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Myeloid-Derived Suppressor Cells (MDSCs) as modulators of Immune Responses after experimental Blunt Chest Trauma (Txt)

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ABBREVIATIONS

°C = degree Celsius

µl = microliter

7-AAD = 7-Aminoactinomycin D

ALI = acute lung injury

ARDS = acute respiratory distress syndrome

ATP = adenosine triphosphate

B cells = B lymphocytes

BAL = bronchoalveolar lavage

BMC = bone marrow cells

BSA = bovine serum albumin

CARS = compensatory anti-inflammatory response syndrome

CD = cluster of differentiation

CFSE = carboxyfluorescein diacetate succinimidyl ester

ConA = Concanavalin A

CSB = cytokine stabilization buffer

DAMPs = danger associated molecular patterns

DMSO = dimethylsulfoxide

DNA = deoxyribonucleic acid

DTT = dithiothreitol

e.g. = for example (exempli gratia)

EDTA = ethylendiaminetetraacetic acid

ELB = erythrocyte lysis buffer

FACS = fluorescence activated cell sorting

FCS = fetal calf serum

G-CSF = granulocyte colony stimulating factor

GFP = green fluorescent protein

GM-CSF = granulocyte macrophage colony stimulating factor

gMDSCs = granulocytic MDSCs

Gr-1 = granulocyte differentiation antigen 1

GVHD = graft versus host disease

GVT = graft versus tumor

HMGB1 = high mobility group box 1 protein

IFN = interferon

IL = interleukin

iNOS = inducible nitric oxide synthase

Ly-6C = lymphocyte antigen 6 complex, locus C

Ly-6G = lymphocyte antigen 6 complex, locus G

M-CSF = macrophage colony stimulating factor

MDSCs = myeloid-derived suppressor cells

mMDSCs = monocytic MDSCs

MODS = multi-organ dysfunction syndrome

MOF = multiorgan failure

N = number of experiments

n = number of mice

n.d. = no data

NK cells = natural killer cells

$PaCO_2$ = partial pressure of carbon dioxide

PaO_2 = partial pressure of oxygen

PBS = phosphate-buffered saline

PGE2 = prostaglandin E2

PHA = phytohaemagglutinin

RBC = red blood cell

Rpm = rotations per minute

RT = room temperature

SD = standard deviation

SIPA = stimulus induced proliferation assay

SIRS = systemic inflammatory response syndrome

SPF = specific-pathogen-free

T cells = T lymphocytes

TBI = traumatic brain injury

TCR = T cell-receptor

TGF-β = transforming growth factor β

T_H1 cells = T helper 1 cells

T_H2 cells = T helper 2 cells

TLR = toll like receptor

TNF = tumor necrosis factor

T_{Regs} = regulatory T cells

Txt = blunt chest trauma

VEGF = vascular endothelial growth factor

α-CD28 = anti-mouse CD28 antibody

α-CD3 = anti-mouse CD3 antibody

α-MEM = alpha Minimum Essential Medium

1. INTRODUCTION

Traumatic injuries (e.g. blunt chest trauma) highly impact the morbidity and mortality of patients by activating a strong pro-inflammatory immune response, which is counterbalanced by immunosuppression. Trauma danger signals not only induce a local and systemic innate immune response, but frequently lead to an impaired adaptive immunity, which in turn can increase the risk of subsequent infections. The specific changes in immunohomeostasis after trauma, and their pathogenesis, are still poorly understood and a better understanding of the underlying mechanisms is urgently required.

Immunoregulatory cells strongly modulate the immune response. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells, which are induced by inflammation and known to have immunomodulatory functions both on innate and adaptive immunity in various pathological conditions. Although it is known that MDSCs are induced after traumatic injuries their influence on the trauma-induced immune response is not clear.

1.1. TRAUMA

1.1.1. Relevance of Trauma in our Society

Even though trauma mortality constantly decreases due to modern diagnostic and treatment options, trauma is still the leading cause of death in young adults (<40 years) in high income countries and a frequent cause for disability [10, 89, 124]. In the USA and Germany, injuries account for more years of life lost than diseases like cancer or heart disease [79, 106]. Injuries from traffic accidents alone account for an estimated economic cost of 15,226 billion Euro/year in Germany [7].

Leading causes of trauma deaths include polytrauma (45.7%), traumatic brain injury (TBI) (38%), exsanguination (9.5%) and chest trauma (3.2%) e.g. in Berlin [50]. Along with increased survival rates through improved intensive care treatment certain complications, namely acute respiratory distress syndrome (ARDS) and

multi-organ dysfunction syndrome (MODS) are more frequently observed in trauma patients. Their treatment poses a challenge due to lack of causal therapies [89].

While chest trauma rarely occurs in an isolated manner [88, 97], it is part of the injury pattern in 40% of polytrauma patients, and their most common relevant injury [6, 31]. Treatment of blunt chest injuries still poses a medical as well as economic challenge in the western world [45].

1.1.2. Blunt Chest Trauma

Blunt chest trauma (Txt) is a severe condition, affecting major vital organs important for oxygenation and circulation, and often occurs in the context of polytraumatic injuries [97]. Traffic accidents are the leading cause for thoracic trauma in industrial nations, followed by falls from great heights [6, 17, 37, 45, 110].

Although chest trauma is only the second most common cause of death among polytrauma patients following TBI [6, 65], mortality significantly increases when a chest injury is part of the injury pattern [31]. About one-fourth of trauma deaths are due to a chest injury, and include causes like ARDS, heart failure, sepsis and multiple organ failure (MOF) [17, 97]. Patients with an injury pattern that includes a severe chest injury also suffer from more post-traumatic complications such as sepsis, organ failure and respiratory failure [37] and often depend on a prolonged mechanical ventilation [45]. Every third patient with a relevant trauma to the chest develops respiratory and every fourth circulatory failure [6].

The combination of posttraumatic systemic inflammatory response syndrome (SIRS) and a chest injury seems to potentiate organ dysfunction. This results in a higher morbidity and mortality rate among patients suffering multiple injuries, including chest injury, compared to those without chest injury but equally severe injuries [107].

1.1.3. Immune Reactions after Trauma

Patients deaths in the hospital later after the traumatic injury (days and weeks after trauma), are usually not due to the initial injury, but rather correlate with advanced age and complications such as sepsis and MOF [61]. Traumatic injuries do not only locally affect the function of injured organs but also severely disturb systemic immunohomeostasis. Clinical data show that patients with a severe blunt injury

suffer from an ongoing increase in inflammation and suppression of adaptive and innate immune responses [108]. Polytrauma patients also suffer primarily from the direct impact of their injuries, including blood loss or organ damage but also from systemic inflammation and infections caused by posttraumatic immune depression, and are at high risk of MOF [42, 62].

Trauma triggers a systemic reaction in the complex and highly interconnected inflammation network. The inflammatory response is essential for defense mechanisms such as elimination of pathogens and for starting tissue repair processes. After a strong impact, failure to restore integrity of the inflammation network may lead to an imbalanced response with profuse systemic inflammation and immune paralysis [62, 100].

Trauma leads to the simultaneous development of the opposite immune responses SIRS and CARS [93]. Hemorrhage and initial and secondary tissue damage lead to a local as well as systemic release of damage associated molecular patterns (DAMPs) such as adenosine triphosphate (ATP), high mobility group box 1 (HMGB1) protein and mitochondrial deoxyribonucleic acid (DNA) [43, 69, 119]. DAMPs recognized by the innate immune system prompt a strong pro- as well as anti-inflammatory immune response [46]. DAMPs activate immune cells, inflammasomes and complement, thus triggering the systemic release of inflammatory mediators [8, 66]. Pro-inflammatory cytokines (e.g. tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-8) released early after trauma play a central role in the development of SIRS, while anti-inflammatory cytokines (e.g. IL-10) contribute to a compensatory anti-inflammatory response syndrome (CARS) [42, 115]. SIRS and CARS present early after trauma, causing changes in adaptive and innate immunity. Their prolonged simultaneous occurrence contributes to complications [16, 115]. The strong inflammation drives further tissue injury, while the anti-inflammatory response may lead to impaired adaptive immunity and sepsis [16, 66]. Both contribute to a high risk of MOF, especially acute lung injury (ALI), and result in a higher trauma mortality rate [42, 93] .

Clinical studies revealed a link between an increase in plasma levels of IL-6, IL-8 and soluble TNF receptors and morbidity as well as mortality in patients with an ALI

[114]. IL-6 plasma concentrations may even serve as a diagnostic tool to determine the extent of the injury in trauma patients [33].

Despite awareness of limited immune function being a crucial factor for trauma morbidity, established therapeutic options infrequently aim to restore host defense mechanisms [93]. This is mainly due to a lack of deep understanding of the pathophysiology and factors contributing to host susceptibility for post-traumatic infections, sepsis and other injury related complications [46]. Thus treatment options for restoring immune functions are few and symptomatic rather than pathophysiologically-oriented [86].

1.1.4. Impact of Experimental Blunt Chest Trauma in Mice

Animal models are needed to investigate immune responses after trauma. The pathologies after experimental blunt chest trauma in mice resemble the clinical appearance of ALI patients. Previous experiments using a Txt model have shown that exposure of mice to a standardized pressure wave to the chest leads not only to organ injury, membrane dysfunction and inflammation, but also initiates systemic inflammation [87].

Txt leads to physiological changes in mice such as initial apnea and a drop in heart rate and mean arterial pressure. Txt also leads to a decrease in the partial pressure of oxygen (PaO_2) and an increase in the partial pressure of carbon dioxide ($PaCO_2$) values in arterial blood gas analysis in the first three hours after trauma [53]. Microscopic analysis of the lung after Txt shows intraalveolar, intrabronchial and subpleural hemorrhage, interstitial edema and atelectasis, whereas the chest wall and abdominal organs usually remain uninjured [53]. The infiltration of lung tissue with inflammatory cells such as macrophages, neutrophils and inflammatory monocytes peaks at 24h after trauma [23].

Txt leads to an early inflammatory response reflected by elevation in plasma levels of IL-6 and TNF-α two and three hours after trauma which return to normal levels after 24 hours [52, 53, 87]. IL-6 levels were also elevated in bronchoalveolar lavage (BAL) fluids early after Txt [87]. Intratracheal instillation of recombinant human Il-6 into Txt animals led to a significant increase in plasma levels, showing that Txt causes endothelial barrier dysfunction [87]. Besides the local inflammation in the lung, Txt promotes a general early systemic inflammatory response even influencing

immune cells remote from the actual injury site [52, 87]. At 24 hours after Txt, production of cytokines from splenic cells (IFN-γ, IL-2, IL-3, IL-10, IL-12, IL-18) and splenic macrophages (TNF-α, IL-10, IL-12) are reduced, pointing to a strong impact of local injuries on general immune changes which are often associated with elevated immunosuppression [52]. Animals exposed to Klebsiella pneumoniae in addition to trauma were not able to generate the same inflammatory response as the non-injured animals, were therefore less successful in clearing bacteria and more likely to die from the infection [23].

All these findings point to a strong initial local and systemic inflammatory response after Txt, with subsequent impairment of the adaptive immunity where cellular immune responses are mainly affected [52, 86].

1.2. T CELLS

Normal T cell function is crucial for a balanced and efficient immune response. T cells are cluster of differentiation (CD) 3^+ lymphocytes and central effector cells in the immune system. They subdivide into many different T cell populations. Most important in the context of this work are $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells.

$CD4^+$ T helper cells are further classified according to their cytokine secretion pattern. Besides the two major $CD4^+$ subsets T helper 1 (T_H1) cells, that mainly produce interferon-γ (IFN-γ), IL-2 and TNF-α, and T helper 2 (T_H2) cells that produce IL-4, IL-5 and IL-13, nowadays other subsets are identified such as Th9, Th17, Th22 or regulatory T cells (T_{Regs}) [73, 123]. T_H1 cells assist cell-mediated immunity, while T_H2 cells are important for humoral immunity. The two T helper cell differentiation pathways can down-regulate one another [1, 123].

A disproportion between the two types of $CD4^+$ T cells is considered as an important factor in the pathogenesis of numerous diseases. It is known that an excessive T_H1 response can lead to autoimmune diseases, whereas a mainly T_H2 based response has been found in the context of allergies [98]. The type of immune response induced is dependent on the antigen but can also be influenced by other immune cells.

T cell dysfunction has been shown to be an important contributor to the impairment of host defense mechanisms following traumatic injury. It is identified by low expression of the TCR, decreased proliferation and T cell anergy [20, 48]. Trauma typically supports a T_H2 immune response and suppresses T_H1 immunity. T cell dysfunction and the imbalanced T_H1/ T_H2 response contributes to the impaired pathogen defense mechanisms of trauma victims leading to infectious complications, sepsis and successive multiorgan failure [20, 67, 78]. It has been shown that the course of ALI after Txt depends on the presence and function of certain T cell populations and that treatment options might include balancing certain T cell populations [112].

T cells are known to express different markers on their surface in certain stages of activation. These markers can then be used to determine the activation status of T cells. The main markers used to clarify the T cell activation status in different settings are CD25 (an IL-2 receptor α-chain expressed by early progenitors of T cell lineage and activated mature T lymphocytes), CD44 (a cell adhesion molecule expressed on hematopoietic and non-hematopoietic cells), CD69 (an early activation antigen and cell adhesion molecule expressed on many cell types in the immune system) and CD122 (an IL-2 receptor β-chain expressed on NK, T, B cells, monocytes and T_{Regs}) [4, 5].

In an experimental setting T cells can be activated in different ways. They can either be stimulated by lectins that act through cross-linking of parts of the T cell-receptor (TCR), by antibodies binding directly to TCRs and costimulatory receptors or by antigen presented in the center of MHC molecules. Phytohemagglutinin (PHA) is a lectin purified from red kidney beans with a high affinity for lymphocyte surface receptors, and acts as a T cell mitogen [77]. ConcanavalinA (ConA) is a lectin purified from jack-beans that initiates T cell mitogenesis [101]. Anti-mouse CD3 and anti-mouse CD28 antibodies activate T cells via specific binding to the costimulatory receptors CD3 and CD28 [105].

1.3. MYELOID-DERIVED SUPPRESSOR CELLS

1.3.1. Characterization of MDSCs

Myeloid-derived suppressor cells are a heterogeneous population of immature myeloid cells including precursors of macrophages, granulocytes and dendritic cells [30]. They are described to modulate functions of both the innate as well as the adaptive immune system [30].

Their differentiation is favored in pathological conditions associated with chronic inflammation such as cancer, sepsis, trauma and also during pregnancy [21, 29, 32, 55, 68]. The inflammatory microenvironment leads to their accumulation and activation and prevents further differentiation in mature myeloid cells [12].

In mice, MDSCs are defined as Gr-1^{+}CD11b^{+} cells. As Gr-1 and CD11b are markers also expressed on mature myeloid cells such as neutrophils, the ability to inhibit T cell functions is what finally defines the cell population as MDSCs [85]. The Gr-1 marker consists of the two molecules Ly-6C and Ly-6G. Furthermore, MDSCs are subdivided into granulocytic and monocytic MDSCs by the expression pattern of Ly-6G and Ly-6C. Monocytic MDSCs (mMDSCs) are characterized as Ly-6G^{neg}Ly-6C^{high} and granulocytic MDSCs (gMDSCs) (also called as polymorphonuclear MDSCs) as Ly-6G^{high}Ly-6C^{low} CD11b^{+} cells [117]. The capacity to suppress T cells has been found to differ between the two MDSC subtypes but the role each subtype plays in distinct pathological conditions remains largely unclear [30, 74].

Human MDSCs lack a marker equivalent to Gr-1 in mice [85]. Nonetheless human MDSCs are generally defined as myeloid cells, which express CD11b and HLA-DR^{low}. Monocytic MDSCs are characterized as CD33^{+}CD14^{+}HLA-DR$^{-/lo}$CD15^{-} cells whereas polymorphonuclear MDSCs are generally described as being CD33^{dim}CD14^{-}CD15^{+} [9].

1.3.2. Expansion and Activation of MDSCs

MDSC expansion and activation are induced by inflammation associated factors and mediators secreted for example by bone marrow, tumors and T cells such as vascular endothelial growth factor (VEGF), GM-CSF, macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), IL-1β, IL-6, IL-10, IL13, IL-17, TNF-α, transforming growth factor β (TGF-β), IFN-y, HMGB1,

prostaglandin E2 (PGE2), toll-like receptor (TLR) ligands, calcium binding proteins S100A8 and S100A9, estrogen, glucocorticoids and complement component C5a [18, 30, 36, 64, 80, 91, 121].

Condamine and Gabrilovich suggest a two-signal model in which different pathways are responsible for MDSC expansion, leading to proliferation of immature myeloid cells, and their subsequent activation and promotion of their immunosuppressive functions by upregulation of e.g. arginase-1 and inducible nitric oxide synthase (iNOS) [18]. Factors such as GM-CSF, M-CSF, G-CSF, IL-6 and VEGF mainly promote MDSC expansion whereas pro-inflammatory molecules such as IFN- y, IL-1 β, IL-13 and TLR ligands lead to MDSC activation [18]. It is highly likely that these pathways overlap and interact. Various factors might contribute to the predominant accumulation of one of the two MDSC subpopulations [18].

In addition to MDSC expansion and recruitment during inflammation, elevated MDSC numbers are also due to an increase in their survival time mediated by apoptosis resistance or due to the ability of differentiated cells to convert into MDSCs (e.g. natural killer cells) [81, 83] .

1.3.3. Role of MDSCs in Immune Reactions

A population of suppressor Bone Marrow Cells was first described in the context of cancer in 1987 [118]. Knowledge and theories about the heterogeneous immune cell population and its impact on immunoregulation have since evolved. The commonly used term – myeloid-derived suppressor cells – is partially misleading, since MDSCs do not only suppress immune functions. Their influence on immunohomeostasis is far more complex and substantially depends on surrounding conditions [96].

Most of the knowledge about the impact of MDSCs on immune responses comes from studies regarding cancer. MDSC numbers increase in cancer patients and the tumor-induced MDSCs favor tumor growth and suppress adaptive immune responses [103]. The role of MDSCs in other pathological conditions such as autoimmune diseases or trauma is less clear [30].

MDSCs interfere with a vast number of immune responses but are mostly known for and mainly characterized by their potential for modulating T cell functions. The

capability of MDSCs to suppress T cell proliferation and function has been demonstrated in various in vivo and in vitro studies in humans and mice. MDSCs have been shown to suppress T cell responses in pregnancy and models for asthma or autoimmune diseases, traumatic stress and sepsis [13, 21, 55, 56, 68].

MDSCs use multiple mechanisms to suppress T cell immune responses as for example the production of reactive oxygen species (ROS), nitric oxide (NO) and peroxynitrite. They exhibit an increased expression of arginase-1 (depletes L-arginine, an amino acid essential for T cell function) and of iNOS (depletes L-arginine, produces NO). In addition MDSCs lead to the seclusion of cystine (amino acid crucial for T cell activation and function), the secretion of TGF-β, IL-6 and IL-10 and the induction and recruitment of regulatory T cells (T_{Regs}) [18, 19, 36, 68, 80].

Suppressive mechanisms differ between the MDSC subsets mainly in their ability to produce ROS and NO. Granulocytic MDSCs produce increased levels of ROS, while monocytic MDSCs mainly produce NO. There is no difference detectable in their arginase activity [117].

MDSCs do not merely suppress T cell functions but also modulate the type of T cell response and thereby the cytokine environment. They modify the T_H1/ T_H2 balance and thus influence the quality of the T cell mediated immune responses. Whether a T_H1 or T_H2 response is promoted by the presence of MDSCs seems to depend on the nature of the disease. An association with a preferentially T_H1 mediated T cell response has been found in the context of allergic airway inflammation [3, 13] . In contrast to those findings, MDSCs seem to promote a T_H2 response in viral infections, different cancers, sepsis, pregnancy and bone marrow transplantation [21, 29, 32, 56, 72, 99, 111].

Besides their major target – T cells – MDSCs also influence other immune cells such as B cells, natural killer cells (NK cells) and dendritic cells [2, 25, 51, 75]. For example in a model of central nervous system autoimmunity polymorphonuclear MDSCs selectively control the accumulation of B cells and their cytokine secretion [51]. In mice infected with Mycobacterium avium monocytic MDSCs inhibit the capacity of dendritic cells to stimulate T cells [2]. MDSCs extracted from tumor-bearing mice were shown to act as potent suppressors of NK cell functions [25].

Whether the presence of MDSCs is detrimental or beneficial for the host cannot generally be answered and might be dependent on the disease entity. Some examples demonstrate the diversity of MDSC functions in immune regulation. In certain cancers high levels of MDSCs have been shown to be an independent prognostic factor correlating with inferior survival of the patient [29]. In contrast, low MDSC numbers in pregnant women correspond to a high risk of spontaneous abortion [56]. Adoptive transfer of MDSCs in experimental models of allogeneic bone marrow or solid organ transplantation has shown that MDSCs promote immune tolerance thus leading to an increased survival [72, 122].

Nowadays the classification of MDSCs as mainly T cell suppressive cells is under consideration, since the understanding of their vast functions regarding immunohomeostasis and repair processes is continuously evolving. Especially functions such as favoring tumor angiogenesis, tissue regeneration and creating pre-metastatic-niches, classify MDSCs as cells with many facets and point to their importance as not merely immunosuppressive cells, but as regulators of immune functions [84, 109, 113].

1.4. MDSCS IN TRAUMA

Cytokines released after trauma (e.g. IL-1β, IL-6, and IL-10) are potent inducers of MDSCs [30, 42]. Thus MDSCs accumulate after traumatic stress such as a laparotomy, spinal cord injury, Txt and peripheral tissue trauma [47, 68, 91, 113].

There are only limited data available on the effect of MDSCs after traumatic injury. The consequences of the expansion of MDSCs following trauma for the host is widely debated. There are studies describing the effects of MDSCs after trauma or sepsis as beneficial [76, 92, 121], and others concluding that their accumulation is harmful for the patient [21, 113].

MDSCs are crucial for regeneration and angiogenesis after trauma. In spinal cord injury, transplantation of MDSCs to the site of injury led to reduced inflammation and promoted tissue regeneration [113]. Depletion of MDSCs in a femoral bone defect model led to impaired fracture healing further demonstrating their importance in healing processes [63].

On the other hand, trauma-induced MDSCs inhibit T cell proliferation and function [64, 68]. MDSC accumulation after a surgical trauma leads to suppressed T cell proliferation and has even been shown to promote tumor progression by creating an immunosuppressive environment [68, 116]. After Txt and the depletion of Gr-1$^+$ cells with a monoclonal antibody treatment, the plasma levels of the pro-inflammatory cytokine IL-6 stayed altered for longer periods [86]. Reducing MDSC accumulation through a COX-2 inhibitor after traumatic stress leads to increased T cell proliferation and a reduction of apoptosis [64]. Furthermore, the depletion of arginine (one of the main immunosuppressive mechanisms of MDSCs) has been shown to contribute to septic infections in burn patients [26]. All these studies demonstrate that MDSCs are at least partially responsible for posttraumatic immunosuppression.

MDSCs accumulate due to inflammation and strongly modulate the immune responses after trauma. Examples demonstrate that the role of MDSCs after trauma is versatile and might be dependent on the model used. Effects of in vitro- generated and systemically applied MDSCs on trauma immune responses are largely unknown.

1.5. MDSCS IN EXPERIMENTAL BLUNT CHEST TRAUMA

In previous experiments regarding the role of MDSCs in posttraumatic immune suppression after Txt our group showed, that MDSCs are induced in the spleen and lung early after Txt. We could also show, that the depletion of cells with an anti-Gr-1 antibody has little effect on the early local pro-inflammatory response but leads to a systemic increase in pro-inflammatory factors such as IL-6, MCP-1 and G-CSF. MDSCs isolated from mice after Txt were shown to efficiently prevent CD4$^+$ and CD8$^+$ T cell proliferation in vitro. Trauma-induced MDSCs were also shown to inhibit the proliferation of T cells in vivo after injection of animals with staphylococcus enterotoxin B. Even though MDSCs induced after Txt clearly act immunosuppressive by preventing T cell proliferation the blunt chest trauma does not affect T cell numbers in the lung or spleen. Txt induced MDSCs influence the T_H1/ T_H2 balance by supporting a T_H1 response which is necessary for activating cellular immunity [47].

Currently there is no antibody available to specifically deplete MDSCs. The commonly used anti-Gr-1 antibody does not only deplete MDSCs but also other Gr-1^+ cells such as neutrophils. Therefore, we decided to use a model where in-vitro generated MDSCs are adoptively transferred before Txt induction to further elucidate the effect MDSCs have on trauma immune responses.

1.6. AIM OF THE STUDY

Death and disability following injury account for many years of life lost and have an immense social and economic impact on our society [79, 106]. Treatment options for reestablishing immunohomeostasis after traumatic injuries are rare. This is due to insufficient understanding of the pathophysiology of post-traumatic inflammation and immunosuppression.

Txt causes a strong local as well as systemic inflammatory response leading to inflammation associated organ damage and long-term immunosuppression with septic complications [62, 108]. MDSCs have diverse effects on our immune system and play an important role in immune regulatory mechanisms [30]. They have been shown to accumulate after trauma and have an influence on the immune imbalance following injury.

The blunt chest trauma model in mice is well established, partially resembles the human situation and has been shown to lead to a systemic inflammatory response and accumulation of MDSCs [47]. Previous results regarding the role of MDSCs in posttraumatic immune suppression after Txt have shown that MDSCs, when induced early after experimental blunt chest trauma, suppress T cell proliferation in vitro and in vivo and support the production of T_H1 associated cytokines. After Gr-1^+ cell depletion, a decrease in systemic pro-inflammatory factors such as IL-6, G-CSF and MCP-1 occurs while their absence has no influence on the early local pro-inflammatory response [47].

Further clarification of their impact on the Txt-induced immune response is limited, due to the lack of an antibody selectively depleting MDSCs. Therefore, instead of in vivo elimination of MDSCs, we generated MDSCs from BMC in vitro with the help of GM-CSF and adoptively transferred the in vitro-generated MDSCs into Txt mice to further study their effects on the immune response after blunt chest trauma.

Several questions regarding the role of adoptively transferred MDSCs on immune responses after blunt chest trauma in mice were addressed in this thesis:

1. Where do in vitro- generated MDSCs migrate after adoptive transfer and Txt and how long do they remain detectable?
2. Do adoptively transferred MDSCs influence the early systemic pro-inflammatory immune response in trauma animals?
3. Does the adoptive transfer of MDSCs before Txt affect the number, activation status, proliferation capacity, and viability of T cells and the type of T cell immune response?

Immune reactions after severe trauma are on the one hand essential, e.g. to remove debris and destroy pathogenic bacteria, but on the other hand partially detrimental for the injured host. Understanding the mechanisms of immune imbalance following traumatic injuries may lead to the development of therapy options and further increase patient survival. Clarifying the functions of MDSCs in the post-traumatic immune response will help to determine whether MDSCs contribute to the trauma-associated immune suppression or whether they support the re-establishing of normal immune functions.

2. MATERIAL AND METHODS

2.1. MATERIAL

2.1.1. Materials

The used laboratory equipment and the maker are alphabetically listed as follows:

1 mL syringe	BD Plastikpak, USA
10 mL syringe	BD Plastikpak, USA
70 µm cell strainer	BD Falcon, USA
96-well plate (U-bottom)	Sarstedt, Germany
Animal lancet	Goldenrod, USA
Cell culture dish (Ø10 cm)	Sarstedt, Germany
Cell culture dish (Ø15 cm)	BD Falcon, USA
Disposable injection canula sterican (size 1)	Braun, Germany
Disposable injection canula sterican (size 12)	Braun, Germany
Disposable injection canula sterican (size 20)	Braun, Germany
FACS tubes	Sarstedt, Germany
Multichannel pipette	Thermo Fisher Scientific, USA
Perfusion needles ECOFLO/41023	Dispomed, Germany
Pipetman; 10, 20, 100, 200, 1000 µL	Thermo Fisher Scientific, USA
Pipette boy Pipetus „accu-jet® pro”	Brand, Germany
Reaction tubes (0.5 mL, 1.5 mL, 2 mL)	Eppendorf, Germany
Reaction tubes (15 mL, 50 mL)	Sarstedt, Germany
Sterile Serological Pipet (5 mL, 10mL, 25 mL)	Corning, USA

2.1.2. Machines

The technical equipment and maker that was used in this work is alphabetically listed in the following:

Bio-Plex™200 System	Bio-Rad, USA
Centrifuge 5417C	Eppendorf, Germany
Deep freezer -20°C	Liebherr, Germany
Deep freezer -80°C	Thermo Fisher Scientific, USA
Flow cytometer LSR II	BD Bioscience, USA
Hemocytometer	Brand, Germany
Ice-machine AF 10	Scotsman, Italy
Incubator (37°C)	WTC Binder, Germany
Laminar flow sterile bank	Heraeus Instruments, Germany
Microscope „CK2“	Olympus, Japan
Refrigerator	Liebherr, Germany
Refrigerator	Bosch, Germany
Thermo cycler	PTC 100™ MJ Research, USA
Varifuge 3.0 RS	Heraeus Instruments, Germany
Water bath „MWB 10“	GK Labortechnik Medingen, Germany

2.1.3. Reagents and Chemicals

All reagents and chemicals that were used in this work are listed as follows:

1x PBS (phosphate-buffered saline)	Gibco by Life Technologies, USA
7-Aminoacetinomycin D (7-AAD)	Sigma-Aldrich, Germany
99,8 % Ethanol	Sigma-Aldrich, Germany
Ammonium chloride	Merck, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Germany

Cytokine stabilization buffer	U-CyTech Biosciences, Netherlands
Dimethylsulfoxide (DMSO)	Merck, Germany
Dithiothreitol (DTT)	Sigma-Aldrich, Germany
Easycoll	Biochrom, Germany
Ethylenediaminetetraacetic acid (EDTA)	Roth, Germany
Fetal calf serum (FCS)	BioWhittaker, Lonza, Switzerland
GM-CSF	Peprotech, Germany
Hepes buffer	Biochrom, Germany
L-glutamin (200mM)	Gibco by Life Technologies, USA
Liver digest medium	Gibco by Life Technologies, USA
Liver perfusion medium	Gibco by Life Technologies, USA
Methanol	Sigma-Aldrich, Germany
Penicillin-Streptomycin	Gibco by Life Technologies, USA
Potassium hydrogen carbonate	Merck, Germany
Sodium acetate	Sigma-Aldrich, Germany
Sodium-pyruvate	Gibco by Life Technologies, USA
TRIzol®	Zymo Research, USA
Disinfection Spray	DESOMED, Germany
Trypan blue stain (0.4 %)	Sigma-Aldrich, Germany
α-MEM (-)	BioWhittaker, Lonza, Switzerland
β-Mercaptoethanol (50mM)	Gibco by life Technologies, USA
Phytohemagglutinin (PHA)	Sigma-Aldrich, Germany
Concanavalin A (ConA)	Sigma-Aldrich, Germany
α-CD3/28:	
- anti CD3 purified	BD Bioscience, USA
- anti CD28 purified	BD Bioscience, USA
Fc Block: α-CD16/32	BD Bioscience, USA
Annexin V FLUOS Staining Kit	Sigma-Aldrich, Germany

2.1.4. Kits

The used kits and maker are alphabetically registered in the following:

CFSE cell proliferation Kit	eBioscience, USA
ProcartaPlex™ Mouse Basic Kit	eBioscience, USA
ProcartaPlex™ Mouse IFN-γ Simplex	eBioscience, USA
ProcartaPlex™ Mouse IL-13 Simplex	eBioscience, USA
ProcartaPlex™ Mouse IL-4 Simplex	eBioscience, USA
ProcartaPlex™ Mouse IL-5 Simplex	eBioscience, USA
ProcartaPlex™ Mouse TNF-α Simplex	eBioscience, USA

2.1.5. Trauma Equipment

The used trauma equipment and maker are listed as follows:

Sevoran (Sevoflurane)	Abbvie Deutschland, Ludwigshafen
Temgesic (Buprenorphinhydrochlorid)	RB Pharmaceuticals Limited, Slough, Great Britain
Pressure Wave generator for Txt	Feinwerkstatt Ulm University, Germany
Mylar Polyester Film 50a	DuPont, Wilmington USA

2.1.6. Media and Buffer

All media and buffer used in the study are described in the following:

α-MEM

α-MEM(-) supplemented with 10 % FCS, 2 mM L-glutamine, 1 mM sodium-pyruvate,100 U/mL Penicillin-Streptomycin and 0.05 mM β-Mercaptoethanol

Erythrocyte lysis buffer (ELB)

8.29 g/L ammonium chloride, 1 g/L potassium hydrogen carbonate, 0.037 g/L EDTA in Aqua bidest; pH 7.2 – 7.4

FACS buffer

1x PBS, 10 % FCS, 0.1 % sodium acetate

2.1.7. Antibodies

In the following, all used antibodies are listed:

Target Protein	Supplier	Clone	Isotype	Fluorochrome
CD3	eBioscience	17A2	rIgG2bkappa	APC
CD3	eBioscience	145-2C11	arm hamster IgG	PeCy7
CD4	Invitrogen	RM4-5	rIgG2akappa	APCeF780
CD8a	eBioscience	53-6.7	rIgG2akappa	APC
CD11b	eBioscience	M1/70	rIgG2bkappa	PE
CD25	eBioscience	PC61.5	rIgG1lambda	PeCy7
CD44	eBioscience	IM7	rIgG2bkappa	PeCy7
CD45.1	eBioscience	A20	mIgG2akappa	APC
CD69	BD Pharmingen	H1.2FR	mIgG1kappa	FITC
CD122	eBioscience	TM-b1	rIgG2bkappa	eFluor450
Gr-1	eBioscience	RB6-8C5	rIgG2bkappa	eFluor450

2.1.8. Mice

All animal experiments were conducted in accordance with the guidelines approved by the Regierungspräsidium Tübingen (1196, 1297, 0.100-3).

The used mice strains are listed as shown below. All mice were maintained in the animal facility under specific-pathogen-free (SPF) conditions.

Male C57BL/6 mice (CD45.2+)	Janvier, France
Male B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (CD45.1+)	breeding pairs from The Jackson Laboratory; bred at Ulm University

2.1.9. Software

Software used for this work is listed as follows:

BD FACS Diva Software 6.0

Bio-Plex Manager 4.1.1

FlowJo 10.0.6

Microsoft Office 2016

Libre Office Calc

GraphPad Prism 6

RefWorks ProQuest

2.2. METHODS

2.2.1. Cell Culture

MDSCs were cultured and in vitro assays were performed in a humidified atmosphere at 7.5% CO_2 and 37°C in α-MEM.

2.2.2. Cell Counting

Living cells were counted with a Neubauer chamber after staining with trypan blue stain to exclude dead cells.

2.2.3. Tissue Preparation

2.2.3.1. Bone marrow (BM)

Mice were sacrificed by cervical dislocation. The femur and tibia were exposed under sterile conditions. BM was extracted by rinsing the medullary cavity with a syringe. A single cell suspension was prepared by separating cell clumps with a small syringe. Erythrocytes were lysed in red blood cell (RBC) lysis buffer at 37°C for 5 minutes. Cells were washed and kept on ice in phosphate-buffered saline (PBS) until further analysis.

2.2.3.2. Spleen

Mice were sacrificed by cervical dislocation. Spleens were harvested, and a single cell suspension was prepared by passing them through a cell strainer (⌀ 70µm). Erythrocytes were lysed in RBC lysis buffer at 37°C for 5 minutes. Cells were washed and kept on ice in PBS until further analysis.

2.2.3.3. Liver

Mice were sacrificed and directly prepared for liver perfusion by exposing the portal vein. The liver was slowly perfused via the portal vein with 5mL liver perfusion medium followed by 5mL liver digest medium. Afterwards the liver was removed and digested in 10mL of liver digest medium at 37°C. After 30 minutes of digestion, liver was pressed through a cell strainer (⌀ 70µm) to prepare single cell solution. Cell solution was centrifuged at 500 rpm for 5 minutes to remove debris. The supernatant was collected, and the pellet resuspended in PBS and centrifuged at 500 rpm for another 5 minutes. The second supernatant was collected, and the pellet discarded. The two supernatants were centrifuged at 1400 rpm for 5 minutes to obtain pellets

containing lymphocytes. Both new pellets were resuspended in 1% FCS and then mixed with 3mL of 70% Percoll. This mixture was then slowly layered onto 3 mL of 70% Percoll in a new falcon. After centrifugation for 20 minutes at 2000 rpm and slow deceleration, lymphoid cells were collected from the interphase. Cells were washed, and erythrocytes were subsequently lysed in RBC lysis buffer at 37°C for 5 minutes, followed by several washing steps. Cells were kept on ice in PBS until further analysis.

2.2.3.4. Lung

Mice were sacrificed by cervical dislocation. Lungs were harvested after opening the thorax and pressed through a cell strainer (⌀ 70µm) to produce single cell suspensions. Erythrocytes were lysed in RBC lysis buffer at 37°C for 5 minutes. Cells were washed, and the pellet was re-suspended in 1% FCS and then mixed with 3mL of 70% Percoll. This mixture was then slowly layered onto 3mL of 70% Percoll in a new falcon. After centrifugation for 20 minutes at 2000 rpm and slow deceleration, lymphoid cells were collected from the interphase. Cells were washed and kept on ice in PBS until further analysis.

2.2.4. Generation of MDSCs in vitro

MDSCs were generated from BMC of male B6.SJL mice ($CD45.1^+$). The in vitro generation of MDSCs was performed as previously described in Messmann et al. [72]. In brief, 30 mL single cell suspension with a total of $3x10^5$ BMC/mL were seeded in culture dishes (⌀ 15cm) in α-MEM with GM-CSF (200U/mL) at 37°C. After

4 days of cultivation, MDSCs were harvested with the help of a cell scraper. They were washed with PBS, counted and kept on ice until injection or further analysis.

2.2.5. Animal Experiments

2.2.5.1. Blunt Chest Trauma

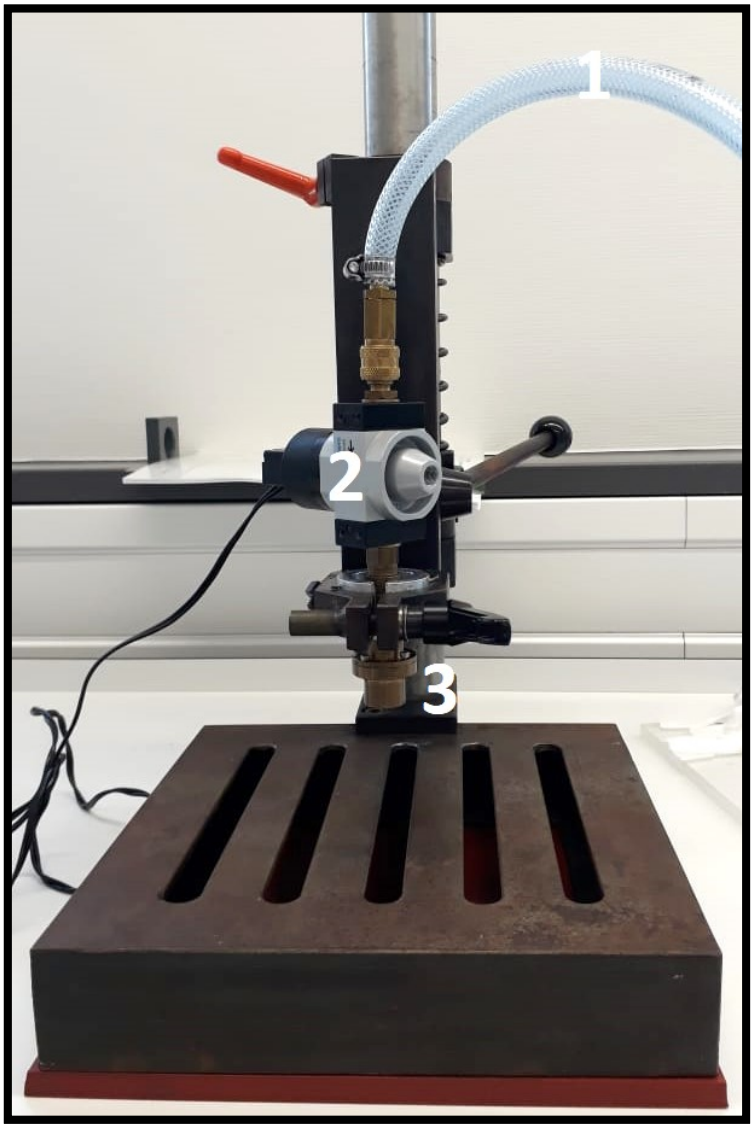

Figure 1. Blunt chest trauma Apparatus. *1= compressed air, 2= pressure reservoir, 3= nozzle. Distance between device and thorax of animals was adjusted to 1.5 cm.*

Male C57BL/6 mice ($CD45.2^+$) were treated at an age between 11 and 17 weeks. Blunt chest trauma was induced by a single blast wave centered on the thorax as previously described by Knöferl et al. [53]. The device consists of two sections. The upper section serves as a pressure reservoir and is separated from the lower section ("nozzle") by a Mylar® polyester film (50 µm thickness) (see Figure 2). A storage

tank of compressed air is connected to the pressure reservoir. A high-speed valve and a pressure reducing valve set to 13 bar control the flow of air. The nozzle was directed to the animal's chest. By opening the high-speed valve, compressed air was delivered to the pressure reservoir. As soon as the pressure exceeded the resistance of the membrane, it ruptured towards the nozzle and released a reproducible pressure wave toward the thorax.

Mice under sevoflurane anesthesia (see 3.2.5.2.) were fixed on an acrylic glass plate on their back. Their chests were shaved and marked at the junction between the xiphoid process and the body of the sternum to allow reproducible placement under the device for the generation of the blast wave. The distance between the sternum and the cylinder was adjusted to 1.5 cm. Sevoflurane anesthesia was stopped after the trauma, and mice were returned to their cages after regaining consciousness.

2.2.5.2. Sevoflurane Anesthesia

Txt mice were anesthetized via a mask covering their snouts with a mixture of 2.5% sevoflurane and 97.5% oxygen at a continuous flow of 0.5 L/min for the duration of the procedure.

2.2.5.3. Analgesic Treatment

Mice were treated with 0.03mg/kg Buprenorphine 30 minutes before, as well as every 8 hours up to 24 hours after trauma induction.

2.2.5.4. Transplantation of in vitro-generated MDSCs

To clarify the function of MDSCs in the Txt model, $2x10^7$ in vitro-generated MDSCs ($CD45.1^+$) were re-suspended in 100µl sterile PBS and injected into the tail vein of B6 mice approximately 1 hour before blunt chest trauma (Txt).

2.2.5.5. Blood Sampling

Blood was collected after puncture of the mandibular vein with an animal lancet. The blood was then kept at room temperature for 30 minutes to coagulate before being centrifuged at 13.000 rpm for 15 minutes at 4°C. Serum was separated from the cell pellet and transferred into a new reaction tube. Cytokine stabilization buffer (CSB) at a ratio of 1:20 (CSB:serum) was added to the serum. Samples were stored at -80°C until further analysis.

2.2.5.6. Homing of MDSCs

The homing of in vitro-generated and adoptively transferred MDSCs was analyzed by staining for $CD45.1^+$ cells in the spleen, liver and lung at 2,4 and 7 days after Txt and adoptive transfer of in vitro-generated MDSCs. (Tissue preparation see 3.2.3.)

2.2.6. In vitro Assays

2.2.6.1. CFSE Labeling

Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling was used to determine cell proliferation in vitro. The fluorescent cell staining dye attaches to intracellular molecules and is divided as cells proliferate. Therefore, a drop in intensity can be used to determine cell proliferation.

$2x10^7$ spleen cells/10mL were labeled in PBS/5%FCS with 5µM CFSE in a 50mL reaction tube. Cells were incubated for 10 minutes at 37°C in the dark. The reaction was stopped by adding ice-cold PBS/5%FCS. Cells were incubated on ice for 5 minutes, then washed 3 times with cold PBS/5%FCS and immediately used for proliferation assays.

2.2.6.2. Stimulus Induced Proliferation Assay (SIPA)

$2.5x10^5$ CFSE labeled spleen cells in 100µl α-MEM were used as effector cells and seeded in a 96 well round bottom plate. Various stimulators (PHA, ConA or α-CD3/CD28) in titrated concentrations, diluted in α-MEM or α-MEM alone were added to the different wells (Table 1).

Table 1. *Pipette scheme for SIPA in the presence of different stimulating agents. Example for one mouse.*

$2.5x10^5$ CFSE labeled spleen cells		
+100µl αMEM (x3)	+2.5µg/mL PHA (x3)	+1.25µg/mL PHA (x3)
+100µl αMEM (x3)	+2.5µg/mL ConA (x3)	+1.25µg/mL ConA (x3)
+100µl αMEM (x3)	+ 0.1µg/mL αCD3/αCD28 (x3)	+ 0.01 µg/mL αCD3/αCD28 (x3)

SIPA was cultivated for either 2 or 4 days at 37°C in an atmosphere with 7.5% CO_2. T cell proliferation was then analyzed by flow cytometry. Since each cell

division decreases the amount of CFSE in the cell, $CFSE^{low}$ T cells are identified as proliferated cells.

Antigen specific proliferation was calculated with the following formula: $\%\ \text{specific proliferation} = 100 \times \frac{(\%\mathbf{CFSE^{-}CD3^{+}\ cells\ after\ stimulation}) - (\%\mathbf{CFSE^{-}CD3^{+}\ cells\ medium\ control})}{\mathbf{100} - (\%\mathbf{CFSE^{-}CD3^{+}\ cells\ medium\ control})}$

2.2.6.3. Preservation of SIPA supernatants

After centrifugation of the 96-well plate described in 3.2.6.2. for 2 minutes at 2000 rpm, 50µl of the supernatants were transferred to a new plate. Supernatants were then kept at -80°C until cytokine concentrations were determined by Multiplex Immunoassay.

2.2.7. Flow Cytometry

Flow cytometric analysis of blood, spleen, liver, lung, MDSCs and T cells for different surface markers was performed on a LSR flow cytometer.

2.2.7.1. Cell Surface Staining

A total of $5x10^5$-$1x10^6$ cells were stained in 100µl FACS buffer in the dark, on ice for 30 minutes with different fluorochrome-conjugated antibodies (see chapter 2.1.7). Afterwards, cells were washed with FACS buffer three times and then transferred into a reaction tube with 300µl FACS buffer. 1µg 7-AAD (7-aminoactinomycin D) was added to each sample in order to exclude non-viable cells (=7-AAD^{+}) from measurements. Dead cells lack an intact plasma membrane and therefore cannot prevent the dye from entering the cell and binding to intracellular DNA.

Expression of Fc Receptors is often responsible for unspecific antibody staining of cells. Therefore, liver, lung and spleen cells were pretreated with 2µl Fc-Block (α-CD16/32)/$1x10^6$ cells for 10 minutes before staining with the relevant fluorochrome-conjugated antibodies

2.2.7.2. Analysis

Flow cytometry samples were measured on LSR II flow cytometer (BD Bioscience) and analyzed with FlowJo10.0.6 software.

2.2.8. Cytokine Analysis

2.2.8.1. ProcartaPlex™ Multiplex Immunoassay

The multiplex immunoassay to analyze cytokines in the serum and in the supernatants of the SIPA was performed according to the manufacturer's instructions (eBioscience).

Cytokine concentrations were determined by ProcartaPlex™ based on magnetic microsphere Luminex®. 12.5µl of collected serum samples (see chapter 3.2.5.5) or SIPA supernatants (see chapter 3.2.6.3) were diluted with 12.5µl of 1x universal assay buffer (eBioscience) and added to a 96-well plate, coated with 100µl analyte specific antibody magnetic beads. To quantify the amount of cytokines in the samples, defined concentrations of cytokines were used to create a standard curve. The plate was incubated light-protected on a shaker for 2 hours at room temperature (RT). After incubation, the 96-well plate was fixed in a magnetic plate and samples were washed with 75µl 1x wash buffer (eBioscience) three times. Analyte-specific detection antibody mixture was prepared according to the manufacturer's protocol (eBioscience) and 12.5µl of mixture were added to each well. The plate was incubated light-protected on a shaker for 30 minutes at RT. Three washing steps with 75µl 1x wash buffer were performed (96 well plated fixed on the magnetic plate). For analyte quantitation, 25µl Streptavidin-PE solution was added to each well. The plate was incubated light-protected on a shaker for 30 minutes at RT. Three washing steps with 75µl 1x wash buffer were performed (96-well plate fixed on the magnetic plate). 120µl reading buffer (eBioscience) were added to each well and the plate incubated light-protected on a shaker for 5 minutes to resuspend beads. Cytokine concentrations were determined with a Bio-Plex™ 200 System (BioRad), powered by Luminex xMAP™ technology.

2.2.9. Statistical Analysis

Quantitative datasets were analyzed by using GraphPad Prism 6. All results are expressed as means ± standard derivation (SD) and analysis were performed by using unpaired student's t-test. A value of $*p < 0.05$ $**p < 0.01$ and $***p < 0.0001$ was accepted as statistically significant.

3. RESULTS

Parts of these results were already published in Klingspor and Kustermann et al (2019) [58].

Traumatic injuries strongly disturb immunohomeostasis and lead to a malfunction of the innate and adaptive immune system. MDSCs might be a promising therapeutic target to influence immune responses in patients with an impaired immune system after traumatic injuries. Trauma-induced MDSCs are known to be immunosuppressive, since they inhibit the expression of pro-inflammatory factors and suppress T cell proliferation [47].

To better understand the influence of MDSCs on posttraumatic inflammation and T cell functions in vivo, in vitro-generated MDSCs were adoptively transferred 1 hour before mice received a blunt chest trauma. Subsequently, the effect of adoptively transferred MDSCs on immune responses after Txt was analyzed.

3.1. GENERATION OF MDSCS AND ADOPTIVE TRANSFER IN TXT MICE

MDSCs were generated by culturing BMCs from B6.SJL ($CD45.1^+$) mice with GM-CSF for four days. Cells of B6.SJL mice are $CD45.1^+$ cells and are otherwise genetically identical to $CD45.2^+$ cells from B6 mice. This CD45.1 marker allowed distinction of the transplanted MDSCs from endogenous cells after adoptive transfer.

After 4 days in culture with GM-CSF, more than 90% of the cells are $CD11b^+/Gr1^+$ compared to BMC where only 56% are $CD11b^+/Gr1^+$ cells. By staining Ly-6G and Ly-6C, MDSC subpopulations were defined. After culturing BMC with GM-CSF 74% of cells represent gMDSCs while 24% are mMDSCs (Figure 2A). MDSC generation was controlled before transplantation for each individual experiment. Cells were stained with anti CD11b and anti Gr-1 antibodies and analyzed via fluorescence activated cell sorting (FACS). If MDSC cell suspension included >90% of CD11b+ and Gr-1+ cells the $CD45.1^+$ MDSCs were transplanted into B6 ($CD45.2^+$) mice via

the tail vein (Figure 2B). Approximately 1 hour later mice received blunt chest trauma (Txt) [58].

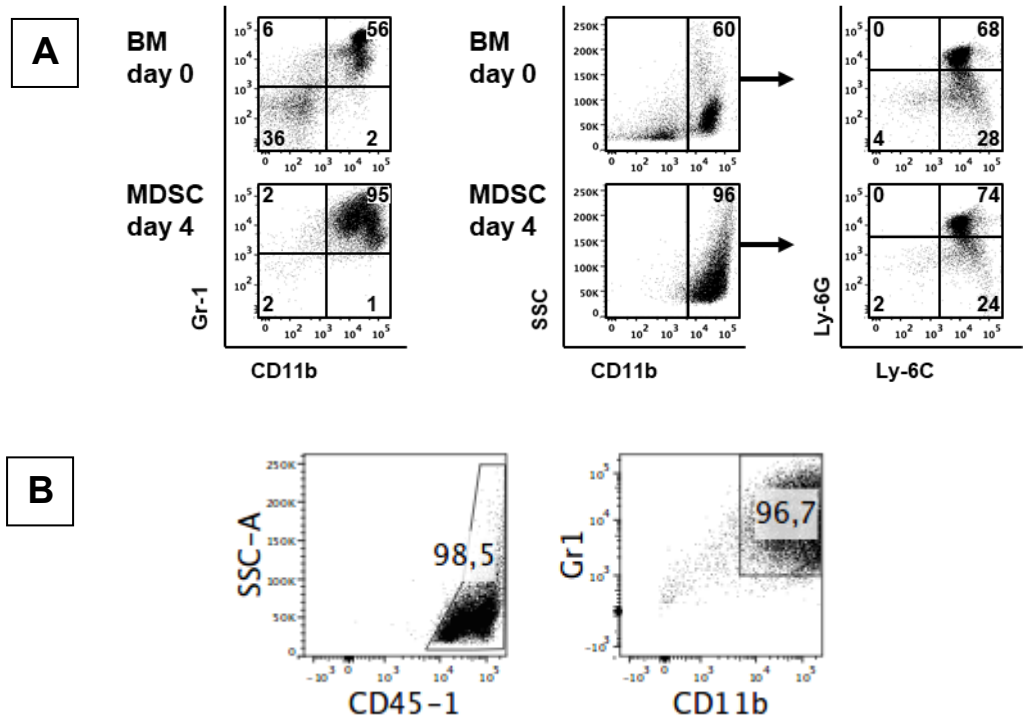

Figure 2. In vitro generation of MDSCs. (A) *BMC of B6.SJL mice were incubated with GM-CSF for 4 days or left untreated. Directly after extraction from BM or after 4 days in culture with GM-CSF cells were stained with CD11b, Gr1, Ly-6G and Ly-6G antibodies. Cells were gated for Gr-1 and CD11b or CD11b only. CD11b⁺ cells were further gated for Ly-6G and Ly-6C to determine the distribution of the two MDSC subpopulations. Graph shows representative flow data from 4 independent experiments performed. This figure was created together with Monika Kustermann. [58]* ***(B)*** *Representative FACS staining of in vitro-generated MDSCs before transplantation is shown. BMC of B6.SJL mice were treated with GM-CSF to induce MDSCs. After 4 days in culture cells were stained with CD45.1, CD11b and Gr1 antibodies. Cells were gated for CD45.1⁺ cells or Gr-1⁺ and CD11b⁺ cells.*

3.2. HOMING OF IN VITRO-GENERATED MDSCS

First, we defined the homing of MDSCs into different organs of Txt treated mice by staining for CD45.1 expression. Blood samples were analyzed 1 and 5 hours after transfer, while MDSC homing in liver and spleen was analyzed 2, 4 and 7 days after transfer. To distinguish between adoptively transferred and own cells in the FACS staining, we used the marker CD45.1, which was only expressed on the transplanted cells.

3.2.1. Blood

Blood was taken from the mice at 1 and 5 hours after injection of MDSCs to check for successful cell transplantation and spreading of the cells in the circulation. Blood cells were stained for CD45.1 and the classic MDSC markers Gr-1 and CD11b. Gr-1$^+$ and CD11b$^+$ cells were then gated for CD45.1$^+$ to define adoptively transferred cells. 1 hour after injection, blood contains 1.41% of CD45.1+ MDSCs. The percentage decreases to 0.56% at 5 hours after transplantation, indicating that adoptively transferred MDSCs extravasate with time (Figure 3).

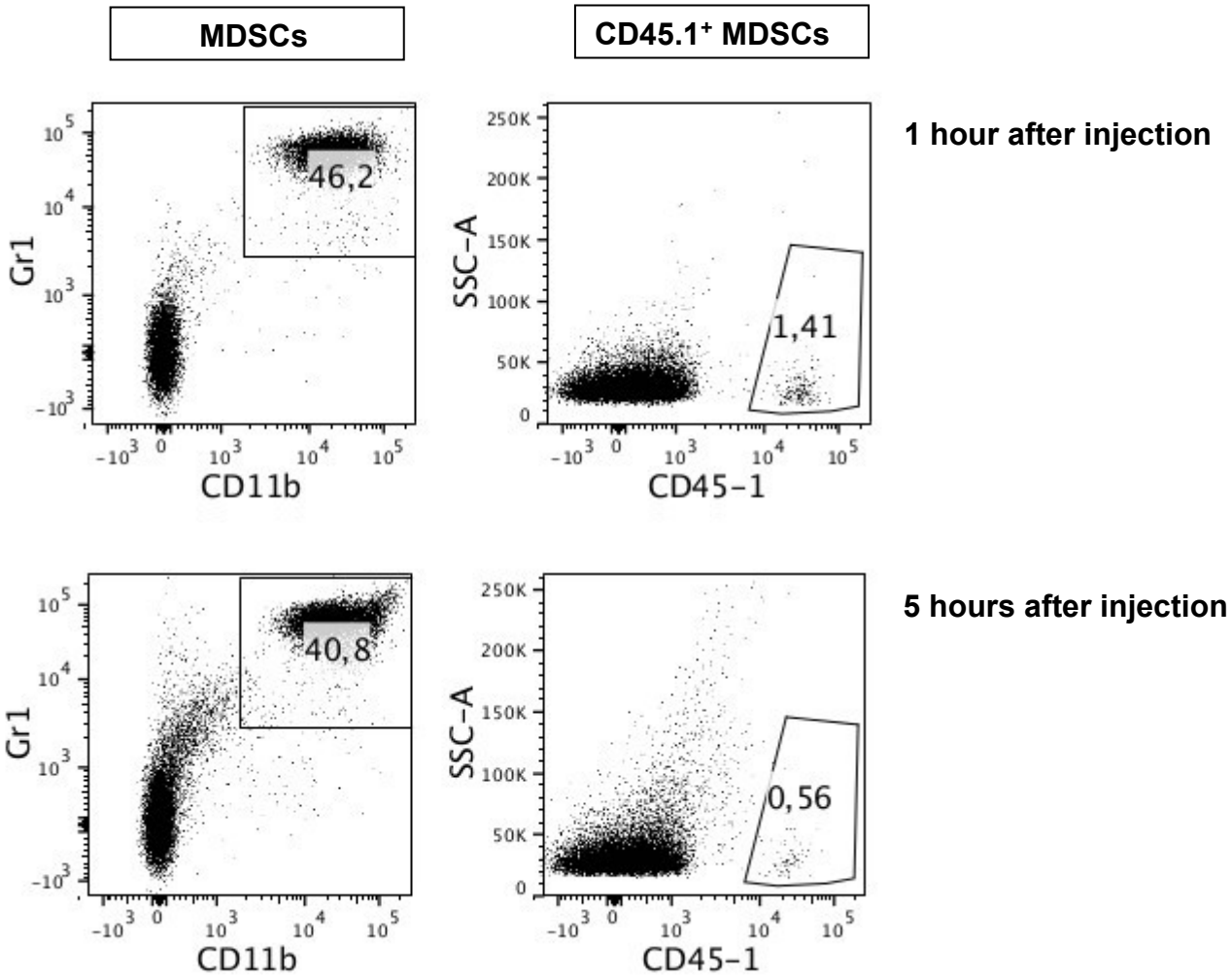

Figure 3. Adoptively transferred MDSCs are detectable in the blood. *MDSCs were generated in vitro from BMC of B6.SJL mice (CD45.1$^+$) by incubation with GM-CSF for 4 days. CD45.1$^+$ MDSCs were adoptively transferred into B6 mice (CD45.2$^+$). Blood was taken from MDSC treated mice 1 and 5 hours after adoptive transfer of cells. Blood cells were stained with CD11b, Gr1 and CD45.1 antibodies. Cells were gated for Gr-1 and CD11b to determine MDSCs. Gr-1$^+$ and CD11b$^+$ cells were further gated for CD45.1 to show adoptively transferred MDSCs only. Two representative FACS stainings for MDSCs and CD45.1$^+$ MDSCs in the blood at 1 and 5 hours after injection shown. Graph shows representative flow data from 3 independent experiments performed.*

3.2.2. Liver and Spleen

The decrease of %CD45.1$^+$ MDSCs in the circulation over time led to the conclusion that transplanted MDSCs probably home into organs. Therefore, we analyzed the presence of adoptively transferred MDSCs (CD45.1$^+$) in the spleen and liver of MDSC treated mice at 2, 4 and 7 days after Txt.

Interestingly, adoptively transferred MDSCs were found in the spleen predominantly 7 days after injection. CD45.1$^+$ cells were detected in the spleen of Txt treated mice (day 2: TM: 0.07±0.02% n=7; day 4: TM: 0.19±0.27% n=7; day 7: TM: 2.07±0.37% n=6) in 2 independent experiments for each time point (Figure 4).

Adoptively transferred MDSCs were also found in the liver at all time points, although at lower percentages. CD45.1$^+$ cells were detected in the liver of Txt mice (day 2: TM: 0.19±0.02% n=3; day 4: TM: 0.26±0.10% n=7; day 7: TM: 0.21±0.09% n=6) in 1-2 independent experiments for each time point (Figure 4).

Since blunt chest trauma treatment leads to barrier dysfunction and local inflammation in the lung, we expected to find MDSCs in the lung after transplantation, but surprisingly, after MDSC transplantation and Txt treatment, no CD45.1$^+$ MDSCs were detectable in the lung (data not shown).

These results show that in vitro-generated MDSCs survive after transplantation, and preferentially home to the spleen with a maximum at day 7 after trauma. Having clarified the path of MDSCs after transplantation into the Txt mice, we were interested to see whether they modulate immune response after Txt treatment [58].

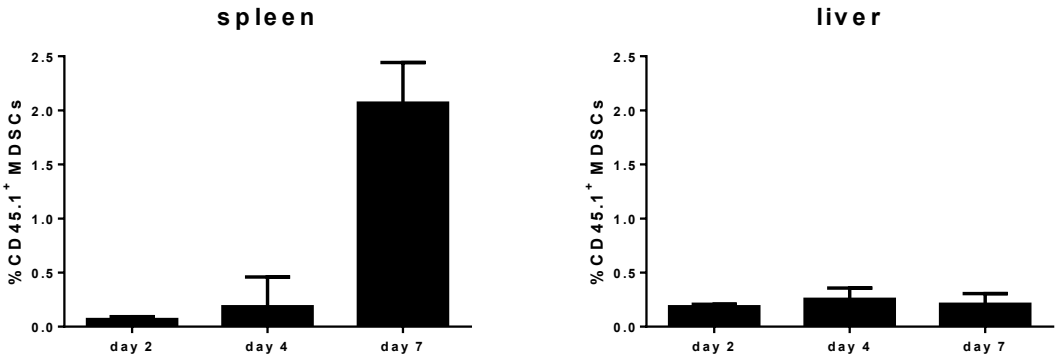

Figure 4. Transplanted MDSCs migrate to the spleen and liver. *MDSCs were generated in vitro from BMC of B6.SJL mice (CD45.1⁺) by incubation with GM-CSF for 4 days. CD45.1⁺ MDSCs were adoptively transferred into B6 mice (CD45.2⁺) 1 hour before Txt. Homing was analyzed in the spleen and liver by analyzing the percentage of CD45.1 expressing cells. Bar graph shows %CD45.1⁺ MDSCs in spleen and liver at 2, 4 and 7 days after Txt treatment. Data show mean values ±SD of n=3-7 in 1-2 independent experiments per time point.*

Modified from [58], CC BY 4.0, https://creativecommons.org/licenses/by/4.0/

3.3. EARLY SYSTEMIC PRO-INFLAMMATORY IMMUNE RESPONSE

Since it is known that Txt induces an early local and systemic pro-inflammatory response, blood was taken from the mandibular vein at different time points after Txt treatment to analyze the impact of adoptively transferred MDSCs on serum cytokines in Txt mice. Cytokine levels in the serum were measured via the ProcartaPlex® Multiplex Immunoassay (eBioscience) in Txt mice treated or untreated with MDSCs 3-6 hours and 24 hours after Txt (Figure 5).

Pro-inflammatory cytokines are known to increase in the serum after blunt chest injury. In our Txt study the treatment with in vitro-generated MDSCs does not influence IL-6 or MCP-1 levels and merely shows a tendency to decrease the serum concentration of IL-1β and increase the concentration of G-CSF 3-6 hours after trauma. No difference is detectable at 24 hours after Txt indicating that MDSC treatment after Txt does not substantially modulate the systemic pro-inflammatory immune response.

Since MDSCs are known to influence the type of T cell response, we also analyzed the concentration of T_H1/T_H2-specific cytokines in the serum. The concentration of the T_H1-specific cytokine IL-5 is slightly increased in MDSC-treated mice at 3-6

hours after Txt, but no change is detectable at 24 hours after Txt compared to untreated animals. The concentrations of all other T_H1- and T_H2-specific cytokines analyzed (IL-2, IL-10, IL-13, TNF-α, IFN-γ) are slightly decreased in MDSC-treated mice at 3-6 hours after Txt but show no difference compared to untreated animals 24 hours after Txt. These data show that in vitro-generated and adoptively transferred MDSCs transiently effect the levels of T_H1- and T_H2-specific cytokines in the serum early after Txt without showing a clear tendency towards either of the two types of immune responses and do not have a long-term effect.

Having shown that the transplanted MDSCs seem to have little influence on the systemic inflammation, we were interested to investigate the potential effects of MDSCs on the adaptive immune response. Since MDSCs homed preferentially to the spleen, and organ where interactions with T cells might occur, we investigated the impact of MDSCs on the cellular immune response in the spleen [58].

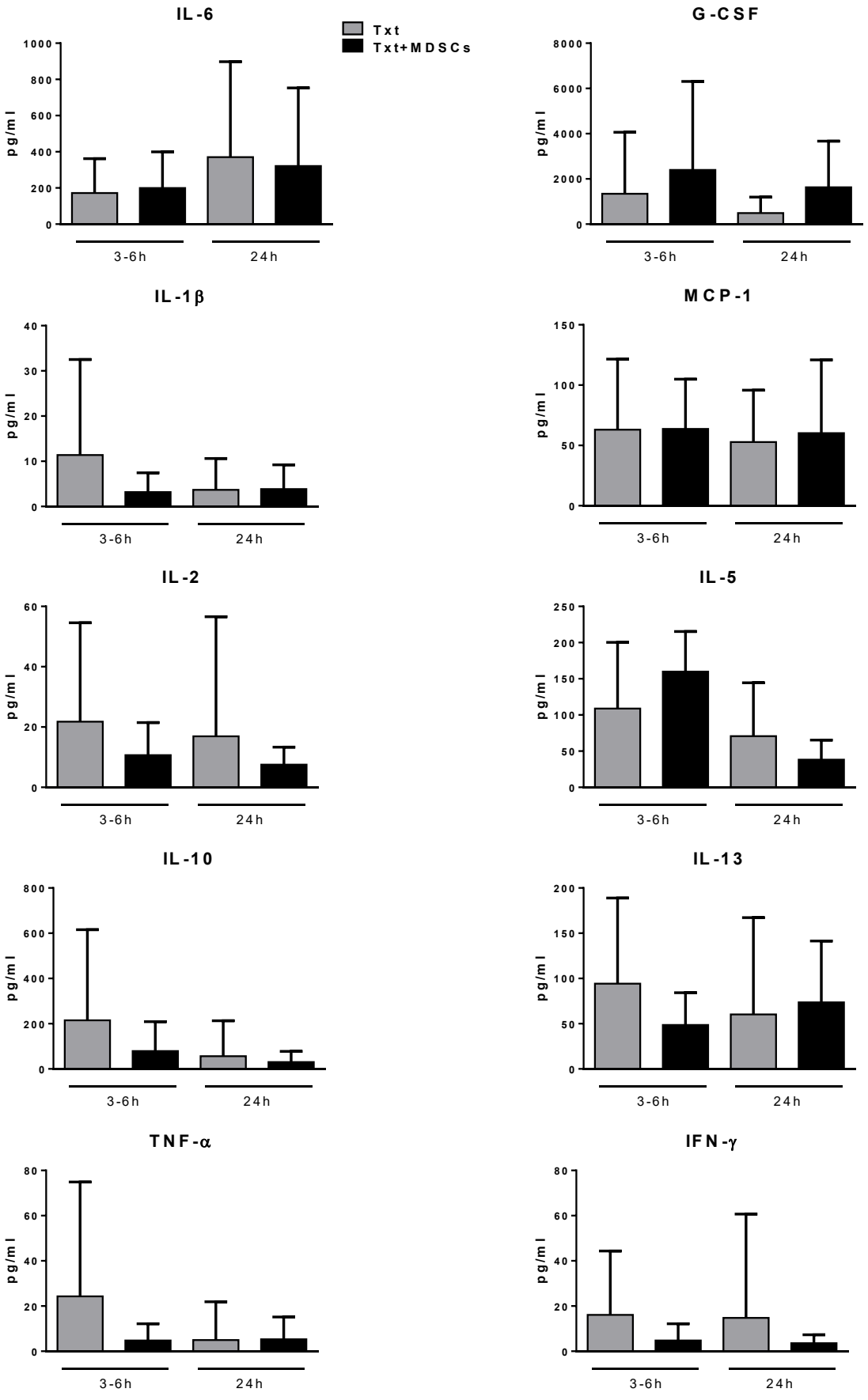
IL-6
Txt
Txt+MDSCs
pg/ml
1000
800
600
400
200
0
3-6h
24h
G-CSF
8000
6000
4000
2000
IL-1β
40
30
20
10
MCP-1
150
100
50
IL-2
60
IL-5
250
IL-10
800
IL-13
TNF-α
80
IFN-γ

Figure 5. Adoptively transferred MDSCs do not influence cytokine and chemokine levels after Txt. *MDSCs were generated in vitro from BMC of B6.SJL mice (CD45.1$^+$) by incubation with GM-CSF for 4 days. CD45.1$^+$ MDSCs were adoptively transferred into B6 mice (CD45.2$^+$) 1 hour before Txt or mice received Txt without previous transfer of MDSCs. Blood was taken from the mice at 3-6 hours and 24 hours after Txt. Levels of IL-6, IL1-β, IL-2, IL-10, TNF-α, G-CSF, MCP-1, IL-5, IL-13 and IFN-γ were measured via the ProcartaPlex® Multiplex Immunoassay (eBioscience) in the serum samples from Txt and MDSC-treated Txt mice. Data represent means ± SD of n=11-22 from 9 independent experiments. A part of the experiments was performed by Monika Kustermann.*

3.4. ADOPTIVELY TRANSFERRED MDSCS INCREASE NUMBER OF SPLEEN CELLS

After demonstrating that adoptively transferred MDSCs home to the spleen, we also noticed that the spleens of mice analyzed 2 days after MDSC transplantation were macroscopically enlarged (Figure 6). Enlargement of spleens of MDSC-treated Txt mice was due to an increase in splenocyte numbers compared to untreated Txt animals. Splenocyte numbers of Txt (T) and MDSC-treated Txt (TM) mice were counted 2, 4 and 7 days after Txt. Spleen cell numbers are significantly higher in MDSC-treated mice at all time points analyzed (day 2: T: $4.8x10^7$±0.7 cells n=11; TM: $8.8x10^7$±1.5 cells n=10; day 4: T: $6.8x10^7$±1.8 cells n=7; TM: $12.3x10^7$±1.8 cells n=7; day 7 T: $4.9x10^7$±1.0 cells n=7; TM: $9.6x10^7$±2.0 cells n=6) (Figure 7) [58].

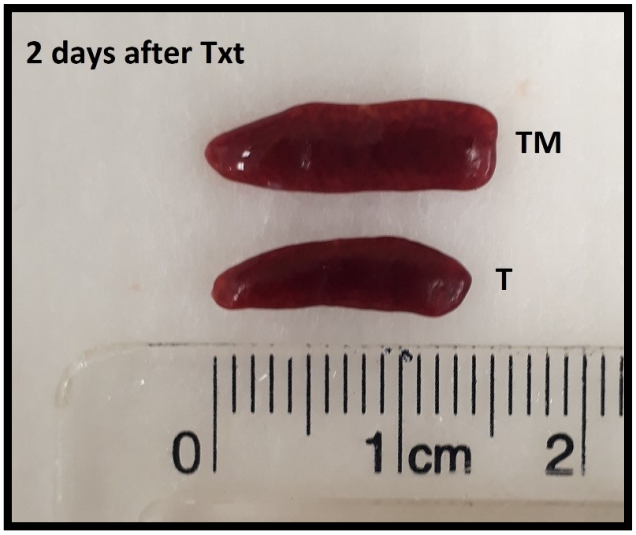

***Figure 6. Adoptively transferred MDSCs increase spleen size**. MDSCs were generated in vitro from BMC of B6.SJL mice (CD45.1⁺) by incubation with GM-CSF for 4 days. CD45.1⁺ MDSCs were adoptively transferred into B6 mice (CD45.2⁺) 1 hour before Txt or mice received Txt without previous transfer of MDSCs. Spleens of Txt mice after MDSC transplantation (TM) were macroscopically enlarged compared to spleens of untreated Txt animals (T). Photo was taken 2 days after Txt and shows 2 representative spleens out of 10 (TM) or 11 (T) analyzed.*

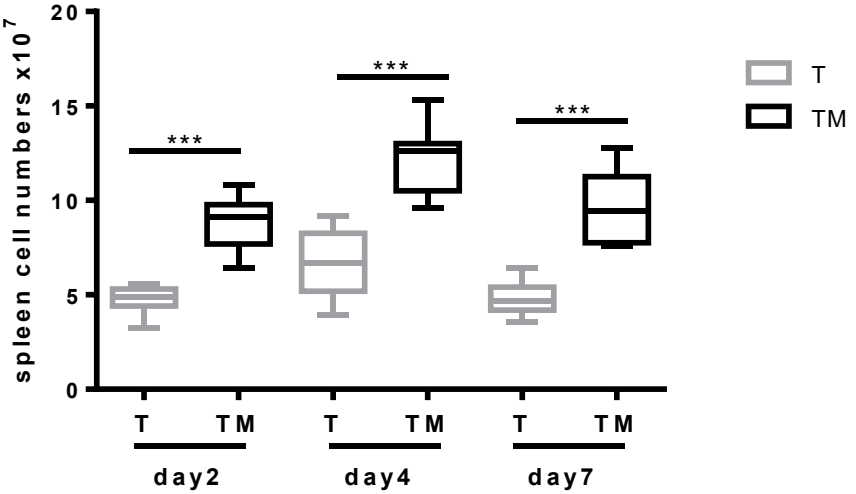

***Figure 7. Transplantation of in vitro-generated MDSCs increases number of spleen cells.** MDSCs were generated in vitro from BMC of B6.SJL mice (CD45.1⁺) by incubation with GM-CSF for 4 days. CD45.1⁺ MDSCs were adoptively transferred into B6 mice (CD45.2⁺) 1 hour before Txt or mice received Txt without previous transfer of MDSCs. Graph shows statistical analysis of splenocyte numbers of Txt (T) and MDSC-treated Txt mice (TM) at 2, 4 and 7 days after Txt. Data represent means ± SD of n=6-11 in 2-3 independent experiments per time point. Unpaired student's t-test, *** p-value<0.0001.*

3.5. MDSC TREATMENT IN TXT MICE INCREASES SPLENIC T CELL NUMBERS

Since adoptive transfer of in vitro-generated MDSCs is associated with a significant increase in splenocyte numbers, we analyzed which lymphocyte subsets were responsible for the increase of splenocytes. Of note, transplanted MDSCs themselves do not significantly contribute to elevated spleen cell numbers since the 2% $CD45.1^+$ MDSCs found in the spleen 7 days after transplantation (Figure 4) only account for an increase of roughly $1x10^6$ cells. As it is known that MDSCs influence T cell functions and proliferation, we stained spleen cells for different lymphoid T cell markers at 2 and 7 days after Txt in order to define whether T cells - and which subsets of T cells - are increased in MDSC treated Txt mice. The adoptively transferred MDSCs lead to an increase in total T cell numbers ($CD3^+$) and an increase in both subpopulations ($CD4^+$ and $CD8^+$). The ratio of $CD4^+/CD8^+$ T cells is not altered by MDSC treatment. We observed an increase in T cell numbers even 7 days after MDSC transplantation indicating a long-term effect of MDSCs despite their low numbers in the spleen (Figure 8) [58].

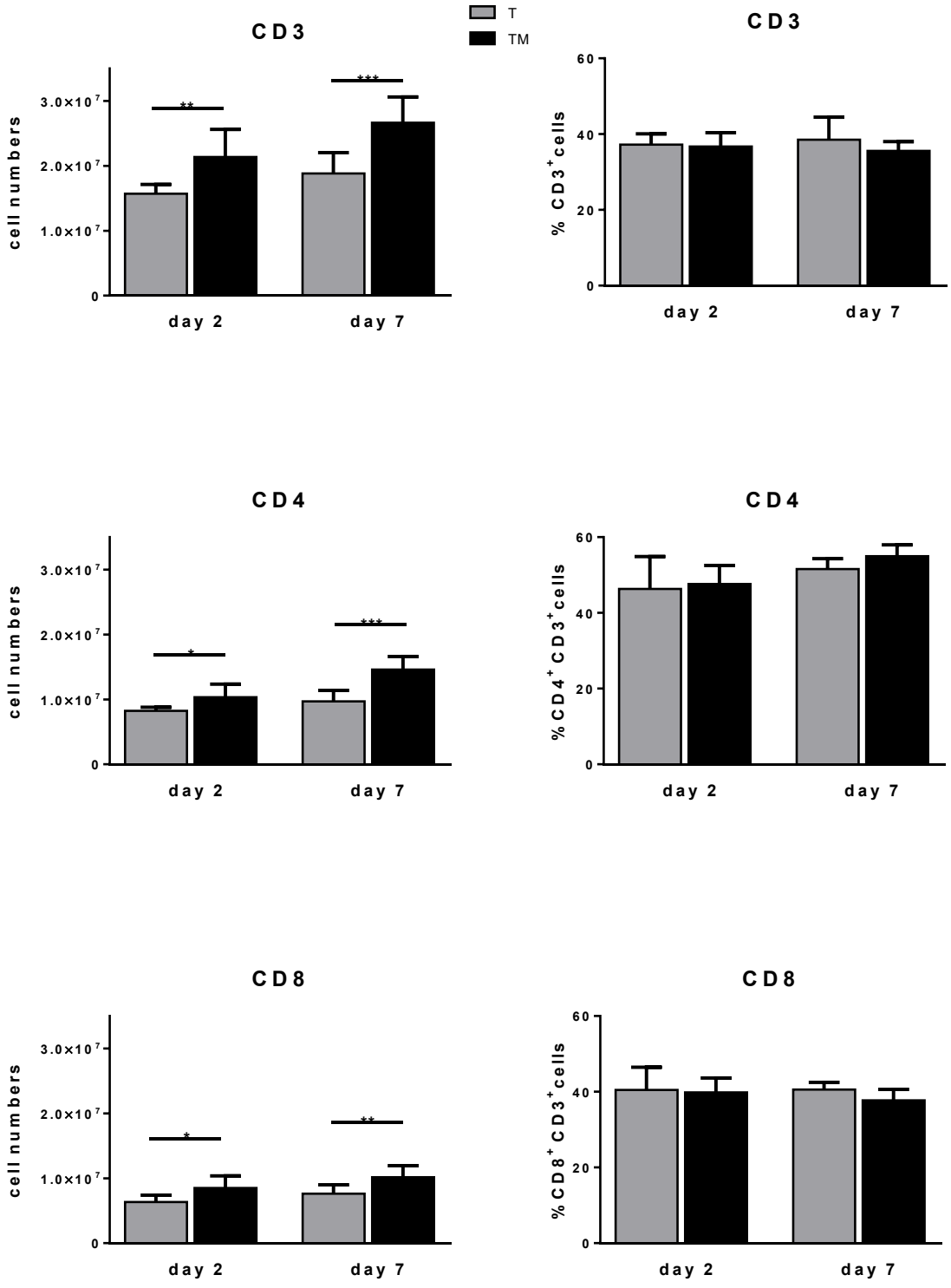
T
TM
CD3
cell numbers
3.0×10^7
2.0×10^7
1.0×10^7
0
**
***
day 2
day 7
CD3
% CD3+cells
60
40
20
0
day 2
day 7
CD4
cell numbers
3.0×10^7
2.0×10^7
1.0×10^7
0
*
***
day 2
day 7
CD4
% CD4+CD3+cells
60
40
20
0
day 2
day 7
CD8
cell numbers
3.0×10^7
2.0×10^7
1.0×10^7
0
*
**
day 2
day 7
CD8
% CD8+CD3+cells
60
40
20
0
day 2
day 7

Figure 8. T cell numbers increase in the spleen of MDSC-treated Txt mice. *MDSCs were generated in vitro from BMC of B6.SJL mice (CD45.1$^+$) by incubation with GM-CSF for 4 days. CD45.1$^+$ MDSCs were adoptively transferred into B6 mice (CD45.2$^+$) 1 hour before Txt or mice received Txt without previous transfer of MDSCs. Spleens were harvested at day 2 and 7 after Txt. Spleen cells were stained for CD3, CD4 and CD8. By defining the percentage of CD3, CD4 and CD8 populations total cell numbers were calculated. The presence of adoptively transferred MDSCs increases T cell numbers in the spleen but does not influence the distribution of the lymphocyte subsets. Bar graphs show number of CD3$^+$, CD4$^+$ or CD8$^+$ splenocytes (left) or % CD3$^+$, CD4$^+$ or CD8$^+$ splenocytes (right) of Txt (T) and MDSC-treated Txt (TM) mice. Data represent means ± SD of n=7-12 from 2-4 independent experiments. Unpaired student's t-test, *p-value<0.05, **p<0.001, *** p-value<0.0001.*

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Despite the massive increase in T cell numbers in the spleen after transplantation of MDSCs, it does not account for the entire difference in splenocyte numbers of untreated and MDSC treated mice. Staining of more lymphoid markers in future experiments will clarify which other cell populations are induced by the presence of MDSCs.

3.6. EXPRESSION OF ACTIVATION MARKERS ON T CELLS

Increased cell numbers can be explained either by an increased proliferation rate or a decreased apoptosis rate. Therefore, we first analyzed whether T cells of MDSC-treated Txt mice exhibit increased proliferation. Proliferation is always associated with activation reflected by the expression of activation markers and the appearance of effector cells. Thus, we analyzed different markers (CD25, CD69, CD44, CD122) known to be expressed on activated T cells.

T cells of MDSC-treated Txt mice show higher expression of the T cell activation markers CD25, CD69, CD44 and CD122 compared to untreated Txt mice at 2, 4 and 7 days after Txt (Figure 9A). When being kept in culture for 24h in medium or with additional T cell stimulation through an anti-CD3 and anti-CD28 (α-CD3/28) antibody, T cells of MDSC treated Txt mice also show a higher expression of CD25 and CD69. Due to low number of experiments these results merely show a tendency and are awaiting further analysis (Figure 9B). These findings suggest that the presence of adoptively transferred MDSCs activates T cells in the spleen after Txt [58].

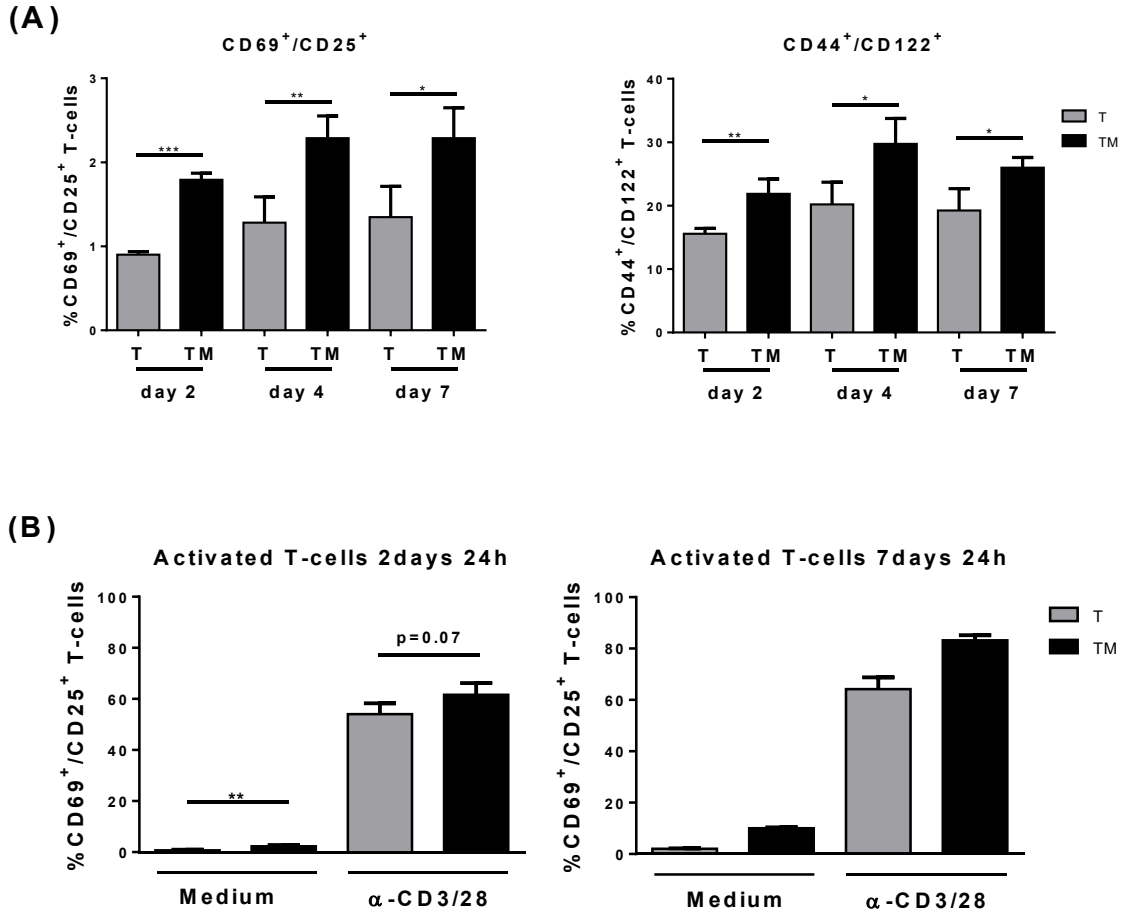

Figure 9. MDSC-treated Txt mice show higher expression of T cell activation markers. *MDSCs were generated in vitro from BMC of B6.SJL mice (CD45.1+) by incubation with GM-CSF for 4 days. Txt mice received MDSCs 1h before Txt (TM) or were left untreated (T).* ***(A)*** *Mice were sacrificed 2, 4 or 7 days after Txt and splenocytes were stained immediately for the expression of CD25, CD69, CD44 or CD122. Data represent means ± SD of n=4 per time point.* ***(B)*** *Mice were sacrificed 2 or 7 days after Txt and splenocytes were cultured in the presence of α-CD3/28 or in medium alone. 24h later cells were stained for CD25 and CD69. Data represent means ± SD of 2d: n=3-4 and 7d: n=1 (SD calculated from pipetted replicates). Unpaired student's t-test, *p-value<0.05, **p<0.001, *** p-value<0.0001. Due to low number of experiments, results merely show a tendency and are awaiting further analysis.*

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3.7. PROLIFERATION CAPACITY OF T CELLS

Since we could show that the T cells of MDSC-treated Txt mice exhibit a more activated phenotype compared to non-treated Txt mice, we further analyzed the potential of the T cells to react to different stimuli in a proliferation assay.

3.7.1. Stimulus Independent Proliferation of T cells

Activated T cells are intended to undergo proliferation and clonal expansion. Since the transplantation of in vitro-generated MDSCs shortly before Txt elevates the activation status of T cells, we investigated their proliferative capacity. First, we analyzed whether T cells expand in culture without further activation signals. Splenocytes were isolated from Txt (T) and MDSC-treated Txt (TM) mice 2, 4 and 7 days after Txt. CFSE stained spleen cells of T and TM were cultured in medium for 4 days without adding further T cell stimulating agents and then analyzed by flow cytometry. $CFSE^{low}$ T cells were considered as proliferated.

Interestingly T cells derived from MDSC-treated Txt mice exhibit increased proliferation without any further stimulation, which corresponds to the increased expression of activation markers in T cells derived from MDSC-treated animals. $CD3^+$ T cells from MDSC treated Txt mice exhibit a proliferation rate between 50% and 80%, while T cells from untreated mice show a proliferation of between 15% and 30% dependent on the time point analyzed (day 2: T: 13.6±5.4% n=8; TM: 56.2±8.7% n=7; day 4: T: 29.2±6.4% n=7; TM: 67.5±4.1% n=7; day 7 T: 43.1±19.8% n=7; TM: 78.8±3.0% n=6) (Figure 10). These data show, that MDSC treatment leads to an activation of T cells [58].

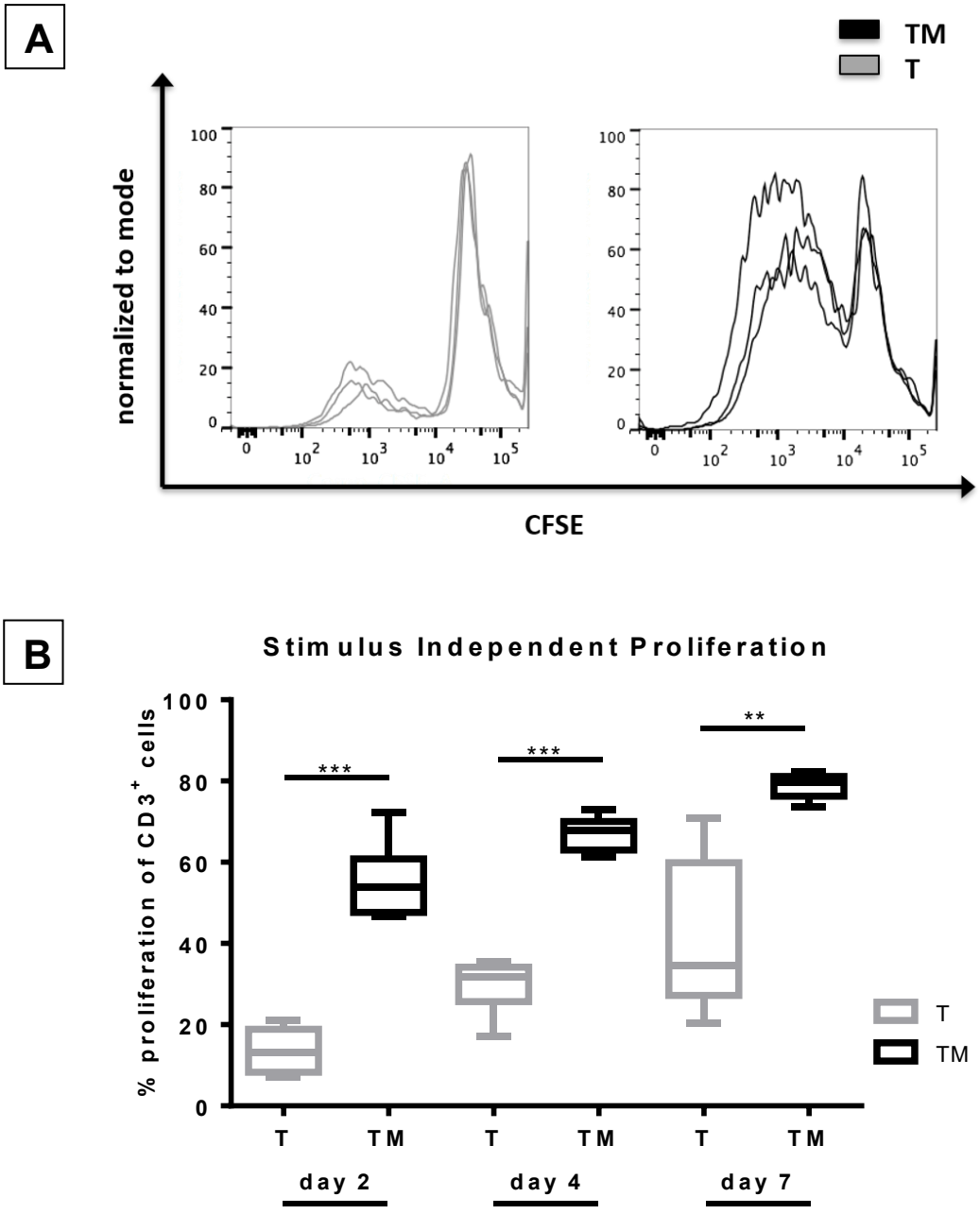


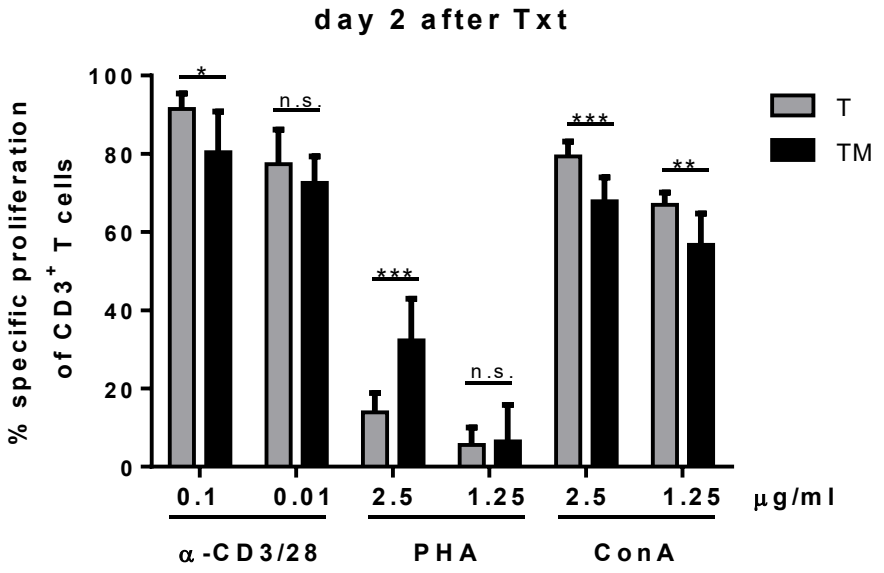
Figure 10. CD3⁺ T cells of Txt-mice exhibit increased stimulus independent proliferation after MDSC treatment. *MDSCs were generated in vitro from BMC of B6.SJL mice (CD45.1⁺) by incubation with GM-CSF for 4 days. Txt mice received MDSCs 1h before Txt (TM) or were left untreated (T).* ***(A)*** *4 days after Txt spleen cells were harvested and labeled with CFSE. CFSE stained spleen cells were cultured in medium for 4 days and then stained for CD3. Graph shows CFSE expression level of CD3⁺ cells for one experiment with 4 mice.* ***(B)*** *2, 4 and 7 days after Txt spleen cells were harvested and labeled with CFSE. CFSE stained spleen cells were cultured in medium for 4 days and then stained for CD3. $CFSE^{low}$ cells were considered as proliferated cells. Graph shows $\%CFSE^{low}CD3^{+}$ spleen cells. Data represent means ± SD of N=2, n=6-8 for all time points. Unpaired student's t-test, *p-value<0.05, **p<0.001, *** p-value<0.0001.*

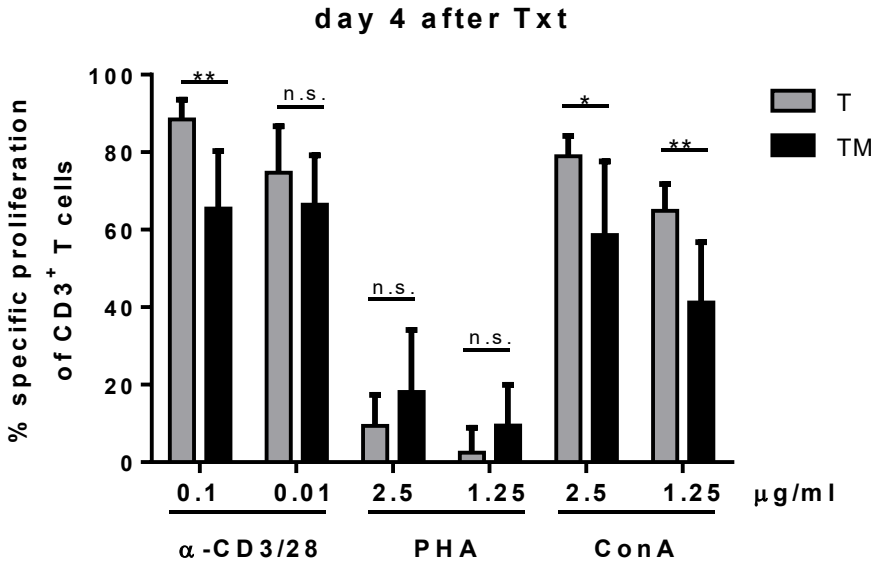
3.7.2. Stimulus Induced Proliferation of T cells

Since MDSC treatment activates T cells from Txt mice, we next clarified whether the T cells are still sensitive for stimulation signals. For the Stimulus Induced Proliferation Assay (SIPA), CFSE stained spleen cells were stimulated with α-CD3/28, ConA or PHA and specific proliferation was analyzed via FACS staining after 4 days of stimulation.

By trend, T cells of MDSC-treated Txt mice show a reduced stimulus induced specific proliferation compared to T cells of mice that were not treated with MDSCs after stimulation with different concentrations of α-CD3/28 (0.1 and 0.01µg/mL), PHA (2.5 and 1.25µg/mL) or ConA (2.5 and 1.25µg/mL) for four days (Figure 11).

In conclusion, these results demonstrate that in in trauma-induced mice adoptively transferred MDSCs do not exhibit a significant immunosuppressive effect, but rather support T cell expansion without impairing the proliferative capacity [58].


day 2 after Txt
% specific proliferation of CD3+ T cells
100
80
60
40
20
0
*
n.s.
***
n.s.
***
**
T
TM
0.1
0.01
2.5
1.25
2.5
1.25
µg/ml
α-CD3/28
PHA
ConA


day 4 after Txt
% specific proliferation of CD3+ T cells
100
80
60
40
20
0
**
n.s.
n.s.
n.s.
*
**
T
TM
0.1
0.01
2.5
1.25
2.5
1.25
µg/ml
α-CD3/28
PHA
ConA

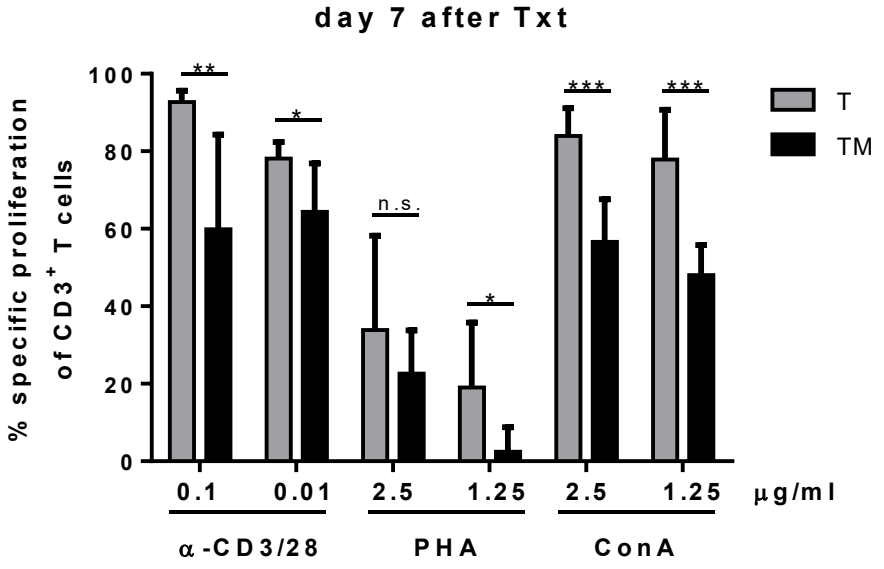

day 7 after Txt
% specific proliferation of CD3+ T cells
100
80
60
40
20
0
**
*
n.s.
*
***
***
T
TM
0.1
0.01
2.5
1.25
2.5
1.25
µg/ml
α-CD3/28
PHA
ConA

Figure 11. Presence of adoptively transferred MDSCs negligibly inhibits antigen specific proliferation. *MDSCs were generated in vitro from BMC of B6.SJL mice (CD45.1$^+$) by incubation with GM-CSF for 4 days. Txt mice received MDSCs 1h before Txt (TM) or were left untreated (T). 2, 4 and 7 days after Txt spleen cells were harvested and labeled with CFSE. CFSE stained spleen cells were cultured in medium with different concentrations of either α-CD3/28 (0.1 and 0.01µg/mL), PHA (2.5 and 1.25µg/mL) or ConA (2.5 and 1.25µg/mL) for 4 days to stimulate T cells and then stained for CD3. CFSE^{low} cells were considered as proliferated cells. Graph shows % antigen specific proliferation of CD3$^+$ cells calculated with the help of %CFSE^{low}CD3$^+$ spleen cells after stimulation and in medium controls. Data represent means ± SD of N=2, n=6-8 for all time points. Unpaired student's t-test, *p-value<0.05, **p<0.001, *** p-value<0.0001.*

3.8. DETECTION OF TYPE 1 AND 2 CYTOKINES IN IN VITRO EXPERIMENTS

MDSCs are known to influence the differentiation of T cells depending on the type of inflammation. Adoptive transfer of in vitro-generated MDSCs shifts the immune response towards T_H2 immunity in the context of allogeneic bone marrow transplantation, while MDSCs are reported to induce a T_H1 immune response in inflammatory lung diseases [3, 72].

To further define whether adoptively transferred MDSCs also modulate the type of T cell response after Txt, we analyzed the expression of T_H1 and T_H2 specific cytokines in a SIPA, in which T cells were derived either from MDSC-treated Txt mice or untreated Txt mice. T_H1 cells mainly express IFN-γ, IL-2 and TNF-α, while T_H2 cells are known to express higher levels of IL-5, IL-10 and IL-13. SIPA supernatants were taken after 4 days of stimulation and cytokines measured via the ProcartaPlex® Multiplex Immunoassay (eBioscience).

Adoptively transferred MDSCs lead to a strong increase in all measured cytokine levels (TNF-α, IFN-γ, IL-2, IL-5, IL-10 and IL-13) in supernatants of the proliferation assay even in the absence of additional stimulation compared to supernatants derived from splenocytes of mice that were not treated with MDSCs (medium controls in Figure 12). Further stimulation with α-CD3/28 or PHA does not increase cytokine levels in supernatants of splenocytes from MDSC-treated mice, except for TNFα. In untreated Txt mice further stimulation with 0,05 µg/ml α-CD3/28 leads to an increase in all measured cytokines compared to the medium control (Figure12).

In summary, MDSC-treatment of Txt mice induces a strong increase in cytokine expression in splenocytes, further confirming that MDSCs exhibit and activating function in the context of Txt. However, a clear shift in favor of a T_H1 or T_H2 immune response is not detectable [58].

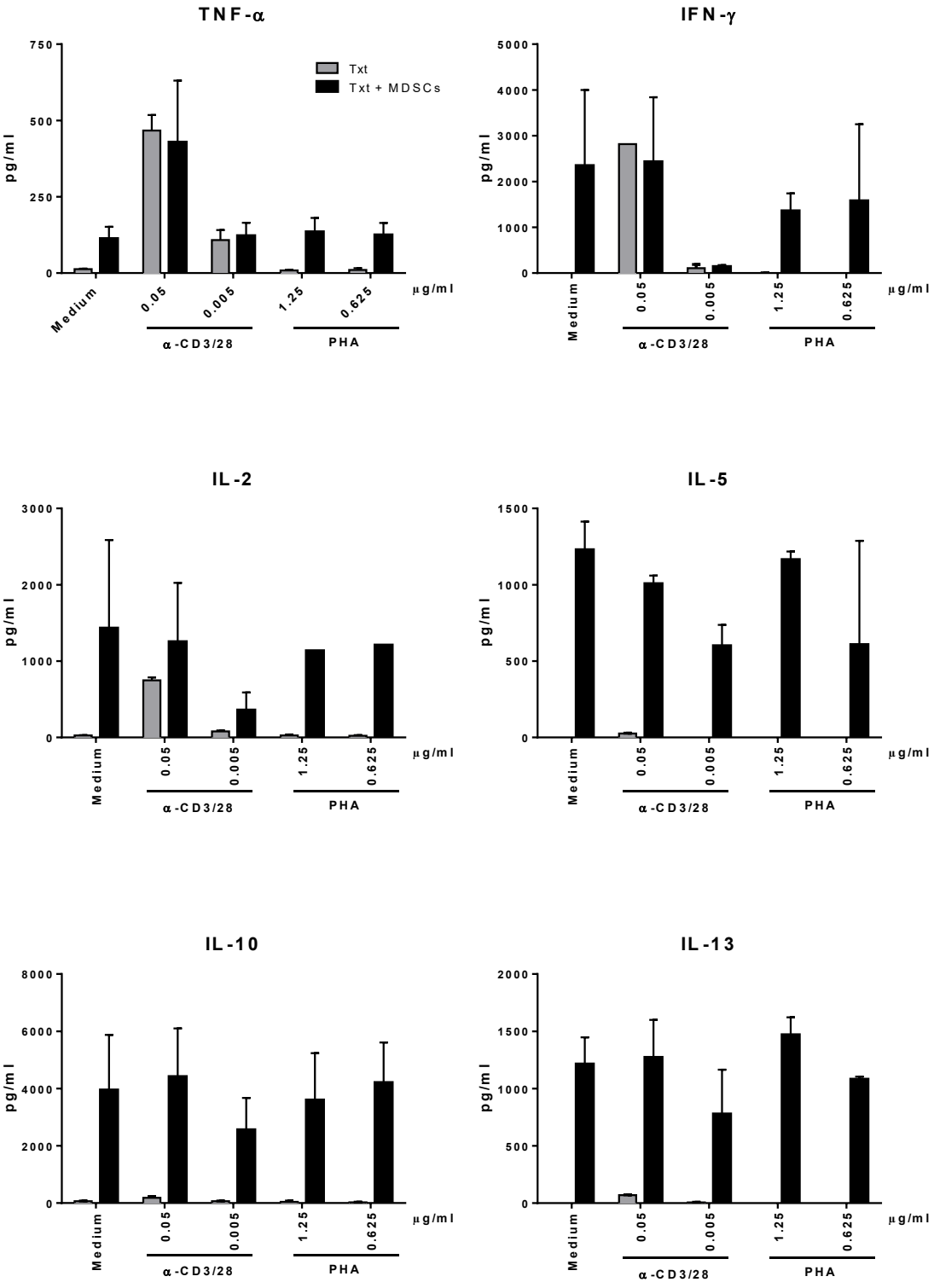

TNF-α
Txt
Txt + MDSCs
pg/ml
750
500
250
0
Medium
0.05
0.005
1.25
0.625
μg/ml
α-CD3/28
PHA
IFN-γ
5000
4000
3000
2000
1000
IL-2
3000
2000
1000
IL-5
1500
1000
500
IL-10
8000
6000
4000
2000
IL-13
1500

Figure 12. Increased cytokine expression in SIPA supernatants from MDSC-treated Txt mice. *MDSCs were generated in vitro from BMC of B6.SJL mice (CD45.1$^+$) by incubation with GM-CSF for 4 days. Txt mice received MDSCs 1h before Txt (TM) or were left untreated (T). 7 days after Txt spleen cells were harvested and labeled with CFSE. CFSE stained spleen cells were cultured in medium only or with different concentrations of either α-CD3/28 (0.1 and 0.01µg/mL) or PHA (2.5 and 1.25µg/mL) for 4 days. Cytokine levels of TNF-α, IFN-γ, IL-2, IL-5, IL-10 and IL-13 were measured via the ProcartaPlex® Multiplex Immunoassay (eBioscience) in supernatants of SIPA from day 7 Txt mice and MDSC-treated Txt mice at 4 days after in vitro culture. Data represent means ± SD of N=1, n=2.*

3.9. MDSC-TREATMENT DOES NOT INDUCE APOPTOSIS IN T CELLS

Since T cells from MDSC-treated mice exhibit an increased activation status, we also analyzed the viability of T cells in MDSC-treated vs. untreated Txt mice. Annexin V was used to detect externalization of phosphatidylserine, which is a marker for apoptosis. Spleen cells were stained with Annexin V directly after sacrificing the animals (0h) and after being cultured for additional 24h. Subsequently, T-cells were stained for CD3 expression and Annexin V positivity was defined. Adoptively transferred MDSCs did not alter the percentage of Annexin V$^+$ T cells in Txt mice, further indicating that the increase in T cell numbers is mediated by T cell expansion and not by an increased apoptosis resistance (Figure 15).

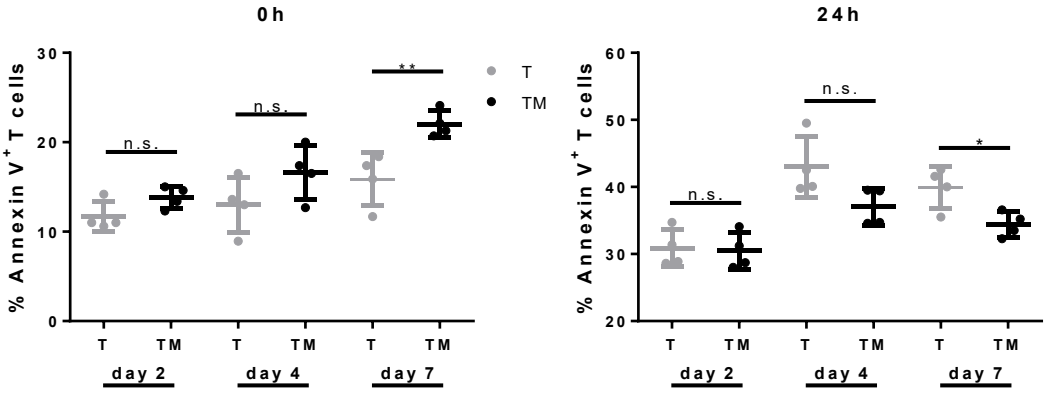


Figure 13. The presence of adoptively transferred MDSCs does not alter the expression of Annexin V on CD3⁺ T cells. *MDSCs were generated in vitro from BMC of B6.SJL mice (CD45.1⁺) by incubation with GM-CSF for 4 days. CD45.1⁺ MDSCs were adoptively transferred into B6 mice (CD45.2⁺) approximately 1 hour before Txt (TM) or mice were left untreated (T). Spleen cells were harvested at days 2, 4 and 7 after Txt. Spleen cells were then stained for CD3 and Annexin expression at 0 or 24 hours after harvesting the spleens. Graph shows statistical analysis of % Annexin V⁺ T cells in Txt (T) and MDSC-treated Txt (TM) mice. Data represent means ± SD of n=3-4. Unpaired student's t-test, *p-value<0.05, **p<0.001, *** p-value<0.0001.*

4. DISCUSSION

Parts of this discussion were already published in Klingspor and Kustermann et al (2019) [58].

Traumatic injuries cause severe changes in immunohomeostasis. An initial strong activation of the inflammatory system leads to an imbalanced and overwhelming innate immune response and to a suppression of adaptive immunity, sensitizing the host for infectious complications, sepsis and MODS.

Therapeutic options to strengthen the immune response and prevent immune dysfunction remain scarce due to an uncomplete understanding of what drives the failure of post-traumatic immune responses and which cells are responsible. Thus, it is important to identify post-traumatic changes in immune cells known to regulate immune responses such as myeloid-derived suppressor cells.

Since MDSCs are known to regulate innate and adaptive immune responses, we analyzed the impact of MDSCs on the post-traumatic immune response. Therefore, we adoptively transferred in vitro-generated MDSCs in mice which underwent Txt and defined changes in the early systemic pro-inflammatory response and in T cell immunity.

4.1. HOMING OF MDSCS AFTER TXT

It has been shown that endogenous MDSCs accumulate preferentially in the lung and also to some extent in the spleen after Txt [47]. Therefore, we were first interested to see to which organs the in vitro-generated MDSCs migrate and how long they can be detected after transfer. Little is known about the path of in vitro-generated MDSCs after adoptive transfer into trauma mice. MDSCs isolated from tumor-bearing mice migrate to the lung after injection into mice in a model of asthma-related airway inflammation [13]. Furthermore, in vitro-generated MDSCs home to the primary breast tumor and the spleen, lung, liver and bone marrow in a breast cancer model [94]. These results suggest that adoptively transferred MDSCs migrate to inflamed tissue as well as other organs.

We could show that in vitro-generated and adoptively transferred MDSCs home to the liver at all time points investigated but are mainly found in the spleen at 7 days

after adoptive transfer and blunt chest trauma induction. Surprisingly, there were no adoptively transferred MDSCs detectable in the lung, even though the lung is targeted by the Txt, which causes barrier dysfunction and local inflammation, usually promoting the invasion of different immune cell populations.

After trauma, other adoptively transferred cells such as mesenchymal stem cells have been shown to migrate primarily into the lung and were only later found at the injury site (e.g. tibia fracture) [34]. MDSCs injected into adjacent tissue after spinal cord injury migrate to the injury site, alleviate inflammation and promote tissue regeneration [92].

It remains to be definitely defined whether in vitro-generated MDSCs really do not migrate to the lung after Txt, or whether our detection methods were merely insufficient. There are some conceivable explanations for us not being able to detect them. It may be that we could not detect the transplanted MDSCs in the lung due to low total extractable lymphoid cell numbers and the already low number of transplanted MDSCs possibly present in the lung. The most likely explanation might be that our failure to detect the adoptively transferred MDSCs is because we transplanted MDSCs one hour before the blunt chest trauma. Findings might be completely different if we transplanted MDSCs after Txt into animals already suffering from barrier dysfunction and inflammation in the lung.

Future studies with different detection methods, such as using MDSCs generated from green fluorescent protein (GFP) or luciferase transgenic mice, might make detection of adoptively transferred MDSCs easier and help further elucidate their fate after transplantation.

4.2. SYSTEMIC INFLAMMATORY RESPONSE AFTER ADOPTIVE TRANSFER OF MDSCS AND TXT

After showing that adoptively transplanted MDSCs travel via the circulation and preferentially home to the spleen, we analyzed whether MDSCs influence the systemic inflammatory response. Given that MDSCs are immunosuppressive cells, we expected that in vitro-generated and adoptively transplanted MDSCs would suppress the early immune response e.g. by decreasing the amount of pro-inflammatory factors.

Surprisingly, the transplanted in vitro-generated MDSCs had no influence on the systemic expression of pro-inflammatory factors or cytokines, and therefore did not modulate the early post-traumatic inflammation. This is in contrast to the finding that MDSCs induced endogenously after Txt decrease the amount of IL-6, G-CSF, and MCP-1 in the serum, thus suppressing the secretion of pro-inflammatory cytokines [47]. However, our findings suggesting that in vitro-generated and adoptively transferred MDSCs do not influence the systemic inflammatory response are in line with the findings of Derive et al. showing that the adoptive transfer of MDSCs in a sepsis model had no effect on serum cytokine levels, but helped control microbial sepsis by promoting bacterial clearance [22].

The strong discrepancy between the effect of trauma-induced and in vitro-generated MDSCs might also be explained by their differentiation under very different conditions, possibly leading to distinct activation stages and thus differing functions in immune regulation. Other findings in our study support this idea of alternative functions of MDSCs when induced under non-inflammatory conditions (see also 5.6.).

4.3. IMMUNE CELL FUNCTION AFTER ADOPTIVE TRANSFER OF MDSCS

In the blunt chest trauma model, adoptively transferred MDSCs do not act immunosuppressive but have immunostimulatory functions. We could show that the adoptive transfer of MDSCs shortly before Txt leads to the activation and expansion of T cells neither inhibiting their proliferative capacity nor influencing the balance of T_H1/T_H2 immune responses.

4.3.1. Splenocyte Numbers after Adoptive Transfer of MDSCs

MDSCs home mainly to the spleen seven days after adoptive transfer and blunt chest trauma. In contrast to the idea that MDSCs act as mainly immunosuppressive cells, we could show, that adoptively transferring MDSCs one hour before Txt strongly increases splenocyte numbers, leading to spleens of treated trauma animals almost doubling in size. This is in contrast to a study showing that spleens of MDSC-treated lupus erythematosus mice are significantly smaller [82].

In our study the number of all T cell subpopulations ($CD3^+/CD4^+/CD8^+$T cells) analyzed is increased while their composition is not altered. An increase of all splenic T cells after adoptive transfer of MDSCs has not been previously described, but adoptively transferred MDSCs have been shown to alter certain immune cell subpopulations in distinct pathological conditions.

An increase in regulatory B cells but decrease in activated T cells after adoptive transfer of MDSCs has been shown in a model for autoimmune encephalomyelitis and systemic lupus erythematosus [14, 82]. The influence of adoptively transferred MDSCs on the population of regulatory T cells depends on the disease entity. Their presence leads to an increase in T_{Regs} in experimental viral myocarditis and asthma [27, 102], while no change is seen in models for autoimmune encephalomyelitis or collagen-induced arthritis [14, 28], and no change or even a decrease was shown in different studies regarding systemic lupus erythematosus [49, 82].

MDSCs generated in exactly the same way as we did, do not affect T cell numbers in the spleen when injected into allogeneic bone marrow transplanted mice, demonstrating that their effect most likely depends on the inflammatory conditions in the host [72]. Since the increase of splenocytes can not only be explained by elevated T cell numbers, it is of major interest to define which other cell populations are induced by adoptively transferred MDSCs.

4.3.2. T cell Function after Adoptive Transfer of MDSCs

Post-traumatic T cell dysfunction is one of the main reasons for trauma-induced morbidity and mortality. Although myeloid-derived suppressor cells are well known for their immunosuppressive functions in several disease entities, especially in the tumor setting, they exhibit alternative functions rather than acting immunosuppressive when transferred into Txt mice. Apart from T cell numbers strongly increasing in the spleen after treatment with MDSCs, splenic T cells exhibit a strong proliferative capacity when cultured for several days. Interestingly, after MDSC treatment, T cells from Txt mice show a higher expression of activation markers (CD25, CD69, CD44, CD122) and an increased proliferative capacity in vitro without further stimulation.

The rather immunostimulatory role of MDSCs through promoting immune cell expansion is in line with findings by other groups. Makarenkova et al. showed that

MDSCs isolated from mice after traumatic stress can stimulate proliferation of naive T cells [68]. In skin-transplanted mice, adoptively transferred MDSCs cause an increase of $CD4^+$ T cells in the transplant and of antigen-presenting cells as well as activated T cells ($CD25^+/CD69^+$) in the spleen. MDSC transfer seems to be beneficial, leading to reduced skin graft rejection [24]. Rather than suppressing immune responses, the MDSCs induce an activation of the immune system comparable to the results we obtained in our Txt study. Immunostimulatory functions augmenting T cell proliferation have also been demonstrated for $CD11b^+/Gr1^+$ cells from malignant ascites and MDSCs from patients with inflammatory bowel disease [54, 104].

Elevated proliferation might lead to exhaustion of T cells and interfere with their ability to respond to activation signals. We tested different stimuli such as α-CD3/28, ConA or PHA, but no significant decrease in responsiveness was observed. Thus, in contrast to MDSCs being widely described as acting T cell suppressive, we could show that transferring in vitro-generated MDSCs does not impair the T cell response to specific stimulation in a Txt model. Enhanced T cell function through MDSCs has also been shown in tumor models. T cells conditioned with MDSCs before transfer show increased anti-tumor activity [90].

In our studies it was shown that endogenous MDSCs isolated from animals early (24 and 48 hours after blunt chest trauma) are immunosuppressive. Therefore, we were surprised that in contrast to the described immunosuppressive functions of the endogenous MDSCs, our in vitro-generated and adoptively transferred MDSCs act as a strong stimulus on the immune system. They promote T cell activation and stimulus independent proliferation, while the antigen specific stimulation is hardly impaired. Endogenous MDSCs isolated 72 hours after blunt chest trauma do not exhibit immunosuppressive functions [47]. This demonstrates that the immunosuppressive capacity might be a short-term effect of MDSCs in response to the Txt. In our adoptive transfer study, we looked at a rather long-term effect, analyzing immune functions at 2, 4 and 7 days after adoptive transfer and Txt. In addition to the probably very different activation status of endogenous MDSCs induced after Txt compared to the in vitro-generated MDSCs, the time point of analysis might also partially explain why results differ significantly between the two

Txt studies. Transcriptome analysis of the endogenously induced and in vitro-generated MDSCs at different time points will help identify the differences.

4.3.3. T_H1/ T_H2 Balance after Adoptive Transfer of MDSCs

After traumatic injuries patients often develop an imbalance of the T_H1/T_H2 immune responses. Various studies regarding traumatic injuries have shown that trauma promotes a T_H2 mediated immune response [59]. This T_H2 mediated response with concurrent suppression of the T_H1 response contributes to impaired pathogen defense mechanisms, and a high risk for infectious and septic complications in trauma patients.

In various pathological conditions, MDSCs are known to strongly influence the type of T cell response induced and hence modify the response to infections and interfere with wound healing. They have been shown to promote either a T_H1 or T_H2 response depending on the type of inflammation. MDSCs mediate a preferentially T_H1 mediated T cell response in the context of allergic airway inflammation [3, 13] but promote a T_H2 response in viral infections, different cancers, sepsis, pregnancy and bone marrow transplantation [21, 29, 32, 56, 72, 99, 111].

Even though the adoptively transferred MDSCs after Txt strongly increase T cell proliferation, they do not shift the T_H1/T_H2 balance. Thus, the transplanted MDSCs do not impede Th1 immunity after Txt, crucial for fighting infections. This differs from our findings for blunt chest trauma-induced MDSCs. They support the production of T_H1-associated cytokines without changing the number of T_H2-cytokine producing T cells, hence promoting cellular immunity [47]. Interestingly, in allogeneic bone marrow transplanted mice, in vitro-generated and adoptively transferred MDSCs lead to the induction of T_H2 cells, further demonstrating that the influence of MDSCs on the type of T cell response induced, might also depend on the inflammatory setting in the host [72].

4.4. BLUNT CHEST TRAUMA MODEL

An isolated blunt chest trauma causes only a mild and short-lived inflammatory response in mice compared to other murine disease models such as GVHD, in which the functions of MDSCs have already been analyzed.

Blunt chest trauma-induced MDSCs possess an immunosuppressive potential for up to 48 hours after Txt [47]. In our adoptive transfer study, we were looking at a rather long-term effect of the transplanted MDSCs, checking immune functions at 2, 4 and 7 days after Txt. The inflammation caused by Txt might not be strong enough for transplanted in vitro-generated MDSCs to maintain their immunosuppressive function for a longer period. It would be interesting to analyze immune functions of MDSC-treated Txt mice early after trauma to check whether the transplanted MDSCs act immunosuppressive and then later switch to a more stimulating phenotype, or rather act immunostimulatory at all time points.

Investigating the functions of MDSCs in a more severe trauma model, e.g. combining blunt chest trauma with other injuries, such as sepsis or aspiration of gastric acid, might help to further elucidate the function of MDSCs in trauma immune responses. All these are known co-morbidities or complications of Txt, which further enhance tissue injury and thus cause a more severe and sustained inflammatory response.

4.5. DEFINING MDSCS AND THEIR FUNCTIONS

We are still far from being able to exactly define the cell population Young et al. first described in 1987 as a population of Suppressor Bone Marrow Cells in a model of cancer [118]. Even though a lot of progress has been made in unraveling the characteristics of MDSCs, their origin and plasticity are still highly debated, and no ultimate definition of their phenotype and function has been agreed upon. Many different functions of this heterogeneous cell population have been demonstrated under diverse inflammatory conditions such as trauma, cancer, autoimmune diseases and infections.

Even the name myeloid-derived suppressor cell is heavily debated. Cells are now also being described as immunosuppressive neutrophils, a population of myeloid progenitors, or as MDSC-like cells [15, 39]. Since our in vitro-generated MDSCs, transferred shortly before Txt, rather act immunostimulatory, we would preferably define them as myeloid-derived regulatory cells instead of myeloid-derived suppressor cells.

Currently there is no antibody available to specifically deplete MDSCs. Gr-1 is also expressed on other myeloid cells. Therefore, the commonly used anti-Gr-1-antibody also depletes other cells such as granulocytes and macrophages apart from MDSCs [95]. Since MDSCs and neutrophils share markers and are both able to act as immunosuppressive cells, further data is needed to clearly distinguish between these subpopulations and their functions. The different effects of a Gr-1 antibody on e.g. serum cytokine levels or the balance of T_H1 or T_H2 responses shown in previous studies of our Txt model, might not only be attributed to the depletion of MDSCs alone but also the depletion of other myeloid cells [47].

It will also be interesting to analyze whether transplanted MDSCs further expand in vivo and whether they maintain their phenotype when being re-isolated ex vivo and subsequently transplanted into mice with distinct pathological conditions, or if they further differentiate into other myeloid cells in the new inflammatory environment. In a GVHD model, the in vitro-generated MDSCs have been shown to further proliferate in lymphoid organs and organs targeted by GVHD after transplantation [72]. In a study regarding cancer it was shown that a significant number of immature myeloid cells isolated from tumor-bearing mice and transferred into untreated mice remain $Gr1^+CD11b^+$, whereas cells isolated from tumor-free mice rapidly differentiate into mature myeloid cells after transfer [57].

4.5.1. MDSC Function depends on Microenvironment and Type of Immune Response

The function of MDSCs seems to depend on conditions under which they differentiate and expand. A complex network of strongly interwoven influence factors seems to control activation status and consequently the function of MDSCs.

MDSCs have been shown to accumulate after traumatic stress [47, 68, 91, 113]. These MDSCs develop under far more complex and constantly changing conditions compared to the MDSCs generated in vitro from BMC in the presence of GM-CSF. Circumstances in which MDSCs are generated might strongly impact their impact on immune responses, possibly explaining the differences we observe between the function of endogenously-induced MDSCs in mice after Txt and the adoptively transferred in vitro-generated MDSCs [47].

The impact of the environment is further demonstrated by the finding that the function of MDSCs differs greatly depending on whether the transferred MDSCs are isolated from early (day 3) or late (day 12) septic mice. MDSCs isolated from early septic mice increase early sepsis mortality, leading to elevated production of pro-inflammatory cytokines such as IL-6 and TNF-α, produce high amounts of NO, express low arginase activity and have a more mature phenotype. In comparison, MDSCs isolated from late septic mice decrease early sepsis mortality, leading to elevated production of anti-inflammatory cytokines such as IL-10 and TGF-β, express high arginase activity, only produce little NO and tend to be more immature and have less potential to differentiate into immunocompetent dendritic cells or macrophages [11]. That the stage of a disease influences MDSC functions has also been shown for MDSCs isolated from tumor bearing mice early vs. late after tumor injection [60].

Many of the factors released after a traumatic injury, such as DAMPs and inflammatory cytokines, have been shown to induce MDSCs. Some of the factors that are elevated after traumatic injuries, and known to induce MDSCs, are IL-6, IL-1β, HMGB1, TNF-α and IL-10 [46, 66, 80]. Impaired T cell function after trauma has been shown to be associated with high levels of HMGB1. Depletion of HMGB1 in a peripheral tissue trauma model leads to a decrease in MDSC accumulation, demonstrating that HMGB1 might play a role in MDSC induction following trauma [91]. Traumatic injuries including experimental blunt chest trauma lead to immediate activation of the complement system. High levels of circulating complement proteins correlate with poor outcome and risk of septic complications [38]. Anaphylatoxin C5a especially has been shown to be elevated in BAL after lung contusion [44]. In a tumor model, C5a seems to be crucial for recruitment of MDSCs and for enhancing their inhibitory functions [71]. In a tumor therapy study, C5a improves inhibitory functions of MDSCs but does not promote their accumulation [120]. C5a is also elevated after trauma and lung contusion. Due to the influence of C5a on MDSC functions in cancer, C5a might also be a promising candidate for future investigations in the context of MDSC functions in the Txt model.

Apart from inducing MDSCs many factors have also been shown to influence MDSC functions. Different protocols exist for cultivating MDSCs from BMC ex vivo that add factors to the culture medium apart from GM-CSF used in our study. Adding

dexamethasone to the culture medium in addition to GM-CSF has been shown to augment differentiation and suppressive capacity of MDSCs [122]. Stimulating BM-derived MDSCs with LPS and IFN-γ in addition to GM-CSF promotes their suppressive potential and prevents their differentiation into dendritic cells [35]. In vitro suppressive capacity of MDSCs increases even further when cells are generated with G-CSF or IL-6 in addition to GM-CSF [70]. Adding IL-13 in addition to G-CSF and GM-CSF further augments the percentages of $Gr1^+/CD11b^+$ cells and improves T cell suppressive capacity by an increase in arginase activity [41].

Culture conditions also determine suppressive mechanisms used by MDSCs. MDSCs generated in vitro from BMC with GM-CSF and G-CSF suppress T cell proliferation mainly via upregulation of iNOS [72]. In contrast, trauma-induced MDSCs exert their immunosuppressive functions mainly by depleting arginine, while an increase in iNOS expression is only observed at day 1 after Txt [47, 64, 68, 121]. Arginase 1 is also been upregulated when IL-13 is added during ex-vivo differentiation [41].

Hengartner et al. defined a poly-trauma cocktail to clarify the role of mesenchymal stem cells in trauma immune responses and how cell functions are influenced by the inflammatory environment. The serum-free polytrauma cocktail consists of the key pro-inflammatory factors expressed in poly-trauma patients in early stages of SIRS. It contains IL-1β, IL-6, IL-8, C3a and C5a [40].

It will be interesting to define how MDSCs generated under circumstances resembling the conditions in trauma patients influence the subsequent trauma-induced immune responses compared to MDSCs generated in vitro with GM-CSF. This could be achieved by adding certain inflammatory cytokines, complement factors, a poly-trauma cocktail or even serum from Txt-treated mice to the culture medium. Would they achieve inhibitory functions comparable to MDSCs induced endogenously after Txt? Further analysis might clarify mechanisms responsible for determining whether MDSCs act immunostimulatory or immunosuppressive and elucidate which trauma-induced factors are responsible for creating immunosuppressive MDSCs.

In comparison to the in vitro-generated MDSCs it would also be interesting to define how endogenously induced MDSCs isolated ex vivo after Txt and subsequently transferred into other Txt mice influence immune reactions following blunt chest trauma. In addition, clarifying whether their functions depend on the time point of isolation from the mice and therefore on the stage of the disease, as it has been described in models of late and early sepsis, and in the tumor setting, could help further understand the immunoregulatory role of MDSCs [11, 60].

But even MDSCs generated under the same conditions may exhibit completely different functions. In the blunt chest trauma model, they promote T cell expansion and do not impair stimulus dependent T cell responses, whereas in an allogeneic bone marrow transplantation model they act immunosuppressive, impair T cell functions and thus prevent GVHD [72].

In summary, all these data indicate that in addition to the culture conditions the environment to which the BM-derived MDSCs are exposed strongly influences their functions. This implies that it is urgently required to analyze MDSC functions in each pathological condition in order to define their effects on the immune response.

Pathways of immunoactivation by MDSCs and their interaction with lymphocytes in Txt are currently unclear. Future investigations will help to further understand the complex immune regulatory role and underlying mechanisms of MDSCs. Adoptively transferring alternatively activated MDSCs by either adding activating factors to the culture medium or by transferring MDSCs isolated from mice with chronic inflammation, will show how the surrounding conditions influence MDSC functions. Also, the function of different MDSC subsets (monocytic and granulocytic MDSCs) known to strongly differ from one another, remains to be investigated in the Txt model. Analyzing gene expression patterns of MDSC subgroups exhibiting different immune functions, as well as determining whether MDSCs maintain their phenotype or differentiate into other cells after adoptive transfer will identify promising candidates for further research. This could in turn help to further understand the versatile mechanisms of immunomodulation used by MDSCs such as secretion of soluble factors, induction of cells and direct interaction with target cells.

4.6. CONCLUSION

Although there are many publications reporting the induction of MDSCs after trauma, it is still not clear whether the presence of MDSCs is beneficial or detrimental for the patient. After showing that immunosuppressive MDSCs appear early after trauma and possibly dampen the trauma-induced immune response, we tried to further clarify the influence of MDSCs on adaptive and innate immunity after trauma by performing adoptive transfer experiments with in vitro-generated MDSCs [47].

In contrast to the trauma-induced MDSCs, the adoptively transferred MDSCs do not suppress T cell responses but rather promote T cell expansion without impairing stimulus induced T cell action. Transplanted MDSCs have no effect on early pro-inflammatory serum cytokines or the balance of the T_H1/ T_H2 response.

These findings point to an immunostimulatory role of in vitro-generated MDSCs in a post-traumatic setting. Thus, adoptive transfer of MDSCs might be beneficial for the host after traumatic injuries through dampening T cell apoptosis and malfunctions that often cause complications in trauma patients.

Future experiments challenging MDSC-treated Txt mice with pathogens frequently infecting trauma patients and subsequent analysis of their immune response will define whether MDSC-treatment improves or stimulates the host immune response. If this turns out to be true, MDSCs may be used therapeutically in the future to alter immune functions in pathological situations, e.g. in patients with failure to return to normal immunohomeostasis after sepsis or trauma. Adoptive transfer of MDSCs might then also be beneficial for patients with other pathologies that lead to impaired host immunity such as e.g. HIV, malnutrition or pharmacological side effects.

5. SUMMARY

Traumatic injuries account for more years of life lost than other pathologies such as cancer or heart disease. Blunt chest trauma (Txt) is a frequent injury in poly-trauma patients and negatively impacts morbidity and mortality. Late trauma deaths are often attributed to complications such as sepsis and multiorgan failure (MOF), which can occur as a consequence to an immune imbalance following trauma. When trauma patients fail to restore immunohomeostasis, treatment options remain limited due to a lack of understanding of underlying mechanisms. Therefore, it is of crucial importance to analyze changes in cell populations regulating immune functions.

Myeloid-derived suppressor cells (MDSCs) have been shown to act as immunosuppressive cells in studies mostly concerning cancer, but also expand in other conditions that lead to chronic inflammation such as traumatic injuries including Txt. However, it remains unclear whether the presence of MDSCs is beneficial or detrimental for the host in the context of trauma immune responses.

Therefore, we adoptively transferred in vitro-generated bone marrow derived MDSCs to further clarify their functions in the context of Txt. MDSCs were generated with the help of granulocyte macrophage colony stimulating factor (GM-CSF) and transferred into the mice shortly before Txt. Since many trauma patients suffer from long-lasting immunosuppression after their injuries, we analyzed the effect on T cell functions until 1 week after transfer.

Surprisingly, the adoptively transferred MDSCs do not exhibit immunosuppressive functions after Txt, but rather act immunostimulatory. MDSCs transferred before Txt travel via the blood stream, preferentially home to the spleen and are undetectable in the injured lung. Spleens of MDSC-treated animals strongly increase in cell numbers, while invading MDSCs are not the reason for increased splenocyte numbers since they only contributed to up to 2% of the cells. MDSCs strongly increase T cell numbers in the spleen however without altering the composition of different T cell subpopulations. MDSC transfer not only promotes T cell expansion but also induces an activation of T cells. T cell activation markers such as CD25, CD44, CD69 and CD122 are increased after adoptive transfer of MDSCs. Most interestingly, T cells from MDSC-treated mice proliferate in culture without any further activation signal. This intrinsic activation of T cells, however, does not

abrogate their ability to respond to further activation signals, since T cells from MDSC-treated Txt mice exhibit similar proliferation ratios after stimulation compared to T cells from untreated Txt mice. Although MDSCs are reported to influence the T_H1/T_H2 balance, we did not observe an influence of the in vitro-generated MDSCs on the polarization of T cells in Txt mice.

All together these results show for the first time, that adoptively transplanted MDSCs in the context of Txt exhibit a rather immune activating than suppressive function and might counteract trauma-induced T cell malfunctions without impeding the T_H1 response required to prevent post-traumatic infections and support tissue regeneration.

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