreased apoptosis, a relative larger subgroup of neutrophils exhibits signs of apoptosis in whole blood (34).

Neutrophils are essential in the pathophysiology of trauma-related organ failure (35). Blocking or depletion of neutrophils in experimental models results in a reduction of organ failure in the pro-inflammatory (early) phase. However, overall organ failure increased due to an increased incidence of organ failure caused by severe infections during the anti-inflammatory (late) phase (36). For future studies it seems more favorable to regulate the neutrophil compartment instead of shutting this important defense mechanism down.

CELLULAR RESPONSE: MACROPHAGES

Neutrophils are important in the first response to injury, as they form the first natural immunological defense against micro-organisms and occur within 10 minutes after injury is sustained. Subsequent to the initial responders, macrophages are recruited. These cells orchestrate the mechanisms involved in wound healing (37). They function in wound debridement and secrete biologically active substances, called growth

factors (e.g. TGF). TGF plays an important role in cell growth and tissue repair and thus essential in the wound repair after trauma (38). Macrophages have a lasting influence on the subsequent phases of proliferation and tissue differentiation. Most of the macrophages are derived from blood monocytes. Differentiation of monocytes into macrophages and activation of macrophages takes place at the wound site. The cells reach the wound area in great numbers, attracted by chemotactic signals from injured tissue, the cytokines produced by immune cells and the presence of bacterial products,. A macrophage can phagocytose micro-organisms and, in addition, is also capable of modulation of the adaptive immune response by mediating antigen presentation to lymphocytes. Antigens are taken up and partially degraded by the macrophage and then presented to a T-lymphocyte for recognition by MHC-II molecules. In injured patients, macrophages form the bridge between innate and adaptive immunity.

Downregulation of MHC-II expression leads to decreased antigen presentation capacity and therefore higher susceptibility for infectious complications. Several authors have shown MHC-II suppression after trauma, which correlated with the incidence of infectious complications. MHC-II suppression on monocytes and macrophages is considered to be one of the most important features of immune suppression after injury. Some authors have suggested CARS to be defined as less than 30% expression of MHC-II on monocytes (29).

CYTOKINES AND CHEMOKINES

In past years many studies focused on the relation between pro- and anti-inflammatory cytokines and the development of SIRS and CARS. Tissue damage causes the endothelial cells, fibroblasts, lymphocytes and tissue-macrophages to produce these cytokines (39). At first, pro-inflammatory cytokines, such as TNF-α, GM-CSF, interleukin 1β (IL-1β), IL-6 and IL-8 are produced (40).

TNF-α and IL-1β

TNF-α and IL-1β are situated at the beginning of the pro-inflammatory cascade (Figure 3). IL-1β acts primarily locally, but induces a systemic release of TNF-α and IL-6 by stimulation of hepatic cells. IL-1β and TNF-α increase the concentration of

neutrophils in the circulation, trigger an increased chemotactic response, decrease the apoptosis ratio, amplify phagocytosis and cause an increased permeability of the endothelium. These actions lead to accumulation of activated inflammatory cells in the tissue (41,42). IL-1β has been identified as an important cytokine in patients with the acute respiratory distress syndrome (ARDS), a neutrophil mediated disease. Only small amounts of biological active IL-1β are necessary to induce inflammation in the pulmonary compartment (41,43). TNF-α has a more ambiguous role as its function is depending on the context of the tissue. It participates in an adequate immune response in its physiological role in the circulation. TNF-α depleted or inhibit mice were incapable of handling an infectious threat (44). In addition, administration of TNF-α reduces mortality in a sepsis model performed on rats (45). In a clinical situation however, increased serum concentrations of TNF-α correlate with the development of septic shock in trauma patients. It is unclear whether this is a causal relationship, or whether this is merely an epiphenomenon and the high levels of TNF- α are a sign of the host coping with tissue injury or invading micro-organisms (46).

IL-6 and IL-8

Both IL-1β and TNF-α stimulate the production of IL-6 and IL-8. IL-8 is an important chemokine in the cascade that leads to leukocyte recruitment and activation in the tissues (47). Production of IL-8 induces an influx of neutrophils towards the site of production, for example in patients with ARDS to the lung. The IL-8 concentration in the pulmonary fluid of patients with a thoracic trauma is seen as an indicator for the occurrence of ARDS, as increased levels correlate with the incidence (48). IL-6 is an acute phase protein such as C-reactive protein (CRP). The protein's role in the pathophysiology of trauma-related organ failure remains unclear due to the non-specificity of IL-6. However, epidemiological data shows evidence of a correlation between increased IL-6 levels after trauma and the Injury Severity Score (ISS), the incidence of complications and mortality. A correlation also exists between the IL-6 concentrations after intramedullary osteosynthesis and the development of ARDS (49). IL-6 can be seen as marker for the severity of trauma and, despite its indistinct role in the pathophysiology, can be a resource in triage, diagnosis and prognosis.

MIF

Macrophage migration inhibitory factor (MIF) is a pleiotrophic molecule exerting its functions as an anterior pituitary hormone, a pro-inflammatory cytokine and high activity enzyme. It is produced abundantly by monocytes/macrophages and acts in an autocrine/paracrine manner to up-regulate and sustain the activation responses of diverse cell types (50). MIF is present in preformed, cytoplasmic pools within the macrophage and is *in vitro* rapidly released to microbial products (both lipopolysaccharide and Gram-positive exotoxins) (51). This is also seen *in vivo* as high circulating levels of MIF were found in septic and septic shock patients, in contrast to normal levels in non-septic, multi-traumapatients (52). In addition, circulating levels of MIF correlated with positive tests for bacterial cultures (53). MIF induces vascular hyporeactivity and could be the threshold protein in the occurrence of septic shock.

MIF overrides the anti-inflammatory actions of glucocorticoid and acts via the stimulation of pro-inflammatory cytokines like TNF-α, IL-1β and IL-8 via the NF-κB pathway. MIF prevents apoptosis by reduction of the p53 tumor suppressor gene. Therefore, high concentrations of MIF lead to a sustained pro-inflammatory response and delayed apoptosis of cells of the innate immune system. High concentrations of MIF have been found in the alveolar spaces of patients with ARDS (54). Those authors suggest that MIF acts as a mediator sustaining the inflammatory response in ARDS and that an anti-MIF strategy may represent a novel therapeutic approach in inflammatory diseases like ARDS.

HMGB-1

High-mobility group box (HMGB)-1 was originally identified as a nuclear DNA-binding protein that functions as a structural cofactor for proper DNA-transcriptional regulation and gene expression (55). Recent studies indicate that immune cells can liberate HMGB-1 into the extracellular milieu where it functions as a pro-inflammatory cytokine. HMGB-1 is recognized by cells of the immune system as a necrotic marker to signal tissue damage. It can be passively released by damaged or necrotic cells or actively secreted by macrophages and neutrophils. It is seen as a late mediator as it is secreted by macrophages *in vitro* 20 hours after stimulation. Increased levels of HMGB-1 result in the disruption of endothelial barrier functions, leading to vascular leakage and tissue hypoperfusion, similar to that observed in sepsis. *In vivo* increased levels of HMGB-1 are shown in patients with severe sepsis (56). In experimental

studies inhibition of HMGB-1 prevents endotoxin- and bacteremia-induced multiple organ failure and improves survival (57). In an experimental model intratracheal administration of recombinant HMGB-1 induces a dose-dependent interstitial and intra-alveolar neutrophil accumulation and lung edema at 8 and 24 hours post-administration (58). Neutralizing HMGB-1 antibodies have been reported to reduce mortality in experimental models of acute lung injury or ischemia/reperfusion injury (55).

IL-10

IL-10 plays an important role in the anti-inflammatory response. This protein is produced simultaneously with the pro-inflammatory cytokines, but peaks hours later. One of the functions of IL-10 is the negative feedback on the production of TNF-α, IL-6 and IL-8. The cytokine IL-10 plays a pivotal role in the suppression of monocyte function as it directly decreases MHC-II expression (59). IL-10 causes the MHC-II molecules on the surface of monocytes and macrophages to be internalized (60). Increased levels of IL-10 have been shown to correlate with the development of sepsis or adverse outcome during sepsis. However, IL-10 is unable to discern outcome or severity of illness on an individual level. In addition, the biological activity of IL-10 is dependent on the pH and temperature, which is often altered in severely injured or septic patients (61). It is unclear, whether increased IL-10 levels have a causal relationship with the development of complications, or whether it is a sign of a struggling host.

COMPLEMENT FACTORS

Complement is a collection of proteins, which are involved in the protection against micro-organisms. It is one of the most preserved defense mechanisms during the evolution of the immune system. Next to activation by immune complexes complement can bind conserved bacteriological compounds (e.g. bacterial carbohydrates, bacterial antigens) and altered self-products (e.g. free DNA) via mannose binding lectin, ficolins or complement factor C1q (62). Complement can opsonize bacteria by complement factor C3b, a split product of C3. Opsonisation leads to attraction of leukocytes to these bacteria. In the absence of bacterial or altered self products, the complement system can be activated by a connection with the coagulation

system. The coagulation cascade and the complement cascade are connected through plasmin, a product of the trombolytic route that regulates homeostasis in the coagulation. Due to injury large scale activation of the coagulation cascade occurs. In trauma both coagulation factors and tissue damage activate the complement cascade (63). This leads to neutrophil homing to the tissues and activation on the site of injury. Several studies have shown a correlation between activated complement factors (C3a/C3 ratio and C5a) and mortality after trauma (64). *In vitro* is shown that C5a regulates two important aspects of neutrophil function; i) adhesion associated processes and ii) cytotoxic associated processes (65). Complement is one of the most important factors contributing to neutrophil dysfunction, likely due to this dual function. In recent experimental studies, blocking of complement lead to a reduction in pulmonary and intestinal permeability (66). The accumulation of neutrophils in the lung was reduced by blocking the complement factor C5. This is a promising finding, which can lead to novel therapeutic probabilities.

TISSUE INVOLVEMENT

Trauma not only activates the innate immune response, but also alters the barrier integrity of several organs. Intramedullary osteosynthesis of femur fractures is thought to stimulate the innate immune response on a systemic level and is associated with an increased incidence of ARDS (67). On the other hand, isolated thoracic injury induces local injury but is associated with the occurrence of ARDS as well (68,69). When additional injury to the lungs is present during intramedullary osteosynthesis, the incidence of ARDS can increase two-fold (70). This phenomenon suggests a synergistic mechanism between the activation of innate immunity and the loss of tissue barrier function (Figure 4). The contribution of the loss of barrier function comes to attention not only in pro-inflammatory complications such as ARDS, but also in anti-inflammatory complications such as sepsis. A correlation has been shown between increased intestinal permeability and the occurrence of infectious complications (71). It is thought that bacterial translocation due to increased intestinal permeability cause septic complications in an immune-compromised host (72). In the pro-inflammatory phase, organ failure often precedes infection and an additional infection “only” deteriorates the remainder of the organ functions. This can be explained by the danger model, which states that innate immunity is already

triggered after trauma, but can receive an additional stimulus in the form of invading bacteria. During the anti-inflammatory phase infection often precedes organ failure, giving it a more prominent role in the development of this severe complication. Despite the clear correlations between increased intestinal permeability and the incidence of sepsis in experimental settings, the relation in the clinical setting is less clear (73,74). It is also known that the interpretation of immunological signals by cells of the innate immune system is dependent on environmental and tissue specific factors and for complications to become clinically evident, a threshold needs to be reached in specific tissues.

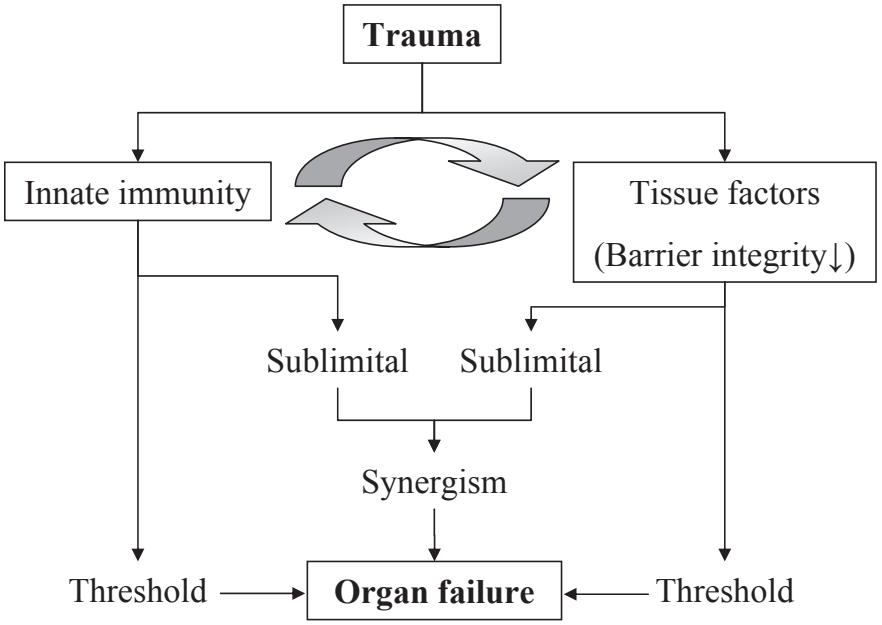

Figure 4. Relation between innate immunity and tissue factors following trauma.
Shows the synergistic relation between the activation of the innate immune system and the loss of organ barrier functions. Both can act independently to promote organ failure, or when working together (synergize) induce clinical evident organ failure.

A cut-off point of >800 pg/ml IL-6 has been proposed as a prognostic marker and has been suggested for immune-monitoring in the damage control strategy. Unfortunately, at present no scoring system or prognostic tool is conclusive enough to adequately predict an adverse outcome on an individual level. The complexity of organ failure and the often ambiguous role of the different factors prevents a clear cut

target for therapy. Many studies investigated individual mediators or effectors, which limits the interpretation of effector function in the tissues. Furthermore, cytokines often have crosstalk or cumulative effect and insight in the group effect of cytokines and chemokines would provide more accurate information about the net effect.
The scoring systems ought to be used to define the appropriate therapy. Damage control surgery and damage control orthopedics are currently used strategies to limit the incidence of organ failure after trauma (75,76). Timing of surgery is essential in this damage control approach and recent literature provides a timeframe for planning interventions (77,78). This timeframe, which is based on database analysis, is not fully complementary with the activation status of the innate immune system. According to the measurements of neutrophils (oxidative burst and L-selectin) hyper-inflammation is at its maximum 6 hours after trauma, whereas according to the damage control timeframe hyper-inflammation is present between day 2-4 (20,26). Despite this problem in defining the timeframe, solutions are sought to prevent the excessive inflammation. A recent therapy that became available, hemoglobin based oxygen carriers as alternative for packed red blood cells, show promising results in limiting the inflammatory response (28). The start of hypo-inflammation is less well defined and more individual determined which makes therapy more difficult.

CONCLUSION

Several studies have shown a relationship between the severity of trauma and the resulting immune response (79). The injury to the host can be expressed in scoring systems and these have become important prognostic tools to calculate the risk based on clinical signs and symptoms in combination with inflammatory parameters (68). It is likely that a threshold needs to be reached before clinical symptoms become evident. The loss of barrier integrity of different organs seems to play a major role in the development of complications in both the pro-inflammatory period and the anti-inflammatory period. Studies which focus on the interaction between host and innate immunity are to be performed to resolve the post-traumatic complications resulting in organ failure. Immune-monitoring with interpretation of group effects of cytokines or analysis of effector cells in interaction with tissue may lead to more intensive immune-monitoring and the adjustment of therapeutic and supportive strategies for the optimalization of care for trauma-patients.

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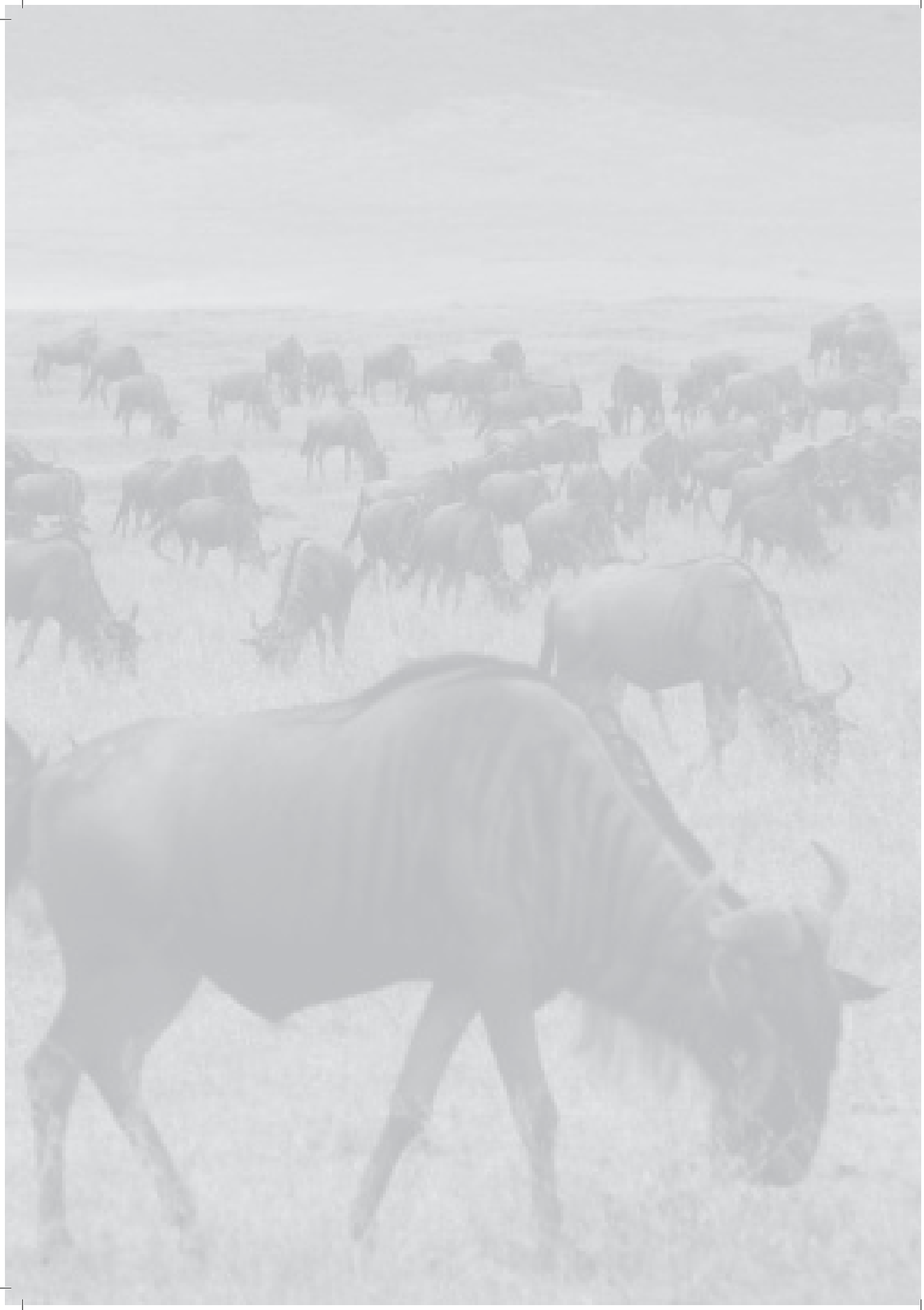

CHAPTER 3

ABERRANT REGULATION OF PMN RESPONSIVENESS IN MULTITRAUMA PATIENTS

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ABSTRACT

A systemic inflammatory response often follows severe trauma. Priming (pre-activation) of polymorphonuclear phagocytes (PMNs) is an essential first step in the processes that lead to damage caused by the systemic activation of innate immune response. Until recently priming could only accurately be measured by functional assays, which require isolation of cells, thereby potentially inducing artificial activation. The aim of this study was to identify primed PMNs in response to trauma by using a whole blood analysis with a broad detection range. Twenty-two traumapatients were analyzed for PMN priming with novel developed antibodies recognizing priming epitopes by flowcytometric analysis. Expression of priming epitopes on PMNs was analyzed with respect to time, injury and disease severity. Expression of priming epitopes in the circulation was compared with expression profiles of PMNs obtained from lungfluid. Fourteen healthy volunteers served as controls. Expression of priming epitopes on peripheral blood PMNs of injured patients was similar as found in healthy controls, whereas highly primed cells were found in the lungfluid of injured patients (>50 times increase as compared to peripheral blood cells). In fact, the responsiveness of PMNs towards the bacterial derived stimulus fMLP was markedly decreased in traumapatients. Lack of expression of priming epitopes and the unresponsiveness to fMLP demonstrates the presence of partially refractory cells in the circulation of trauma patients. An increased expression of epitopes found on pulmonary PMNs suggests that optimal (pre)activation of these cells only occurs in the tissues.

Trauma is the number one cause of death for people under the age of 50 in the Western World. Death can occur in the first hours as a direct result of the injuries caused by the trauma. Death can also occur at a later stage caused by multiple organ failure (MOF) mediated by a dysfunctional immune system. About 5% of all patients admitted after severe trauma will develop MOF, a syndrome in which neutrophils are thought to be the main executioners of tissue damage (1,2,3). Stress signals from the injured tissue, activation of the complement system and/or production of chemokines and cytokines are factors thought to contribute to this hyperactive immune response seen in traumapatients (4). Although much research has been focused on the presence of these modulating factors in blood and broncho-alveolar lavage, surprisingly little is known regarding the basic immune mechanisms causing this injury induced MOF.

In normal immune homeostasis, production of pro-inflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) is balanced by the production of anti-inflammatory mediators such as IL-10. It is generally accepted that severe trauma induces an over-stimulation of the immune system, which can cause the early form of MOF. This auto-destructive systemic inflammatory response syndrome (SIRS) is caused by (over)production of pro-inflammatory mediators by both resident and inflammatory cells in the injured tissue (2). On the other hand, a late disproportionate production of anti-inflammatory mediators after trauma can lead to immune suppression. This is generally referred to as the compensatory anti-inflammatory response syndrome (CARS) (5). This anti-inflammatory reaction is thought to cause severe immune suppression, which can facilitate sepsis and the associated late form of MOF (2,6,7,8).

Activated polymorphonuclear phagocytes (PMNs) are instrumental in the development of early MOF in traumapatients. It is shown that PMNs have a higher oxidative response towards the bacterial product N-formyl-methionyl-leucyl-phenylalanine (fMLP) after *in vitro* pre-activation with granulocyte macrophage colony stimulating factor (GM-CSF) or TNF-α (9). This process of enhanced functional response is generally referred to as “priming” or pre-activation. Unfortunately, priming is still a poorly-defined concept in terms of signal transduction. Therefore, the most widely used definition of priming is an increase in functional responses of PMNs to stimuli after pre-exposure of the cells to priming-agents (10). This phenomenon also

occurs *in vivo* under the control of local and/or systemically produced mediators. In traumapatients the oxidative burst, after stimulation with fMLP, was increased as compared with controls and was seen as a determinant of *in vivo* priming (11).

Unfortunately, no accurate cell surface markers are available that allow visualization of priming using flowcytometry. Some authors have reported the up regulation of integrins (CD11b/CD18) and CD66 or down regulation of L-selectin in traumapatients (12,13,14). However, the range of induced receptor expression was small which complicates the application of these markers for clinical studies. Therefore, up to now, priming could only be investigated by functional assays.

Recently, we developed two human monoclonal phage antibodies, designated A17 and A27 (15). These antibodies recognize epitopes that are up-regulated on phagocytes, including neutrophils, which are primed *in vitro* and *in vivo* (16). *In vitro* cytokine induced expression of these priming epitopes mirrors the dose response curves of functional priming induced by the same cytokines in with a sufficient detection range (17). These antibodies proved capable of identifying primed PMNs in the peripheral blood of COPD patients *in vivo* (15). This direct FACS method is performed on whole blood and, therefore, gives a better indication of PMN priming in the traumapatient in comparison with functional assays of isolated cells (18). Isolation of PMNs induces clear differences in the phenotype of the cells (19). In addition, it has been shown that isolation of PMNs alters the behaviour of these cells, which complicates extrapolation to *in vivo* PMN function (18). In this study, we have investigated the differences in priming of peripheral blood PMNs from traumapatients, by measuring the expression of priming epitopes recognized by the A17 and A27 monoclonal phage antibodies.

MATERIALS AND METHODS

A group of thirteen patients was analyzed in time and disease severity was measured. Nine healthy volunteers served as control. A second cohort of six patients and six controls provided blood samples for comparison between functional responses and expression of priming epitopes. A third group consisted of three patients who developed acute lung injury and provided lung aspiration samples. So, a total of twenty-two patients and fifteen controls were enrolled in this study. The local ethical committee approved the study and informed consent was obtained from all patients or their spouses, in accordance to the protocol.

Patients: Time series and severity of disease

Thirteen traumapatients (Injury Severity Score [ISS] > 16) admitted at the Department of Traumatology, University Medical Center Utrecht, were included in this study (table 1). The ISS > 16 was chosen based on findings in previous studies, which showed increased oxidative burst in this group of patients (20). The median ISS was 21 (range 16-38). The patients were all males with a median age of 40 (range 20-78). The median APACHE II score on admission was 10 (range 0-22) (21). Three patients died as a result of severe head trauma. None of the patients with neurological injuries received corticosteroids for their treatment. One patient died as a result of cardiac arrest based on a severe myocardial contusion with arrhythmia. All four patients died between day 2 and 4 after admission. Infectious complications were registered. The Sepsis Score and APACHE II Score were calculated on a daily basis to assess the severity of illness (21-23). Nine healthy adults with a median age of 26 (range 20-31) provided blood samples that served as controls. This control group consisted of 4 males and 5 females.

The day of injury was defined as day 0. Blood samples were taken at admission, day 1 and every other day during the first week after trauma. The timing of sampling was chosen based on findings in other studies, in which priming index (oxidative burst) was most increased between day 2 and 5 after trauma (11).

Table 1. Trauma patient characteristics.

	Age	ISS	Major injuries	Surgery	Follow up	Blood products
Time series						
1	65	17	Neurological injury	Trepanation day 1	Deceased: brain damage	None
2	52	20	Severe chest injury	None	Deceased: cardiac arrest	None
3	38	38	Chest injury; spinal cord	ORIF day 1	ALI day 1; Pneumonia day 4	6 PRBC; 4 FFP
4	26	21	Chest injury; spinal fracture	None		None
5	58	16	Chest injury	None		None
6	39	21	Fractures extremities	ORIF day 1 + 7		3 PRBC
7	40	25	Chest injury; fractures extremities	ORIF day 1 + 4		None
8	33	18	Chest injury; facial fractures	ORIF day 5		None
9	78	32	Neurological; chest injury	None	Deceased: brain damage	2 PRBC; 2 FFP
10	50	25	Neurological injury	Trepanation day 1	Deceased: brain damage	None
11	47	25	Chest injury; fractures extremities	ORIF day 4		None
12	20	26	Neurological; chest injury	None	ALI day 1	None
13	38	16	Chest injury (penetrating)	Thoracotomy day 1		23 PRBC; 2 FFP
Functional response						
1	24	18	Fractures extremities	ORIF day 1		None
2	18	29	Pelvic fracture; fractures extremities	ORIF day 1		4 PRBC; 21 FFP
3	79	17	Fractures extremities; chest injury	ORIF day 1		None
4	65	18	Neurological injury; chest injury	None	ALI	None
5	18	16	Abdominal injury	None	ALI	None
6	40	18	Chest injury	None	ARDS day 1, pneumonia day 7	None
Lung aspiration						
1	20	25	Chest injury; fractures extremities; abdominal injury	CREF day 1, TPL		2 PRBC
2	38	16	Fractures extremities	ORIF day 1		8 PRBC
3	33	25	Chest injury	None		4 PRBC

Listed are the characteristics of the traumapatients who were admitted. The region of injury is conform the different headings of the abbreviated injury score (AIS). Pt = Patient number; ISS = Injury Severity Score; ALI = Acute lung injury; ARDS = Acute respiratory distress syndrome; PRBC = Packed red blood cells; FFP = Fresh frozen plasma; ORIF = Open reduction, internal fixation; CREF = Closed reconstruction, external fixation; TPL = Thoracophrenicolaparotomy.

Patients: Functional response

It has been shown that the oxidative burst is up-regulated after trauma. To confirm that our patient population and/or our assay conditions were similar compared to these studies, we set out experiments evaluating the priming of the oxidative burst in a subpopulation of our patients. Six patients provided blood samples the day after trauma for patient-typing. Their median age was 32 (range 18-79) and their median ISS was 18 (range 16-29). The group consisted of 5 males and 1 female. Six healthy adults with a median age of 25 (range 23-28) provided blood samples that served as controls. This group consisted of 4 males and 2 females.

Patients: Lung aspiration

Three patients, who had developed acute lung injury the first day after trauma, provided lung aspiration samples. Their median age was 33 (range 20-38) and their median ISS was 25 (range 16-25). They were all male. A non-directed broncho-alveolar lavage was performed, which is standard of care at the intensive care unit (24). The cells from the alveolar compartment were analyzed and compared with the expression on peripheral blood PMNs, obtained from the same patients.

Procedure for staining of PMNs

Blood was collected in sodium heparin as anticoagulant and cooled immediately after vena puncture and kept on ice during the whole staining procedure. The analysis of the PMN priming was started within three hours after the blood sample was obtained. The expression of the priming markers recognized by Mophabs A17 and A27 was compared with expression of αmβ2 (CD11b/CD18), a more widely used marker for PMN activation, as described above (12,13,25). These markers were also measured after 5 minutes 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 a bacterial derived activating agonist. After stimulation, the samples were put on ice again and analyzed.

Blood samples were stained with fluorescein isothiocyanate (FITC) directly labeled phage antibodies A17 and A27 as described previously (15). In short, monoclonal phage antibodies (MoPhabs) A17 and A27 were diluted 1:15 with PBS 0.38% tri-sodium citrate, 10% pasteurized plasma solution and 4% milk powder. 100 micro liter of FITC–labeled (MoPhab) A17 or A27 solution was added to 50μl of whole blood

and incubated for 60 minutes on ice. The CD11b antibody (Clone 2LPM19c, DAKO, Denmark) was added to whole blood at a concentration of 0.5µl/50µl and incubated for 60 minutes on ice. Hereafter, the binding of this antibody was counter stained with GAM-FITC as described (26).

After incubation, the red cells were lysed with ice-cold isotonic NH_4Cl (12). After a final wash, the cells were analyzed in a FACSvantage Flowcytometer (Becton & Dickenson, Mountain view. CA). The PMNs were identified according to their specific side-scatter and forward-scatter signals. Data from individual experiments are depicted as fluorescence intensity in arbitrary units (AU) or summarized as the median channel fluorescence (MCF) of at least 10.000 events.

Data of alveolar PMNs were acquired by using the flowcytometry analysis of PMNs from non directed broncho alveolar lavage (ND-BAL) fluid obtained from a patient with acute lung injury. The unprocessed ND-BAL fluid containing alveolar cells was incubated and PMNs were identified on the basis of scatter characteristics.

Isolation of PMNs and measurement of respiratory burst activation

PMNs were isolated from 5 ml of whole blood. Blood was diluted with 2 ml PBS, supplemented with 0.38% tri-sodium citrate and 10% pasteurized plasma solution (PBS2+). A layer of 5 ml Ficoll was added under the cells with a curved needle. The cells were centrifuged for 20 minutes at 1000g at room temperature. The PMN fraction was isolated together with the red cells and the red cells were lysed using ice-cold isotonic NH_4Cl. After a final wash the cells were resuspended in HEPES-buffer, supplemented with 20% human serum albumin, 1% $CaCl_2$ and 0.2% glucose. The reactive oxygen species (ROS) production was measured as follows (27). One series of samples were incubated for 20 minute with granulocyte macrophage colony stimulating factor (GM-CSF) 10^{-10}M, while the other series of samples remained untreated. Dihydrorhodamine (DHR123) was added 0.1 µg/ml and the incubation was continued for another 10 minute at room temperature. Hereafter, the cells were stimulated with fMLP 1 µM for 30 minutes at 37°C. The stimulation was stopped by washing the cells with ice-cold PBS supplemented with 0.38% tri-sodium citrate and 10% pasteurized plasma solution. The cells were analyzed in the FACSvantage Flowcytometer (Becton & Dickenson, Mountain view. CA).

Statistical analysis

Results in figures are generally expressed as means ± standard error of mean (SEM). Statistical analysis was performed with the non-parametric Mann-Whitney U test, to compare the healthy controls with the trauma patients. A Kruskal-Wallis H test was used for analysis between groups of patients. Statistical significance was defined as $p < 0.05$.

RESULTS

Lack of expression of priming markers on peripheral blood PMNs after trauma

No increased expression of the epitopes A17 and A27 was found on PMNs from traumapatients as compared to controls. Even on cells from the most severely injured patients (ISS > 25) no increased expression was found the day after injury (Figure 1A). This lack of increased expression was continued during the first week of admission (Figure 1B). Results of A27 epitope expression were complementary with A17 epitope expression (results not shown). CD11b was slightly increased during the first week, but with a high variability (Figure 2A and Figure 2B). In addition, no relation was found between the severity of illness and the expression of the epitopes A17, A27 and CD11b (Figure 3 and Figure 4) (21,22). Two patients developed a fever (>38,5 degrees Celsius) without an identified origin and one patient developed acute lung injury (ALI) on the second day of admission. Several patients needed surgery for their injuries. However, under these conditions no significant differences were seen in the expression of A17 and A27 on PMNs. One patient received 25 units of packed red blood cells and showed a slight increase in A17 expression, no increase in A27 expression and a moderate increase (1.7 times) in CD11b expression. This returned to normal after the third day of admission. Other patients who received packed red blood cells (less than 10 units) did not show any difference in the priming epitope expression.

Decreased responsiveness of PMNs from traumapatients to the innate immune stimulus fMLP

The expression of the priming epitopes on PMNs after fMLP stimulation was dramatically lower in traumapatients during the first week after trauma, when compared to controls ($p < 0.001$, power = 0.969; Mann Whitney U Test) (Figure 1). Again, there was no correlation with the severity of injury or the severity of illness; PMNs of all traumapatients showed a lower maximal epitope expression (Figure 1 and Figure 3). Furthermore, there was no relation with the amount of blood transfusion or type and time of surgery.

This decreased responsiveness was not restricted to the expression of epitopes recognized by A17 and A27, maximal CD11b expression after *in vitro* activation

with fMLP was significantly lower when severity of disease increased (Figure 4). In traumapatients with a severe sepsis score (> 9) or high APACHE II Score (>14), the decrease in fMLP-induced CD11b expression on peripheral blood PMNs was more pronounced compared to traumapatients with less severe disease (Kruskal-Wallis H Test = 0.013).

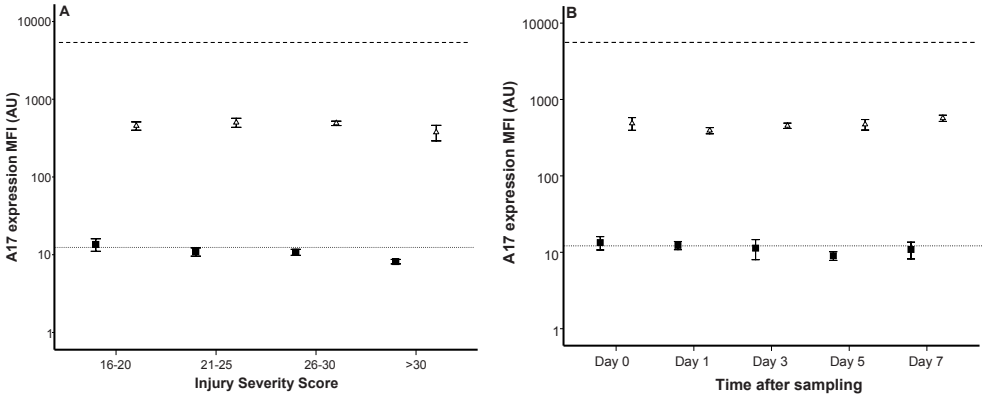

Figure 1. A17 epitope expression by injury severity score and in time.
Shows the intrinsic A17 epitope expression (■) and maximal inducible A17 epitope expression (Δ) in arbitrary units (AU). The epitope expression is shown (**A**) in relation with the Injury Severity Score on the first day of admission and (**B**) over time during the first week after admission. Traumapatients are compared with controls. All values for intrinsic A17 (and A27) epitope expression were within the normal range (normal value is expressed as:....) during the first week. All values for maximal inducible A17 (and A27) epitope expression were decreased (normal value is expressed as:__). No differences were found for patients having different injury severity scores. Data are presented as mean ± SEM.

The decreased responsiveness of PMNs in the context of expression of priming epitopes recognized by Mophabs A17 and A27 was not restricted to fMLP stimulation. Blood samples of 4 patients were analyzed for epitope expression after both fMLP and TNF-α (tumor necrosis factor α) stimulation, which showed similar results (results not shown). Blood samples of 4 other patients were analyzed for epitope expression after both fMLP and PMA (Phorbol Myristate Acetate) stimulation. PMA was utilized because this potent stimulus bypasses classical membrane bound receptors. Again, these data showed no differences in impairment of maximal inducible A17 and A27 epitope expression (results not shown).

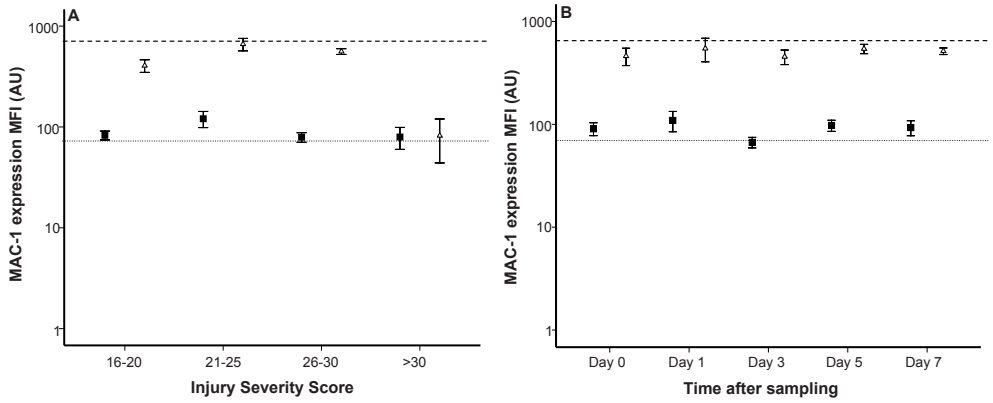

Figure 2. CD11b expression by injury severity score and in time.
Shows the relation between intrinsic CD11b expression (■) and maximal inducible CD11b expression (∆) in arbitrary units (AU). CD11b expression is shown (**A**) in relation with the Injury Severity Score on the first day of admission and (**B**) over time during the first week after admission. Traumapatients are compared with controls. The intrinsic epitope expression was highly variable (normal value is expressed as:....). On the day after trauma CD11b was significantly increased as compared with controls. Patients showed normal values for maximal inducible CD11b expression as compared with controls (normal value is expressed as:__). Data are presented as mean ± SEM.

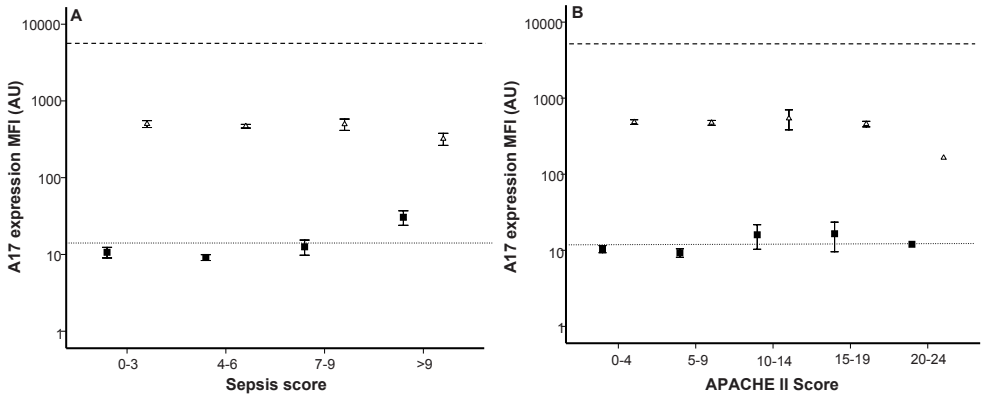

Figure 3. A17 epitope expression by APACHE II Score and severity of illness.
Shows the intrinsic A17 epitope expression (■) and maximal inducible A17 epitope expression (∆) in arbitrary units (AU). The epitope expression is shown (A) by the severity of illness (sepsis score) and (B) in relation with the APACHE II Score. Traumapatients are compared with controls. All values for intrinsic A17 (and A27) epitope expression were within the normal range (normal value is expressed as:....). All values for maximal inducible A17 (and A27) epitope expression were decreased (normal value is expressed as:__). No differences were found for patients having different APACHE II Scores or Sepsis Scores. Data are presented as mean ± SEM.

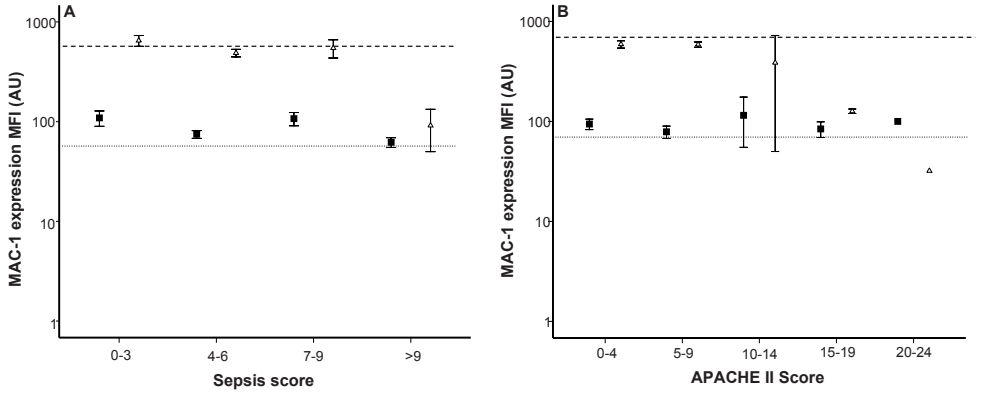

Figure 4. CD11b expression by APACHE II Score and severity of illness.
Shows the relation between intrinsic CD11b expression (■) and maximal inducible CD11b expression (Δ) in arbitrary units (AU). CD11b expression is shown (A) by the severity of illness (sepsis score) and (B) in relation with the APACHE II Score. Traumapatients are compared with controls. The intrinsic CD11b expression was overall slightly increased, but not related with disease severity (normal value is expressed as:....). Patients with mild disease showed normal values for maximal inducible CD11b expression as compared with controls (normal value is expressed as:__). However, as severity of illness increased (by APACHE II Score or Sepsis Score), maximal inducible CD11b expression on neutrophils decreased significantly as compared with controls. Data are presented as mean ± SEM.

Production of reactive oxygen species by PMNs is functionally up-regulated in isolated PMNs

The lack of expression of priming markers on PMNs from traumapatients was not anticipated and this prompted us to study priming of these cells in a functional context to perform patient characterization. Activation of the fMLP induced respiratory burst in isolated PMNs was studied in cells of 6 patients and 6 healthy controls in parallel with the measurements with A17, A27 and CD11b. There was no difference in the background signal of rhodamine 123 between patients and controls. After stimulation with fMLP, the rhodamine assays showed a small but significant increase in oxidative burst in the isolated PMNs from traumapatients as compared to the controls. A Wilcoxin Signed Ranks test was used for the parallel measured patients and controls. This showed significant difference ($p = 0.027$), as all the patients had increased oxidative burst as compared with controls (results not shown).

PMNs with a fully primed phenotype are found in the lungfluid of patients with acute lung injury

A sample of lungfluid was obtained from three patients who developed acute lung injury. On average, the median A17 or A27 epitope expression on the PMNs in

this fluid was approximately 50 (A17) and 10 (A27) times higher respectively as compared to the expression in the systemic circulation (Figure 5). This expression was not further increased by *in vitro* stimulation with fMLP. CD11b expression on the PMNs from the lung showed a 5 times increase and was not increased by *in vitro* fMLP stimulation.

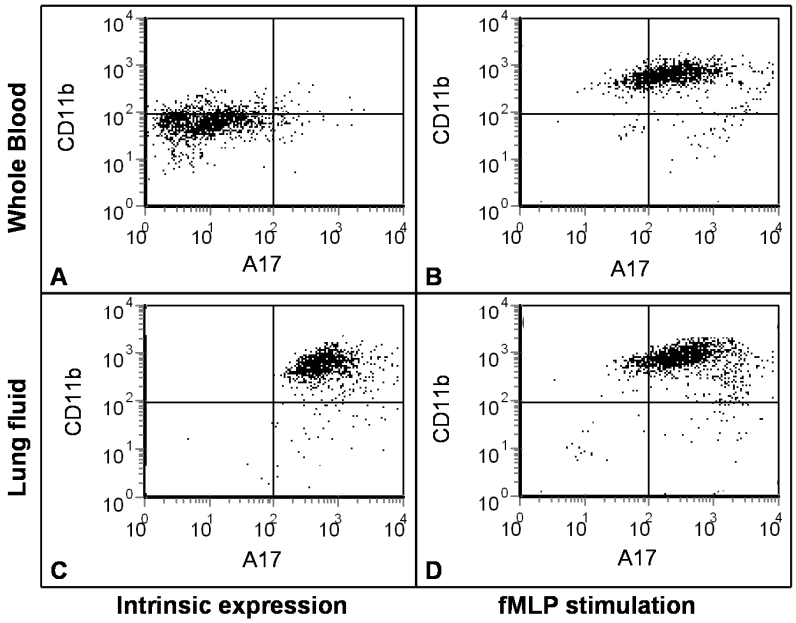

Figure 5. Example of flowcytometric profile of A17/CD11b staining in whole blood and lungfluid.
Shows the profile of A17 and CD11b expression on neutrophils harvested from the lungfluid of a patient with acute lung injury. This plot is representative for the two other patients with acute lung injury. The scatterplot was obtained with flowcytometry in which the neutrophils were gated by their specific forward and siteward scatter signal. The right under quadrant represents low expression of both epitopes, the left upper quadrant represents high expression of both epitopes. The results are compared with the blood samples obtained from the same patient.

DISCUSSION

Priming of PMNs in traumapatients

The phenotypical and functional alterations of PMNs induced by the process of isolation hamper the extrapolation of data of current studies with these isolated cells to the situation *in vivo* (18,19). Therefore, we designed experiments to study priming without the need for isolation of PMNs from the peripheral blood. We applied our recently developed phage antibodies which recognize epitopes on cytokine primed PMNs in whole blood. This enables us to identify primed PMNs in traumapatients by whole blood analysis with a wide detection range (15). Full priming in the context of expression of these epitopes can be induced *in vitro* by adding cytokines to PMNs in the physiologically relevant (picomolar) range (15,16). In marked contrast to our expectation in the current study, no primed PMNs were found in the systemic circulation of traumapatients (Figure 1 and Figure 3). This seems in contrast to the results obtained from studies which focused on functional responses of isolated cells or CD11b expression (25,28). A slight increase in the expression of CD11b was seen in this study and this trend was considered consistent with other reports of larger patient populations (12,13,28). The moderate (1.7 times) increase of CD11b expression seen in one patient the day after massive blood transfusion, was congruent with findings of increased CD11b expression after massive administration of red blood cells (29).

The apparent lack of priming in the context of both A17 and A27 epitope expression as well as the fMLP unresponsiveness of PMNs in the circulation of traumapatients can be explained by an alternative hypothesis in which cells with certain priming phenotypes home for the tissues, leaving partially refractory cells in the circulation. This hypothesis sheds light on the poorly understood mechanisms underlying CARS which is often seen in the period after severe trauma. However, one could argue that the priming epitopes are not yet expressed by PMNs, recently mobilized from the bone marrow. This seems unlikely, because PMNs from the peripheral blood of the injured patients did not show consistent characteristics of “young“ cells with banded nuclei (results not shown). To characterize the lack of a priming phenotype of PMNs in more detail, we studied the responsiveness of PMNs for the priming sensitive bacterial derived stimulus fMLP in the context of whole blood. The lack of a priming phenotype was even more pronounced in this assay as the PMN population

was relatively unresponsive to the stimulus. This decreased expression after *in vitro* stimulation showed no correlation with the severity of injury or the severity of disease during admission. Maximum induction of epitopes recognized by A17 and A27 was already significantly decreased in patients with mild disease, whereas maximal CD11b expression only decreased in patients with more severe disease. Our results are complementary with the clinical data of Flores et al. They show that an APACHE II score above 14 is associated with the development of sepsis (30). The decrease in maximum CD11b expression with broad variation seen in patients with an APACHE II Score near 14 shows that this is a possible borderline situation for immune dysfunction.

The functional assays performed in parallel with the measurement of A17 and A27 on the day after admission, showed an increased functional activity of the *isolated* PMNs from traumapatients as compared to the isolated PMNs from healthy controls. These tests were performed to characterize the included patients. The increased expression of CD11b and oxidative burst imply to the presence of PMNs in the circulation of traumapatients with increased functional (cytotoxic) activity. Although significant, the differences between cells from healthy controls and patients were only minor.

Unresponsiveness of peripheral PMNs in traumapatients

Gundersen et al showed in a pig-model (penetrating trauma-model) that PMNs are capable of an increased production of cytokines (TNF-α, IL-1β) after trauma (31). Furthermore, these PMNs showed an increased oxidative burst to Phorbol Myristate Acetate (PMA) after trauma, most likely due to *in vivo* priming. Complementary to our results for A17 and A27 epitope expression, the group of Gundersen found a decreased capability of the PMNs to produce TNF-α, IL-1β or IL-6 after trauma, when stimulated *ex vivo* with the bacterial component lipopolysaccharide (LPS). In addition to these results, Johnson et al found similar results for maximum CD11b expression. They compared a group of traumapatients who received hemoglobin based oxygen carriers (HBOC) with a group of traumapatients who received normal packed red blood cells. These authors reported an increase in oxidative burst for the group which was resuscitated with packed cells, indicative for *in vivo* priming. They also showed decreased CD11b responsiveness towards *in vitro* stimulation with platelet activating factor (PAF) as compared to the group treated with HBOC (28).

The unresponsiveness of PMNs to *in vitro* stimulation is not restricted to the production of cytokines or adhesion related epitopes, but for cytotoxic responses as well. Botha et al showed that after 24 hours after trauma the oxidative burst (spontaneous and after fMLP stimulation) was increased in traumapatients as compared with healthy controls (32). However, the oxidative burst after *in vitro* priming with PAF (platelet activating factor) in traumapatients could not be induced to the levels found in PMNs of healthy control donors. Thus, despite enhanced functions at baseline, the maximum capacity was found to be decreased for several functions of PMNs in the peripheral blood of traumapatients.

Extravasation of primed PMNs

Pallistar et al showed an increased migratory capacity of PMNs of traumapatients, which might lead to enhanced homing of these cells to the tissues (33). To investigate the hypothesis that activated or primed PMNs home to the site of inflammation, samples were obtained from the lung of traumapatients during a period of clinically diagnosed acute lung injury. We investigated the expression of priming epitopes on the PMNs extravasated to the lung. PMNs harvested from the lung fluid by lung aspiration were characterized by a very pronounced priming phenotype both in the context of expression of priming epitopes (A17/A27) and expression of the integrin αmβ2 (CD11b). The PMNs exhibited a fully primed phenotype, which could not be further increased by *in vitro* fMLP stimulation (example shown in Figure 5). These data implicate the presence of fully primed PMNs in the pulmonary tissue and are consistent with the hypothesis that primed cells home to the tissue, leaving partially refractory cells in the circulation.

Aberrant regulation of neutrophils

The finding that trauma is associated with responsiveness of PMNs towards *in vitro* activation demonstrates the presence of PMNs in the peripheral blood with impaired functionality for some PMN-functions, visualized with impaired expression of priming associated markers. The fully primed phenotype found on pulmonary PMNs, suggest optimal (pre)activation of these cells only in the tissues. This difference not found in the context of cytotoxic responses of PMNs in the peripheral blood. Therefore, it is tempting to speculate that in severely injured traumapatients primed cells are likely to leave the circulation for the tissues, leaving partially refractory cells in the circulation. This subgroup of PMNs comes to attention only under the extreme circumstances

caused by trauma, as enhanced homing of the adequately functioning PMNs to the tissues has occurred. This state of immunologic impairment could make the patient more prone for later infectious complications.

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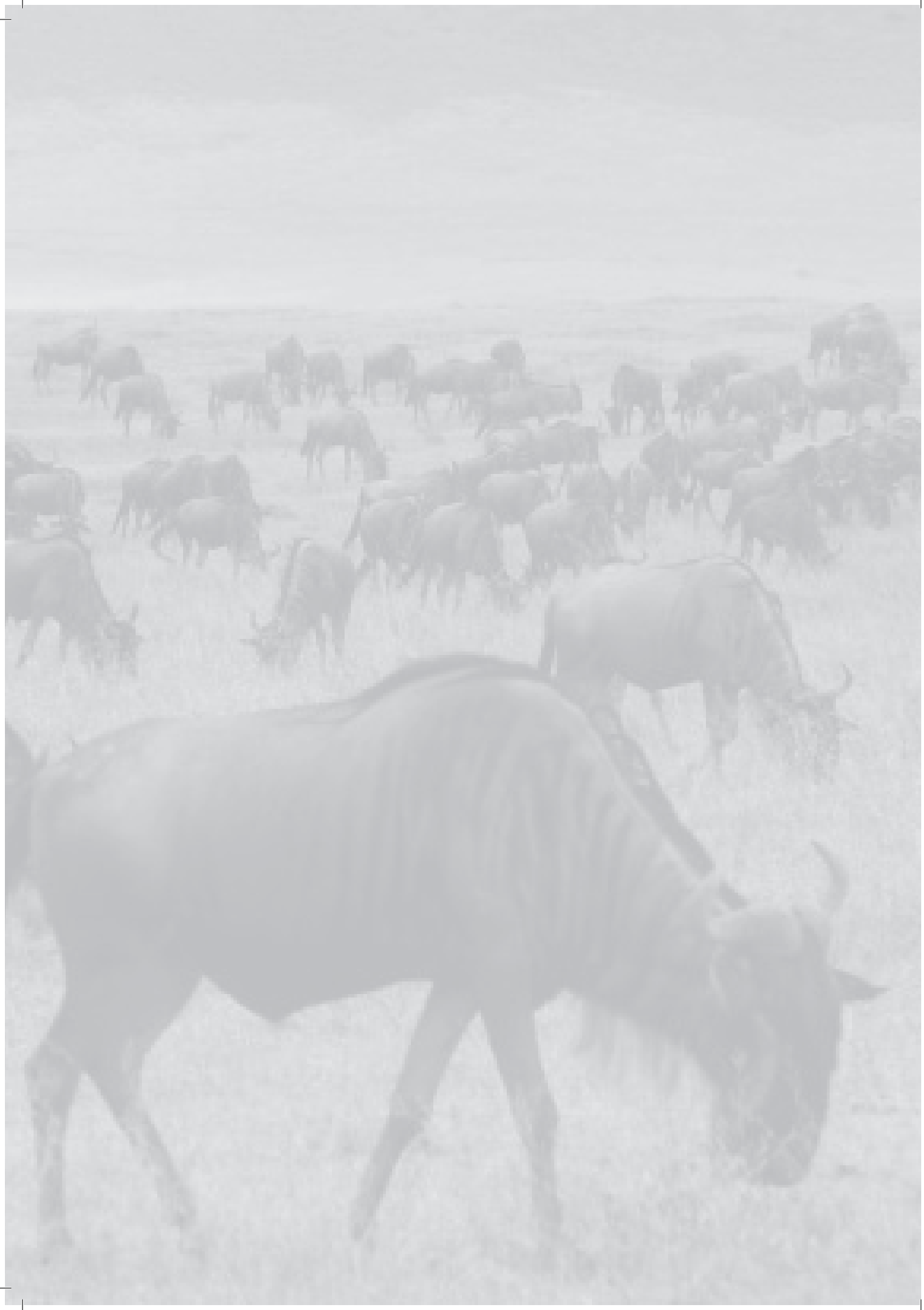

CHAPTER 4

MODULATION OF THE INNATE IMMUNE RESPONSE AFTER TRAUMA VISUALIZED BY A CHANGE IN FUNCTIONAL NEUTROPHIL PHENOTYPE

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Submitted

ABSTRACT

Acute Respiratory Distress Syndrome (ARDS) is a frequent and severe complication after trauma, caused by an excessive inflammatory response mediated by polymorphonuclear granulocytes (PMNs). Early identification of patients with increased PMN activity will aid in the prevention of ARDS. We tested the hypothesis that a correlation exists between injury severity and phenotypic changes of circulating PMNs.

Fifty-two patients were included and injury severity was assessed by clinical injury severity scores. Complications were recorded on a daily basis and the changed PMN phenotype was assessed by FACS analysis within 24 hours after injury. Results were compared with 10, age matched healthy controls.

The membrane expression of Mac-1/CD11b and active FcγRII/CD32 was not correlated with injury severity. Levels of the acute phase protein IL-6 correlated significantly with injury severity, indicating that a range in severity of the inflammatory response was present in the studied population. A significant correlation between the PMN responsiveness toward the bacterial derived peptide fMLP (visualized by up-regulation of active FcγRII) and injury severity was demonstrated. In addition, the largest change in PMN responsiveness was found in patients who developed ARDS.

Sustained injury is reflected by systemic inflammation and the subsequent PMN activation status can be determined by analysis of fMLP-induced active FcγRII on these cells. FMLP-induced active FcγRII was associated with the occurrence of ARDS. Therefore, the extent of the injury-induced systemic inflammatory response can be determined by phenotyping PMNs in the peripheral blood.

The Acute Respiratory Distress Syndrome (ARDS) is a frequent and severe complication after trauma. ARDS has a mortality and morbidity rate of up to 40% and 80% respectively (1,2). Polymorphonuclear granulocytes (PMNs) play an essential role in the development of ARDS. In experimental animal models high numbers of PMNs are found in the pulmonary interstitium (3). Activation of these cells leads to increased production of reactive oxygen species (ROS), which causes increased vascular permeability, interstitial edema, reduced surfactant concentrations to maintain normal surface tension and reduced oxygen diffusion (4). PMN depletion or blocking of PMN extravasation, protects animals for the development of ARDS in severe trauma models (5-7). In addition, an increasing amount of ischemia reperfusion injury correlates with increasing accumulation of PMNs in the pulmonary interstitium. These data are consistent with the hypothesis that a direct relation is present between injury severity, PMN extravasation/activation and subsequent tissue damage (8,9).

Translating the data from these animal models to the clinical situation is difficult, as both pulmonary retention of PMNs and ROS induced tissue injury can not be measured prior to the development of clinical symptoms. Alternative approaches are sought to accurately identify patients at risk for the development of ARDS. Early identification of patients with pronounced activation of the PMN compartment could aid in the prevention of this complication after trauma. However, limited knowledge about the underlying pathophysiological processes impedes the development of new diagnostic and therapeutic strategies.

The acute phase protein IL-6 (interleukin 6) has been extensively studied as a marker of systemic inflammation and shown to be related with the development of organ failure (including ARDS) after trauma (10-13). An enhanced acute phase response (characterized by e.g. increased IL-6 levels) is associated with occurrence of activated PMNs in the peripheral blood and studies have shown that IL-6 can modulate the function on human PMNs (14,15). However, IL-6 is not a direct marker for systemic activation of the innate immune response. In addition, other pro- and anti-cytokines will contribute to the final modulation of the immune response. Changes in PMN phenotype and function *in vivo* will be the sum of the effect of these interacting cytokines. Analysis of the final common pathway, which is associated with a complex modulation of PMN phenotype and function, will provide more insight

into the pathophysiological processes which lead to inflammatory complications and organ failure after trauma.

Unfortunately, determination of the reactivity of PMNs of trauma patients *in vitro* by e.g. activation of the NADPH-oxidase, can easily be biased by artifacts caused by isolation of PMNs (16,17). Whole blood analysis of the phenotype(s) of circulating PMNs circumvents many of these problems and can be better used as a read out of changed PMN functionality. We have recently demonstrated that a relation exists between changes in receptor expression of circulating PMNs and altered PMN phenotype/function in the interstitium (18).

Some studies have investigated changes in expression of single PMN receptors in relation to injury severity (9,19,20). Only the expression of the alpha chain of MAC-1 (CD11b) showed a weak correlation with the burden of trauma (expressed by base deficit) (21). We have recently demonstrated that the responsiveness towards the bacterial derived N-formyl-methionyl-leucyl-phenylalanine (fMLP) was impaired in severely injured patients (18). This was visualuzied by the fMLP-induced *in vitro* up-regulation of the PMN receptors, such as MAC-1 and active FcγRII, which are well known activation markers. Reduced MAC-1 up-regulating capacity after trauma has been related to the development of Pseudomonas aeruginosa infections (22). However, a relation between PMN receptor expression and injury severity was not further detailed.

The aim of the present study was to investigate whether the extent of systemic inflammatory reaction on trauma can be visualized by expression of single PMN receptors or PMN responsiveness towards the innate immune stimulus fMLP. In addition, it was investigated whether these putative PMN characteristics were associated with the development of ARDS.

MATERIALS AND METHODS

Patients

In our previous study only severely injured patients were included, thus no relation between trauma severity and PMN receptor expression of responsiveness could be analyzed. Therefore, patients with a wide range of injury severity were chosen for the present study. Fifty-two trauma patients with an ISS (Injury Severity Score) >3 and admitted at the Department of Traumatology, University Medical Center Utrecht were included. Exclusion criteria were age < 16 years or > 80 years and patients with an altered immunological status (e.g. corticosteroid use or chemotherapy). A blood sample was taken prior to any surgical procedure and within 24 hours after admission. Ten healthy volunteers served as a control group, which was matched for age and gender. The local ethical committee approved the study and written informed consent was obtained from all patients or their legal representatives in accordance with the protocol.

Clinical parameters

The Injury Severity Score (ISS), New Injury Severity Score and APACHE II Score were calculated on admission (23,24). Within the first 72 hours after injury the presence of systemic inflammation (i.e. systemic inflammatory response syndrome [SIRS]), or the occurrence of pulmonary complications (e.g. acute lung injury [ALI], or acute respiratory distress syndrome [ARDS]) were assessed according to their clinical criteria as determined in the consensus conferences for SIRS and ARDS (2,25). The presence of pneumonia was determined by a positive sputum culture, an infiltrate on the chest X-ray and clinical symptoms of infection (26). Transfusion related data and intensive care support days were recorded.

Materials

For analysis of PMN receptor expression by flowcytometry the following monoclonal antibodies were commercially purchased: RPE-labeled IgG2a negative control (clone MRC OX-34, Serotec, Dusseldorf, Germany) and RPE-labeled CD11b (clone 2LPM19c, DAKO, Glostrup, Denmark). A FITC-labeled monoclonal phage antibody, which recognizes active FcγRII (CD32) designated as FcγRII*, was manufactured at the Department of Respiratory Medicine at the University Medical Center Utrecht (MoPhab A27, UMC Utrecht, The Netherlands) (27,28). Interleukin 6 (IL-6) was

measured by ELISA (Pierce Biotechnology Inc., IL, United States) as described by the manufacturer. Hematology parameters were determined at the Clinical Laboratory Department of the University Medical Center Utrecht.

Flowcytometry

Blood was collected in a vacutainer® with sodium heparin as anticoagulant cooled immediately and kept on ice during the whole staining procedure. The analysis of the PMN receptor expression was started within two hours after the blood sample was obtained. The expression of the above mentioned antibodies was measured as described previously (18). The expression of MAC-1 (CD11b) and FcγRII* (active CD32) was also measured after 5 minutes 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 a bacterial derived protein products/peptides. After stimulation, the samples were put on ice again and analyzed.

Blood samples were stained with the fluorescein isothiocyanate (FITC) labeled phage antibody A27 (recognizing FcγRII*) as described previously and with the commercial antibodies as described by their manufacturer (18). In short, the directly labeled antibodies were added 1:20 to whole blood and incubated for 60 minutes on ice. After incubation, the red cells were lysed with ice-cold isotonic NH_4Cl. After a final wash with PBS2+ (phosphate buffered saline supplemented with sodiumcitrate (0.4% wt/vol) and pasteurized plasma protein solution (10% vol/vol), the cells were analyzed in a FACScalibur Flowcytometer (Becton & Dickenson, Mountain View. CA). The PMNs were identified according to their specific side-scatter and forward-scatter signals. Data from individual experiments are depicted as fluorescence intensity in arbitrary units (AU) or summarized as the median channel fluorescence (MCF) of at least 10000 events.

IL-6 analysis

Blood was collected in a vacutainer® with EDTA as anticoagulant, cooled immediately and kept on ice during the procedure. Plasma was isolated by spinning the sample down at 1000 G. IL-6 was determined using a human IL-6 sandwich ELISA (Endogen, Pierce Biotechnology, IL, United States) according to the procedures prescribed by the manufacturer.

Statistics

Results are expressed as means ± standard error of mean (SEM). Statistical analysis was performed with the non-parametric Mann-Whitney U test or Kruskall Wallis H test to compare two or multiple groups respectively. Spearman correlation analysis was performed for comparison of two continues variables. Statistical significance was defined as $p < 0.05$.

RESULTS

Patient demographics

52 Trauma patients were included with varying severities of their injuries. Their mean age was 38 years (SD = 20) and the mean ISS was 11 (SD = 9). Demographics are summarized in Table 1.

Table 1. Patient demographics

	Mean (range)
Number of patients (n)	52
Male / Female (n)	31 / 21
Age (years)	38 (16-80)
Injury Severity Score	11 (4-43)
New Injury Severity Score	13 (4-63)
APACHE II Score	4 (0-24)
Time to sampling (< 12 hrs / 12-24 hrs)	35 / 17
Time on ICU (days)	2.5 (0-31)
Time on ventilation (days)	2.2 (0-29)
Packed red blood cells before sampling (units)	0.7 (0-15)
Fresh frozen plasma before sampling (units)	0.2 (0-4)
Cause of trauma (n)	
- MVA	36
- Assault	0
- Fall of height	15
- Penetrating trauma	1
Complications (n)	
- None	33
- SIRS	9
- Pneumonia	2
- ALI / ARDS	6

Expression of Mac-1 (CD11b) and active FcγRII* (CD32) visualize PMN activation in peripheral blood of trauma patients: lack of correlation with injury severity.

In line with previous reports, a trend of increased expression FcγRII* and MAC-1 on PMNs was found after trauma as compared to healthy controls. However, the data failed to reach statistical significance, because the variation in expression of these epitopes was large (Table 2). This was likely caused by the broad range of injury severities. Plasma IL-6 concentrations showed a statistically significant correlation with all three calculated injury severity scores (Table 3), indicating more pronounced

acute phase response in patients with higher injury severity scores. However, no significant correlation was found between the injury severity scores and expression of Mac-1 (CD11b) and active FcγRII* (CD32) on peripheral PMNs (Table 3).

Table 2. Expression of single PMN receptors.

	Active FcγRII MFI (AU)	MAC-1 MFI (AU)
Controls	176 (48)	321 (33)
Patients	310 (245)	421 (47)

Mean fluorescence intensity ± SEM (in arbitrary units = AU). Mann Whitney U test; No significant differences were found between patients and controls.

Table 3. Lack of correlation between single PMN receptor expression and injury severity.

	IL-6	Active FcγRII	MAC-1
ISS	p=0.001 / r=0.378*	p=0.120 / r=0.049	P=0.092 / r=0.093
NISS	p=0.001 / r=0.344*	P=0.115 / r=0.036	p=0.188 / r=0.072
APACHE II Score	p=0.014 / r=0.219*	p=0.828 / r=0.026	P=0.862 / r=0.021

Spearman correlation: p-value and correlation coefficient. Significant values are marked *. A significant correlation was found between the clinical injury severity scores and plasma IL-6 levels, indicating a more pronounced inflammatory response in more severely injured patients. However, no correlation between injury severity and MAC-1 or FcγRII* (active CD32) expression on blood PMNs was found.

Neutrophil responsiveness for fMLP in the context of FcγRII (CD32) activation correlates with injury severity*

In concordance with previous reports, trauma patients were characterized by a statistically significant reduction in PMN responsiveness towards fMLP, when compared to cells from healthy controls. This reduced responsiveness was found in the context of expression of both FcγRII* and MAC-1 (Table 4). This reduced PMN responsiveness towards fMLP in the context of expression of FcγRII* correlated significantly with injury severity (Spearman; r=0.226 / p=<0.001). On the other hand, the reduced PMN responsiveness of MAC-1 up-regulation was not significantly correlated with injury severity (Spearman; r=0.003 / p=0.297). (Table 5).

Table 4. PMN responsiveness towards the bacterial peptide fMLP.

	Active FcγRII MFI (AU)	MAC-1 MFI (AU)
Controls	9832 (168)	5325 (267)
Patients	4686 (527)*	3744 (256)*

Mean fluorescence intensity ± SEM (in arbitrary units = AU), Mann Whitney U test; * = p<0.05 compared to controls. Patients demonstrated a significantly decreased responsiveness of MAC-1 expression and FcγRII* towards fMLP when compared to healthy controls.

Table 5. Correlation of fMLP induced PMN receptor expression with injury severity.

	fMLP induced active FcγRII	fMLP induced MAC-1
ISS	p=0.000 / r=0.226*	p=0.297 / r=0.003
NISS	P=0.000 / r=0.177*	p=0.373 / r=0.025
APACHE II Score	p=0.032 / r=0.082*	P=0.151 / r=0.008

Spearman correlation: p-value and correlation coefficient. Significant values are marked *. A significant correlation was found between the clinical injury severity scores and fMLP induced FcγRII* on PMNs.

PMN responsiveness is related to the inflammatory complication ARDS

Patients were analyzed in the context of the systemic inflammatory response visualized by a change in PMN phenotype and the development of inflammatory complications within the first 48 hours after sampling. Two patients developed ALI and four patients fulfilled the ARDS criteria. Five patients developed pneumonia and nine patients fulfilled the SIRS criteria. Plasma IL-6 levels were statistically significant increased (p=0.033) in patients who later developed ALI or ARDS (Figure 1A). The responsiveness of the fMLP-induced FcγRII* expression gradually decreased (Kruskall Wallis H test; p=0.023) when severity of inflammatory pulmonary complications increased (Figure 1).

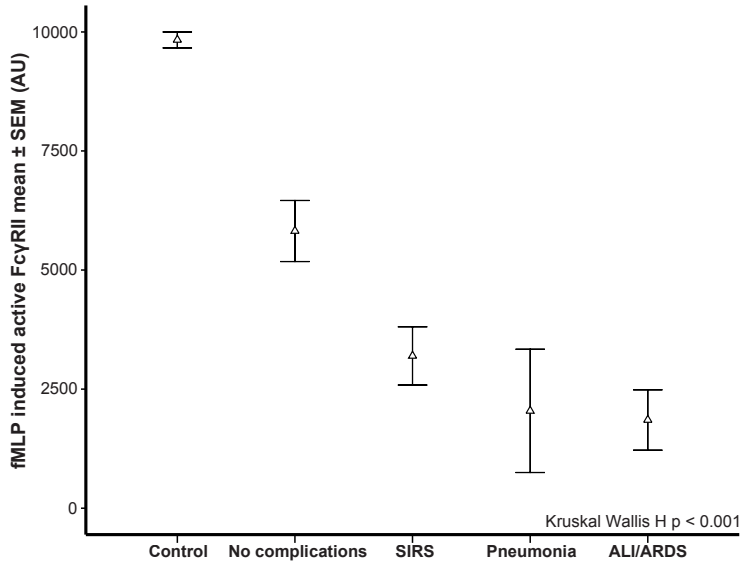

Figure 1. FMLP induced FcγRII* and inflammatory complications
The expression of FcγRII* (active CD32) on PMNs after fMLP stimulation decreased when the severity of complications increased. Patients who later developed ALI/ARDS demonstrated the most impaired responsiveness (Kruskal-Wallis H test p=0.023).

DISCUSSION

PMNs change their phenotype during priming and activation in vivo. This change can be visualized by FACS analysis of the expression of activation markers on the membrane of these cells. In our hands analysis of the expression of FcγRII* and the alpha-chain of MAC-1 (CD11b) is the most sensitive method to visualize activation of neutrophils *in vivo.* (18,21,27-29). However, expression of these markers does not allow a sufficient quantification of the innate immune response, as no correlation was present between expression of these markers and the injury severity. A range in severity of the injury-induced inflammatory response was present, which was demonstrated by the correlation between the acute phase protein IL-6 (Table 3) and the calculated clinical scores.

In marked contrast, the functional responsiveness towards the bacterial peptide fMLP in the context of induction of active FcγRII* clearly visualized a change in the circulating PMN population. In addition, the extent of modulation of this PMN response correlated with the extent of injury (Table 5).

The use of MAC-1 expression (CD11b/CD18) on PMNs as a single marker for injury severity has been proposed previously (3,21,30,31). In these studies, a correlation was shown between initial base excess and initial CD11b expression. However, the correlation coefficient appeared low as significant increase in PMN CD11b expression only occurs during severe physiological disturbances, as demonstrated by a low base excess (32). The expression of FcγRII* has previously been suggested by our group as a marker for injury severity. However, only critically ill patients were included and a relation with injury severity could not be further detailed (18).

As shown by us previously, the responsiveness of peripheral neutrophils towards fMLP in the context of up-regulation of FcγRII* was markedly decreased in severe trauma patients. This study confirms this counterintuitive finding and even shows a significant correlation between the extent of injury severity and the decrease in responsiveness for fMLP in this context. This finding did not reflect an overall unresponsiveness for fMLP as this correlation was not found for expression of CD11b (see Table 5). In addition, we and others have found that fMLP-induced activation of the NADPH-oxidase in isolated peripheral PMNs is up-regulated in

trauma patients (18,33,34). Although a mechanism explaining this finding is lacking, fMLP-induced activation of PMNs is very complex as induction of chemotaxis and activation of the respiratory burst occurs at completely different IC50's: 10 nM for chemotaxis and 1μM for oxidase activation (35,36). The dose response curve for fMLP-induced FcγRII* expression on PMNs is close to 10 nM (37). These findings are consistent with the hypothesis that after trauma PMNs mainly express the low affinity fMLP receptor coupled to cytotoxic responses. This hypothesis is supported by a decreased chemotaxis response found in whole blood analysis of PMNs obtained from trauma patients (17)

Studies focusing on the systemic innate immune response in response to trauma are complicated by the fact that the clinical scorings systems have been created as mortality prediction rules (23,24,38). The changed PMN FcγRII* responsiveness towards fMLP identifies the inflammatory response induced by the burden of trauma and does not predict mortality. It is, therefore, not surprising that the correlations between the clinical scores and PMN responsiveness, though significant, were weak. PMN responsiveness reflects a final common pathway of systemic inflammation, which is part of the complex clinical picture in trauma patients and a known risk factor for the development of organ failure such as ARDS. The responsiveness of PMN FcγRII* towards fMLP is not a static parameter and allows monitoring of the inflammatory response in individual patients over time, which is in marked contrast to the calculated admission scores (39). In addition, analysis of PMN phenotype allows the determination of kinetics and extent of additional inflammation caused by surgical procedures.

In conclusion, a changed responsiveness in fMLP-induced active FcγRII* represents a marked change in PMN phenotype, which reflects an important mechanism in the pathophysiology of the systemic inflammatory response after trauma. The changed PMN phenotype correlates with the amount of sustained injury and is related to the incidence of inflammatory complications such as ARDS. Systemic PMN inflammation is an important therapeutic target for treatment of inflammatory complication inflicted by injury. There is, however, an important unmet need in future treatment of trauma patients, as no potent neutrophil antagonists are available for clinical application yet.

ACKNOWLEDGEMENT

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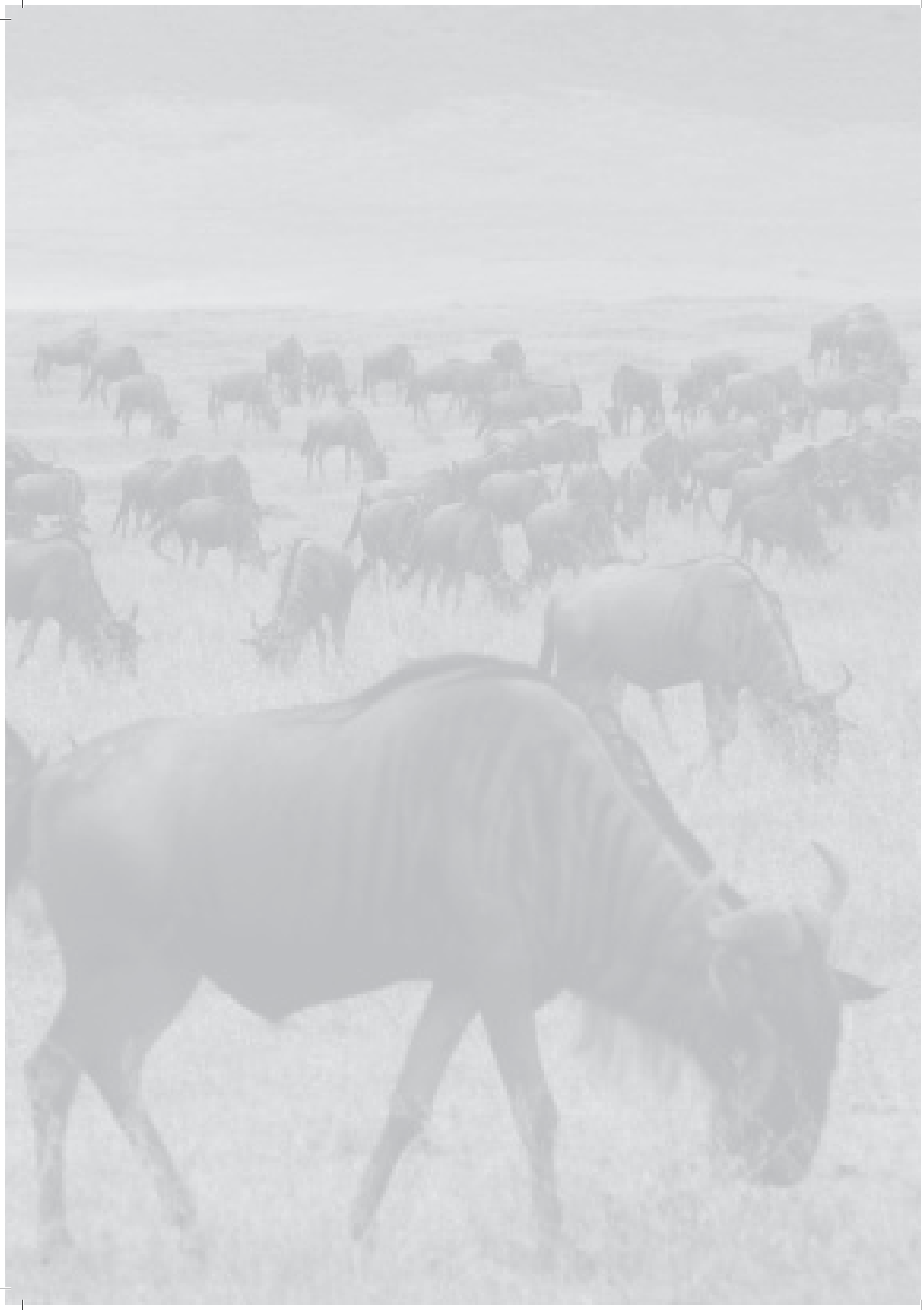

Chapter 5

Synergism between tissue injury and systemic inflammation leads to ARDS: Influence of intramedullary nailing

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In preparation

ABSTRACT

ARDS is a severe complication in patients receiving intramedullary nailing (IMN) for femur fractures. It is hypothesized that ARDS is mediated by synergism between severe inflammation and pulmonary endothelial damage. Damage control orthopedics has been developed to limit the exacerbation of the inflammatory response and thereby preventing ARDS. Although clinical reports show promising results, the underlying mechanisms have not been revealed.

Sixty-eight trauma patients who required primary or secondary lower extremity IMN were included. The choice for treatment strategy was made by the attending surgeon. The development of ARDS was recorded. Blood samples were taken prior, 15 minutes after and 18 hours after IMN. Inflammation was analyzed by plasma IL-6 levels and changes in neutrophil phenotype. Triglyceride levels were determined as a risk factor for pulmonary endothelial injury.

Thirteen patients underwent damage control orthopedics and 55 patients early total care. Nine patients developed pulmonary failure. Plasma IL-6 levels increased 18 hours after IMN for femur fractures. FMLP-induced FcγRII* was most decreased in severely injured patients, but did not alter during IMN. Triglyceride levels increased during IMN in severely injured patients. These changes in IL-6, PMN phenotype and triglyceride levels were most prominent in patients who developed pulmonary failure, regardless of treatment strategy.

Although IL-6 levels increased during IMN, the cellular inflammatory state was determined by the initial trauma. IMN does not further change the PMN phenotype, but releases factors which potentially can damage the pulmonary endothelium. IMN should therefore be performed in patients who are inflammation controlled as pulmonary endothelial injury will most likely occur.

ARDS (Acute Respiratory Distress Syndrome) is a frequent complication after trauma. ARDS has a mortality rate and morbidity rate of up to 40% and 80% respectively (1,2). Several risk factors have been identified for the development of ARDS, such as intramedullary osteosynthesis (IMN) of a femural fracture, massive blood transfusion and thoracic injury (3,4). When IMN is performed in the presence of a risk factor mentioned before, the incidence of ARDS can be over 40% (5-7).

It has been suggested that the incidence and course of complications such as ARDS can be altered by performing a staged approach of surgical interventions (i.e. damage control surgery) (8). In damage control orthopedics (DCO), patients who require IMN of long bone fractures are initially treated by external fixation, which then, at a later stage, is converted to IMN (9). In this strategy, a recovery period is allowed after the initial trauma, easing of the initial inflammatory response. Definitive osteosynthesis (e.g. IMN) is postponed until the patient is in a more stable phase. The disadvantage of this strategy is that fracture healing is hampered compared to the early total care (ETC) strategy (10). Therefore, it is essential to identify and allocate the correct patients for the ETC or DCO strategy. However, this is currently difficult as the underlying pathophysiological processes remain largely unknown.

An enhanced inflammatory response and the occurrence of fat embolisms have been associated with the risk of developing ARDS (11-13). This hypothesis is supported by experimental animal models in which polymorphonuclear granulocytes (i.e. PMNs or neutrophils) play an essential role in the development of ARDS. When PMN extravasation is blocked or animals are depleted of PMNs no ARDS occurs after a sufficient insult (14,15). In addition, animals without local pulmonary tissue injury (e.g. not ventilated) do not have accumulation of PMNs in the pulmonary interstitium, which supports the hypothesis that endothelial damage/activation facilitates PMN extravasation into the pulmonary interstitium (16).

Recently, we have shown that the extent of systemic inflammation can be identified using PMN receptor expression (e.g. fMLP induced FcγRII*) (**chapter 3 and 4**). Little is known regarding activation of damage of local endothelium in the lung tissue. Several endothelial markers have been studied in trauma patients of which the selectins and vascular adhesion molecules have gained the most attention. However, none of these markers has been sufficiently studied to safely conclude on

their prognostic value or direct relation with pulmonary endothelial damage (17,18). Endothelial damage or activation can be induced by direct injury, increased levels of triglycerides or massive blood transfusions. These parameters can be analyzed as potential risk factors for endothelial damage or activation (4,19,20).

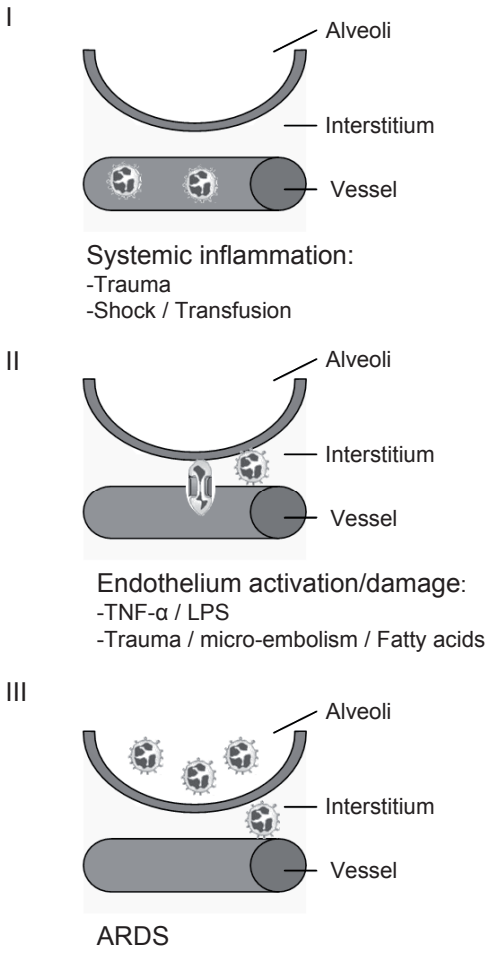

Figure 1. Hypothesis of synergism
We state the hypothesis that pulmonary failure (ALI or ARDS) can only develop when both activation of PMNs and pulmonary endothelial damage or activation is present. In severely injured patients PMNs will be activated on a systemic level. In addition, systemic factors such as triglycerides, TNF-α or massive blood transfusion and local factors such as thorax injury will damage or activate the pulmonary endothelium. The activated or damaged endothelium will facilitate the extravasation of activated PMNs. Besides the assumption that PMN activation and pulmonary endothelial damage/activation are independent processes, interaction will take place. Activated PMNs can damage or activate the pulmonary endothelium and activated endothelium can activate PMNs. Thus, when one of the two factors is severe enough, the other factor will be involved as well.

This study was designed to test the hypothesis that both systemic inflammation and local tissue injury synergize for the development of ARDS (Figure 1); In addition, it was investigated what the effect of IMN is on both the inflammatory status and risk factors for local pulmonary tissue injury and whether the timing of IMN (DCO versus ETC) influences the IMN effect on both the inflammatory status and the induction of the amount of triglycerides in the peripheral blood as risk factor for the development of ARDS.

PATIENTS AND METHODS

Patients

Seventy-five trauma patients with a tibia or femur fracture which required primary or secondary intramedullary nailing, admitted at the Department of Traumatology, University Medical Center Utrecht were included in this study. Exclusion criteria were age < 16 years or > 80 years and patients with an altered immunological status (e.g. corticosteroids use or chemotherapy). The local ethical committee approved the study and written informed consent was obtained from all patients or their spouses in accordance to the protocol.

Clinical parameters and sampling

On admission the Injury Severity Score and APACHE II Score were calculated (21,22). During their admission patients were stratified by development of pulmonary complications or uncomplicated course. During admission the presence of systemic inflammation (e.g. systemic inflammatory response syndrome [SIRS]), or the occurrence of pulmonary complications (i.e. acute lung injury [ALI] or acute respiratory distress syndrome [ARDS]) were assessed according to their clinical criteria as determined in the consensus conference (2). In addition, multitrauma patients were analyzed by treatment strategy: ETC or DCO. The choice for treatment strategy was made by the attending surgeon.

Blood samples were taken at distinct time points: one hour prior to IMN and 15 minutes and 18 hours after the intramedullary nail was introduced. To investigate the influence of IMN, patients were stratified by tibia or femur fracture and by isolated fracture and multitrauma. Patients were compared with healthy controls as described previously (**chapter 4**).

Materials

For analysis of PMN receptor expression by flowcytometry the following monoclonal antibodies were commercially purchased: IgG1 negative control (clone DD7, Chemicon, Hampshire, United Kingdom) and IgG2a negative control (clone MRC OX-34, Serotec, Dusseldorf, Germany). An antibody, which recognizes an active FcyRII/CD32 (designated FcyRII*), is manufactured at the Department of Pulmonary Science at the University Medical Center Utrecht (MoPhap A27, UMCU, Utrecht, The Netherlands)(23).

Interleukin 6 (IL-6) was measured by ELISA (Pierce Biotechnology Inc., IL, United States) as described by the manufacturer. Hematologic parameters and triglycerides, were determined at the Clinical Laboratory Department of the University Medical Center Utrecht.

PMN phenotype: fMLP induced FcγRII*

The inflammatory status of a patient can be assessed by assessing the phenotype of PMNs in the peripheral blood (**chapter 4**) (16). The PMN phenotype was based on the expression of active FcyRII upon activation with fMLP (fMLP induced FcyRII*).

Blood was collected in a vacutainer® with sodium heparin as anticoagulant, cooled immediately and kept on ice during the whole staining procedure. The analysis of the PMN receptor expression was started within two hours after the blood sample was obtained. The expression of the above mentioned markers was measured as described previously (24). Expression of active FcγRII by MoPhab A27 was measured after 5 minutes 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 a bacterial derived activating agonist (25). After stimulation, the samples were put on ice again and analyzed.

Blood samples were stained with fluorescein isothiocyanate (FITC) directly labeled phage antibody A27 as described previously and the commercial markers as described by their manufacturer (**chapter 3**). In short, directly labeled antibody was added 1:20 to whole blood and incubated for 60 minutes on ice. After incubation, the red cells were lysed with ice-cold isotonic NH_4Cl. After a final wash with PBS2+ (phosphate buffered saline with added sodium citrate (0,38 % wt/vol) and isotonic pasteurized plasma proteins (10% vol/vol), the cells were analyzed in a FACScalibur Flowcytometer (Becton & Dickenson, Mountain view. CA). The PMNs were identified according to their specific side-scatter and forward-scatter signals. Data from individual experiments are depicted as histograms of fluorescence intensity in arbitrary units (AU) or summarized as the median channel fluorescence (MCF) of at least 10000 events.

Interleukin 6

IL-6 was determined using a human IL-6 sandwich ELISA (Endogen, Pierce

Biotechnology, IL, United States) according to the procedures prescribed by the manufacturer.

Triglycerides

Blood was collected in a vacutainer® with EDTA as anticoagulant, cooled immediately and kept on ice during the procedure. Plasma was isolated by spinning the sample down at 1000 G. Triglyceride levels were determined as triglycerides have been documented to damage or activate the pulmonary endothelium (19,20).

Statistical analysis

All data were analyzed using SPSS version 12.0 software (The Apache Software Production 2004, Chicago, Illinois). Results are expressed by means ± standard error of the mean. Statistical analysis was performed using a non-parametric Mann Whitney U Test for two groups and a Kruskall Wallis H test for multiple comparisons. Paired analysis (before and after surgery) was performed using Wilcoxon Signed Ranks test. Statistical significance was defined as $p < 0.05$.

RESULTS

Demographics

A total of 90 patients fulfilled the inclusion criteria of which 75 patients were included (83%). Of the non-included patients 1 patient suffered from osteogenesis imperfecta, and in 14 cases there were logistical problems. The demographics of the not included patients did not differ from the included patients (results not shown). Of the 75 patients included, 2 patients underwent external fixation initially, but did not ultimately receive conversion to intramedullary osteosynthesis, 1 patient did not give consent and in 4 patients sampling or analysis was flawed. Thus, 68 patients were adequately followed up. Their mean ISS was 14 ± 10 (mean ± SD) and their mean APACHE II Score was 6 ± 7 (mean ± SD) ad admission in the ICU. Further demographics are listed in Table 1.

Table 1. Patient demographics.

	Mean (range)
Number of patients (n)	68
Male / Female (n)	41 / 27
Age (years)	39 (16-80)
Injury Severity Score	14 (4-43)
New Injury Severity Score	(4-92)
APACHE II Score	6 (0-25)
Time on ICU (days)	5.8 (0-60)
Time on ventilation (days)	4,9 (0-55)
Packed red blood cells before first blood sample (units)	3 (0 – 54)
Fresh frozen plasma before first blood sample (units)	2 (0 – 20)
Treatment strategy	
– Early total care	55 (< 24 hrs)
– Damage control	13 (2 – 8 days)
Type of trauma (n)	
– Tibia fracture	22
– Femur fracture	21
– Tibia fracture in multitrauma	7
– Femur fracture in multitrauma	18
– Severe chest injury	15
Complications (n)	
– ALI	3
– ARDS	6

Systemic inflammation measured by IL-6

Pre-operative plasma IL-6 levels were not statistically significant different between patients who developed ALI or ARDS and patients who did not. Plasma IL-6 levels increased 18 – 24 hours post-operative (p = 0.027), an even more pronounced increase in plasma IL-6 was seen in patients who developed ALI or ARDS (p = 0.011, Figures 2A and 3A).

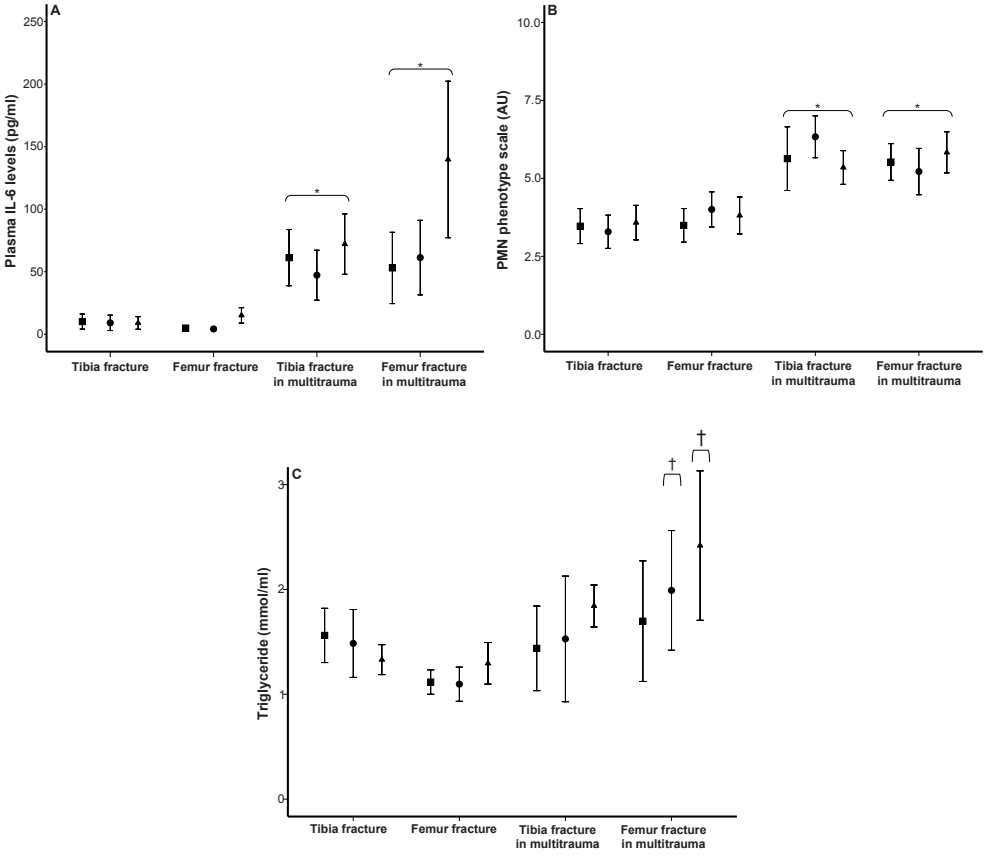

Figure 2. Influence of IMN on IL-6, fMLP induced FcyRII* and triglyceride levels.
Plasma IL-6 levels are increased (p<0.05) in multitrauma patients (**A**). Inaddition, a statistically significant increase was seen after intramedullary nailing in patients with an isolated femur fracture (p=0.027). The largest changes in PMN phenotype (p<0.05) were seen in multitrauma patients (**B**), no statistically significant increase was seen during intramedullary nailing of either tibia or femur fracture. No difference between the patient groups was found in the initial triglyceride levels (**C**), however, a statistically significant increase was seen during intramedullary nailing of femur fractures in multitrauma patients (p=0.002). Squares ■ represent pre-operative samples, circles ● represent samples taken 15 minutes after introduction of the nail and triangles ▲ represent samples taken 18 – 24 hours post-operative. Bars represent mean ± SEM. * = p < 0.05 and ** = p < 0.01 between groups of tibia fracture, femur fracture or multitrauma patients (Mann Whitney U test). † = p < 0.05 within groups (Kruskal Wallis H test).

Systemic inflammation by PMN phenotype

The innate immune activation was based on PMN active FcγRII expression after fMLP stimulation (fMLP induced FcγRII*) (**chapter 4**). The changes in PMN phenotype were statistically significant larger in patients who suffered from multiple injuries compared to patients with only a tibia or femur fracture ($p < 0.05$ on all time points). During IMN no statistically significant alteration of the fMLP induced FcγRII* was detected (Figure 2B). The changes in PMN phenotype were statistically significant larger ($p < 0.05$ on all time points) in patients who developed ALI or ARDS compared to patients without pulmonary complications (Figure 3B).

Triglyceride levels as risk factor for pulmonary endothelial injury

In patients with isolated tibia or femur fractures, no increased triglyceride levels were observed. However, in patients with multiple injuries a statistically significant increase ($p = 0.002$) was seen in triglyceride levels during IMN of the femur (Figure 2C). In patients who developed ALI/ARDS triglyceride levels increased statistically significant ($p < 0.05$) at 15 minutes and 18 hours after IMN (Figure 3C).

Treatment strategy

In the patients undergoing early total care 7/55 (13%) developed ALI/ARDS, compared to 2/13 (15%) in patients undergoing damage control orthopedics. The clinical decision to follow DCO or ETC protocol was evaluated afterwards by plasma IL-6 levels, which were increased ($p < 0.041$) in patients undergoing DCO compared to patients undergoing ETC (Figure 4A). In patients undergoing ETC, an increase in plasma IL-6 was seen 18 hours after IMN ($p < 0.001$). This increase was less pronounced in DCO patients undergoing their delayed surgical procedure, as IL-6 levels were already high. In contrast, no difference in PMN phenotype was found between the two treatment strategies (Figure 4B). Triglyceride levels were statistically significant increased in patients undergoing DCO (Figure 4C) and increased markedly during IMN regardless of the timing of IMN ($p = 0.038$).

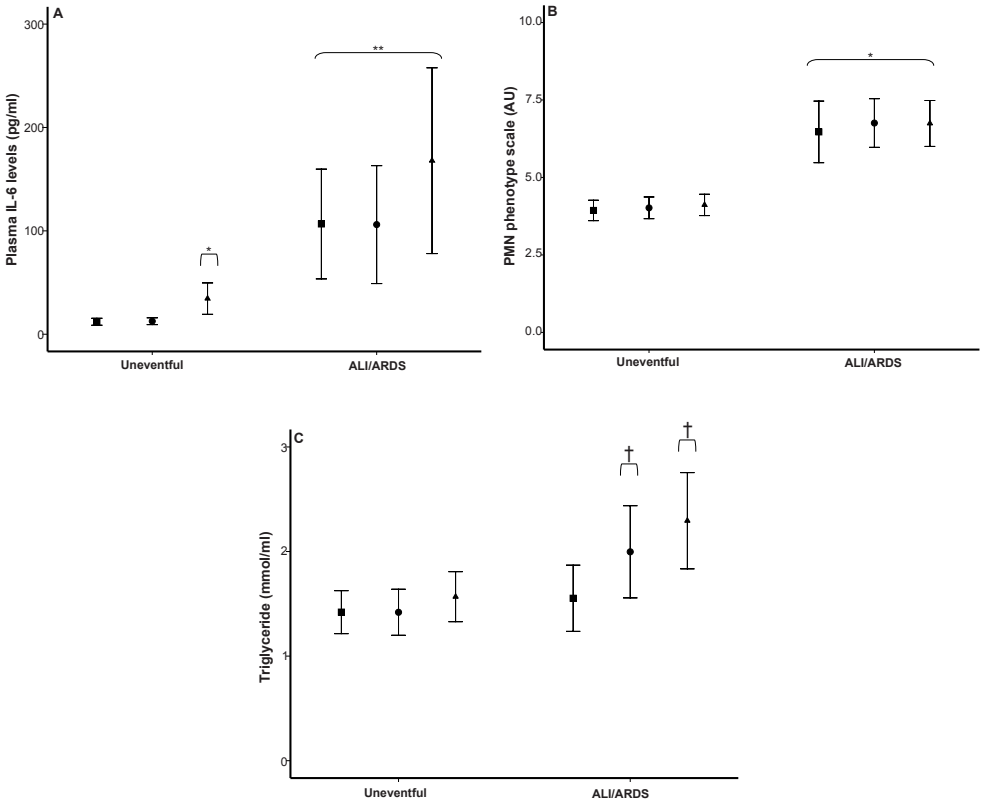

Figure 3. Development of ALI/ARDS and IL-6, fMLP induced FcγRII* and triglyceride levels during IMN.
Initial plasma IL-6 levels are not increased in patients who developed ALI/ARDS (**A**). Although in patients without ALI/ARDS plasma IL-6 levels increased significantly 18 hours after intramedullary osteosyntheses (p=0.006), plasma IL-6 levels were even higher in patients with ALI/ARDS (p=0.011). PMN phenotype was statistically significant changed at all time points (p<0.05) for patients who developed ALI/ARDS (**B**), no statistically significant increase was seen during intramedullary nailing. Triglyceride levels were statistically significant increased 15 minutes and 18 hours after intramedullary osteosynthesis (p<0.05) for patients who developed ALI/ARDS (**C**). Squares ■ represent pre-operative samples, circles ● represent samples taken 15 minutes after introduction of the nail and triangles ▲ represent samples taken 18–24 hours post-operative. Bars represent mean ± SEM. * = $p < 0.05$ and ** = $p < 0.01$ between groups of patients with or without ALI/ARDS (Mann Whitney U test). † = $p < 0.05$ within groups (Kruskal Wallis H test).

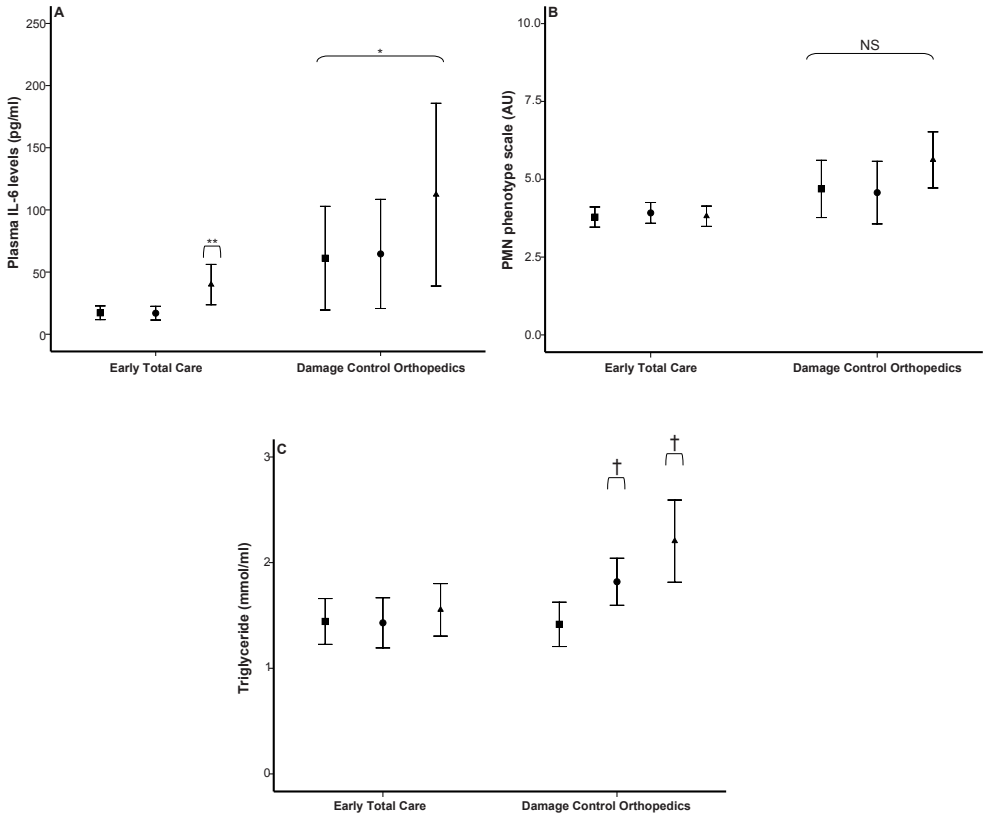

Figure 4. Treatment strategy and IL-6, PMN phenotype and triglycerides during IMN.
Initial plasma IL-6 levels were increased (p=0.041) in patients undergoing damage control orthopedics (**A**), in patients undergoing early total care a increase was seen after intramedullary nailing (p<0.001). No differences were found in PMN phenotype on any time point between patients undergoing damage control orthopedics or early total care (**B**), no increase was seen during intramedullary osteosynthesis. Triglyceride levels were increased in patients undergoing damage control orthopedics on all time points (p<0.05) (**C**), in addition, in patients who underwent damage control orthopedics triglyceride levels increased statistically significant 18 hours after intramedullary osteosynthesis (p=0.035). Squares ■ represent pre-operative samples, circles ● represent samples taken 15 minutes after introduction of the nail and triangles ▲ represent samples taken 18–24 hours post-operative. Bars represent mean ± SEM. * = p < 0.05 and ** = p < 0.01 between ETC and DCO groups (Mann Whitney U test). † = p < 0.05 within groups (Kruskal Wallis H test).

DISCUSSION

Although some additional inflammation occurs during intramedullary osteosynthesis, as measured by a moderate increase in plasma IL-6 levels, apparently this was not sufficient to alter the already activated PMNs, measured by phenotype changes. The severity of inflammation was determined by trauma and as we demonstrated in this study only moderately increased by surgery. IMN induces increased levels of triglycerides in the circulation, which are potentially harmful during a state of severe inflammation. Therefore, before IMN is performed first inflammation should be controlled.

Inflammation occurs after trauma and severe inflammation has been related to the development of ALI/ARDS, which was confirmed in this present study (26,27). Additional inflammation during surgery has been suggested as a possible cause of organ failure. Based on this theory damage control strategies have been developed. Prevention of the additional inflammatory response was hypothesized to reduce the risk for (pulmonary) organ failure (9,28). Application of DCO in “borderline” (i.e. multitrauma) patients who required IMN for femoral fractures resulted in a lower incidence of ALI/ARDS (29). In contrast, increased incidence of complications was reported when DCO strategy was applied in “stable” patients, which confirmed previous reports on the beneficial effects for early definitive care in these “stable” patients (30,31). We corroborated with previous reports that plasma IL-6 levels increased after IMN (32). Though, its’ role in the development of ALI/ARDS has not been elucidated and PMN phenotype was not altered by the increase in plasma IL-6 levels. This lack of change in PMN phenotype during IMN has been suggested previously (7,33). Although no clear explanation could be given, those reports suggest an important role for factor(s) other then the innate immune one.

High levels of triglycerides have been suggested to be involved in the development of ALI/ARDS (19). Micro fat embolisms have been demonstrated in the pulmonary vasculature during IMN, therefore triglyceride levels were designated as the most likely factor to induce pulmonary endothelial damage/activation during IMN (20). We demonstrated that triglyceride levels increased during IMN of femur fractures and during IMN in multitrauma patients. Even more, elevation of triglyceride levels was most pronounced in patients who developed ALI/ARDS. Although several studies

have provided supportive evidence for a pathophysiological role of fat embolisms in ARDS, this is the first study which demonstrates a clinical relation between increased triglycerides levels during IMN and the development of ALI/ARDS. The increase in triglyceride levels during IMN was present in both treatment strategies, ETC and DCO. Therefore, the risk for pulmonary endothelial injury/activation by triglycerides is similar in ETC and DCO.

In total, 2 patients in the DCO group and 7 patients in the ETC group developed ALI/ARDS. The 2 patients who developed ARDS after DCO, received their IMN on different time points. One patient was deemed stable by the attending surgeon, and received his IMN, on the second day after trauma. The second patient was considered fit for IMN on the seventh day after trauma. Both patients demonstrated severe systemic inflammation by severely changed PMN phenotypes. This indicates that a general stated window for secondary surgery of 4 – 10 days after trauma, should be replaced by tailor made therapy (34,35). This finding calls for more adequate laboratory tests to assist the clinical decision of the attending physician. Additional pulmonary endothelial damage/activation by triglycerides is likely to occur frequently during IMN of femur fractures, however additional inflammation barely takes place. Therefore, DCO can only be successful when the patient undergoes his/her secondary surgical procedure (IMN) during an attenuated inflammatory state. However, finding an adequate clinical applicable tool has proven difficult.

Complications in medicine are often the result of an accumulation of events (36,37). ARDS is among the most complex clinical syndromes, harboring multiple disease entities (38). Therefore, combinations of multiple etiologic and pathophysiological factors would most likely lead to the best predictive model. The clinical applicability is related to the availability and complexity of the predictive model. Pathophysiological factors which form a common final pathway of several cascades are best suited for such tools. PMNs are an excellent example of a common endpoint, indicating the inflammatory state of the patient. In contrast, finding the optimal marker for local pulmonary endothelial injury or activation appears more challenging. Triglycerides are thought to induce endothelial damage or activation but are not a marker of the pulmonary endothelial damage or activation itself. Therefore, analysis of pulmonary endothelial damage or activation remains circumstantial and most likely incomplete. Thus, a predictive model that combines systemic inflammation and local pulmonary

injury would profit tremendously from a common endpoint marker for local pulmonary damage. Unfortunately, this study lacks such a pathophysiological factor.

In conclusion, we demonstrated that both systemic activation of PMNs and local pulmonary endothelial damage or activation synergize for the development of ARDS. We therefore suggest not to perform intramedullary osteosynthesis in patients with severe inflammation, identified by changed PMN phenotype. Trauma patients should undergo Inflammatory Control Orthopedics, since pulmonary endothelial damage (/activation) is likely to be induced during IMN.

ACKNOWLEDGEMENTS

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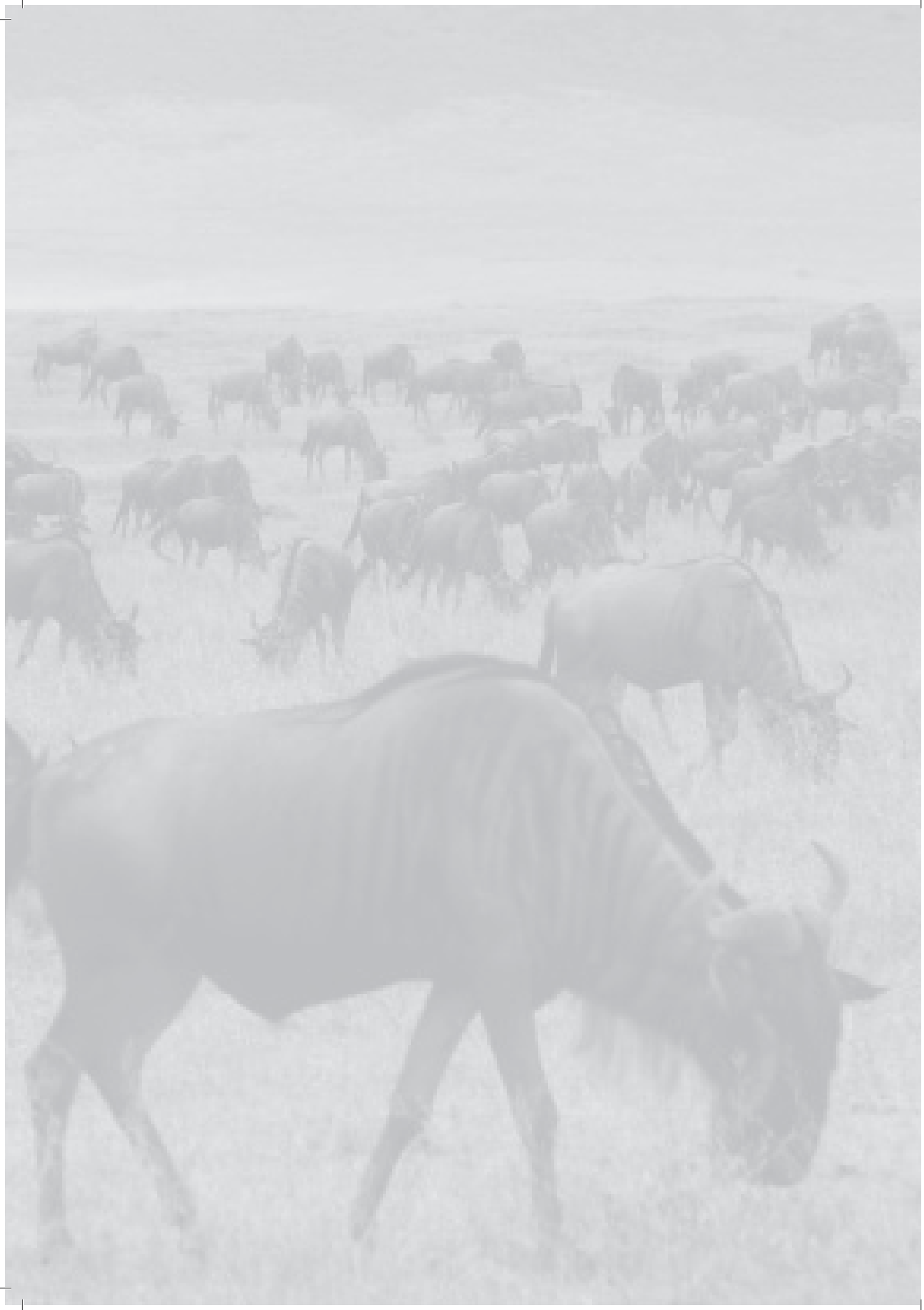

CHAPTER 6

THE INITIAL INFLAMMATORY RESPONSE AFTER TRAUMA IS RELATED TO THE OCCURRENCE OF LATE PHASE SEPTIC SHOCK

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Submitted

ABSTRACT

Severe trauma is frequently followed by sepsis. After an initial pro-inflammatory response induced by the injuries sustained, a compensatory anti-inflammatory state is seen. This immune suppressive state facilitates the development of sepsis. The hypothesis was tested if the initial immunological response after injury is related to the late development of immune dysfunction and septic shock.

A consecutive series of thirty-six severely injured patients was included and followed for 14 days. Admission scores were calculated and sepsis criteria were assessed daily. A blood sample was taken daily and analyzed for neutrophil phenotype, characterized by expression of adhesion receptor MAC-1, chemotaxis receptor CXCR-1, opsonin receptor active FcγRII and inside-out control of fMLP induced active FcγRII.

A profound inflammatory reaction was noted in all trauma patients by the statistically significant differences from controls for IL-6, MAC-1, CXCR1 and active FcγRII expression. Ten out of 36 patients developed septic shock, invariably 8 – 10 days after admission. FMLP induced active FcγRII was significantly decreased in patients who later developed septic shock. CXCR-1 and fMLP induced active FcγRII showed a gradual decrease in expression prior to clinical signs of septic shock, while no additional activation measured by MAC-1 up-regulation was present.

In conclusion, phenotyping blood neutrophils enables identification of the kinetics and magnitude of the initial systemic inflammatory response after injury. The inability of the PMN to react to fMLP is related to the subsequent development of late phase septic shock.

Multiple Organ Failure (MOF) is a frequent complication of critically ill patients and goes with a high mortality (50-80%) and morbidity rates (1). Patients suffering from severe trauma are at risk for this complication in two distinct phases; an early phase 1 - 4 days after injury and a late phase 8 - 14 days after injury (2). Early MOF is thought to be the result of an excessive inflammatory response (Severe Inflammatory Response Syndrome [SIRS]) ensuing the sustained injuries (3,4). Late MOF is thought to be a consequence of sepsis or uncontrolled infection during a state of immune paralysis (Compensatory Anti-inflammatory Response Syndrome [CARS]) (5). These clinical findings have led to the hypothesis of a biphasic inflammatory response after trauma (6). However, this hypothesis awaits experimental foundation.

In the pursuit for a pathophysiological explanation several approaches were used. The initial pro-inflammatory response, characterized by soluble markers e.g. IL-1β, IL-6 or CRP, has been qualitatively identified. Although a general increased inflammatory state was found, the quantification of its magnitude has remained difficult due to the large interpersonal variation of these markers (7). Most research on the development of sepsis during CARS determined the role of lymphocytes and monocytes in this process, but these studies remained inconclusive (8,9). Polymorphonuclear granulocytes (i.e. neutrophils or PMNs) are important in the pathogenesis of organ damage during early organ failure, but play an essential role in the clearance of bacterial pathogens under normal immunological conditions (10,11). We recently demonstrated that PMNs are partially dysfunctional after trauma, as measured by the inside-out control of these cells (12). Although basic PMN functions such as cytotoxic capacity (reactive oxygen species production) were increased, activation of FcγRII in response to bacterial products was decreased. Therefore, it was hypothesized that the loss of inside-out control by PMNs might play a role in the susceptibility of a patient in the development of sepsis and the additional damage to organs during severe sepsis (12,13).

In the present study we tested, whether an excessive initial systemic pro-inflammatory response after severe trauma was related to the late development of immune dysfunction and septic shock.

MATERIALS AND METHODS

Patients

A consecutive series of severely injured patients who required intensive care support (ICU) in the University Medical Centre Utrecht was included. Patients had to be between 18 and 80 years old, with an expected ICU stay of ≥ 3 days. Exclusion criteria were chronic diseases influencing the immune system and/or the use of immunosuppressive medication. The patients were followed for a maximum of 14 days or as long as their stay in the ICU lasted. The institutional ethics committee approved the study and written informed consent was obtained from all patients or their legal representatives in accordance with the protocol. When needed, results obtained from these patients were compared with results from 10 healthy controls.

Clinical parameters

The APACHE-II score and Injury Severity Score (ISS) were calculated on admission (14,15). Criteria for SIRS, sepsis or septic shock were assessed on a daily basis as defined by the criteria proposed by the International Sepsis Definitions Conference (16,17).

Sampling and phenotyping

A first blood sample was taken 3 – 12 hours after the patients' admission to the ICU (day zero). Serial blood samples were taken on a daily basis during the following 14 days. Blood was collected in a vacutainer® with sodium heparin as anticoagulant, cooled immediately and kept on ice during the whole staining procedure, which started directly. The functions of the studied PMN surface receptors have been described (10,13). The adhesion molecule MAC-1 (CD11b) was chosen as representative for neutrophil activation, because this receptor is up-regulated during granule release (18-20). CXCR-1 receptor (CD181) was chosen, as its role in chemotaxis has been well documented. CXCR-1 is only down-regulated after strong stimulation, while CXCR-2 is a low-affinity receptor and is rapidly down-regulated (21,22). In addition, a pathophysiological role of CXCR-1 has been suggested in the development of severe infections and sepsis, as chemotaxis would be impaired (22). Focus was given to the expression of active FcγRII (active CD32 or FcγRII*) as read-out for a very sensitive marker for activation: inside-out control of FcR's (23){Kanters, 2007 700 /idThe expression was measured directly, to study the intrinsic expression as

indication of activation of the cells in the blood. The intrinsic activation of FcγRII was compared with the responsiveness of circulating PMNs for the innate stimulus fMLP in induction of active FcγRII *in vitro* (24,25).

Materials

For analysis of PMN receptor expression by flowcytometry, the following standard monoclonal antibodies were commercially purchased: FITC-labeled IgG1 negative control (clone DD7) from Chemicon, Hampshire, United Kingdom; RPE-labeled IgG2a negative control (clone MRC OX-34), RPE-labeled CD11b (clone 2LPM19c) from DAKO, Glostrup, Denmark and FITC-labeled CD181 (clone 42705) from R&D Systems, McKinley Place. A monoclonal phage antibody, which recognizes active FcγRII (active CD32), was manufactured in the Department of Respiratory Medicine at the University Medical Center Utrecht (MoPhap A27, UMCU, Utrecht, The Netherlands) (24,26). Interleukin 6 (IL-6) analysis was performed using a sandwich ELISA (Pierce Biotechnology Inc., IL, United States) as described by the manufacturer. C reactive protein (CRP) was measured in the clinical diagnostic laboratory of the UMC Utrecht by immunoturbidimetric analysis (27).

Flowcytometer analysis

Blood samples were stained with directly-labeled antibodies as described previously (12). In short, labeled antibodies were added 1:20 to whole blood and incubated for 60 minutes on ice. After incubation, the red cells were lysed with ice-cold isotonic NH_4Cl. After a final wash with PBS2+ (phosphate buffered saline with added sodiumcitrate (0.38 % wt/vol) and isotonic pasteurized plasma proteins, 10% vol/vol), the cells were analyzed in a FACScalibur flowcytometer (Becton & Dickinson, Mountain View. CA). The PMNs were identified according to their specific side-scatter and forward-scatter signals. Data from individual experiments are depicted as median fluorescence intensity in arbitrary units (AU) of at least 10000 events.

To determine the responsiveness of neutrophils for N-formyl-methionyl-leucyl-phenylalanine (fMLP) the expression of active FcγRII/CD32 (designated FcγRII*) was also measured after 5 minutes of stimulation of whole blood at 37 °C with (fMLP 10^{-6}M). After stimulation, the samples were put on ice and analyzed.

Soluble proteins

Plasma IL-6 levels have been correlated with the severity of the inflammatory response on admission (28,29). In addition, the initial plasma IL-6 levels have been related to the development of both early and late phase organ failure (7). However, longitudinal measurements of plasma IL-6 levels did not provide additional information to the initial sampling (7). Therefore, we analyzed plasma IL-6 levels between 3 – 12 hours after trauma. Plasma was isolated by spinning the sample down at 1000 x g. IL-6 was determined using a human IL-6 sandwich ELISA according to the procedures prescribed by the manufacturer.

C-reactive protein (CRP) is increased in nearly all trauma patients. However, an ongoing rise in plasma CRP levels after several days have been associated with the development of severe infections and subsequent sepsis (30,31). Therefore, CRP levels were measured on a daily basis by the clinical diagnostic laboratory of the UMC Utrecht using immunoturbidimetric analysis (27).

Statistics

Receptor expression was analyzed as median fluorescence intensity (MFI) in arbitrary units (AU). Results in figures are expressed as means ± standard error of mean (SEM). Statistical analysis was performed with the non-parametric Mann-Whitney U test for two groups. Kruskal Wallis H analysis was used to analyze changes between different days. Statistical analysis for PMN receptor expression between patients with septic shock and without septic shock during longitudinal measurements was performed using generalized estimating equations (GEE), analyzing from day 1 to 8 per patient. First order autoregressive correlation was used as model. Statistical significance was defined as $p < 0.05$.

RESULTS

Demographics

Forty patients fulfilled the inclusion criteria. Two patients were transferred to another hospital and two patients did not provide informed consent. These four patients suffered slightly less severe injuries ($p > 0.05$) compared to the thirty-six included patients admitted to the ICU after trauma. Thirty-two of the analyzed patients fulfilled the SIRS criteria, 21 patients met the sepsis criteria and 10 patients developed septic shock (17). All patients who developed septic shock (n = 10) fulfilled the criteria between days 8 – 10 after admission. Two patients died during their admission, one of them during the study period. Cause of death for both patients was multiple organ failure (Table 1).

Table 1. Demographics

	Mean (± SD)
Number of patients (n)	36
Male / Female (n)	30 / 6
Age (years)	45 (18-73)
Injury Severity Score	24 (9-57)
APACHE II Score	14 (0-35)
Time on ICU (days)	16 (3-45)
Time on ventilation (days	15 (0-42)
Complications (n)	
- SIRS	32
- Sepsis	21
- Septic shock	10
- Mortality	2

Disease severity on admission and ensuing course

No statistically significant difference was found in the admission scores for patients who developed sepsis or septic shock as compared to patients without complications (APACHE II score $p = 0.154$; ISS $p = 0.376$; initial leukocyte counts $p = 0.810$) (Table 2). Plasma IL-6 levels were increased in all patients on admission and slightly more pronounced in patients who developed septic shock, however, with large interpersonal variation (Figure 1A). CRP demonstrated comparable levels in both groups during the first week of admission. In the second week of ICU stay, CRP

levels in patients with septic shock remained elevated, whereas patients without septic shock demonstrated decreasing levels of CRP (Figure 1B).

Table 2. Admission severity and occurrence of events.

Mean ± SD	No sepsis (n = 15)	Sepsis (n = 11)	Septic shock (n = 10)	P-value
APACHE II Score	12 (± 6.3)	14 (± 8.4)	18 (± 7.2)	NS
ISS	24 (± 10.1)	21 (± 9.2)	29 (± 12.7)	NS
Leukocytes	10.5 (± 3.7)	11.8 (± 7.2)	13.0 (± 6.9)	NS

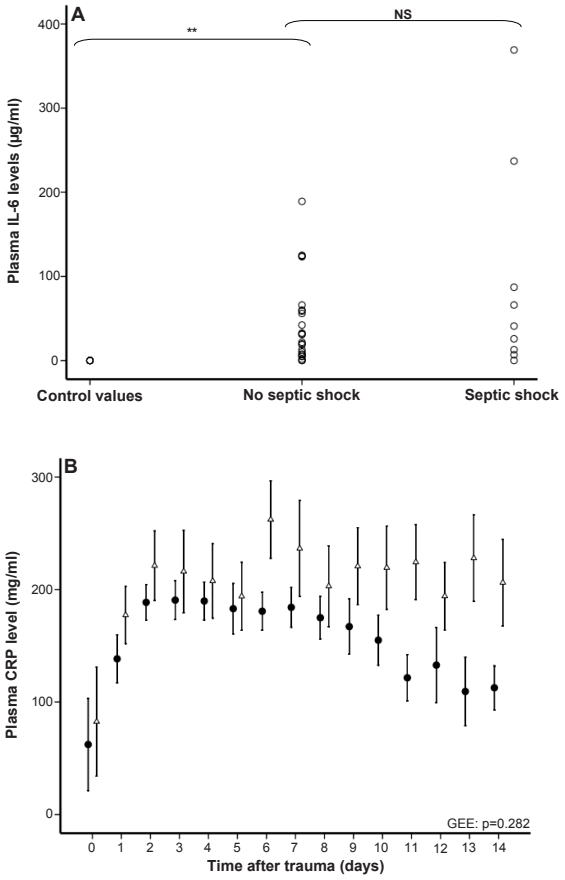

Figure 1. Severity of inflammation assessed by acute phase proteins.
Initial plasma IL-6 levels were increased in trauma patients. However, in this relative small population, no statistically significant differences were found between patients who later developed septic shock and patients who did not (**A**). CRP increased during the first days of ICU admission for all patients, but was only discriminative just prior to the presence of clinical evidence of septic shock (**B**). Differences were seen from day 7 on and septic shock developed in all patients between days 8 – 10. Patients without septic shock are depicted as closed circles ● and patients with septic shock (n = 10) are depicted as open triangles Δ. Bars represent mean ± standard error of mean. * represent $p < 0.05$ and ** represent $p < 0.01$.

Initial inflammatory response within 24 hours after trauma

By evaluating basic PMN phenotype changes, the following results were obtained. The expression of the activation marker MAC-1 (CD11b) was statistically significant increased ($p = 0.009$) compared to controls. Chemotaxis receptor CXCR-1 (CD181) was statistically significant decreased in patients compared to controls ($p = 0.027$). However, no significant difference for both receptors was found between patients who developed septic shock and patients who did not (Figure 2A and 2B).

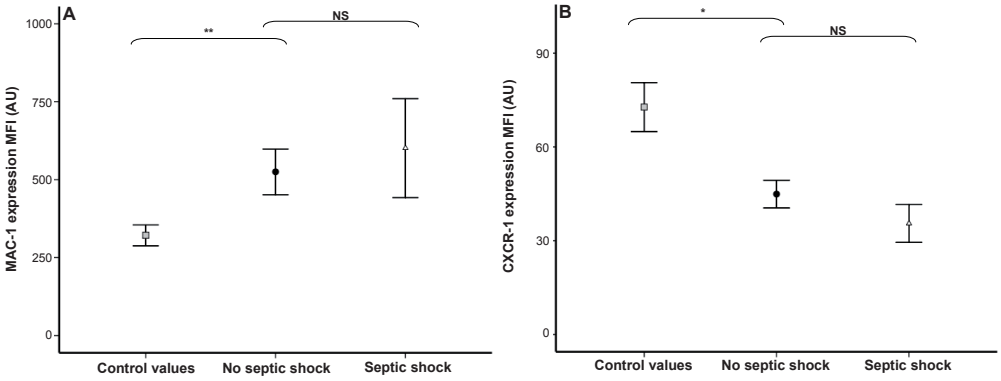

Figure 2. Expression of MAC-1 and CXCR-1 on PMN's does not relate to late onset sepsis.
Initial levels of (**A**) PMN MAC-1 and (**B**) PMN CXCR-1 expression were statistically significant altered in patients compared to controls. Septic shock developed in all patients after 8 – 10 days. Control values are presented as blocks □. Patients without septic shock are depicted as closed circles ● and patients with septic shock (n = 10) are depicted as open triangles Δ. Bars represent mean ± standard error of mean. * represent $p < 0.05$ and ** represent $p < 0.01$.

Evaluating a more sensitive PMN phenotype, active FcγRII (FcγRII*), revealed also decreased intrinsic expression of this active receptor complex in all patients (Figure 3A, $p < 0.05$). However, we failed to demonstrate any statistically significant difference between septic and non-septic patients. In contrast, the responsiveness of neutrophils for fMLP in inducing inside-out control of FcγRII* was statistically significant ($p < 0.001$) decreased in patients compared to controls. More importantly, the responsiveness fMLP could differentiate between patients who developed septic shock and those who did not (Figure 3B; $p < 0.05$).

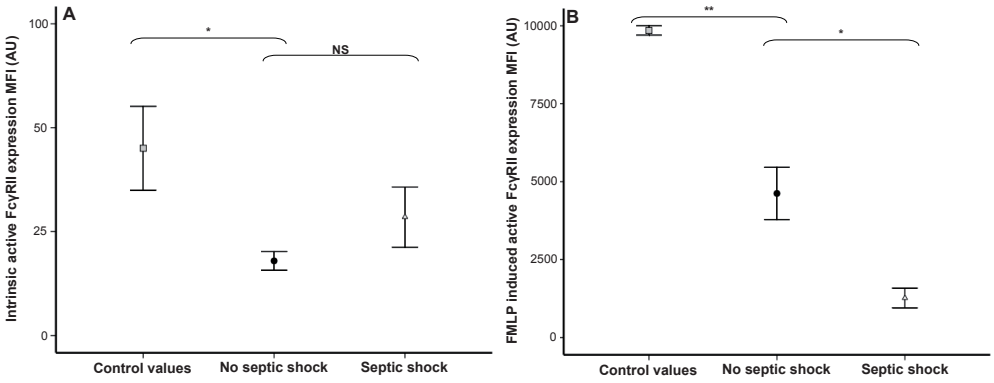

Figure 3. fMLP-induced expression of active FcγRII on neutrophils is suppressed in patients that develop septic shock.
Initial levels of (**A**) PMN active FcγRII expression and (**B**) inside-out control of fMLP induced FcγRII* were statistically significant altered in patients compared to controls. In addition, fMLP induced FcγRII* was significant decreased in patients who later developed septic shock compared to patients without this complication. Control values are presented as blocks □. Patients without septic shock are depicted as closed circles ● and patients with septic shock (n = 10) are depicted as open triangles Δ. Bars represent mean ± standard error of mean. * represent $p < 0.05$ and ** represent $p < 0.01$.

Decrease in inside-out control without further activation during first week after trauma

The PMN phenotype was further analyzed during the first week of admission. In this first week, no further PMN activation was detectable, as measured by MAC-1 and FcγRII* (Figure 4A and 4B). Also, no statistically significant differences were found between patients who developed septic shock and patients without this complication.

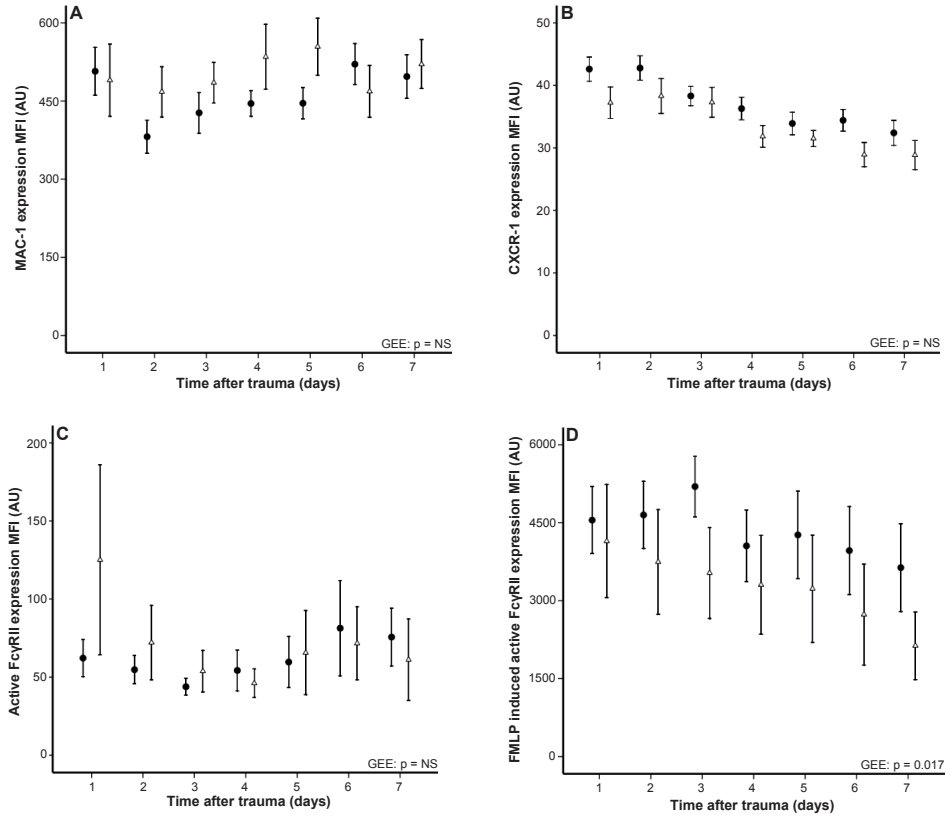

Figure 4. Course of PMN surface receptor expression during the first week of admission.
Alterations in PMN phenotype of (**A**) adhesion associated activation marker MAC-1, (**B**) chemotaxis receptor CXCR-1, (**C**) opsonin receptors FcγRII and (**D**) inside-out control by fMLP induced FcγRII*. Patients without septic shock during admission are depicted as closed circles ● and patients who developed septic shock during admission are depicted as open triangles ∆. Bars represent mean ± standard error of mean.

CXCR-1 demonstrated no statistically significant differences between patients who developed septic shock and patients without sepsis (Figure 4C). However, CXCR-1 showed a gradual decrease in expression during the first week of admission ($p < 0.001$). Although fMLP stimulated FcγRII* showed a recovery during the first day after trauma in patients who developed septic shock, longitudinal analysis of inside-out control of FcγRII* showed a gradual decrease during the first week ($p < 0.001$). As a group, patients who developed septic shock demonstrated a more pronounced decrease in fMLP stimulated FcγRII* on all days (Figure 4D, GEE: $p = 0.017$).

Septic shock developed in 10 patients in the period thereafter (8 – 10 days after admission). MAC-1 and CXCR-1 expression did not demonstrate any statistically significant differences after the onset of septic shock. In contrast, fMLP induced FcγRII* showed a significant recovery in the group of patients who did not developed septic shock (Figure 5, GEE: p = 0.05).

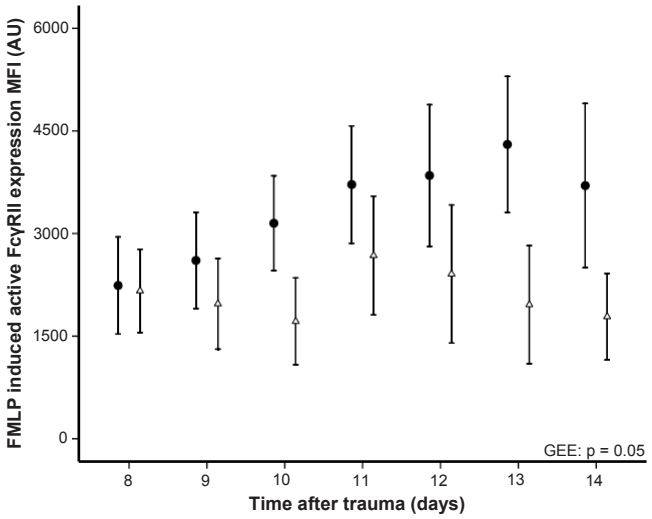

Figure 5. Recovery of fMLP induced FcγRII* in patients without septic shock.
Patients without septic shock during admission are depicted as closed circles ● and patients who developed septic shock during admission are depicted as open triangles △. Bars represent mean ± standard error of mean.

DISCUSSION

In this study we demonstrated a marked inflammatory response after severe trauma, shown by the acute phase response (measured by IL-6 and CRP) and an increase in expression of the activation epitope MAC-1 on PMNs. Moreover, on the first day after trauma we could demonstrate a statistically significant difference between those patients developing septic shock and those with a less severe complication or course, evaluating the responsiveness of PMNs for fMLP in stimulating FcγRII*.

All markers (plasma IL-6 and CRP levels, leukocyte count, but also expression of activation epitopes on PMN's) found in the trauma patients showed statistically significant changes compared to controls, demonstrating a severe systemic inflammatory response after trauma. However, these markers could not differentiate between patients who developed septic shock and those who did not. Analysis of PMN functionality by changes in fMLP induced FcγRII*, showed a significantly decreased in responsiveness for fMLP of peripheral blood PMNs in patients who developed septic shock. A straightforward explanation for these findings on the day of trauma could be that those patients, ultimately developing septic shock, have a more profound initial systemic (cellular) innate immune response after injury.

This decreased functionality partly recovered in the first 24 hrs after trauma, however, reduced again during the first week after trauma. The lowest PMN expression levels were found between 6 – 7 days after injury. For all septic shock patients initial shock symptoms became evident between days 8 – 10 after admission. Therefore, the impaired responsiveness for fMLP and other changes in receptor expression clearly *preceded* clinical symptoms. In patients who did not develop septic shock, a statistically significant recovery of fMLP induced FcγRII* was observed in the second week after injury.

Several authors have evaluated the relevance of analysis of changes in expression of single PMN receptors (19-22,32), as well as levels of single cytokines and acute phase proteins such as CRP (7,28,30,31). The current study confirms that changes occur both in expression of activation markers on PMN's and in levels of acute phase proteins after trauma. However, our study uniquely shows a decreased response of circulating PMNs to fMLP, a bacterial derived product. Other authors

have demonstrated increased cytotoxic activity (ROS production) in isolated cells (33,34), however, these studies could have been biased by the isolation of PMNs (35). The present study, performed by whole blood analysis, lacks *ex vivo* leukocyte manipulation, limiting isolation artifacts. Therefore, these results allow a more direct interpretation of the cellular innate immune processes taking place after trauma and might open new avenues to develop new prognostics. Although it is tempting to extrapolate our data and suggest that fMLP induced FcγRII* could be used as a predictor for the development of septic shock, this hypothesis should be tested in a prospective study with sufficient power. This study to evaluate the prognostic capacities of this finding is currently underway.

It has been suggested that after injury (i.e. acute severe inflammation) PMNs home to the tissues, leaving behind PMNs with an altered or even refractory phenotype (3,12,36). It tempting to speculate that the refractory PMNs we found are the result of a profound extravasation of non-refractory PMNs in these patients. After the initial decrease in inside-out control, a slight recovery in fMLP induced FcγRII* was seen on the first day after trauma, which might be the result of functional competent cells from the bone marrow as large amounts of functional PMNs are released from the bone marrow and marginated pool (13). This rapidly mobilization of a functional pool of PMNs from the bone marrow and homing to the tissues is thought to be mediated by chemotactic signals (12,36). Due to the ongoing migration, a persistent loss of functional PMNs takes place by homing, leaving relatively refractory cells behind in the circulation. These refractory PMNs will lead to a progressively less competent immune system and a poorer handling of any new bacterial threat. Our findings described above, provide additional pathophysiological evidence that supports the hypothesis that more pronounced SIRS is associated with more severe CARS and therefore the development of septic shock (37).

Although mortality after trauma has decreased over the last decade, the incidence of sepsis remains high. In this cohort, the development of septic shock was very frequently encountered; an incidence of septic shock of 28% was found. In comparison, an incidence of septic shock of 10 – 15% is generally reported in literature (38,39). The high incidence in the present study can be attributed to the selection of patients. Patients with minor injuries and no post-operative complications, who were expected to have a short uneventful intensive care stay, were excluded from this study. In

addition, patients who died within three hours after trauma were excluded as well. Therefore, the studied population suffered from severe injuries, however survived the first hours and was consequently at an increased risk for the development of infectious complications and sepsis. Yet, even in this small population with a severe inflammatory response initial fMLP induced FcγRII* differences between patients who ultimately developed septic shock and patients who did not with sufficient power and statistical significance.

In conclusion, this study creates a new perspective on the pathophysiological processes of severe inflammation after trauma. The responsiveness of peripheral blood PMNs to respond to bacterial derived products is diminished after severe trauma, which is likely related to the development of septic shock 8 – 10 days after injury.

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Chapter 7

Occurrence of activated HLA-DR negative monocytes preceding development of ARDS in trauma patients

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ABSTRACT

Organ failure after trauma can develop early (1-3 days) mediated by an overactive innate immune system, or late (>6 days) due to sepsis as a result of a suppressed immune system. Monocytes are important as precursor cells for both antigen presenting HLA-DR positive dendritic cells and tissue macrophages. Traditionally, changes in monocyte phenotype are thought to be related with second phase organ failure (e.g. sepsis). In this study the hypothesis was tested if composition of HLA-DR positive/negative blood monocytes relates to the early phase (e.g. ARDS) of organ failure after trauma.

Fifty-five trauma patients who underwent lower extremity intramedullary nailing (IMN) were included. The development of ARDS during the admission was recorded. Blood samples were taken prior, 15 minutes after and 18 hours after IMN. The phenotype of monocytes in the peripheral blood was determined.

The percentage of HLA-DR positive monocytes was decreased prior to IMN compared to controls and correlated with the Injury Severity Score. A significant decrease in the percentage of HLA-DR positive monocytes was seen 18 hours after IMN in all patients. Patients who developed ARDS after IMN showed a significantly lower percentage of HLA-DR positive monocytes prior to surgery. This monocyte population demonstrated an activated phenotype characterized by increased CD11b expression. In addition, these cells were refractory to activation with fMLP in the context of active FcγRII (CD32).

In conclusion, an early shift in the composition of the monocyte population towards an activated phenotype, rich in HLA-DR negative cells, was related to the development of ARDS after IMN. This finding is in line with the hypothesis that initial hyper- or aberrant activation of the innate immune system induced by trauma is a risk factor for organ failure.

INTRODUCTION

Monocytes, as precursor cells for both dendritic cells and macrophages, play an important role in the control of immune homeostasis, tissue repair and the host defence against invading macro-organisms (1-3). Monocytes enter a site of tissue injury within one hour after the initial insult (4). A pool of monocytes is present in the peripheral circulation allowing an immediate response to infection, inflammation and/or tissue damage.

Monocytes also play an important role in the connection between the innate and adaptive immune system, as these cells can express HLA-DR (MHC-II), a receptor complex that allows antigen presentation to CD4+ lymphocytes (5,6). This allows these cells to prime the immune response of the host for an amplified response to recall antigens.

Several studies have linked a reduction of HLA-DR expression on monocytes and/or a reduction in the percentage of HLA-DR positive monocytes in the circulation with the incidence of late phase infections after injury (7-9). It was demonstrated in patients with severe sepsis, that circulating monocytes with a reduced HLA-DR expression have a pro-inflammatory phenotype characterized by increased cytokine production and increased expression of adhesion receptors (10).

Presently, there is a general consensus that a hyper-activated innate immune response can lead to the systemic inflammatory response syndrome (SIRS), responsible for early organ failure after trauma (11). Interestingly, little is known regarding these HLA-DR negative, pro-inflammatory monocytes in relation with this early phase of organ failure such as pulmonary failure (Acute Lung Injury [ALI] or Acute Respiratory Distress Syndrome [ARDS]). We hypothesized that a relation is present between a reduction in HLA-DR positive monocytes and severity of injury. We investigated if HLA-DR expression and expression of activation markers on circulating monocytes was related to the development of early phase organ failure. In addition, it was investigated if intramedullary osteosynthesis (a well documented risk factor for ARDS) (12) influenced the percentage of HLA-DR positive monocytes in the circulation during and after the surgical procedure.

MATERIALS AND METHODS

Patients

Fifty-five trauma patients (admitted at the Department of Traumatology, University Medical Center Utrecht) with a tibia or femur fracture who required primary intramedullary nailing, were included in this study. Exclusion criteria were age < 16 years or > 80 years and patients with an altered immunological status (e.g. use of corticosteroids or chemotherapy). The local medical ethical review board approved the study and written informed consent was obtained from all patients or their spouses in accordance with the protocol. Ten healthy volunteers were included for comparison with control values.

Clinical parameters

The Injury Severity Score (ISS) and APACHE-II score were calculated on admission (13,14). During the hospital stay, presence of systemic inflammation (e.g. systemic inflammatory response syndrome [SIRS]), or the occurrence of pulmonary complications (i.e. acute lung injury [ALI], or acute respiratory distress syndrome [ARDS]) were assessed by their clinical criteria as determined in the consensus conference (15,16). The presence of pneumonia was recorded by a positive sputum culture, an infiltrate on the chest X-ray and clinical symptoms of infection (17). Pulmonary problems due to cardiac failure were determined by chest X-ray, high venous pressure (as determined by Swahn-Ganz catheter) and clinical signs of cardiac pump failure.

A first blood sample was taken within 3-24 hours after the patients' admission. Consecutive blood samples were taken during intramedullary nailing and 18 hours after intramedullary osteosynthesis. Blood was collected in a vacutainer® with sodium heparin as anticoagulant cooled immediately and kept on ice during the whole staining procedure.

Materials

For analysis of monocyte HLA-DR expression by flowcytometry the following monoclonal antibodies were commercially purchased: FITC-labeled IgG1 negative control (clone DD7, Chemicon, Hampshire, United Kingdom) and FITC-labeled HLA-DR (YE2/36-HLK, Serotec, Dusseldorf, Germany), RPE-labeled CD11b

(clone 2LPM19c, DAKO, Glostrup, Denmark) and FITC labeled active FcγRII (A27; Respiratory Medicine, UMC Utrecht, Netherlands). Routine hematology parameters were determined at the Clinical Laboratory Department of the University Medical Center Utrecht (18). The analysis of the monocyte HLA-DR expression was started within two hours after the blood sample was obtained.

Flowcytometry

Blood samples were stained with directly labeled antibodies as described previously (19). One sample of whole blood was stimulated for 5 minutes at 37˚C with 10^{-6} fMLP, after which active FcγRII was measured. For all samples the directly labeled antibodies were added 1:20 to whole blood and incubated for 60 minutes on ice. After incubation, the red cells were lysed with ice-cold isotonic NH_4Cl. After a final wash with PBS2+ (phosphate buffered saline supplement with sodiumcitrate 0.38% wt/vol and pasteurized plasma protein solution 10% vol/vol), the cells were analyzed in a FACScalibur Flowcytometer (Becton & Dickenson, Mountain view. CA). The monocytes were identified according to their specific side-scatter and forward-scatter signals. Data from individual experiments are depicted as fluorescence intensity in arbitrary units (AU) or summarized as the median channel fluorescence (MCF) for all monocytes and/or for HLA-DR positive monocytes. The percentage of HLA-DR positive monocytes was defined as the percentage of monocytes with a higher expression of HLA-DR than its negative control value.

Statistics

Results in figures are expressed as means ± standard error of mean (SEM). Statistical analysis was performed with the non-parametric Mann-Whitney U test to compare two groups and Kruskal-Wallis H test to compare multiple groups. Correlation analysis was performed using non-parametric Spearman correlation analysis. Statistical significance was defined as $p < 0.05$.

RESULTS

A total of 55 patients fulfilled the inclusion criteria. Three patients were excluded due to technical problems with the analysis of the monocytes: 1 multitrauma patient and 2 patients with isolated tibia fractures. A total of 52 patients were further analyzed. Their mean age was 38 (range 16-80) and the mean ISS was 11 (range 4-43). Further demographics are listed in table 1. Eight patients had an ISS > 20, while an additional 8 patients suffered multi trauma as defined by ISS > 16 and 2 affected AIS regions.

Table 1. Patient demographics.

	Mean (range)
Number of patients (n)	52
Male / Female (n)	31 / 21
Age (years)	38 (16-80)
Injury Severity Score	11 (4-43)
APACHE II Score	4 (0-24)
Time on ICU (days)	2.5 (0-31)
Time on ventilation (days)	2.2 (0-29)
Packed red blood cells before sampling (units)	0.7 (0-15)
Fresh frozen plasma before sampling (units)	0.2 (0-4)
Cause of trauma (n)	
- MVA	36
- Assault	0
- Fall of height	15
- Penetrating trauma	1
Complications (n)	
- None	46
- ALI / ARDS	6

A relation between HLA-DR positive monocytes and injury severity (ISS) was found (Kruskal-Wallis H p=0.005). Patients with only mild trauma demonstrated a slight reduction in HLA-DR positive monocytes, whereas patients suffering severe trauma showed the most pronounced decrease in HLA-DR positive monocytes (Figure 1A). This difference in HLA-DR positive monocytes between the 3 groups disappeared after IMN (Figure 1B), as all patients demonstrated a decreased percentage of HLA-DR positive monocytes.

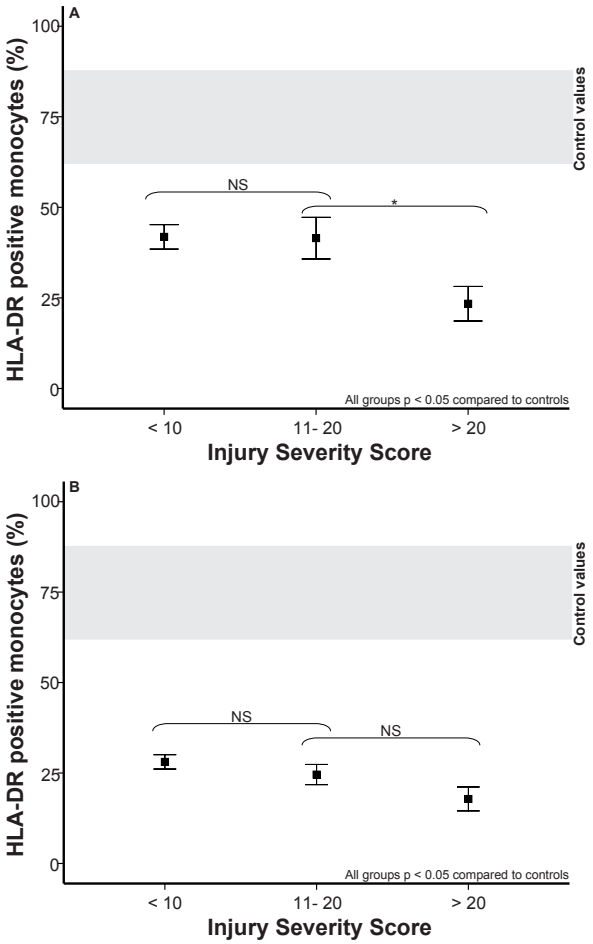

Figure 1. Injury severity and percentage HLA-DR positive monocytes.
A statistically significant difference was found before surgery (**A**) between patients with a low or high Injury Severity Score and the reduction in HLA-DR positive monocytes (p=0.005). However, this difference disappeared 18 hours after intramedullary nailing (**B**). Gray shaded area depicts normal control values.

All patients underwent intramedullary nailing. Patients were stratified according to their injury severity and surgical procedure (intramedullary nailing of tibia or femur fracture). Interestingly, all patients demonstrated reduced percentage HLA-DR positive monocytes 18 hours after the surgical procedure, regardless of their initial injuries (Figure 2).

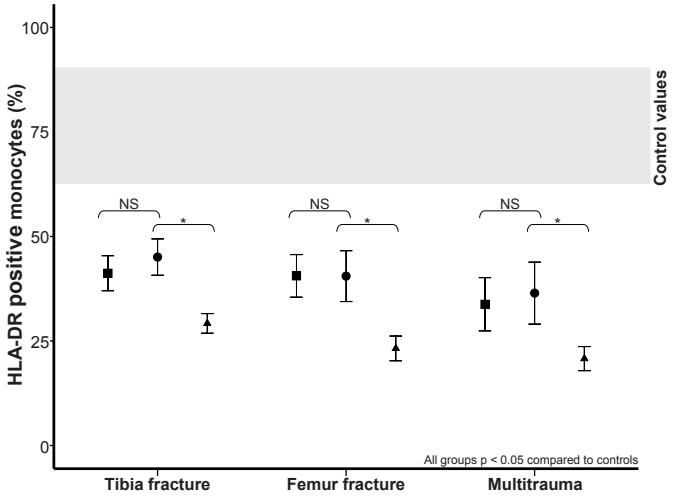

Figure 2. HLA-DR positive monocytes and intramedullary nailing.
Patients were stratified in 4 groups according to their type of surgery (intramedullary nailing of either tibia or femur fracture) and the presence of other severe injuries. Prior to surgery, no statistically significant differences were found between the 4 groups. After the surgical procedure, virtually all patients demonstrated a reduction in HLA-DR positive monocytes. No differences were found for reamed versus unreamed or local versus general anesthesia. Squares ■ represent pre-operative samples (all within 24 hours after admission), circles ● represent per-operative samples and triangles ▲ represent samples taken 18 hours post-operative.

Severe systemic inflammation is thought to be related to the development of inflammatory complications such as ALI and ARDS. Along this line, a reduction in HLA-DR positive monocytes can be viewed as a consequence of this inflammation. Therefore, we analyzed our patients in the context of presence HLA-DR positive monocytes in the circulation in relation to the development of complications within the first 48 hours after sampling. Two patients developed ALI and four patients fulfilled the ARDS criteria. The severity of the complications of these patients with ALI/ARDS is expressed in their days on intensive care support (0-11 vs 5-31) and mechanical ventilation (0-9 vs 4-29). The percentage HLA-DR positive monocytes was

statistically significant decreased in the initial sampling preceding the development of ALI/ARDS (Mann Whitney U test p=0.001, Figure 3). After surgery, no significant differences between patients with ALI/ARDS and without ALI/ARDS were detected.

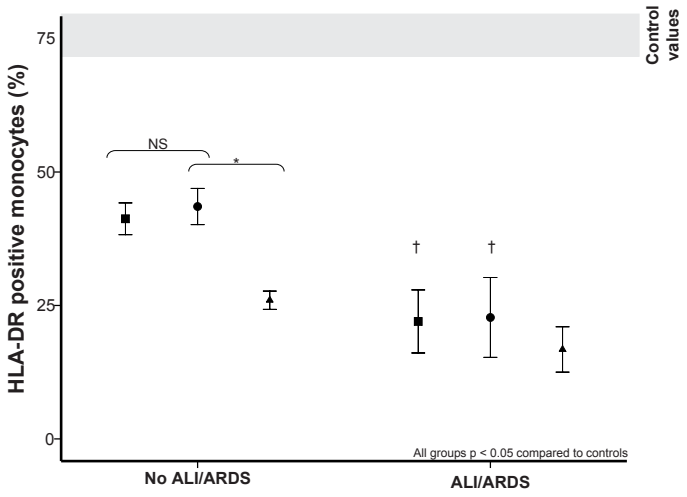

Figure 3. HLA-DR positive monocytes and development of ARDS.
When patients were stratified by the development of acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) within 48 hours after the surgical procedure a statistically significant lower percentage of HLA-DR positive monocytes was found pre-operative (Mann Whitney U test; p=0.035) and during surgery (Mann Whitney U test; p=0.039). 18 hours after the surgical procedure no statistically significant difference was found. Squares ■ represent pre-operative samples (all within 24 hours after admission), circles ● represent per-operative samples and triangles ▲ represent samples taken 18 hours post-operative.

Although CD11b (MAC-1) was initially not increased on monocytes in injured patients, an increased expression (p=0.02) was found on monocytes 18 hours after IMN in patients who developed ALI/ARDS (Figure 4A). In addition, a decreased responsiveness for the formylpeptide fMLP visualized by FMLP induced expression of active FcγRII was seen after IMN (Figure 4B).

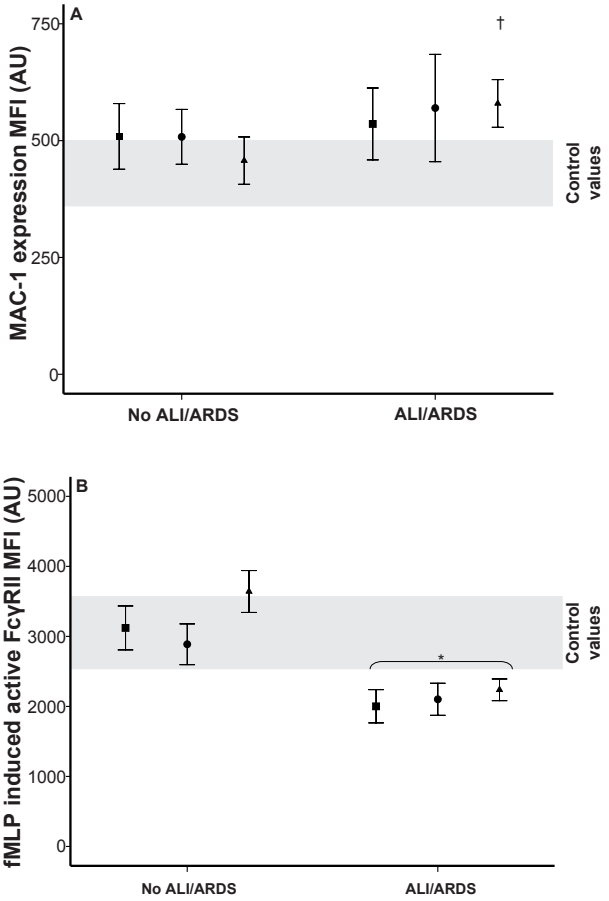

Figure 4. Activation markers on monocytes after injury.
Expression of (**A**) MAC-1 (CD11b) and (**B**) fMLP induced active FcγRII on monocytes in patients who developed pulmonary complications and patients with an uneventful course after trauma. Patients who developed ALI/ARDS demonstrated significantly increased MAC-1 (p=0.02) and decreased fMLP induced active FcγRII (p=0.023) after surgery. Squares ■ represent pre-operative samples (all within 24 hours after admission), circles ● represent per-operative samples and triangles ▲ represent samples taken 18 hours post-operative. Gray shaded area depicts normal control values.

DISCUSSION

This is the first study that describes a relation between a decreased percentage of HLA-DR positive monocytes and injury severity in trauma patients. A marked decrease of the number of HLA-DR positive monocytes at admission precedes the occurrence of inflammatory complications such as ALI or ARDS after IMN. Based on these data, the percentage of HLA-DR positive monocytes prior to IMN might allow staging of the systemic inflammatory response in these patients. Interestingly, post-operative measurements demonstrated similar reduced percentages HLA-DR positive monocytes in virtually all patients and are, therefore, not suitable as read-out factor for the development of inflammatory complications early after IMN.

The phenotype of circulating monocytes shifts during the injury induced initial inflammatory response. More pronounced injury is accompanied by the occurrence of monocytes characterized by the absence of HLA-DR, increased MAC-1 expression and a low responsiveness to fMLP. It is tempting to speculate that these cells represent a shift from monocytes which are precursors of antigen presenting cells to precursors of inflammatory phagocytic cells (e.g. macrophages). The clinical consequence of this change in phenotype of the circulating population of monocytes remains to be determined.

Identification of *all* monocytes in total leukocyte preparations is a matter of concern as gating the cells on either scatter characteristics (as in Figure 1) or on the basis of immunophenotype (20-22) can induce bias by missing subpopulations of these cells. Therefore, no golden standard is available. Aggregation of monocytes might shift these cells out of the FSC/SSC gates, but this process is minimized as the staining is performed in a cold buffer without Ca^{2+} present. In addition, a small number of NK-cells can be present in the monocyte gate but this low amount of cells (<2%) could not have affected our data.. Other authors have provided reliable results by analyzing monocytes based on their forward sideward scatter characteristics (23). In addition, our results corroborate with previous reports on the presence of HLA-DR negative, activated monocytes (10,24).

To circumvent the above mentioned problem, monocytes are also identified by CD14 in combination with other markers, such as CD16 or CD45(20,21). However,

the use of these markers under pathological conditions can still not guarantee an accuracy which is better than identification on FSC/SSC characteristics. A decrease of >30% in CD14 expression on monocytes after *in vitro* incubation with streptotoxin and during clinical endotoxemia has been demonstrated (22,25). Identification of monocytes can be biased by CD14 expression on both NK cells and PMNs (21).

Reduced monocyte HLA-DR has been demonstrated after burns, trauma and during sepsis (23,26-29). Even the existence of the specific phenotype we analyzed has been shown previously during surgical patients with sepsis (10). This reduced HLA-DR expression is thought to lead to deregulated communication between the adaptive and innate immune responses resulting in an impaired immune status and the development of severe infections or sepsis. However, a relation between altered monocyte phenotype in the peripheral circulation and the development of ARDS has not been published yet. Monocyte changes have been related to ARDS, but these cells were harvested from the pulmonary interstitium (30,31). Early phase organ failure (ARDS) is thought to be the consequence of an *excessive* inflammatory response (32-35). The relation between the reduced percentage of HLA-DR positive monocytes and the development of ARDS, further confirms this hypothesis. The exact mechanisms and consequences need to be further addressed, but this study gives a new perspective on the pathophysiological role of monocytes during the early stages after trauma.

This study shows a relation between injury severity and subsequent systemic inflammation measured by a change in monocyte phenotype. This shift to a more macrophage type of cell, was related to the development of early inflammatory complications such as ALI or ARDS. Therefore, by limitation of the occurrence of HLA-DR negative monocytes is likely to prevent early onset organ failure.

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Chapter 8

Redistribution of monocyte population during the systemic inflammatory response after injury

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ABSTRACT

Monocytes connect the innate immune system with the adaptive immune system by phagocytosis and antigen presentation. Reduced HLA-DR expression on monocytes after injury is thought to mediate immune suppression and, thereby, the development of sepsis. However, it is unknown if this is due to down-regulation of HLA-DR receptors or by redistribution of monocytes that are HLA-DR positive or negative.

A consecutive series of surgical ICU patients were included and monitored daily for both HLA-DR expression per individual monocyte as well as the percentage and number of HLA-DR positive monocytes. During this 14 day period, criteria for sepsis were daily assessed.

Forty-one patients were included of which twelve patients developed septic shock during the second week of admission. HLA-DR expression was decreased in all patients throughout the study period both at the individual cell and the population level. This was the result of both a reduction in HLA-DR expression per monocyte and a reduction in the number of HLA-DR positive monocytes. A normalization in the absolute number of HLA-DR positive monocytes was found in patients without septic shock, which was accompanied by a major increase in HLA-DR negative monocytes.

Patients who develop late phase septic shock following major injury are characterized by (1) a lack of normalization of HLA-DR positive monocytes and (2) no increase of HLA-DR negative monocytes during the recovery phase. The absence of this monocyte response points at an exhausted innate immune response in patients who develop late onset sepsis after injury.

Monocytes are precursors of tissue macrophages and myeloid dendritic cells, which are important effector cells in clearance of invading pathogens and necrotic tissue as well as antigen presentation (1,2). Monocytes and macrophages enter a site of inflammation and/or tissue damage within one hour after the initial inflammatory insult (3). This rapid response is mediated by homing of cells from a circulating pool in the peripheral blood. Upon homing to specific tissues or organs, monocytes can differentiate into tissue-specific macrophages, such as Kupfer-cells in the liver or alveolar macrophages in the lung (4).

Monocytes belong to the interphase between the innate and adaptive immune system. They can phagocytose and kill targets as true phagocyte-cells and can activate CD4+ lymphocytes by antigen presentation via HLA-DR (MHC-II) (5,6). By acting as antigen presenting cells (APC's), these cells can steer T-cells in the direction of Th1 or Th2 type immune responses. A reduction in HLA-DR expression on monocytes is supposed to be an important determinant in the immune suppression after severe injury leading to late phase sepsis (7-9). These findings led to the interpretation that reduction in HLA-DR expression is involved in the compensatory anti-inflammatory response syndrome (CARS).

Current consensus is that the reduced expression of HLA-DR on monocytes during sepsis is caused by an active down-regulation of the molecule from individual cells initiated by anti-inflammatory cytokines. Indeed, after injury high concentrations of the anti-inflammatory cytokine IL-10 (interleukin 10) have been found (10). This is consistent with the finding that IL-10 exposure to monocytes *in vitro* induces an internalization of the HLA-DR receptors (11). In addition, a correlation has been found between the concentrations of plasma IL-10 and the reduction of HLA-DR expression on monocytes (12). However, these in vivo findings are not necessarily causative related (13). In literature, most studies regarding HLA-DR expression on monocytes focused on the whole population of cells. Several reports have described that both HLA-DR expression of the *whole population* as well as the percentage of HLA-DR positive monocytes are related to the development of septic shock after injury. However, it has not been proven if (1) monocyte HLA-DR expression is actively down-regulated, (2) HLA-DR positive monocytes leave the circulation or (3) HLA-DR negative monocytes are mobilized from tissues such as the bone marrow. Therefore, we analyzed HLA-DR expression both on the whole population and on individual monocytes in the circulation of severely injured patients.

MATERIAL AND METHODS

Patients

A consecutive series of trauma and post-operative patients, who required admission to the intensive care unit of the University Medical Centre Utrecht were included. Patients were between 18 and 80 years old, with an expected ICU stay of ≥ 3 days. Exclusion criteria were chronic disease influencing the immune system and the use of immunosuppressive medication. The patients were followed for 14 days or as long as their stay on the ICU lasted.

Informed consent was obtained as soon as possible from the patient self or by a legal representative. The local ethical committee approved the study and written informed consent was obtained from all patients or their spouses in accordance with the protocol.

Clinical parameters

The APACHE-II score was calculated on admission (14). The criteria for SIRS (Systemic Inflammatory Response Syndrome), sepsis or septic shock were defined as proposed by the International Sepsis Definitions Conference and were assessed on a daily basis (15).

Sampling

A first blood sample was taken within 3 – 12 hours after the patients' admission to the ICU; this was defined as day zero. Serial blood samples were taken on a daily basis during the next 14 days of the patient's admission. Blood was collected in a vacutainer® with sodium heparin as anticoagulant, cooled immediately and kept on ice during the whole staining procedure. The analysis of the monocyte HLA-DR expression was started within two hours after the blood sample was obtained.

Materials

Monocyte HLA-DR expression was analyzed by flowcytometry. The following monoclonal antibodies were commercially purchased: FITC-labeled IgG1 negative control (clone DD7, Chemicon, Hampshire, United Kingdom) and FITC-labeled HLA-DR (YE2/36-HLK, Serotec, Dusseldorf, Germany). Routine hematology parameters were determined at the Clinical Laboratory Department of the University Medical Center Utrecht.

Flowcytometer analysis

Blood samples were stained with directly labeled antibodies (16). The directly labeled antibodies were added 1:20 to whole blood and incubated for 60 minutes on ice. After incubation, the red cells were lysed with ice-cold isotonic NH_4Cl. After a final wash with PBS2+ (phosphate buffered saline supplemented with sodiumcitrate (0.38% wt/vol) and pasteurized plasma proteins (10% vol/vol)), the cells were analyzed in a FACScalibur Flowcytometer (Becton & Dickenson, Mountain view. CA). The monocytes were identified according to their specific side-scatter and forward-scatter signals. Data from individual experiments are depicted as median fluorescence intensity (MFI) in arbitrary units (AU) for all monocytes and/or for HLA-DR positive monocytes. The percentage of HLA-DR positive monocytes was defined as the percentage of monocytes with a HLA-DR expression above the 99%-CI of negative control values.

Measurements

Results of a single sample are expressed in five different forms; 1) median HLA-DR expression of all monocytes, 2) percentage of monocytes expressing HLA-DR above the 99%-CI of negative control value (= HLA-DR positive monocytes), 3) median HLA-DR expression of monocytes with HLA-DR expression above the 99%-CI of negative control value (e.g. percentage HLA-DR positive monocytes), 4) number of HLA-DR positive monocytes and 5) number of HLA-DR negative monocytes.

Statistical analysis

Results are expressed as means ± standard error of mean (SEM). Statistical analysis was performed with the non-parametric Mann-Whitney U test to compare 2 groups, Kruskal Wallis H test for multiple groups and Pearson correlation for comparison of two continues values. Statistical significance was defined as $p < 0.05$.

RESULTS

Demographics

Forty-one patients were analyzed. Thirty-six patients were admitted after (multi)trauma, the other 5 were post-operative patients after major surgery. The mean APACHE-II and ISS score were 15 (SD = 7.7) and 24 (SD = 10.8) respectively. Thirty-five of the included patients developed a SIRS and 24 patients met the sepsis criteria (15). Twelve patients developed septic shock. All trauma patients (10) that developed septic shock fulfilled the septic shock criteria between days 8-10 after admission. One post-operative patient developed septic shock on the second day and one post-operative patient developed septic shock on the seventh day of ICU admission. Four patients died during their admission, of whom one died during the 14 day study period. Causes of death were multiple organ failure for 3 patients and cardiac arrest for 1 (Table 1).

Table 1. Demographics

	Mean (range)
Number of patients (n)	41
Male / Female (n)	32 / 9
Age (years)	49 (18-73)
Injury Severity Score	24 (9-57)
APACHE II Score	15 (0-35)
Time in ICU (days)	17 (2-67)
Time on ventilator (days	16 (0-65)
Cause of admission (n)	
- Trauma	36
- Post-operative	5
Complications (n)	
- SIRS	35
- Sepsis	24
- Septic shock	12
- Mortality	4

Number of monocytes

Monocytes numbers remained within the normal range (0.3 – 0.810^6/ml) during the first week after trauma. However, a massive increase in monocytes numbers was seen during the second week of admission, more pronounced in patients who did not develop septic shock ($p < 0.05$) (Figure 1A).

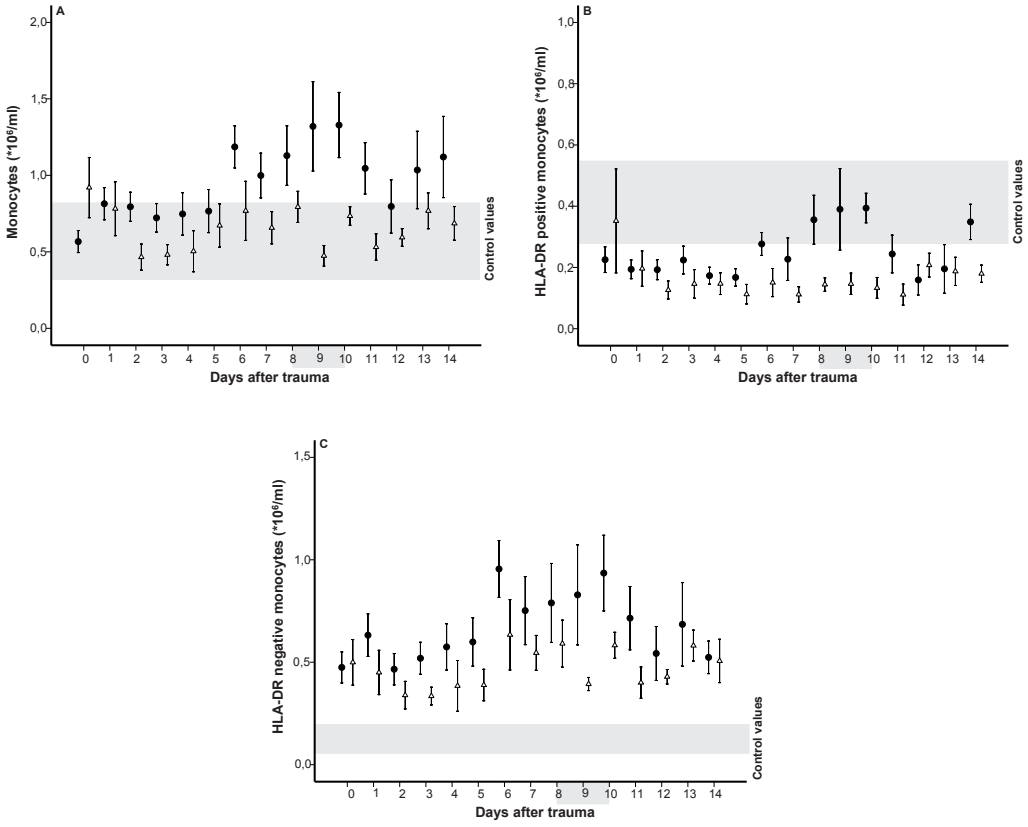

Figure 1. Number of monocytes in the circulation
The number of monocytes was within the normal range (0.3 – 0.8 10^6/ml, indicated by gray shaded area) for all patients during their first week of ICU admission. Patients who did **not** develop septic shock showed a marked **increase** ($p < 0.05$) in total number of monocytes during the second week of admission (**A**). This increase in monocyte number consisted of monocytes with HLA-DR and monocytes (**B**) without HLA-DR expression (**C**), while patients who developed septic shock showed low numbers of monocytes expressing HLA-DR. Closed circles ● represent patients without development of septic shock and open triangles Δ represent patients who developed septic shock. Data are presented as mean ± standard error.

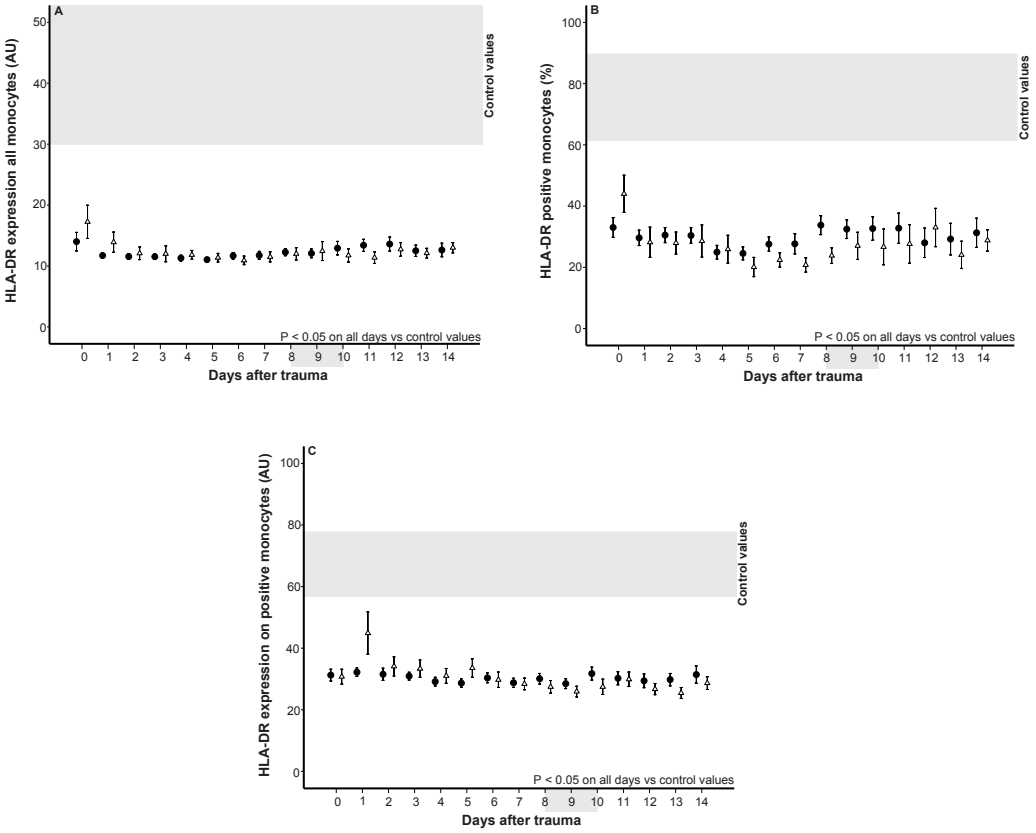

Figure 2. HLA-DR expression on monocytes throughout admission.
Both the HLA-DR expression on the whole monocyte population (**A**) and the percentage HLA-DR positive monocytes (**B**) reduced significantly during the first three days of admission. Median HLA-DR expression remained decreased in all patients throughout admission. No significant difference was found between patients who developed septic shock and patients without septic shock. Gray shaded area indicate reference values, closed circles ● represent patients without development of septic shock and open triangles ∆ represent patients who developed septic shock. Data are presented as mean ± standard error, both patient groups demonstrated significantly lower HLA-DR expression as compared to controls (Mann Whitney U test P < 0.05 on all days).

Control values

HLA-DR expression on monocytes

Compared to controls total monocyte HLA-DR expression was reduced directly after injury and further decreased during the first days of admission ($p < 0.05$). No significant differences were found between patients that developed septic shock and patients without sepsis (Figure 2A). Additionally, compared to controls the percentage of HLA-DR positive monocytes was decreased throughout the study period. During the study period no significant differences were found between patients with or without later development of septic shock (figure 2B). Furthermore, the relative expression of HLA-DR was decreased on monocytes actually expressing the receptor. Again, no differences were found between patients who developed septic shock and patients without septic shock (Figure 2C).

HLA-DR expression on HLA-DR positive monocytes

In order to study the cause of the massive reduction of HLA-DR expression on monocytes, correlation analysis was performed to differentiate between down-regulation of HLA-DR on the entire monocyte population versus the reduction of HLA-DR per individual cell. A statistically significant correlation (Pearson $p = 0.000$; $r = 0.595$) was found between these two parameters (figure 3A). A decrease in numbers of 50% in HLA-DR positive monocytes (from 75% to 25%) resulted in a 50% reduction in HLA-DR expression (from 30 AU to 15 AU) for the whole monocytes population.

In contrast, no correlation was found between the percentage of HLA-DR positive monocytes and the median HLA-DR expression on these HLA-DR positive monocytes (figure 3B). Thus, the magnitude of HLA-DR expression on individual monocytes was not related to the number of monocytes expressing HLA-DR. Even when only 2% HLA-DR positive monocytes were present in a sample, monocytes with a high expression of > 500 AU of HLA-DR were present (results not shown).

Number of HLA-DR positive monocytes

The increase in number of monocytes, seen in patients without septic shock during the second week of admission, also led to a normalization in the *absolute number* of HLA-DR positive monocytes (Figure 1B). The low *percentage* of HLA-DR positive monocytes in the total monocyte population could completely be attributed to the massive increase in the total number of monocytes (Figure 1C).

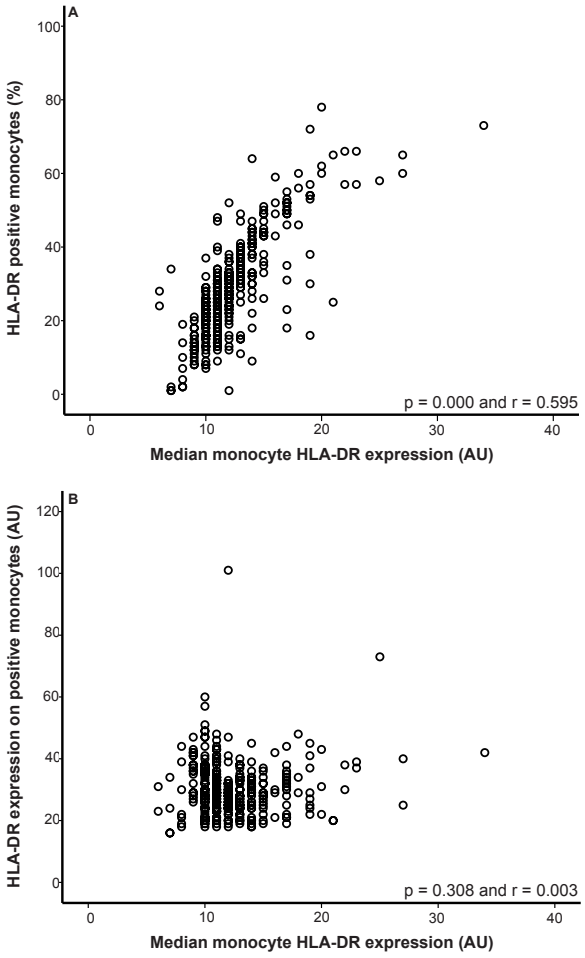

Figure 3. Cause for reduction in relative expression of HLA-DR on monocytes.
A statistically significant correlation was found between the relative median HLA-DR expression on monocytes and the percentage of HLA-DR positive monocytes (**A**), with p = 0.000 and r^2 = 0.595 (Pearson). No correlation was found between the relative median HLA-DR expression on monocytes and relative median HLA-DR expression on HLA-DR positive monocytes (**B**), with p = 0.308 and r^2 = 0.003 (Pearson).

DISCUSSION

In this study, a correlation was found between HLA-DR expression on the total monocyte population and the percentage monocytes positive for HLA-DR, which corroborates with previous studies on this subject. However, our data do not support the hypothesis from those studies that immune suppressive cytokines, such as IL-10, cause active down regulation of HLA-DR on individual monocytes under these conditions. Our data support the idea that massive redistribution of monocytes is at the basis of the decrease in percentage of HLA-DR positive cells (17,18). This conclusion is based on the following.

No correlation was found between the extent of HLA-DR expression on HLA-DR positive monocytes and the percentage of monocytes expressing HLA-DR in the monocyte population during the whole study period. This suggests that the reduction in HLA-DR expression in the monocyte population is primarily the result of redistribution of HLA-DR positive monocytes from the circulation rather than a decreased expression per cell.

This is supported by analysis of absolute numbers of monocyte populations rather than percentages of these population. It turned out that the major increase in the number of HLA-DR negative monocytes caused the decreased percentage of HLA-DR positive monocytes during the recovery phase in trauma patients. In fact, the absolute number of HLA-DR positive monocytes normalized in the recovery phase of the patients who did not develop septic shock (Figure 1B) despite the fact that percentage HLA-DR positive cells was markedly decreased (Figure 2B). Although the expression of HLA-DR on individual monocytes was also decreased in patients compared to controls, this phenomenon was not correlated with the HLA-DR reduction on the whole monocyte population. Thus, additional suppression of HLA-DR on monocytes by e.g. anti-inflammatory cytokines might be present, but apparently plays a minor role in the HLA-DR reduction on the circulating monocyte population.

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Although there occurs restoration to normal numbers of HLA-DR positive monocytes in patients without septic shock, the HLA-DR positive monocyte population is outnumbered by a population of HLA-DR negative monocytes (17,18). These HLA-

DR negative monocytes have been described previously as HLA-DR negative ("angry") macrophages. In contrast to the normally required antigen presentation function of monocytes in their dendritic cell precursor function, a functional shift occurs in severely injured patients towards a more phagocytic phenotype (19,20). It is tempting to speculate that these patients require a large amount of phagocytotic cells to clear the necrosis and inflammation from the injured tissues(18).

Some studies have shown that a low median HLA-DR expression of the whole monocyte population or a low percentage of HLA-DR positive monocytes at admission is indicative for the development of late sepsis after trauma (8,9). We could not demonstrate such an early relation. On the other hand, we corroborated the finding that a continuous low number of HLA-DR positive monocytes (both percentage and absolute number) was related with the development of septic shock. These data should be interpreted in the context of two not necessarily connected phenomena: modulation of HLA-DR+ and HLA-DR- monocytes.

All our patients were severely injured and exhibited a severe inflammatory response resulting in a very high incidence of SIRS and a high incidence of infectious complications presumably caused by a CARS (21,22). This latter period of immune suppression in these patients was characterized by two phenomena: low expression of HLA-DR+ cells and a lower supra-normal amount of HLA-DR- cells. Previous studies focusing on the modulation of HLA-DR+ cells, found moderate significances and had to analyze larger patient cohorts. Therefore, these findings are of limited applicability for the individual trauma patient. In line with this reasoning are studies performed in patients with sepsis on inclusion. These studies showed a trend of increased mortality in patients characterized by < 30% of HLA-DR positive monocytes, but these findings failed to reach statistical significance(12,23-25). Our study was not powered for mortality, but took into account the fact that the number of HLA-DR+ normalized during the recovery phase and that the number of HLA-DR- negative monocytes drastically increased. Studies focusing purely on percentage HLA-DR+ monocytes will be confounded by the modulation of numbers of HLA-DR- monocytes.

Our study could not demonstrate a significant difference in HLA-DR expression on monocytes during the first 6 days upon admission in patients who developed septic

shock. These data question the importance of immune suppressive cytokines such as IL-10 in the first 7 days of trauma (11,12). In addition, it has recently been shown that IL-10 concentrations decrease rapidly after its peak in the first 48 hours, thus after 3 days do not correlate with decreased HLA-DR expression (13,26). On the eight day of sampling patients who developed late phase septic shock demonstrated significantly lower percentages as well as absolute numbers of HLA-DR positive monocytes, which is in line with the aforementioned studies.

We can only speculate as to why monocytes with high HLA-DR expression disappear during the first 48-72 hours after injury in all patients and why HLA-DR+ cells normalize and large numbers of HLA-DR negative monocytes appear in the circulation during the recovery phase of patients without septic shock (Figure 1). It is tempting to speculate that a overzealous innate immune response after trauma leads to exhaustion of monocyte mediated immune response. The lack of HLA-DR positive monocytes during the CARS phase might be part of a “paralyzed” immune response to infectious agents such as broadly speculated in the literature. The low numbers of HLA-DR- monocytes might lead to improper repair of injured tissue leading to impaired barrier function, contributing to impaired responses to infectious conditions. Thus, the magnitude of redistribution of the monocyte populations can be used as marker for the risk of development of severe infections and subsequent sepsis.

In conclusion, we have demonstrated that the early reduction in HLA-DR expression in the monocyte population in response to injury in all trauma patients is largely due to the loss of HLA-DR positive monocytes from the circulation. The risk for developing late onset sepsis coincides with an impaired monocyte response during the recovery phase: no normalization of HLA-DR positive cells together with a low mobilization of HLA-DR- cells. Therefore, therapy should be aimed at maintaining normal monocyte populations.

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CHAPTER 9

KINETICS OF SYSTEMIC NEUTROPHIL PHENOTYPES AFTER TRAUMA

Occurrence of VLA-4 positive neutrophils coincides with suppressed functionality of the innate immune system

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In preparation

Septic shock is a leading cause of death (30% mortality) with a world wide annual incidence of 18 million affected individuals. Sepsis resulting from a severe inflammatory insult such as burns and trauma typically occurs after 8–14 days and is facilitated by a compensatory anti-inflammatory response syndrome (CARS) caused by a dysfunctional innate immune system. Deregulated neutrophils (PMNs) play an essential role in the pathogenesis of CARS and characterization of these PMNs was the subject of this study.

Forty-seven trauma patients who required ICU support were included and followed for 14 days in terms of both clinical and immunological parameters. The study focused on the morphology, immuno-phenotype and functionality of PMNs during this study period. The functionality of PMN phenotypes was assessed on cells that were isolated by cell sorting. The characteristic of these systemic PMNs was compared with cells isolated (by sorting) from lung fluid, bone marrow and lymph fluid of critically ill patients. Tissue samples were obtained post mortem from patients who died of severe sepsis.

A complex mixture of PMN subpopulations was identified in the peripheral circulation in the 14 days study period after trauma. These phenotypes were identified by differences in FcγRIII (CD16)/ VLA-4 (CD49d) expression. Two VLA-4 positive PMN phenotypes were found both early and late after major trauma. Sorting revealed that (1) $CD16^{Intermediate}/CD49d^{Intermediate}$ cells were (meta)myelocytes and (2) $CD16^{Bright}/CD49d^{Bright}$ cells were end stage toxic PMNs. This latter phenotype was also found in the lymph and lung fluid of patients with organ failure. The VLA-4 expressed on these phenotypes was functional as the cells could bind to VCAM-1 coated beads. None of the peripheral PMN phenotypes showed any indications for apoptosis. PMN apoptosis was only found in spleen, liver and bone marrow in post mortem tissue from patients with severe sepsis.

The appearance of VLA-4 positive PMNs coincides with periods of acute severe systemic inflammation directly caused by trauma and/or during development of sepsis. Functionally these cells have a suppressed phenotype and are very similar to cells obtained from the tissues. These data support the hypothesis that development of CARS 8-10 days after trauma is mediated by development of neutrophils with a low functionality from the tissues together with insufficient suppletion of functional neutrophils from the bone marrow.

During the systemic inflammatory response syndrome (SIRS) that follows early after injury, PMNs (polymorphonuclear granulocytes or neutrophils) home to both injured *and* non-injured tissues (1,2). In addition, a clear leukocytosis is present in the peripheral blood (**chapter 6**)(2). During this period of acute inflammation, a marked decrease in functionality of PMN was found (3-5). Recently, we could show that at approximately 7 days after trauma the impairment of neutrophil function was maximal (**chapter 6**). Strikingly, the kinetics of this process overlaps with the compensatory anti-inflammatory response syndrome (CARS) (6). This syndrome is an important risk factor for the development is of late onset (>8 days after) sepsis and septic shock (1,6-9) and is characterized by a marked suppression of the innate immune system of unknown aetiology. One hypothesis suggests that CARS is induced by the production of anti-inflammatory cytokines (e.g. IL-10), but this conclusion lacks solid experimental support (10,11).

Despite the multitude of studies regarding functionality of neutrophils *in vitro* surprisingly little is know regarding the kinetics and functionality of these cells in humans *in vivo*. The current paradigm describes that neutrophils originate from the bone marrow, are distributed through the blood and home to tissues, where they eventually are cleared by apoptosis and phagocytosis by resident macrophages (12-14). Once released from the bone marrow the half life of neutrophils in the peripheral blood is short (8-24 hrs) (15,16). This situation allows PMNs to accurately participate in immune surveillance by rapidly responding to an infectious threat and fast clearance upon completion of their task.

Under normal homeostatic conditions neutrophils are found in the peripheral blood as a uniform population, which is homogenous in responsiveness to stimuli. Neutrophils can acquire a primed phenotype in peripheral blood under conditions of both acute and chronic inflammation (17-19). Strikingly, these conditions are still characterized by a homogenous pool of primed cells; i.e. all cells in the blood had a primed phenotype and no separate subpopulations were found at the same time. The most convincing data that subpopulations of neutrophils can coexist in blood was found under extreme conditions found during sepsis (20-22). In this study, Ibottson *et al.* showed the existence of a distinct subpopulation of blood neutrophils that was characterized by the expression of VLA-4 next to cells not expressing this integrin. This phenotype could be induced in cells from normal donors by incubation of these cells with serum

of septic patients. The study did not provide any information on the morphology and functionality of these VLA-4 positive neutrophils. In addition, a lower expression of FcγRIII (CD16) was demonstrated on a part of the neutrophil population after extra-corporal circulation during bypass surgery, which was attributed to the presence of young PMNs in the circulation (23-26).

We tested the hypothesis that after severe injury PMN subpopulations can be identified in the peripheral circulation by characterization of expression of FcγRIII (CD16) and VLA-4 (CD49d/CD29) and that these subpopulations are characterized by different kinetics, morphology and function.

MATERIAL AND METHODS

For all different patient populations, the institutional medical ethical review board approved the studies and written informed consent was obtained from all patients or their legal representatives in accordance with the protocol.

Patients: severely injured trauma patients

A series of 47 consecutive severely injured patients was included who required intensive care support (ICU) in the University Medical Centre Utrecht. Patients had to be between 18 and 80 years old, with an expected ICU stay of ≥ 3 days. Exclusion criteria were chronic diseases influencing the immune system and/or the use of immunosuppressive medication. The patients were followed for a maximum of 14 days or as long as their stay in the ICU lasted. Results obtained from these patients were compared with results from 10 healthy controls.

Study set up

Longitudinal sampling

The study was set up as a longitudinal study. Multitrauma patients were included as soon as possible after admission in the ICU. The first blood sample was taken 3 – 12 hours after trauma (day zero). Serial blood samples were taken on a daily basis during the following 14 days or as long as the patient stayed in the ICU. Criteria for SIRS, sepsis or septic shock as defined by the criteria proposed by the International Sepsis Definitions Conference, were assessed during the ICU stay (27,28). Also the SOFA (Sequential Organ Failure Assessment) score as a measurement for disease severity was determined on a daily basis (29). Blood was collected in a vacutainer® with sodium heparin as anticoagulant and was cooled immediately and kept on ice during the whole staining procedure, which started directly.

Sampling of PMNS from different locations

1. *Bone marrow:* Bone marrow from 5 healthy donors was obtained by crista puncture. One milliliter of bone marrow was obtained before further handling of the sample for therapeutic reasons. This bone marrow sample was put in a vacutainer® with sodium heparin as anticoagulant. Phosphate buffered saline (PBS = 0.5% wt/vol) with added sodiumcitrate (0.38 % wt/vol) and isotonic pasteurized plasma proteins (10% vol/vol) (PBS2+) was added to

the bone marrow aspirate. The bone marrow PBS2+ solution was filtered with 500 µm pores, leaving behind larger peaces of bone or medulla. The filtered fluid was put on ice and directly analyzed.

2. *Lung fluid:* Five patients, who had developed acute lung injury the first day after trauma, provided lung fluid aspiration samples. A non-directed broncho-alveolar lavage was performed, which is standard of care at the intensive care unit (3). The lung fluid sample was put in a vacutainer® with sodium heparin as anticoagulant, which was directly put on ice and analyzed.
3. *Lymph fluid:* Five patients, who had developed chylothorax in the first days after trauma or surgery, provided thoracic chylus samples. Chylus was obtained by aspiration from the thoracic cavity by guidance of the drain which was already *in situ*. The fluid was drained in a vacutainer® containing sodium heparin as anticoagulant.

Flowcytometer analysis

For analysis of PMN receptor expression by flowcytometry, the following standard monoclonal antibodies were commercially purchased: FITC-labeled IgG1 negative control (clone DD7) from Chemicon, Hampshire, United Kingdom; RPE-labeled IgG2a negative control (clone MRC OX-34), Alexa-labeled CD16 (clone 3G8, BD Pharmingen, Franklin Lakes, USA) and RPE-labeled CD49d (clone 9F10, Ebioscience, San Diego, USA). Blood samples were stained with directly-labeled antibodies as described previously (3). In short, red cells were lysed with with ice-cold isotonic NH_4Cl. Directly labeled antibodies were added 1:20 and samples were incubated for 60 minutes on ice. After a final wash with phosphate buffered saline (PBS = 0.5% wt/vol) supplemented with sodiumcitrate (0.38 % wt/vol) and isotonic pasteurized plasma proteins (10% vol/vol), the cells were analyzed in a FACScalibur flowcytometer (Becton & Dickinson, Mountain View. CA). The PMNs were identified according to their specific side-scatter and forward-scatter signals. Data from individual experiments are depicted as median fluorescence intensity in arbitrary units (AU) of at least 10000 events.

Characterization of sorted PMN populations

Granulocytes in 5 ml of whole blood were analyzed. Red cells were lysed with with ice-cold isotonic NH_4Cl. Leukocytes were washed and resuspended in PBS supplemented with human serum albumin (0.5% wt/vol) and sodium citrate

(0.38% wt/vol). Leukocyte samples were directly analyzed by staining of cytospins with May-Grunwald-Giemsa as well as by flowcytometry using a FACScalibur flowcytometer. The presence of subpopulations was analyzed by flowcytometry. When subpopulations were present, $5*10^6$ fresh cells were stained with FITC-labeled CD16 (LNK16, Serotec, Dusseldorf, Germany) and RPE-labeled CD49d (clone 9F10, Ebioscience, San Diego, USA) and sorted. Granulocyte populations were sorted with a FACSvantage flowcytometer (Becton & Dickenson, Mountain view. CA). The PMNs were identified according to their specific side-scatter and forward-scatter signals and sorted based on their CD16 and CD49d expression (Figure 1). Five phenotypes of granulocytes were sorted; 1) $CD16^{Bright}/CD49d^{Minus}$, 2) $CD16^{Bright}/CD49d^{Bright}$, 3) $CD16^{Intermediate}/CD49d^{Dim}$, 4) $CD16^{Intermediate}/CD49d^{Intermediate}$, 5) $CD16^{Minus}/CD49d^{Bright}$ (see also Figure 2).

Histological examination of post mortem tissue samples

Approval for pathological examination was obtained from the legal representatives of five patients who died of organ failure during severe sepsis. Organs were inspected, weighted and a specimen per organ was obtained. The specimens were fixed in 4% buffered formalin, embedded in paraffin until further analysis. Staining of specimens was performed as described before (30). Samples were sliced in 4 µm sections, which were placed at 56 ˚C overnight. Endogenous peroxidase was blocked with blocking buffer ($C_6H_8O_7$, Na_2HPO_4 and NaN_3 with a pH of 5.8 diluted 1:1000 in H_20) for 15 minutes. Coupes were pre-treated before the primary antibody was added and incubated at room temperature for 1 hour. For MPO and Caspase 3 samples were pre-treated with citrate and for Lactoferrin with EDTA. Giemsa and HE staining did not require pre-treatment. Caspase 3 was added 1:20. The secondary directly labelled antibody was added for 30 minutes at room temperature. Hereafter, the staining was developed with DAB substrate for 10 minutes and haematoxylin for 10 seconds. Finally, the coupes were dehydrated and stored.

Functional analysis: beads assay

In three patients with VLA-4 positive PMNs, the functionality of the α4 integrins expressed on PMN's was determined by binding to VCAM-1-coated fluorescent microbeads as described before (31). Briefly, TransFluor-Spheres (488/645 nm, 1.0 µm; Molecular Probes) were covalently coupled to streptavidin using 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide in MES buffer (pH 6). Then, beads were coated

with biotin-SP-Affinipure goat anti-human Fc(γ) $F(ab')_2$ and subsequently coated with Fc-VCAM-1. Mixed granulocytes were isolated as described previously (32). In short, mononuclear cells were removed by centrifugation over isotonic Ficoll (1.077 g/ml). After lysis of erythrocytes with an isotonic icecold NH_4Cl solution, granulocytes were washed and resuspended in incubation buffer (HEPES supplemented with glucose 0.16% wt/vol, calcium 0.8% wt/vol and serum albumin 2% vol/vol). Cells (40 –50,000/well) were pre-incubated with control anti-HLA-ABC (W6/32) mAb, anti-α4-integrin blocking mAb HP2/1 (10 µg/ml), or pharmacological inhibitors. The ligand-coated beads were washed twice and added in a 96-well V-shaped-bottom plate. Next, the pre-incubated PMNs were added and incubated for 30 min at 37°C. The cells were washed and resuspended in incubation buffer (4°C) and kept on ice until measurement. Binding of the fluorescent beads to the PMNs was determined by flow cytometry using the FACSCalibur and results reported as the percentage of PMNs positive for VCAM-1-coated beads.

Statistical analysis

Receptor expression was analyzed as median fluorescence intensity (MFI) in arbitrary units (AU). In addition, cells were analyzed as percentage of positive cells. Results in figures are expressed as means ± standard error of mean (SEM). Statistical analysis was performed with the non-parametric Mann-Whitney U test for two groups. Kruskal Wallis H analysis was used to analyze changes between multiple groups. Statistical significance was defined as $p < 0.05$.

RESULTS

Patient characteristics

Of the 47 included surgical intensive care (ICU) patients, 13 patients developed septic shock during admission. All but 1 patient remained at least 7 days at the ICU, illustrating the high severity of trauma these patients suffered (Table 1). In analogy to the study of Ibottson *et al.* the VLA-4 expression on neutrophils was determined and analyzed in the context of severity of disease. As is clear from Figure 1A more severe disease during admission is associated with more VLA-4 positive neutrophils in peripheral blood ($p = 0.042$). Figure 1B shows that patients with this high percentage of VLA-4 positive neutrophils during admission (>10%) exhibit sustained severe disease during the first 5 days after admission, demonstrated by the increased SOFA score.

Table 1. Patient demographics

	Mean (range)
Number of patients (n)	47
Male / Female (n)	36 / 11
Age (years)	45 (18-73)
Injury Severity Score	28 (9-75)
APACHE II Score	16 (0-35)
Time on ICU (days)	16 (3-67)
Time on ventilation (days	15 (0-65)
Complications (n)	
- SIRS	37
- Sepsis	25
- Septic shock	13
- Mortality	3

Demonstration of multiple immune-phenotypes of PMN's in trauma patients

Five PMN subpopulations were identified in the peripheral blood of these critically ill patients. These populations can be identified on the expression of only two receptors: FcγRIII and VLA-4 (see below). The kinetics of occurrence of these subpopulations in the different patients differed during the 14 days sampling period demonstrating the complexity of the regulation of the innate immune response under these conditions. The phenotypes varied in timing of occurrence as well as in magnitude, depending on the type and severity of injury. In patients with severe septic shock up

to 40% (20x10^9 cells/L) VLA-4 positive PMNs were found in the circulation. Detailed analysis demonstrated the presence of 5 distinct PMN subpopulations characterized by 1) CD16^{Bright}/CD49d^{Minus}, 2) CD16^{Bright} /CD49d^{Bright}, 3) CD16^{Intermediate}/CD49d^{Dim}, 4) CD16^{Intermediate}/CD49d^{Intermediate}, 5) CD16^{Minus}/CD49d^{Bright} (Figure 2).

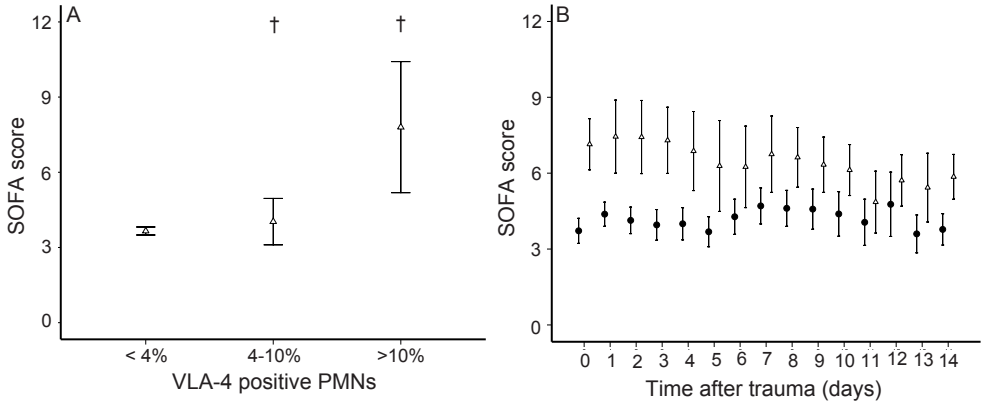

Figure 1. VLA-4 positive PMNs and severity of disease.
Subpopulations based on CD16/CD49d were identified in the investigated population. However, the percentage of VLA-4 positive cells varied widely. Patients in who most VLA-4 positive cells were seen, demonstrated more severe illness (**A**) compared to patients without large amounts of VLA-4 positive cells (p=0.042, Kruskal Wallis H test). In addition, patients with toxic PMNs during their admission demonstrated a more severe and prolonged course of illness (**B**). †: $p < 0.05$.

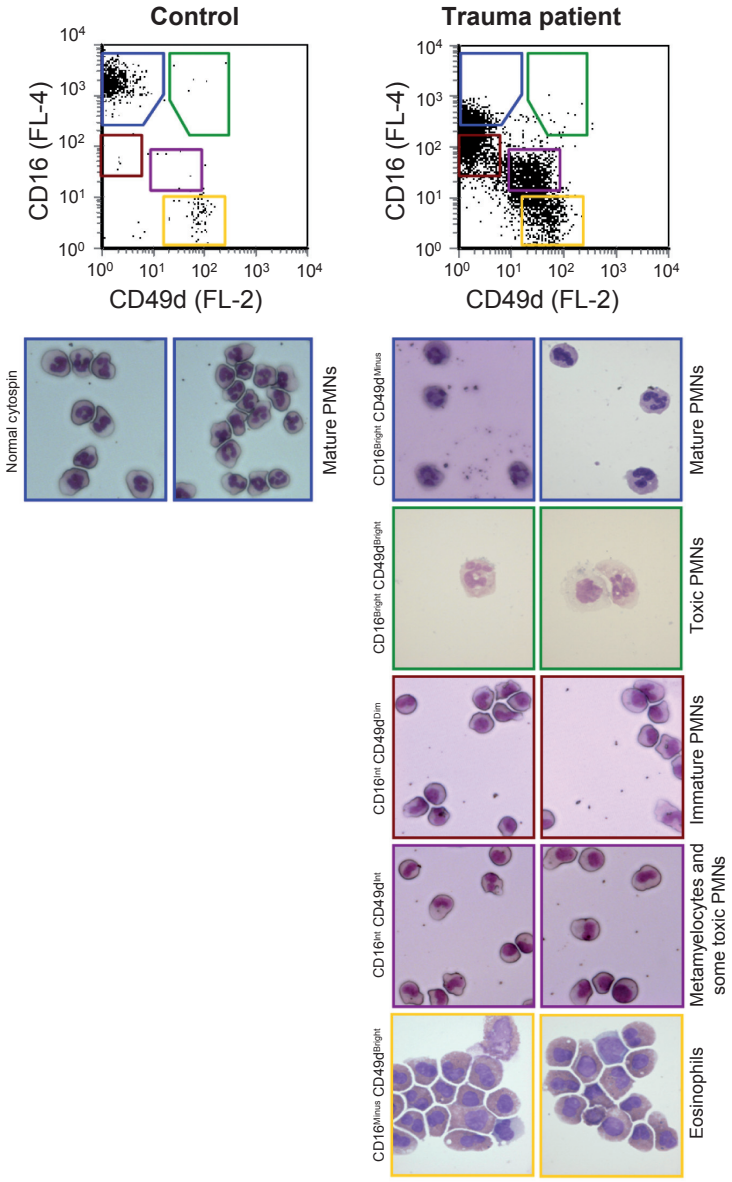

Figure 2. Subpopulation morphology.
Healthy volunteers have mostly $CD16_{Bright}$/$CD49d_{Minus}$ PMNs. In contrast, critically ill patients demonstrate 5 subpopulations based on CD16 and CD49d: 1) $CD16_{Bright}$/$CD49d_{Minus}$ = mature (segmented) neutrophils; 2) $CD16_{Bright}$/$CD49d_{Bright}$ = toxic neutrophils; 3) $CD16_{Intermediate}$/$CD49d_{Dim}$ = banded neutrophils and metamyelocytes; 4) $CD16_{Intermediate}$/$CD49d_{Intermediate}$ = metamyelocytes or toxic neutrophils; 5) $CD16_{Minus}$/$CD49d_{Bright}$ = eosinophils.

Kinetics and histological characteristics of PMN phenotypes in peripheral blood of trauma patients

Immediately (3-12 hours) after trauma only $CD16^{Bright}/CD49d^{Minus}$ and $CD16^{Intermediate}/CD49d^{Dim}$ PMN subpopulations were identified. Hereafter, the most abundant PMN subpopulations were characterized by $CD16^{Intermediate}/CD49d^{Intermediate}$ and $CD16^{Bright}/CD49d^{Bright}$ which typically occurred during days 4-8 after trauma (Figure 3). The different PMN subpopulations were sorted according the gates indicated in Figure 2A. Histological assessment of this $CD16^{Intermediate}/CD49d^{Intermediate}$ subpopulation showed multiple PMN maturation stages, ranging from myelocytes, metamyelocytes to cells with banded phenotypes. Interestingly, the (meta)myelocytes characterized by a $CD16^{Intermediate}/CD49d^{Intermediate}$ phenotype contained nuclei with a chromatin density which was comparable to that of mature (segmented) PMNs.

Histological examination of the other sorted subpopulations revealed unique features (Figure 2). $CD16^{Bright}/CD49d^{Minus}$ appeared to be mature PMNs with a normal nuclear shape and density. $CD16^{Minus}/CD49d^{Bright}$ granulocytes were eosinophils. $CD16^{Intermediate}/CD49d^{Dim}$ were more immature banded PMNs with occasional metamyelocytes. $CD16^{Bright}/CD49d^{Bright}$ cells had toxic PMN characteristics (i.e. toxic neutrophils), with multiple vacuoles and toxic granules. Most cells harvested from patients during (or just before) septic shock demonstrated toxic granules in the cytoplasm. In contrast to the $CD16^{Bright}/CD49d^{Bright}$ cells, the $CD16^{Intermediate}/CD49d^{Dim}$ and $CD16^{Minus}/CD49d^{Bright}$ populations did not demonstrate a comparable abundant number of vacuoles. $CD16^{Bright}/CD49d^{Bright}$ toxic neutrophils contained mainly hyper-segmented nuclei.

Functionality of VLA-4 expressed on neutrophils of trauma patients

In order to analyze the functionality of VLA-4 expressed on PMNs, cells were sorted and analyzed in adhesion assays. Hereto, the specific binding of VLA-4 positive cells to VCAM-1 coated fluorescent micro-spheres was evaluated. PMN phenotypes characterized by $CD49d^{Bright}$ and $CD49d^{Intermediate}$ bound VCAM-1 coated beads. This response was blocked by the specific VLA-4 blocking antibody (clone HP2/1, see Figure 4). Binding could not be up-regulated by *in vitro* stimulation by fMLP (1 μM). The presence of functional VLA-4 was found on both the (meta)myelocytes and the toxic PMNs.

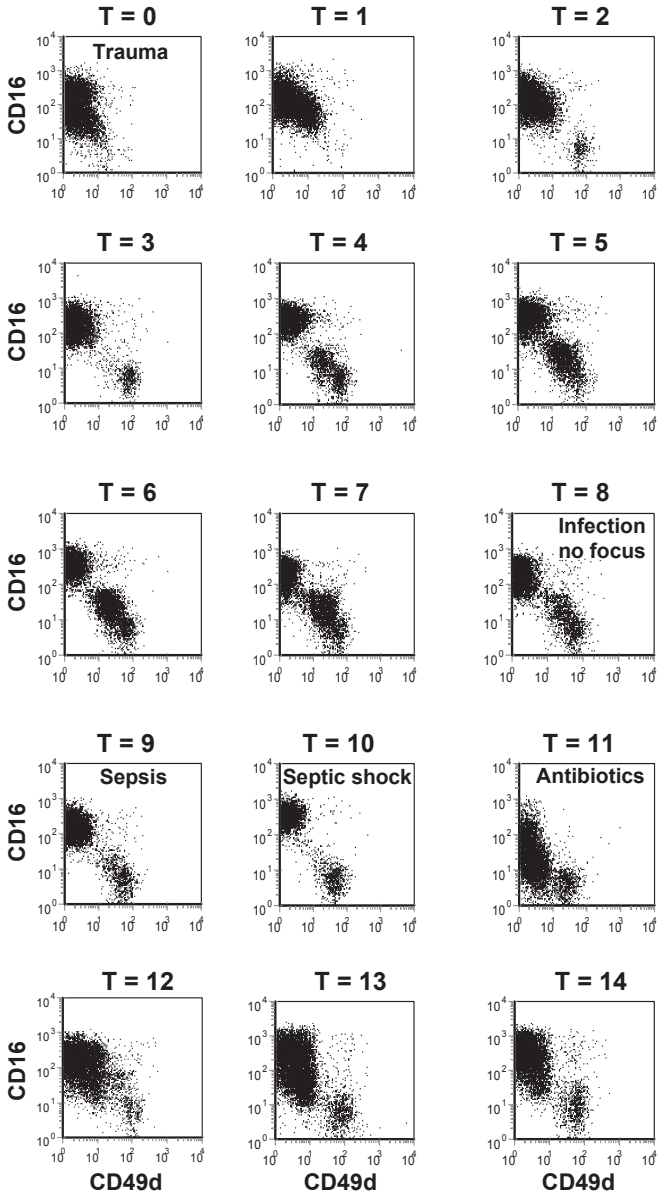

Figure 3. Subpopulation kinetics by flowcytometry.
Demonstrated scatter-plots are representative for the occurrence and disappearance of CD16/CD49d based granulocyte subpopulations after severe trauma. Although no standard pattern was seen in all patients, the occurrence of a $CD16^{Intermediate}/CD49d^{Intermediate}$ and/or $CD16^{Bright}/CD49d^{Bright}$ population was most frequently seen between days 5-8 after injury.

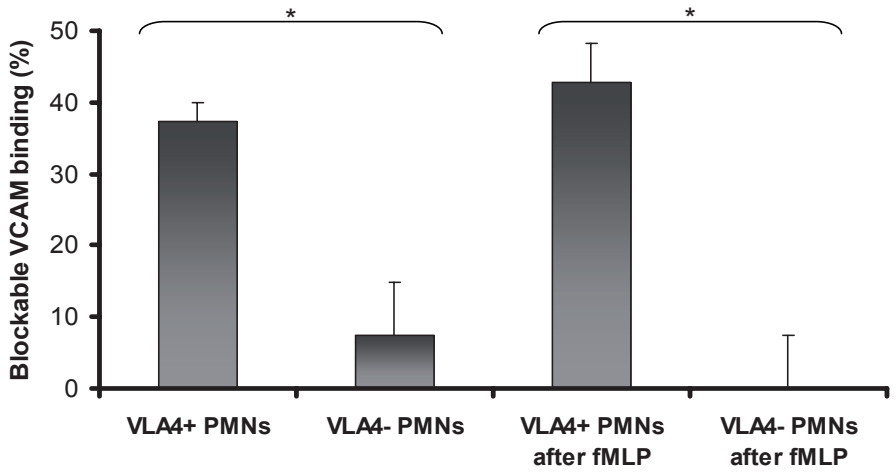

Figure 4. Specific VCAM beads binding of PMNs is related to VLA-4 expression.
After sorting VLA-4 positive and VLA-4 negative PMNs, binding of VCAM beads was compared before and after fMLP stimulation of the cells. The blocking antibody HP 2/1 was compared with it's negative control W632. VCAM binding could be blocked by HP 2/1 in VLA4 positive cells with and without prior stimulation by fMLP. Results are expressed as specific binding (percentage of binding in the context of W632 minus binding in the context of HP 2/1). *: $p < 0.05$, VLA-4 positive PMNs versus VLA-4 negative PMNs.

Ibottson *et al* have demonstrated that treatment of control PMN's with plasma of septic patients induced binding to VCAM coated beads. Although we confirmed the induction of binding of PMNs to VCAM coated beads after stimulation of the cells with plasma from septic patients, this increase was also found with plasma of healthy controls. More importantly, this binding could not be blocked by HP2/1 (Figure 5A) nor did these cells show increased expression of VLA-4 by FACS analysis (see Figure 5B).

Presence of toxic neutrophils in peripheral blood and other body fluids late after trauma

Toxic PMNs were found in blood of 14/47 ICU patients 8 – 10 days after trauma. Patients who were characterized by these toxic PMNs during the study period suffered a more profound illness (Figure 1B). In order to investigate the possible origin of this subpopulation, granulocytes were harvested from different body fluids of patients with severe injury. Large amounts of VLA-4 positive granulocytes were found in both the lung fluid of patients with acute respiratory distress syndrome (ARDS) and patients with chylothorax at the time of multiple organ failure (Figure

6A). Histological assessment after sorting revealed these cells to be end stage (hyper)segmented toxic PMNs (Figure 6B).

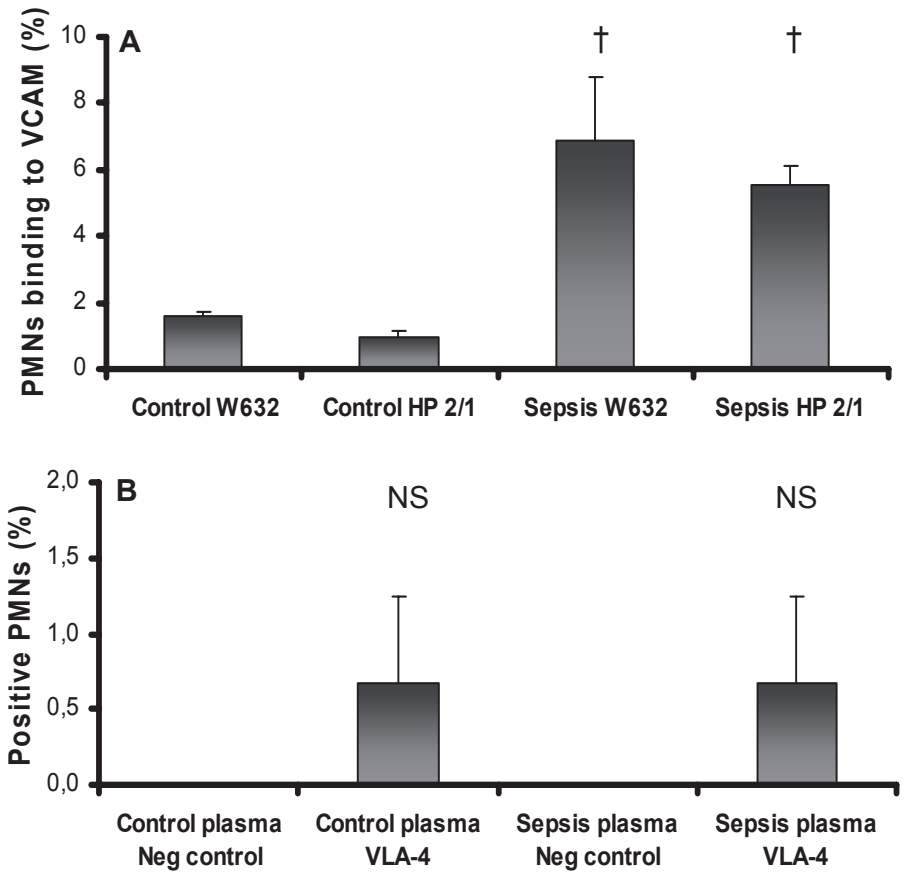

Figure 5. VLA-4 expression on PMNs not inducible by plasma from patients with sepsis, but VCAM binding is.
Although binding of PMNs to VCAM coated beads could be induced after stimulation of the cells with plasma from septic patients (**A**), this increased binding could not be blocked by HP2/1 nor did these cells show increased expression of VLA-4 by FACS analysis (**B**). †: $p < 0.05$ for plasma from septic patients versus plasma from healthy controls.

Localizaton of tissue neutrophils in patients died of septic shock

Tissue samples obtained post mortem from patients who died from septic shock and organ failure were analyzed in the context of presence of neutrophils. Although histological examination of these tissues did not clearly show the typical multilobular

nucleus of mature PMNs, expression of MPO (myeloperoxidase) and lactoferrin were used to identify tissue PMNs (Figure 7A). In the bone marrow of all patients a shift of granulocytes towards progenitor cells was found with a complete depletion of mature PMNs (Figure 7A). Leukostasis was present in both the lungs and spleen (Figure 7A). PMNs were found in the lymph nodes of patients with septic shock, especially in the cap of the germinal zone (Figure 7A).

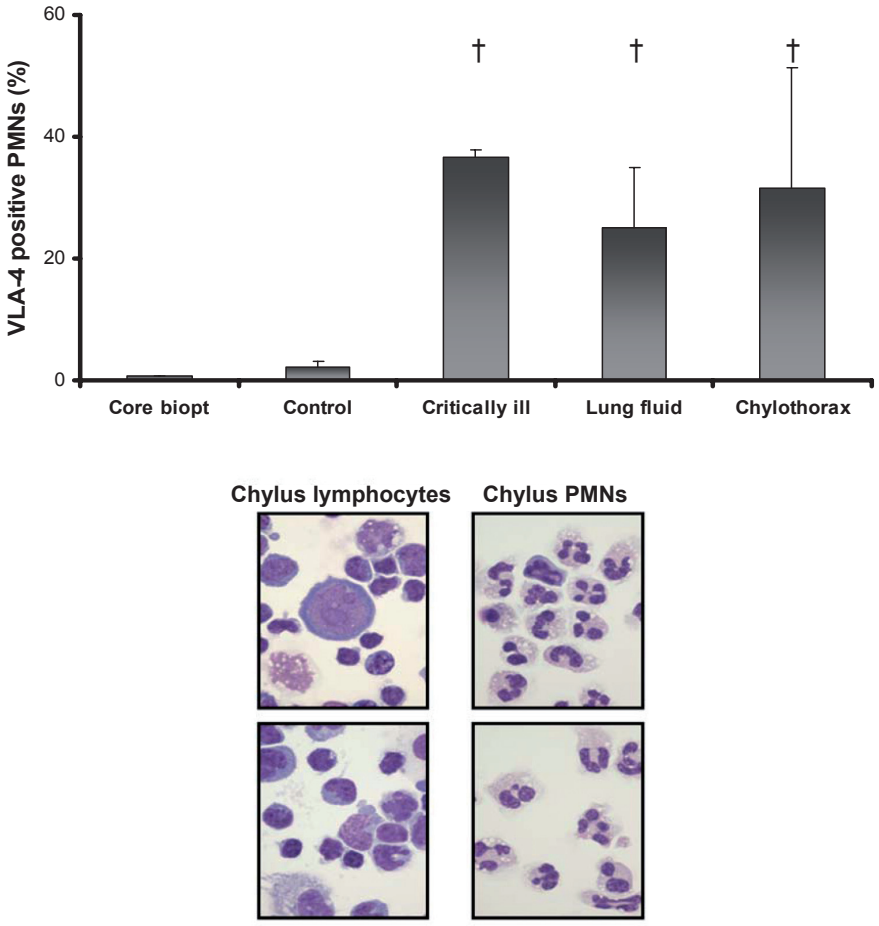

Figure 6. Percentage VLA-4 positive granulocytes in different body compartments.
Fluids and cells were harvested from different body compartments demonstrated statistically significant (Kruskal Wallis H; p=0.02) more VLA-4 positive PMNs in critically ill patients, patients with ARDS and in chylothorax samples of patients with MOF (**A**). In addition, VLA-4 positive cells were isolated by sorting and then divided (by sorting) according to their forward (FSC) and sideward (SSC) scatter characteristics. Normal FSC and SSC characteristics for PMNs and lymphocytes demonstrated lymphocytes and PMNs on histological examination (**B**). †: $p < 0.05$ versus control values.

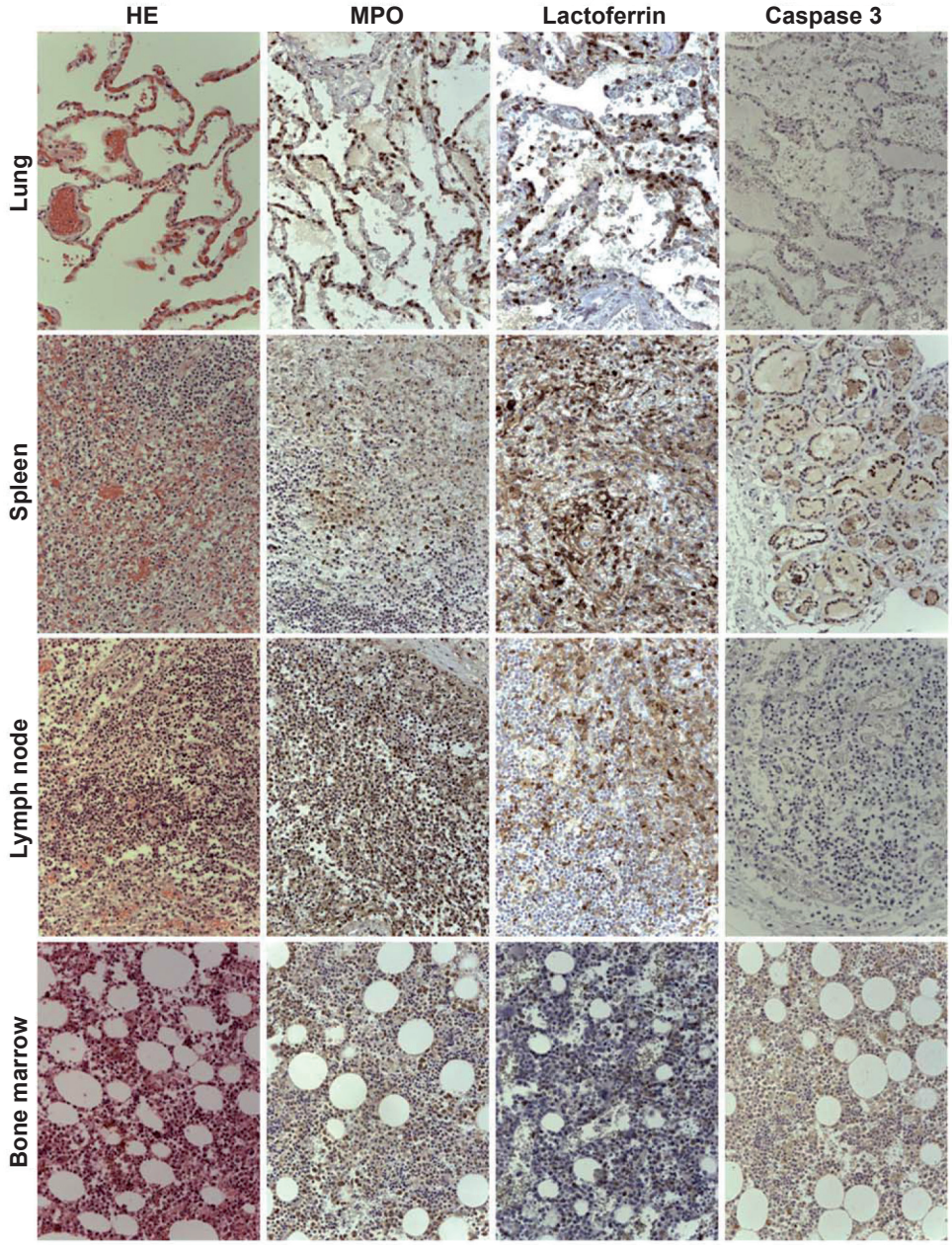

Figure 7A. Histological assessment of PMNs in tissues.
Large amounts of PMNs were seen in the lungs, spleen lymph nodes and bone marrow (**A**), as characterized by both MPO and lactoferrin positive staining. Apoptotic PMNs were found in the spleen and bone marrow.

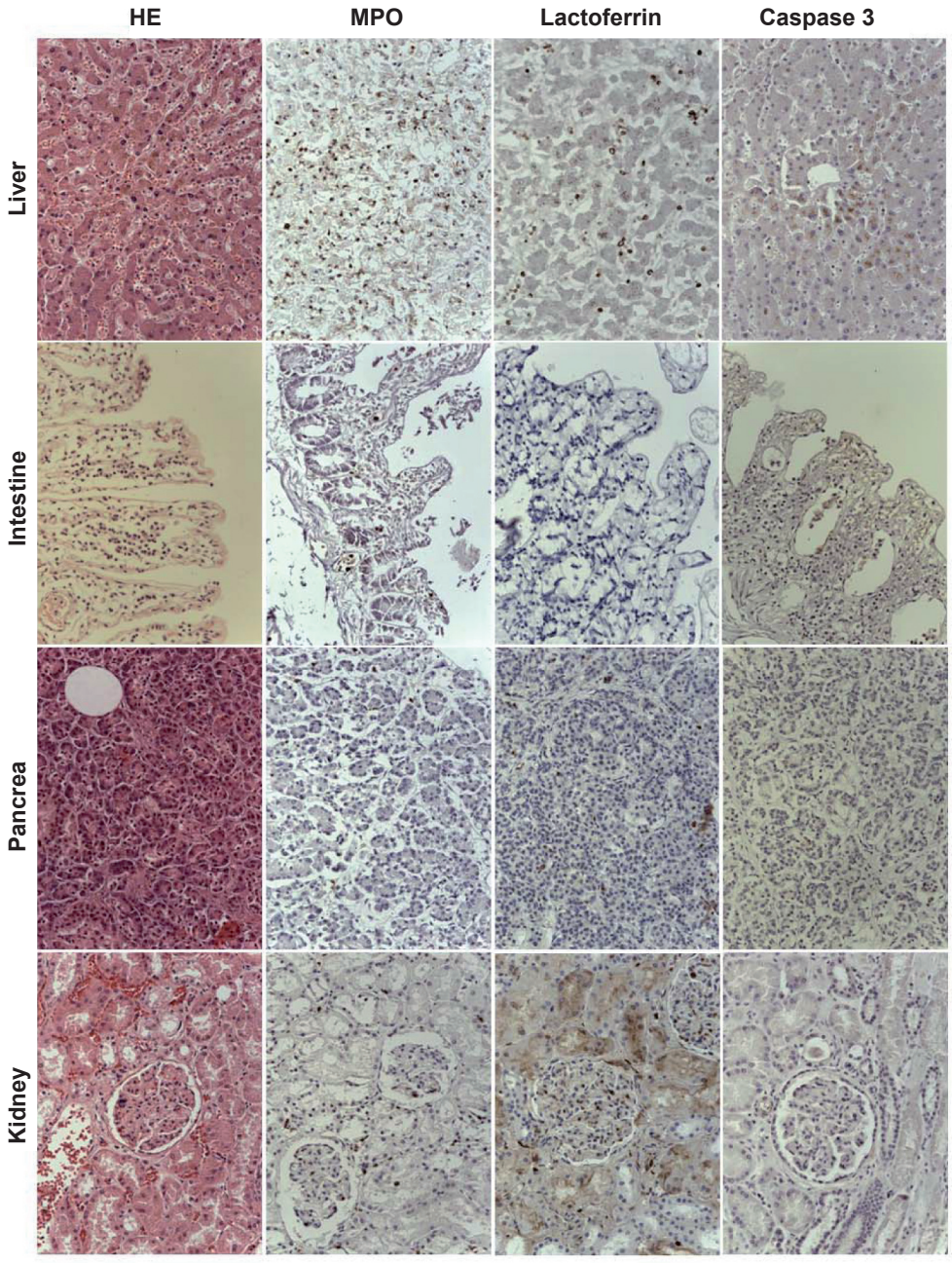

Figure 7B. Histological assessment of PMNs in tissues. Relatively small numbers of PMNs were found in other organs of these patients such as intestine, liver, kidney and pancreas.

Large amounts of apoptotic (caspase 3 positive) PMNs were found in the spleen and bone marrow and were absent in lungs and lymph nodes. The other organ samples from these patients (bowel, liver, kidney and pancreas) showed relatively small numbers of PMNs (Figure7B). A strong staining of lactoferrin was observed in the kidneys, while no MPO staining was present thereby ruling out the presence of PMNs (33).

Expression of HLA-DR on CD16^{Bright}/CD49d^{Bright} toxic neutrophils

The presence of PMNs in lymph fluid and lymph nodes suggested an additional role for these cells other than the established antimicrobial function. Therefore, we tested the hypothesis that these neutrophils expressed HLA-DR. Analysis of the PMN subpopulations for HLA-DR (MHC-II) expression by triple staining revealed that neither CD16^{Bright}/CD49d^{Minus} nor CD16^{Intermediate}/CD49d^{Intermediate} expressed HLA-DR, which was similar to the normal phenotype of neutrophils found in the blood of controls. However, the CD16^{Bright}/CD49d^{Bright} PMNs demonstrated an increased expression of HLA-DR (Figure 8).

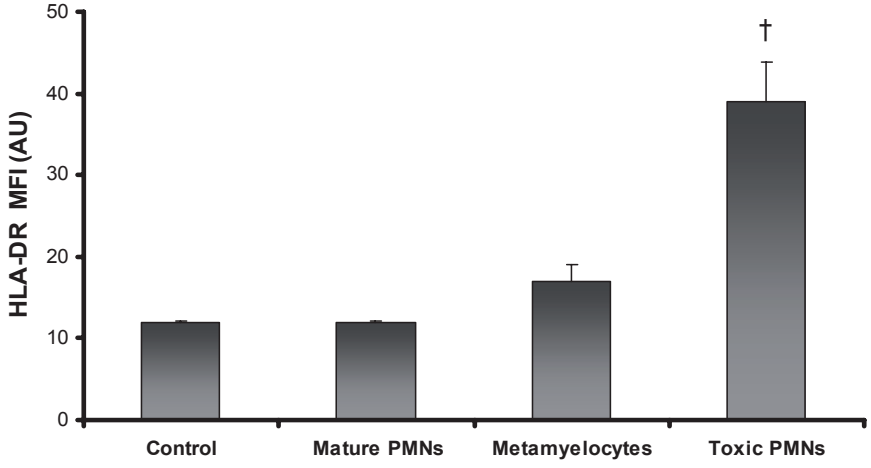

Figure 8. HLA-DR expression on PMN subpopulations.
Expressed are the mean fluorescence intensity (MFI) for PMN HLA-DR ± SEM. HLA-DR is under normal conditions not present on peripheral PMNs. The negative control antibody induces normal background fluorescence of 10 AU. A statistically significant (Paired sample T-test; $p<0.001$) increased expression of HLA-DR was found on toxic PMNs (CD16^{Bright}/CD49d^{Bright}). †: $p < 0.05$ versus control values.

DISCUSSION

PMN subpopulations exist in humans *in vivo*, but only a few studies have characterized them in any detail (24,34,35). With analysis of only two receptors, VLA-4 and FcγRIII, we identified five different subpopulations of granulocytes that can occur preceding, during and after severe acute inflammation in severely injured patients. One can only speculate on the origin of these subpopulations, but (meta)myelocytes and banded neutrophils likely originate from the bone marrow (36,37) (38). The source of the other cells remains to be identified. The study of this important research question is complicated by the fact that no consensus is currently present regarding the "life cycle" of neutrophil in health and disease. Classical studies provided evidence that tissue neutrophils are cleared by apoptosis and phagocytosis by reticular macrophages (12,39,40).

Our data regarding the occurrence of VLA-4 positive toxic neutrophils does not fit this hypothesis. In fact more recent studies provide a mechanism that better fits our findings. These studies have shown PMNs can recirculate from the tissues back to blood and bone marrow. Firstly, *in vitro* it has been shown that PMNs can retro-migrate over endothelial cells in the basal to apical direction and cells with the retro-migrated phenotype were found in the circulation under chronic inflammatory conditions (41). Phenotypical analysis of these cells, however, showed that they did not express VLA-4 on their surface. Secondly, PMNs injected into the footpad of mice were shown to travel through lymphatic vessels towards the draining lymph nodes (42) . Thirdly, in a clinical setting, PMNs have been identified in the lymph fluid of the thoracic duct of surgical intensive care patients with multiple organ failure due to sepsis (43). Finally, PMNs could emigrate from inflamed glomeruli in patients with glomerulonephritis (44). This more modern view of granulocyte physiology is important in interpretation of the data of this study.

Our data show that the kinetics of occurrence of the different populations differed markedly during the cause of disease in the different patients. Generally. two populations of VLA-4 positive granulocytes were seen occurring at different times after trauma. The first group comprised of VLA-4 positive (meta)myelocytes, which were typically seen between 2-7 days after trauma. These progenitor cells, which were not detectable in patients without inflammatory complications, comprised up to 40% of all blood PMNs in patients that developed inflammatory complications after

trauma. The second group of VLA-4 positive PMNs was typically seen between days 5 – 10 after trauma and consisted of toxic PMNs (see Figure 2). These cells had all the characteristics of end stage PMNs. Other authors have shown the presence of bacteria in these toxic PMNs, suggesting these cells have participated in antimicrobial responses (45). These data fit the hypothesis that VLA-4 positive (meta)myelosites originate from the bone marrow and the VLA-4 positive toxic neutrophils are redistributed cells from the tissues.

Both VLA-4 expressing populations, (meta)myelocytes and toxic PMNs, express functional VLA-4. This was demonstrated by analysis of binding of FACS sorted VLA-4 positive PMNs to VCAM-1 coated fluorescent beads. Our data show that VLA-4 on these PMNs is present in a partially activated state, as activation with fMLP hardly induced additional binding (5-10%, see Figure 4). The partial functionality of VLA-4 is very similar to the situation found on other leukocytes such as human eosinophils (46). It has been suggested that granulocyte expression of activated VLA-4 is involved in constitutive homing of these cells to VCAM-1 expressing vascular beds such as lymph vessels, bone marrow and spleen (47).

VLA-4 is expressed on PMN progenitors, however is normally not expressed on PMNs in the peripheral circulation (48). Some studies suggest that VLA-4 is used by leukocytes to migrate to connective tissue, lymph and bone marrow (47,49,50). Extending these findings, we demonstrated VLA-4 positive toxic PMNs both in the peripheral circulation, lung fluid and lymph fluid in patients suffering from septic complications. Therefore, it is likely that cells originate from the tissues. To test this hypothesis we tested several tissues obtained from patients *post mortem* who died from septic complications after trauma. PMNs were difficult to identify on nuclear morphology in these tissue samples stained with either HE or Giemsa. Therefore, we studied their presence by immunohistochemistry of the tissue samples for both myeloperoxidase (MPO) and lactoferrin which are specific markers for PMNs (51,52). Neutrophils were preferentially found lymph nodes, spleen, liver and lung. On the other hand, they were absent in bone marrow as well as several other tissues such pancreas, kidney and gut (see Figure 7). This preferential location of PMN's under these conditions fits with the hypothesis that neutrophils are not merely antimicrobial effector cells, but also immune modulatory cells that can travel in between tissues and even back to the bone marrow.

Several recent studies suggest that PMNs can modulate the cells of the adaptive immune systems. In a murine model Maletto *et al.* showed that application of PMNs together with antigen to the footpad of can steer the adaptive immune response in the draining lymph node towards (42). This view was supported by a study providing some evidence of differentiation of mature PMNs towards cells expressing co-stimulatory molecules for T-cells during pathological inflammation (e.g. tuberculosis). During *in vitro* culture PMNs can lose their chemotactic capacity, but gain the ability to present antigens to T-cells (53-55).

Our data strongly support this hypothesis for an additional immune modulatory role of PMNs: 1) large amounts of PMNs can be present in the lymph fluid (see Figure 6) (43,56); 2) large numbers of PMNs were found in the cap of the germinal centre in lymphoid tissues of patients died from septic complications (see Figure 7); and 3) the $CD16^{Bright}/CD49d^{Bright}$ PMNs found in the blood preceding the development of septic complications expressed HLA-DR, which is essential in the steering of the adaptive immune response.

Accepting the hypothesis that neutrophils redistribute through the body the question arises where the cells are cleared by apoptosis. We did not find indications that neutrophils exhibit features of apoptosis in peripheral blood, lung fluid or tissue, lymph fluid or lymph nodes, kidney, pancreas and gut of patients died from septic complications (see the absence of caspase3 staining in these tissues in Figure 7). In marked contrast, clear apoptotic neutrophils were found in spleen and bone marrow. It is tempting to speculate that these hematopoietic tissues are preferentially used for clearance of neutrophils *at least* under conditions of acute inflammation.

The number of circulating PMNs remains normal or is even increased during the CARS induced after trauma, neutropenia is seldom seen (16,57). Therefore, it is not the number of peripheral PMNs that is associated with the dysfunctional immune response during CARS, but rather the functionality of the individual cells (**chapter 6**) (3-5). We have shown previously that PMNs after severe trauma are less responsive towards fMLP in the context of inside-out control of active FcγRII (3,4). In addition, several surface proteins used for chemotaxis or opsonisation demonstrated a gradual decrease during 6-7 days after trauma.

The hypotheses described above do not seem to explain as to why septic complications occur 8-10 days after trauma. However, this time is very similar with the transit time through the post-mitotic pool of PMNs in the bone marrow (58-60). It takes around 7 days for the whole blood PMN compartment to be depleted when mitotic myelopoiesis is acutely stopped (60). Our data are consistent with the idea that the severe systemic inflammatory following multi-trauma leads to mobilization of the majority of functional PMNs from the bone marrow, via the blood to the injured tissues. This process can deplete the bone marrow from functional granulocytes which is illustrated by the “empty” bone marrow from mature neutrophils found in post mortem in these patients (see Figure 7). During the first days after trauma the massive homing of cells to the tissues can be compensated by the release of young PMNs and even (meta)myelocytes from the bone marrow but a gradual decrease in neutrophil functionality is still initiated. After around 7 days the bone marrow seems to fail to release functional neutrophils. During this phase we found mainly old or even toxic PMNs, which are less functional (**chapter 6**). Therefore, the increase in VLA-4 positive PMNs seems to be a sign of innate immune exhaustion, which gives the impression to be a main risk factor for CARS and septic complications.

CONCLUSION

Our data are in line with the hypothesis that long term (> 6 days) severe systemic inflammation induced by multi trauma leads to failing of the output of functional neutrophils from the bone marrow, which coincides with the clinical onset of CARS. Prevention of this “immune exhaustion” should be focused on antagonism of the initial exaggerated inflammatory response rather than blocking putative anti-inflammatory mediators produced during the CARS.

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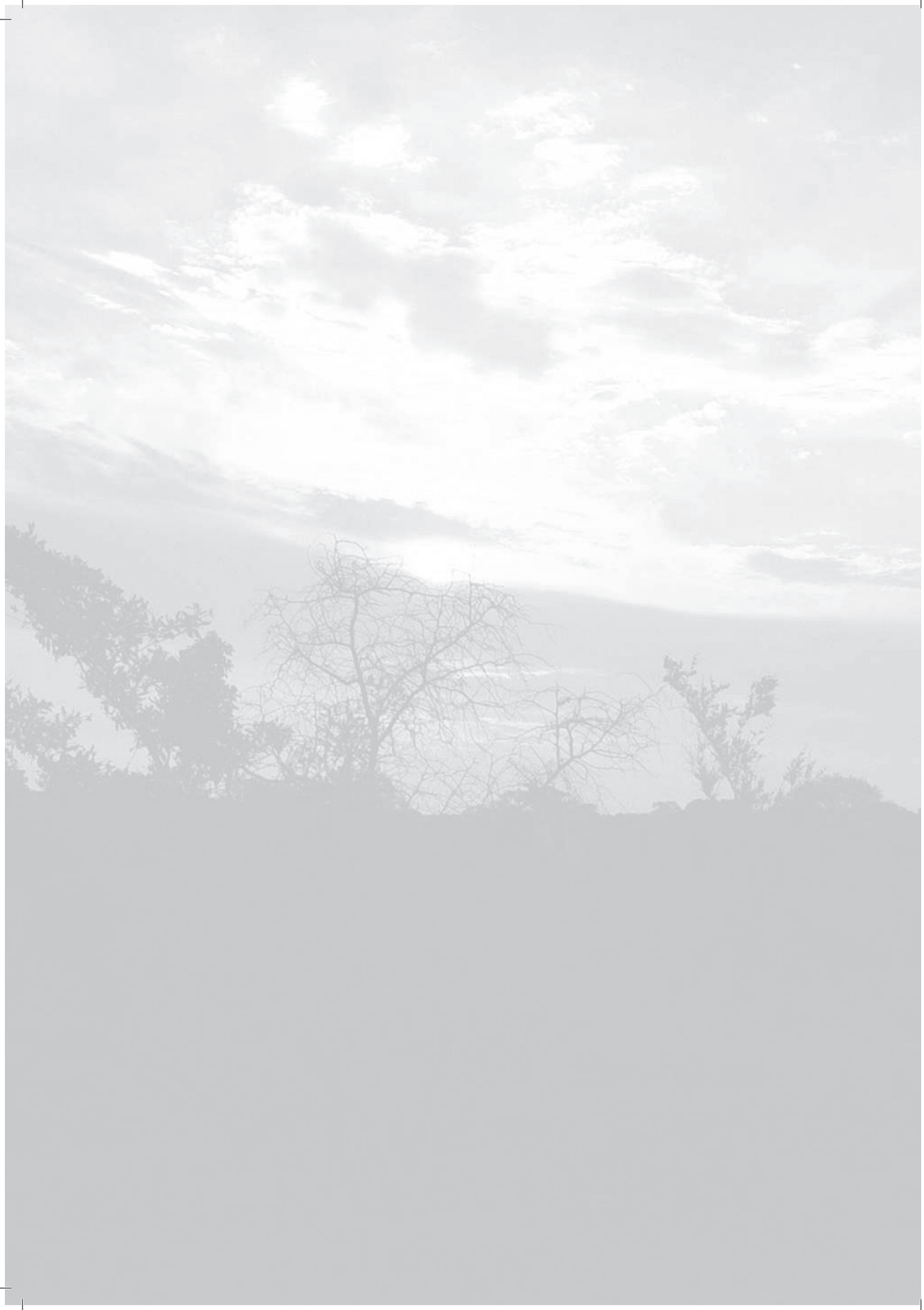

Chapter 10

Discussion and future perspectives

The course of patients suffering severe trauma is often complicated by (multiple) organ failure (MOF), an innate immune system driven disease with devastating consequences. It has been suggested that tailor-made treatment strategies reduce the incidence of organ failure after trauma (1-5). However, the limited knowledge of the pathophysiological processes impedes the correct allocation of patients to the different surgical options, or the development of new immune modulatory drugs (6-11). The pathophysiological process of inflammation after trauma should be further analyzed to identify new leads for novel therapeutic strategies. For this, an extensive analysis was performed of the kinetics of the cellular innate immune response to injury in a clinical setting.

EXCESSIVE INFLAMMATION AND PMN PHENOTYPE

Cells of the innate immune system form the integrated endpoint of several immunological cascades (12-15). In addition, these very cells cause additional tissue damage leading to MOF (16-22). Therefore, we investigated innate immune cells, neutrophils and monocytes, by measuring sensitive activation markers in relation with severity of trauma and development of organ failure (23,24). Individual PMN receptors or functions (i.e. MAC-1 and oxidative burst) have been previously investigated, but were only related to severe trauma and thus severe inflammation (19,25-28). In accordance with previous reports we found an enhanced expression of MAC-1. However, no correlation with injury severity was found. In addition, we analyzed the active FcγRII complex on PMNs (A17/A27) after *in vitro* fMLP stimulation as a read-out for the functionality of neutrophils (29-32). An increased functionality after trauma was anticipated, however, in contrast a decreased responsiveness was demonstrated even after mild or moderate injury (**chapter 3**). Although no satisfactory explanation could be provided at that point, this analysis method proved to be a sensitive detection method for systemic inflammation after trauma. In order to further analyze this seemingly contradictory finding additional studies were performed.

PMN PHENOTYPE AS READ OUT FOR THE INFLAMMATORY RESPONSE

Several authors have tried to correlate the initial inflammatory response to the development of early phase organ failure. Soluble inflammatory markers, such as IL-1β, IL-6, IL-8 and complement factors have been evaluated and most factors demonstrated a relation with the development of early phase organ failure, though with a large interpersonal variation (13,33-44). For cellular markers, such correlations have not been shown. Only a slight relation between MAC-1 and the base deficit (BE) in a small population of trauma patients has been demonstrated (27). Until now, no other surface marker for the cellular innate immune system has been correlated with the severity of trauma and thus the severity of inflammation (19,24,28,45-47). Functional analysis of innate immune cells demonstrated a moderate relation between the development of organ failure and PMN cytotoxic capacity, but a relation with severity of inflammation was not further detailed (6,28,48-50). Functional analysis often requires granulocyte isolation (51,52), which has been shown to alter cellular functionality (53,54). In addition, measurement of reactive oxygen species (ROS) in whole blood remains difficult with large variations between samples and protocols (55-57). Thus, alternative approaches are needed to analyze cellular innate immune function after trauma in relation to the severity of inflammation and development of organ failure.

Although the changes in phenotype of circulating PMNs are not the changes that occur in the tissues (**chapter 3**), the changes of circulating PMNs can be used as a read-out for the inflammatory processes taking place in those tissues. These changes in cellular phenotype represent in our opinion the integration of the pro- and anti-inflammatory signals (12,58-62). With this analysis of functional phenotypes a relation between the severity of trauma by clinical scores and the magnitude of changes in fMLP induced active FcγRII on PMNs was demonstrated (**chapter 4**). Thus, a relation exists between the changes in functional PMN phenotype and the inflammatory response of the host.

Furthermore, specific phenotypic changes of circulating PMNs had a close relation to the development of early phase pulmonary organ failure, such as ALI and ARDS (**chapter 4**). Other authors have demonstrated a relation between changes in

individual PMN receptors or functions and the development of early phase organ failure before (19,24,28,46,47). However, this could not be translated to a dose-dependent mechanism between the magnitude of receptor changes and the severity of trauma or the incidence of ARDS. In **chapter 4** a relation was found between the magnitude of changes in PMN responsiveness towards fMLP in the context of active FcγRII and the severity of inflammatory pulmonary complications.

This indicates that the functional phenotype of blood PMNs represents the severity of the inflammatory response in the individual patient. By identifying the cellular innate immune response after trauma, the effect of several inflammatory cascades becomes visible. This new tool enables analysis of the impact of surgical procedures and therapeutic strategies on the inflammatory status of the individual patient.

SURGERY DOES NOT SEEM TO HAVE IMPACT ON THE SYSTEMIC CELLULAR IMMUNE RESPONSE

It has been suggested that surgery adds to the inflammatory burden of a patient and thus the risk for early phase organ failure (1,63). The prognostic value of immunological parameters has been investigated, in order to identify high risk patients. High levels of IL-6 (interleukin 6) have been associated with early phase organ failure. Unfortunately, due to its' large individual variation, IL-6 has not been widely implicated in a clinical setting. However, in several trauma centers, plasma IL-6 levels are used in combination with clinical parameters to identify "stable" or "borderline" patients for treatment allocation (34,37,42,64). Plasma IL-6 levels have been used to analyze the impact of intramedullary nailing (IMN) in cohort studies and this provided ground for the development damage control orthopedics (DCO), as IMN increased plasma IL-6 levels (42,65-68).

Allocation of patients to ETC (early total care) or DCO by clinical parameters plus IL-6 levels demonstrated improved outcome of severely injured patients ("borderline patients") treated with DCO (69). Nevertheless, still 10-30% of the patients, which were presumed stable by these clinical parameters plus IL-6 levels, developed severe pulmonary complications.

In **chapter 5** it was demonstrated that, although plasma IL-6 levels increased during IMN of femoral fractures, *the measured changes in functional PMN phenotype were determined by the initial trauma and did not change during the surgical procedure*. This discrepancy between soluble and cellular innate immune components has been suggested previously in a single case, based on MAC-1 and IL-6 (70). Pathophysiological mechanisms provide a putative explanation of this discrepancy: cytokines by themselves do not induce ARDS, but are involved in the modulation of activation of leukocytes. These leukocytes, which integrate the signals of the cytokines into a activation prone or refractory phenotype. Activation prone neutrophils are essential in the alveolar destruction underlying the clinical symptoms of ARDS (71). In this view, cytokines are risk factors and the increase of single cytokines, such as IL-6, do not necessarily lead to activation of innate immune cells. The absence of changes in PMN phenotype suggests that no significant additional inflammation occurs during surgery, but alternative processes might add to the development of ARDS.

In **chapter 5** it was shown that the nailing procedure releases factors, such as triglycerides, as well as IL-6 and TNF-α. These factors can damage or activate the (pulmonary) endothelium (72-76). When these factors are produced at the same time that neutrophils and monocytes become activated, this will lead to homing to and activation of leukocytes in the tissue (77). The increase of triglyceride levels or the occurrence of fat embolisms during IMN has been studied extensively (72,73,78). Fat embolisms particles are frequently seen during IMN, but seldom lead to clinical signs or symptoms (73). It appears that the combination between pulmonary endothelial activation/damage and an activated cellular innate immune system leads to ARDS. Thus, IMN on itself does not seem to increase the inflammatory burden, but might decrease the threshold for pulmonary complications to develop by damaging or activating endothelium by release of triglycerides and further increase of IL-6 and TNF-α levels.

INITIAL EXCESSIVE INFLAMMATION IS RELATED TO LATE PHASE SEPSIS

It has been suggested that the pro-inflammatory response evokes an anti-inflammatory response. This implies that the magnitude of the pro-inflammatory response is related to the compensatory anti-inflammatory response syndrome or CARS (13,79). Indeed, after injury both pro-inflammatory cytokines such as IL-6, TNF-α and IFN-γ as well as anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β appear in increased levels in the circulation (12,37,80,81). However, until now, no correlation has been demonstrated between the pro-inflammatory cytokine levels and the anti-inflammatory cytokine levels, thereby questioning the hypothesized mechanism (67).

In **chapter 6** it was demonstrated that in severely injured patients the initial inflammatory response determines the extent of ongoing inflammation and thereby the later state of immune paralysis. By determination of the functional PMN phenotype, a direct relation was found between the magnitude of the “pro-inflammatory” reaction and the development of late phase (>7 days) septic shock. This suggests that the initial cellular innate immune response after trauma determines the development of both early phase and late phase organ failure.

Acute phase proteins such as C-reactive protein (CRP) and pro-calcitonin (PCT) have been associated with late phase sepsis and subsequent organ failure, but these were only discriminative 7 days after trauma (33,37,82-85). In **chapter 6** a similar pattern for CRP was seen, which allowed discriminating between patients with or without septic shock 6 days after trauma. This finding has little clinical consequences as the time to septic shock is much too short. In contrast, the initial (< 1 day after trauma) decrease in PMN functionality (fMLP induced active FcγRII) after trauma is related to late phase organ failure, thus 7 days prior to the onset of clinical symptoms. However, our study investigated the pathophysiological mechanisms which lead to septic shock. The prognostic value of these PMN phenotype changes has yet to be determined. If these changes prove valuable, septic shock in severely injured patients can be anticipated 7 days before its’ onset.

Some authors have shown similar findings in monocytes during inflammation (62,86-89). They demonstrated that low median HLA-DR expression of the whole monocyte population or a low percentage of HLA-DR positive monocytes at admission is indicative for the development of late sepsis (7 days) after trauma (90,91). In **chapter 7** it was found that the percentage of HLA-DR positive monocytes was decreased after trauma and that the magnitude of HLA-DR positive monocyte redistribution was related to the severity of trauma. In contrast to the PMNs, no studies have been published on monocyte HLA-DR expression and the development of ARDS. However, similarly to the situation with PMNs, a relation between an activated monocyte phenotype and the development of early phase pulmonary organ failure was eminent (**chapter 7**). Furthermore, **chapter 8** corroborated the finding that a continuous low number of HLA-DR positive monocytes (both percentage and absolute number) was related with the development of septic shock.

A more activated phenotype of monocytes was found in **chapter 7** characterized by increased expression of MAC-1 and decreased responsiveness of active FcγRII to fMLP. The hypothesis was tested that similar to leukocytosis a monocytosis would occur after trauma,. Although initially there was a change in composition of HLA-DR positive and negative monocytes, no monocytosis was present during the first week after trauma. On the other hand, a large increase in HLA-DR negative monocytes was observed during the second week after trauma in patients who did not develop septic shock. Thus, the lack of restoration of normal levels of HLA-DR positive monocytes indicates the occurrence of sepsis and late phase organ failure, while the occurrence of large amounts of HLA-DR negative monocytes suggests a protective role for these cells in the second week after trauma.

The kinetics of monocytes and of PMNs in peripheral blood suggest an opposite role of these cells in response to injury. This is exemplified for instance by the finding that PMN numbers increased in patients with late phase sepsis (second week), whereas monocyte numbers remained low. On the other hand, monocyte numbers (especially HLA-DR negative monocytes) increased dramatically in patients without late phase complications, while the number of neutrophils remained constant or increased slightly. It is tempting to speculate that in patients with septic shock, massive increased PMN numbers respond excessive to the presence of a new bacterial threat, which leads to collateral damage to the tissue with MOF as a result.

Increased numbers of HLA-DR negative, MAC-1 high monocytes with altered Fc-receptor function have been described previously in patients with sepsis (92,93). However, the authors concluded that in their population of patients with sepsis, these activated monocytes contribute to the development of additional tissue damage. In **chapter 8** low numbers of these monocytes were found in patients with sepsis, but the number of HLA-DR negative monocytes was much higher in patients without sepsis, suggesting a protective rather that harmful role for these cells in the later phase after trauma. It is tempting to speculate that the presence of these cells during sepsis is a physiological consequence of the initial monocyte redistribution during excessive inflammation.

INNATE IMMUNE PARALYSIS CAUSED BY EXHAUSTION

General consensus is that immune paralysis during CARS develops by active down-regulation of the immune system by anti-inflammatory processes (12). However, this hypothesis has not been proven by clinical and immunological data. The alternative hypothesis that the low functionality of the neutrophils in the blood during CARS is caused by exhaustion of the immune system. However, this latter hypothesis is not in line by the current consensus on the life cycle of the PMN. Many authors claim that PMNs are produced in the bone marrow, enter the circulation, leave for the tissues where they are cleared by apoptosis and subsequent phagocytosis by reticular macrophages (94-96). If this view on the lifecycle of PMNs is true: (1) the low functionality of blood neutrophils found after trauma would likely be induced by anti-inflammatory mediators (2) leukocytosis is caused by high mobilization of bone marrow derived neutrophils. Indeed, after trauma and during sepsis large amounts of young or even premature PMNs have been found in the peripheral circulation (28,87,97-99). The half-life of PMNs in the circulation is presumably several hours (100,101). Following this view, new PMNs are released into the circulation in large numbers on a daily basis. In addition, anti-inflammatory cytokines found in the blood during CARS down-regulate neutrophil functionality (102).

Detailed analysis of neutrophil functionality points at a more complex mechanism. The refractory phenotype of PMNs found in the peripheral circulation of trauma patients during sepsis is characterized by suppressed inside-out control of

FcγRII (**chapter 6**) and chemotaxis (103). On the other hand, cytotoxic capacity was enhanced characterized by an increased capacity to produce ROS (reactive oxygen species) (28,104). The initial increased homing of cells with primed cytotoxic capacity during severe systemic inflammation will lead to an enhanced oxidative stress in the affected tissues (14,19,28,105). The characteristics of PMN function directly after trauma and during sepsis are summarized in table 1, which displays a clear discrepancy between functions that are up-regulated and down-regulated. In summary, neutrophils acquire a mixed refractory phenotype characterized by an enhanced cytotoxic capacity and lowered chemotaxis and Fc-receptors.

Table 1. Phenotype and function changes in PMNs from trauma to septic shock

PMN functions	Trauma	Sepsis	Septic shock
Chemotaxis	Increased (19)	Decreased (103)	Decreased (103)
Adhesion / MAC-1	Increased (6,27)	Increased (87)	Increased (87)
Opsonin-receptors	Decreased (**chapter 3-5**)	Decreased (**chapter 6**)	Decreased (**chapter 6**)
Phagocytosis	NA	Increased (50,99)	Increased, (< sepsis) (99)
Oxidative burst	Increased (28)	Increased (50)	Increased, (< sepsis) (99)
Apoptosis	Decreased (159)	Increased (50)	Increased (50)

Based on the findings presented in this thesis, we propose an alternative hypothesis. In **chapter 6** it was demonstrated that immune paralysis occurs during a period of 7 days, measured by PMNs with a refractory phenotype in the circulation. In **chapter 9** it was shown that this might be the process of exhaustion of the innate immune system. The innate immune system can, during ongoing (>6 days) severe systemic inflammation, no longer produce fully functional PMNs and monocytes from the bone marrow to challenge a new bacterial threat. This process of ongoing inflammation causes depletion of the rapid mobilizing bone marrow pool of PMNs as well as the normal post-mitotic pool and accumulation of activated PMNs in the tissues In **chapter 9** it was demonstrated that severely injured patients after several days no longer produce significant amounts of new PMNs The data imply that PMNs which have entered the tissues presumably re-enter the lymph and circulation. We demonstrated multiple subpopulations of PMNs, which can only be seen under these extreme conditions:

- **VLA-4 positive end stage neutrophils:** The re-circulated PMNs were found in the blood, lymph and lung fluid in multi trauma patients and can be recognized histologically as toxic PMNs with toxic granules and vacuoles(106,107). The cells were also characterized by the expression of VLA-4, an integrin which is lacking on normal neutrophils. These characteristics suggest that these cells are end-stage and need to be cleared. However, in most patients no sign of apoptosis was found in these cells. In two patients with severe septic shock 3-6 hours before these patients died, triple staining revealed part (8-40%) of the VLA-4 positive cells to be annexin V positive, suggesting these VLA-4 positive PMNs to be (pre-)apoptotic (results not shown).

- **VLA-4 positive metamyelosites:** During detailed analysis we found another VLA-4 positive neutrophil population with a histological shape of (meta)myelocytes. Based on their nuclear shape and cytoplasm these (meta)myelocytes seem normal progenitors, but their nuclear density and toxic cytoplasm indicates these cells to be older (Figure 2, **chapter 9**) (108-110). In contrast to the presence of similar cells during hematological malignancies, after trauma this phenomenon seems to be completely reversible in trauma patients, matching the finding of these cells during tuberculosis infection (108,109). Nevertheless, this maturation dissociation is another warning that the demand for fresh PMNs is larger than the PMN bone marrow production. Further analysis of these PMN subpopulations in different fluids and post-mortem tissues suggested specific PMN subpopulations (the VLA-4 positive toxic PMNs) to be able to re-circulate and may have an additional immunological function. These VLA-4 positive cells were found in the lymph fluid and large numbers of PMNs were found in an organized fashion surrounding the germinal center of lymph nodes (**chapter 9**). The relatively high expression of HLA-DR on these cells suggest an immune modulation role for these PMNs (111,112).

Even though the initial PMN subpopulations kinetics were not studied in this thesis, Pillay *et al.* demonstrated that at least three different subpopulations have very distinct kinetics (personal communication). Though based on a different receptor expression profile, they showed that “normal” (segmented), “young” (banded) and “old” (hyper-segmented) PMNs all appear after a standardized insult with LPS (*E. coli* derived lipopolysaccharide) in healthy volunteers (113-115). In conclusion, we

suggest that the initial inflammatory response determines the process of ongoing inflammation, bone marrow depletion, PMN recirculation and thereby immune paralysis for the PMN population.

This questions the importance of immune suppressive cytokines, such as IL-10 in the first 7 days after trauma (61,89). Monocytes with high HLA-DR expression are outnumbered by HLA-DR negative monocytes after 48-72 hours after injury in all patients. In addition, the number of HLA-DR positive cells normalize, while large numbers of HLA-DR negative monocytes appear in the circulation during the recovery phase of patients without septic shock (**chapter 8**). It is tempting to speculate that an overzealous innate immune response after trauma not only leads to exhaustion of the PMN population, but the monocyte mediated immune response as well. The lack of HLA-DR positive monocytes during the immune paralysis phase might be part of the “paralyzed” immune response to infectious agents, as broadly speculated in the literature (116-118).

Monocytes are important in the clearance of necrosis and PMNs undergoing apoptosis (96,119). Through this process, monocytes play an essential role in the homeostasis of inflammation. Relatively low numbers of HLA-DR negative monocytes were found in patients with septic shock compared to patients without sepsis. This might lead to improper repair of injured tissue and impaired barrier function, which creates or maintains an entrance for pathogenic micro-organisms (90,120).

IN CONCLUSION: EXCESSIVE INFLAMMATION LEADS TO EXHAUSTION OF THE INNATE IMMUNE SYSTEM

The results in this thesis corroborate the hypothesis that severe injury leads to a marked inflammatory response. Patients suffering excessive cellular innate immune activation are at risk for ARDS when additional risk factors such as local endothelial damage or activation are present. In addition, patients with excessive inflammation will undergo a process of a marked ongoing inflammation, which will lead to innate immune exhaustion. This situation can facilitate the development of septic shock in the presence of a bacterial threat (Figure 1). A limitation of our studies should be taken into account.

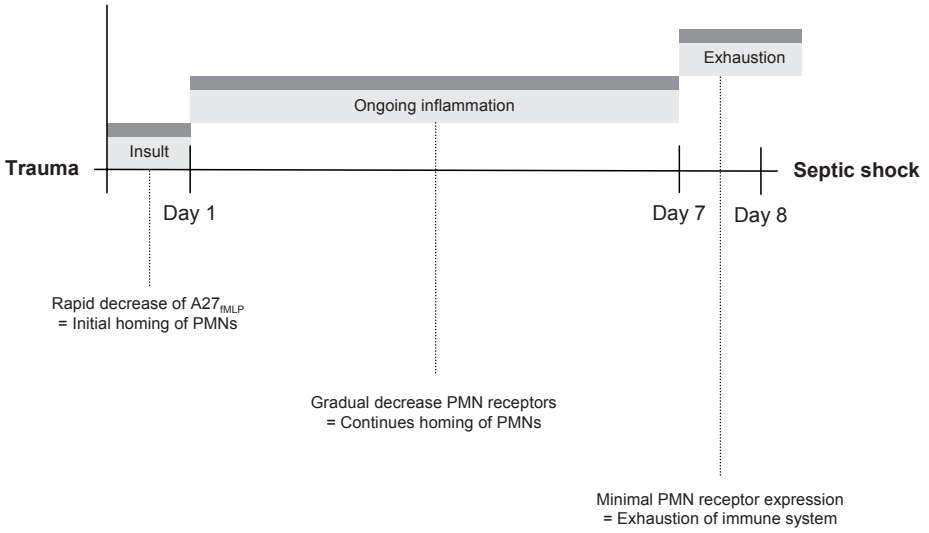

Figure 1. Concept of exhaustion after initial severe inflammation
Injury evokes an inflammatory response. When excessive, this response can result in early phase organ failure like ARDS. In addition, the excessive inflammation leads to an ongoing immunological response, finally causing exhaustion of the innate immune system. This phase of innate immune exhaustion facilitates the development of septic shock.

Excessive cellular innate immune activation is thought to occur mainly in the tissues. In our study the functionality of cellular phenotypes was analyzed to evaluate the integrated inflammatory response of the innate immune system. However, the innate immune cells in the tissues actually causing the damage were not studied for obvious clinical and ethical reasons. Our data show, however, that the phenotypes and subpopulations of the circulating cells are the result of the processes taking place in the tissues, which are directly related to both functionality and life cycle of these innate immune cells. The inflammatory response to trauma can, therefore, be quantified by analyzing disturbances in the normal cellular innate immune dynamics. With the additional knowledge on the life cycle of PMNs shown in this thesis, we can explain the seemingly contra-dictionary findings in our first study, as decreased responsiveness to the innate immune stimulus fMLP was related to severe trauma

The hypothesis that excessive inflammation after trauma leads to organ failure, which fits with the pathophysiology of experimental ischemia/reperfusion models of heart, lungs, skin and cerebrum (121-126). In these latter models, total blocking of PMNs by e.g. antibodies leads to a massive reduction in ARDS, indicating the importance

of these cells in the pathogenesis of this inflammatory complication (21). Integrins are essential in the extravasation of PMNs during reperfusion to the tissues except the lung (see below). Therefore, therapeutic strategies against these receptors have been developed, but has not reached clinical application yet. This is mainly due to a not-expected finding. Homing of neutrophils to lung is not mediated by beta-2 integrins or selectins (127). In knock-out models where integrins and selectins were knocked out (even by creating triple knock-out mice) secondary injury to brain and heart was prevented, whereas homing of neutrophils to the lung and additional injury to the lungs could not be prevented (128). In addition, CD11b (MAC-1) high PMNs do not necessarily sequester in the lungs (129). This implicates that integrins are not essential in the the extravasation of PMNs to the lungs and that the activation of neutrophils in the lung is not mediated by beta-2 integrins. In order to prevent ARDS, PMN activation and migration should be countered at an early stage.

Ideally, the systemic inflammatory response syndrome is to be attenuated. Unfortunately, attempts to prevent SIRS by mono-therapy with anti-inflammatory mediators either did not result in a reduction of ARDS or resulted in the development of severe sepsis because CARS was induced (9,130,131). The rapid increase and decrease of anti-inflammatory mediators does not reflect the gradual development of cellular innate immune paralysis (37,132). The results shown in this thesis suggest that active down-regulation is not the primary cause of PMN and monocyte dysfunction. Ongoing severe systemic inflammation results in the depletion of bone marrow of functional neutrophils. This leads even to the release of (meta)myelocytes from the bone marrow. Figure 7 in **chapter 9** shows that under these conditions the bone marrow is devoid of mature neutrophils (133). Most of these (meta)myelocytes were characterized by the expression of VLA-4.

It has been suggested that the presence of VLA-4 positive PMNs is a sign of reactive PMNs, which acquire the VLA-4 on the surface by responding to a serum factor present in the blood of patients with sepsis (134). This hypothesis does not fit the dynamics of our findings and our longitudinal analysis suggest an alternative physiological process.

1. Serum obtained from our trauma patients obtained during different time points after trauma did not induce any VLA4 expression on neutrophils isolated from normal donors (see Figure 5, **chapter 9**). The finding of Ibbotson *et al.* that serum of severe sepsis patients did induce VLA-4 on normal neutrophils might be explained by a mechanism not operational post trauma.
2. VLA-4 positive toxic PMNs were found in the circulation, lung fluid and lymph fluid. These data strongly suggest recirculation of VLA-4 positive PMNs. In addition, the presence of small numbers of $CD16^{Bright}/CD49d^{Bright}$ (meta)myelocytes with a toxic neutrophil appearance allows the speculation that a subpopulation of PMNs is released from the bone marrow expressing VLA-4, which remain VLA-4 positive, even after recirculation.

The function of these VLA-4 positive PMNs remains elusive. The toxic VLA-4 positive PMNs could be involved in immune modulation as they express HLA-DR. Unfortunately, The co-stimulatory proteins CD80 and CD86 were not measured, which are needed for activation of lymphocytes. However, previous reports have shown that activated PMNs with up-regulated HLA-DR expression also express co-stimulatory proteins (135). These HLA-DR positive PMNs could well be involved in the steering and regulation of the adaptive immune system.

Interestingly, it has been demonstrated that subpopulations of PMNs can return to the bone marrow (136). where part of these cell go into apoptosis. Upon phagocytosis by bone marrow macrophages these cells produce G-CSF, resulting in increased PMN production and release from the bone marrow (137). This provides a mechanism how the bone marrow can sense the status of the innate immune system even in the absence of cytokines.

FUTURE PERSPECTIVES

Limiting the excessive inflammatory response by (pharmacological) intervention without paralyzing the innate immune system will prove challenging. Anti-inflammatory therapeutic interventions often lead to immune suppression (9,130,131). Short-term limiting the initial inflammatory response appears beneficial, however, there is only circumstantial evidence (6). Thus, therapy should be aimed at modulating the innate immune system rather then paralyzing it. One of the key steps in the development of early phase organ failure and ongoing inflammation is the extravasation of activated PMNs. Several authors have blocked PMN adhesion or created ROS (Reactive Oxygen Species) knockout mice, all with adverse outcomes (21). Although the incidence of ARDS is severely decreased in those studies, the incidence of severe infections and sepsis was unacceptably increased. How PMN extravasation should be blocked remains unclear. It can even be questioned if ARDS can be prevented at all. Elegant studies have shown that upon activation, PMNs become more rigid and get struck in the small pulmonary capillaries. In studies where PMN integrins were blocked, secondary damage to the brain, heart, skin and peritoneum could be prevented, but pulmonary damage could not (121-126,128). In addition, current treatment regimes do not adequately attack PMNs and monocytes. Studies on the applicability of corticosteroids in the treatment of sepsis, brain injury or ARDS demonstrated increased incidence of infections and mortality in the group treated with corticosteroids (9,131). Part of this pathology might be attributed to the stimulating effects corticosteroids have on PMNs (138).

Inhibition of cellular innate immune function could harbor severe side effects. Most trauma patients suffer large wounds which can easily be invaded by micro-organisms (139,140). In addition, mechanical ventilation leads to decreased respiratory barrier function and alters the inflammatory response even further (141,142). Although we did not provide the solution to bypass this delicate balance between innate immune activation and paralysis, we have shown new details concerning the pathophysiological processes leading to organ failure. Initial excessive inflammation leads to ARDS, ongoing inflammation, immune exhaustion and sepsis. By limiting the initial excessive inflammatory response, the entire cascade will be attenuated. In order to prevent late phase organ failure it should be assured the innate immune system of the patient can sufficiently handle a new threat. According to the hypothesis

described above, the initial excessive inflammatory response should be minimized in order to limit late phase immune paralysis (Figure 2). When pulmonary leukocytosis and ARDS develops, future treatment strategies should be aimed at stimulation of the recirculation process rather than blocking of homing. Immune modulation should be aimed at inducing sufficient myelopoiesis after 7 days in patients that show initial excessive activation of the innate immune system (143-147). Again, identification of suitable patients is essential.

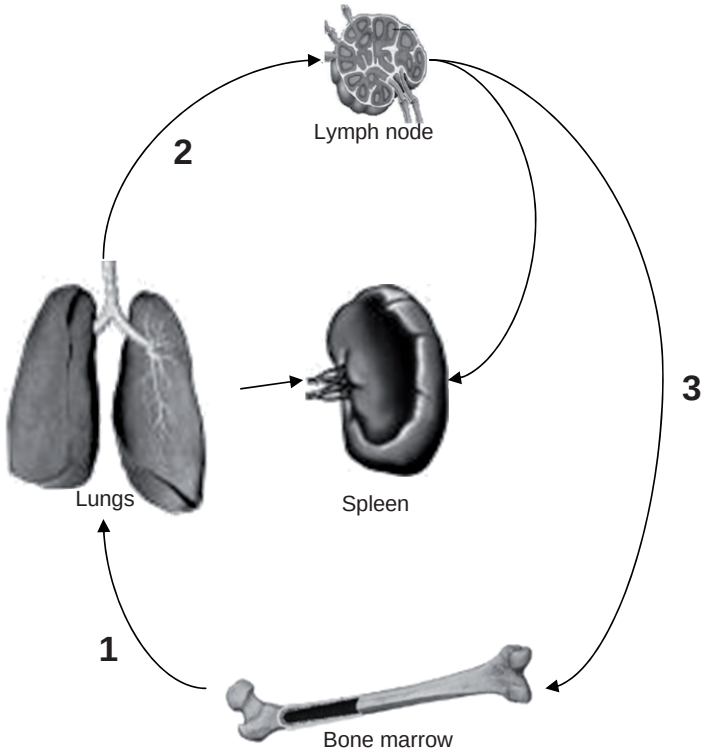

Figure 2. Possibilities for intervention by immune modulation
After trauma, massive release of fresh PMNs from the bone marrow is induced to compensate the massive homing and extravasation of circulating PMNs. This phase of extravasation and bone marrow release determines the development of early phase organ failure, innate immune exhaustion and subsequent late phase septic shock. Blocking or modulating PMN extravasation might reduce the incidence of both early and late phase organ failure (**1**). When the extravasation has already occurred, the process of recirculation should be stimulated. Clearing the tissues from the potentially dangerous PMNs could aid in the reduction of secondary organ damage. In addition, it is tempting to speculate that PMNs re-circulating to the lymph nodes may fulfill their immune-modulatory role and inhibit the excessive inflammatory response (**2**). Finally, re-circulation of PMNs might reduce the need for bone marrow release of young PMNs as well as the stimulation of bone marrow granulopoeisis (**3**).

Alternatively, therapeutic strategies for the prevention of septic shock could be aimed at the monocyte population. However, we question the use of monocyte HLA-DR restoration drugs, as the absolute number of HLA-DR positive monocytes is only limited affected. Treatment strategies, such as application of IFN-y or hemofiltration, were designed to normalize HLA-DR expression on circulating monocytes and were successful in this respect (118,148). However, the clinical effect of this seemingly improved immunological function was not further detailed. The interventions appeared beneficial, but large scale therapeutic trials have not been conducted to draw firm conclusions. In addition, caution should be taken as IFN-γ leads to activation of monocytes and in **chapter 7** we demonstrated monocyte activation was related to the development of ARDS.

Both trauma and subsequent surgery are well documented risk factors for the development of ARDS. It was hypothesized that surgery amplifies the inflammatory burden after trauma (**general introduction**). However, in our study surgery did not seem to add significantly to the inflammatory burden, as determined by the PMN responsiveness towards fMLP. It could be argued that the presented studies were performed on relatively small patient groups and additional cellular innate immune activation could have been missed with the measurements used. However, conventional measurements with MAC-1 did not demonstrate increased activation and in **chapter 5** PMNs were measured with a more sensitive method focused on analysis of inside out control of FcR's. Surgery might in fact induce factors which damage or activate the endothelium, which would act as risk factors for enhanced homing and activation of innate immune cells. Although the results might be considered circumstantial evidence, the suggestion is supported by several reports in literature. Unfortunately, no adequate marker to quantify the extent of endothelial damage or activation is currently available to further analyze this hypothesis (149).

Based on the only limited contribution of surgery to the inflammatory response, we suggest that surgery is safe in patients with a limited inflammatory response: *inflammation control surgery*. This has a direct impact on the current debate on non-discriminative ETC or DCO protocols all over the world (4,150-152). Adequate quantification of the cellular innate immune response would allow correct allocation of patients to the different treatment regimes.

Supplementary to the treatment strategies for the prevention of early phase organ failure, several treatment regimes have attempted to limit late phase complications. The “Surviving Sepsis Campaign” has been organized to improve outcome of septic patients (153). An essential challenge in the “Surviving Sepsis Campaign” is the early identification of patients with sepsis or patients at risk for sepsis (8,154). Immediate application of treatment has shown to reduce mortality and morbidity, which has been implemented in the “early goal directed therapy”. Therapeutic measures are mostly aimed at limiting the inflammatory response, such as the use of corticosteroids or activated protein C (9,155). However, the results of these studies are still the subject of debate as some therapeutic measures proved detrimental. Part of this adverse outcome might be attributed to the allocation of patients to specific treatment strategies. Identification of high risk patients eight days prior to the onset of symptoms, would greatly aid in prevention of this clinical condition. The analysis of fMLP induced active FcγRII provides a new pathophysiological insight, allowing the development of prognostic tools which might predict the development of septic shock 7 days in advance.

FMLP induced active FcγRII on PMNs on admission is related with severe complications. However, the studies presented in this thesis were developed to investigate the pathophysiology of acute inflammation after trauma leading to organ failure. In order to use this parameters as a prognostic value a large prospective cohort study is required (156). In addition, a future prediction rule for these complications based on PMN and monocyte phenotype changes is likely to be improved by combining these factors with patient characteristics such as location of trauma or age. Combining several PMN markers (with or without stimulation by fMLP) would provide additional power of such a prediction rule. Currently, the prognostic value of the parameter (fMLP induced active FcγRII) is evaluated in a large prospective international cohort series.

In addition to the determination of the prognostic value of the described PMN characteristics, it is currently investigated if there is a close correlation between PMN phenotype changes and current standard clinical laboratory investigations. Analysis of the PMN phenotype is still slow and time consuming (approximately 1 hour), time which is not available in the acute phase after trauma (157,158). Leukocyte analysis in standard clinical care can be performed in minutes and can provide a

tremendous amount of information. If fMLP induced active FcγRII on PMNs proves a valuable prognostic factor, it would aid tremendously if a surrogate parameter is readily available in the current clinical setting.

In conclusion, the research described in this thesis provides new insight in the pathophysiological processes that leads to organ failure after trauma. With this new information, tools can be developed to improve treatment allocation for patients at risk and new ground for therapeutic interventions has been created. By limiting the excessive inflammatory response after trauma, the incidence of both early and late phase organ failure could be reduced. Thereby, mortality rates, morbidity rates and annual health care costs can be reduced tremendously.

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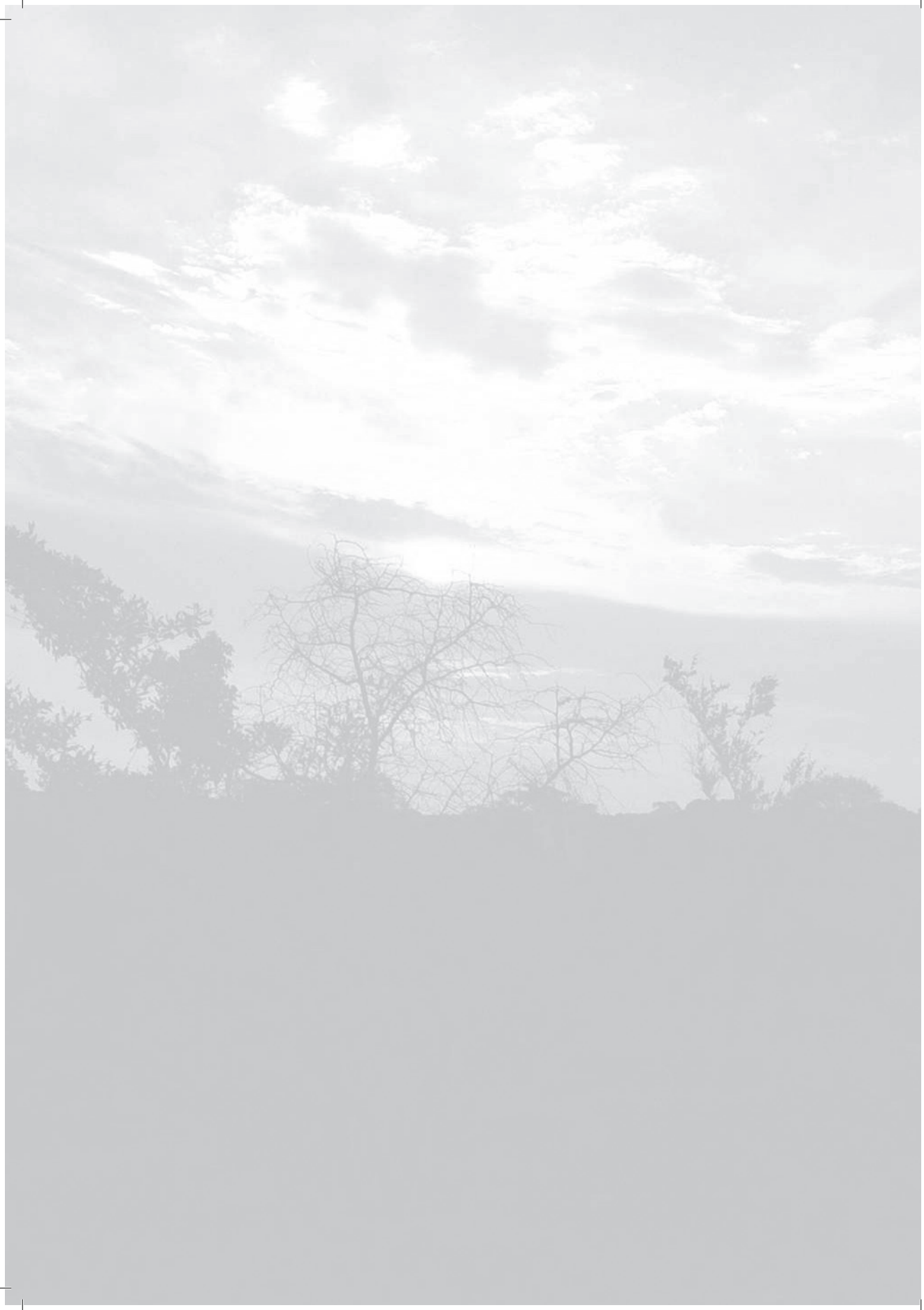


Chapter 11

Samenvatting in het Nederlands

(Summary in Dutch)

KERN: IMMUUN SYSTEEM PUT ZICHZELF UIT NA TRAUMA

Bij patiënten met ernstig letsel treedt een overmatige activatie van het immuun systeem op. Leukocyten (witte bloedcellen) gaan dan naar de organen en veroorzaken extra schade, waardoor orgaanfalen kan optreden. Tijdens deze activatie worden leukocyten massaal verbruikt en is de productie van verse witte bloedcellen door het beenmerg onvoldoende om een adequaat aantal cellen in het bloed te houden. Als gevolg hiervan komen oude, gebruikte leukocyten terug in het bloed, waardoor patiënten extra vatbaar zijn voor ernstige infecties.

PROBLEEMSTELLING: MAATSCHAPPIJ

Wereldwijd is trauma de belangrijkste doodsoorzaak en in de Westerse wereld de belangrijkste oorzaak van invaliditeit en verlies van arbeidsjaren bij personen onder de 50 jaar. Patiënten kunnen direct overlijden aan de uitgebreidheid van het letsel (bijvoorbeeld verbloeding of hersenletsel), maar een belangrijk deel (50%) van de mortaliteit ontstaat in de fase na de resuscitatie (initiële opvang). In deze fase kunnen organen uitvallen die niet noodzakelijkerwijs zijn aangedaan door het primaire letsel, ook wel (meervoudig) orgaanfalen genoemd. Orgaanfalen komt voornamelijk voor bij patiënten die een stomp trauma hebben ondergaan (in tegenstelling tot de steek en schotverwondingen). In Nederland betreft dit meestal verkeersslachtoffers en ongevallen in de bouw. Hoewel het aantal verkeersdoden de laatste jaren is afgenomen, blijft het aantal ziekenhuisgewonden vrijwel gelijk. Patiënten overlijden tegenwoordig minder vaak aan orgaanfalen na trauma, maar de incidentie (voorkomen) van deze ernstige complicatie is niet significant afgenomen. Patiënten die orgaanfalen ontwikkelen kunnen met de huidige medische kennis beter worden behandeld voor hun symptomen. Ruim 15% van de opgenomen patiënten met ernstig trauma ontwikkelt enkel of meervoudig orgaanfalen. Deze patiënten hebben vaak langdurig mechanische beademing en intensive care behandeling nodig. Hierdoor gebruiken patiënten met orgaanfalen jaarlijks 1,7% van het Nederlandse zorgbudget (170 miljoen euro).

PROBLEEMSTELLING: OORZAAK VAN ORGAANFALEN

Hoe meervoudig orgaanfalen na trauma ontstaat, is nog altijd niet volledig bekend. Initiële studies lieten zien dat het immuun systeem betrokken is bij deze aandoening. Bij obductie van patiënten die overleden waren aan orgaanfalen werden grote hoeveelheden witte bloedcellen (leukocyten) gevonden in de organen die faalden. Opvallend was dat dit ook vaak het geval was in organen zonder duidelijke infectiebron. Verdere studies toonden aan dat orgaanfalen voorkomt in 2 fasen. De verdeling van orgaanfalen over deze 2 fasen is ieder 50%. De vroege fase (tot 4 dagen na trauma) gaat vrijwel nooit gepaard met een infectie, terwijl de late fase (vanaf 8 dagen na trauma) vrijwel altijd voorafgegaan wordt door een infectie. Op basis van deze studies werd gesteld dat de vroege fase van orgaanfalen het gevolg is van een buitensporige ontstekingsreactie (excessieve inflammatie) op de weefselschade die door het trauma is geïnduceerd. Symptomen van patiënten die deze reactie hebben worden samengevat in het systemische inflammatoire respons syndroom (SIRS). Tijdens dit syndroom is de long het orgaan dat het meest frequent faalt, dit wordt het acuut respiratoir distres syndroom genoemd (ARDS). ARDS wordt veroorzaakt door overmatige activatie van neutrofielen (i.e. PMNs), cellen van het immuunsysteem die normaal gesproken bacteriën en dood weefsel opruimen. Deze cellen produceren stoffen die de long kunnen beschadigen. Door de reactie van het lichaam op het letsel worden grote hoeveelheden neutrofielen geactiveerd die massaal in de longen vastlopen en dit orgaan vervolgens beschadigen.

De huidige theorie is dat bij gezonde mensen neutrofielen aangemaakt worden in het beenmerg en 12-24 uur in het bloed zijn voor immuun surveillance. Zodra deze cellen het juiste signaal krijgen treden ze uit naar de weefsels om daar hun werk te doen: fagocyteren (opeten en vernietigen) van necrose (dood weefsel) en bacteriën. Als hun werk is voltooid gaan de neutrofielen in apoptose (gereguleerde celdood) en worden ze door weefselmacrofagen (gedifferentieerde monocyten) opgeruimd.

In de late fase is er juist sprake van een te slecht werkend immuunsysteem, waardoor bacteriën vrij spel krijgen. Deze situatie wordt het compensatoire anti-inflammatoire respons syndroom (CARS) genoemd. Deze naam omvat de huidige theorie, waarin het ontstaan van CARS gezien wordt als een actief proces. Een overmatige ontstekingsreactie na trauma zou een compensatie mechanisme uitlokken, wat op zichzelf weer door kan slaan en zo een inactief immuunsysteem kan veroorzaken.

De monocyte wordt gezien als een belangrijke witte bloedcel in deze fase. Monocyten zijn ongedifferentieerde cellen, wat betekent dat ze nog van vorm en functie kunnen veranderen in de loop van hun leven. De hoofdtaak van monocyten is het fagocyteren van bacteriën en dode cellen. Daarnaast vormen monocyten een brug met het aangepaste immuun systeem. Monocyten maken, zoals alle leukocyten, gebruik van specifieke eiwitten op hun celoppervlak (receptoren), om te communiceren met andere cellen. Zo kunnen monocyten met HLA-DR receptoren specifieke antigenen (bijvoorbeeld stukjes bacterie) presenteren aan lymfocyten en zo deze cellen instrueren. Lymfocyten zijn witte bloedcellen die van belang zijn voor immuniteit tegen bacteriën en virussen. Doordat de monocyten direct invloed op deze lymfocyten uit kunnen oefenen kunnen ze als het ware het immuunsysteem sturen.

Meervoudig orgaanfalen tijdens SIRS (vroege fase) wordt dus gezien als een passieve reactie op het letsel. Door dit excessieve proces ontstaat additionele schade aan de organen door het eigen immuunsysteem. Meervoudig orgaanfalen tijdens CARS ontstaat door een actief proces. In reactie op de excessieve inflammatoire respons treedt down-regulatie van het immuunsysteem op. Belangrijke immunologische cellen (o.a. monocyten) functioneren hierdoor niet meer optimaal.

BEHANDELING VAN ORGAANFALEN

De huidige therapieën om orgaanfalen na trauma te behandelen zijn gebaseerd op bovenstaande theorieën over de ontstaanswijze van het orgaanfalen en de levenscyclus van neutrofielen en monocyten. Van oudsher werd de ernstig gewonde traumapatiënt te “ziek” geacht om uitgebreide chirurgische interventies te ondergaan. Dit leidde vaak tot complicaties, zoals een longembolie of infectie. Door de vooruitgang op het gebied van anesthesie werd het vanaf de jaren 80 mogelijk uitgebreide interventies toe te passen. Volgens deze uitgebreide strategie ondergaat een patiënt met bijvoorbeeld meerdere fracturen direct operatieve fixatie van al zijn/haar fracturen. Dit beleid van volledige directe behandeling in de acute fase is effectief gebleken. Het leidt gemiddeld tot afname van de genoemde complicaties en sneller herstel. Echter, een deel van de patiënten bleef ernstige complicaties, zoals orgaanfalen, ontwikkelen. Na een analyse van de Duitse ongevallen database,

bleek dat patiënten met een ernstig letsel een slechte prognose hebben bij de directe volledige strategie. Er werd gesteld dat een operatie als additioneel trauma fungeert bij deze patiënten die al een excessieve inflammatoire reactie hebben en zo bijdraagt aan het ontstaan van orgaanfalen. Minimale operatieve belasting of uitstel van operaties heeft bij deze patiënten de voorkeur. Echter, dit beleid leidt tot een slechter lokaal resultaat met meer infecties en slechte genezing van de fracturen. Bovendien wordt de identificatie van de geschikte patiënt voor de twee uiteenlopende behandelingsstrategieën bemoeilijkt door de beperkte kennis van de pathofysiologie (ontstaanswijze) van orgaanfalen na trauma. Samenvattend is er voor vroege fase orgaanfalen na letsel nog geen effectieve preventie of therapie voor handen.

Ook voor orgaanfalen tijdens CARS (late fase) is nog geen effectieve therapie voor handen. Door de “Surviving Sepsis Campaign” worden patiënten met sepsis (bloedvergiftiging) eerder herkend en behandeld op de intensive care, maar het ontstaan van sepsis wordt niet voorkomen. Experimentele therapieën zijn o.a. het up-reguleren van HLA-DR op monocyten en het remmen van neutrofielen om extra schade aan de organen te voorkomen. Ondanks adequate up-regulatie van HLA-DR op monocyten is het klinische effect hiervan nog niet aangetoond. Het remmen van neutrofielen tijdens sepsis lijkt eerder averechts te werken, er treden meer ernstige infecties op. Meer kennis van de pathofysiologie (ontstaanswijze) van orgaanfalen is dus nodig om hoogrisico patiënten te identificeren en orgaanfalen tijdens SIRS en CARS beter te behandelen.

STUDIE OPZET

Voor de ontwikkeling van nieuwe preventieve en therapeutische strategieën is eerst meer kennis van de onderliggende processen nodig. Zoals hierboven uitgelegd spelen neutrofielen en monocyten een belangrijke rol in zowel de vroege als late fase van orgaanfalen na letsel. Het onderzoek heeft zich dan ook voornamelijk op deze cellen van het immuunsysteem gericht.

In eerste instantie moet de juiste analyse methode voor beide soorten cellen gekozen worden, zodat de veranderingen die optreden na trauma in kaart kunnen

worden gebracht. Met de gekozen methode kunnen vervolgens de veranderingen van de neutrofielen en monocyten in relatie tot het ontstaan van orgaanfalen worden onderzocht. Daarnaast kan de invloed van chirurgie worden geanalyseerd. Tot slot kan door neutrofielen en monocyten in de tijd te vervolgen het dynamische proces dat leidt tot late fase orgaanfalen worden onderzocht.

ANALYSE VAN NEUTROFIELEN

Bovenstaande hypotheses en therapieën zijn gebaseerd op analyses van cytokinen (hormonen van het immuunsysteem). Echter, de cellen, neutrofielen en monocyten, zijn de uitvoerende factoren in het ontstaan van orgaanfalen. Analyse van deze cellen kan meer inzicht verschaffen in de ontstaanswijze van deze ernstige complicatie. Neutrofielen zijn slechts beperkt onderwerp van onderzoek geweest, maar zijn wel de belangrijkste cel in vroege fase orgaanfalen. De activiteit van neutrofielen kan op meerdere manieren onderzocht worden. Ten eerste kan de productie van schadelijke stoffen (ROS) kan gemeten worden. Echter, hiervoor moeten cellen geïsoleerd worden wat de betrouwbaarheid van de test beïnvloed. Ten tweede kan het receptorprofiel van de cel kan bepaald worden, maar dit laat vaak een grote variatie zien en is meestal niet erg gevoelig. Echte veranderingen treden pas op als een patiënt ernstig ziek is. Ten slotte kan de inside-out controle van neutrofielen kan geanalyseerd worden. Hierbij wordt onderzocht in hoeverre een cel zijn fenotype (totaal aan eiwitten op het oppervlak van de cel) kan reguleren in reactie op bepaalde signalen. Dit kan een erg sensitieve maat zijn en cellen hoeven niet geïsoleerd te worden. Wij hebben dan ook neutrofielen onderzocht door het functionele fenotype te meten.

Volgens de huidige theorie worden neutrofielen geactiveerd door trauma, treden deze cellen massaal uit in de long en veroorzaken daar extra schade. Er zal dus sprake zijn van een toename in het functionele fenotype. Echter, in **hoofdstuk 3** wordt aangetoond dat het functionele fenotype na trauma juist verlaagd is. Er is een lagere up-regulatie van FcγRII na stimulatie met fMLP bij traumapatiënten dan bij gezonde controle personen. De afname in functioneel fenotype was wel gerelateerd aan een toename in functioneel fenotype van neutrofielen in de longen van deze patiënten. In een vervolg studie wordt in **hoofdstuk 4** gedemonstreerd dat de mate

van afname in functioneel fenotype van neutrofielen in het bloed gerelateerd is aan de ernst van de complicaties in de longen. Er is dus een verband tussen wat er met de cellen in de weefsels gebeurt en wat er aan de cellen in het bloed gemeten kan worden.

INVLOED VAN CHIRURGIE

Chirurgie wordt gezien als risicofactor voor het ontstaan van orgaanfalen, doordat chirurgie de excessieve inflammatoire reactie nog verder opjaagt. We anticipeerden dan ook een verdere afname in het functionele fenotype tijdens het plaatsen van een femur (bovenbeen) pen, een operatie waarvan bekend is dat deze een hoog risico geeft op het ontstaan van ARDS. In **hoofdstuk 5** laten we zien dat er inderdaad een toename is van cytokinen en factoren die het endotheel (binnenwand van de vaten) kunnen activeren of beschadigen. Echter, een afname in het functionele fenotype van neutrofielen werd niet gezien tijdens of na de operatieve ingreep. Dit suggereert dat er geen extra invloed is van chirurgie op neutrofielen, maar wel op andere factoren voor het ontstaan van ARDS.

FUNCTIONEEL FENOTYPE EN SEPSIS

Neutrofielen laten na trauma een afname zien in het functionele fenotype. Er werd als hypothese gesteld dat deze afname in functie van neutrofielen gerelateerd zou kunnen zijn aan het ontstaan van CARS en daarmee ernstige infecties en sepsis. Hiervoor werd het functionele fenotype van neutrofielen bij ernstig gewonde traumapatiënten gedurende 2 weken geanalyseerd. In **hoofdstuk 6** wordt aangetoond dat er na de eerste dag na letsel een langzame afname is in het functionele fenotype en dat deze afname meer uitgesproken is bij patiënten die uiteindelijke ernstige sepsis ontwikkelen. De afname duurt tot dag 7, sepsis begon bij alle patiënten tussen dag 8 – 10. Direct na trauma werd al een meer uitgesproken afname gezien in het functionele fenotype van patiënten die uiteindelijk sepsis ontwikkelen. Een directe relatie tussen de mate van afname in neutrofiel functioneel fenotype en het ontstaan van sepsis werd gevonden, maar wellicht belangrijker is de bevinding dat de initiële afname gerelateerd is aan het ontstaan van sepsis 7 dagen later.

MONOCYTEN NA TRAUMA

Na trauma is er sprake van een afname in HLA-DR op monocyten, een afname die in de literatuur al gerelateerd werd aan het ontstaan van sepsis. In aanvulling hierop wordt in **hoofdstuk 7** aangetoond dat deze afname in HLA-DR gerelateerd is aan het ontstaan van ARDS. Ook het functioneel fenotype van monocyten was verlaagd bij patiënten die ARDS ontwikkelden. In patiënten die geen ARDS ontwikkelden had chirurgie wel invloed op de HLA-DR expressie van monocyten. Een verdere reductie werd gezien 18 uur na de operatie. Opvallend was dat de afname in HLA-DR voornamelijk toe te schrijven was aan een toename van HLA-DR negatieve cellen en niet zozeer een afname van HLA-DR positieve monocyten. In **hoofdstuk 8** wordt gedemonstreerd dat niet actieve down-regulatie door cytokinen (bijvoorbeeld IL-10) de oorzaak is voor een verlaagde HLA-DR, maar dat het grootste deel van reductie het gevolg is van redistributie van monocyten. In patiënten die sepsis ontwikkelen lijkt de monocyten populatie uitgeput te zijn. Er vindt geen toename in het aantal monocyten plaats tijdens de herstel fase in de tweede week na trauma.

UITPUTTING VAN NEUTROFIELEN NA TRAUMA

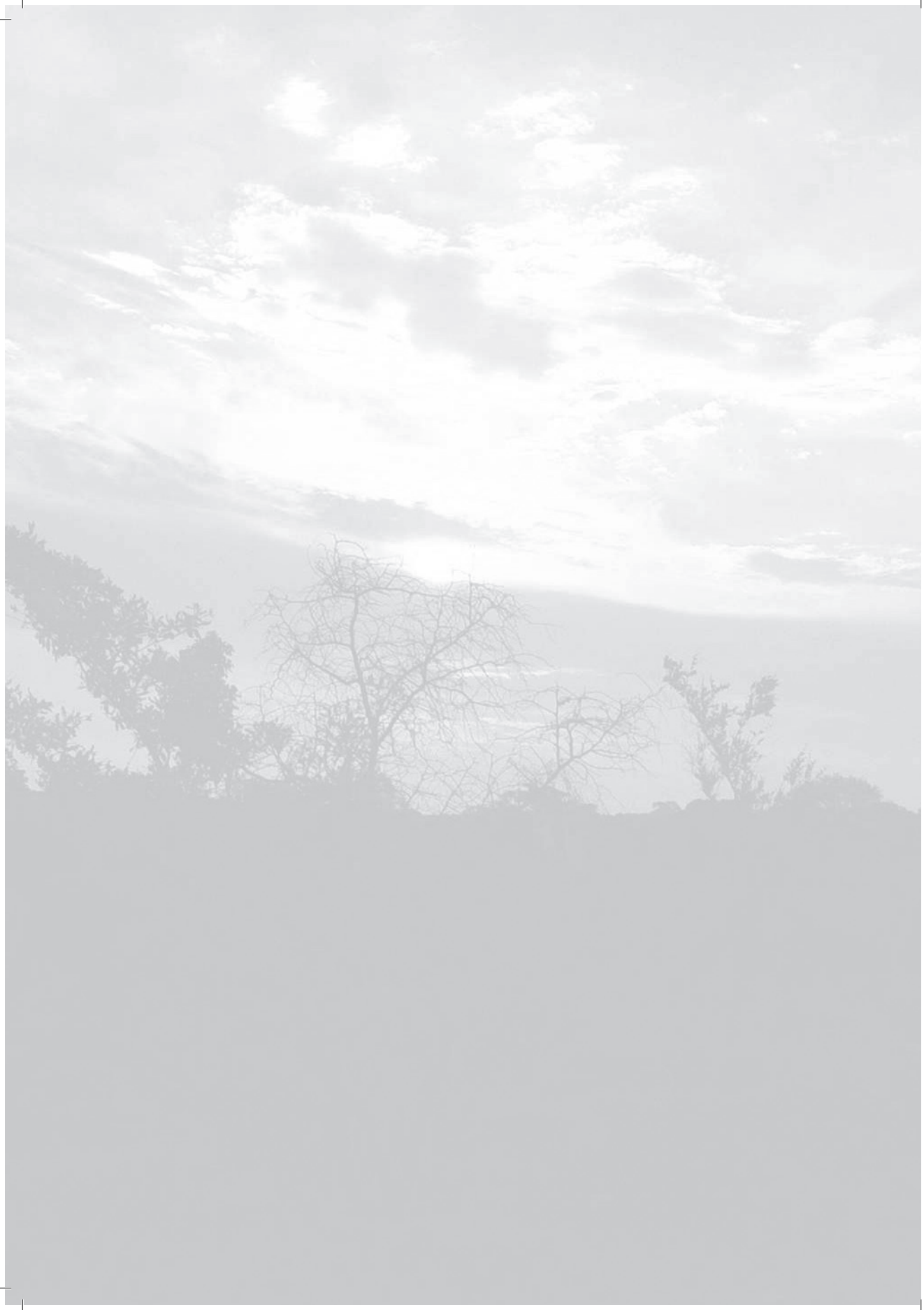
Doordat de monocytenpopulatie van traumapatienten uitgeput lijkt te zijn, vroegen wij ons af of dit bij de neutrofielenpopulatie van septische patiënten ook het geval kon zijn. Een langzame uitputting zou de geleidelijke afname in fenotype kunnen verklaren. In **hoofdstuk 9** demonstreren we dat er meerdere soorten neutrofielen na trauma in het bloed voorkomen. Initieel na letsel komen er jonge (banded) neutrofielen in het bloed. Bij excessieve inflammatie komen er zelfs (meta)myelocyten in de circulatie terecht. Dit zijn premature cellen die eigenlijk nog verder zouden moeten uitrijpen in het beenmerg. Cellen met die specifieke uiterlijke kenmerken worden normaal gesproken alleen bij mensen met leukemie gezien. In tegenstelling tot leukemie verdwijnen deze cellen bij traumapatiënten vanzelf weer. Het beenmerg heeft ongeveer 7 dagen nodig om nieuwe neutrofielen te maken. Door de hoge vraag zijn de te jonge cellen eerder het bloed ingestuurd. Na een paar dagen verschijnen geleidelijk aan toxische neutrofielen in het bloed. Dit zijn neutrofielen met toxische korreling, gaten in het cellichaam en gefagocyteerde bacteriën. Ze hebben dus het uiterlijk van gebruikte, oude cellen. In **hoofdstuk 9** wordt aannemelijk gemaakt

dat deze toxische cellen uit de weefsels komen; bij gebrek aan verse cellen uit het beenmerg. Dit proces van uitputting van de neutrofielen populatie is te meten met specifieke eigenschappen van deze cellen.

Deze geleidelijke uitputting in 7 dagen komt overeen met het ontstaan van complicaties bij de patiënt op de intensive care. Sepsis ontstaat in de door ons onderzochte populatie vrijwel altijd na 8 – 10 dagen. De uitputting gaat dus vooraf aan sepsis. Daarnaast duurt ook de langzame afname in functioneel fenotype van neutrofielen ongeveer 7 dagen. Hierdoor wordt de theorie dat CARS het gevolg is van actieve down-regulatie door cytokinen aangetast. De resultaten gepresenteerd in dit proefschrift suggereren een fysiologisch proces dat alleen optreedt onder extreme omstandigheden.

CONCLUSIES

Trauma leidt tot buitensporige inflammatie. Tijdens dit proces treden neutrofielen uit naar de weefsels. Hierbij kan ARDS ontstaan. De achtergebleven cellen in het bloed hebben een verlaagd functioneel fenotype. De mate van afname in het bloed is gerelateerd aan de schadelijke processen in de weefsels. Voorschrijdende inflammatie leidt tot uitputting van het immuunsysteem. Neutrofielen en monocyten reageren dan niet meer adequaat op een infectie. Hierbij kan sepsis ontstaan. De initiële immuun respons bepaalt dus het verdere beloop. Ter voorkoming van orgaanfalen dient de initiële respons te worden geremd en de redistributie van monocyten gemoduleerd.



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CURRICULUM VITAE

Falco Hietbrink was born on March 7, 1981 in Ede, The Netherlands. In June 1999 he graduated from the Pallas Athene College in Ede. After 3 months pharmaceutical sciences he enrolled in Medical School at the University of Utrecht just before 2000. In 2003 he performed his first prospective clinical study during an elective course at the department of Surgery and Respiratory Medicine under supervision of his later promotors prof. dr. L.P.H. Leenen and prof. dr. L. Koenderman. In 2004 – 2005 he participated and performed in several studies concerning gastro-intestinal permeability and the development of sepsis, under supervision of prof. dr. L.M.A. Akkermans and prof. dr. H.G. Gooszen. For an elective in trauma surgery and emergency medicine he arranged an internship at the GF Jooste Hospital and Groote Schuur Hospital in Cape Town in 2004.

After graduating from medical school in August 2005, he committed to full time research on the development of organ failure after major trauma as a PhD-student, resulting in this thesis. He contributed to the founding and development of a dedicated research group, which has the capabilities of performing translational research on the inflammatory response after injury.

Based on the results described in this thesis, additional etiological, prognostic and therapeutic studies were developed, in which he is still involved. For several of these studies he sought international collaboration with the Tygerberg Hospital, University of Stellenbosch South Africa.

With pleasure he started his surgical training in January 2008 at the University Medical Center Utrecht, Utrecht, The Netherlands (prof. dr. I.H.M. Borel Rinkes). This program will be completed in 2013 at the Department of Surgery, Twee Steden Ziekenhuis, Tilburg, The Netherlands (dr. S.E. Kranendonk).

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