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CROSSTALK OF HUMAN MESENCHYMAL STROMAL CELLS WITH THE CELLULAR COMPONENTS OF THE IMMUNE SYSTEM

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For my parents

ABSTRACT

Using the potential of immune regulatory cell populations for cellular therapy constitutes an attractive tool to obliterate imbalances of immune responses in inflammatory disorders. In this context, adoptive transfer of mesenchymal stromal cells (MSCs) represents a relatively novel approach and its impact on the immune system has not been completely clarified. In this thesis we aimed to study the effects of MSCs on key immune cell types, which led us amongst others to investigate regulatory T-cells (T_{Regs}), and myeloid cells.

We show that MSCs utilize the anti-oxidative, immune regulatory enzyme hemeoxygenase-1 (HO-1) for suppressing T-cell activation directly and for inducing T_{Regs} (=indirect T-cell suppression). An inflammatory milieu generated by alloreactive T-cells led to the so-called 'licensing' of the MSCs boosting their regulatory capacity. Interestingly, HO-1 expression was substantially diminished during this process and its functions were taken over by other (up-regulated) molecules such as cyclooxygenase-2 thereby highlighting (functional) MSC plasticity.

Most MSC-based trials lack a systemic immune monitoring, which is key for interpreting the *in vivo* effects of MSCs. Performing a comprehensive flow cytometry-based immune screening in patients with acute graft-versus-host disease (aGVHD), treated with either third-party MSC or placebo infusions (in a double-blinded fashion), we were - most importantly - able to further corroborate the notion that MSCs function *in vivo* partly by promoting T_{Reg}-subsets. Thereby, our data underscores the need for accompanying extensive immune analyses to better comprehend such "bench-to-bedside" approaches. Accordingly, we carried out thorough, laboratory investigations when we were the first to apply MSCs in a patient with treatment-refractory hemophagocytic lymphohistocytosis. Upon MSC infusion we could observe an increase of the immune modulating cytokine interleukin (IL)-10 in the serum and a preferential appearance of regulatory type 2 macrophages in the patients' bone marrow. Altogether, this data confirmed previous findings from *in vitro* and animal model studies regarding the MSCs' impact on myeloid cell populations. Driven by these observations we sought out to assess whether MSCs induce so-called myeloid derived suppressor cells (MDSCs) in aGVHD patients. Although we did not find an MSC-associated effect, we were the first to identify monocytic $CD14^{+}HLA\text{-}DR^{low/neg}$ MDSCs accumulating after allogeneic hematopoietic transplantation. We characterized their suppressive function (*via* indoleamine-2,3-dioxygenase) and established a significant association with inflammatory cytokines and aGVHD. In fact, our data indicates that MDSCs are part of an immune regulating feedback mechanism that is activated during hyper-inflammations (such as in aGVHD).

Overall, our results indicate that immune regulatory populations play a decisive role in various inflammatory diseases and MSCs could boost their responses. Furthermore our work suggests that combining basic and translational research is pre-requisite for understanding the MSCs' multifaceted interactions and for optimizing their clinical use.

LIST OF PUBLICATIONS

I. The impact of inflammatory licensing on heme oxygenase-1-mediated induction of regulatory T cells by human mesenchymal stem cell.
Mougiakakos D*, **Jitschin R*,** Johansson CC, Okita R, Kiessling R, Le Blanc K. Blood. 2011 May 5;117(18):4826-35. doi: 10.1182/blood-2010-12-324038.

II. Treatment of familial hemophagocytic lymphohistiocytosis with third-party mesenchymal stromal cells.
Mougiakakos D*, Machaczka M*, **Jitschin R***, Klimkowska M, Entesarian M, Bryceson YT, Henter JI, Sander B, Le Blanc K. Stem Cells Dev. 2012 Nov 20;21(17):3147-51. doi: 10.1089/scd.2012.0214.

III. Immunosuppressive CD14+HLA-DRlow/neg IDO+ myeloid cells in patients following allogeneic hematopoietic stem cell transplantation.
Mougiakakos D*, **Jitschin R***, von Bahr L, Poschke I, Gary R, Sundberg B, Gerbitz A, Ljungman P, Le Blanc K. Leukemia. 2013 Feb;27(2):377-88. doi: 10.1038/leu.2012.215.

IV. Alterations in the cellular immune compartment of patients treated with third-party mesenchymal stromal cells following allogeneic hematopoietic stem-cell transplantation.
Jitschin R*, Mougiakakos D*, Von Bahr L, Völkl S, Moll G, Ringden O, Kiessling R, Linder S, Le Blanc K. Stem Cells. 2013 Apr 4. doi: 10.1002/stem.1386.

* contributed equally

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LIST OF ABBREVIATIONS

AICD	Activation induced cell death
Allo	Allogeneic
APC	Antigen presenting cell
ARG1	Arginase 1
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CARD	caspase recruitment domain-containing protein
CMV	Cytomegalovirus
COX-2	Cyclooxygenase-2
CTL	Cytotoxic T-lymphocyte
CTLA4	Cytotoxic lymphocyte antigen 4
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
FGL2	Fibrinogen like protein 2
FHL	Familial hemophagocytic lymphohistiocytosis
FOXP3	Forkhead box protein 3
G-CSF	Granulocyte colony stimulating factor
GVHD	Graft-versus-host disease
GVT	Graft-versus-tumor
HLA	Human leukocyte antigen
HLH	Hemophagocytic lymphohistiocytosis
HO-1	Hemeoxygenase-1
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IDO	Indoleamin-2,3-dioxygenase
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthetase
ITG	Integrin
iT_{Regs}	Induced regulatory T-cells
JAK	Janus kinase
L-NMMA	L-NG-monomethyl-Arginine acetate
LAG-3	Lymphocyte activation gene 3
LPS	Lipopolysaccharide
MDSCs	Myeloid derived suppressor cells
MS	Multiple sclerosis
MSC	Mesenchymal stromal cell
NADPH	Nicotinamide adenine dinucleotide phosphate
NK-cell	Natural killer cell
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain-containing protein
Nor-NOHA	Nω-hydroxy-nor-Arginine
nT_{Regs}	Naturally occurring regulatory T-cells

$ONOO^-$	Peroxynitrite
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PGE_2	Prostaglandin E_2
PMSCs	Peripheral mobilized stem cell
ROS	Reactive oxygen species
STAT	Signal transducer and activator of transcription
TBI	Total body irradiation
T_{Conv}-cells	Conventional T-cells
TCR	T-cell receptor
TGF-β1	Transforming growth factor- β1
T_H	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
T_{Regs}	Regulatory T-cells

1 INTRODUCTION

A functioning and balanced immune system is key to our existence. It efficiently protects us from invading pathogens, eliminates aberrant (malignant) cells and constantly evolves and adapts to new challenges. Cellular defects or dysfunctions in various diseases, but also iatrogenic conditions unhinge this tightly regulated system. This can render afflicted persons defenseless against pathogens and can drive the system out of balance leading to autoreactive, potentially life threatening processes. Besides traditional, drug-based approaches adoptive cellular therapy has emerged as an auspicious strategy. It aims to replace deficient or diseased parts of the immune system respectively to reestablish balance by introducing potent immune regulators into an unbalanced system. Cellular therapy's most successful representation is at the same its most holistic form: the allogeneic hematopoietic stem cell transplantation (alloHSCT).

AlloHSCT allows the replacement of an entire hematopoiesis and immune system and permits the cure for various hematological diseases. On the other hand, it exposes us to problems of unique complexity as we need to cope (a) with (transient) immunodeficiency, (b) a reconstituting immune system, and (c) potential rejection reactions (against the graft or the host). A plethora of innovative drugs and an emerging number of cell-based approaches have been introduced for the treatment of the complications following alloHSCT. As yet, studies based on adoptive T-cell transfer have dominated the field, such as virus-specific T-cells to boost host defense, donor lymphocyte infusion to ensure tumor eradication, and infusion of *ex vivo* expanded regulatory T-cells (T_{Regs}) in order to attenuate the reactivity of the newly transplanted immune system against the host. In some settings natural killer (NK)-cell infusion was utilized for promoting anti-tumor immunity. However, cells other than lymphocytes are steadily gaining momentum, when it comes to restore immune homeostasis in rejection reactions and autoimmunity, with mesenchymal stromal cells (MSCs) being the most prominent representative and subject of this thesis.

Since their initial introduction ten years ago MSCs' safety and efficacy has been shown in several pre-clinical and clinical studies for the treatment of various inflammatory conditions (e.g. multiple sclerosis (MS), rheumatoid arthritis, and sepsis). However, it is still scarcely understood how exactly MSCs impact the immune system and how they exert their immune regulatory effects *in vivo*. We were in the privileged position to study the interaction of human MSCs with the innate and adaptive immune system at the 'bench' and then to try to validate our findings at 'the bedside' by analyzing unique sample collections from patients that had received MSC treatment.

The aim of all adoptive cell therapies is to exert very specifically the desired effect without systemic toxicity and without compromising the immune system in other ways. A profound understanding of the underlying immunologic mechanisms is *sine qua non* to improve cellular therapies and to allow further individualization. Much knowledge can be undeniably gained from *in vitro* and animal-models, however effective cellular therapy necessitates thorough evaluation of patient samples and clinical data for comprehending the *in vivo* processes in one of the most complex biologic systems – the human.

2 MESENCHYMAL STROMAL CELLS

2.1 DISCOVERY

Nowadays, multipotent mesenchymal stromal cells (MSCs) are widely known for their regenerative and immune regulatory properties. In the 60s of the last century Friedenstein et al. identified a small subpopulation of colony-forming unit fibroblasts (CFU-F) among bone marrow cells that are capable to form ectopic bone tissue [2, 3]. These cells could be easily distinguished from the rest of the bone marrow cells based on their plastic adherence, a spindle-shaped appearance, and a rapid expansion [3]. In humans, MSCs constitute about 0.001 to 0.01% of the bone marrow mononuclear cells isolated from Percoll gradient [4] and their number steadily decreases over lifetime [5]. MSCs can virtually be isolated from all mammalian connective tissues [4, 6] but to date bone marrow still remains the primary source while cord blood [7] and adipose tissue [8] gain more and more importance.

2.2 DEFINITION OF MSCs

As yet, no specific marker has been identified for MSCs. In 2006 the International Society of Cellular Therapy (ISCT) proposed a panel of so-called minimal criteria in terms of required function and surface antigens for classifying candidate cells as MSCs [9]. MSCs need to be plastic adherent under standard culture conditions and must display *in vitro* trilineage multipotency by differentiating into bone, fat, and cartilage. The markers CD73 (ecto-5'-nucleotidase), CD90 (Thy-1) and CD105 (endoglin, SH2) have to be expressed on over 95% of the cells. Additionally, MSCs lack myeloid markers (CD11b, CD14), hematopoietic progenitor and endothelial cell marker (CD34 (mucosialin)), the common leukocyte antigen (CD45), B-cell markers (CD19 or CD79a) and human leukocyte antigen (HLA)-DR [9].

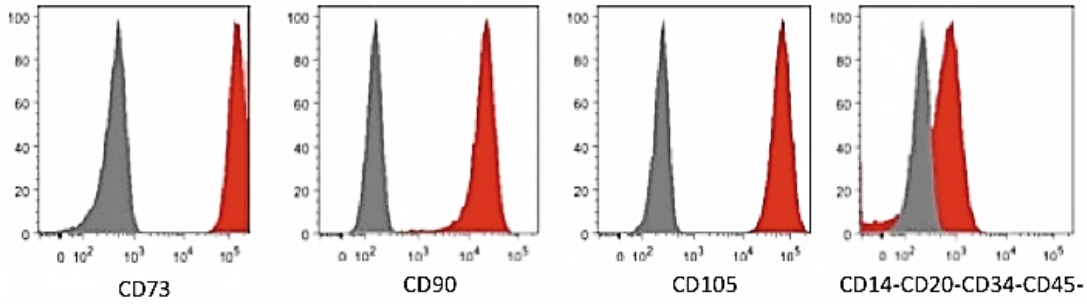

Figure 1: Phenotypical characterizations of MSCs. MSCs are defined by the expression of CD73, CD90, CD105 and the absence of CD14, CD20, CD34 and CD45. This representative FACS analysis shows the characteristic expression profile of these markers (= red) in human bone marrow derived MSCs (grey= isotype control).

Adhesion molecules	
Immunoglobulin superfamily	ALCAM (CD166), ICAM-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50), NCAM (CD56), HCAM (CD44), VCAM (CD106)
Integrins (ITG)	ITG-α1 (CD49a), ITG-α2 (CD49b), ITG-α3 (CD49c), ITG-α5 (CD49e), ITG-α6 (CD49f), ITG-αV (CD51), ITG-β1 (CD29), ITG-β3 (CD63), ITG-β5
Transmembrane superfamily	Tetraspanin (CD9)
Toll-like receptors	TLR1, TLR2, TLR4, TLR5, TLR6, TLR9
Chemokine Receptors	
C-C chemokine receptor (CCR)	CCR1, CCR2, CCR4, CCR6, CCR7, CCR8, CCR10
C-X-C chemokine receptor (CXCR)	CXCR1 CXCR2, CXCR4, CXCR5, CXCR6
CX3 chemokine receptor (CXCR1)	CX3CR1
Growth factor receptors	EGFR, FGFR, IGFR, PDGFR, TGFβRI, TGFβRII, NGFR
Cytokine receptors	
Interleukin (IL) receptors	IL-1R, IL-3R, IL-4R, IL-6R, IL-7R, Prolactin receptor (PRLR)
Prostaglandin (PG) E receptors	E prostanoid (P)1, EP2, EP3, EP4
Interferon (IFN)-γ-, tumor necrosis factor (TNF) - receptors	IFN-γR, TNFRI (CD120a), TNFRII (CD120b)
Ligands	
Programmed death (PD) ligands	PD-L1 (CD274), PD-L2 (CD273)
	Notch ligand Jagged 1
Signaling receptors	
Wnt receptors	Fz2, Fz3, Fz4, Fz5, Fz6
Notch	Notch 1, 2, 3,
Proteins	STRO-1, MUC18 (CD146)

Table 1: Receptors and molecules that have been identified to be expressed on MSCs.

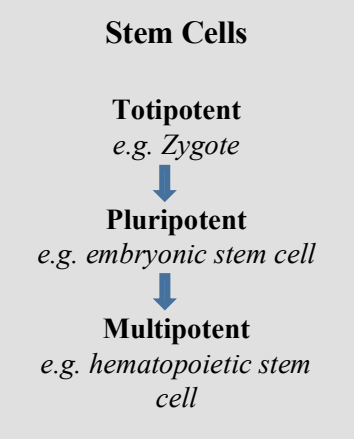

Figure 2: Differentiation capability of stem cells.

Based on their assumed capacity for self-renewal, the term mesenchymal stem cell was introduced in the 1980s [5] and became widely popular in the 1990s [10]. Stem cells per definition differentiate under the appropriate conditions into various cell types and replenish lifelong tissues with new cells. According to their plasticity and differentiation versatility, they are classified as totipotent, pluripotent and multipotent stem cells (Figure 2).

MSCs retain for up to 40 cell divisions their trilineage multipotency [11]. However, not all individual MSCs exhibit the same level of multipotency. MSCs seem to rather comprise a mixture of cells being at different maturation levels, of which many are solely mono- or bipotent [12]. Furthermore, excessive expansion of MSCs *in vitro* has been associated with a loss of differentiation capability, telomere shortening along with genetic or epigenetic modifications, resulting in senescence and apoptosis [13-15]. It is therefore still an ongoing debate whether MSCs truly represent stem cells. Currently, the term 'multipotent mesenchymal stromal cells' more and more replaces the potentially equivocal term 'multipotent mesenchymal stem cells' [11].

2.3 IMMUNOGENICITY

MSCs were considered an attractive tool for adoptive cell therapy early on. This is partially owed to a key immunological feature: their so-called immune-privilege, which means that they do not elicit a specific immune response when infused in HLA-non-identical hosts.

HLA-Class I and II are the two major classes of HLA-molecules on cell surfaces that present peptides of processed antigens to immune cells. HLA-Class I molecules present peptides of cytosolic antigens that have been synthesized within the cell. If these self-antigens are a flawless presentation of 'self', they inhibit NK-cell mediated toxicity. However, if the presented self-antigens exhibit alterations e.g. due to viral infections, tumors or if HLA-Class I is down-regulated in malignant diseases, cytotoxic T-cells (CTLs) along with NK-cells are activated and ideally eliminate the aberrant cell. HLA-class II molecules are expressed at high densities on phagocytizing cells and antigen presenting cells (APCs). Alloantigens are taken up, processed, and presented to immune cells thereby initiating their activation leading to potent antigen-specific immune responses.

Un-stimulated MSCs are positive for HLA-Class I molecules and can therefore inhibit NK-cell mediated lysis [16]. Furthermore, un-stimulated MSCs are negative for HLA-Class II molecules [17]. This remarkable characteristic allows cell-transfer across HLA-barriers and makes them a *bona fide* tool for an "off the shelf" cell therapy [18, 19].

2.4 CROSSTALK OF MSCs WITH THE IMMUNE SYSTEM

MSCs exhibit numerous immune regulatory mechanisms interfering with the innate and the adaptive immune system (Table 2). To describe all the different populations and immune compartments in detail is beyond the scope of this introduction. Therefore, this chapter focuses mainly on the aspects that have been addressed within this thesis.

The majority of the observed immune regulatory effects can be attributed to a plethora of enzymes and secreted immune modulatory factors encompassing cytokines, chemokines and interleukins (Figure 3). Most data on the interaction of MSCs with immune cells originate from preclinical studies, which did not always produce consistent results. Over time it became apparent that these mostly *in vitro* observed effects depend on the origin of MSCs (tissue as well as species), condition and duration of culture and the activation status of responder cells as well as MSCs.

Innate immune system		[11]
	Complement	[20, 21]
	TLR-signaling	[11]
	Macrophages	[22]
	Dendritic cells	[23, 24]
	Neutrophils	[25]
	Mast cells	[26]
	NK-cells	[27, 28]
Adaptive immune system		
	T-cells	[29]
	T_H1-T_H2 Balance	[30]
	Induction of T_{Regs}	[31]
	B-cells	[32]

Table 2: Overview over the identified interactions of MSCs with the different parts of the innate and adaptive immune system.

Recent data suggests that an inflammatory environment, in particular abundance of cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, Interleukin (IL)-1α and IL-1β, activates MSCs [33]. This process is called "licensing" [34] and results in an increased immune suppressive potency due to an elevated expression of immune regulatory molecules (Table 3) such as indoleamine-2,3-dioxygensase (IDO) or cyclooxygenase-2 (COX-2) [35, 36]. In fact, it can be speculated that MSC-"licensing" is part of physiological negative-feedback mechanisms that are activated for preventing the exacerbation of inflammatory responses. Actually, there are even efforts to integrate the MSCs' responsiveness to *in vitro* "licensing" (with IFN-γ and TNF-α) in the evaluation process for screening the most potent MSCs to be used in clinical approaches [33].

Immune modulatory molecules	B7-H1 / PD-L1 / CD200 / CD274
Cytokine / chemokine receptors	CD119 / IFN-γ receptor, CXCR3, 4, 5, CCR7
Adhesion molecules	CD54, CD106
DNAM ligands	CD112, CD115
NKG2D ligands	Macrophage inflammatory complex A/B, UL binding protein 1, 2, 3
Notch receptors	Jagged-1
TLR	TLR-3, TLR-4
Cytokines	IDO, COX-2

Table 3: Upregulated molecules and cytokines upon inflammatory licensing [33].

Lymphoid cells were the first to be identified as preferential targets of MSC-mediated effects. Soluble factors (e.g. IL-10 and prostaglandin (PG) E_2 [37, 38]), as well as cell-to-cell contact-dependent mechanisms (interaction of programmed death (PD)-1 with its respective ligands (PD-L1, PD-L2) [39]) are both involved in T-cell inhibition. MSCs suppress in a dose-dependent fashion the proliferation, IFN-γ production, and cytotoxicity of activated $CD4^+$ and $CD8^+$ T-cells [24, 40, 41].

Induction of T-cell apoptosis as mean of MSC-mediated T-cell suppression is still under debate [42, 43]. However, activation induced cell death (AICD) of T-cells is shown to be decreased as a consequence of the attenuated T-cell activation in the presence of MSCs. Furthermore, MSCs indirectly diminish T-cell responses by preventing maturation and thereby antigen priming function of the main APCs: the dendritic cells (DCs) [44-47].

The different types of differentiated T-cells regulate the immune response. Increased frequencies of IL-17-producing T-cells have been associated with various inflammatory diseases. MSCs were shown to prevent the differentiation of naïve $CD4^+$ T-cells into pro-inflammatory Th17-cells [48, 49]. Furthermore, by inducing T_{Regs}, MSCs amplify their immune regulatory capacity. They directly induce T_{Regs} by expressing immune modulatory enzymes or molecules such as HLA-G [50] and hemeoxygenase-1 (HO-1) [31]. Furthermore, by e.g. secretion of IL-10 and PGE_2, MSCs skew other cell types (e.g. monocytes and DCs) towards regulatory phenotypes capable of T_{Reg} induction [24, 47]. Recently, we confirmed the positive effect of MSCs on T_{Regs} induction *ex vivo* in patients receiving a third-party MSC infusion [51].

Similar to the inhibitory effect on T-cells, co-culturing MSCs with (CpG oligonucleotide 2006, anti-immunogloblin (Ig) antibodies, IL-2, IL-4, IL-10) stimulated B-cells leads to an inhibition of B-cell proliferation, impaired Ig-secretion, and lack of CXCL-12 driven B-cell chemotaxis *in vitro* [32]. Furthermore, MSCs impair the maturation of B-cells into Ig-producing plasma cells [52]. Candidate molecules for mediating these B-cell suppressive effects are tumor growth factor (TGF)-β1, hepatocyte growth factor (HGF), PGE_2 and IDO [32]. These observations have recently been challenged by studies showing that MSCs actually support survival, proliferation and differentiation of B-cells [53, 54]. The effect on B-cells is a typical example of how the type of effect elicited by MSCs varies depending on the activation status of responder cells (in this case B-cells) and MSCs: MSCs present in a mixed lymphocyte

culture can inhibit IgG, IgA and IgM production, an effect that is abrogated, if B-cells are stimulated with CD40L [55]. Furthermore, as Rasmusson et al. demonstrated, the grade of MSC activation is critical. There seems to be a direct correlation of the potency of the stimuli present in the co-culture and the MSC mediated effect. Under low stimulation with lipopolysaccharide (LPS) or viral antigens MSCs increase Ig-production by B-cells, but have an Ig-reducing function in the presence of high levels of the aforementioned stimuli [54].

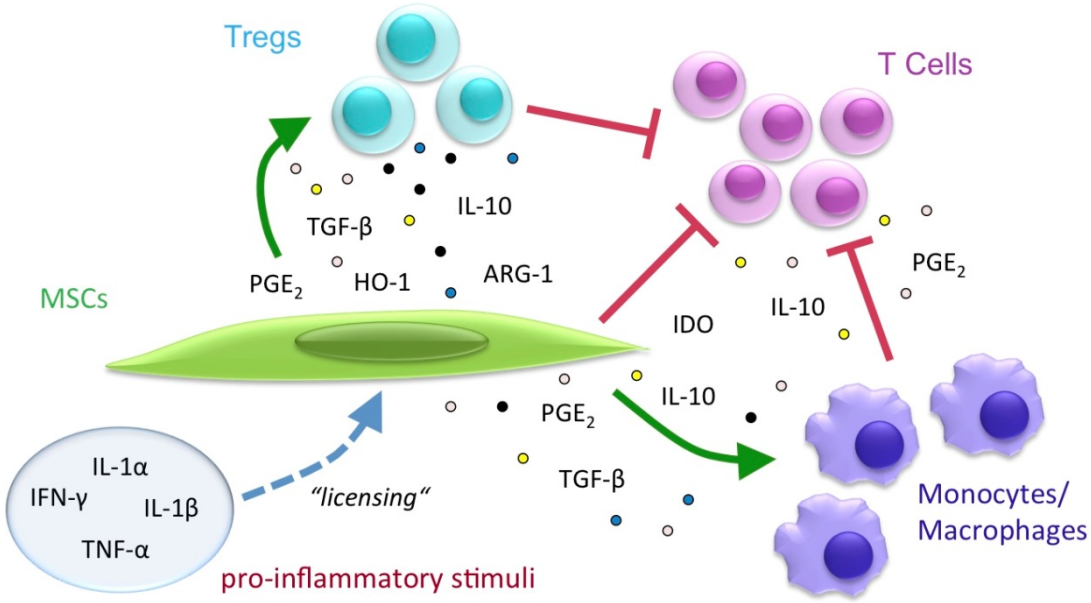

Figure 3: Impact of MSC-derived soluble factors on T-cells and myeloid cells. MSCs express and secrete a plethora of molecules that have direct inhibitory effects on myeloid and T-cells. Furthermore, MSCs induce an immune regulatory phenotype in both, lymphoid and myeloid cells. Inflammatory cytokines such as IFN-γ and TNF-α elicit an inflammatory licensing of MSCs, which leads to the up-regulation of *inter alia* IDO and COX-2 thereby further potentiating their immune regulatory potency.

Two key cell populations for anti-viral and anti-tumor immune defense are the $CD8^+$ CTLs and NK-cells . Since MSCs express only low levels of HLA-Class I molecules ("missing self") and several NK-cell activating ligands (ligands for NKG2D e.g. ULBPs, MICA and ligands for DNAM-1 PVR, Nectin-2), they should be targeted by NK-cells [27]. Whether MSCs are lysed or not by NK-cells depends on (1) the NK-cell pre-activation status [16, 28, 56, 57] and (2) the tissue origin of MSCs [58], which additionally also determines the elicited death pathways (TRAIL by fetal MSCs or FasL by adult MSCs) [59]. Then again, it has been shown that MSCs lead to the down-regulation of activating NK-receptors (NKp30, NKp44, NKG2D) [28] and impact negatively by COX-2 [57] and IDO [60] the IFN-γ production [24], proliferation [28] and cytoxicity of NK-cells.
Peptides of cytosolic processed antigen of virus-infected cells and tumor cells presented by HLA-Class I lead to the activation and proliferation of $CD8^+$ CTLs. $CD8^+$ CTLs

play a pivotal role for the elimination of these diseased cells by releasing e.g. cytotoxic granules and pro-apoptotic surface receptors [61, 62]. Despite HLA-Class I expression, allogeneic MSCs exhibit a low susceptibility to CTL-mediated lysis and do not lead to CTL-activation, even if pulsed with synthetic peptide [61].

The inhibiting role of MSCs seems to be limited to the initial stimulation phase of CTLs, where they inhibit formation of antigen-specific CTLs and target cell lysis [63, 64]. MSCs do not have an effect on the cytotoxic phase [47, 61, 64] and interfere only to a small extent with the lysis mediated by viral-antigen-specific memory CTL *in vitro* and *in vivo* [47, 63].

Despite this *in vitro* evidence, if MSCs impair anti-viral responses *in vivo* has not been completely resolved. In patients that were treated with MSCs for steroid refractory graft-versus-host disease (GVHD) a higher incidence of cytomegalovirus (CMV) infections in GVHD affected organs was observed [65]. However, compared to a historic control MSC treated GVHD patients did not experience a higher rate of viral infections and exhibited a sufficient anti-viral response [66].

MSCs have a (1) chemotactic as well as an (2) immune regulatory effect on myeloid cells. MSCs attract macrophages and monocytes in case of tissue damage and/or mobilize them from the bone marrow by secreting monocyte chemotactic protein 1 (MCP-1) in response to generalized inflammatory reactions as seen during sepsis [67]. This potentially serves two effects: first, macrophages and monocytes boost the clearance of invading organisms and cell debris, which is a prerequisite for wound healing [68, 69]. Next, MSCs can regulate inflammation by shifting the type of cytokines produced by myeloid cells. Induction of IL-10 by e.g. IDO [70] or PGE_2 [22] can lead, as convincingly demonstrated in a murine sepsis model, to a significantly better control of inflammation and finally survival [22]. In a recent study it has been shown that MSCs induce a myeloid derived suppressor cell (MDSC)-like phenotype in monocyte-derived DCs. These cells were immune suppressive and capable of skewing conventional T-cells towards a tolerogenic immunophenotype [71].

Monocytes can be polarized by a the surrounding environment in an acute inflammatory phenotype (M1), which plays an important role in host defense for example by phagocytosis of bacteria, and an alternative type of monocytes (M2), which is immune-regulatory and important for maintaining immune tolerance as well as tissue repair.

Taken together, MSCs exert a multifaceted immune regulatory response in various cells of the immune system. Overall, they seem to induce a more tolerogenic milieu, which is even more potentiated in an inflammatory environment. MSCs elicit inhibiting effects, directly by e.g. impairing inflammatory immune responses or indirectly by inducing immune regulatory populations such as T_{Regs} and alternative type monocytes.

2.5 MSCs AS A THERAPEUTIC TOOL

As discussed above MSCs exhibit a potent capacity for immune regulation and tissue regeneration. MSCs can be isolated with relative ease from healthy donors and quickly multiplied *in vitro*. Together with their low immunogenicity, they can be transplanted across HLA-barriers and can be used as a third-party off-the-shelf product to treat patients. This section will briefly describe the different current applications of MSCs.

2.5.1.1 Regenerative medicine

Since their differentiation capability into various tissues was the first well-defined feature of MSCs, their clinical application was initially focused on tissue replacement and regeneration. MSCs secrete many factors that promote wound healing and restoration of physical barriers [72]. Furthermore, in murine models, it was demonstrated that MSCs quickly home to sites of tissue injury [73, 74]. Infusion of MSCs was for example successfully used to substitute defective bone tissue in osteogenesis imperfecta patients [75]. Osteogenesis imperfecta is a congenital bone disorder characterized by the defective production of Type I collagen. This defect leads amongst others to frequent bone fractures resulting in bone deformities. In a seminal study, fetal MSCs were transplanted *in utero* in a human female fetus with severe osteogenesis imperfecta, manifesting already with multiple intrauterine bone fractures. The engraftment of the transplanted MSCs was successful despite existing immune competence of the host [76].

A further scope of the regenerative intended application of MSCs constitutes a very common disease of the western world: myocardial infarction. Myocardial infarction is accompanied by scaring of the affected heart tissue leading to diminished contractility of the heart muscle and hence a less effective pumping function. In pig models of myocardial infarction, it was shown that MSCs infused in the affected coronary artery had a beneficial effect on the recovery of heart function [77]. MSCs release a broad array of trophic and immune regulatory molecules (thereby limiting inflammation of the damaged tissue) but may also stimulate the endogenous cardiac stem cells recruitment and differentiation [78, 79].

Recently, the research in tissue engineering has focused on integrating bioartificial scaffolds to potentiate tissue regeneration. Maccarini et al. demonstrated the great potential this approach holds by using stem cells grown on a scaffold to replace a tracheobronchial airway tube in a patient after its own trachea had been removed due to tumor resection [80].

2.5.1.2 Immunomodulation

The discovery of the immune regulating functions of MSCs heralded their era as a potential cellular therapy for (hyper-) inflammatory diseases. In fact, *in vitro* evidence suggests that MSCs migrate towards inflammatory cytokines and in response to complement namely the component 1 subcomponent q (C1q) [81], C3a and C5a [20]. This feature might indicate their tropism towards the site of damage and inflammation. This is very useful in terms of their migration towards the preferential areas of action upon infusion.

MSCs are currently evaluated for the treatment of several autoimmune disorders [11], such as autoimmune arthritis [82] and autoreaction-driven

Hemophagocytic lymphohistiocytosis (HLH) is a rare autoimmune disease. Two forms exist: the primary, familial HLH, which is based on genetic mutations and the secondary, reactive form, which is triggered by viruses, bacteria and parasites.
HLH patients present with hepatosplenomegaly and fever, hyperactivation of T-cells and macrophages, which results in a cytokine storm. This leads to the hyperactivation of macrophages and increased production of TNF-α, IL-6, ferritin and ultimately the phagocytosis of leukocytes accompanied by the typical cytopenia.

neurological diseases amongst others amyotrophic lateral sclerosis and MS. In animal models of MS and amyotrophic lateral sclerosis the beneficial effect of MSCs has been demonstrated [43, 83] and currently, adoptive transfer of MSCs is evaluated in clinical trials [84]. Furthermore, MSCs have been anecdotally used as rescue therapies or for "bridging" a desolate inflammatory situation (until allogeneic hematopoietic stem cell transplantation (alloHSCT) could be performed), in which standard treatment was ineffective e.g. in rare autoimmune diseases such as HLH [85] and autoimmune enteropathy [86].

2.5.1.2.1 MSCs in hematopoietic stem cell transplantation

In the bone marrow niche, stromal cells (including MSCs) support hematopoiesis. MSC function can be impaired after chemotherapy [87] leading to a prolonged reconstitution harboring an increased risk for infections. Therefore the hope for an accompanying infusion of *in vitro* expanded human MSCs together with hematopoietic stem cells (HSCs) was to achieve a faster reconstitution. This is especially of interest for patients that receive HSCs from a haploidentical donor or cord blood, which are regularly associated with delayed engraftment (see section 3 of this thesis). Indeed, the first studies in transplanted breast cancer patients showed an improved reconstitution after alloHSCT if MSCs were co-infused [88]. Equally important, no adverse effects were registered [89]. Also in haploidentically-transplanted patients, infusion of MSCs led to quicker lymphocyte recovery [90]. In pediatric patients a co-infusion of *in vitro* expanded MSCs failed to prevent graft rejection, however MSCs appeared to prevent another life threatening complication after alloHSCT: the development of acute GVHD (aGVHD) [91]. Severe aGVHD is one reason for the high morbidity and mortality after HSCT (see section 3.5.3). In short, aGVHD is characterized by hyperactivated T-cells of the graft, which react against healthy tissue of the host. Steroid treatment constitutes the first-line therapy to which about 50-60% of all patients respond [92]. The response to treatment correlates with severity of disease and patients with milder GVHD show a better response rate (> 60%), than severe GVHD (Grade IV 33%) [92]. However, in the event of steroid unresponsiveness, aGVHD can become treatment-resistant and is associated with high morbidity and mortality (up to 90%) [93].

Haploidentical means that the donor and recipient are genetically identical for half of the HLA molecules

Cellular therapy has emerged as a promising tool for the complications (including GVHD) that occur during the post-transplant period. One of them is the adoptive therapy of MSCs. Le Blanc et al. were the first to administer cryopreserved, third-party MSCs for severe aGVHD with gut and liver involvement in a pediatric patient that did not respond to conventional treatment. The infusion of haploidentical MSCs from the patients' mother led to a remarkable clinical improvement and durable complete remission. Importantly, no toxic side effects were observed [94].

The adoptive transfer of MSCs still remains a relatively novel experimental approach to treat aGVHD. Despite the vast knowledge gained during the last decade many questions remain unresolved and observations are not always coherent. In contrast to the unequivocal beneficial effects demonstrated in clinical phase I and II trials in aGVHD carried out at European academic facilities, a commercial, large phase III study by Osiris (Therapeutics Inc Columbia, MD, USA) failed the reach the primary end-point of the study. It is important to stress out that in this Osiris-led study MSCs of

only few donors were heavily expanded, which is a major contrast to the studies carried out at academic houses, where MSCs from one donor and at low passages were transferred into one patient [95]. Low passages seem to be a prerequisite for a more effective MSC function [65]. In the future, it will be of great importance to develop, integrate, and harmonize protocols, which will allow us the identification of the most potent, e.g. in terms of immune regulation, MSCs for clinical application. Consensus regarding MSC selection criteria, culturing protocols and expansion rate would furthermore result in a higher comparability among studies.

2.5.2 Safety

Every novel treatment has to be critically reviewed in regards of any occurring adverse events and in comparison to established standard treatment. MSCs exhibit low toxicity and no adverse side effects have been reported during or right after MSC administration irrespective of treatment indication [89, 94, 96, 97].

Since MSCs are a relatively new cellular therapy, long-term risks are still very poorly evaluated in large cohorts. No spontaneous formation of ectopic bone or cartilage formation was shown for the infusion of autologous human MSCs together with autologous HSCs [88, 89] and even more importantly no ectopic tumor formation was observed for MSCs [98].

In particular, aside from an elevated risk of infections due to impaired immune responses, any immune suppressive treatment might lead to increased relapse rates in patients that underwent HSCT to treat a malignant disease. As described above MSCs have been shown to impact $CD8^+$ CTLs [61] and NK-cells [27, 28]. Both are critical for the prevention of (malignant) diseases and virus-clearance, but on the other hand activated CTLs also drive GVHD [61]. *In vitro* evidence shows that although MSCs suppress the primary alloantigen-induced proliferation and IFN-γ production by human peripheral T-cells, they seem to be able to exert a selective T-cell control. They do not impair expansion of CMV and Epstein-Barr virus (EBV) pentamer-specific T-cells nor the proliferation or cytolytic-killing in established CMV- and EBV-specific CTLs [63].

2.5.3 Engraftment

One yet not completely resolved puzzle is the way of redistribution of MSCs upon infusion respectively engraftment. In animal models a quick distribution to the lungs was shown immediately after infusion [99]. In patients with cirrhosis that were infused with 111-In-oxine labeled MSCs, cells accumulated initially in the lungs, and then redistributed to spleen and liver, where they could be detected up to 10 days [100]. In case of non-human primates, MSCs were detected in gastrointestinal tissue, lung, liver, kidney, thymus and skin after several months at a range of $1x10^3$ to 2.7×10^4 cell equivalents per microgram of DNA [101]. If tissue was injured prior to infusion for example due to irradiation, even higher rates (up to 10%) were to be observed [102]. In HSCT-patients, MSC long-term engraftment was extremely low and scarce as assessed in autopsies [103-105]. Overall, these observations indicate that MSCs do not integrate into host tissue thereby exerting long-term activity. MSCs appear to mediate their effects in a rather

Engraftment is the successful integration of transplanted cells in the recipient bone marrow niche. In stem cell transplantation this refers to the time point when the transplanted

"hit-and-run" fashion. Upon infusion MSCs might represent "only" the trigger for initiating a cascade of immunological events that lead to a more tolerogenic immune profile (=tolerogenic immune memory) and thereby indirectly shift (or even restore) the balance of immunological processes.

2.6 CONCLUSION

Compared to other cellular therapies like HSCT discussed in the next chapter, the adoptive transfer of MSCs for inflammatory diseases is a very novel approach. Despite the repetitively documented clinical responses, mechanisms by which MSCs exert *in vivo* their immune regulatory effect have not been fully deciphered not allowing us to predict which patient would benefit the most from such treatment or to monitor treatment efficacy at a cellular level. In order to develop more individualized protocols and to achieve the most effective MSC therapy possible, a profound understanding is therefore indispensable. Due to the lack of convincing preclinical models (as rodent MSCs are vastly different from human MSCs), basic research on MSCs has overall occurred *in vitro*, and was furthermore confused by the big inter-study variances. For the future, synchronizing manufacturing and culturing protocols would immensely help to transfer the knowledge gained *in vitro* to the clinical setting. We are of the opinion that it represents a key step for ensuring that the beneficial effects of MSCs stay in the limelight and are not overshadowed by potential inconsistent results.

3 HEMATOPOIETIC STEM CELL TRANSPLANTATION

Hematopoietic stem cell transplantation (HSCT) is the most successful and routinely used form of cellular immunotherapy [106]. It allows replacing a failing or diseased hematopoiesis and immune system with a new, healthy one. Furthermore, it represents the only curative option for many hematological malignancies [107] with patients suffering from leukemia and lymphoma accounting for the largest patient cohort.

3.1 HISTORY OF HEMATOPOIETIC STEM CELL TRANSPLANTATION

HSCT was initially developed for two reasons: (1) to treat patients with immune deficiencies and inherited anemia and (2) as a rescue therapy after myeloablative cancer therapy. In 1951 the seminal study by Lorenz et al. demonstrated for the first time in lethally irradiated mice that the transplantation of syngeneic as well as autologous bone marrow allows reconstitution of a sufficient hematopoiesis that ensures survival [108]. This reconstitution can be attributed to a small number of HSCs in the bone marrow that is characterized by the expression of the cell surface protein CD34, which seems to function as a cell-to-cell adhesion factor. Furthermore, CD34 might also impact cell proliferation and maturation but its function has not yet been completely understood [109]. These $CD34^+$ HSCs regenerate primitive progenitors, which reproduce less-differentiated precursors and finally develop into mature blood cells. Since peripheral blood cells exhibit a limited life span, a constant replenishment of the peripheral blood pool is important to maintain sufficient blood cell numbers throughout the body.

Myeloablation describes the situation in which the bone marrow is completely depleted of bone marrow cells by high doses of chemotherapy or irradiation. This leads to a complete failure of hematopoiesis and is therefore lethal.

The first HSCT in humans was performed in the late 1950ies in a patient with end-stage leukemia [110]. Even though hematological recovery was achieved in some transplanted patients, the outcome of the early transplantations remained poor. A study investigating the survival of patients that received transplants between 1958 and 1968 showed that in 1970 only three out of 203 patients were still alive [111]. This can partly be attributed to the advanced stages of disease of the treated patients, but also due to the limited understanding of the immunological processes initiated by the transplantation of a whole immune system.

3.2 HLA-SYSTEM

The discovery of the importance of the HLA-system by J. J. van Rood and J. Dausset in the 1960s was one of the most decisive developments towards an individualized stem cell therapy and made transplantation of third-party stem cells feasible. HLA-molecules are expressed on the surface of cells and present peptides recognized by immune cells allowing the distinction from self- and non-self-antigens [112, 113]. This plays an arbitrative role for the whole function of the immune system in every human being, but also explains many of the immunological adversities faced upon alloHSCT.

HLA-genes consist of three main classes that are structurally and functionally different. All nucleated cells express HLA-Class I genes however at varying levels. HLA-Class I

molecules present peptides of processed cytosolic antigen and are essential for regulating the NK-cell mediated cytotoxicity and for triggering $CD8^+$ CTLs. Twenty different genes exist, the most important ones are HLA-A, -B, and C.

HLA-Class II genes present endo- and phagocytized extracellular antigens and are normally expressed by specialized immune cells as e.g. professional APCs, B-cells, activated T-cells, and thymic epithelial cells. The class II genes encode for the alpha and beta polypeptide chains of the class II molecules. The most significant ones are HLA-DR, HLA-DQ, and HLA-DP. So far, HLA-Class III plays a minor role in transplantation and is involved in immunity by expression complement factors and cytokines.

Since each person has a specific combination for his or her histocompatibility complexes, it is essential to thoroughly evaluate the HLA-region for matching recipients with adequate donors. Taken together, the rate of rejection, occurrence and severity of GVHD as well as the grade of delay in immune reconstitution is proportional to the degree of mismatch between donor and recipient [114, 115].

3.3 IMMUNOLOGICAL ERADICATION OF TUMOR CELLS

Although patients undergoing alloHSCT are overall well pretreated and at best disease free or exhibit minimal residual disease, it is always possible that some tumor cells survive the therapy leading to the relapse of disease. This is especially true for stem cell diseases such as leukemia. Cancer stem cells are quiescent, metabolically inactive cells that remain in G_0-state and do not proliferate [116]. As most chemotherapeutic agents act almost exclusively on proliferating cells, some malignant stem cells might successfully evade even lethal doses of total body irradiation (TBI) and chemotherapy.

Cancer immunosurveillance: F.M. Burnet and L. Thomas postulated the hypothesis that cancer cells are targeted and recognized by the immune system namely lymphocytes.

Every healthy, well-functioning immune system recognizes and targets malignant cells to a certain degree (concept of cancer immunosurveillance) [117, 118]; however soon after alloHSCT was introduced, it became apparent that the transplantation of a third party donor-derived graft results in lower relapse rates (35 percent) [119] than the transplantation of a syngeneic or autologous graft (40 to 75 percent) [120]. In subsequent studies, it was elucidated that the potential to target and eliminate residual tumor cells (graft-versus-tumor (GVT) effect) was mediated by the alloreactivity of the allogeneic graft against the host. An allogeneic graft consequently does not only recognize cancer cells as malignant (based on the expression of tumor antigens), but also as foreign and therefore kills them even more efficiently [121]. This effect is predominantly conveyed through host-reactive co-transplanted donor-derived CTLs that react against recipient HLA-complexes. [122]. Increasing evidence suggests that in certain haploidentical donor-recipient constellations observed GVT effect can also be mediated by NK-cells [123].

In a seminal study, H.J. Kolb demonstrated that the infusion of additional donor derived lymphocytes so-called DLI in the post-transplant period can potentiate respectively boost the GVT effect [124]. This has laid the foundation to an effective immunological targeting of cancer cells by the transplanted immune system. It revolutionized cancer

therapy respectively changed the perception of conditioning therapy allowing the introduction of a non-myeloablative conditioning therapy (see section 3.4.2).

3.4 HSCT PROCEDURE

In principle a successful HSCT follows three consecutive phases (1) a suitable graft has to be selected first. Depending on underlying disease, the transplant can be the (A) patient's own stem cells (autologous), (B) stem cells from genetically identical siblings (syngeneic) or (C) third-party derived stem cells (allogeneic). (2) Before receiving the transplant, each patient has to undergo a therapy that creates the right conditions ('conditioning therapy') in the host to receive the transplant. (3) Transplantation is followed by a post-transplant period characterized by immunodeficiency until the new immune system reconstitutes resulting in a healthy, functioning immune system.

3.4.1 Graft and sources of stem cells

The primary graft source for the first HSCTs was bone marrow. Bone marrow cells are collected under local or general anesthesia by repeated aspiration at the posterior iliac crest. It still remains the source of choice for pediatric patients. The discovery of G-CSF and its mobilizing function of $CD34^+$ cells from the bone marrow into the peripheral blood have resulted in the predominate use of peripheral mobilized stem cells (PMSCs) in adults. Sufficient numbers of PMSCs are easily collected via the antecubital veins by leukapheresis and can be subsequently frozen in liquid nitrogen (< -160°C) until further use. Storage of HSCs in liquid nitrogen warrants high viability after thawing and facilitates e.g. the usage of autologous HSCs that are collected prior to conditioning therapy. Another positive feature of PMSCs is a quicker engraftment and immune reconstitution as compared with bone marrow grafts [125].

Leukapheresis is a process in which white blood cells are filtered out of the blood stream and collected while the remaining blood components are returned to the donor.

Umbilical cord blood constitutes the third source of graft and was first used in 1988 for alloHSCT between matched siblings in a child with Fanconi's anemia [126]. Cord blood is rich in HSCs and contains more naïve, immature cells [127] as well as immunosuppressive T_{Regs} [128]. Potentially due to this multitude of immune regulatory and naïve cells (=more tolerogenic milieu), cord blood transplants cause less GVHD and require less restrictive HLA-matching, however in contrast to autologous transplants a GVT effect is still observed. Despite these premises the success of treatment still increases with better HLA-matching and increasing numbers of HSCs [129]. Since volume of cord blood and number of HSCs is limited, double-unit cord blood transplants are regularly used in order to achieve sufficient HSC numbers suitable for transplantation in adults. In recent studies the co-transplantation of cord blood HSC with HLA-haploidentical peripheral stem cells [130] as well as the *ex vivo* expansion of cord-blood stem cells [131] are under investigation.

3.4.2 Conditioning therapy

For each patient that undergoes HSCT a specific regimen depending on underlying disease and co-morbidities is chosen that conditions or prepares the body for receiving the new immune system. Conditioning therapy comprises three goals: (A) the reduction

of the underlying disease, (B) the depletion of the recipient's bone marrow from the (diseased) hematopoietic system making room for the new immune system, as well as the (C) immunosuppression to avoid immune-rejection especially in alloHSCT. Traditionally, conditioning therapy encompassed a combination of TBI, chemotherapy with cyclophosphamide, which was thought to constitute the main tumor eradicating effect of the treatment. This combination is rather harsh and accompanied with high toxicity that led to the limited application of HSCTs to younger individuals with no severe co-morbidities.

The discovery of the aforementioned curative potential of the GVT effect paved the way for a reduced intensity conditioning (RIC). RIC does not primarily aim to exert an anti-cancer effect but rather aims to prepare the immune system for the transplantation. It therefore solely relies on the immunological eradication of residual cancer cells after transplantation [132]. RIC avoids the high morbidity and mortality caused by early organ toxicity of a standard conditioning regimens thereby allowing alloHSCT in elderly patients with comorbidities. It has however been associated with a higher relapse rate, indicating the importance of a good remission status prior to transplantation [133].

3.4.3 Reconstitution of the immune system

The reconstitution of the immune system in terms of versatility, rapidity and quality determines the success of alloHSCT and cancer eradication [134]. The profound immunodeficiency as prevalent in this post-transplant phase holds many severe risks for the patient: (1) occurrence of (opportunistic) infections (see Section 3.5.2 of this chapter), (2) recurrence of the underlying malignant disease due to a missing cancer immunosurveillance and (3) long term development of secondary malignancies [135].

Opportunistic infections are infections by pathogens that would not cause infections in a healthy host but take advantage of the in immune-compromised situations of the patient e.g. after HSCT but also AIDS.

Reconstitution is influenced by many factors and events: they can occur (1) before transplantation namely conditioning regimen and type of GVHD prophylaxis, (2) at the time point of transplant (type of transplantation, choice of graft, manipulation of any sort, the degree of histocompatibility (HLA, mHAg, NOD/CARD15 [136])) or (3) after HSCT (presence and grade of GVHD, relapse, infections [137-139]).

The first cells that reconstitute within the first months are the innate immune cells such as neutrophil (mostly first 30 days) and myeloid cells, which are important cells to fight bacterial infections. Interestingly, host derived macrophages are mostly not impacted by conditioning therapy and persist in the tissue. They are replaced by donor-derived macrophages over time [140]. Reconstitution has been predominantly studied in terms of numerical alterations; however decisive for e.g. host defense is the efficient function of the cells. In spite of increasing numbers, neutrophils can be inoperable due to insufficient chemotaxis and phagocytic-bactericidal function for several months [141].

The rapid post-transplant recovery of NK-cells is mostly attributed to an expansion of cytokine producing $CD56^{bright}CD16^{-}$ NK-cells and takes about 3 months [142].

T- (at least 4 months) and B-cells (at least 9-12 months) take much longer to reconstitute and the complete regeneration of the T- and B-cell compartments can take 1-2 years after HSCT.

In adults the first subtype of T-cells that expands in response to IL-2, IL-7 and IL-15 in the post-HSCT period are the memory T-cells, particularly $CD8^+$ T-cells [143, 144]. Overall, $CD8^+$ T-cells exhibit a quicker reconstitution than $CD4^+$ T-cells [137] leading to an inversed $CD4^+/CD8^+$ T-cell ratio compared to healthy persons in the first months [145, 146]. Both residual host memory T-cells that survived conditioning therapy as well as donor memory T-cells in a non-T-cell-depleted graft can give rise to this population [147]. Memory T-cells react against previously encountered pathogens such as herpes viruses (CMV and EBV) and enter more easily tissue than naïve T-cells [148]. However, in order to respond adequately to new immune challenges towards pathogens and tumor antigens, the naïve T-cell repertoire needs to be reconstituted (neothymopoiesis). Since for a complete regeneration, the whole T-cell ontogeny is thymus dependent, involution of thymus due to age seems to play a decisive role. This seems to be especially true for $CD4^+$ T-cells [149] and as a consequence elderly patients never manage to fully restore the peripheral naïve T-cell receptor repertoire [150, 151]. The population of these lymphocytes leaving the thymus is named recent thymic emigrants (RTE) and can nowadays be monitored according to the signal-joint T-cell receptor rearrangement or T-cell receptor excision circles (TREC) [152]. Low TREC levels have been associated with the aforementioned diminished thymus function due to age, but also by impaired function during opportunistic infections and after alloreactive immune responses in the course of GVHD. A dysfunctional thymus entails an even enhanced GVHD disease activity, since it results in an incorrect selection of T-cell clones respectively fails to effectively deplete auto-reactive T-cells.

Humoral immune responses require a functioning B-cell lineage encompassing B-cell derived plasma cells and memory B-cells. B-cells reconstitute within the first 6 to 9 months [153] and seem to be dependent on a functioning, immaculate bone marrow micro environment. Infiltration of the bone marrow by alloreactive T-cells (GVHD) as well as GVHD immunosuppressive treatment results in a diminished B-cell reconstitution [139]. Furthermore, similar to the T-cell receptor (TCR) repertoire also the immunoglobulin variety (B-cell antibody) seems to be decreased after transplantation [154].

The reconstitution of the immune system is the most decisive step towards cure. A profound understanding of involved underlying processes as well as associated immune impairments will help to adequately cope with many of the subsequently described complications, which originate from an incompetent immune system.

3.5 COMPLICATIONS AFTER HSCT

3.5.1 Early complications

Early negative effects of HSCT are largely associated with the toxic side effects of the conditioning radio- and chemotherapy and the diminished immune function. In the following paragraphs, some of the most common early complications are described.

Mucositis is a one of the most prevalent problems in patients after HSCT. It is caused by conditioning therapy (= direct tissue damage) and also constitutes a common side

effect of the widely used immunosuppressive agent methotrexate, which preferably targets the fast dividing cells of the mucosa. Oropharyngeal mucositis is very painful regularly requiring opioid-based pain medication. Intestinal mucositis often necessitates parenteral substitution of fluids and calories. Keratinocyte growth factor (KGF) has been used to treat and/or prevent mucositis in some studies showing mixed results [155]. Interestingly, KGF seems to possess a positive effect on thymus function in terms of enhanced thymopoiesis [156].

A very severe, early complication caused by endothelial damage is the hepatic veno-occlusive disease (VOD) also known as sinusoidal obstruction syndrome. It originates from the hepatotoxic effects of the conditioning regimen, which affects the sinusoidal capillary endothelium. Toxic metabolites that are retained in the liver facilitated by e.g. preexisting liver impairment or due to interactions with other drugs lead to an obstruction of hepatic circulation that causes hepatomegaly, fluid retention and jaundice. Treatment is very difficult and prevention remains the main goal [157]. Defibrotide, which is a deoxyribonucleic acid derivative, has shown some promising results in the treatment of VOD [158].

HSCT-associated thrombotic microangiopathy (TMA) is characterized by anemia, thrombocytopenia, schistozytes and elevated lactate dehydrogenase. It is associated with high mortality (75%) [159]. In contrast to other treatment related adverse events, the intensity of treatment seems to play a minor role in the incidence of TMA. Certain drugs e.g. calcineurin inhibitors, sirolimus [160], viral as well as fungal co-infections, HLA-mismatched donor grafts [161] and aGVHD [162] seem to drive it. Endothelial dysfunction causes microangiopathic hemolytic anemia and platelet consumption. The most decisive therapeutic action is to immediately and completely abrogate calcineurin inhibitors and change of GVHD prophylaxis respectively therapy to e.g. mTOR-inhibitors. The beneficial effect of plasmapheresis and application of thrombolytics has been described in some cases [163].

An early onset of hemorrhagic cystitis is associated with direct toxic effects of the conditioning regimen. Clinical presentation can range from microscopic asymptomatic hematuria, but also very painful, heavy hemorrhage of the entire urine tract has been observed. A later onset is mostly mediated by viral infections (mostly BK polyomavirus, but also adenovirus and CMV) and aGVHD. Hemorrhagic cystitis is normally treated by consequent irrigation and supportive platelet infusions.

3.5.2 Infections

The post-transplant period is coined by an increased risk of infections. It reflects the immune compromised situation of the patient as well as the consequences of intensive pretreatment during conditioning and iatrogenic immunosuppression. The disruption of protective anatomical barriers as skin and mucosa by radio-chemotherapy, mucositis and GVHD together with the usage of plastic catheters further facilitate the entry of pathogens into the body.

The first thirty days after HSCT are characterized by functional asplenia, absent B- and T-cells and most predominantly neutropenia. Neutrophils represent the first line of defense against invading pathogens. Patients are therefore prone to bacterial infections, candida and Aspergillus species as well as Herpes simplex virus (HSV). These pathogens often originate from the patient's endogenous gastrointestinal flora, which

led to the prophylactic usage of antibiotics for the so-called selective intestinal decontamination. However, the benefit of this approach has become heavily disputed, since it seems to disrupt the microbial balance of the gut impacting the onset of aGVHD (see following section GVHD).

With rising numbers of neutrophils the incidence of bacterial and fungal infections decreases and infections that are related to the impaired T- and B-cell function dominate. T-cells are vital to prevent viral infections as well as invasive fungal infections. HSCT patients are prone to develop viral infections namely CMV, EBV, adenovirus, BK polyomavirus and respiratory viruses. In addition to newly acquired infections, viruses that reside permanently (and under previous immunologic control) in the patients' tissues such as CMV, EBV, and human herpes virus 6 (HHV-6) reactivate and become pathogenic. CMV and EBV are very common viruses and infections are wide spread among the population. The danger of a life-threatening CMV re-activation has substantially been decreased due to CMV-specific PCR screenings that allow (quantitative) detection of the virus already at a subclinical level and the early initiation of antiviral treatment. Similarly, screening assays for EBV are routinely applied allowing early interventions (by using the anti-CD20 antibody Rituximab), which has significantly reduced the rate of a very severe EBV-associated complication, the post-transplant lympho-proliferative disease (PTLD) [164]. In severe (treatment-resistant) cases of CMV and EBV infections approaches of infusing virus specific T-cells are currently evaluated.

In this phase very intensive prophylaxis is undertaken to prevent fungal infections. It encompasses prophylactic medication, structural precautions (e.g. disinfection routines), and since mold infections are predominantly airborne, isolation rooms and air filters. Again, cellular therapies have been also utilized for treating invasive fungal infections, namely the transfusion of third-party granulocytes but without striking success [165].

3.5.3 Graft-versus-host disease

During the first alloHSCT attempts and especially before the importance of the HLA-system was recognized, severe adverse immunological events were regularly observed and resulted in a deteriorated, life threatening immune reaction. To current understanding, this is mostly mediated by alloreactive T-cells of the graft that react against the host's healthy tissues giving rise to the term graft-versus-host disease (GVHD). The underlying patho-mechanisms share some common features with the beneficial effect of the GVT reaction (Figure 4). The reaction however is amplified to such an extent that it affects healthy tissue and becomes harmful, even life threatening to the host. GVHD remains the greatest cause of morbidity and mortality after alloHSCT and constitutes the biggest hindrance for a successful HSCT.

It occurs depending on the GVHD prophylaxis, mismatch status and previous therapies in 30-60% of all transplanted patients being fatal in about 15% of the cases [119]. The most affected organs are the skin, the gut and the liver, but GVHD can theoretically involve all areas of the body. Traditionally, GVHD was defined as acute when it occurred in the first 100 days after transplantation and chronic GVHD with an onset past day 100 after transplantation. In recent years, it has been challenged whether this classification still applies and there is a tendency to base the classification on the

symptom complex rather than the time-point of onset. Furthermore, a better understanding of the different underlying immunological processes that drive acute and chronic GVHD respectively gains more significance and recognition.

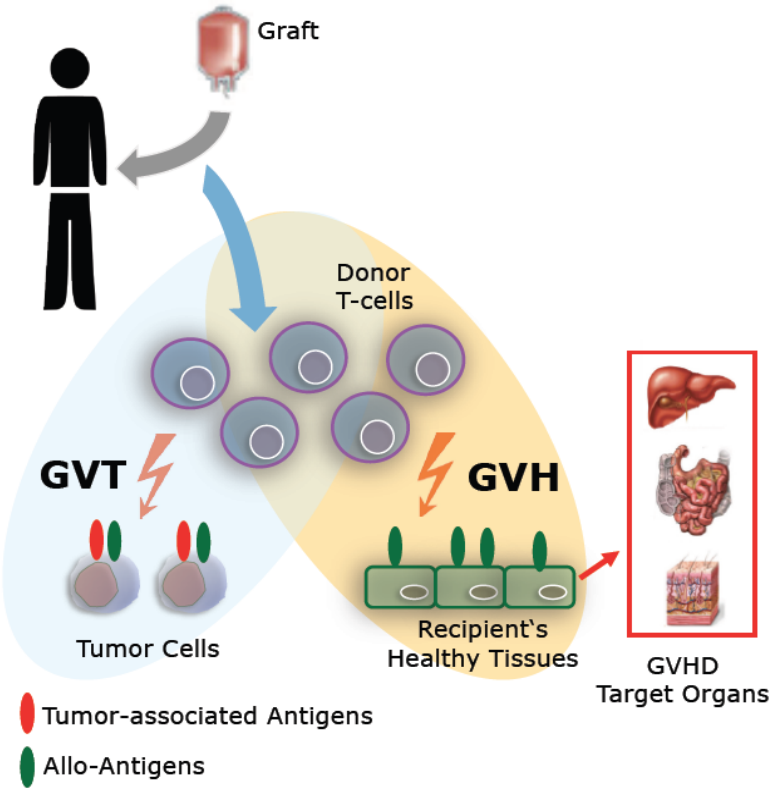

Figure 4: Graft-versus-tumor (GVT) effect and graft-versus-host disease (GVHD). Both reactions are mediated by alloreactive donor T-cells. The GVT effect is strongly desired, since it represents the cornerstone for the elimination of residual tumor cells and results in lower relapse rates and better overall survival. GVHD on the other hand represents one of biggest causes for mortality and morbidity after alloHSCT. Highly activated donor T-cells target healthy recipient's tissue with skin, intestine, and liver being the most affected organs.

The pathophysiology of aGVHD is subject to intense research. Still many mechanisms driving aGVHD remain elusive. In the following paragraphs a short outline of todays' understanding of the aGVHD pathophysiology is attempted.

GVHD seems to constitute a constringent immunological process that spans three phases [166]: (Phase 1) the initiation of GVHD starts even before transplantation during the conditioning regimen. Radiation as well as chemotherapy damages the host tissue encompassing disruptions of protecting mucosal barriers amongst other in the gut facilitating the entry of pro-inflammatory microorganisms into the body. The resulting inflammation and release of microbial peptides enhances the activation of innate immune cells and results in increased secretion of inflammatory cytokines. These augmented cytokine levels consequently promote an enhanced recognition of host antigens by donor T-cells (Phase two) triggering the activation and proliferation of T-cells. Finally all these processes disembogue into (Phase 3) effector cell responses that

damage target organs of GVHD forming a self-amplifying vicious cycle (the GVHD's "perpetuum mobile").

GVHD is predominantly a T-cell driven disease [167], but also NK-cells and macrophages were identified to be involved [123]. Recent evidence indicates that the innate immune system reacting towards microbial peptides holds a pivotal role for GVHD pathopysiology [168].

Several factors that increase the risk of developing GVHD have been identified: they mostly concern matching of recipient and donor (HLA-mismatch, female donors for male recipients, preceding alloimmunisation of the donor), age of recipient, administered conditioning regimen as well as the type of GVHD prophylaxis [169, 170]. Recently, more genetic factors have been found to be associated with the onset of GVHD. Polymorphisms of cytokine-encoding genes [171], differences regarding the pathogen recognition receptors (PPRs) (NOD2/CARD15) [172] and how the body copes with antigens of infective organisms seem to have an important impact on the development of GVHD.

A recent study highlighted the importance of the microbiome respectively its regulators such as paneth cells in the development of aGVHD. Paneth cells reside in the small intestine and regulate the microbial flora by secreting anti-microbial peptides such as defensins and regenerating islet-derived protein 3 alpha (Reg3α) [173]. Lower numbers of paneth cells as well as lower levels of Reg3α correlate with severity of aGVHD and reduced response to GVHD treatment [173, 174]. The loss of regulation in the gut results in a loss of bacterial diversity in favor of increasing prevalence of enterococci [175].

Microbiome is a term coined by J. Lederberg "to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" [1].

Despite intensive research in terms of developing elegant means for specifically targeting established key players of aGVHD, nowadays steroids that act in a rather non-specific immune suppressive fashion remain the first-line treatment of choice. Taken together, most of the approaches for the treatment and prevention of GVHD have focused on reducing the alloreactivity of donor T-cells. The most potent effect is achieved by depleting donor T-cells from the graft or *in vivo*.

Today most commonly the function of donor T-cells is controlled by immunosuppressive agents firstly the aforementioned steroids, but also other drugs such as calcineurin inhibitors, sirolimus, cyclosporine and methotrexate. Each of them is accompanied by characteristic side effects, ranging from increased infection rates, mucositis, to even renal or liver failure. Overall, 50-60 % of the afflicted patients will respond to an immunosuppressive treatment [92].

Cellular therapy for the treatment of aGVHD is a comparatively novel approach and its use is discussed in the respective sections for MSCs (see section 2), T_{Regs} (see section 5) and MDSCs (see section 4).

3.6 CONCLUSION

To date, HSCT transplantation is the most successful immune therapy. It constitutes a grand chance for terminally ill persons to be cured and return to an active life. However, despite the substantial progress that has been made in the last fifty years, many HSCT associated complications remain unresolved and not fully understood. Infections, delayed reconstitution, graft rejection and relapse are often life threatening and remain together with GVHD the major cause of HSCT related morbidity and mortality. Prognosis of high-grade aGVHD is still extremely poor and since the response rate to any treatment decreases with advancing degrees of aGVHD, it appears obligatory to develop means for preventing or at least alleviating the occurrence of severe aGVHD without impairing the GVT effect. The adoptive transfer of MSCs seems to constitute an attractive tool to rebalance the immune system. In the following two chapters, two more cellular populations are introduced that have been implicated to have a beneficial effect on hampering GVHD.

4 MYELOID DERIVED SUPPRESSOR CELLS

4.1 MDSCs IN HEALTH AND DISEASE

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of myeloid cells at different stages of maturation. They encompass myeloid progenitors, immature granulocytes, DCs and macrophages. Owing to their heterogeneity, human MDSCs cannot be identified by a sole cell-surface-marker guided classification, but only in due consideration of their T-cell suppressive function [176, 177].

MDSCs were first described in the metastatic lymph nodes of head and neck cancer patients [178]. Since early studies focused on tumor diseases [179, 180] elevated frequencies of MDSC and their immunosuppressive effects have been best characterized in malignant diseases of various entities such as hepatocellular carcinoma, renal cancer, melanoma, and breast cancer [181-184].

Increasing evidence suggest that malignant diseases are not a prerequisite for MDSC accumulation. Many immunological situations that involve some sort of inflammatory response such as autoimmunity [185], sepsis and trauma [186], chronic inflammation [187, 188], and also alloHSCT [189-191] can elicit increased frequencies of these suppressive cell phenotype. In fact, Hoechst et al. recently identified MDSCs with regulatory properties in the peripheral blood of healthy individuals [192] further corroborating the notion that MDSCs hold a physiological role in immune homeostasis similar to T_{Regs}. In healthy individuals MDSCs constitute only a very low percentage (about 0.5%) of the total peripheral blood mononuclear cells (PBMCs) [180, 193] and quickly differentiate into mature granulocytes, macrophages and DCs. However, immunological conditions as found in cancer and inflammation seem to create an environment that leads to the enhanced mobilization and accumulation of these cells.

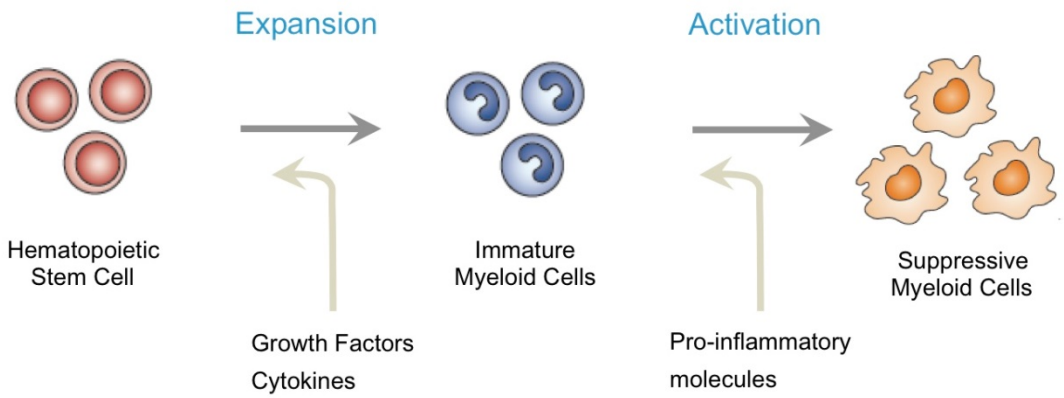

Figure 5: Two-step model of expansion and activation of MDSCs. Cytokines and growth factors, which are present in inflammatory processes such as TNF-α, IFN-γ, G-CSF and interleukins, mediate the expansion and activation of MDSCs.

Accumulation of MDSCs appears to involve two consecutive steps (Figure 5):

1. Cytokines and growth factors that are abundant during inflammation such as TNF-α, G-CSF, GM- or M-CSF [194], IL-6 [195], PGE_2 [196, 197], but also complement factors (C5a) [198] and cytokine-like pro-inflammatory proteins such as S100A8/A9 [199] cause an expansion and mobilization of immature myeloid cells [200, 201].
2. The second signal, which leads to the activation and inception of their suppressive function, occurs in the periphery. Myeloid progenitors come in contact with inflammatory cytokines such as IL-4, IL-6, IL-13, IFN-γ, and TGF-β that induce suppressive pathways. These triggering cytokines are secreted by activated cells and in the case of malignant disease by tumor (associated) cells. Besides activation, this cytokine-"cocktail" also promotes a differentiation arrest (by e.g. activation of the transcription factor signal transducer and activator of transcription (STAT)3) [200-202]. On the basis of inter-individual biological variability respectively the distinct biology of various malignancies, the composition of these cytokines differs. This might explain the plethora of described (some time inconsistent) MDSC-phenotypes.

Self-evidently it is extremely difficult to prove this two-signal model of expansion and activation *in vivo* [203]. The pivotal role of the surrounding immunological milieu however has been demonstrated indirectly in *in vitro* studies. Bone marrow derived MDSCs exhibit an increased proliferation rate in the presence of activated T-cells, which also imped further maturation of MDSCs [204]. Furthermore, immunosuppressive MDSCs can be induced from healthy donor derived $CD14^+$ monocytes in the presence of tumor cells or by addition of a cocktail including several of the aforementioned cytokines and growth factors [195, 196, 200, 201, 205]. Upon removal from the tumor milieu, MDSCs can partially differentiate into normal DCs and macrophages [206-208] indicating the importance of the extrinsic signals and lack of a genetic imprinting.

Besides the initiation, activation and differentiation arrest of MDSCs, activated T-cells seem to represent a checkpoint for their expansion. Similar to T-cell homeostasis in which activation always also partially results into an increased cell death (AICD), it was shown for murine granulocytic, Fas^+ MDSCs that Fas-FasL interaction with activated T-cells led to a caspase mediated apoptosis in Fas^+MDSC [209].

Interestingly, MDSCs are not only 'passively' expanded in response to their environment. MDSCs themselves might actively promote their own accumulation within an autocrine feedback loop, as paradigmatically shown for MDSCs isolated from ovarian and prostate cancer patients. These cells secrete IL-10, which amongst other effects, causes the sequestration of HLA-DR and consequently leads to higher frequency of $HLA\text{-}DR^{low/neg}$ monocytic MDSCs [210].

4.2 PHENOTYPE

In the mouse MDSCs are defined by the expression of the myeloid lineage differentiation antigens Gr-1 and CD11b [211]. Based on the level of Gr-1 expression,

MDSCs are subdivided in a $CD11b^{+}Gr1^{high}$ ($CD11b^{+}$Ly-$6G^{+}$ Ly-$6C^{low}$) expressing granulocytic and a $CD11b^{+}Gr1^{low}$ ($CD11b^{+}$Ly-$6C^{high}$Ly-$6G^{-}$) monocytic MDSC subset [212]. In contrast to murine MDSCs their human counterparts lack specific markers and are therefore much harder to define phenotype wise [187]. As yet, a steadily growing number of phenotypes have been described in various pathological conditions [213]. Consensus is that human MDSCs are displaying a lack or at least significantly decreased levels of markers characteristic for mature myeloid cells [214]. Furthermore, they regularly have a pronounced expression of CD11b an integrin required for monocytes and neutrophils to interact with the endothelium, the transmembrane receptor CD33 and display - in a non-typical fashion for myeloid cells - an absence or a very low expression of HLA-DR [193, 215].

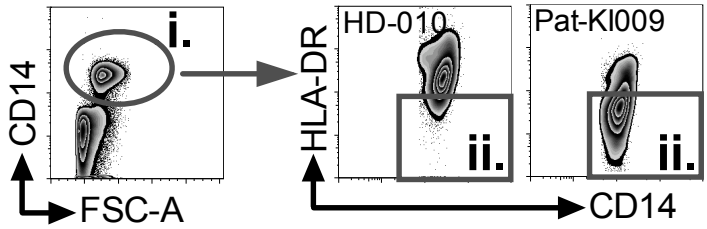

Figure 6: Gating strategy to identify $CD14^{+}$HLA-$DR^{low/neg}$ MDSCs. This FACS blot exemplifies how frequencies of MDSCs can vary between a healthy donor (HD) and a person that experiences some kind of inflammatory reaction in this case alloHSCT (Pat) [190].

In recent attempts to categorize human MDSCs, the cells have been grouped based on the expression of the LPS co-receptor CD14, into monocytic ($CD14^{+}$HLA-$DR^{low/neg}CD33^{+}$) [216-218] and granulocytic MDSCs (lineage negative (CD3, CD14, CD19, CD56)) $CD33^{+}$ HLA-$DR^{low/neg}$ [176, 177]. These two subpopulations can occur exclusively but overlaps have also been reported [219, 220]. Multiple phenotypes have been described in different diseases [221] indicating that the population of MDSCs is shaped by their surrounding micro milieu as afore described in more detail.

Marker	Function	Reference
CD80, CD83, DC sign	Co-stimulatory molecules	[183, 222]
IL-4 receptor α (CD124)	Activation and suppression	[223-225]
M-CSFR (CD115)	Growth factor receptor	[224, 226]
TNFRII	MDSC accumulation, apoptosis inhibition	[203, 227]
VEGFR1		[177, 228]
CD15, CD66b	Granulocytic marker	[177, 229, 230]
CD62L	Adhesion molecule	[177, 190, 231]
CD49d	Integrin	[232]
CD16	Fc receptor	[177]
CCR2 (CD192)	Chemokine receptor	[227]

Table 4: Phenotypic markers that have been described for human MDSCs.

4.3 T-CELL SUPPRESSIVE ACTIVITY

MDSCs exhibit a plethora of direct and indirect mechanisms to suppress T-cell activation and proliferation [233, 234] (Figure 7). This diversity seems to be partially attributed to their monocytic or granulocytic nature, maturation and activation status and is consequently shaped by their surrounding microenvironment similar to their phenotype. Most likely, MDSCs constitutively exhibit more than one suppressive pathway, however since most investigators concentrated on the most distinguished, protruding suppressive mechanism the co-existence of several suppressive mechanisms has only been evaluated in few studies [183, 235].

The basic question regarding an antigen-specific MDSC-mediated suppression has not been completely resolved. Interestingly, MDSCs are able to take up and process soluble antigens and to subsequently present them to T-cells [231, 236]. Since antigen-presentation by MDSCs is - unlike in professional APCs inadequate (e.g. lack of co-stimulation) - it is speculated whether it represents a very elegant method for inducing tolerant $CD4^+$ and $CD8^+$ T-cells in an antigen-specific manner [204].

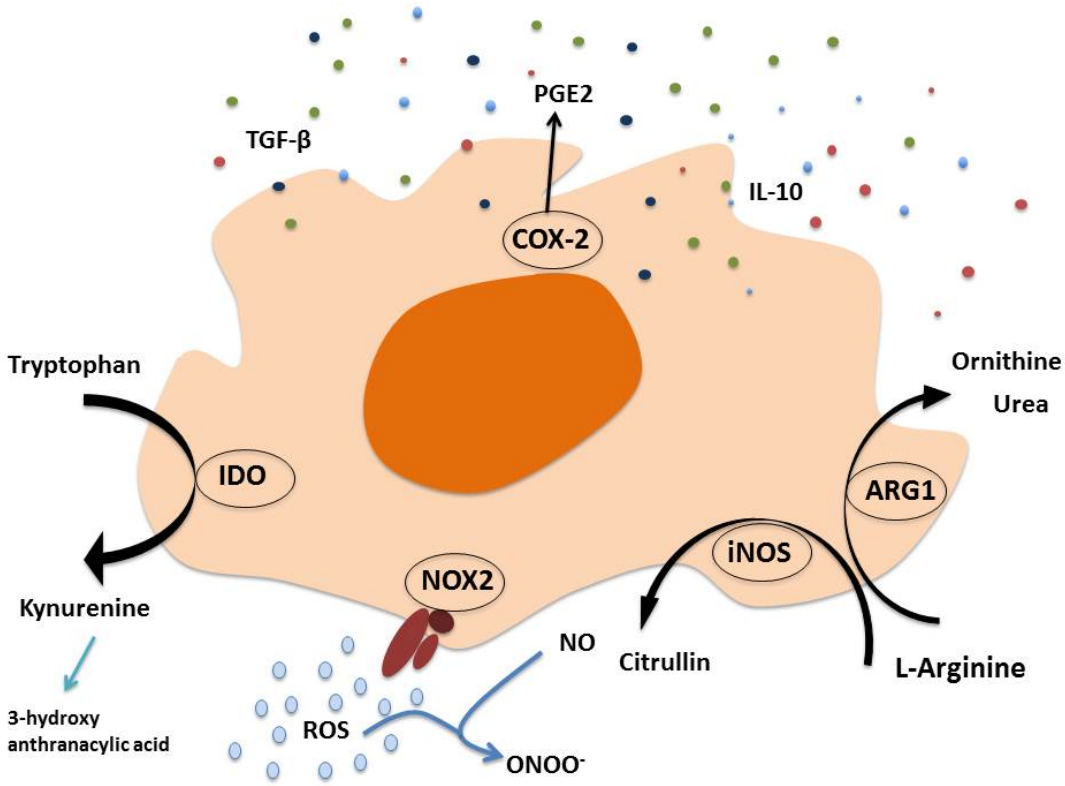

Figure 7: Immune suppression exerted by MDSCs. MDSCs release a plethora of immune regulatory factors such as IL-10, TGF-β and express enzymes such as inducible nitric oxide synthetase (iNOS) and Arginase-1 (ARG1) that deplete arginine an essential amino acid for T-cell activation from the environment. MDSCs produce furthermore reactive oxygen species (ROS) via NADPH oxidase (NOX2) that react with nitric oxide (NO), which is released by the metabolization of L-arginine by iNOS. This forms the radical peroxynitirite ($ONOO^-$).

4.3.1 Lymphocyte nutrient depletion

One of the first identified mechanisms of MDSC-mediated suppression involved the metabolism of the essential amino acid arginine. L-Arginine is utilized for protein synthesis and plays a decisive role for T-cell activation (TCR-signaling) and proliferation.

In MDSCs two enzymes have been identified that lead to arginine depletion: arginase 1 (ARG1) [237] and inducible nitric oxide synthetase (iNOS). First, cells take up arginine by the cationic membrane transporters (CATs). Next, ARG1 metabolizes arginine into ornithine and urea thereby depleting it from the environment and additionally generating immune regulatory metabolites (e.g. putrescine, L-proline) [215, 238]. Up-taken arginine is converted by iNOS [200] into citrullin while nitric oxide (NO) is released. Interestingly, the expression of these two enzymes seems to be competitively regulated [239] and they are normally not simultaneously active in the same cell. Furthermore, in some cell populations (e.g. macrophages) expression of ARG1 and iNOS seem to be linked to maturation [240, 241].

CD3ζ-chain (CD247) is a key component for signal transduction of the TCR. It contains three immunoreceptor tyrosin-based activation motifs that transduce activation upon antigen recognition to intracellular signaling. Low expression of CD3ζ-chain results in impaired T cell activation.

MDSC-mediated arginine deficiency impairs the proliferation and IFN-γ production of T-cells [181, 183] and leads to a decreased CD3ζ-chain expression. CD3ζ-chain is decisive for T-cell signaling and its decreased expression results in impaired T-cell signal transduction [242]. In a murine GVHD model, ARG1 activity of MDSCs could significantly inhibit T-cell activation, proliferation and inflammatory cytokine release [189]. One underlying mechanism of the observed effects of L-arginine starvation on T-cells could be an impaired up-regulation of cell cycle regulators cyclin D3 and cyklin dependent kinase 4 (cdk4) which results in a cell cycle arrest in the G_0-G_1 phase [238]. ARG1 mediated effects could be abolished *in vitro* by the exogenous L-arginine addition or inhibition of ARG1 with nor-NOHA (Nωr-hydroxy-nor-arginine) and L-NMMA (L-NG-monomethyl arginine acetate) [183, 243].

A cell cycle or cell division cycle encompasses sequential events involved in cell division:
(1) quiescent G_0-Phase,
(2) Interphase, ('preparatory phase') encompassing G_1-Phase, S-Phase, G_2 Phase and the cell division itself (3) mitosis

Nitric oxide (NO) release leads to the impaired activation and function of T-cells. It inhibits for example Janus kinase (JAK) 3 and STAT5 signaling [244] and suppresses the expression of HLA-DR [245]. It also has been associated with the induction of T-cell apoptosis [246]. Furthermore, in the presence of reactive oxygen species (ROS), NO is converted to peroxynitirite (ONOO-). Peroxynitirite is a radical that randomly interacts with proteins causing nitration. In a mouse model, it was demonstrated that this nitrotyrosine accumulation also occurs in the TCR-region. It impairs HLA-class mediated antigen recognition resulting in an impaired T-cell response [236].

ARG-1 and iNOS expression seems to be impacted by the surrounding environment. Inflammation accompanied by the secretion of cytokines such as IFN-γ, IL-4 and IL-13 by activated effector cells might be involved in the up-regulation of ARG-1 activity respectively the induction of iNOS [189, 247]. IFN-γ is known for its activating impact

on the transcription factor STAT1, which is responsible for the up-regulation of iNOS and ARG1 in MDSCs. Furthermore the link of IFN-γ and STAT1 could be one reason that strongly activated T-cells are more easily suppressed by MDSC [204] and blocking of IFN-γ abolishes the suppressive capacity of MDSCs [231, 248]. Up-regulation of iNOS/ARG1 has also been associated with STAT6 activation increasing the suppressive function of MDSC [247] (Figure 8).

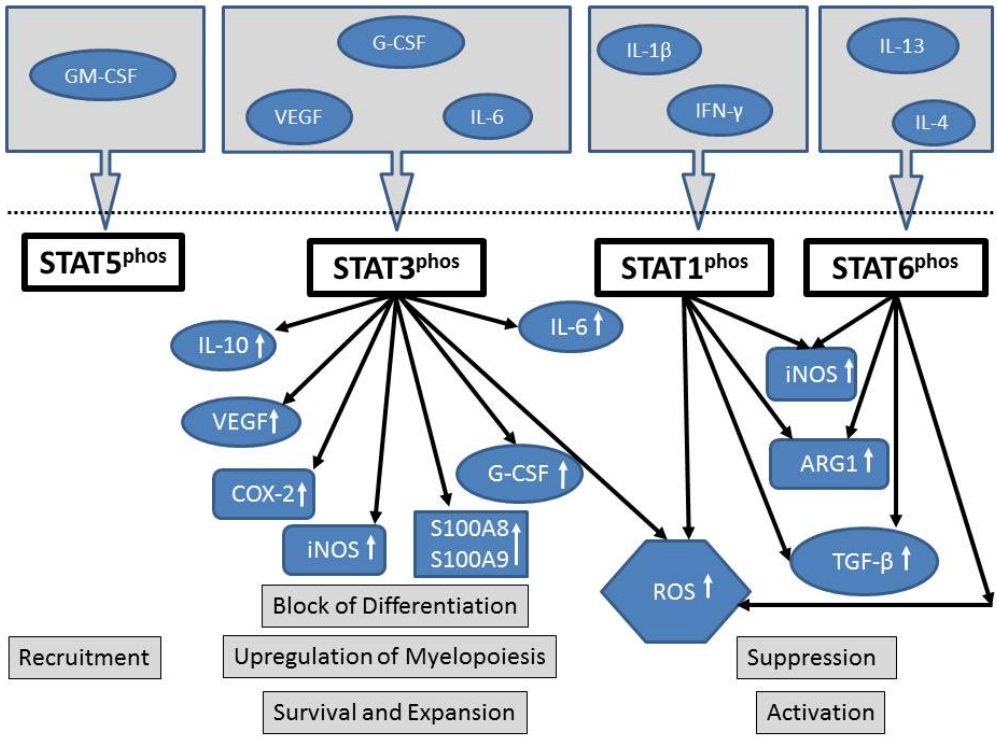

Figure 8: STAT signaling pathways involved in the expansion and activation of MDSCs. Cytokines and growth factors that are released in inflammation and malignant diseases such as IL-6, IL-4, G-CSF and VEGF activate the family of STAT transcription factors. STAT3 plays a central role for a differentiation arrest, which together with an enhanced myelopoiesis leads to MDSC accumulation. STAT1, STAT3, and STAT6 activation promote the up-regulation of immunosuppressive enzymes such as iNOS, ARG1, and COX-2 as well as to the increased production of suppressive cytokines such as TGF-β and MDSC-inducing factors such as IL-6, IL-10 and G-CSF. Increased ROS production, which has been shown to favor myeloid differentiation arrest and T cell suppression, is observed upon STAT1, STAT3 and STAT6 activation.

4.3.2 Oxidative stress

Reactive oxygen species (ROS) such as hydrogen peroxides or superoxide anions are main by-products of the cellular respiration (in the mitochondria) and furthermore produced by the membrane bound enzyme complexes of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Figure 9). ROS is a double-edged sword in immunology [249]:

Reactive Oxygen Species (ROS) encompass superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and instable products that result from lipid peroxidation. ROS readily damage intracellular targets such as DNA, carbohydrates and proteins and has to be balanced by the cellular anti-oxidants.

ROS play a central role in cell signaling as an important second messenger and also possess a direct role in immune function, in particular in the elimination of pathogen by phagocytes via the so-called oxygen burst. However, a constant, increased production of ROS, which outweighs the cellular anti-oxidative capacity epitomized by the thiol-containing molecules glutathione, and several ROS-metabolizing enzymes such as catalase or superoxide dismutase, results in oxidative stress. Oxidative stress causes inter alia potent immunosuppression and constitutes one of the known tumor escape mechanisms. It inhibits the recruitment of CTLs [250] and suppresses T-cell function [251]. Interestingly, immune suppressive T_{Regs} exhibit an enhanced resistance to oxidative stress [235].

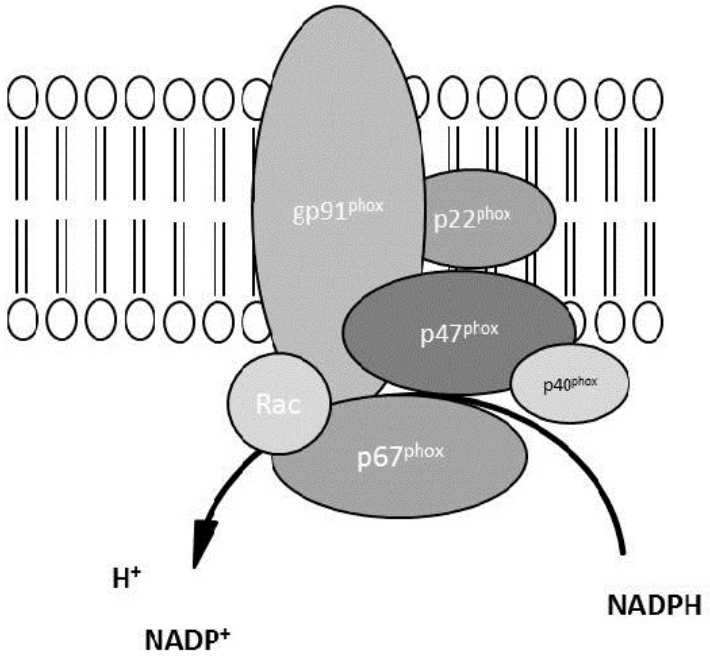

Figure 9: Composition of nicotinamide adenine dinclueotide (NADPH) oxidase. NADPH oxidase is a transmembrane enzyme complex that produces superoxide anions upon activation. It consists of six subunits: Rho related C3 (Rac) and five phagocytic oxidase (phox) units: gp91, p22, p47, p40 and p67. NADPH oxidase plays a pivotal, physiological role in microbial defense by neutrophils. In various malignant and inflammatory diseases, an immoderate activity of NADPH oxidase is observed in various cell types and tissues. This results in an excessive production of superoxide leading to oxidative damage of DNA, proteins, and lipids and immune alterations

MDSCs, especially the granulocytic subsets have been shown to regularly exhibit an increased production of ROS [211, 229]. MDSCs produce ROS mainly via the NADPH oxidase [252] und an up-regulation of its subunits gp91 und p47 was observed for MDSCs in patients with advanced melanoma [183]. ROS serves several functions for MDSCs: (1) suppression of the immune system as demonstrated for antigen specific $CD8^+$ T-cells [211, 252], and induction of apoptosis in activated T-cells by decreasing

Bcl-2 expression [253]. In fact, antagonizing ROS by using for example the ROS metabolizing enzyme catalase can revert MDSC-mediated suppression by increasing IFN-γ production of T-cells in presence of MDSCs [211, 220]. The second function ROS holds is the (2) promotion of MDSC expansion: ROS abets the phosphorylation of the transcription factor STAT3. STAT3 plays a pivotal role for several aspects in MDSC immunology. It controls the suppressive activity of MDSCs as well as the myelopoiesis and blocks their further differentiation into granulocytes, macrophages and DCs [183, 200, 252] (Figure 8).

Inflammatory cytokines such as IL-3, IL-6, IL-10, GM-CSF [254], but also cytokines secreted by MDSCs themselves as TGF-β promote ROS production within an (autokrine) feedback loop [183].

4.3.3 Alternative suppressive mechanisms

The suppressive activity of MDSC has been associated with the production of various immune regulatory cytokines such as IL-10 [197], TGF-β [255, 256] and PGE_2 [257]. These cytokines play a role in direct immunosuppression and have been associated with the MDSC driven tumor promotion [255, 258, 259], and induction of T_{Regs} (see following section).

Recently, $CD14^+HLA\text{-}DR^{low/neg}$ MDSCs have been identified in breast cancer [184], but also in GHVD which exhibited an increased expression of IDO [190]. IDO is an enzyme, which metabolizes tryptophan an essential amino acid to kynurenine-pathway metabolites. Low tryptophan levels leads amongst other to the inhibition of the mTOR kinase pathway. Furthermore, the resulting kynurenine metabolites namely, 3-hydroxyanthranilic acid and quinolinic acid, hold immune regulatory properties [260]. IDO plays an important role for acquired peripheral tolerance and the control of severe inflammation [261]. One characteristic is its quick upregulation respectively inducement upon inflammatory stimuli such as IFN-γ [262].

4.3.4 T-cell differentiation and trafficking

MDSCs do not only directly suppress T-cell responses but also amplify their immunosuppressive capacity by the T_{Regs} induction [225, 263] and expansion of antigen-specific $nT_{Regs.}$ The exact mechanisms have not been elucidated but production of cytokines such as IFN-γ, TGF-β and IL-10 [225, 263] as well as direct cell-cell interactions via CTLA4 [222] and CD40-CD40L interactions [264] seem to play a role. T_{Regs} play an important role in regulating the immune responses and their expansion in cancer constitutes one of the well-established immune escape mechanisms [265]. This T_{Regs} inducing function is albeit not limited to MDSCs in cancers [181, 225, 263], but also utilized by immature myeloid cells in healthy persons to orchestrate the immune system: It was shown that $CD14^+HLA\text{-}DR^-$ cells induce in $CD4^+$ T-cells the production of IL-10 and convert conventional T-cells into $FoxP3^+$T-cells while promoting the transdifferentiation of Th17-cells into iT_{Reg} [192].

Furthermore, MDSCs confine the trafficking of T-cells to secondary lymphoid organs, which is a key step for the T-cell priming. Expression of CD62L (L-Selectin) allows cells to enter secondary lymphoid organs. MDSCs have been shown to decrease the CD62L expression on naïve T-cells by tumor necrosis factor-α-converting enzyme (TACE) thereby hampering their priming in secondary lymphoid organs [266]

4.4 CONCLUSION

MDSCs have emerged as an important immune regulatory population that exists physiologically at low frequencies. In situations of immune activation such as inflammation, cancer or within the framework of an emergency myelopoiesis MDSCs can accumulate. Due to the plethora of identified phenotypes in human diseases, a sole phenotypic identification does not suffice to reliably identify these cells. To experimentally prove their immunosuppressive function towards T-cells is pivotal for naming them MDSCs.

Taken together, similar to T_{Regs}, described in the next chapter, MDSCs might be - according to the given condition - a friend (trying to control acute inflammation) or a foe (suppressing immune responses against cancer cells). In the future, it will be of great interest to further delineate the underlying mechanisms responsible for their expansion and activation as well as their immune suppressive strategies. These insights could provide the sound basis for developing strategies to target specifically MDSCs or to exploit their therapeutic potential (e.g. in GVHD).

5 REGULATORY T-CELLS

The maintenance of self-tolerance and immune regulation is largely attributed to a suppressive subpopulation of T-cells. The existence of so-called suppressor T-cells was first described by Gershon et al. in 1970 [267]. It took however another 25 years until Sakaguchi et al. could identify a specialized subset of $CD4^+CD25^+$ T-cells that could prevent autoimmune disease in a murine model [268]. Based on their immune regulatory function in maintaining tolerance to auto- and alloantigens, these cells were named regulatory T-cells (T_{Regs}) [269].

T_{Regs} are primarily classified according to their origin (Figure 10): T_{Regs} that derive from the thymus are named natural occurring T_{Regs} (nT_{Regs}) and T_{Regs} that are induced from activated conventional T-cells (T_{Conv}) in the periphery are called induced T_{Regs} (iT_{Regs}). The exact mechanisms how iT_{Regs} are induced from T_{Conv} have not yet been deciphered, but two pivotal aspects have been ascertained to promote their generation: (1) inadequate antigen presentation (2) and the presence of high levels of cytokines such as IL-2, IL-10, and TGF-β [265]. Several studies have reported that T_{Reg}-induction can also be cell-mediated. Immunosuppressive cell populations such as MSCs [31, 48] (see section Chapter 2), MDSCs [192] (see Chapter 4) and even nT_{Regs} [270] appear to amplify their own suppressive capacity (in terms of cell numbers but also duration of suppressive activity) by inducing additional regulatory phenotypes.

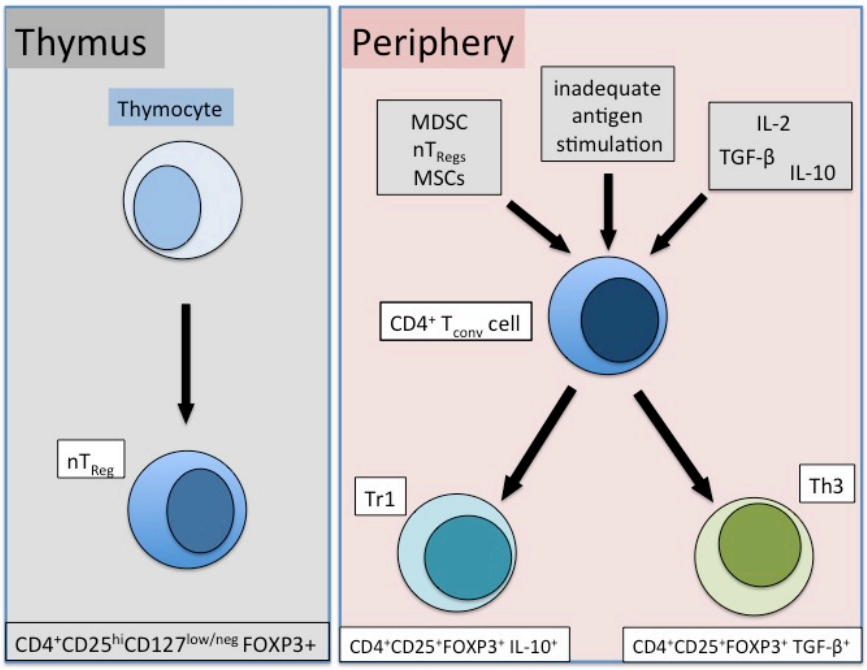

Figure 10: Origin of regulatory T cells (T_{Regs}). Natural occurring T_{Regs} develop in the thymus from T cell progenitors (thymocytes). Furthermore, T_{Regs} can be induced from mature conventional T cells in the periphery as here depicted for $CD4^+$ T cells, which can be induced to become e.g. IL-10^+ Tr1 cells or TGF-β^+ Th3 cells. Several suppressive cells such as MSC and MDSC as well as the presence of high levels of cytokines namely IL-2, TGF-β and IL-10 in combination with inadequate antigen stimulation mediate this process.

5.1 PHENOTYPES

One of the first phenotypic markers for defining nT_{Regs} was the high expression of CD25, which is the alpha-chain of the high-affinity receptor for IL-2 [271]. IL-2 is secreted by stimulated T-cells [272] and drives their proliferation and clonal expansion [273]. Although nT_{Regs} themselves are normally anergic and do not produce IL-2 upon stimulation, IL-2 plays an essential role for T_{Regs} function, proliferation and survival. IL-2 seems to reconstitute a feedback circuit: activation of T-cells simultaneously promotes the existence of T_{Regs} and furthers their immunosuppressive function [274]. Furthermore, the high consumption of IL-2 by T_{Regs}, results in shortage of IL-2 for naïve and effector T-cells leading to their apoptosis [275]. This has been proposed to represent an immunosuppressive feature of T_{Regs}.

Since CD25 expression can also be increased on activated T_{Conv} cells, CD127 (IL-7Rα chain) was added to the panels for identifying nT_{Regs}. It has been shown that nT_{Regs} (unlike activated T-cells) express CD127, if at all only at low levels [276], which in addition correlates inversely with the expression levels of the transcription factor FoxP3 [277]. FoxP3 has been identified as the prototypic master regulator for nT_{Regs} and represents one of the most reliable (intracellular) phenotypic markers of nT_{Regs} [278]. nT_{Regs} depend on a high and continuous expression of FoxP3 for their development and suppressive function [279]. The key function of FoxP3 and nT_{Regs} for immune homeostasis can be observed in the so-called IPEX syndrome (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome), which is associated with heavy autoimmunity and is frequently lethal in the first years of life [280]. The human IPEX locus was identified as Xp11.3-Xq13.3 [281] and IPEX mutations may lead to alterations in the forkhead motif in the FOXP3 coding region. Recent studies emphasize the role of the methylation status of the FOXP3 promoter region [282]. This data suggests that methylation analyses could represent an even more accurate method (than FACS) for identifying and even quantifying "pure" nT_{Regs}. But obviously more research and development is required for introducing such method into the clinical or laboratory routine.

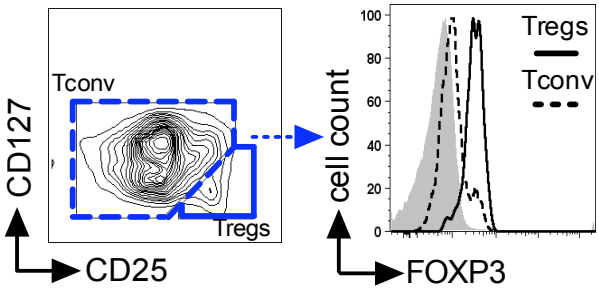

Figure 11: Phenotypical characterization of naturally occurring T_{Regs}. This figure shows a representative example for the FACS Blot gating strategy for nT_{Regs}. Gating on $CD3^{+}CD4^{+}$ T-cells is followed by the identification of the $CD25^{hi}CD127^{low/neg}$ subpopulation, which expresses the transcription factor FOXP3 (depicted as histogram, grey = isotype control) [51].

The afore described phenotypic markers are characteristic for nT_{Regs} and do not necessarily apply for iT_{Regs} (Table 5), which sometimes express only low levels respectively no FOXP3 and CD25 [265, 283]. The two most frequently described subsets of iT_{Regs} are mostly defined by their high expression of the immune modulating cytokines IL-10 (IL-10^+ T_{Reg}1 cells (Tr1 cells)) and TGF-β (TGF-β^+ T helper (Th) 3) [265] upon antigen-stimulation.

Cell Type	Origin	Phenotype	Suppressive mechanisms
nT_{Regs}	**Thymus**		
CD4 nT_{Regs}		$CD4^+CD25^+FOXP3^+CD127^{-/low}$ $CTLA4^+$ LAG-3^+GITR^+	contact, cytotoxicity, IL-10, TGF-β
CD8 nT_{Regs}		$CD8^+CD25^+FOXP3^+CTLA4^+CD122^+$	contact
Adaptive/ Induced T_{Regs}	**Periphery**		
CD4 nT_{Regs} like		$CD4^+CD25^+FOXP3^+CTLA4^+GITR^+$	contact (requires IL-2 and TGF-β)
Tr1		$CD4^+CD25^+FOXP3^+$	IL-10
Th3		$CD4^+CD25^+FOXP3^+$	TGF-β, IL-10 (to a lesser extent)
CD8 iT_{Regs}		$CD8^+CD25^+FOXP3^+$	IL-10, TGF-β
CD8 iT_{Regs}		$CD8^+CD25^+CD28^+FOXP3^+CTLA4^+$ $GITR^+$	contact, IL-10, ILT3, ILT4

Table 5: Regulatory T-cell subsets and suppressive mechanisms. Different regulatory T-cells subsets have been identified which differ in phenotype as well as their mode of immunosuppression. *Adapted from* [265].

5.2 SUPPRESSIVE MECHANISMS

T_{Regs} suppress as demonstrated in numerous *in vitro* and *in vivo* studies the activation and expansion of cells from the innate and adaptive immune system, thereby impacting cellular and humoral immune responses. Several prerequisites have to be met for T_{Regs} mediated suppression as (1) physical proximity to target cells, (2) sufficient number of T_{Regs} and (3) preceding activation of T_{Regs} by TCR-ligation [284-286]. T_{Regs} activation is antigen specific, however once they are activated their suppression is antigen independent ("bystander suppression") [285]. Interestingly, in order to prevent autoimmune disease and maintain immunological self-tolerance, T_{Regs} might even be "auto-reactive" in terms of (self-) antigen specificity, which warrants a continuous stimulation by self-antigens [284].

Several soluble factors such as immune regulatory cytokines most notably IL-10 and TGF-β are released by activated T_{Regs}. Both cytokines seem to play a pivotal role in

T_{Regs} mediated suppression *in vivo,* as it has been e.g. shown for IL-10 in a murine model for autoimmune diseases [287, 288]. In most *in vitro* models, secretion of TGF-β and not IL-10 was observed in most nT_{Regs}-cultures [289]. The existence of TGF-β^+ Th3 cells were first mentioned in association with the inhibition of Th1-cells via secretion of TGF-β in a murine MS model [290] with their importance being recognized in several autoimmune disease models in the following years [291].

Besides direct suppression, TGF-β and IL-10 foster the induction of additional immune regulatory cells such as IL-10^+ Tr1 cells and TGF-β^+ Th3 cells from naïve T-cells at the site of action [265, 270]. Present data indicates that these iT_{Regs} play a physiological role in the maintenance of tolerance in the gut environment and inflammatory responses. A similar effect as IL-10 and TGF- β, has PGE_2, which is generated by COX-2 and mainly released by iT_{Regs}: it leads to the induction of an immune regulatory phenotype in $CD4^+$ T-cells [292] and furthermore suppresses effector T-cells [293].

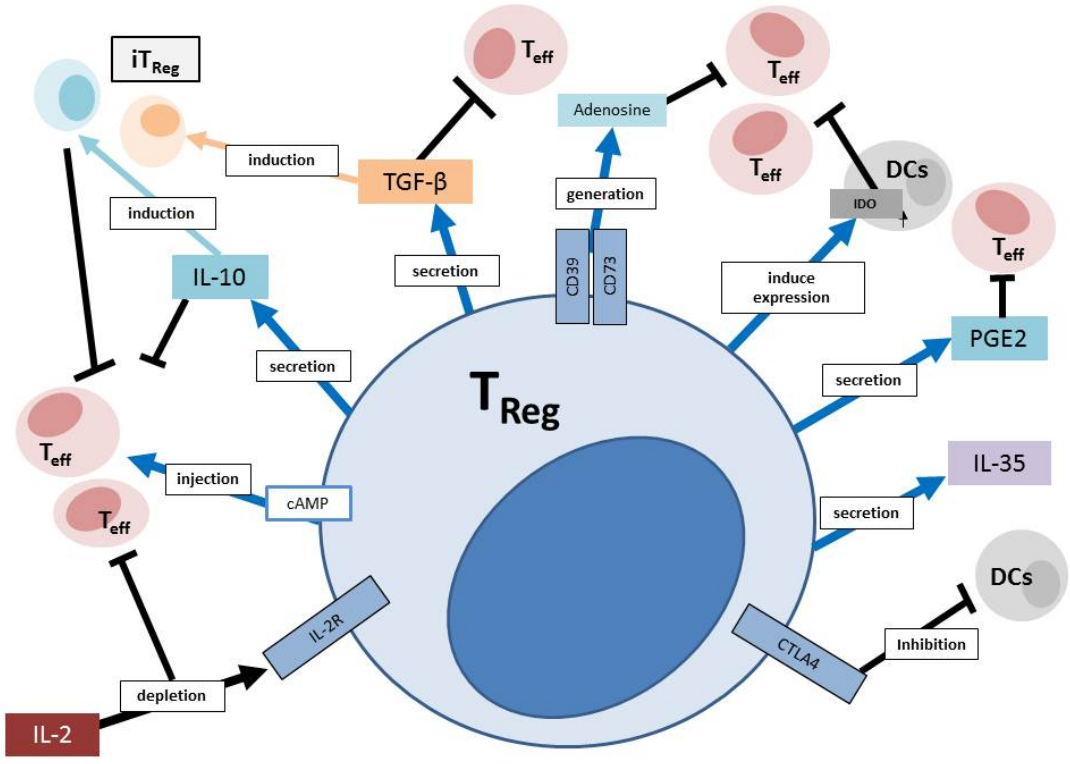

Figure 12: Suppressive mechanisms identified for regulatory T cells (T_{Regs}). T_{Regs} secrete suppressive cytokines such as IL-10, PGE_2 and TGF-β, which directly suppress effector T-cells (T_{eff}), but also further promote induction of iT_{Regs}. CTLA4 expressed on the T_{Reg}-surface directly inhibits DCs. Furthermore T_{Regs} express high levels of CD25 that is part of a high affinity receptor for IL-2 (IL-2R). Thereby they can outcompete activated T-cells in terms of IL-2 uptake leading to the anergy of the activated T-cells and cell death. T_{Regs} can also directly kill conventional T-cells by injecting toxic levels of cyclic adenosine monophosphate (cAMP) via membrane gap junctions or in a granzyme B/perforin dependent way. T_{Regs} furthermore deplete the environment of adenosine triphosphate (ATP) by metabolizing it into adenosine a potential suppressant of T-cells by ectoenzymes (CD39 and CD73).

Furthermore, the elevated levels of all these cytokines lead to a suppressive, tolerogenic milieu by reducing the capacity of APCs to induce alloantigen-specific T-cells [289, 290]. These suppressive effects can also be observed in the tumor microenvironment, which in this case hinders anti-tumor immune responses [265]. Recently, IL-35, which belongs to the IL-12 heterodimeric family was identified as an essential player for the full suppressivity of T_{Regs} [294]; however so far its role has been mostly identified in the murine system, while its impact on the suppressive capacity of human T_{Regs} as well as its effect on other cells than T-cells has not been elucidated.

The role of IL-2 consumption by high affinity receptor CD25 leading to IL-2 starvation as a mean of T_{Reg} mediated suppression has been subject of debate. A recent study has shown that IL-2 deprivation leads to apoptosis [275], however IL-2 depletion does not seem to be the premise for effector T-cell suppression [295].

T_{Regs} express several inhibitory receptors such as fibrinogen-like protein 2 (FGL2), cytotoxic T-lymphocyte antigen 4 (CTLA4) and lymphocyte-activation gene 3 (LAG-3). CTLA4 and LAG-3 are constitutively expressed on nT_{Regs}. They have direct inhibitory effects on T-cells and also indirect T cell regulating effects by modulating APC function: CTLA4 is a ligand for accessory molecules (such as CD80/CD86) expressed on activated T-cells and its binding to the T-cell transmits an inhibitory signal. Furthermore, CTLA4 binding to CD80/CD86 on DCs seems to lead to their down-regulation of these co-stimulatory molecules in a negative feedback manner. This leads to a decreased T-cell stimulation. T_{Regs} furthermore impair DC maturation by e.g. binding of LAG-3 to HLA-Class II molecules, which are expressed on several types of APCs. This LAG-3 binding induces a negative signal for the maturation of APCs [265]. CTLA4 on the other hand can amplify its suppressive effects on T-cell activation and function by the induction of IDO in APCs [296]. IDO metabolizes tryptophan into kynurenine and several immune regulatory metabolites. FGL2 has been shown to induce B-cell apoptosis thereby decimating their interaction with activated T-cells [297, 298].

Cyclic adenosine monophosphate (cAMP) is an important second messenger for cell signaling. T_{Regs} utilize cAMP injections via membrane gap junctions into target cells to impair T-cell proliferation and IL-2 production [299]. T_{Regs} can also alter the levels of cAMP by influencing the extracellular nucleotide/nucleoside levels of for example adenosine by CD39 (ecto-nucleoside triphosphate diphosphohydrolase) and CD73 (ecto-5'-nucleotidase) [300] expressed on their cell membrane. Adenosine inhibits function of T-cells through the adenosine receptor 2A [301]. Furthermore, it has been shown that T_{Regs} also utilize ROS produced by NADPH oxidase in conjunction with TGF-β to suppress $CD4^+$ effector cells [302].

Direct cytolysis of effector cells mediated by T_{Regs} is accomplished by the perforin-granzyme pathway or by the induction of apoptosis by TRAIL-DR5 pathway [301]. Granzymes are serine proteases that utilize perforin to enter target cells, where they induce apoptosis by cleaving important substrates. Several studies have shown that T_{Regs} induce cytolysis in monocytes T-, B- and NK-cells as well as CTLs in a granzyme B dependent manner [265, 301, 303, 304]. However, if the induction of apoptosis is a pivotal suppressive mechanism *in vivo*, has yet to be clarified.

5.3 THERAPEUTIC POTENTIAL FOR T_{REGS} IN THE TREATMENT OF GVHD

The adoptive transfer of T_{Regs} has gained momentum as a mean to treat GVHD following the seminal studies carried out by Edinger et al. In murine mismatched alloHSCT models, it has been shown that the ratio of T_{Regs} to $CD4^+$ T_{conv}-cells strongly determines the outcome of GVHD [305, 306]. Furthermore, it has been shown that this ratio (T_{Regs} to $CD4^+$ T_{conv}-cells) plays an important predictive role for long-term graft tolerance in alloHSCT and also liver and kidney transplantations [307]. Many immune monitoring *ex vivo* studies concluded that humans that have lower frequencies of T_{Regs} in the peripheral blood after HSCT hold a higher risk of developing GVHD [308, 309]. This was not only the case for diminished thymic derived nT_{Regs} but also IL-10^+ Tr1 T_{Regs} [310], which has been associated with increased intestinal inflammation [289].

In animal models it has been shown that T_{Regs} suppress hyperactive, autoreactive T-cells (GVHD) while maintaining the GVT effect [311, 312] and without impairing antiviral T-cell immunity [313].

One important prerequisite for a successful adoptive cellular therapy is to gain sufficient numbers of the transferred cells. Different approaches have been undertaken ranging from in vitro expansion of cord blood units [314], to *ex vivo* isolation of $CD4^+CD25^+$ T_{Regs} [315, 316]. The first clinical studies showed a beneficial effect without an increased relapse or higher infection rate [314-316]. Another issue that has to be resolved which is the best time point, the optimal cell dose for transfusion and what phenotype of T_{Regs} is associated with the highest suppressive capacity and should therefore be preferentially selected. $CD4^+CD25^{high}$ T_{Regs} that also express CD45RA, inducible T-cell costimulator (ICOS) and are negative for CD127 seem to be most suitable [317]. Infusion numbers range from 100,000 to 1,000,000 T_{Regs} per kg body weight [314, 316, 318]. Interestingly, it seems to depend on the utilized expansion protocol, if these numbers are sufficient and effective.

Recently, it was shown that in patients with chronic GVHD low dose treatment with IL-2 ($0.3\times10^{6,}$ to 1×10^6 international units (IU) per square meter of body-surface area) leads to *in vivo* expansion of T_{Regs} [319]. It is thought that this low dose of IL-2 stimulates specifically T_{Regs} due to their higher affinity for IL-2 as compared to conventional T-cells. The clinical effects were promising and therefore further studies have to show whether the *in vivo* induction of T_{Regs} could represent a (more convenient) Treg-based approach than *ex vivo* expansion with following adoptive cell transfer.

5.4 CONCLUSION

Regulatory T-cells (T_{Regs}) represent a pivotal checkpoint for the immune system preventing auto-immunity and chronic inflammation. In addition to the well-established thymus-derived naturally occurring nT_{Regs}, T_{Regs} can be induced from regular T_{Conv} cells by a number of cytokines (e.g. IL-10) and other cell types (e.g. MDSCs). This so-called "on-site"generation is an elegant mean for a timely and most likely locally restricted reaction against inflammatory processes.

T_{Regs} exhibit a plethora of immune regulating functions that have been mostly studied in terms of their impact on T-cell-responses. However, most components of the innate and adaptive immunity appear to be affected directly or indirectly by T_{Regs}. Taken

together, T_{Regs} keep the immune system in balance. Consequently, reduced or deficient T_{Regs} are associated with autoimmunity and an increased risk for developing aGVHD after alloHSCT. During the last five years, adoptive T_{Reg} transfer and (pharmacological) means (e.g. low-dose IL-2) for promoting an *in vivo* T_{Regs} expansion have both been heavily investigated and emerging as a promising option for treating GVHD. However, it is important to note that increased T_{Reg} frequencies are not *per se* beneficial. In malignancies the immune suppressive effect of T_{Regs} is regularly "hijacked" by tumor cells, which should be always carefully considered in patients transplanted for underlying neoplasias. Compelling evidence suggests that T_{Reg} accumulation plays a key role for the tumor immune escape – one of the hallmarks of cancer.

Taken together, T_{Regs} are a double-edged sword that can be "boon and bane" to the host depending on the immunological situation and underlying disease(s).

6 AIMS

The aim of this thesis was to further decipher the impact of MSCs on the immune system. The following questions were addressed:

1. MSCs are often transfused into inflammatory environments; a potent protection from oxidative stress is necessary to maintain their immune regulatory function. HO-1 is a potent anti-oxidative, immune regulatory and cytoprotective molecule that, as previously shown, furthermore exerts a T-cell suppressive effect in rat MSCs [320]. The questions addressed in the first study were therefore: does HO-1 serve a dual role in human MSCs as an antioxidant with immune regulating functions? How is its expression and function impacted by inflammatory licensing?

2. HLH is a highly inflammatory disease of often-fatal outcome. The only curative regimen for this disease is allogeneic HSCT. There are cases in which a suitable transplant is not available or the health condition of the patient does not allow the induction of the therapy. Could MSCs therefore constitute a mean to bridge the therapeutic gap and furthermore limit the disease-associated hyper-inflammation until allogeneic HSCT is feasible? Does MSC treatment lead to a measurable immunological impact?

3. MSCs have been applied in studies to treat complications after alloHSCT and aGVHD. Do MSCs transfused in patients lead to measurable immunological alterations compared to placebo controls and for how long are these alterations present?

4. Recently, it was shown for animal models that regulation of inflammation by MSCs has been associated with the induction of immune suppressive myeloid cells [22]. Do infused MSCs lead to the induction of a regulatory phenotype in myeloid cells in patients after with aGVHD after aHSCT and do these myeloid cells hold a suppressive capacity that potentially limits GVHD?

7 MATERIAL AND METHODS

All information regarding material and applied methods is detailed in the respective papers (I-IV). This section addresses some general aspects.

7.1 PATIENTS AND DONORS

The studies included in this thesis were all approved by the ethics committees of the Karolinska University Hospital (Stockholm, Sweden) and the University Hospital of Erlangen (Erlangen, Germany) respectively. Healthy donors and patients respectively their parents signed an informed consent in accordance to the Declaration of Helsinki.

Patients were enrolled from the Hematology Center at the Karolinska University Hospital in Huddinge and the Department of Hematology and Oncology at the University Hospital of Erlangen, Germany. The patients undergoing alloHSCT were treated with standardized protocols following international guidelines and recommendations.

In the presented studies, adoptive MSC transfer was utilized for steroid refractory acute GVHD and HSCT related complications [51] and the treatment of HLH [85]. Steroid refractoriness in GVHD patients was diagnosed, if steroid treatment (≥ 2 mg per kg per day) led (A) to no clinical improvement after at least seven days, or (B) in the case of GVHD progression by at least one grade, within 72h.

7.1.1 Patient material

Peripheral blood mononuclear cells (PBMCs) were retrieved by density gradient-based centrifugation and stored in 10% dimethyl sulfoxide (DMSO) in liquid nitrogen until further use. For subsequent isolations of specific cell subsets such as monocytes or T-cells magnetic bead–based methods (Miltenyi Biotec, Bergisch Gladbach, Germany) were applied.

Serum was immediately processed and stored at -80°C until analysis.

MSCs were isolated from the bone marrow of unrelated unmatched donors harvested from the iliac crest as described previously in detail [94, 321]. In short, heparinized bone marrow was separated according to the density gradient and the bone marrow mononuclear cells were plated in DMEM with 10% FCS. The culture procedure was in line with the recommendations of the MSC consortium (EBMT) and the Swedish Medical Products Agency. The purified and expanded MSCs fulfilled the ISCT criteria: they expressed CD73, CD90 and CD105 and were negative for CD3, CD14, CD31, CD34, CD45, and HLA-DR. They differentiated into cartilage, bone and fat (multipotency) using special culture conditions and inhibited mixed lymphocyte cultures *in vitro* (immune regulation). All cells for clinical and research purposes were cultured according to well-established protocols for three passages [322] and subsequently cryopreserved in 10% DMSO until infusion or further utilization. Release criteria for MSCs included viability of >95%, absence of visible clumps, and sterility. In average two million cells per kg bodyweight (range: 1.5 – 2.2) diluted in saline solution supplemented with 10% AB plasma were infused for 15 – 20 minutes.

7.2 METHODS

7.2.1 T-cell suppression assays (Paper I and III)

Mixed lymphocyte reactions (MLRs) or bead-triggered (i.e. anti-CD3- and anti-CD28 activating beads) T-cell stimulation have been established to mimic T-cell responses *in vitro* and can be used for evaluating the suppressive activity of regulatory cells such as MSCs. Briefly, the impact of immunosuppressive cells on classical lymphocyte responses to activation such as proliferation (primarily) or cytokine production are assessed.

In this thesis two common experimental set-ups were utilized: (1) for assessing the suppressive capacity of MSCs [31], pooled irradiated (for preventing proliferation) allogeneic peripheral blood lymphocytes (PBLs) from five healthy donors were used as stimulators of non-irradiated PBLs from another donor (responder cells) and co-cultured for five days. Proliferation (measured by quantifying the ^{3}H-thymidine incorporation in T-cells) was comparatively analyzed in cultures in which MSCs were present (ratio of 1:10 to the responder PBLs) or absent. (2) The suppressive capacity of MDSCs [190] was estimated by measuring the proliferation of purified, autologous T-cells in response to anti-CD2/CD3/CD28-T-cell stimulating bead-coupled antibodies. For quantifying T-cell proliferation, T-cells were pre-labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE) before the assay. CFSE diffuses passively into cells, where it interacts with intracellular esterases and amines and becomes fluorescent. The formed dye-protein adduct is maintained in the cell during meiosis and inherited by the daughter cells. The dilution of the dye upon proliferation was assessed by fluorescence activated cell sorting.

7.2.2 FACS (Paper I-IV)

Fluorescence activated cell sorting (FACS) has become an essential method in immunology. It allows the detailed monitoring of many facets of the immune system. In short, cells stained with fluorescence labeled antibodies or dyes are acquired in a laminar stream of fluid. The cells pass through a laser beam that allows (utilizing different lasers, filters and detectors) the simultaneous detection of the cell volume (forwards scatter (FCS)), cell granularity (sideward scatter (SSC)) as well as by measuring the emitting signal of the excited fluorescent dye, the expression of the target of interest. Fluorescent antibodies stain antigenic surface markers. The application of different fluorochromes in combination with the data gained by FCS and SSC makes it possible to distinguish different cells subsets. Furthermore, utilizing fixation/permeabilization reagents also intracellular expressed cytokines e.g. IL-10, IL-17, IFN-γ and transcription factors such as FOXP3 or STAT can be analyzed. Besides phenotype, FACS gives the possibility to examine the viability (7-amino-actinomycin D (7AAD)/AnnexinV), proliferation (Ki67, CFSE) and the metabolism respectively oxidation status of cells. All above described application were utilized at some point in this thesis to analyze cell population in PBMCs [31, 51, 85, 190] respectively bone marrow cells [85] isolated by gradient based centrifugation.

7.2.3 ELISA and multiplex (Paper II and IV)

Enzyme-linked immunosorbent assays (ELISAs) are a standard procedure to measure cytokines in serum or supernatant. The principle of the so-called sandwich ELISA is based on capture antibodies (bound to pre-coated micro plates), which bind one specific target antigen contained in the sample (in our studies IFN-γ, IL-10 and PGE_2 in supernatants [31, 190]. After the binding incubation phase, unbound antigen is washed off and secondary specific antibody is added, to the wells. This secondary antibody is conjugated to an enzyme e.g. horseradish peroxidase. By adding the according substrate, the enzyme produces (depending on the amount of bound secondary antibody) a colored product. The resulting absorbance can be measured in a plate reader and the amount of target antigen quantitated employing a standard curve.

Multiplex cytokine assays on the other hand allow the simultaneous analysis of multiple cytokines even in small volumes. This method was used to analyze serum samples of alloHSCT patients and the HLH patient [51, 85, 190]. This method utilizes microscopic small spheric, polysyrol particle (beads), which serve as binding phase similar to coated ELISA plates described above. Each assay contains a specified mix of bead populations, which are each coated with antibodies on the surface specific for one target analyte. Each bead population is again internally labeled with a defined concentration of a fluorescent dye. These different concentrations result in different emitting fluorescence upon excitation and allowing the discrimination of the population.

In short, beads are incubated with the sample to allow binding of the respective analyte. Subsequently a biotinylated detection antibody is added, followed by the incubation with a streptavidin conjugate. This conjugate is bound to a fluorescent, which allows the specific detection of the amount of binding of the analyte. The conjugate has a high specific affinity to the analyte and emits a defined signal. Integrating all this information, allows the simultaneous classification of the beads and the quantification of the analyte in the sample.

7.2.4 RT-PCR (Paper I, III)

Real time polymerase chain reaction (RT-PCR) often synonymously termed quantitative real time PCR (qPCR) allows the detection and quantification of genes on the messenger RNA (mRNA) level utilizing complementary DNA (cDNA) synthesized from isolated total RNA.

Cells were lysed utilizing RLT Buffer containing 0.01% β-Mercaptoethanol according to manufactures instruction (RNeasy Mini Kit, Qiagen Hilden). Cell lysates were stored at -80°C, if RNA isolation was not subsequently carried out. The technology of RNeasy Kits relies on (1) the lysis by denaturing guanidine thiocyanate which also inactivates RNases and (2) subsequent purification of RNA involving several centrifugation and washing steps in which RNA is bound to silica membranes. In the last step total RNA is eluted with RNAse free water. The obtained total RNA was stored at -80°C or immediately used for cDNA synthesis. cDNA synthesis requires two steps: (1) single stranded RNA is transcribed by the enzyme reverse transcriptase utilizing a RNA independent DNA-Polymerase and short oligonucleotide (primer); (2) obtained cDNA is amplified by conventional PCR (reverse-transcription PCR). Yielded cDNA was stored at -20°C.

During RT-PCR the amount of cDNA is measured in „real time“ with quantification at the end of each cycle. The quantification is detected by utilizing a fluorescent dye such as SYBR Green. SYBR Green intercalates with double stranded DNA (dsDNA) and emits a signal that correlates with the amount of dsDNA that was amplified by the according primer pair for the target gene.

The gene expression can be normalized to the expression of a reference gene (house keeping gene). For all studies included in this thesis this was *β-actin*. Relative gene expression was calculated using the ΔΔ-C_T-formula:

relative gene expression = $2^{\wedge(\Delta CT \text{ Target gene} - \Delta CT \text{ Reference gene})}$

We used relative gene expression to monitor the impact of inflammatory licensing on the expression of immune regulating molecules such as HO-1, COX-2 and IDO in MSCs [31] and to elucidate the immunosuppressive mechanism of MDSC after alloHSCT [190].

8 RESULTS AND DISCUSSION

MSCs have been widely evaluated within clinical studies and their efficacy and safety, in terms of an increased risk for infectious complications and/or acute toxicity, has been well demonstrated [11, 94, 323]. Since it is yet not unequivocally clarified, which mechanistic effects take place upon an adoptive transfer of MSCs, it is a difficult task to identify biomarkers that reflect the response to and/or the efficacy of MSC treatment. To date, most studies use the clinical response as the only parameter to value the (biological) impact of MSCs [94]. This obviously represents a hurdle, which needs to be overcome in order (1) to discriminate biological from clinical responses, (2) to develop biomarkers that identify good and bad responders towards MSC treatment, and (3) to optimize our application protocols and the patients' outcome.

As yet, most of our knowledge regarding the immunological effects of MSCs is based on *in vitro* observations and pre-clinical models. This self-evidently does not necessarily reflect the situation in patients. Aim of this thesis was to address the mode of action of MSCs focusing on the modulation of the adaptive immunity. Moreover, we attempted to transfer our *in vitro* observations into the translational clinical setting: (1) to better understand the in-patient MSC effects and (2) to deduct from these observations potential immunological biomarkers, which would allow to objectively assess the biological efficacy of transferred MSCs in the future.

Briefly, in this thesis we were able to show that MSCs via T_{Reg}-induction directly and indirectly regulate T-cell responses. In doing so MSCs display a remarkable plasticity in terms of switching between different suppressive strategies, which is governed by their environment **(Paper I)**. Furthermore, we observed that MSCs promote the skewing of monocyte polarization towards a regulatory M2 phenotype. This is of great importance for the experimental MSC treatment of diseases that are actually driven by inflammatory monocytes (such as HLH) **(Paper II)**. In order to translate our findings from bench to bedside, we performed comprehensive immunome analyses in patients receiving MSCs and could *de facto* detect immune responses that were MSC-associated **(Paper II, III, IV)**. Taken together our data support the notion that MSCs act in a "hit and run"-fashion [103]: infused MSCs elicit an immediate response (by e.g. releasing cytokines) that initiates an immunological cascade resulting in long lasting immune regulatory effects (Figure 3), which are active even after the disappearance of transfused MSCs.

In the following sections the main results of my thesis are summarized. The information provided should help the reader to understand the scope of the performed published studies and the general implications of these findings. For more details on experimental set-up, results, and specific discussion of the findings, please refer to the appended articles.

8.1 T_{REG}-INDUCTION BY MSCs - A MATTER OF PLASTICITY (PAPER I)

The MSC-triggered promotion of immunosuppressive phenotypes in various immune cells, including the induction of T_{Regs} from conventional T-cells has been demonstrated in several (*in vitro* and animal) studies [11, 29, 324].

Our initial interest was to investigate the impact of HO-1 on the MSC-mediated control of T-cell responses. HO-1 is a stress-responsive molecule with known anti-oxidative and immune modulating properties [325]. We found HO-1 to be highly expressed in human MSCs contributing to their resilience towards oxidative stress-induced cell death. This key feature allows them to remain operational in inflamed environments similar to the also immune modulating T_{Regs} [235]. Since there were reports on HO-1 being involved in the suppression of T-cells by rat MSCs [320], we speculated that human MSCs express also HO-1, which as a prototypical stress-responsive molecule might be up-regulated within an inflammatory environment leading to a stronger suppressive activity.

In fact, we could show that human MSCs (1) inhibit T-cell proliferation and (2) promote the induction of T_{Reg}-(subsets) in a HO-1 dependent fashion. In contrast to our initial hypothesis, we noticed a significant reduction of the HO-1 expression when MSCs encountered an *in vitro* alloreactive, highly inflammatory environment. Counter intuitively this HO-1 down-regulation elicited by soluble factors was not accompanied by a reduced capacity for T-cell regulation. In fact, MSCs suppressed more potently T-cells and induced more T_{Regs} albeit HO-1 levels were very low. In order to understand this phenomenon we evaluated the impact of the inflammatory milieu on other important immune modulating molecules (e.g. IDO or COX-2) [33, 70]. Inflammatory conditioning of MSCs led to a dramatic up-regulation of COX-2, which was then central for their boosted ability to promote T_{Reg} generation. These findings are very much in line with the current dogma of MSCs being activated by inflammatory cytokines (e.g. IFN-γ or TNF-α) in a process termed as MSC-"licensing" [33, 35]. This is important for two reasons: (1) therapeutically applied MSCs will most likely encounter an inflammatory environment since nowadays inflammatory diseases represents their main indication and (2) it can be reasoned that a MSC-"licensing" prior administration could harness their therapeutic efficacy. Of course more *in vitro* and preclinical data as well as addressing regulatory issues (e.g. compliance with GMP standards) are obligatory before considering the clinical translation. In addition, we demonstrate that the same (suppressive) function within one cell type (MSCs) can be mediated by different molecules, in our case HO-1 or COX-2, depending on the current environment further highlighting the unique functional plasticity of MSCs.

8.2 MYELOID CELLS AS A TARGET FOR MSC-BASED THERAPY (PAPER II)

The failure to treat a disease with the means provided by conventional therapy is one of the strongest motivations to employ experimental approaches. This was the case in a young patient suffering from a rare autosomal recessive immunological disorder that is known as familial hemophagocytic lymphohistiocytosis (FHL). Defects in lymphoid as well as myeloid cells lead to hyper-inflammation, which is often triggered by normally harmless viral infections. The name FHL is derived from a characteristic bone marrow infiltration with monocytes/macrophages that phagocyte the hematopoietic cells

leading to severe cytopenias. Without immune suppressive treatment (e.g. chemotherapy and steroids) survival time is very low and the only curative treatment remains alloHSCT.

The particular patient was treated for over several months with a combination of chemotherapy, steroids, and targeted anti-inflammation (=anti-TNF-α antibodies) without achieving disease control and while facing severe infectious complications. An alloHSCT was at this time not foreseeable due to the lack of a matched donor and sufficient disease control. After careful consideration we decided to treat the patients with an adoptive transfer of third-party MSCs. The transferred MSCs inhibited in *in vitro* pre-testing T-cell activation and more importantly promoted the differentiation of regulatory $CD206^{+}HLA\text{-}DR^{low}$IL-10 producing M2 monocytes. This observation is very important since monocytes/macrophages play a decisive role in FHL pathophysiology. In fact and in addition to their hemophagocytic activity, it is widely assumed that suppressive M2 monocytes/macrophages in FHL patient represent a reaction - in terms of an inherent feedback-mechanism – to the systemic inflammation [326, 327]. Based on these promising biological properties we infused the MSCs without any signs of toxicity. In fact, we observed a fast decline of inflammatory cytokines (e.g. IL-15, which is produced by activated phagocytes) and FHL activity markers (e.g. serum triglyceride and ferritin levels), less IFN-γ producing T- and NK-cells, and elevated IL-10 levels. Overall, several of the anticipated beneficial effects (based on the *in vitro* tests) in terms of T-cell and monocyte/macrophage modulation were confirmed *ex vivo* further strengthening our motivation for performing similar bench to bedside approaches always on the grounds of previously well-studied basic biology. In fact, treatment with MSCs might represent an option for patients with refractory FHL at least for bridging the time to alloHSCT and for potentially reducing the need for chemotherapeutics and the accompanying severe toxicities.

8.3 REGULATORY CELLS AS BIOMARKERS IN MSC TREATMENT (PAPER III AND IV)

As pointed out several times, we still lack an objective (bio-) marker for assessing the biological (in addition to the clinical) response of MSC treatment. In order to address this translational issue we performed a comprehensive analysis of the immune system of patients receiving therapeutic MSC infusions. These (screening) efforts included almost all main populations (T-cells, B-cells, NK-cells, DCs, and monocytes) and several key subsets (e.g. nT_{Regs}, Tr1 cells, and MDSCs) of immune cells and several relevant cytokines (e.g. IL-2, IL-4, IL-10, and TNF-α) that could have been affected by MSCs based on previous reports from *in vitro* and/or animal studies [11, 22, 27, 28, 46, 47, 49, 52, 54, 71, 328].

Of note, we had the opportunity to perform our experiments on unique patient material: steroid-refractory GVHD patients enrolled in a randomized double blinded study receiving either MSC or placebo infusions. To our knowledge this was the first time a randomized control study available for investigating MSC associated effects on the immune system. Blood was drawn at 7, 30, 60 and 180 days following MSC or placebo application respectively. Shortly after MSC infusion we observed a significant drop of endothelial cell death products (serum cytokeratin 18 fragments), which represent a novel activity marker for GVHD [329]. This finding was very important for us, since it

proved that MSCs lead to a measurable (at least) non-specific reduction of the ongoing inflammation. Albeit our immune screening was rather broad T-cell analysis remained our focus since (alloreactive) donor T-cells hold a key role in initiating and driving GVHD but at the same time are decisive for elimination of residual tumor cells (=GVT effect) and protection from pathogens (e.g. viral infections).

Reconstitution of T-cells in terms of numerical alterations was not affected by MSC infusion. However, we found a rather normal $CD4^+/CD8^+$ T-cell ratio in the MSC group. The placebo group on the other hand displayed an inversed ratio, which is often seen in alloHSCT patients. This observation together with a shift of the T_H1/T_H2-balance towards T_H2-responses further strengthens the notion that MSCs might act as a "reset button" leading to restoration of immunological homeostasis. Both, impact on $CD4^+/CD8^+$ as well as T_H1/ T_H2-ratio comes as a nice validation of results from animal studies [29, 30]. The induction of T_{Regs} including their main subsets (nT_{Regs}, Tr1 and Th3 cells) (**Paper I**) represents a key MSC feature for us. It would allow MSCs to prolong their suppressive action by generating another type of suppressive cells that most likely outlives the transferred cells [103] leading to some kind of "immunologic imprinting" (by MSCs). As a matter of fact, we found significant higher levels of nT_{Regs} and Tr1 cells in the circulation of MSC treated patients. This observation is very much in line with findings from several *in vitro* and animal studies performed by others and us (**Paper I**).

Our data strongly indicates that the reason for the elevated T_{Reg} levels was not an increased thymic output, but rather the proliferation of already pre-existing T_{Regs} and/or their conversion from conventional T-cells respectively. In this context we found increased IL-2 levels in patients receiving MSCs, which has already be seen under *in vitro* conditions [330], but still requires a convincing mechanistic explanation. This finding is of particular importance taking into consideration that IL-2 is central for fitness and homeostatic proliferation of T_{Regs} [331]. Remarkably, T_{Reg} frequencies correlated positively with the IL-2 serum concentrations in our treated patient cohort and current trials are actually testing the use of low-dose IL-2 in GVHD patients for promoting T_{Regs}. Based on this data, one could speculate that MSCs might in the future redundantise approaches that exclusively aim in boosting the T_{Reg} compartment (e.g. IL-2 or adoptive T_{Reg} transfer). One outstanding commonality of all altered immune parameters was their transient nature as both, the placebo and MSC group, were almost equal six months after treatment supporting the more and more emerging concept of using sequential MSC applications.

The so-called MDSCs are thought to represent the myeloid counterpart of T_{Regs}. Our data (**Paper II**) and a recently published study [71] indicate that human MSCs when cultured together with monocytes promote a phenotype that resembles monocytic MDSCs as found accumulated in several tumor entities [187]. Of course we were interested in confirming these observations in the GVHD patients that had received MSCs (**Paper III**). Before doing so we first had to identify and characterize MDSCs in alloHSCT patient, which led to a number of interesting results (**Paper IV**). AlloHSCT patients have increased levels of monocytic $CD14^+HLA\text{-}DR^{low/neg}$ cells that suppress activation of autologous T-cells allowing us to term them MDSCs. The MDSC frequency peaked early after transplantation and early engraftment during a period that is characterized by (1) high levels of inflammatory cytokines (=so-called "cytokine

storm") and (2) (myeloid) growth factors as well as an (3) increased ("emergency") myelopoiesis. These parameters have already been shown to be involved in MDSC accumulation and we could effectively notice a significant link between IL-6 (=inflammatory cytokine) and G-CSF (=growth factor) with MDSC frequency. The MDSCs' T-cell suppressive activity was mediated by IDO, which appears to be also systemically activated in alloHSCT patients [261]. An inhibition of IDO led *in vitro* to a partial restoration of T-cell function, which was corroborated by the *ex vivo* data that MDSC numbers were negatively associated with T-cells' proliferative capacity, CD3ζ chain levels, and the expression of activation markers. Interestingly and despite their obvious T-cell suppressive function MDSCs were more abundant in more severe inflammatory GVHD, which is in line with a generalized activation of tryptophan metabolism during GVHD [332]. This finding could be indicative of a compensatory increase in regulatory MDSCs that however fail to control the overwhelming immune response that underpins GVHD.

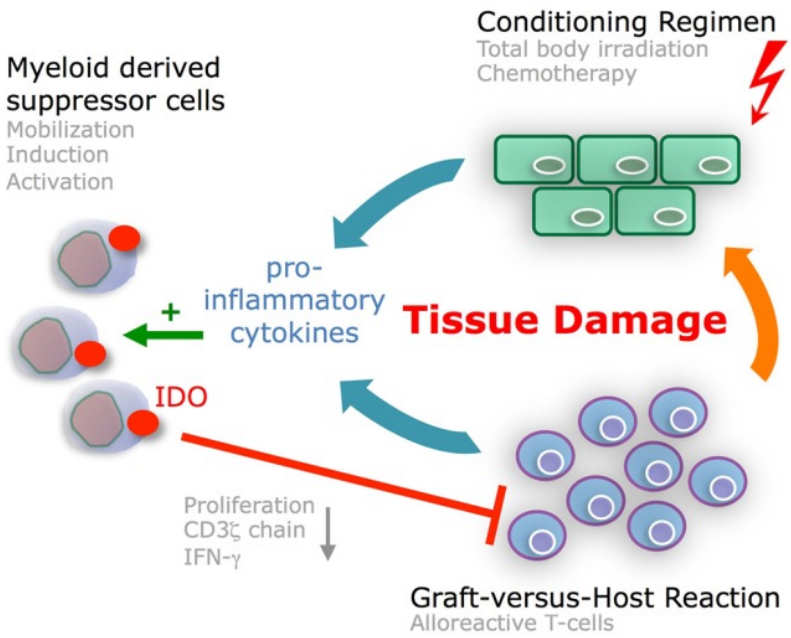

Figure 13: Accumulation of MDSCs in alloHSCT. Preparatory (radio-) chemotherapy (conditioning regimen**)** for alloHSCT leads to tissue damage and subsequent inflammation. In addition, alloreactive donor T-cells, which are fundamental for establishing the GVT effect regularly target healthy tissue (preferably the liver, skin, and gut) of the host, resulting in a highly inflammatory condition known as GVHD. The high levels of pro-inflammatory cytokines released in this setting stimulate MDSCs as part of a physiological mechanism for the control of inflammatory damage. This stimulation most likely represents a multistep process, including an increased mobilization of myeloid progenitors from the bone marrow, a differentiation arrest and the re-programming of mature myeloid cells mediate immunosuppressive functions. Accumulating monocytic MDSCs following alloHSCT express the enzyme IDO, which exerts suppressive effects on T-cells by depleting tryptophan and via its metabolites (e.g. kynurenine). Such immunosuppressive effects include the inhibition of T-cell proliferation and IFN-γ production as well as the downregulation of CD3ζ [333].

Based on these results it can be speculated whether MDSC-based therapeutic approaches might represent an additional option for treating GVHD. Nevertheless, studies evaluating larger patient cohorts for longer observational periods are required to elucidate the actual potential of MDSCs in alloHSCT patients. First, we need to unequivocally clarify the impact of MDSCs on the risk of disease relapse (=GVT effect). Second, we have to assess whether MDSCs are linked to viral infection and/or reactivation, since an increasing number of publication suggests that viruses (e.g., hepatitis C virus) utilize MDSCs for escaping immunosurveillance. However, when returning back to our initial question regarding the impact of MSC infusion on the patients' MDSC we did not detect any interconnection, which might be due to the strong MDSC-promoting inflammatory signals that overshadow the effects of MSCs.

9 CONCLUSIONS

Nowadays, cellular therapy has emerged as a promising tool for boosting immune responses (e.g. T-cell transfer in cancer) or restoring immunological homeostasis (e.g T_{Reg} transfer in aGVHD). However, transferring living cells into patients naturally implies the risk for substantial, often unforeseen obstacles and consequences. A prerequisite for a successful cellular therapy is a thorough understanding of the involved immunological processes. Compared to HSCT, adoptive immunotherapy of MSCs is still in its infancy and its potential has certainly not been fully exploited. In the future, it will be an important task to elucidate the mode of action of MSCs in detail to ascertain the best way to employ them.

In this thesis the following aspects of MSC biology and function were observed:

MSCs possess potent antioxidative protective mechanisms and are not hampered by being cultured in an inflammatory milieu with abundant reactive oxygen species.

MSCs utilize the antioxidative, cytoprotective enzyme HO-1 to induce T_{Regs} *in vitro*. This function is even potentiated upon so-called "inflammatory licensing". MSCs display a remarkable functional plasticity and while HO-1 loses its role during the licensing process, other molecules such as COX-2 take over its T_{Reg}-inducing capacity

MSCs transferred in a patient suffering from HLH exhibited an immune regulating potential. The infusion of MSCs led to a fast reduction of inflammatory cytokines and reduced activation of T- and NK-cells. This was accompanied by a higher prevalence of myeloid cells with an immune regulatory phenotype and an increased release of IL-10.

Upon infusion into patients suffering from steroid-refractory aGVHD, we found that patients that had received MSCs exhibited higher levels of regulatory T-cells which correlated with increased IL-2 levels. The ratio of Th1/Th2-T-cell responses appeared more balanced and lower frequencies of Th17-cells were prevalent. The observed effect was transient as MSC treated patients exhibited similar levels after 6 months as placebo treated controls. MSCs did not negatively impact the immune reconstitution.

When evaluating the myeloid compartment of patients that underwent alloHSCT we found regardless of MSCs a higher frequency of $CD14^{+}HLA\text{-}DR^{low/neg}$ cells. These cells correlated with time point after transplant as well as grade of aGVHD. Elevated frequencies correlated with reduced T-cell activation as well as reduced CD3ζ chain expression. These $CD14^{+}HLA\text{-}DR^{low/neg}$ cells suppressed T-cells *in vitro* utilizing IDO. Inhibition of IDO restored T-cell function and lead to upregulation of CD3ζ chain expression.

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