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Impaired interferon-γ production and associated interleukin-12 signalling of CD56^{bright} Natural killer cells following severe trauma

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Introduction

This study intends to give new insight into aspects of postinjury immunosuppression and the underlying pathophysiology. This introduction defines severe trauma, explains aspects of immunoregulation in the post-traumatic phase, and focuses on relevant cellular and soluble components needed for an adequate immune response upon pathogen challenge.

1.1 Severe injury

1.1.1 Definition of the term Polytrauma and the Injury Severity Score

The term Polytrauma is widely used to describe patients with severe multiple injuries and is associated with high risk for morbidity and mortality. In 1984 Tscherne et al. defined the Polytrauma as a simultaneous occurrence of injuries to multiple physical regions or organ system, where at least one or the combination of several injuries is life threatening (Tscherne et al., 1984). Even before that Baker et al. introduced the Injury Severity Score (ISS) in 1974 which allows categorizing and comparing patients with multiple injuries (Baker et al., 1974). An ISS score of 16 and higher was found to identify patients who are at high risk of trauma related death. Accordingly, these patients should be defined as major trauma and be treated at a designated trauma center (Long et al., 1986). In 2001 the Guideline Committee of the German Registered Society of Trauma Surgery published their recommended guidelines for diagnostics and therapy in trauma surgery. They defined the Polytrauma as: "Injury to several physical regions or organ systems, where at least one injury or the combination of several injuries is life threatening, with the severity of injury being ≥ 16 on the scale of the Injury Severity Score" (Guideline Committee of the German Registered Society for Trauma Surgery, in collaboration with The Polytrauma Association of the German Registered Society for Trauma Surgery, 2001).

The ISS is a solely anatomical scoring system and is based on the Abbreviated Injury Score (AIS). The AIS was introduced by the Committee on Medical Aspects of Automotive Safety in 1971 and has been revised several times after its introduction (Committee on Medical Aspects of Automotive Safety, 1971). The AIS classifies each injury to the body from mild to non-survivable. For the calculation of the ISS the AIS scores are allocated to body regions defined in the ISS.

The ISS correlates very well with the survival of patients. In cases of multiple injuries, death rates increase with injuries in additional body regions even if these injuries themselves are not life threatening (Baker et al., 1974).

Since the ISS only considers the worst injury to a certain body region it may underscore patients with multiple severe injuries confined to the same body region. Osler, Baker and Long addressed this issue in 1997 by introducing the New Injury Severity Score (NISS). They eliminated the anatomical body regions and just considered the most severe injuries represented by the highest AIS scores. They found the NISS easier to calculate and a significantly better predictor of survival. The authors concluded that the NISS should replace the ISS in trauma scoring (Osler et al., 1997).

1.1.2 Epidemiology of Polytrauma

Most recent data from the World Health Organization (WHO) show that injuries account for almost 10 % of deaths worldwide with the age group from 15 to 49 years being at the highest risk for injury related death. Injuries are by far the most common cause of death in the 15 to 29-year-old age group with over 50 % of deaths related to injury. With 67 % of all death caused by injuries, men are more often subject to injury related death than women. Road injuries, before falls, are by far the most common cause of death among the group of injuries. Overall, they are among the top ten leading causes of death globally (WHO [2], 2018).

Looking at disability adjusted live years (DALY) as a measure of the overall disease burden, the WHO data demonstrates that even though more than three times as many people die from cardiovascular disease than as a consequence of injury, in terms of DALY the effect of cardiovascular disease is only slightly bigger than that of injury (WHO [1], 2018). This data especially emphasizes the tremendous impact that injury as a disease of the young, has on the overall disease burden of society.

In contrast to the data for the worldwide population, data for Europe show a major shift in death due to injury towards higher ages. The majority of deaths due to injury occurs in the group of 70 years and above. In elderly people falls replace road injury as the most common mechanism leading to injury and the number of women dying as a consequence of injury exceeds the number of men (WHO [2], 2018). Regarding the overall disease burden though, injury in younger age groups still is the main contributor to DALY (WHO [1], 2018).

In 1983 the analysis of 425 trauma fatalities led Trunkey et al. to the introduction of a trimodal concept of trauma related death. The distribution describes three distinct peaks that represent immediate, early and late death. Immediate death on the scene or within the first hour is primarily caused by injury to the central nervous system or the cardiovascular system leading to severe haemorrhage. Immediate death accounts for 45 % of fatalities. The second peak describes death within 1 to 4 hours after trauma, which accounts for 34 % of all deaths and has a similar pattern of injury as immediate death. Further 20 % of trauma related deaths occur more than one week after injury. These are mainly due to infection and multiple organ failure (MOF) (Trunkey, 1983).

Due to the introduction of many novel strategies in trauma treatment such as the Advanced Trauma Life Support (ATLS), specialised trauma centers as well as better diagnostic and therapeutic options, the mortality due to trauma was reduced within the last decades (Nast-Kolb et al., 2001). More recent studies did not confirm that trimodal distribution postulated by Trunkey et al. but rather found a steady decline of death over time (de Knegt et al., 2008; Lansink et al., 2013).

Nevertheless, death due to infection, sepsis and MOF becomes a relevant contributor to overall death at the end of the first week. Lansink et al. report pneumonia / respiratory insufficiency to be to be the third most common cause of overall trauma death. Pneumonia / respiratory insufficiency and MOF combined account for more than one third of all late fatalities (Lansink et al., 2013).

Even though mortality related to MOF decreased, it is still a major complication in the treatment of trauma patients since the reduced mortality cannot be attributed to a lower incidence but rather to improved treatment strategies (Ciesla and Moore, 2005; Nast-Kolb et al., 2001). The incidence of MOF in trauma patients ranges from 15 to 25 % (Ciesla and Moore, 2005; Dewar et al., 2013). Reduction in MOF incidence and related mortality can be achieved by further reducing the incidence of infection and sepsis, which are major contributors to the development of MOF (Cohen, 2002; Dewar et al., 2009).

Even though a constant decline in trauma-related death and sepsis was demonstrated, no decline in mortality of patients with manifest sepsis was seen. Sepsis and MOF are associated with longer intensive care unit (ICU) and hospital stays as well as an increased mortality. Wafaisade et al. report increased rates of single organ failure (SOF) and MOF in septic compared with non-septic trauma patients. SOF was observed in 83 % of septic versus 35 % of non-septic patients while MOF developed in 63 % of septic versus 17 % in non-septic patients (Wafaisade et al., 2011).

Hoover et al. report an incidence of 45 % for nosocomial infection in their cohort of trauma patients with a mean time to onset of infection of 8 days. Infections of the respiratory system followed by genitourinary infections were the most common sites of infection (Hoover et al., 2006). A multi center study from Europe conducted in 2002 showed *Staphylococcus aureus* (*S. aureus*) to be the most common organism isolated in septic patients followed by *Pseudomonas species* and *Escherichia coli* (Vincent et al., 2006).

1.2 Pathophysiology of Polytrauma

Severe trauma causes tremendous injury to soft tissue, the skeletal system and organs. Those who survive the initial insult are at great risk to develop secondary immunological dysfunction, increasing the risk for infectious complications.

1.2.1 Systemic inflammatory response syndrome

In 1992 the American College of Chest Physicians / Society of Critical Care Medicine Consensus Conference introduced the term systemic inflammatory response syndrome (SIRS) to describe an inflammatory process, independent of its cause (Bone et al., 1992). Researchers found that MOF could be triggered by inflammation in the absence of infection and hypothesised that MOF is caused by the massive release of inflammatory mediators rather than by bacteria (Goris et al., 1985).

In 1994 Matzinger introduced the concept of damage associated molecular patterns (DAMPs). She proposed that the immune response was stimulated by endogenous danger signals originating from cells exposed to pathogens, toxins or mechanical damage rather than by foreign components (Matzinger, 1994).

Independent of whether immune cells are activated by endogenous or microbial ligands, SIRS is characterised by a severe inflammatory response. Cytokines like tumor necrosis factor (TNF)-α interleukin (IL)-1β, IL-6, IL-8, IL-12, IL-18, granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) mediate that pro-inflammatory response aiming to clear pathogens and restore tissue integrity. Physiological effects of those cytokines include the activation of intracellular signalling pathways that mediate an activation and recruitment of immune cells, an increased expression of adhesion molecules as well as increased permeability of endothelial cells promoting immune cell migration into tissue (Keel and Trentz, 2005; Lenz et al., 2007). This physiological reaction is associated with tremendous stress for local tissue. It can

eventually lead to multiple organ dysfunction or failure via disseminated intravascular coagulation (DIC) as well as endothelial and parenchymal cell injury (Keel and Trentz, 2005). If compensatory mechanisms cannot restore homeostasis, persistent SIRS is the consequence. Clinically SIRS can be diagnosed when two of the following apply (Bone et al., 1992):

- Body temperature > 38 °C or < 36 °C
- Heart rate > 90 /min
- Respiratory rate > 20 /min or $PaCO_2$ < 32 mmHg
- White blood cell count > 12.000 /µl or < 4.000 /µl, or > 10 % immature leukocytes

1.2.2 Compensatory anti-inflammatory response syndrome

Introduced by Bone et al. in 1996 the term compensatory anti-inflammatory response syndrome (CARS) describes a strong anti-inflammatory response overweighing the pro-inflammatory mechanisms and thus being responsible for immunodeficiency following severe trauma (Bone, 1996).

Several cytokines like IL-4, IL-10, IL-13 and transforming growth factor (TGF)-β as well as receptor antagonists like soluble TNF receptors and IL-1 receptor antagonists have been shown to participate in the anti-inflammatory response. Originally CARS was considered to follow SIRS in a two-wave process. Further research revealed that pro- and anti-inflammatory processes occur at the same time but in different compartments of the body (Cavaillon et al., 2001; Munford and Pugin, 2001). Posttraumatic immunodepression is characterised by several changes in the innate and adaptive immune system as well as cytokine expression profiles that will be discussed in chapter 1.3.

1.2.3 Sepsis

The dysfunctional immune system after trauma predisposes to nosocomial infections and in severe cases to the development of sepsis (Bone, 1996). Sepsis was defined as a systemic inflammatory response to infection. Criteria for the systemic inflammatory response are the same as for SIRS defined in chapter 1.2.1. Infection is described as an inflammatory response towards a microorganism or the presence of those organisms in normally sterile tissue. If sepsis is complicated by organ dysfunction, hypotension or hypoperfusion it is termed “severe sepsis”. A septic shock is diagnosed if hypotension persists despite adequate fluid resuscitation and is accompanied by perfusion abnormalities (Bone et al., 1992).

The physiological response to invading organisms is similar to that seen in SIRS. Activation of the innate immune system by invading microorganisms leads to a cellular and humoral response creating a pro-inflammatory environment at first. In contrast it is not endogenous DAMPs that initiate the immune response, but rather foreign pathogen associated molecular patterns (PAMP) expressed by microbes (Cohen, 2002).

1.2.4 Multiple organ failure

As discussed in chapter 1.1.2 major infection / sepsis and MOF are important complications in the treatment of severely injured patients. MOF occurs in a bimodal distribution with early and late onset MOF (Moore et al., 1996). While early onset MOF is rather not associated or followed by infection / sepsis, late onset MOF is frequently associated with infection / sepsis. A more recent study showed a three to four-fold higher risk of lethal outcome when comparing the frequency of injured people dying from early MOF (7.5) versus late MOF (38.7 %) (Maier et al., 2007).

Taking the bimodal distribution and differences in outcome into account researchers argued that early and late MOF were two differing entities with distinct underlying pathophysiology. While early MOF is mainly due to haemorrhagic shock and resuscitation, late MOF is considered to develop in a two-step process. The first hit putting the immune system into a status of imbalance and susceptibility and a second hit resulting in uncontrolled inflammation. Possible second hits include mechanical ventilation, surgery, and sepsis (Moore et al., 2005).

1.3 The immune system

The immune system consists of cellular and soluble components. Complex interactions enable the organism to recognize and eliminate foreign pathogens as well as abnormal host cells. Initial immune defence is carried out by the innate immune system, which includes surface barriers, acute phase proteins, the complement system as well as cellular components and the mediators they secrete. The evolutionary more advanced adaptive immune system mainly depends on specific antigen recognition and has a memory function. Both systems enhance each other's potency to eliminate a potential thread to the host organism.

1.3.1 The innate immune system

The innate immune system is made up of different subsystems with specific functions. Very first line defence is the skin and mucosal epithelia representing a physical, chemical and biological barrier. Those organisms that overcome the first barrier are detected and eliminated by host innate immune cells. Macrophages, dendritic cells (DCs) and neutrophils are essential phagocytes for bacterial clearance. Macrophages and DCs also serve as antigen presenting cells (APCs) linking the innate to the adaptive immune system. Natural killer (NK) cells detect stressed cells and function as cytotoxic as well as cytokine-producing cells. Interferon (IFN)-γ is the main cytokine produced by NK cells. Further eosinophils protect the host from parasites while basophils and mast cells secrete histamine and heparin, which promotes the inflammatory process (Medzhitov, 2007).

Microorganisms express highly conserved specific molecular structures called PAMPs that include lipopolysaccharide (LPS) on gram-negative and lipoteichoic acid on gram-positive bacteria. Innate immune cells recognise those structures through certain germ line encoded receptors referred to as pattern recognition receptors (PRR) (Janeway, 1989).

The cellular immune response is supported and enhanced by cytokines like interleukins and interferons as well as acute phase proteins. Acute phase proteins opsonize pathogens and activate the complement system which itself has opsonizing, chemotactic and direct killing function (Medzhitov, 2007).

1.3.1.1 Monocytes, macrophages and DCs

Circulating monocytes are precursor cells of tissue macrophages and may differentiate into DCs. They have direct antimicrobial as well as signalling function. These cells are part of the innate immune system and key effector cells in bacterial clearance. As APCs they can phagocytise foreign microorganisms and present them to T cells via major histocompatibility complex (MHC) II molecules (Gordon and Taylor, 2005).

Macrophages can be activated on the classic way by IFN-γ or Toll like receptor (TLR) ligands creating highly microbicidal cells that release reactive oxygen species (ROS) and produce high levels of pro-inflammatory IL-1, IL-6, IL-12 and IL-23 as well as low levels of IL-10 (Gordon and Taylor, 2005; Mosser and Edwards, 2008). A lack of NK cell or Th 1 derived IFN-γ is associated with higher susceptibility to pathogen-induced infections (Filipe-Santos et al., 2006). If activated on the alternative way by IL-4 and IL-13, tissue repair macrophages will develop. Anti-inflammatory macrophages result from stimulation with IL-

10 and TGF-β and will themselves be potent source of IL-10, TGF-β, prostaglandin E2 and show decreased MHC II expression (Gordon and Taylor, 2005; Mosser and Edwards, 2008).

In human studies the MHC equivalent human leucocyte antigen (HLA) was investigated. After trauma monocytes show impaired expression of HLA-DR as well as impaired production of IL-12. While HLA-DR is necessary for potent antigen presentation, IL-12 activates NK cells and induces differentiation of T cells into Th1 cells. Both studies found impaired monocyte function to be associated with increased risk for septic complications and death (Venet et al., 2007; Spolarics et al., 2003). To achieve a detectable IL-12 response, monocytes had to be preincubated with IFN-γ (Spolarics et al., 2003).

Muehlstedt et al. proved prolonged HLA-DR suppression of alveolar macrophages to predispose for nosocomial pneumonia in injured patients (Muehlstedt et al., 2002). A study performed with burn patients showed HLA-DR expression of circulating monocytes to be impaired on the protein as well as mRNA level and found a maximum in HLA-DR suppression 7 to 10 days after injury (Venet et al., 2007).

IL-10 is a major suppressor of monocyte HLA-DR expression. Inverse correlation of high plasma or alveolar IL-10 and low monocyte HLA-DR expression was described (Lekkou et al., 2004; Muelstedt et al., 2002). IL-10 levels are elevated early after trauma and the risk of developing septic complications as well as the outcome of sepsis correlate with IL-10 levels (Giannoudis et al., 2000; Lyons et al., 1997). Neutralization of IL-10 can restore NK cell specific IFN-γ production upon bacterial challenge in thermally injured mice (Toliver-Kinsky et al., 2002).

1.3.1.2 NK cells

NK cells are lymphocytes of the innate immune system and belong to the group of innate lymphoid cells (ILC). They provide first line cytotoxic defence by killing virus-infected as well as malignantly transformed cells and have immunomodulatory properties exerted by chemokine and cytokine production (Spits et al., 2013).

NK cells account for 10 to 15 % of human peripheral blood lymphocytes and are phenotypically defined as CD3 negative (-) and CD56 positive (+) lymphocytes (Robertson and Ritz, 1990). They are derived from a $CD34^+$ hematopoietic precursor cells (HPC) that over various intermediates lose CD34 and express CD56 (Colucci et al., 2003). As early development takes place in bone marrow, developmental studies found secondary lymphatic

tissue (SLT), the spleen and peripheral blood to play key roles in further development and maturation (Eissens et al., 2012).

Mature $CD56^{+}$ NK cells are divided in two functionally distinct groups that differ in their expression of the CD56 surface molecule (Lanier et al., 1986). Approximately 90% of NK cells in peripheral blood express low density CD56 ($CD56^{dim}$), high density CD16 and are known for their cytotoxic activity. The remaining 10% of peripheral blood NK cells express high density CD56 ($CD56^{bright}$) and either low levels or no CD16 at all (Cooper et al. [1], 2001; Cooper et al. [2], 2001). Even though both NK cell subsets are considered distinct mature cells, recent studies indicate that in terms of maturation they have a linear relationship with $CD56^{bright}$ being precursors of $CD56^{dim}$ NK cells (Romagnani et al., 2007).

$CD56^{dim}$ NK cells exert their cytotoxicity through degranulation of cytoplasmic granules containing perforin and granzymes. Those cytoplasmic granules are abundantly found in the $CD56^{dim}$ but not in the $CD56^{bright}$ NK cell subset, giving reason for their superior ability to kill target cells. The authors additionally describe $CD56^{dim}$ NK cells to be twice as effective in forming conjugates with target cells, which further supports their superior killing potential when compared to the $CD56^{bright}$ subset (Jacobs et al., 2001).

$CD56^{bright}$ NK cells, even though they only make up a small proportion of peripheral blood NK cells, account for more than 95 % of NK cells in SLT and produce high amounts of IFN-γ upon stimulation. Within the SLT they are primarily found in the parafollicular T cell region (Fehniger et al., 2003). Frey et al. compared the two NK cell subtypes in terms of L-selectin adhesion molecule (CD62L) expression and binding to peripheral lymph node high endothelial venules (HEV). The authors found $CD56^{bright}$ NK cells to express CD62L to a higher percentage and in higher density compared to the $CD56^{dim}$ subtype. This circumstance results in more efficient binding of $CD56^{bright}$ NK cells to CD62L ligands expressed in peripheral lymph node HEVs (Frey et al., 1998).

$CD56^{bright}$ NK cells produce abundant amounts of cytokines upon monokine stimulation. The repertoire of cytokines produced includes IFN-γ, GM-CSF, TNF-α, TNF-β, IL-10 and IL-13. The production profile depends on the monokine which NK cells are stimulated with (Cooper et al. [2], 2001). Due to its various effects on shaping an immune response IFN-γ is considered critical for immune competence. Monocyte, macrophage and DC derived IL-12

is the most potent inducer of NK cell IFN-γ production (Cooper et al. [2], 2001; Fehniger et al., 2003).

NK cell dependent killing of target cells and cytokine production is regulated through a complex integration of signals transduced by numerous inhibitory and activating NK cell receptors (Bryceson et al., 2006). Inhibitory human NK cells receptors like the killer cell immunoglobulin-like receptor (KIR) family, recognize MHC class I molecules and their activation prevents target cell killing or cytokine production. Activating receptors include the natural killer group 2D (NKG2D) receptor, a type 2 transmembrane receptor signalling via phosphatidylinositol 3 (PI-3) kinase activation and eventually leading to target cell perforin-dependent killing or cytokine production. Ligands for NKG2D are expressed through pathways associated with cellular stress, infection, or tumorgenesis (Raulet et al., 2013). CD16, a low affinity Fcγ receptor III (FcγRIII), binds to the Fc region of antibodies which opsonise pathogens and its activation induces target cell lysis. This mechanism is described as antibody-dependent cellular cytotoxicity (ADCC) (Leibson, 1997).

Due to their distinct features of enhancing the innate immune response as well as linking the innate to the adaptive immune system, NK cells play a crucial role in viral and bacterial clearance. Two studies conducted in the 1980s were able to show impaired NK cell cytotoxicity in burn and trauma patients (Blazar et al., 1986; Bender et al., 1988). Blazar et al. found the degree of thermal injury to correlate with the duration of NK cell impairment (Blazar et al., 1986). Bender et al. applied burn patient serum to healthy volunteer mononuclear cells and showed that a suppressive effect is mediated via that serum. The authors ascribed that effect to endotoxin within the serum (Bender et al., 1988). Animal models further supported the suppressive effects of thermal injury on NK cell IFN-γ production. Splenic NK cells of thermally injured mice showed reduced IFN-γ secretion upon stimulation with *Pseudomonas aeruginosa* (Toliver-Kinsky et al., 2002).

1.3.1.3 NK cell / DC interaction

An adequate immune response requires complex interactions among innate immune cells and an effective linking of the innate to the adaptive immune system. Their close proximity within the SLT put NK cell interactions into the focus of many researchers. NK cells and APCs where found to enhance each other's function in clearing pathogens and killing virally

infected or transformed cells (Gerosa et al., 2002). Those effects depend on cell-to-cell contact via specific receptors as well as soluble molecules (Borg et al., 2004). Furthermore, NK cells play a critical role in maturation of DCs (Gerosa et al., 2002; Vitale et al., 2005).
IL-12 is a key cytokine enhancing $CD56^{bright}$ specific NK cell IFN-γ production (Borg et al., 2004). The active IL-12 p70 is a heterodimeric cytokine composed of a p40 and p35 subunit. The corresponding IL-12 receptor is made up from two β type subunits. Only a receptor composed of a β1 (IL-12Rβ1) and β2 (IL-12Rβ2) subunit exerts high affinity IL-12 binding and signal transduction. The two subunits individually show low affinity for IL-12 (Presky et al., 1996). Expression profiles of the IL-12 reeptor subunits differ among cell types and are highly regulated (Watford et al., 2004).

IL-2 was found to be a major contributor to IL-12 induced NK cell activity. This effect is mediated via IL-2 induced IL-12Rβ1 and IL-12Rβ2 chain expression (Wang et al., 2000). Fehniger et al. showed that recombinant and T cell derived endogenous IL-2 acts in synergy with IL-12. IL-2 reduces the amounts of IL-12 needed for NK cell specific IFN-γ production (Fehniger et al., 2003).
Downstream IL-12 signalling depends on phosphorylated signal transducer and activator of transcription (STAT) 4 (Thierfelder et al., 1996). STATs were identified as a cytoplasmic transcription factor family mainly activated by receptor associated Janus kinases. Activation results in phosphorylation of specific tyrosine residues leading to subsequent dimerization, translocation to the nucleus and DNA binding (Ihle, 1995). IL-2 was shown to maintain downstream STAT4 expression (Wang et al., 2000).

NK cell activating IL-12 is mainly derived from activated Monocytes and its production is further enhanced by positive feedback through NK cell derived IFN-γ (Moretta et al., 2006). IFN-γ production is closely controlled by various cytokines with IL-12 and IL-18 being the most potent inductors and IL-4, IL-10, TGF-β and glucocorticoids exerting suppressive effects on IFN-γ production (Schroder et al., 2004). Among its various functions IFN-γ induces up-regulation of MHC class I and II molecules, promotes a Th 1 shifted immune response, is involved in cell trafficking to sites of inflammation and facilitates anti-viral as well as microbicidal properties. Signalling defects in the pathways of IL-12 as well as IFN-γ have long been associated with increased susceptibility to bacterial infections (Dorman and Holland, 1998; de Jong et al., 1998).

Positive enhancement of the NK cell / DC exchange of IL-12 and IFN-γ is negatively controlled by IL-10. Aste-Amezaga et al. showed that peripheral blood mononuclear cells (PBMC) pre-treated with IL-10 produced significantly less IL-12 and TNF-α upon stimulation with *S. aureus* or LPS even if primed with IFN-γ (Aste-Amezaga et al., 1998).

1.3.2 The adaptive immune system

T cells and B cells are the key effector systems of the adaptive immune system. The hallmark features are a protection that is specific for a certain pathogen and an immunological memory. High specificity is achieved through somatic recombination of genes encoding for antigen receptors (Murphy et al., 2014).

T cells maturate in the thymus and mature to single positive $CD4^+$ T helper (Th) cells or $CD8^+$ cytotoxic T cells. Th cells further differentiate into either Th1, Th2 or Th17 cells. An IL-12 predominant milieu will favour Th1 cell development and the production of IFN-γ and IL-2. This promotes a cell mediated immune response through activation of macrophages and cytotoxic T cells. The Th2 cell polarization results from IL-4 stimulation. Th2 cells mediate a B cell dominated humoral immune response as they produce IL-4, IL-5, IL-10 and IL-13 (Seder and Paul, 1994).

B cells mature in the bone marrow and if activated develop into antibody producing plasma cells. B cells express MHC-II molecules and have the ability to present antigens. B cells and T cells activated by their specific antigen develop into effector as well as memory cells.

Studies on trauma patients described a shifted pattern towards Th2 cells and cytokine pattern. This shift causes cell mediated immune suppression and thus, increases the risk for infectious complications (O´Sullivan et al., 1995, Spolarics et al., 2003).

Other $CD4^+$ cells involved in the immune response to trauma are T regulatory cells (Treg) phenotypes. Tregs possess a major anti-inflammatory potential by producing IL-10 and TGF-β. MacConmara et al. demonstrated that even though the number of $CD4^+$ cells fell after trauma, Tregs increased relative to all $CD4^+$ cells. They found a suppression of IFN-γ cytokine production in the presence trauma patient Tregs. Additionally, Treg specific IL-4 and IL-10 production on day 1 correlated with the development of sepsis. (MacConmara et al., 2006).

1.4 Aim of the study

Worldwide severe injury is a major cause of death and even if not fatal, trauma is an enormous socioeconomic burden for society. Risk for immediate and early death were tackled by prophylactic measures like for example improving road and car safety as well as the implementation of mature trauma systems with advanced diagnostic and therapeutic opportunities. Late trauma death due to secondary complications could likewise be reduced but remains a challenging field of in hospital care.

Alterations of the immune system were found to predispose to secondary complications. Following severe injury, the immune systems reacts with a systemic inflammatory response called SIRS. At the same time our immune system tries to counteract in an anti-inflammatory manner through the development of CARS. Due to this severe dysregulation the risk for bacterial infection and accompanied complications like sepsis and MOF is increased.

Several studies on trauma, burn and surgical patients proved various cellular and plasmatic alterations. Monocytes and DCs as innate immune cells were found to express reduced levels of HLA-DR and produce less IL-12. NK cells, as further innate immune cells with cytotoxic and cytokine producing function depend on sufficient IL-12. The $CD56^{bright}$ NK cell subtype, when stimulated with IL-12, produces abundant IFN-γ that in return stimulates monocytes and DCs and thus enables an efficient elimination of the infection. While IL-2 is described to enhance NK cell IFN-γ production via enhancing the IL-12 effect, IL-10 is a well-studied suppressor of the IFN-γ innate immune response

This study wanted to clarify whether $CD56^{bright}$ NK cell IFN-γ production is impaired after severe trauma. Blood samples of trauma patients were tested on consecutive days following the incident. Isolated PBMC were stimulated with inactivated *S. aureus* and IFN-γ production was assessed. Since IL-12 is the main activator of NK cell IFN-γ production, additional probes supplemented with IL-12 were set up. Further set ups with supplementation of IL-2 and neutralization of IL-10 were analysed. To assess the underlying pathophysiology, IL-12 receptor expression profiles as well as downstream signalling via STAT4 were analysed. In a last step the effect of trauma patient serum on cells of healthy controls was investigated in the aspect of IFN-γ production, IL-12 receptor expression and STAT4 phosphorylation.

2 Material und Methods

2.1 Material

2.1.1 Consumables

Consumables used during cell separation, culture and staining are listed in table 2.1.

Consumable	Manufacturer
Cellstar® Polypropylen Tubes 15/50ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Cellstar® Serologic Pipettes 5/10/25ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Leucosep® 12ml/50ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Microplates 96 Well, U-bottom	Greiner Bio-One GmbH, Frickenhausen, Germany
Microtest™ Cell culture plate 96 Well, flat bottom	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
Natural Flat Cap Microcentrifuge Tubes 1,5ml	STARLAB GmbH, Ahrensburg, Germany
Pipette tips 0,5-20 µl	Brand GmbH & Co KG, Wertheim, Germany
Pipette tips 10-200 µl, Universal	Greiner Bio-One GmbH, Frickenhausen, Germany
Pipette tips 200-1000 µl, Universal	Greiner Bio-One GmbH, Frickenhausen, Germany
Round-Bottom Tubes 5 ml, Polystyrol	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
S-Monovette® 9ml Z	Sarstedt AG & Co, Nümbrecht, Germany
S-Monovette® 4,5ml Z	Sarstedt AG & Co, Nümbrecht, Germany
S-Monovette® 9ml LH	Sarstedt AG & Co, Nümbrecht, Germany
Transfer Pipettes	Sarstedt AG & Co, Nümbrecht, Germany

Table 2.1 Consumables.

2.1.2 Laboratory equipment

Laboratory equipment used for cell separation, culture and analysis are listed in table 2.2.

Equipment	Manufacturer
Centrifuge Heraeus Megafuge 40R	Thermo Electron LED GmbH, Osterode, Germany
Centrifuge Heraeus Varifuge 3.0RS	Heraeus Instruments GmbH, Hanau, Germany
Centrifuge Labofuge 2000	Heraeus Instruments GmbH, Hanau, Germany
CO2 Incubator Forma Scientific Model 3548	Forma Scientific, Inc., Marietta, Ohio, USA

Equipment	Manufacturer
Absorbance Microplate Reader ELx808	Biotek, Winooski, VT, USA
Flow cytometer FACSCalibur®	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
Reflected Light microscope Axiostar plus	Carl Zeiss Jena GmbH, Jena, Germany
Reflected Light microscope Axiovert 25	Carl Zeiss Jena GmbH, Jena, Germany
Workbench antair BSK	Bio-Flow Technik, Meckenheim, Germany

Table 2.2 Laboratory equipment.

2.1.3 Media, Buffer and Ficoll

Products used for isolation, preparation of culture media, cell permeabilization for intracellular staining and analysis are listed in table 2.3.

Media, Buffer, Ficoll	Manufacturer
Aqua	B. Braun Melsungen AG, Melsungen, Germany
BD FACS™ Lysing Solution	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
CellWASH™	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
Fetal Bovine Serum, F7524	Sigma-Aldrich, St. Louis, MO, USA
Ficoll-Paque™ PLUS	GE Healthcare, Chalfont St Giles, GB
Gibco® Dulbecco´s Phosphate-Buffered Saline (DPBS), no magnesium, no calcium	Life Technologies, Carlsbad, CA, USA
Penicillin-Streptomycin, 10000 units penicillin and 10 mg streptomycin/ml	Sigma-Aldrich, Co., St. Louis, MO, USA
Red Blood Cell Lysing Buffer	Sigma-Aldrich, Co., St. Louis, MO, USA
Trypan Blue Solution (0,4%)	Sigma-Aldrich, Co., St. Louis, MO, USA
VLE RPMI 1640 Medium w 2.0g/L $NaHCO_3$, w stable glutamine, very low endotoxin	Biochrom AG, Berlin, Germany
Intracellular IFN-γ staining	
1. Protein Transport Inhibitor (Containing Monensin), BD GolgiStop™ 2. Fixation and Permeabilization Solution, BD Cytofix/Cytoperm™ 3. Perm/Wash Buffer, BD Perm/Wash™	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
Intracellular STAT4 staining	
Foxp3/Transcription Factor Staining Buffer Set 1. Fixation/Permeabilization Concentrate 2. Fixation/Permeabilization Diluent 3. Permeabilization Buffer	eBioscience, Inc., San Diego, CA, USA
Quantikine® ELISA, Human IL-12 p70 Immunoassay	R&D Systems, Minneapolis, MN, USA

Table 2.3 Media, Buffer and Ficoll.

2.1.4 Stimuli and neutralizing antibodies

Stimuli and neutralizing antibodies used for cell culture are listed in table 2.4.

Stimuli and neutralizing antibodies	Manufacturer
Human IL-10 Antibody	R&D Systems, Minneapolis, MN, USA
Pansorbin® Cells Standardized, Calbiochem®	Merck KGaA, Darmstadt, Germany
Recombinant Human IL-12 (p70) (carrier-free)	BioLegend, Inc., San Diego, CA, USA
Recombinant Human IL-2	PeproTech, Rocky Hill, NJ, USA

Table 2.4 Stimuli and neutralizing antibodies.

2.1.5 Antibodies and isotypes

Antibodies used for extracellular and intracellular stating as well as their corresponding isotypes are listed in table 2.5 and 2.6.

Antigen	Fluorchrome	Clone	Isotype	Manufacturer
CD3	FITC	MEM-57	Mouse IgG2a	ImmunoTools, Friesoythe, Germany
CD16	PE/Cy7	3G8	Mouse IgG1, κ	Biolegend Inc., San Diego, CA, USA
CD56	APC	CMSSB	Mouse IgG1, κ	eBioscience, Inc., San Diego, CA, USA
CD62L	PE	DREG-56	Mouse IgG1, κ	BioLegend, Inc., San Diego, CA, USA
IL-12 receptor β1 subunit, CD212	PE	2.4E6	Mouse IgG1, κ	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
IL-12 receptor β2 subunit	PE	2B6/12 β2	Rat IgG2a, κ	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
CD314 (NKG2D)	PE	1D11	Mouse IgG1, κ	BioLegend, Inc., San Diego, CA, USA
IFN-γ	PE	4S.B3	Mouse IgG1, κ	BioLegend, Inc., San Diego, CA, USA
Stat4 (pY693)	PE	38/p-Stat4	Mouse IgG2b, κ	Becton, Dickinson and Company, Franklin Lakes, NJ, USA

Table 2.5 Antibodies.

Isotype	Isotype for	Manufacturer
PE Mouse **IgG1, κ** Isotype Control	CD212, IL-12 receptor β1 subunit	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
PE Mouse **IgG1, κ** Isotype Ctrl	CD62L, CD314 (NKG2D), IFN-γ	BioLegend, Inc., San Diego, CA, USA
PE Rat **IgG2a, κ** Isotype Control	CD212, IL-12 receptor β2 subunit	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
PE Mouse **IgG2b, κ** Isotype	Stat4 (pY693)	BioLegend, Inc., San Diego, CA, USA

Table 2.6 Isotypes.

2.1.6 Software

Software used for data collection via FACS and data analysis as well as graphic illustration is listed in table 2.7.

Software	Manufacturer
BD CellQuest Pro™ Software	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
GraphPadPrism 5.0	GraphPad Software Inc., La Jolla, CA, USA

Table 2.7 Software.

2.2 Methods

2.2.1 Allocation of patients

Severely injured patients (n=10) represented by an ISS equal or above 16 with a minimum age of 18 years were enrolled in the study. All patients were admitted to the University Hospitals of Essen or Düsseldorf directly after the incident leading to severe trauma. Transferals from other hospitals were not included in the study population. Patients with cardiovascular instability, where additional blood withdrawal would have potentially worsened their situation, where excluded. The study was conducted between January and October 2013.

Informed consent was obtained from the patients or legal representatives as soon as possible. An independent physician verified inclusion criteria. The declaration of Helsinki laid the basis for the study and all aspects of the study were conducted to meet their requirements. The ethic committees of both University Hospitals approved the study design.

Healthy volunteers (n=8) matched by age and sex served as control group.

2.2.2 Assessment of the Injury Severity Score

As discussed in chapter 1.1.1 the ISS was shown to be a reliable instrument in measuring severity of injury in trauma patients. The scoring basis for the ISS is laid by the AIS. The AIS separates 10 different regions and weighs injuries to a certain region by assigning a score ranging between 1 and 6 (see Table 2.8).

AIS	Severity
1	Minor
2	Moderate
3	Serious
4	Severe
5	Critical
6	Unsurvivable

Table 2.8 The AIS. Grading of the AIS ranging from minor injury to unsurvivable.

The ISS applies this classification of scoring injury severity to 6 body regions:

1. Head / Neck including cervical spine
2. Face
3. Thorax including thoracic spine
4. Abdomen including lumbar spine
5. Extremities and pelvis
6. Soft tissue

The final ISS is calculated as the sum of the squares of the 3 highest AIS scores, while for each region only the single highest score is considered.

$$ISS = AIS1^2 + AIS2^2 + AIS3^2$$

The ISS score can vary between 1 and 75. An AIS of 6 in any body region is automatically considered a total score of 75. The NISS was also assessed. This score is simply calculated as the sum of the squares of the 3 highest AIS Scores independent of the body region.

2.2.3 Withdrawal of blood

The first blood sample was drawn 24 hours after admittance. Subsequent blood samples were drawn on the 4th, 6th and 8th day after the trauma as well as on the day of discharge or transferal to an institution carrying on treatment.

Blood samples on day 1 and 8 after trauma as well as on the day of discharge were four 9 ml tubes of heparinized blood and one 9 ml tube for serum preparation. On days 4 and 6 after trauma one 9 ml tube of heparinized blood and one 4.5 ml tube for serum preparation were drawn.

All blood samples were drawn via indwelling catheters if applicable. In cases were no indwelling catheters were available peripheral venous puncture was performed. Blood samples from healthy controls were drawn via peripheral venous puncture.

2.2.4 Isolation of peripheral blood mononuclear cells

The time between blood withdrawal and PBMC isolation was kept as short as possible and did not exceed one hour. All of the following procedures were performed under sterile conditions using a workbench.

The process of PBMC isolation and cultivation differed between days 1, 8 and discharge / transferal to days 4 and 6 after trauma due to the different amount of blood samples obtained. All materials used were adapted to the limited probe material on day 4 and 6 to avoid any difference in the process of isolation as well as cell culture.

Before starting the actual process of cell separation, the following steps were carried out to ensure an uninterrupted cell separation:

Preparation of separation tubes: Two 50 ml Leucosep® tubes were prepared by adding 15 ml Ficoll-Paque™ Plus (Ficoll) and subsequent centrifugation at 800 x g and 21 ºC for 1 minute. After the process of centrifugation, the separation medium was found underneath the separation disc integrated in the tubes.

Obtaining patient serum: Serum tubes were centrifuged at 1000 x g and room temperature for 10 minutes. Serum not needed for the preparation of culture medium was transferred to 1.5 ml Eppendorf tubes and deep frozen at -20 °C.

Preparation of washing and culture medium: Both were prepared by adding either patient serum or fetal calf serum (FCS) to VLE RPMI 1640 Medium (RPMI). RPMI was supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin. Wash medium contained 2 % FCS and was prepared by adding 1 ml of FCS to 49 ml of RPMI. Culture medium contained 10 % serum or FCS and was prepared by adding 1 ml of serum / FCS to 9 ml of RPMI.

Cell separation was started by transferring two 9 ml tubes of heparinized blood to one 50 ml Cellstar® polypropylene tube and dilute the content with the same amount of Gibco® Dulbecco´s Phosphate-Buffered Saline (DPBS). 30 ml of the blood / DPBS dilute were transferred to each of the two 50 ml Leucosep® tubes and centrifugation was performed at 800 x g and 21 °C for 15 minutes without acceleration or break. After centrifugation the following layers could be identified from bottom to top:

> Pellet of erythrocytes and granulocytes – separation medium – separation disc – separation medium – enriched cellular fraction containing PBMC – plasma

The PBMC containing interphase was transferred to a 50 ml Cellstar® polypropylene tube via Pasteur-Pipette.

Cells were resuspended and the tube was filled up to 50 ml with DPBS before centrifugation at 300 x g and 21 °C for 10 minutes. Following the process of centrifugation, the tube was decanted and 2 ml of Red Blood Cell Lysing Buffer were added. This was performed since, especially on day 4, 6 and 8, the PBMC fraction contained erythrocytes. PBMC were subjected to erythrocyte lysis using 500 µl of Red Blood Cell Lysing Buffer per 9 ml tube of heparinized whole blood. Erythrocyte lysis remained incomplete in most cases.

Cells were resuspended and transferred to a 15 ml Cellstar® polypropylene tube. The process of red blood cell lysis was stopped by adding wash medium. Subsequent centrifugation was carried out at 300 x g and 21 °C for 10 minutes and followed by decantation of the tube. The remaining cell pellet was resuspended in 2 ml of RPMI, transferred to a new 15 ml Cellstar® polypropylene tube and stored on ice. Cell counts were calculated using the Neubauer counting chamber.

2.2.5 Immediate extracellular staining

Immediate extracellular staining was done directly after PBMC isolation was finished. Staining was carried out for the IL-12Rβ1 and IL-12Rβ2 subunit as well as surface molecules NKG2D and CD62L and the applicable isotypes. One probe without staining was set to exclude autofluorescents. Each of the nine probes had to contain 4×10^5 cells. The appropriate amount of cell / RPMI suspension was calculated, transferred and resuspended in 900 µl CellWASH® minus the amount of cell / RPMI suspension needed. 100 µl per well of the suspension were transferred to a 96 well round-bottom plate and further suspended with additional 100 µl of CellWASH®. Subsequent centrifugation was carried out at 500 x g and 4 °C for 6 minutes. Following the centrifugation, the plate was decanted and the appropriate antibodies were added in 100 µl CellWASH® per well.

Extracellular staining was performed using the following antibodies labelled by Fluorescein isothiocyanate (FITC), Phycoerythrin-Cyanin7 (PE-Cy7), Allophycocyanin (APC) and Phycoerythrin (PE):

All probes: CD3 FITC (3 µl per well), CD16 PE/Cy7 (2.5 µl per well), CD56 APC (5 µl per well)

Probe 01: Negative control
Probe 02: IL-12 receptor β1 chain PE, BD (20 µl per well)
Probe 03: Mouse IgG1, κ PE, BD (10 µl per well)
Probe 04: IL-12 receptor β2 chain PE, BD (20 µl per well)
Probe 05: Rat IgG2a, κ PE, BD (0.3 µl per well)
Probe 06: CD62L PE, BioLegend (5 µl per well)
Probe 07: Mouse IgG1, κ PE, BioLegend (0.156 µl per well)
Probe 08: NKG2D PE, BioLegend (5 µl per well)
Probe 09: Mouse IgG1, κ PE, BioLegend (1.25 µl per well)

Incubation of antibodies was carried out at 4 °C for 12 minutes in the dark and was ended by adding 100 µl CellWASH® per well plus centrifugation at 500 x g and 4 °C for 6 minutes.
After decanting the 96 well round-bottom plate the stained cells were resuspended, taken up in 200 µl CellWASH® and transferred to a 5 ml round-bottom tube with 50 µl CellWASH® pre-titrated.

2.2.6 Cell culture

Isolated PBMCs were cultured in differing environments for 18 or 23 hours depending on the target staining molecule. Cell culture was carried out with 4×10^5 cells per well (each 200-220 µl) at 37 °C and 5 % CO_2 in 96 well flat-bottom cell culture plates. The culture media were prepared from autologous and FCS. IL-12, IL-2 and anti-IL-10 were added where indicated. Those probes for extracellular as well as intracellular phosphorylated STAT4 (pSTAT4) staining were cultured for 18 hours while those for intracellular IFN-γ staining were cultured for 23 hours. Probes for extracellular staining were set up as single

well, while those for intracellular analysis were set up quadruples. Cell culture plates were prepared as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			no stimulus					no stimulus				
C			*S. aureus*					*S. aureus*				
D			*S. aureus* + IL-12					*S. aureus* (isotype)				
E			*S. aureus* + IL-2									
F			*S. aureus* + anti-IL-10									
G			*S. aureus* (isotype)									
H												
			Autologous serum					FCS				

Table 2.9 Cell culture plate 1 (IFN-γ). Set up with 4 x 10^5 cells per well for IFN-γ staining. Six different set ups in culture media prepared from autologous serum and three in culture media prepared from FCS. Each probe was set up as quadruples that were pooled after stimulation.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			no stimulus					no stimulus				
C			*S. aureus*					*S. aureus*				
D			*S. aureus* + IL12					*S. aureus* (isotype)				
E			*S. aureus* (isotype)					no stimulus		*S. aureus*		
F			no stimulus		*S. aureus*							
G												
H												
			Autologous serum					FCS				

Table 2.10 Cell culture plate 2 (pSTAT4, IL-12Rβ1 and IL-12Rβ2 subunits). Set up with 4 x 10^5 cells per well. Four different set ups for pSTAT4 staining in culture media prepared from autologous serum and three in culture media prepared from FCS. Each probe was set up as quadruples that were pooled after stimulation. Probes for extracellular IL-12Rβ1 and IL-12Rβ2 expression were set up as singles. Stimulated and not for autologous serum and FCS, with the corresponding isotype.

For the preparation of all probes as displayed in table 2.9 and 2.10, 19.2 x 10^6 cells needed to be cultured in media prepared from autologous serum and 12.8 x 10^6 cells needed to be cultured in media containing FCS. The corresponding amount of isolated PBMC cells resting in RPMI was taken and the appropriate amount of autologous serum or FCS was added. Before cells were transferred to culture plates at 100 µl per well, the appropriate culture media was added up to the amount needed.

After cells were transferred to cell culture plates, they were rested for 1 hour at 37 °C and 5 % CO_2 before IL-2 and anti-IL-10 were added at a final culture concentration of 5 ng/ml for IL-2 and 10 µg/ml for anti-IL-10. Both were added in 10 µl RPMI per well. Cell stimulation began upon 1 further hour of rest. Pansorbin® Cells Standardized (Pansorbin)

were used for stimulation. Pansorbin cells are heat killed and formalin fixed *S. aureus* cells with surface protein A as antigen. To end up at 0.05 % final cell culture concentration 0.1 µl Pansorbin per well was added to 100 µl of the appropriate culture media per well. 100 µl of the culture media / Pansorbin mix were added to all sets to be stimulated. Negative controls without stimuli were supplemented with 100 µl of the indicated culture media. Cell culture preparation was completed by adding recombinant human IL-12 subunit p70 at a final cell culture concentration of 1 ng/ml in 10 µl RPMI per well where indicated.

Staining for surface two IL-12 receptor subunits and intracellular pSTAT4 was performed after 18 hours of culture as described below. Those probes for intracellular IFN-γ staining were cultured for an additional 5 hours in the presence of 0.5 µl/ml BD GolgiStop™ (GolgiStop) which was added in 10 µl RPMI per well. GolgiStop enhances the detectability for IFN-γ by staining and flow cytometric analysis.

2.2.7 Extracellular staining

Staining of both, the β1 and β2 subunit of the IL-12 receptor was performed after stimulation in order to detect changes in their cell surface expression. Probes were cultured in the presence of *S. aureus* with the culture media containing autologous serum or FCS. Negative controls without *S. aureus* were performed to exclude stimulatory effect of the culture media itself. Isotype controls were performed for each probe.
After 18 hours of culture supernatants were taken of and cells were taken up and transferred to a 96 well round-bottom plate for staining. The plate was centrifuged at 500 x g and 4 °C for 6 minutes. Staining was performed analogously to direct extracellular staining as explained under 2.2.5.

2.2.8 Intracellular staining

2.2.8.1 STAT4

Since intracellular staining needed a considerably larger number of cells, each probe was set up as quadruples. Supernatants were taken off and discarded before the four well of each probe were pooled, transferred to a 96 well round-bottom plate and centrifuged at 500 x g and 4 °C for 6 minutes. Cell surface markers CD3 (5 µl per well) and CD56 (10 µl per well) were set up in 100 µl CellWASH® per well and added to the cells. Incubation was carried

out for 12 minutes at 4 °C in the dark and followed by adding 100 µl of CellWASH® per well before centrifugation at 500 x g at 4 °C for 6 minutes.

All components needed for the following processes of cell fixation and permeabilization as well as the buffer used as carrier for the staining antibodies were delivered with the Foxp3 / Transcription Factor Staining Buffer Set by eBioscience. At first the cells were incubated in 100 µl of the Fixation / Permeabilization Buffer per well for 2 hours at 4 °C in the dark. The Fixation / Permeabilization Buffer was set up according to manufacturer's instructions using 1 part of concentrate with 3 parts of diluent. Cells were fixed and permeabilized by the containing formaldehyde and saponin. Since permeabilization is a reversible process the subsequent steps had to be performed in a saponin containing media. Following the 2 hours of incubation, 100 µl of the Permeabilization Buffer (1 x) were added to each well and centrifugation was performed at 500 x g and 4 °C for 6 minutes. Antibodies targeting pSTAT4 (20 µl per well) as well as the corresponding isotype Mouse IgG2b, κ (3.2 µl per well) were added in 100 µl of Permeabilization Buffer (1 x). Then they were given 30 minutes at 4 °C in the dark to incubate before 100 µl of Permeabilization Buffer (1 x) were added and centrifugation was performed at 500 x g and 4 °C for 6 minutes. Supernatants were decanted and the stained cells were taken up in 200 µl CellWASH® and transferred to a 5 ml round-bottom tube with 50 µl CellWASH® pre titrated.

2.2.8.2 IFN-γ

Since IFN-γ undergoes posttranslational modification within the Golgi apparatus BD GolgiStop was added 5 hours before staining. BD GolgiStop is a monesin containing inhibitor of protein transportation and causes an accumulation of proteins within the Golgi apparatus. Thus, it enhances the detectability of IFN-γ. Staining was performed after 23 hours of culture. Supernatants were transferred to a 96 well round-bottom plate and deep frozen for eventual analysis of IL-12 levels via highly sensitive enzyme linked immunosorbent assay (ELISA). All steps of pooling and cell surface marker staining were performed analogously to staining for STAT4.

As described above cells had to be fixed and permeabilized before actual intracellular staining could be performed. Therefore, cells were incubated in 150 µl BD Cytofix / Cytoperm™ per well for 20 minutes at 4 °C in the dark. BD Cytofix / Cytoperm™ contains formaldehyde for fixation as well as saponin for permeabilization. As mentioned above all subsequent steps of staining had to be carried out in a saponin containing media to avoid reversal of permeabilization. BD Perm / Wash™ buffer is a saponin containing

washing media and was used as a carrier for antibodies in the subsequent steps. Before usage on probes it had to be diluted by distilled aqua to get a 1 x solution from the 10 x concentrate. Fixation and permeabilization were stopped by adding 50 µl of the BD Perm / Wash™ buffer (1 x) solution and subsequent centrifugation at 500 x g and 4 °C for 6 minutes. Antibodies against IFN-γ (2.5 µl per well) and the corresponding isotype Mouse IgG1, κ (0,125 µl per well) were set up in 100 µl of BD Perm / Wash™ buffer (1 x) and added to the probes. Incubation for 20 minutes at 4 °C in the dark was followed by adding 100 µl of BD Perm / Wash™ (1 x) buffer per well and subsequent centrifugation at 500 x g and 4 °C for 6 minutes followed by decantation. The stained cells were taken up in 200 µl CellWASH® and transferred to a 5 ml round-bottom tube with 50 µl CellWASH® pre-titrated.

2.2.9 Healthy control cell culture in patient serum

In order to access the effect of trauma patient serum, healthy control cells were set up in culture media prepared from serum allocated from the 10 trauma patients. Pooled serum of healthy controls served as control media and gave the reference for interpretation of results. Healthy control blood samples were four 9 ml tubes of heparinized blood as well as one 9 ml tube of blood for serum preparation. Isolation of PBMCs was performed as described above. Isolated cells of a single donor were set up in culture media made from a single trauma patient day 1 and 8 serum. Cells of the same single donor were additionally set up in media made from a healthy control serum pool. This was performed for all ten trauma patient sera with the same control serum pool but differing healthy control donor cells.

Surface marker staining as well as intracellular staining for STAT4 and IFN-γ were performed as described above. Cells were set up for as shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	no stimulus				no stimulus				no stimulus			
D	*S. aureus*				*S. aureus*				*S. aureus*			
E	*S. aureus* + IL-12				*S. aureus* + IL-12				*S. aureus* + IL-12			
F	*S. aureus* (isotype)				*S. aureus* (isotype)				*S. aureus* (isotype)			
G												
H												

Serum-Pool Serum day 1 Serum day 8

Table 2.11 Cell culture plate 3 (IFN-γ). Set up with 4 x 10^5 cells per well for IFN-γ staining. Four different set ups in culture media prepared from serum-pool as well as trauma patient serum day 1 and 8. Each probe was set up as quadruples that were pooled after stimulation.

A												
B	no stimulus				no stimulus				no stimulus			
C	*S. aureus*				*S. aureus*				*S. aureus*			
D	*S. aureus* (isotype)				*S. aureus* (isotype)				*S. aureus* (isotype)			
E	no stimulus				no stimulus				no stimulus			
F	*S. aureus*				*S. aureus*				*S. aureus*			
G												
H												

Serum-Pool Serum day 1 Serum day 8

Table 2.12 Cell culture plate 4 (pSTAT4 and IL-12Rβ2 subunit). Set up with 4 x 10^5 cells per well. Three different set ups for pSTAT4 staining. Culture media was prepared from serum-pool as well as trauma patient serum day 1 and 8. Each probe was set up as quadruples that were pooled after stimulation. Probes for IL-12Rβ2 expression were set up as single. Stimulated and not for serum-pool as well as trauma patient serum day 1 and 8 with the corresponding isotype.

2.2.10 Flow cytometry

Flow cytometry is a method used for characterization and quantitative analysis of cell surface as well as intracellular molecules. It is based on the detection of fluorescents emitted by antibodies bound to target structures.

Suspended cells are sucked into a narrow cuvette, are aligned and enter the flow cell one at a time to be analysed. A process called hydrodynamic focusing accomplishes this. The cell suspension is injected into a laminar flowing stream of sheath fluid. This compresses the target cell suspension beam to approximately the size that only one cell can pass at a time. As a cell passes the flow cell, a laser analyses the cell. The detector is located at 90 degrees to the laser source.

Cells itself have different properties of scattering light which is based on their size and intracellular composition. Forward scattered light (FSC) describes the amount of light scattered in the forward direction and is mainly depending on the size of the cell. Side

scattered light (SSC) in contrast describes the light scattered to the sides and is depending on the intracellular composition such as granulation and complexity.

As light reaches the detectors it is converted into a voltage that in turn can be plotted on a histogram. Plotting of leukocytes on a two-dimensional graph with the x- and y-axis being FSC and SCC respectively allows to differentiate lymphocytes, monocytes and neutrophils due to their difference in size and intracellular granulation / complexity.

Further analysis of cell surface receptors and intracellular proteins depend on fluorescence. Specific antibodies binding to target cell structures are linked to certain fluorophores. When activated by light these emit light of a certain wavelength which than can be detected as such. The fluorescence increases with the number of antibodies bound to the target structure. As fluorophores FITC, APC, PE and PE/Cy7 were used in this study.

All data was collected with a FACSCalibur® and displayed and analysed with BD CellQuest Pro software.

Results were graphically illustrated as two-dimensional dot plots. In the FSC / SCC plot NK cells were found among the population of lymphocytes. Gating that population and displaying it on a two-dimensional dot plot, gating for CD3 and CD56, allowed further gating of CD3 negative and CD56 positive NK cells. Gating on the CD56 y-axis performed final differentiation into $CD56^{dim}$ and $CD56^{bright}$ NK cells. The desired population of $CD56^{birght}$ NK cells was displayed with the subject of interest being plotted on the x-axis allowing for quantitative analysis for the desired molecule (s. Results Figure 3.1 A and B).

2.2.11 Enzyme-linked immunosorbent assay

For quantification of IL-12 levels in cell culture, supernatants were collected at 23 hours of culture, before pooling and preparing the cells for flow cytometry. Supernatants were deep frozen at -20 °C until further testing. For analysis the Human IL-12 p70 Quantikine® ELISA Kit by R&D Systems was used according to the manufacturer's instructions. This ELISA is based on the quantitative sandwich enzyme immunoassay technique. The 96 well microplates were pre-coated with a monoclonal antibody specific for IL-12 p70. Adding the prepared standards as well as samples to each well and incubating them for 2 hours gives IL-12 p70 the chance to bind to the immobilized antibodies. Supernatants were aspirated and unbound molecules were removed by washing. For detection of antibody / IL-12 p70 complexes an enzyme-linked polyclonal antibody directed against human IL-12 p70 was added and the probes were incubated for another 2 hours. Horseradish peroxidase served as

enzyme. Supernatants were aspirated and unbound enzyme-linked antibodies were removed by washing. The prepared substrate solution containing hydrogen peroxide and tetramethylbenzidine was added and incubated for 20 minutes protected from light. Tetramethylbenzidine is converted into coloured products by horseradish peroxidase. Colour development was stopped after the appropriate time by adding a sulfuric acid containing stop solution to each well. The intensity of colour development indicates the amount of IL-12 p70 bound and was measured by an absorbance microplate reader.

2.2.12 Statistical analysis

Preparation of graphs and statistical analysis was performed using GraphPad Prism 5.0 Software. Most data were illustrated as scattered dot plots with bars representing the median and interquartile range. Correlation curves were used where indicated. Other data was depicted as box and whiskers plots with the whiskers being plotted in the method of min to max.

Comparing data of patients with controls was performed using non-paired Mann Whitney test. Wilcoxon signed rank test was used to compare two paired groups of patients while Friedman test served as test of choice analysing differences within more than two paired groups of patients. Association of two variables was calculated using the Spearman rank correlation.

3 Results

As described above severely injured patients are at increased risk to develop secondary complications after surviving the acute phase of trauma. A large part of late trauma death is attributed to a dysfunctional immune system predisposing for SIRS / CARS. This status is accompanied with increased susceptibility for infections and possibly sepsis as well as MOF. Focusing on the days following the traumatic event, various alterations of the immune system have been described. The goal of this study was to investigate the role of $CD56^{bright}$ NK cells in post-traumatic immunosuppression. The focus was on the NK cells capability to produce the immunoregulatory cytokine IFN-γ upon bacterial challenge. Additionally, the effect of supplementation or neutralization of cytokines known to influence NK cells IFN-γ production was assessed. For further understanding the underlying pathophysiology several aspects of IL-12 signal transduction, eventually leading to IFN-γ production, were studied. The immunosuppressive effect exerted by the serum of trauma patients on NK cells from healthy donors was also assessed in terms of IFN-γ production and IL-12 signalling.

3.1 Patients included, patterns of injury and in hospital care

A total of 10 severely injured patients (8 male, 2 female) with a median age of 48 (44-55) fulfilled the inclusion criteria and were enrolled in the study. The mechanism of trauma was blunt trauma in all cases and mostly due to car accidents or falls from greater heights. A median ISS of 25 (22-29) and a median NISS of 34 (29-37) were recorded (s. Table 3.1).
Main contributing body regions were the thorax (mean AIS 3.4) and the head / neck (mean AIS 3.4) region with 8 out of 10 patients presenting with corresponding injuries in those body regions. 7 out of 10 patients presented with injuries to the extremities (mean AIS 2.7). Lacerations to the face (4 patients), abdomen (2 patients) and soft tissue (1 patient) were less frequent (s. Table 3.2).
Median time spent on ICU was 27 (17-32) days with a median time spent on ventilation of 13 (8-19) days. None of the patients developed SIRS or septic complication nor did any of the patients die during the observation period.
8 (6 male, 2 female) healthy volunteers at the median age of 43 (38-47) served as control group (s. Table 3.1).

	Patients	Controls	p value
Number of Patients	10	8	
Age [y]	48 (44-55)	43 (38-47)	ns
Gender male	8/10	6/8	
ISS	25 (22-29)		
NISS	34 (29-37)		
Hospitalization [d]	32 (28-35)		
ICU [d]	27 (17-32)		
Respiratory support [d]	13 (8-19)		

Table 3.1 Patient characteristics. Assessment of all patients at admission included age and gender as well as registration of injury pattern including ISS and NISS scores. Duration of hospital stay, time on ICU and on respiratory support were recorded. Quantitative variables are expressed as median with 25^{th} -75^{th} interquartile range. Statistical differences tested using Mann Whitney test.

	Number of Patients	AIS
Head/Neck	8	3.4
Face	4	1.8
Thorax	8	3.4
Abdomen	3	2
Extremities	7	2.7
Soft Tissue	1	2

Table 3.2 Injury pattern. All patients presented with various injuries to different parts of the body. The pattern of injury was recorded according to ISS body regions and severity was documented via AIS coding system**.** AIS scores are expressed as mean.

3.2 $CD56^{bright}$ NK cell IFN-γ production upon *S. aureus* challenge

The immune response upon bacterial challenge is carefully regulated by complex interactions between various cellular and soluble components of the human immune system. Besides physical barriers as the skin, our innate immune system is the first line of defence. NK cells play a crucial role within this system by interacting with for example DCs and macrophages. NK cells do also link the innate to the adaptive immune system. These complex interactions depend on cell-to-cell contact as well as secretion and reception of soluble molecules (s. chapter 1.3).

In this study bacterial infection was simulated by incubating mononuclear cells from trauma patients in the presence of inactivated *S. aureus*. Upon bacterial challenge healthy control NK cells produce large amounts of IFN-γ that in turn plays a critical role in regulating innate and adaptive immunity. To assess the immune competence of trauma patient $CD56^{bright}$ NK

cells, their capacity to produce IFN-γ in the post-traumatic phase of in hospital care was analysed. Blood samples of 10 severely injured patients were analysed following the first 8 days after trauma and on the day of discharge or transferal. Results were compared to healthy controls.

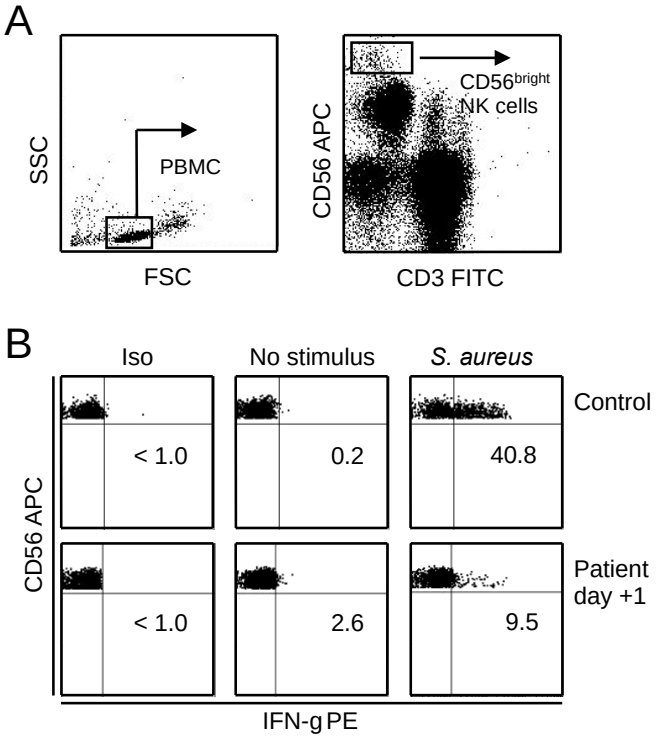

Figure 3.1 Representative gating strategy of CD56^{bright} NK cells and their specific IFN-γ production upon stimulation with *S. aureus*. Isolated PBMC of a trauma patient on day 1 and a healthy control after 23 hours of culture. **A)** Representative gating of CD56^{bright} NK cells by framing PBMCs in the FSC and SSC followed by selecting the CD3 negative and CD56^{bright} positive NK cells. **B)** Representative dot plots showing CD56^{bright} NK cell specific IFN-γ production of a healthy control and a patient on day 1 after trauma. Cells were cultured without any stimulus or in the presence of inactivated *S. aureus*.

Cell culture was done with PBMC which were isolated from whole blood samples by density gradient centrifugation. Blood samples were drawn at fixed time points after trauma. Following isolation some PBMC were directly stained for cell surface molecules while most cells were used for cell culture. Cells were cultured in the presence and absence of *S. aureus*. Cultured cells were then used for cell surface as well as intracellular staining. All cells were stained for CD3 and CD56 to allow identification of CD3 negative CD56^{bright} NK cells and the either extra- or intracellular target molecule. Before staining for intracellular proteins cells underwent a process of permeabilization and fixation. Isotypes were used to show unspecific binding.

Gating was carried out by identifying PBMC in the SSC / FSC scatter before setting them apart by expression of CD3 and C56 (s Figure 3.1 A). Those cells expressing high CD56 were then gated and analysed in terms of target molecule expression (s. Figure 3.1 B).

3.2.1 CD56^{bright} NK cell IFN-γ production upon *S. aureus* challenge is impaired after severe trauma

Upon bacterial challenge healthy control cells were able to produce abundant amounts of IFN-γ. In trauma patients, though, CD56^{bright} NK cell specific IFN-γ production was significantly impaired. The immunosuppression was obvious at all time points of analysis. While a median of 37 % of healthy control NK cells produced IFN-γ upon stimulation, the group of patients did not exceed a median of 5 % IFN-γ producing NK cells on any day. Lowest percentage of IFN-γ producing NK cells was found at day 8 after trauma with a median of less than 3 % of CD56^{bright} NK cells being positive for IFN-γ production (s. Figure 3.2 A).

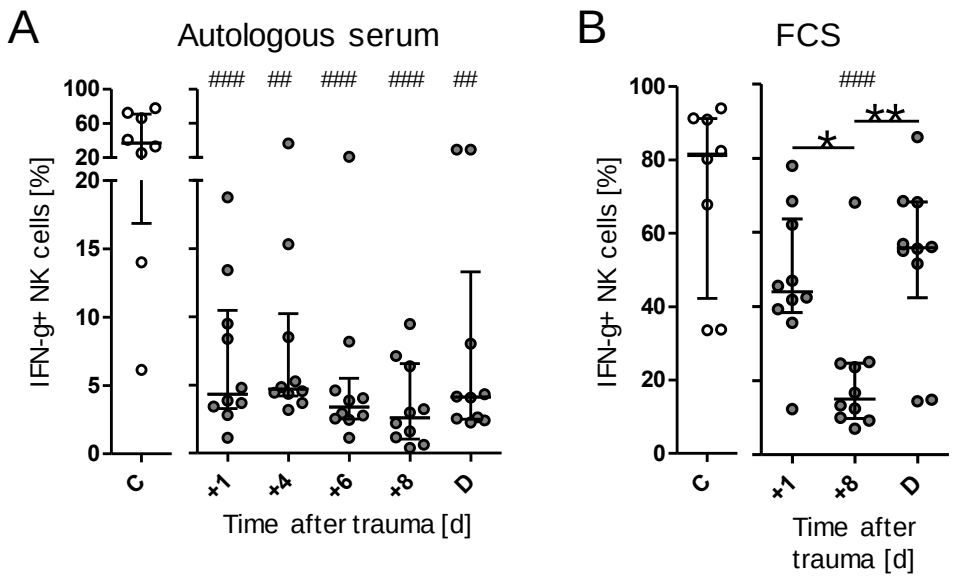

Figure 3.2 CD56^{bright} NK cell specific IFN-γ production upon stimulation with *S. aureus*. Isolated PBMC of trauma patients (n=10) on days 1, (4, 6,) 8 and discharge / transferal (D) plus healthy controls (C) after 23 hours of culture in the presence of inactivated *S. aureus*. **A)** IFN-γ production upon stimulation in culture medium prepared from autologous serum. **B)** IFN-γ production upon stimulation in culture medium prepared with FCS. Horizontal lines represent the median and interquartile range. Statistical analysis was performed using the Friedman test (* $p < 0.05$; ** $p < 0.01$) for comparison within the group of patients and the Mann Whitney test (## $p < 0.01$; ### $p < 0.001$) for comparison of patients with healthy controls.

Since our standard culture media was supplemented with 10 % of the corresponding patient serum, which might itself be an immunosuppressive milieu, an additional, patient-independent culture environment was implemented. FCS substituted the patients' serum. Independent of the culture environment NK cells of patients produced less IFN-γ than those of healthy controls. The difference though was only statistically significant at day 8 following the incident. Among the group of patients IFN-γ production decreased significantly from day 1 to day 8 and showed significant restoration at discharge or transferal (s. Figure 3.2 B). Bacterial challenge induced NK cell specific IFN-γ production was increased upon culture in media prepared from FCS compared to autologous serum. This was true for patients and healthy controls. FCS itself though did not have any stimulatory effect in the absence of *S. aureus*.

The data clearly demonstrated an impaired $CD56^{bright}$ NK cells specific IFN-γ production after trauma. This NK cell suppression was found to be independent of the culture media and most pronounced on day 8 after the incident.

3.2.2 IL-12 can significantly increase $CD56^{bright}$ NK cell IFN-γ production

For further understanding of the pathophysiology of the impaired NK cell immune response upon bacterial challenge, other cytokines involved in NK cell activation were investigated. Monocyte / macrophage and DC derived IL-12 is necessary for and considered to be the most potent cytokine in enhancing NK cell IFN-γ production. An impaired IL-12 synthesis of monocytes following trauma has been shown by various authors (Reinhardt et al., 2015; Borg et al., 2004).

In order to assess the amount of IL-12 available for proper NK cells function, cell culture supernatants of stimulated trauma patient PBMC were analysed for IL-12 levels. The ELISA used did target the biologically active IL-12 p70 heterodimer. Further it was investigated whether supplementation of IL-12 could restore $CD56^{bright}$ NK cell IFN-γ production.

Supernatants of trauma patient PBMC stimulated with inactivated *S. aureus* were found to express significantly reduced levels of IL-12 when compared to supernatants collected from healthy control stimulated PBMC (s. Figure 3.3 A). Additionally, the data indicate the lowest IL-12 production to be on day 8 following the incident (s. Figure 3.3 A). This observation was not significant though.

In order to exclude potential suppressive effects exerted by trauma patient serum, cells were also cultured in FCS. PBMC cultured in FCS produce more IL-12 compared to cells cultured in autologous serum. The suppressive effect of trauma with maximum impairment on day 8 remained true for culture in media prepared from FCS. Compared to healthy controls the reduction in supernatant IL-12 was significant on day 8 (s. Figure 3.3 B).

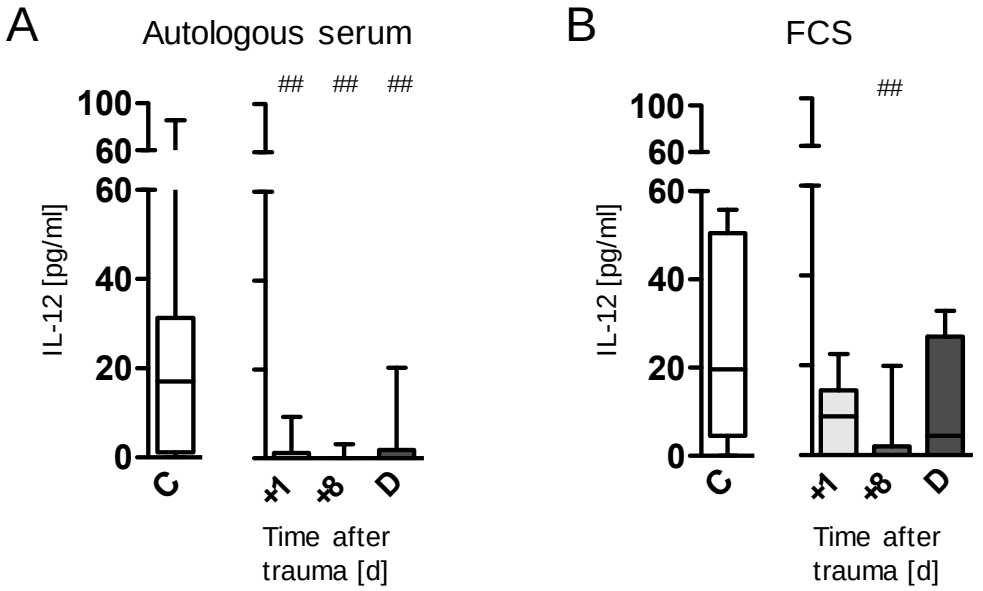

Figure 3.3 PBMC IL-12 p70 production upon bacterial challenge with *S. aureus*. Supernatants obtained after *S. aureus* challenge of isolated PBMC from trauma patients (n=10) on days 1, 8 and discharge / transferal (D) and from healthy controls (C). IL-12 levels after 23 hours of culture in media prepared from **A)** autologous serum and **B)** FCS. Bars depicted as Box and Whiskers, min to max. Statistical analysis was performed using the Friedman test for comparison within the group of patients and Mann Whitney test (## $p < 0.01$) for comparison with control group.

In order to assess the $CD56^{bright}$ NK cell specific IFN-γ producing capacity independent of sufficiently high IL-12 levels in cell culture, supplementation of IL-12 at 1 ng/ml for cell culture process was carried out. The data showed that supplementing IL-12 does significantly increase IFN-γ production on all days following the incident (s. Figure 3.4 A). The highest IL-12 response was seen on day 1 and 4. The relative increase in IFN-γ production indicates a significant decline in IL-12 response on day 8 with only little recovery towards the day of discharge or transferal (s. Figure 3.4 B).

When compared to healthy control NK cells stimulated with equivalent IL-12 levels, the response of trauma NK cells was significantly reduced. As already mentioned above the IFN-γ production reached its lowest levels at day 8 and showed a significant decline compared to day 4. Even though some patients showed signs of a recovered IL-12 response

at time of discharge or transferal, overall IL-12 response remained significantly impaired during the whole observation period compared to healthy controls (s. Figure 3.4 C).

Healthy controls did also show a significant increase in IFN-γ production upon supplementation of IL-12. While simple bacterial challenge led to a median of around 37 % of cells being positive for IFN-γ, around 65 % were positive after adding IL-12. This increase was primarily due to a strong increase in those controls having low cytokine production upon simple bacterial challenge. Those with initially high cytokine production did only respond little causing a wide range of ΔIFN-γ (s. Figure 3.4 B).

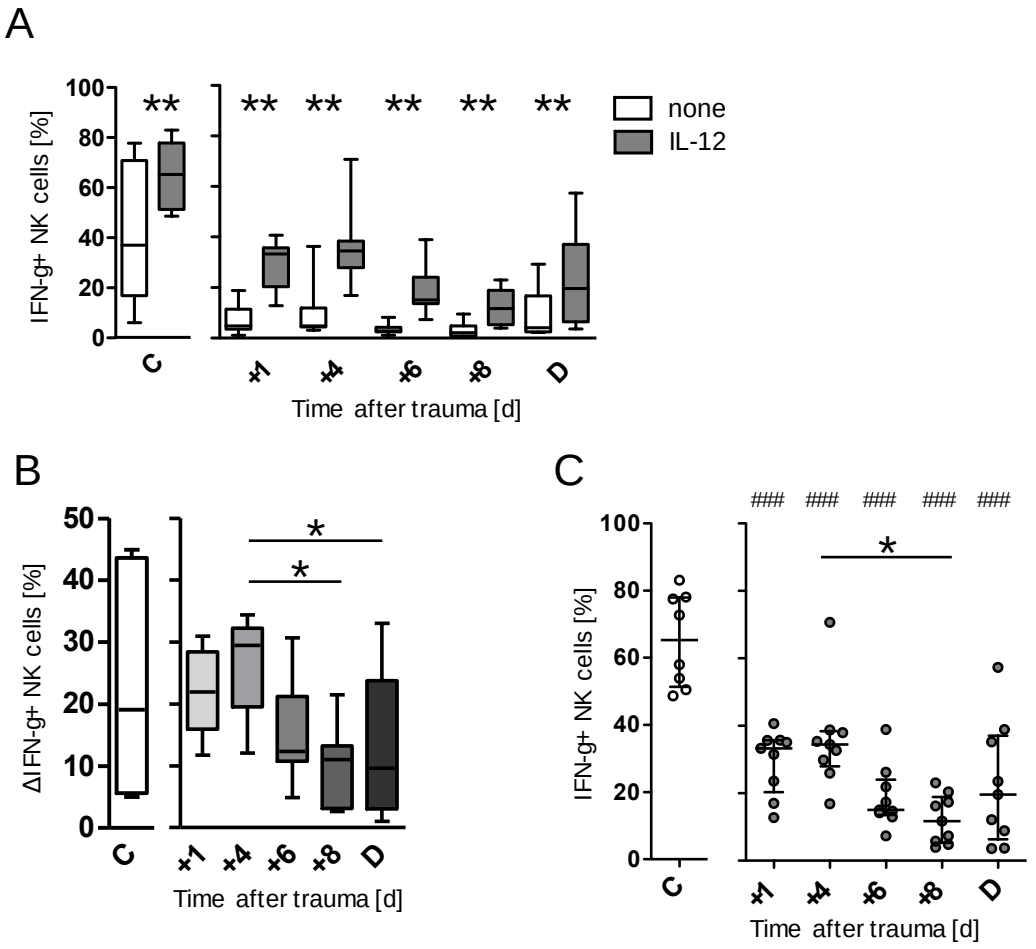

Figure 3.4 Effects of IL-12 supplementation on CD56^{bright} NK cell specific IFN-γ production. Isolated PBMC of trauma patients (n=9) on days 1, 4, 6, 8 and discharge / transferal (D) plus healthy controls (C) after 23 hours of culture. Culture was carried out in the presence of *S. aureus* in autologous serum. **A)** IFN-γ production after simple *S. aureus* challenge and supplementation of 1 ng/ml IL-12. Bars depicted as Box and Whiskers, min to max. Statistical analysis was performed using the Wilcoxon signed rank test (**p<0.01). **B)** Increase of IFN-γ production from baseline *S. aureus* induced IFN-γ production upon supplementation of 1 ng/ml IL-12. Bars depicted as Box and Whiskers, min to max. **C)** IFN-γ production after stimulation with *S. aureus* and 1 ng/ml IL-12. Scattered dot plots with horizontal lines representing the median and interquartile range. Statistical analysis was performed using the Friedman test (* $p < 0.05$) for comparison within the group of patients and Mann Whitney test (### $p < 0.001$) for comparison with control group.

Trauma patient PBMC produce significantly less IL-12 when compared to PBMC isolated from healthy controls. Supplementation with recombinant IL-12 at least partly restored NK cell-derived IFN-γ production. On day 1 and 4 IL-12 supplementation increased IFN-γ production of trauma patients to almost match IFN-γ production by healthy controls without IL-12 supplementation (s. Figure 3.4 A and C). The IL-12 response decreases towards the end of the first week and is lowest on day 6 and 8 following severe injury.

3.2.3 Supplementation of IL-2 can partly restore CD56^{bright} NK cell IFN-γ production, neutralization of IL-10 cannot

Even though IL-12 is known to be the most potent inductor of NK cell IFN-γ production, various other cytokines are involved in regulating NK cell cytokine production and secretion. IL-2 exerts enhancing effects by increasing the NK cell IL-12 response. IL-10 in contrast has been described to be one of the main suppressors of an IFN-γ dominant immune response and was shown to be elevated after trauma. For assessing the effects of IL-2 supplementation and neutralization of IL-10, cell culture was supplemented with IL-2 or with neutralizing antibodies against IL-10.

IL-2 does significantly increase CD56^{bright} NK cell specific IFN-γ production upon *S. aureus* challenge in healthy controls and trauma patients (s. Figure 3.5 A). In trauma patients though this immune enhancing effect was only statistically significant on day 1 and the day of discharge or transferal. The stimulatory effect of IL-2 was not evident on day 8 after trauma (s. Figure 3.5 A and B). The relative increase in IFN-γ production due to IL-2 on day 1 and the day of discharge / transferal in trauma patients does not significantly differ from the healthy control NK cell response towards IL-2 supplementation. Significantly impaired IL-2 response was found on day 8 in trauma patients compared to healthy controls (s. Figure 3.5 B)

Neutralization of IL-10 did not have immune enhancing effects on trauma patient NK cells. While healthy control CD56^{bright} NK cell specific IFN-γ production was significantly increased by blockage of IL-10, it did not increase IFN-γ production by trauma patient NK cells at any time after admission (s. Figure 3.5 C). The NK cell response to anti-IL-10 in trauma patients was found to be significantly impaired compared to healthy controls at each point of time, with maximum impairment on day 8 (s. Figure 3.5 D).

While NK cells responded to IL-2 at least on day 1 after trauma and on the day of discharge / transferal, neutralization of IL-10 remained without any positive effects. Positive effects of IL-2 could not be shown for day 8 after injury. Thus, IL-10 does not play a role in the suppression of NK cells after trauma and NK cells are refractory to IL-12 and IL-2 on day 8 after injury.

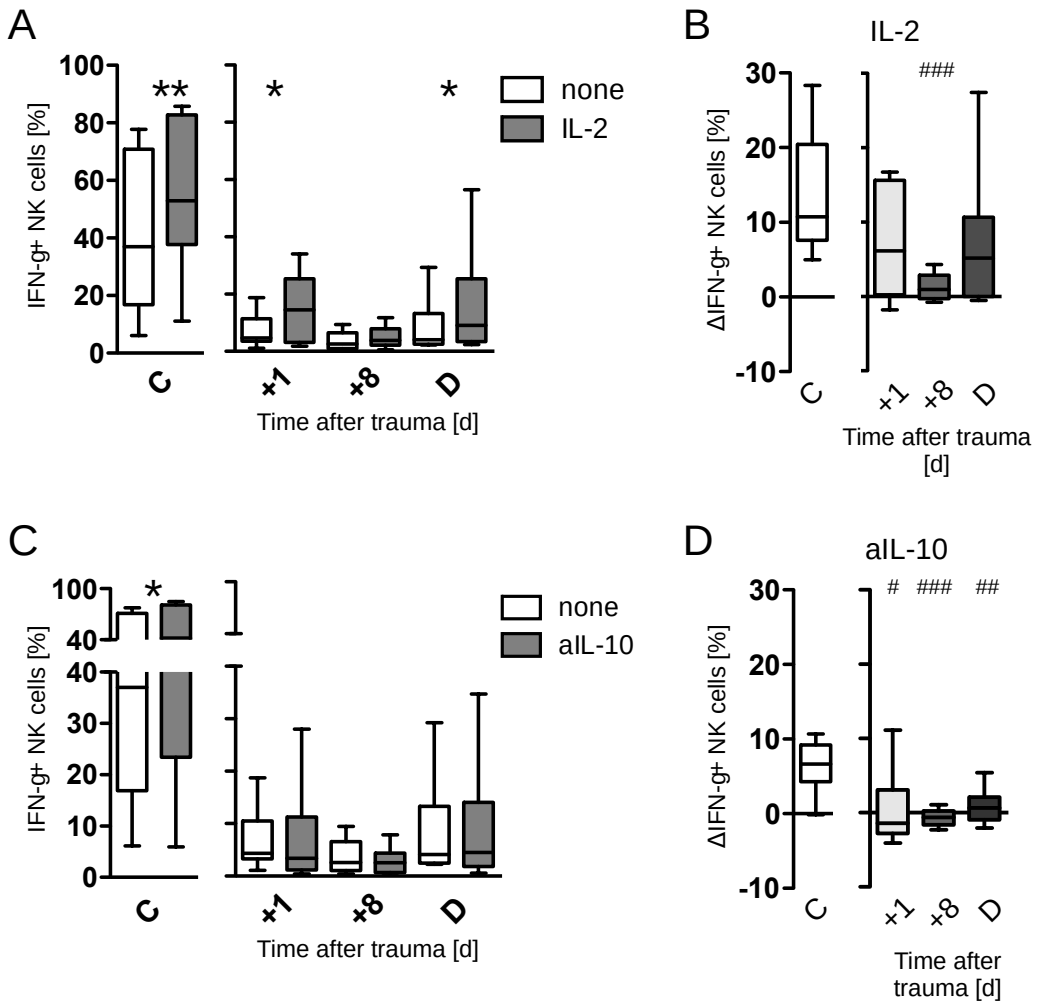

Figure 3.5 CD56^{bright} NK cell specific IFN-γ production upon adding IL-2 and neutralization of IL-10. Isolated PBMC of trauma patients (n=9 (A+B) / n=10 (C+D)) on days 1, 8 and discharge / transferal (D) plus healthy controls (C) after 23 hours of culture. Culture was carried out in the presence of *S. aureus* in autologous serum. **A)** IFN-γ production after simple *S. aureus* challenge and supplementation of 5 ng/ml IL-2. **B)** Increase of IFN-γ production from baseline *S. aureus* induced IFN-γ production upon supplementation of 5 ng/ml IL-2. **C)** IFN-γ production after simple *S. aureus* challenge and supplementation of 10 µg/ml anti-IL-10. **D)** Increase of IFN-γ production from baseline *S. aureus* induced IFN-γ production upon supplementation of 10 µg/ml anti-IL-10. Bars depicted as Box and Whiskers, min to max. Statistical analysis was performed using the Wilcoxon signed rank test (* $p < 0,05$; ** $p < 0.01$) in A and C, the Friedman test for comparison within the group of patients and Mann Whitney test (# $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$) for comparison with control group in B and D.

3.3 CD56^{bright} NK cell IL-12 signalling upon *S. aureus* challenge

3.3.1 CD56^{bright} NK cell IL-12Rβ2 chain expression is reduced after trauma and goes along with impaired IFN-γ production

For further understanding the underling pathophysiology of the impaired IL-12 responsiveness, the IL-12 signalling pathway was investigated. The first step was to analyse reception of IL-12 via NK cell receptors. As described in chapter 1.3.2.3 a functional IL-12 receptor is made up of a β1 and β2 subunit. While the β1 subunit is constituently expressed, CD56^{bright} NK cells do not express the β2 subunit when not stimulated (s. Figure 3.6 A). This observation was independent of the day of investigation.

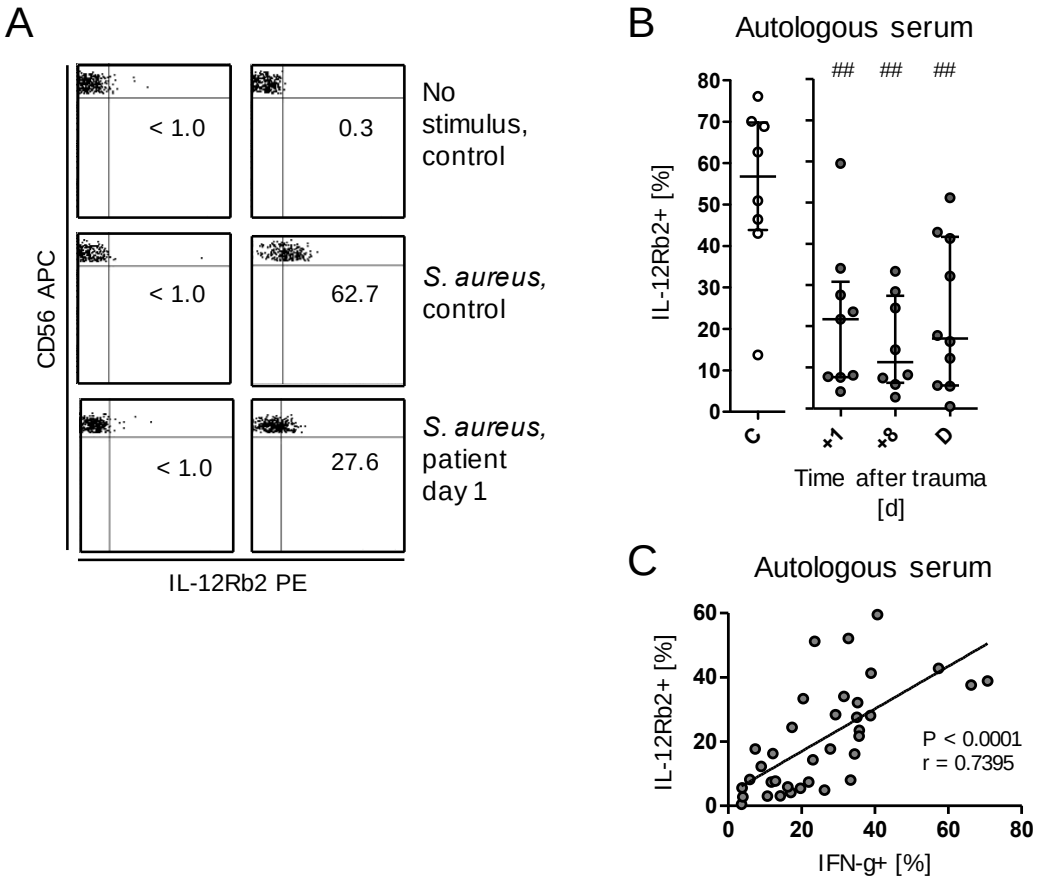

Figure 3.6 CD56^{bright} NK cell-specific IL-12Rβ2 chain expression after trauma and its association with IFN-γ production (autologous serum). Isolated PBMC of trauma patients (n=10) and healthy controls (C) after 18 hours of culture. Culture was carried out in presence of inactivated *S. aureus* in autologous serum. **A)** Representative dot plots showing CD56^{bright} NK cell specific IL-12Rβ2 chain expression of a healthy control and a patient on day 1 after trauma as well as an unstimulated negative control. **B)** Stimulation on days 1, 8 and discharge / transferal (D) plus healthy controls. Horizontal lines represent the median and interquartile range. Statistical analysis was performed using the Mann Whitney test (## $p < 0.01$) for comparison with control group. **C)** Correlation of IL-12Rβ2 chain expression and IFN-γ production after stimulation plus IL-12 (1 ng/ml). Correlation analysis was done using Spearman r.

In order to evaluate the NK cells ability to increase the expression of the IL-12Rβ2 chain, PBMC were cultured in the presence of inactivated *S. aureus* before staining for cell surface receptors. The competence of NK cells to increase IL-12Rβ2 chain expression was significantly impaired following trauma compared to healthy controls. Expression was found to be lowest on day 8. While the ability to increase the IL12-Rβ2 chain expression returned to almost normal at discharge / transferal in some patients, it remained impaired in others (s. Figure 3.6 B).

When looking at the linkage of IL-12Rβ2 chain expression and IFN-γ production upon stimulation with inactivated *S. aureus,* these variables were found to correlate. Those patients with most impaired IL-12Rβ2 chain expression had the lowest capacity to produce IFN-γ and vice versa (s. Figure 3.6 C).

Since the culture medium had been prepared from a possibly suppressive autologous serum, cells were additionally cultured in medium prepared from FCS. Interestingly the IL-12Rβ2 chain expression of trauma patients was about 100% higher when cultured in FCS compared to autologous serum (s. Figure 3.6 B and 3.7 A). This aspect could not be shown for healthy controls where IL-12Rβ2 chain expression was unaffected by culture environment.

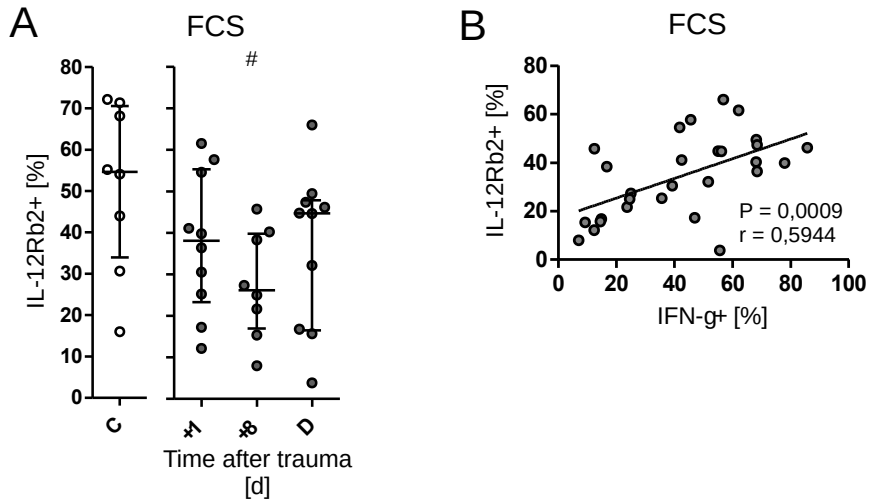

Figure 3.7 CD56^{bright} NK cell specific IL-12Rβ2 chain expression after trauma and its association with IFN-γ production (FCS). Isolated PBMC of trauma patients (n=10) and healthy controls (C) after 18 hours of culture. Culture was carried out in presence of inactivated *S. aureus* in FCS. **A)** Stimulation on days 1, 8 and discharge / transferal (D) plus healthy controls (C). Horizontal lines represent the median and interquartile range. Statistical analysis was performed using the Mann Whitney test (# $p < 0.05$) for comparison with control group. **B)** Correlation of IL-12Rβ2 chain expression and IFN-γ production after stimulation plus IL-12 (1 ng/ml). Correlation analysis was done using Spearman r.

The suppressive effect of trauma on IL-12Rβ2 chain expression upon bacterial challenge could be confirmed for culture in FCS. A significant difference though was only found on day 8 compared to healthy controls (s. Figure 3.7 A). The correlation of IL-12Rβ2 chain expression and IFN-γ production could also be shown for cells cultured in FCS (s. Figure 3.7 B).

Thus, NK cells are clearly suppressed in the expression of the variable IL-12Rβ2 chain upon bacterial challenge on all days following the incident. Independent of the culture medium the IL-12Rβ2 chain expression was lowest on day 8 after the incident. Interestingly this reduced expression correlated with the $CD56^{bright}$ NK cell specific IFN-γ producing capacity, suggesting a substantial link.

3.3.2 $CD56^{bright}$ NK cell IL-12Rβ1 chain expression tends to be reduced early after trauma

Since IL-12 signalling requires both IL-12 receptor subunits to be expressed, $CD56^{bright}$ NK cell specific expression profiles of the β1 chain were also investigated. The IL-12Rβ1 chain in contrast to the IL-12Rβ2 chain is permanently expressed on the NK cell surface. In the early phase after trauma the IL-12Rβ1 cell surface expression tended to be reduced compared to healthy controls and day of discharge or transferal. Those differences though were not statistically significant (s. Figure 3.8 A).

There was no significant difference in IL-12Rβ1 chain expression upon bacterial challenge. Neither compared to unchallenged cells nor compared to healthy control challenged NK cells (s. Figure 3.8 B). There was no correlation between IL-12Rβ1 chain expression and IFN-γ production (data not shown).

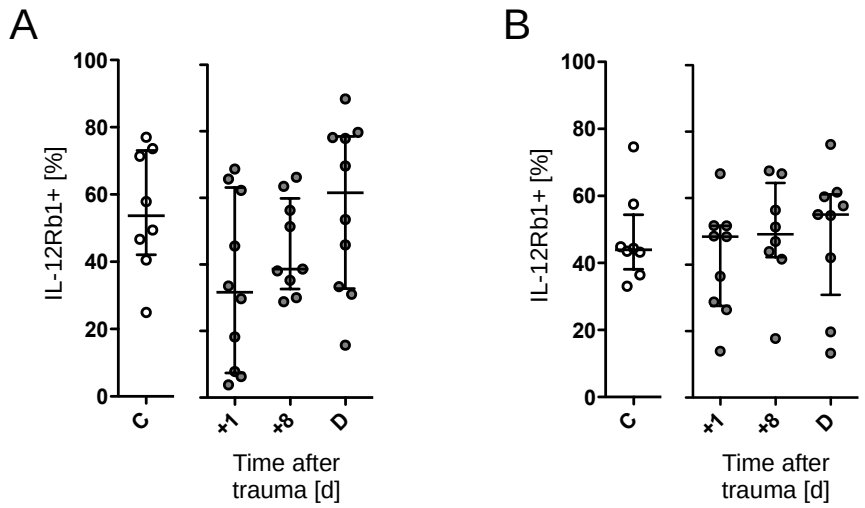

Figure 3.8 CD56^{bright} NK cell specific IL-12Rβ1 chain expression after trauma. Isolated PBMC of trauma patients (n=10) on days 1, 8 and discharge / transferal (D) plus healthy controls (C). **A)** Direct staining following PBMC isolation. **B)** Staining after 18 hours of culture in the presence of *S. aureus*. Scattered dot plots with horizontal lines representing the median and interquartile range. Statistical analysis was performed using the Mann Whitney test for comparison with control group.

3.3.3 STAT4 phosphorylation in CD56^{bright} NK cells is impaired after trauma and correlates with upstream IL-12Rβ2 chain expression and downstream IFN-γ production

When activated by binding IL-12, a functional IL-12 receptor forms a heterodimer and activates a Janus kinase that has tyrosine kinase activity. Phosphorylation of tyrosine residues leads to STAT4 activation, the formation of STAT4 homodimers and subsequent translocation into the nucleus where this complex acts as a transcription factor. Target genes include the genes for cytokines like IFN-γ and cytokine receptors like the IL-12Rβ2 subunit. Since only pSTAT4 has the capacity to form homodimers and function as a transcription factor we investigated whether there was a change in pSTAT4 following trauma.

NK cells were analysed after 18 hours of culture in the absence or presence of *S. aureus.* Due to a wide variance in basal STAT4 levels in trauma patients as well as in healthy controls absolute pSTAT4 values were found not suitable for comparison. Instead, the S. aureus-induced increase in STAT4 phosphorylation (ΔpSTAT4) was calculated (s. Figure 3.9 A).

CD56^{bright} NK cell specific increase in STAT4 phosphorylation was significantly impaired after trauma. Compared to healthy controls the loss of STAT4 phosphorylation was significant on day 1 and 8 with the lowest response to bacterial challenge seen on day 8. No significant difference towards discharge or transferal was shown (s. Figure 3.9 B).

Considering the role of STAT4 within the IL-12 signalling pathway, upstream IL-12Rβ2 chain expression and downstream IFN-γ production were found to correlate with the extent of STAT4 phosphorylation. Those patients showing impaired STAT4 phosphorylation expressed lower cell surface IL-12Rβ2 chain and displayed lower IFN-γ production (s. Figure 3.9 C and D).

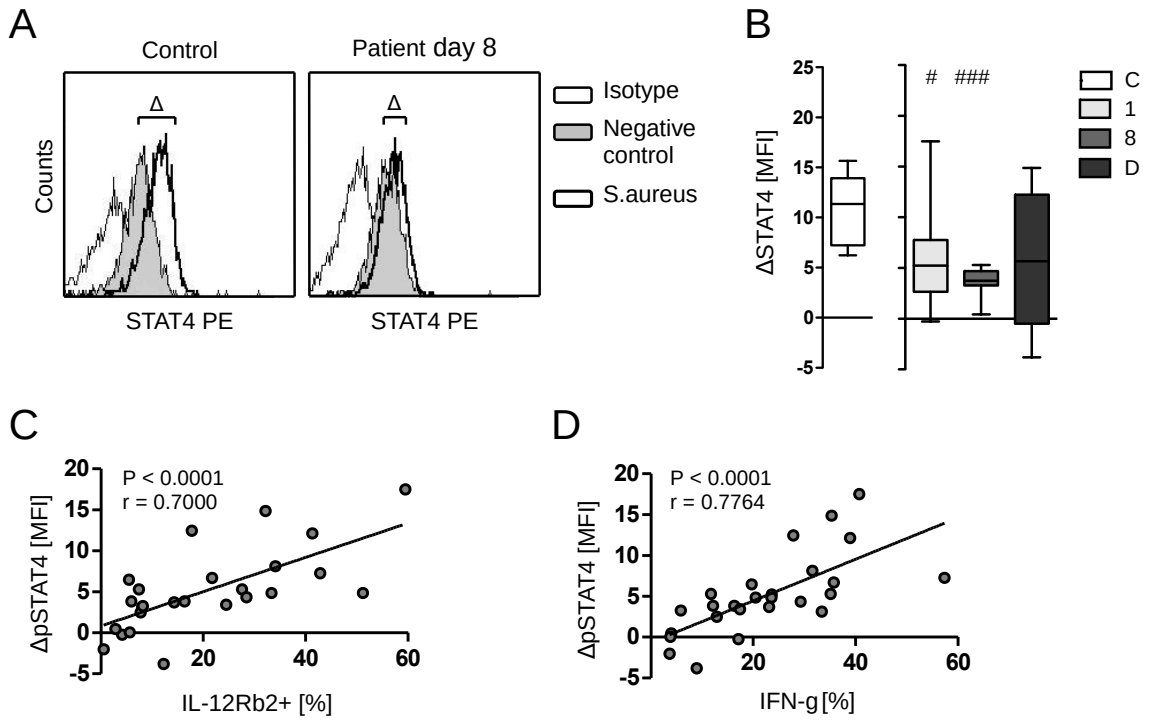

Figure 3.9 CD56^{bright} NK cell STAT4 phosphorylation after trauma and its association with IL-12Rβ2 chain expression and IFN-γ production. Isolated PBMC of trauma patients (n=10) and healthy controls (C) after 18 hours of culture. Culture was carried out in the presence or absence of *S. aureus* in autologous serum. Difference in pSTAT4 between stimulated and unstimulated probe is depicted as ΔpSTAT4. **A)** Representative histogram showing the ΔpSTAT4 after culture with and without stimulation for a healthy control and a patient on day 8. **B)** Increase in pSTAT4 on days 1, 8 and discharge / transferal (D) plus healthy controls. Bars depicted as Box and Whiskers, min to max. Statistical comparison between patients and control group performed using Mann Whitney test (# $p < 0.05$; ### $p < 0.001$). **C)** Correlation of ΔpSTAT4 and IL-12Rβ2 chain expression after stimulation with *S. aureus*. **D)** Correlation of ΔpSTAT4 and IFN-γ production after stimulation with *S. aureus* and IL-12 (1 ng/ml). Correlation analysis was done using Spearman r.

When cultured in FCS, results for STAT4 phosphorylation corresponded to the results found for culture in autologous serum. The increase in phosphorylation of STAT4 was higher comparing culture in FCS to culture in autologous serum. Still this increase in STAT4 phosphorylation was impaired when compared to healthy control cells cultured in FCS. This effect though was only found to be significant on day 8. Healthy control NK cell increase in STAT4 phosphorylation upon bacterial challenge was not influenced by culture media.

Correlation of increase in STAT4 phosphorylation with IL-12Rβ2 chain expression and IFN-γ production paralleled the results for autologous serum (data not shown).

Thus, the suppressed IFN-γ production after trauma correlates with reduced IL-12Rβ2 chain expression and reduced STAT4 phosphorylation. The IL-12 receptor signaling was maximally impaired on day 8 after the incident and was independent of the serum in the culture media.

3.3.4 CD56^{bright} NK cell NKG2D receptor expression is reduced in the initial phase after trauma while CD62L expression remains unaffected

NK cell activation is not only dependent on cytokine stimulation but also on cell-to-cell contact. The NKG2D receptor is a well-known activating receptor expressed on NK cells. It binds ligands that are associated with cellular stress and its activation leads to upregulation of anti-infectious properties.

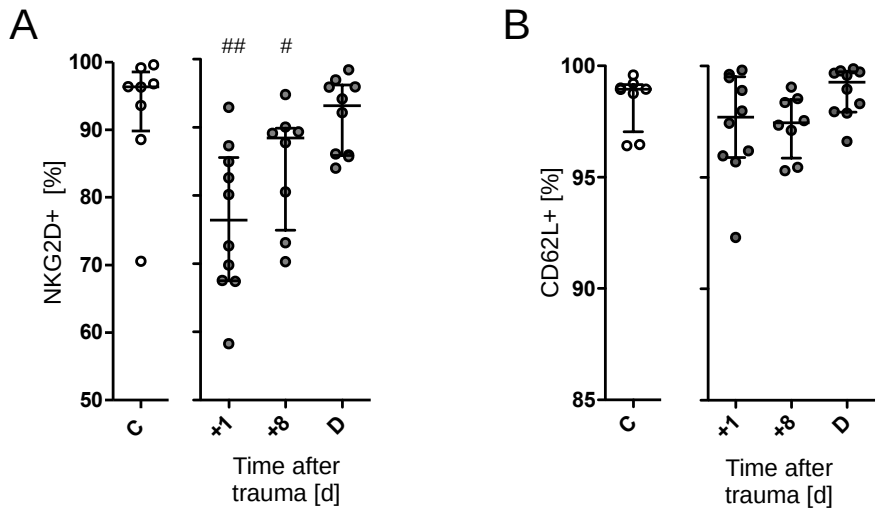

Figure 3.10 CD56^{bright} NK cell NKG2D receptor and CD62L receptor expression after trauma. Isolated PBMCs of trauma patients (n=10) on the days 1, 8 and discharge / transferal (D) plus healthy controls (C). Direct staining for **A)** NKG2D and **B)** CD62L following PBMC isolation. Scattered dot plots with horizontal lines representing the median and interquartile range. Statistical analysis was performed using the Mann Whitney test (# $p < 0.05$; ## $p < 0.01$) for comparison with control group.

Further analyses revealed a significantly impaired NKG2D expression on CD56^{bright} NK cells during the post trauma phase compared to healthy controls, especially on day 1 after trauma. At the time of discharge / transferal NKG2D expression returned to normal (s. Figure 3.10 A).

Since CD56^{bright} NK cells are rather known for their cytokine producing properties than their cytotoxicity, the CD62L cell surface receptor was additionally analysed. This receptor binds to HEVs and allows NK cells to migrate to lymph nodes where they interact with other cells of the innate and adaptive immune system. No difference in the CD62L expression in the phase after trauma was detected (s. Figure 3.10 B).

3.4 Serum of trauma patients exerts immunosuppressive effects

Our studies found impaired CD56^{bright} NK cell specific IFN-γ production and alterations in the underlying IL-12 signaling pathway after trauma. Remarkably, the replacement of autologous serum by FCS significantly increased IFN-γ production, IL-12Rβ2 expression, and STAT4 phosphorylation after trauma. Increase of IFN-γ production due to culture in FCS was also true for healthy control cells but to a much lesser extend (s. Figure 3.11 A). The data used for Figure 3.11 A are a combination of the data displayed in Figure 3.2 A and B. Previous studies have verified that FCS per se does not stimulate NK cells for IFN-γ synthesis (data not shown). These data led to the hypothesis that the serum from trauma patients suppresses NK cell function.

3.4.1 Serum of trauma patients tends to suppress CD56^{bright} NK cell IFN-γ production by healthy control cells

In order to investigate the effect exerted by the serum from trauma patients, PBMC from healthy controls were cultured in serum obtained from an individual trauma patient (n=10) on day 1 or 8 after trauma or in pooled serum from healthy volunteers (s. Figure 3.11 B). Since healthy donor cells were collected from different volunteers, all results were normalized to the value obtained from culture with the serum pool.

CD56^{bright} NK cells tended to produce less IFN-γ in the presence of the serum from trauma patients obtained on day 8. This effect though, was not statistically significant. Especially serum from day 1 partly shows a stimulatory effect on healthy control NK cells in terms of IFN-γ production (s. Figure 3.11 C).

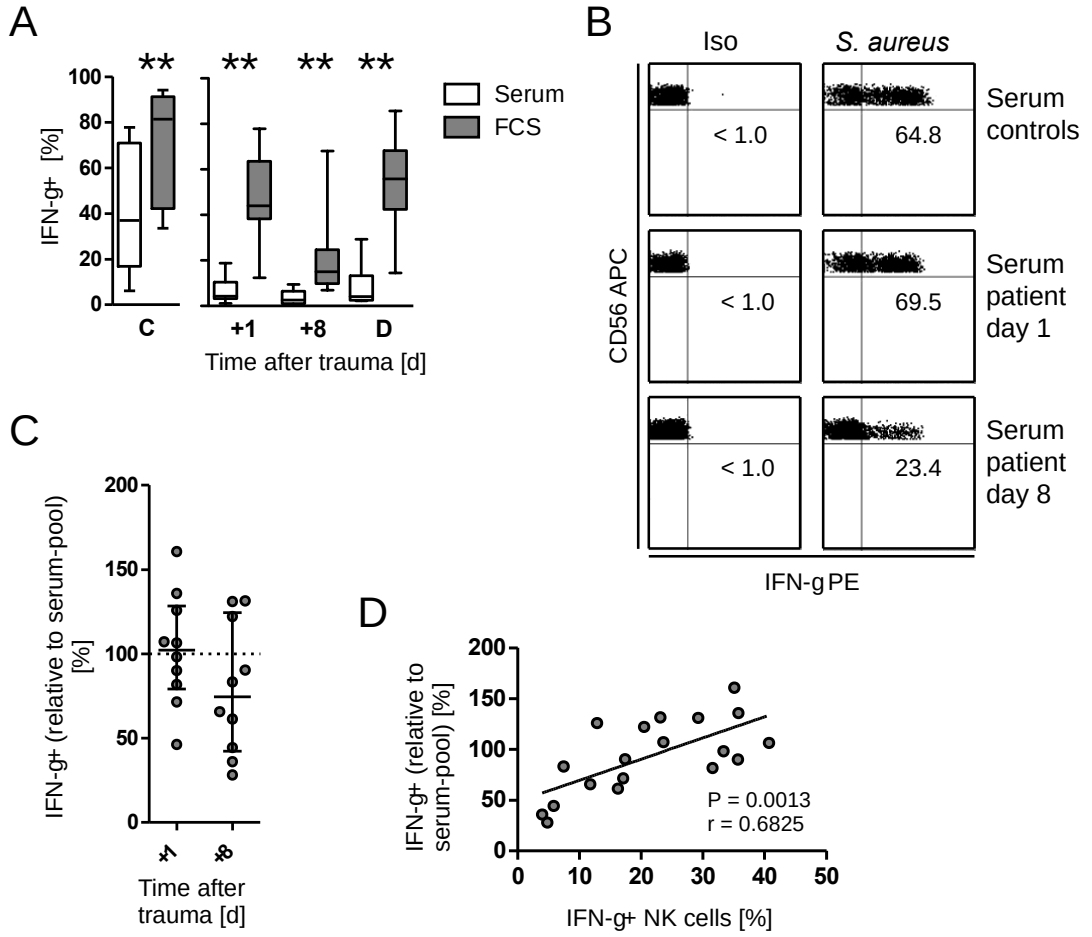

Figure 3.11 Effect of trauma patient serum on IFN-γ production by healthy control CD56^{brigh} NK cells. A) Isolated PBMCs of trauma patients (n=10) on the days 1, 8 and discharge / transferal (D) plus healthy controls (C) after culture in the presence of *S. aureus* in either autologous serum or FCS. Depicted as Box and Whiskers, min to max. Statistical analysis performed using Wilcoxon signed rank test (** $p < 0.01$). **B - D)** Isolated PBMC of healthy controls (n=10) cultured in serum pool and trauma patient day 1 and 8 serum in the presence of *S. aureus*. **B)** Representative dot plots showing IFN-γ production of healthy control cells cultured in serum pool and trauma patient day 1 and 8 serum. **C)** IFN-γ production of healthy control cells cultured with trauma patient day 1 and 8 serum analysed relative to culture in media prepared from serum pool. Scattered dot plots with horizontal lines representing the median and interquartile range. Statistical analysis was performed using Wilcoxon signed rank test **D)** Correlation of IFN-γ production of patients with the effect of their corresponding serum on IFN-γ production by healthy control cells. Correlation analysis was done using Spearman r.

The IFN-γ synthesis of CD56^{bright} NK cells of a given patient correlated with the IFN-γ synthesis of NK cells from healthy donors cultured in the presence of the corresponding serum of that same patient (s. Figure 3.11 D).

Thus, a circulating factor in the serum suppresses NK cells after trauma.

3.4.2 Serum of trauma patients suppresses IL-12Rβ2 expression and STAT4 phosphorylation

In analogy to the analysis of the IL-12 signalling pathway in trauma patients, it was investigated whether the serum from trauma patients exerted suppressive effects on IL-12 signalling. As described above, PBMC of healthy controls were cultured in serum from trauma patients obtained on day 1 or 8 or in pooled serum from healthy controls.

$CD56^{bright}$ NK cells showed significantly impaired IL-12Rβ2 chain expression when cultured in serum from day 8 compared to day 1. While serum from day 8 did mainly suppress receptor expression, serum from day 1 exerted suppressive as well as stimulatory effects relative to the control serum pool (s. Figure 3.12 B).
The positive correlation of IL-12Rβ2 chain expression and IFN-γ synthesis that was observed in NK cells after trauma (s. Figure 3.6 C), was similarly observed for the cells from healthy controls cultured in the serum of trauma patients. The serum that induced lower levels of cell surface IL-12Rβ2 chain expression did also induce less IFN-γ synthesis and vice versa (s. Figure 3.12 C).

Further, the shift in STAT4 phosphorylation was analyzed. In the presence of trauma patient serum, the increase in STAT4 phosphorylation after exposure to inactivated *S. aureus* was severely reduced. Trauma patient serum from both, day 1 and 8, suppressed the change in STAT4 phosphorylation by around 50 %. Though, this effect was significantly stronger in the presence of serum from day 8 (s. Figure 3.12 D). There was no correlation of change in STAT4 phosphorylation with either upstream IFN-γ production or downstream IL-12Rβ2 chain expression (data not shown).

In summary, the data does not only show an immunosuppressive effect of the serum from trauma patient in terms of impaired $CD56^{bright}$ NK cells IFN-γ synthesis, but also demonstrated alterations in IL-12 signaling that is necessary for IFN-γ production.

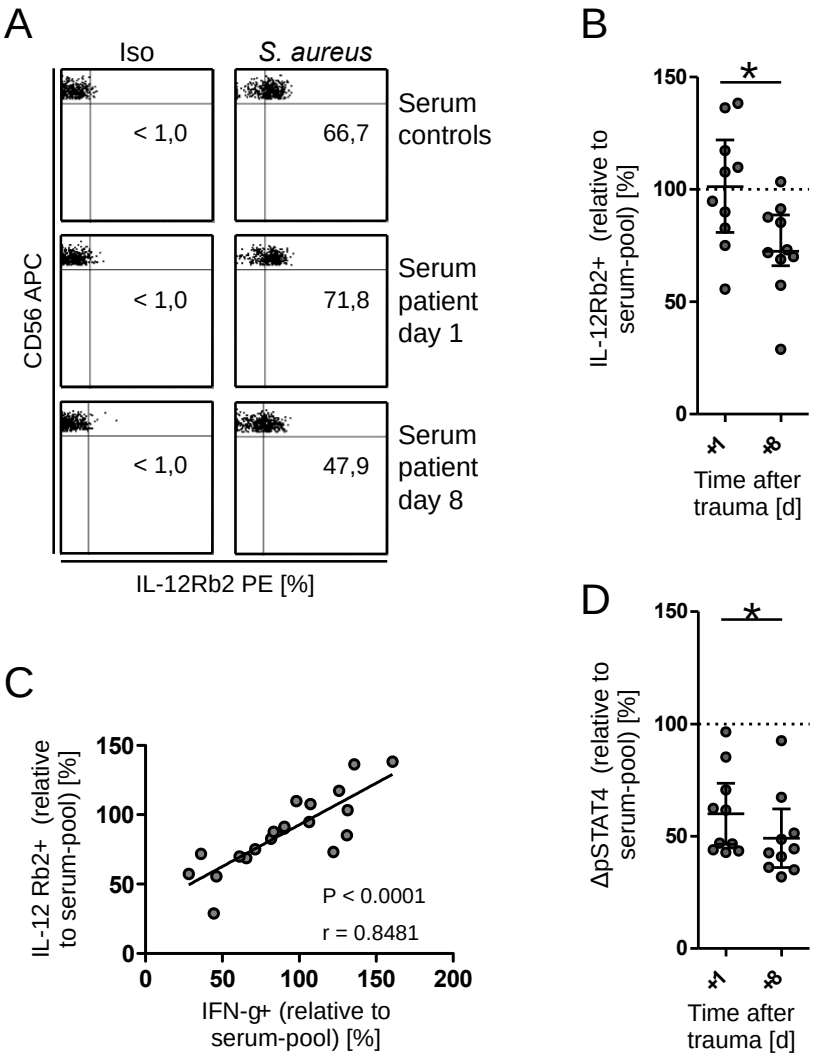

Figure 3.12 Effect of trauma patient serum on IL-12Rβ2 expression and STAT4 phosphorylation by healthy control CD56^{brigh} NK cells. Isolated PBMC of healthy controls (n=10) cultured in serum pool and trauma patient serum day 1 and 8 in the presence of *S. aureus*. **A)** Representative dot plots showing IL-12Rβ2 chain expression after culture in healthy controls as well as patient serum day 1 and 8 **B)** IL-12Rβ2 chain expression after culture in patient serum day 1 and 8 relative to culture in pooled serum of healthy controls. Scattered dot plots with horizontal lines representing the median and interquartile range. Statistical analysis performed using Wilcoxon signed rank test (* $p < 0.05$). **C)** Correlation of the effect of trauma patient serum on IFN-γ production and IL-12Rβ2 chain expression of healthy control cells. Correlation analysis was done using Spearman r. **D)** Change in pSTAT4 after culture in patient serum day 1 and 8 relative to culture in pooled serum of healthy controls. Scattered dot plots with horizontal lines representing the median and interquartile range. Statistical analysis performed using Wilcoxon signed rank test (* $p < 0.05$).

4 Discussion

Severely injured patients who survive the initial phase following severe trauma are at increased risk to develop secondary complications during their hospital stay. Especially an increased susceptibility for infections, sepsis and MOF have been described as main contributors to late trauma-related death (de Knegt et al., 2008; Lansink et al., 2013). The underlying pathophysiology is still incompletely understood.

Researchers have put a lot of effort in analysing the role of various cell types and soluble components in the post trauma phase as well as during sepsis. Most studies focused on mainly monocytes, macrophages, DCs and lymphocytes. During the last couple of years there was a rising interest in NK cells and their role in the immune response. Being part of the innate immune system and having the ability to interact with various immune cells via the production of cytokines and cell-to-cell contacts, NK cells take a central role in the immune response (Gerosa et al., 2002; Borg et al., 2004). More precisely the present study addressed the $CD56^{bright}$ NK cell subtype that is known for its ability to produce abundant amounts of IFN-γ upon activation. The fact that they are primarily found within the lymphatic tissue further supports their importance in immunoregulation as this is where they interact with other immune cells.

The goal of this pilot study was to find out whether $CD56^{bright}$ NK cells are impaired in their ability to produce IFN-γ upon bacterial challenge following tremendous injury. Since IL-12 has been described to be the major cytokine that stimulates NK cells for cytokine production, aspects of the underlying IL-12 signalling pathway, eventually leading to IFN-γ production and secretion, were additionally investigated. 10 severely injured patients were included in the present study.

4.1 Epidemiology of trauma has changed in our sophisticated trauma system

The latest WHO data from 2016 give a very good overview on trauma as a burden for society worldwide. Even though with only 10 patients our sample was very small it did represent the trauma population of European countries very well. Road injuries were the most common mechanism of trauma followed by falls with falls being found more often in elderly patients. This correlates with worldwide as well as data for Europe (WHO [2], 2018).

With 8 out of 10 patients in being male in the present study, men were more often subject to severe trauma than women. The median age of the recruited patients was 48 (44-55) years and blunt trauma was the leading mechanism of injury. These characteristics fit the results of others trauma studies conducted in first world countries (Hoover et al., 2006; Hietbrink et al., 2013).

According to Trunkey et al. trauma death could be categorized in immediate, early and late trauma death (Trunkey, 1983). Only patients who survived the immediate and early phase after trauma were admitted to our ICU and included in our study. Since none of the patients included died, we could not confirm the third peak of late trauma death. This goes along with data of many recent studies arguing that the trimodal system is no longer applicable in a mature trauma system (de Knegt et al., 2008; Lansink et al., 2013). A more applicable outcome parameter would be late trauma complications such as infection with sepsis or MOF. Those represent a major individual and socioeconomic burden (Dewar et al., 2013; Ulvik et al., 2007). Hietbrink et al. included 36 patients with a median ISS of 24 of whom 11 developed Sepsis (Hietbrink et al., 2013). None of the patients in this study developed sepsis.

4.2 Impaired CD56^{bright} NK cell IFN-γ synthesis following severe injury may predispose for pathogen triggered complications

Different authors have described a state of immune paralysis following severe injury. This state of impaired immune defence has been associated with an increased risk for infectious complications that may lead to sepsis (Bone, 1996; Moore et al., 1996). All those adverse events have a possibly fatal outcome and even if not fatal, they prolong hospital stay and long-term morbidity, thus adding to the socioeconomic burden caused by severe trauma (Dewar et al., 2013; Ulvik et al., 2007).

The present study clearly shows an impaired capacity of trauma patient CD56^{bright} NK cells to produce IFN-γ in upon exposure of PBMC to inactivated *S. aureus*. By using intracellular staining of CD56^{bright} NK cells, individual NK cell suppression rather than an overall impairment in PBMC cytokine production was assessed. The results were compared among the days after trauma and to healthy controls.

NK cell suppression was seen throughout the whole observation period. Regardless of whether the cells were cultured in media prepared from autologous serum or in a neutral cell culture environment prepared with FCS. CD56^{bright} NK cell specific IFN-γ production was

found to be the lowest on day 8 postinjury. On the day of discharge most patients' NK cells showed signs of recovery in their ability to produce IFN-γ while few remained severely impaired.

Many authors describe the highest incidence of sepsis and associated complications to be found between 5 and 10 days after trauma (Faist et al., 1983; Hietbrink et al., 2013; Hoover et al., 2006; Waydhas et al., 1992; Moore et al., 1996). This time frame complies very well with the minimum of NK cell IFN-γ production found on day 8 after the insult. The fact that the immune function of NK cells appears to drop towards the end of the first week supports the assumption that an increased susceptibility to late trauma complications goes along with NK cell suppression. Since this study demonstrated that cellular immune dysfunction occurred even in a neutral culture environment, immune suppression cannot be solely attributed to an immunosuppressive milieu.

Hoover et al. conducted one of the most comprehensive studies in terms of patients included. They prospectively included 1277 patients with severe trauma and assessed the occurrence of nosocomial infection. 580 (45.4 %) of the patients developed nosocomial infection. The mean time to the onset was 8.4 days. In accordance with our data on NK cell immune paralysis, infection was seen in 318 and 320 patients during week one and two postinjury (Hoover et al., 2006).

NK cells play a central role in early immune defence against numerous pathogens. Nevertheless, they are only one component in a complex arrangement of cellular and soluble factors forming host defence. Hietbrink et al. could give interesting insight into the kinetics of immune paralysis after trauma. They analysed daily blood samples of 36 trauma patients with a mean ISS of 24 for 10 days after injury. They found the lowest polymorphonuclear neutrophil (PMN) response upon N-formyl-methionyl-leucyl-phenylalanine challenge within the first week. Additionally, they looked at monocyte HLA-DR expression that remained half of that of healthy controls throughout the first week. Those signs preceded the onset of septic shock symptoms which presented between day 8 and 10 in nine out of ten patients (Hietbrink et al., 2013). Taking their data into consideration it appears that immune paralysis towards the end of week one is not NK cell specific but rather a ubiquitous phenomenon of the innate immune system.

Looking at IL-12, the most potent inductor of NK cell IFN-γ production, Spolarics et al. showed that the LPS-induced IL-12 production from monocytes of trauma patients is reduced by 40 to 70%. They took probes of 12 severely injured male patients averaging an

ISS of 26 and a mean age of 35 years. Probes were collected on days 2, 5 and 10 postinjury and culture was carried out in whole blood samples that were preincubated with IFN-γ to achieve maximal IL-12 response. Their data indicates a relatively constant suppression of IL-12 production throughout the observation period without a significant change in circulating blood monocytes (Sporalics et al., 2003). The impaired IL-12 production was confirmed in this study by analysing supernatants of *S. aureus* challenged PBMC. In contrast to the data presented by Spolarics et al., our data rather indicate an increase in suppression towards day 8 following the incident. This difference might be due to the fact that Sporalics at el. looked at IL-12 positive monocytes challenged after preincubation with IFN-γ, while this study analysed cell culture supernatants representing whole PMBC IL-12 production without supplementation.

Sporalics at el. also analysed trauma patient $CD4^+$ and $CD8^+$ T cells in respect to IFN-γ production upon phorbol ester plus ionomycin stimulation. Interestingly they found no reduction in IFN-γ synthesis compared with healthy controls. They propose that impaired monocyte IL-12 production is not associated with lower IFN-γ production by $CD4^+$ and $CD8^+$ cells (Spolarics et al., 2003). Considering the present study, it appears plausible that there is an association of impaired monocyte IL-12 production and $CD56^{bright}$ NK IFN-γ production. Since monocytes need IFN-γ and NK cells depend on IL-12 for proper cytokine production, impairment of one, inevitably leads to a downward spiral.

The stimulatory effect of IL-12 was illustrated by supplementing IL-12 to PBMC cultured in the presence of *S. aureus.* IL-12 increased NK cell IFN-γ production on each day of the study. The IL-12 response though differed throughout the observation period. While supplementation of IL-12 could enhance $CD56^{bright}$ NK cell IFN-γ production on days 1 and 4 to reach a level almost matching healthy controls, the IL-12 response significantly decreased afterwards with only minimal response seen on day 8. Among the group of patients, the variance in the IL-12 response was most pronounced on the day of discharge, indicating recovery of NK cell function in some patients while others remained impaired in their ability to enhance IFN-γ production upon IL-12 supplementation.

In healthy controls supplementation of IL-12 did also increase NK cell IFN-γ production significantly. A big variance in IFN-γ production upon simple *S. aureus* challenge was found. Those controls having low IFN-γ production showed a more pronounced IL-12 response. This suggests a physiological limit to IFN-γ production that cannot be exceeded

by additional stimulation. The limit might be set by saturation of any of the components involved in the IL-12 signalling pathway.

The findings of this study indicate a tremendous decline in NK cells IFN-γ production after trauma. The impairment seems to be at least partly caused by insufficient IL-12 supply. Considering the NK cell dependence on IL-12 and the monocyte dependence on IFN-γ, a lack of either of those immune signals can cause a downward spiral manifesting in severe immune depression.

4.3 Alterations in IL-12 signalling may partly be responsible for the impaired NK cell IFN-γ production after trauma

Our data clearly demonstrate a state of immune paralysis following severe trauma. This condition of impaired IFN-γ synthesis upon bacterial challenge was only partly restored by abundant IL-12 which is the most relevant inductor of NK cell IFN-γ production. Only a minimal IL-12 response was seen on day 8 postinjury indicating impaired IL-12 signalling 1 week after trauma. For further understanding of the underlying pathophysiology the present study looked at certain aspects of the IL-12 signalling pathway in NK cells.

In order to assess the amount of IL-12 available for NK cell IFN-γ production, supernatants of PBMC subjected to bacterial challenge were analysed in regard to IL-12 produced. Since only the heterodimer IL-12 p70, composed of the IL-12 p40 and p35 subunits, induces NK cell IFN-γ production, the production of IL-12 p70 but not of the subunits was addressed in the present study. Accordingly, no statement about the synthesis of the above-mentioned subunits can be made. This though is actually of interest since Weighardt et al. found severely supressed monocyte IL-12 p70 production despite IFN-γ induced recovery of IL-12 p40 production in 35 patients with postoperative sepsis (Weighardt et al., 2000). In mice the IL-12 p40 homodimer was proposed to be a functional antagonist of the IL-12 p70 heterodimer (Gillessen et al., 1995). Other authors in contrast did not find this thesis applicable for humans since the formation of the IL-12 p40 homodimer has never been observed in regular human cells (Carra et al., 2000; Trinchieri, 2003).

According to data from this study, reduced levels of IL-12 could definitely contribute to an impaired immune response upon bacterial challenge. Since the supplementation of abundant levels of IL-12 p70 could not restore the NK cell-derived IFN-γ production, alterations in IL-12 signalling must be considered.

In 1996 Presky et al. described a functional high affinity IL-12 receptor to consist of two β type subunits namely the IL-12Rβ1 and IL-12Rβ2 chain (Presky et al., 1996). In accordance with previous studies, trauma patient NK cells in this study constantly expressed the IL-12Rβ1 chain (Wang et al., 2000; Reinhardt et al., 2015). Data from our group published in 2015 showed a significant decrease in NK cell IL-12Rβ1 expression the day after major visceral surgery compared to the day before surgery (Reinhardt et al., 2015). The present study, in accordance, showed a tendency of reduced IL-12Rβ1 chain expression on day 1 compared to the other days postinjury as well as compared to healthy controls. This impaired expression on day 1 did not correlate with any other variable assessed in this study. Following bacterial challenge, trauma patient Il-12Rβ1 chain expression did not differ on the days postinjury, neither was there any difference to healthy controls. Thus, it is unlikely that this minor alteration in IL-12Rβ1 expression contributes to the impaired signalling of IL-12.

Expression of the Il-12Rβ2 chain in contrast to the Il-12Rβ1 is positively influenced by cytokines such as IL-2 and IL-12. Wang et al. found low IL-12Rβ2 expression on resting NK cells (Wang et al., 2000). In contrast, in the present study neither resting $CD56^{bright}$ nor $CD56^{dim}$ NK cells expressed that inducible component of the IL-12 receptor ex vivo. Only after bacterial challenge with *S. aureus,* NK cells expressed the IL-12Rβ2 chain on the cell surface. The IL-12Rβ2 expression on NK cells from trauma patients was severely depressed compared to healthy controls on all days postinjury. The lowest expression was found on day 8 following the incidence. A significant correlation of receptor expression and IFN-γ response was found, indicating a physiological link.

In contrast to T cells, the IL-12Rβ2 chain expression on NK cells has not been studied in depth so far. In 1997 Rogge et al. published data on IL-12Rβ2 chain dependent signalling in polarised Th1 and Th2 cells using human T cells. Unpolarised T cells were either cultured in an IL-12 or IL-4 predominant milieu favouring Th1 and Th2 polarisation, respectively. They could demonstrate that Th1 and Th2 cells expressed IL-12Rβ1 transcripts in similar amounts, while IL-12Rβ2 transcripts were almost exclusively expressed by Th1 cells. Consequently, Th2 cells were unable of high affinity IL-12 binding. IL-12 could transiently induce IL-12Rβ2 chain expression on Th1 as well as Th2 cells while IL-4 in combination

with neutralizing IL-12 lead to a loss of IL-12β2 chain and downstream STAT4 phosphorylation (Rogge et al., 1997).
Applying this data, it is more likely that the impaired expression of the IL-12Rβ2 chain instead of the IL-12Rβ1 chain contributed to the suppression of NK cells after trauma. Considering that it is the IL-12Rβ2 chain containing cytoplasmic tyrosine residues needed for downstream signal transduction, this thesis becomes even more plausible (Presky et al., 1996; Zou et al., 1997).

As already mentioned, binding of IL-12 to its receptor leads to the phosphorylation of STAT4 through the receptor-associated Janus kinase. Only pSTAT4 can form homodimers that translocate to the nucleus and bind to the corresponding DNA promoter region. There they activate target gene transcription. Important target genes include IFN-γ and the IL-12Rβ2 chain. Studies in mice clarified that IL-12 effects depend on a functional STAT4 in NK cells (Thierfelder et al., 1996). Since the present study found a great variance in basic STAT4 expression among trauma patients as well as among healthy controls, the difference expressed as ΔpSTAT induced by bacterial challenge was determined. NK cells from trauma patients showed an impaired capacity to phosphorylate STAT4 tyrosine residues upon stimulation. In accordance with the data on IFN-γ production and IL-12Rβ2 chain expression, the lowest increase in STAT4 phosphorylation was found on day 8 following the incident.
The current observations on STAT4 phosphorylation is in line with a study on the modulation of NK cells by major surgical interventions published by our group in 2015. Reinhardt et al. found a 5-fold increase in pSTAT4 in $CD56^{bright}$ NK cells before surgery compared to 1.8-fold after surgery upon bacterial challenge. This reduced increase might have been due to a higher baseline pSTAT4 in the absence of bacterial challenge which rose from around 10 % before to roughly 40 % after surgery (Reinhardt et al., 2015). It was hypothesized that the surgery-induced tissue damage caused the phosphorylation of STAT4 through so far unknown signals. Since data on the patients' NK cells before trauma was obviously not available, it remains unclear whether the reduced increase in pSTAT4 in NK cells after trauma was likewise caused by an injury-induced increase in the basal pSTAT4 level. High baseline pSTAT4 does possibly indicate a state of lower responsiveness towards bacterial challenge.

Thus, severe traumatic injury induces a profound disturbance of the IL-12 receptor signalling in $CD56^{bright}$ NK cells.

Besides IL-12, diverse pro- and anti-inflammatory signals are known to modulate the IFN-γ production by NK cells. IL-2 enhances the IFN-γ production by promoting IL-12 receptor and STAT4 expression (Wang et al., 2000). IL-10 is a well-described counter-regulatory cytokine. It inhibits NK cell-derived IFN-γ production. One aim of this study was to clarify whether supplementation of IL-2 or neutralization of IL-10 could restore the capacity of NK cells from trauma patient to synthesise IFN-γ.

The gathered data on immune reconstitution through IL-2 supplementation is controversial. Results from healthy controls clearly demonstrate an enhancing effect on IFN-γ production. In trauma patients the response of NK cells to IL-2 parallels the response to IL-12 supplementation, though to a lower extent. IL-2 significantly increased IFN-γ production on day 1 and at discharge, but only minor response was observed on day 8.

Those results seem logical considering that IL-2 itself does increase the IL-12Rβ2 expression rather than being an inductor of IFN-γ production itself. Fehniger et al. showed NK cell IFN-γ production to increase significantly when incubated with a combination of IL-2 and IL-12 compared to incubation with either one alone. Amounts of IL-12 could be reduced by up to 1000-fold when IL-2 was added. This effect of IL-2 is mediated through the high affinity IL-2 receptor αβγ, which is activated by picomolar amounts of IL-2 (Fehniger et al., 2003).

Previous to the study by Fehniger et al., Wang and co-workers demonstrated two underlying mechanisms in which IL-2 exerts its effect. IL-2 increased the NK cell IL-12Rβ2 chain expression 10-fold over NK cells not primed and 3-fold over NK cells primed with IL-12. Surprisingly the combination of IL-2 and IL-12 was inferior in terms of cell surface receptor expression. In addition, IL-2 enhanced the expression and DNA-binding capacity of STAT4, the main transcription factor mediating IL-12 signalling. In accordance IL-2 primed NK cells had superior IFN-γ producing capacity upon IL-12 treatment (Wang et al., 2000). Thus, priming of PBMC with IL-2 before bacterial challenge and IL-12 supplementation might be a promising approach to restore the IFN-γ production by NK cells after injury.

Applying this knowledge to the obtained results, the increased IFN-γ production achieved by supplementation of IL-2 seems plausible. Since there was some IL-12 produced within the cell culture of PBMC on day 1 and discharge, the IL-12 effects could be enhanced by

IL-2. On day 8 though, IL-12 concentrations in supernatants was lowest and therefore even if IL-12Rβ2 expression had been enhanced, there would have been insufficient IL-12 to activate the IL-12 receptor and induce IFN-γ production. Additionally, the capacity of IL-2 induced IL-12 receptor upregulation and STAT4 signal transduction might be impaired as well. The effects of IL-2 on IL-12 signal transduction though were not investigated in the present study.

According to the literature IL-10 acts as the counterpart to IL-12 in terms of immune modulation. Increased levels of serum IL-10 were shown to predispose for septic complications after blunt trauma (Giannoudis et al., 2000). Lyons et al. demonstrated that LPS-induced IL-10 secretion from PBMC obtained from trauma patients peaked at day 7 to 10 postinjury. The same study describes episodes of SIRS / Sepsis to have occurred from day 10 onward. The authors could further show that treating thermally injured mice with anti-IL-10 could restore their capacity to survive septic challenge by cecal ligation and puncture (Lyons et al., 1997). Other authors found trauma-induced impaired IL-12 and IFN-γ synthesis in mice splenocytes to recover upon neutralization of IL-10 (Toliver-Kinsky et al., 2002).
Since IL-10 is mainly known for its immunosuppressive effects, blocking of that cytokine seemed to be a plausible approach in trying to enhance the IFN-γ synthesis from NK cells. As expected, NK cells of healthy controls pre-treated with anti-IL-10 antibodies showed a slight increase in IFN-γ upon *S. aureus* challenge. Nevertheless, for trauma patient NK cells no positive effect of anti-IL-10 treatment was shown.
Since none of the studies demonstrating positive effects of IL-10 neutralization focussed on human trauma patient NK cells, the described increase in IFN-γ production could very well reflect the recovery of leukocytes distinct to NK cells, such as T cells.

Most of the aspects discussed so far focused on soluble components of intercellular interaction. NK cell interactions do also depend on cell-to-cell contacts. Borg et al. showed that effective DC mediated NK cell activation depends on a functional synapse, which drives DCs to secrete preassembled IL-12 towards NK cells. They found the formation DC / NK cell synapses to depend on cytoskeleton remodelling and raft mobilization (Borg et al., 2004).

Blockage of the NK cell surface receptor NKG2D was shown to impair NK cell IFN-γ production upon influenza virus infection induced NKG2D ligand expression on DCs (Draghi et al., 2007). Further data outlines the pivotal role of NKG2D mediated cell-to-cell contact in the NK cell enhanced clearance of parasites as well as bacteria (Guan et al., 2007; Jiao et al., 2011). The data from this study indicate a decline in $CD56^{bright}$ NK cell surface NKG2D expression early after severe trauma. However, we could not demonstrate a link between NKG2D expression and IFN-γ production. NKG2D expression returned to normal on day 8, the day of maximal NK cell suppression.

The CD62L receptor responsible for homing of NK-cells to secondary lymphatic tissue via high endothelial venules was not affected by trauma. In accordance with data by Frey et al. $CD56^{bright}$ NK cells expressed that surface receptor in high density (Frey et al., 1998).

In terms of NKG2D and CD62L our study only provides information on the cell surface expression, but not on a potential effect of these molecules on NK cell functionality.

4.4 Immunosuppressive effects after trauma are at least partly exerted by a circulating factor

All analyses of the patients NK cells were primarily set up in a culture environment containing the corresponding patient serum. In the context of impaired NK cell IFN-γ synthesis after trauma it is important to differentiate between cell-intrinsic and extrinsic NK cell suppression. Numerous studies and reviews point out various alterations in the serum after trauma and during sepsis, many of which have been linked to an increased risk for infections or worse outcome in case of infection (Bone, 1996; Flohé et al., 2008; Giannoudis et al., 2004; Kimura et al., 2010).

Since the immune response is closely regulated by cell-to-cell contacts as well as soluble components secreted and processed by cells, this study aimed to provide a cell culture environment not affected by trauma. FCS was used for preparation of a neutral cell culture environment. While the absence of autologous serum showed persistent NK cell suppression on day 8, indicating cell-intrinsic changes, it resulted in increased IFN-γ synthesis at earlier and later time points, indicating an inhibitory effect of trauma patient serum on NK cells. Moreover, in the absence of autologous serum NK cells of the trauma patients displayed enhanced IL-12Rβ2 chain expression and STAT4 phosphorylation on all days investigated. This observation points to a suppressive effect of the autologous serum and is supported by

a previous study that showed reduced LPS-induced TNF-α synthesis of healthy donor whole blood samples in the presence of serum of trauma patients (Majetschak et al., 1997).
A cell culture environment using day 1 and 8 trauma patient serum was prepared. Healthy control NK cells challenged with inactivated *S. aureus* in trauma patient serum were compared to the corresponding healthy control NK cells challenged in media prepared from a healthy control serum pool. While day 1 serum presented slightly stimulatory as well as suppressive effects, serum from day 8 did mainly show suppressive effects on healthy control NK cell IFN-γ production. Further, a positive correlation of trauma patient IFN-γ production and the effects of the corresponding serum on healthy control NK cell IFN-γ production was shown. Serum of patients with marked NK cell suppression had a more pronounced effect on healthy control cells than serum of patients presenting with less severe immunosuppression.

The suppressive effects of serum obtained 8 days after trauma were also shown for IL-12Rβ2 expression as well as STAT4 phosphorylation. Interestingly, when compared to control serum, trauma patients' serum from both days clearly supressed STAT4 phosphorylation. The suppressive effect was more pronounced with serum obtained on day 8. Considering that the IFN-γ production was not decreased by day 1 serum additional molecular mechanisms must exist that support IFN-γ production despite impaired STAT4 phosphorylation.

Besides the described IL-12 / STAT4 axis further studies revealed T-bet, a member of the T-box family of transcription factors, to be a potent regulator of an IFN-γ predominant Th1 immune response. T-bet is considered a Th1 favouring inductor and its expression was found to correlate with IFN-γ production in Th1 and NK cells (Szabo et al., 2000). Mullen et al. found T-bet to induce IFN-γ production by chromatin remodelling and enhance lL-12Rβ2 expression independent of IL-12 / STAT4. They concluded that T-bet induces Th1 polarisation while IL-12 dependent STAT4 activation rather fixes or enhances the commitment (Mullen et al., 2001). Townsend et al. were the first to focus on the role of T-bet in NK cells. Using T-bet deficient mice they found impaired maturation as well as target cell lysis and cytokine production. While IFN-γ production upon activation with IL-12 and IL-18 was unaffected in the first 24 hours, maintenance afterwards was clearly reduced in T-bet deficient mice (Townsend et al., 2004). Trauma-induced downregulation of T-bet

could therefor cause prolonged NK cell dysfunction and analysis of T-bet in the post trauma phase could give further insight into the mechanisms causing NK cell immune depression. Nevertheless, this study is the first to demonstrate that the serum from trauma patients suppresses $CD56^{bright}$ NK cells in terms of IFN-γ synthesis. Furthermore, the data obtained give insight into aspects of the underlying pathophysiology of trauma-induced immunosuppression. IL-12Rβ2 expression as well as STAT4 phosphorylation were shown to be impaired by trauma patient serum.

Further research on the trauma-induced suppression of NK cells should focus on additional aspects of inter- and intracellular signalling. Since IL-2 was shown to promote IL-12Rβ2 expression, an assay combining IL-2 and IL-12 would be promising approach to restore NK cell function. Additionally, reception of IL-2 should be analysed in the phase after trauma. Obviously, we proved that the sensitivity to IL-12 was impaired after severe injury. Since all intracellular signalling depends on proper cell surface cytokine sensing, especially the inhibition of IL-12Rβ2 expression should be addressed in future research. The transcription factor T-bet was found to enhance IL-12Rβ2 expression and therefore analysis of T-bet expression in trauma patients is one promising approach. While in this study neutralization of IL-10 did not show positive effects, possibly neutralization of IL-4, a Th2 favouring cytokine known to supress IL-12Rβ2 expression, might influence IL-12 reception positively. This study was conducted with a very limited volume of blood samples obtained from trauma patients. Since material from trauma patients is rare and results from animal studies cannot be simply transferred to humans, the development of an in vitro model of traumatic stress on leukocytes might facilitate the research on the restoration of NK cell function after severe injury.

5 Summary

Patients who survive the initial phase after severe injury are predisposed to develop nosocomial infections. Such infectious complications may lead to sepsis, a life-threatening, mostly bacterial, systemic infection. The present study aimed to give further insight into the underlying pathophysiology predisposing for infectious complications after severe injury.

Natural killer (NK) cells belong to the innate immune system and play a pivotal role in early pathogen clearance. This study focused on the $CD56^{bright}$ NK cells subset that is a main producer of the immune regulatory cytokine interferon (IFN)-γ. Monocyte-derived interleukin (IL)-12 is the most potent inductor of $CD56^{bright}$ NK cell IFN-γ production.

Blood samples were taken of 10 severely injured patients presenting with an Injury Severity Score of 16 and above on consecutive days following the incident. Peripheral blood mononuclear cells were isolated and cultured in the presence of inactivated *Staphylococcus aureus*.

The $CD56^{bright}$ NK cell-derived IFN-γ production was severely impaired after trauma. Supplementation with IL-12 could only partly restore the IFN-γ production. The NK cell suppression as well as the inability to respond to IL-12 were most pronounced on day 8 after trauma. Supplementation of IL-2 enhanced the IFN-γ production from NK cells early after trauma while neutralization of IL-10 failed to do so. Analyses of the IL-12 signalling pathway revealed and impaired expression of the IL-12 receptor β2 (IL-12Rβ2) subunit as well as an impaired ability for signal transducer and activator of transcription (STAT) 4 phosphorylation. Both effects were most pronounced on day 8 following the incidence.

Except for cells obtained on day 8 after trauma, the impaired $CD56^{bright}$ NK cell IFN-γ production, IL-12Rβ2 subunit expression, and STAT4 phosphorylation were at least partly restored when the cells were cultured in the absence of the trauma patient serum. Serum from trauma patients obtained on day 8 exerted a suppressive effect on NK cells from healthy controls.

In summary, cell-intrinsic and cell-extrinsic suppression of $CD56^{bright}$ NK cells is maximal on day 8 after trauma. This coincides with the peak of infectious complications of trauma patients. An impaired NK cell function might contribute to the development of immune depression after severe trauma and the restoration of NK cell function after injury might represent a novel therapeutic target to prevent nosocomial infections and accompanied complications after severe injury.

6 Literature

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7 Appendix

7.1 Index of Abbreviations

A

ADCC	Antibody dependent cellular cytotoxicity
AIS	Abbreviated injury score
APC	Allophycocyanin
APCs	Antigen presenting cells
ATLS	Advanced Trauma Life Support

C

CARS	Compensatory anti-inflammatory response syndrome
CD62L	L-selectin adhesion molecule

D

DALY	Disability adjusted live years
DAMP	Damage associated molecular patterns
DC	Dendritic cell
DIC	Disseminated intravascular coagulation
DPBS	Gibco® Dulbecco´s Phosphate-Buffered Saline

E

ELISA	Enzyme-linked immunosorbent assay

F

FCS	Fetal calf serum
FcγRIII	Fcγ receptor III
FITC	Fluorescein isothiocyanate
FSC	Forward scattered light

G

G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GolgiStop	BD GolgiStop™

H

HEV	High endothelial venules
HLA	Human leukocyte antigen
HPC	Hematopoietic precursor cell

I

ICU	Intensive care unit
IFN	Interferon
IL	Interleukin
IL-12Rβ1	IL-12 receptor β1
IL-12Rβ2	IL-12 receptor β2
ILC	Innate lymphoid cell
ISS	Injury Severity Score

K

KIR	Killer cell immunoglobulin-like receptor

L

LPS	Lipopolysaccharide

M

MHC	Major histocompatibility complex
MOF	Multiple organ failure

N

NISS	New Injury Severity Score
NK cell	Natural killer cell
NKG2D	Natural killer group 2D

P

PAMP	Pathogen associated molecular patterns
Pansorbin	Pansorbin® Cells Standardized
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cyanin7
PI-3	Phosphatidylinositol
PMN	Polymorphonuclear neutrophil
PRR	Pattern recognition receptors
pSTAT4	Phosphorylated STAT4

R

ROS	Reactive oxygen species
RPMI	VLE RPMI 1640 Medium

S

SIRS	Systemic inflammatory response Syndrome
SLT	Secondary lymphatic tissue
SOF	Single organ failure
SSC	Side scattered light
STAT	Signal transducer and activator of transcription

T

TGF	Transforming growth factor
Th	T helper
TLR	Toll like receptor
Treg	T regulatory cell

W

WHO	World Health Organization

7.2 List of Tables

7.3 List of Figures

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